# The C-terminal domain of the Ferric uptake regulator (Fur) binds a [2Fe-2S] cluster to sense the intracellular free iron content in *Escherichia coli*

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## Abstract

*Escherichia coli* Ferric uptake regulator (Fur) binds a [2Fe-2S] cluster, not a mononuclear iron, when the intracellular free iron content is elevated in *E. coli* cells. Here we report that the C-terminal domain (residues 83-148) of *E. coli* Fur (Fur-CTD) is sufficient to bind the [2Fe-2S] cluster in response to elevation of the intracellular free iron content in *E. coli* cells. Deletion of gene *fur* in *E. coli* cells increases the intracellular free iron content and promotes the [2Fe-2S] cluster binding in the Fur-CTD in the cells grown in LB medium under aerobic growth conditions. When the Fur-CTD is expressed in wild type *E. coli* cells grown in M9 medium supplemented with increasing concentrations of iron, the Fur-CTD also progressively binds a [2Fe-2S] cluster with a maximum occupancy of about 36%. Like the *E. coli* Fur-CTD, the CTD of the *Haemophilus influenzae* Fur can also bind a [2Fe-2S] cluster in wild type *E. coli* cells grown in M9 medium supplemented with increasing concentrations of iron, indicating that binding of the [2Fe-2S] cluster in the C-terminal domain is highly conserved among Fur proteins. The results suggest that the Fur-CTD can be used as a physiological probe to assess the intracellular free iron content in bacteria.

## Introduction

Iron is an essential element for living organisms. However, excess intracellular free iron is highly toxic to promote oxidative damage under aerobic conditions (1-4). In *Escherichia coli*, the concentration of the intracellular labile iron pool was estimated to be in the range from 1.0 μM (5), to 10 μM (6), 26 μM (7), 140 μM (8), and 200 μM (9), depending on detection methods. One common approach to probe the intracellular labile iron concentration was based on the membrane-permeable iron chelator desferrioxamine (3, 7). Desferrioxamine binds ferric iron to form the ferric iron-desferrioxamine complex which has an electron paramagnetic resonance (EPR) signal at $g$ = 4.0 (3). However, only the ferric iron-bound desferrioxamine has the EPR signal and intracellular ferrous iron cannot be detected using desferrioxamine. Furthermore, desferrioxamine may extract weakly bound iron or iron-sulfur clusters from proteins (3), resulting in an overestimation of the intracellular free iron concentration in living cells. An alternative approach to probe the intracellular labile iron concentration was the Mössbauer spectroscopy (9). The Mössbauer spectroscopy can reveal the entire iron content in bacteria pre-labeled with $^{57}$Fe. However, it cannot easily resolve the individual iron species in cells (9). Recent eloquent Mössbauer studies have revealed that the intracellular labile iron pool consists of two major iron complexes with masses of ~500 Da (major) and ~1300 Da (minor) in *E. coli* cells (4), mostly iron-citrate and iron-ATP, but not iron-glutathione or aqueous free iron (10). Thus, the concentration of the intracellular free iron in bacteria remains largely elusive.

The ferric uptake regulator (Fur) is a global transcription factor that regulates intracellular iron homeostasis in bacteria (11, 12). Since the discovery of Fur in *Escherichia coli* (13), it has been postulated that Fur binds ferrous iron to repress the expression of its target genes (14-16) when intracellular free iron content is elevated (17, 18). The *in vitro* studies indicated that *E. coli*

Fur is capable of binding Fe(III), Fe(II), Zn(II), Co(II), Cu(II), Cd(II), and Mn(II) (19-22). However, the idea that Fur can be activated by these divalent cation ions *in vivo* has been challenged because the metal binding affinity of Fur is relatively low with the dissociation constants ranging from 1.2 μM to 55 μM (8, 19, 20, 23). Indeed, the proposed iron-bound Fur has never been identified in *E. coli* or any other bacteria. In searching for the putative iron-bound Fur, we unexpectedly found that a small fraction (~4%) of *E. coli* Fur purified from wild-type *E. coli* cells grown in LB medium under aerobic growth conditions binds a [2Fe-2S] cluster (24). The occupancy of the [2Fe-2S] cluster in Fur is increased to about 32% when Fur is expressed in the *E. coli* mutant cells in which the intracellular free iron content is elevated due to deletion of the iron-sulfur cluster assembly proteins IscA and SufA (24). Importantly, the Fur homologs from *Haemophilus influenzae*, *Vibrio cholerae* and *Helicobacter pylori* can also bind a [2Fe-2S] cluster in the *E. coli* mutant cells with an elevated intracellular free iron content (25). Furthermore, binding of a [2Fe-2S] cluster in *E. coli* Fur turns on its specific DNA binding activity to repress its target genes in *E. coli* cells (26). The results suggest that Fur binds a [2Fe-2S] cluster, but not a mononuclear iron, to regulate expression of target genes in response to elevation of intracellular free iron content in bacteria (24-26).

Here we report that the C-terminal domain (amino acid residues 83-148) of *E. coli* Fur (Fur-CTD) is sufficient to bind a [2Fe-2S] cluster in *E. coli* cells, and that deletion of gene *fur* results in elevation of intracellular free iron content (6, 27) and promotes the binding of a [2Fe-2S] cluster in the Fur-CTD in *E. coli* cells grown in LB medium under aerobic growth conditions. When the Fur-CTD is expressed in wild type *E. coli* cells grown in M9 medium supplemented with increasing concentrations of iron, the Fur-CTD also progressively binds a [2Fe-2S] cluster with a maximum occupancy of about 36%. Furthermore, like the *E. coli* Fur-CTD, the C-terminal domain

of the *H. influenzae* Fur (HI-Fur-CTD) also binds a [2Fe-2S] cluster in wild type *E. coli* cells grown in M9 medium supplemented with increasing concentration of iron under aerobic growth conditions. We propose that the C-terminal domain of Fur may be used as a physiological probe to assess the intracellular free iron content in bacteria.

## Materials and Methods

### Preparation of the *E. coli* Fur-CTD and the *H. influenzae* Fur-CTD

The DNA fragments encoding the C-terminal domain of *E. coli* Fur (Fur-CTD) (residues 83-148) (MAQQHHHDHLICLDCGKVIEFSDDSIEARQREIAAKHGIRLTNHSLYLYGHCAEGDCRE DEHAHEGK) and *H. influenzae* Fur (HI-Fur-CTD) (residues 84-146) (MAPTEHHDHLICEDCGKVFEFTDNIIEQRQREISEKYGIKLKTHNVYLYGKCSDINHCDE NNSK) were synthesized (GenScript co) and inserted into plasmid pBAD/His-A (Add gene co.). Because of the endogenous histidine residues in the C-terminal domain of *E. coli* Fur and *H. influenzae* Fur, no extra His-tag was added for protein purification. The cloned plasmid was introduced into *E. coli* wild-type or *fur* mutant cells. Overnight cultures of *E. coli* cells were inoculated 1:100 dilution in freshly prepared LB medium. When cells were grown to O.D. at 600 nm of 0.6 at 37°C under aerobic conditions, protein expression was induced for three hours after adding L-arabinose (0.04%). The *E. coli* cells were also grown in M9 medium supplemented with amino acids (100 μg/ml), thiamine (0.1 μg/ml), glycerol (0.4%), and indicated concentrations of $Fe(NH_4)_2(SO_4)_2$ at 37°C under aerobic conditions. When cells were grown to O.D. at 600 nm of 0.6, protein expression was induced for three hours after adding L-arabinose (0.04%). The C-terminal domain of the *E. coli* Fur (Fur-CTD) or the *H. influenzae* Fur (HI-Fur-CTD) was purified using the Ni-agarose column, followed by passing through a HiTrap Desalting column (GE

Healthcare co.) as described previously for the *E. coli* Fur protein (24). The concentration of purified Fur-CTD was measured at 280 nm after iron-sulfur clusters were removed by adding HCl (10 mM). The extinction coefficients of the *E. coli* Fur-CTD and the *H. influenzae* Fur-CTD at 280 nm are 3.0 and 4.3 $mM^{-1}cm^{-1}$, respectively.

**UV-Visible absorption and circular dichroism (CD) measurements of proteins.**

UV-Visible absorption spectra of purified *E. coli* Fur or the Fur-CTD were taken in a Jasco V-750 UV-Vis absorption spectrometer at room temperature. The circular dichroism (CD) spectra of purified *E. coli* Fur and the Fur-CTD were recorded in a Jasco J-815 CD spectrometer at room temperature.

**Construction of *E. coli fur* mutant**

The gene encoding Fur was deleted in wild type *E. coli* strain MC4100 using the one-step gene inactivation approach (28). Two primers: Fur-A, 5'-TGTCACTTCTTCTAATGAAGTGAACCGCTTAGTAACAGGACAGATTCCGCGTGTAGGCTGGAGCTGCTTC-3', and Fur-B, 5'-CTTGCATAAAAAAGCCAACCCGCAGGTTGGCTTTTCTCGTTCAGGCTGGCCATATGAATATCCTCCTTA-3' were used for deletion of gene *fur*. Deletion of gene *fur* was confirmed by PCR using two primers: Fur-F1, 5'-GTTCAAGTGGCCTTGCCGTTGT-3'; Fur-R, 5'-AGCAGTGTCTGCGTGCAACGCAAAC-3'.

**Measurements of intracellular chelatable iron contents in *E. coli* cells.**

The intracellular chelatable iron content in *E. coli* cells was measured using the membrane permeable iron chelator 2,2'-dipyridyl (24). Briefly, when *E. coli* cells were grown in LB medium or M9 medium to O.D. at 600 nm of 0.4 under aerobic growth conditions, 2,2'-dipyridyl (50 μM) was added to the cell culture. After additional cell growth for one hour, the cells were resuspended in buffer containing Tris (20 mM, pH 8.0) and NaCl (500 mM). The cells were then subjected to French press and centrifuged to remove the cell debris. The supernatants were subjected to UV-Vis absorption measurements. The absorption peak at 522 nm of the 2,2'-dipyridy-Fe(II) complex was used for calculating the chelatable intracellular iron content in *E. coli* cells using an extinction coefficient of 8.6 $mM^{-1}cm^{-1}$ (29).

**Iron and sulfide content determination in purified proteins**

The amounts of iron and sulfide in Fur protein samples were analyzed according to the Fischer's method (30) and the Siegel's method (31), respectively.

## Results and Discussion

**1. The C-terminal domain of *E. coli* Fur (Fur-CTD) binds a [2Fe-2S] cluster in wild type *E. coli* cells.**

*E. coli* Fur contains the N-terminal DNA binding domain and C-terminal regulatory domain (32) (Figure 1A). Previous site-directed mutagenesis studies indicated that *E. coli* Fur binds a [2Fe-2S] cluster via the conserved cysteine residues at the C-terminal domain (24). To test whether the C-terminal domain of Fur (Fur-CTD) is sufficient to bind the [2Fe-2S] cluster, we synthesized a DNA fragment encoding the *E. coli* Fur-CTD (residues 83-148, highlighted in color in Figure 1A) and inserted the DNA fragment into plasmid pBAD. Because the Fur-CTD does not have the

N-terminal DNA binding domain, the Fur-CTD has no DNA binding activity to directly regulate expression of the Fur-repressed genes in *E. coli* cells.

The *E. coli* Fur-CTD was then expressed in wild type *E. coli* cells grown in LB medium under aerobic growth conditions. Figure 1B shows that purified Fur-CTD has small but clear absorption peaks at 325 nm, 410 nm, and 450 nm (spectrum 2), indicative of a [2Fe-2S] cluster binding in the Fur-CTD (24). The amplitudes of the absorption peaks of purified Fur-CTD are essentially identical to these of the full-length Fur purified from wild-type *E. coli* cells (spectrum 1), suggesting that removal of the N-terminal domain does not affect the [2Fe-2S] cluster binding in the Fur-CTD in *E. coli* cells.

Purified *E. coli* Fur-CTD and the full-length *E. coli* Fur were then subjected to the Circular Dichroism (CD) measurement (Figure 1C and D). Simulation of the CD data using the CD simulation software (http://lucianoabriata.altervista.org/jsinscience/cd/cd3.html) revealed that the full-length *E. coli* Fur contains 74.3% alpha helix, 13.7% beta sheets, and 12.0% turns and loops, while the *E. coli* Fur-CTD contains 40.4% alpha helix, 50.7% beta sheets, and 8.9% turns and loops. The estimated secondary structures of the full-length Fur and the Fur-CTD generally agree with the structure models shown in Figure 1A, suggesting that the Fur-CTD is stably expressed in *E. coli* cells.

**2. Deletion of gene *fur* elevates the intracellular free iron content and increases the binding of the [2Fe-2S] cluster in the Fur-CTD in *E. coli* cells.**

The global transcription regulator Fur represses expression of the genes encoding iron acquisition systems (33-35) and stimulates expression of the gene encoding iron storage protein ferritin (36) when the intracellular free iron content is elevated in *E. coli* cells. Thus, deletion of

gene *fur* is expected to elevate the intracellular free iron content in *E. coli* cells grown in LB medium under aerobic growth conditions (6, 27). Here, we constructed an *E. coli* mutant in which gene *fur* was deleted using the one-step gene inactivation approach (28). When the *E. coli fur* mutant and its parental wild type cells were grown in LB medium under aerobic growth conditions to O.D. at 600 nm of 0.4, 2,2'-dipyridyl (50 μM) was added to the cell culture and the growth was continued for one more hour. Quantification of the Fe-2,2'-dipyridyl complex in the *E. coli* cell extracts confirmed that deletion of gene *fur* significantly elevated the intracellular iron content in *E. coli* cells, as reported previously (6, 27).

The Fur-CTD was then expressed in wild type and the *fur* mutant *E. coli* cells grown in LB medium under aerobic conditions. Figure 2A shows that the Fur-CTD expressed in the *E. coli fur* mutant cells (spectrum 2) has much higher amplitudes of absorption peaks at 325 nm, 410 nm, and 450 nm than that in wild type *E. coli* cells (spectrum 1). Using the extinction coefficient of 10 $mM^{-1}cm^{-1}$ at 410 nm for the [2Fe-2S] cluster in Fur (24), we estimated that the occupancy of the [2Fe-2S] cluster in the Fur-CTD is increased from 2.6±0.3% in wild type *E. coli* cells to 7.7±2.3% in the *E. coli fur* mutant cells (Figure 2B). The iron and sulfide content analyses showed that each Fur-CTD purified from wild type *E. coli* cells contains 0.04±0.02 iron and 0.03±0.01 sulfide atoms, while each Fur-CTD purified from the *E. coli fur* mutant cells has 0.13±0.04 iron and 0.10±0.03 sulfide atoms, consistent with the estimated [2Fe-2S] cluster occupancy in the purified Fur-CTD proteins. Thus, deletion of gene *fur* increases the intracellular free iron content and promotes the binding of a [2Fe-2S] cluster in the Fur-CTD in *E. coli* cells.

In the previous studies, we reported that deletion of the iron-sulfur cluster assembly proteins IscA and SufA leads to deficiency of the [4Fe-4S] cluster biogenesis (37) and accumulation of the intracellular free iron content, and promotes the binding of a [2Fe-2S] cluster in Fur in *E. coli* cells

(24). Here, we also expressed the Fur-CTD in the *E. coli* mutant cells with deletion of IscA and SufA. Figure 2A shows that the Fur-CTD purified from the *E. coli* mutant cells with deletion of IscA and SufA also binds a [2Fe-2S] cluster (spectrum 3). Thus, while the intracellular free iron content may be elevated via different mechanisms in the *fur* mutant cells and in the *iscA/sufA* mutant cells, both mutations promote the binding of a [2Fe-2S] cluster in the Fur-CTD in *E. coli* cells, suggesting that the Fur-CTD binds a [2Fe-2S] cluster in response to elevation of the intracellular free iron content in *E. coli* cells.

Interestingly, the Fur-CTD purified from the *E. coli iscA/sufA* mutant cells apparently has a much higher occupancy of the [2Fe-2S] cluster (~21.5±3.5%) than that purified from the *E. coli fur* mutant cells (7.7 ±2.3%) (Figure 2B), indicating that the intracellular free iron content in the *iscA/sufA* mutant cells is much higher than that in the *fur* mutant cells. Deletion of the iron-sulfur cluster assembly proteins IscA and SufA blocks the [4Fe-4S] cluster biogenesis in *E. coli* cells under aerobic growth conditions (37). Because there are a large number of iron-sulfur proteins contain a [4Fe-4S] cluster in bacteria, deletion of IscA and SufA may dramatically elevate the intracellular free iron concentration and promote the binding of the [2Fe-2S] cluster in the Fur-CTD in bacteria.

**3. The *E. coli* Fur-CTD progressively binds a [2Fe-2S] cluster in wild type *E. coli* cells grown in M9 medium supplemented with increasing concentrations of iron.**

M9 medium is known to be iron deficient (containing about 0.05 μM iron) (38). When wild type *E. coli* cells are grown in M9 medium, the intracellular free iron content in *E. coli* cells is very limited. Addition of exogenous iron (10 μM) to M9 medium elevates intracellular free iron content and turns on Fur as an active repressor in *E. coli* cells (26, 39). To evaluate the [2Fe-2S]

cluster binding in the Fur-CTD in wild type *E. coli* cells, we expressed the Fur-CTD in wild type *E. coli* cells grown in M9 medium supplemented with increasing concentrations of iron under aerobic growth conditions. The Fur-CTD was then purified from these cells.

Figure 3A shows that without addition of exogenous iron to M9 medium, only a very small fraction of purified Fur-CTD contains a [2Fe-2S] cluster, consistent with the notion that the intracellular free iron content is low in the *E. coli* cells grown in M9 medium under aerobic growth conditions (38). When iron concentration in M9 medium is increased to 0.5 μM and 1.0 μM, the occupancy of the [2Fe-2S] cluster in the Fur-CTD is increased to 18.3±1.4% and 29.4±2.4%, respectively, indicating that the Fur-CTD progressively binds a [2Fe-2S] cluster in wild type *E. coli* cells in response to increasing iron concentration in M9 medium under aerobic growth conditions. Interestingly, further increase of the iron concentration (2.0 μM or above) in M9 medium does not significantly increase the occupancy of the [2Fe-2S] cluster in the Fur-CTD (Figure 3B), although the chelatable intracellular iron content (40) (which includes intracellular free iron and loosely bound iron centers in proteins and small molecules in cells (10)) still increases (Figure 3C). A simple explanation is that the intracellular free iron concentration reaches the maximum in wild type *E. coli* cells grown in M9 medium supplemented with 1 μM iron (Figure 3B). Thus, the Fur-CTD can progressively bind a [2Fe-2S] cluster in response to elevation of the intracellular free iron content in wild type *E. coli* cells and reaches the maximum when M9 medium is supplemented with about 1.0 μM iron.

**4. The *H. influenzae* Fur-CTD also binds a [2Fe-2S] cluster in wild type *E. coli* cells grown in M9 medium supplemented with increasing concentrations of iron.**

The *H. influenzae* Fur-CTD and the *E. coli* Fur-CTD share 63% identities and 79% similarities. To test whether binding of a [2Fe-2S] cluster in the C-terminal domain is conserved among Fur proteins, we expressed the *H. influenzae* Fur-CTD in wild type *E. coli* cells grown in M9 medium supplemented with increasing concentrations of iron under aerobic growth conditions. Figure 4A shows that like the *E. coli* Fur-CTD, the *H. influenzae* Fur-CTD also progressively binds a [2Fe-2S] cluster in wild type *E. coli* cells grown in M9 medium supplemented with increasing concentrations of iron. The occupancy of the [2Fe-2S] cluster in the *H. influenzae* Fur-CTD expressed in wild type *E. coli* cells reaches the maximum when the iron concentration in M9 medium is about 1.0 μM. Collectively, the results suggest that binding a [2Fe-2S] cluster in the C-terminal domain is highly conserved among Fur proteins, and that the Fur-CTD binds a [2Fe-2S] cluster in response to the elevated intracellular free iron concentration in cells.

## Conclusion

As a global transcript factor, Fur binds a [2Fe-2S] cluster, not a mononuclear iron, to regulate intracellular iron homeostasis in *E. coli* cells (24-26). Here we report that the C-terminal domain of *E. coli* Fur (Fur-CTD) is sufficient for binding a [2Fe-2S] cluster in response to elevation of the intracellular free iron content in *E. coli* cells. When gene *fur* or the iron-sulfur cluster assembly genes *iscA/sufA* are deleted in *E. coli* cells, the intracellular free iron content is elevated, which promotes the binding of a [2Fe-2S] cluster in the Fur-CTD. Importantly, the *E. coli* Fur-CTD progressively binds a [2Fe-2S] cluster in wild type *E. coli* cells grown in M9 medium supplemented with increasing iron concentrations under aerobic growth conditions. Binding of a [2Fe-2S] cluster in the Fur-CTD is apparently saturated when M9 medium is supplemented with

1.0 μM. The results led us to propose that the Fur-CTD may be used as a physiological probe to assess dynamic change of the intracellular free iron content in bacteria.

## Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contribution Statement

CF and HD conceived and designed the study, performed the experiments, analyzed the date, and wrote the manuscript. HD reviewed and edited the manuscript.

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## Data Availability Statement

All data generated and analyzed in the present study are included in the manuscript. Raw data is available on request.

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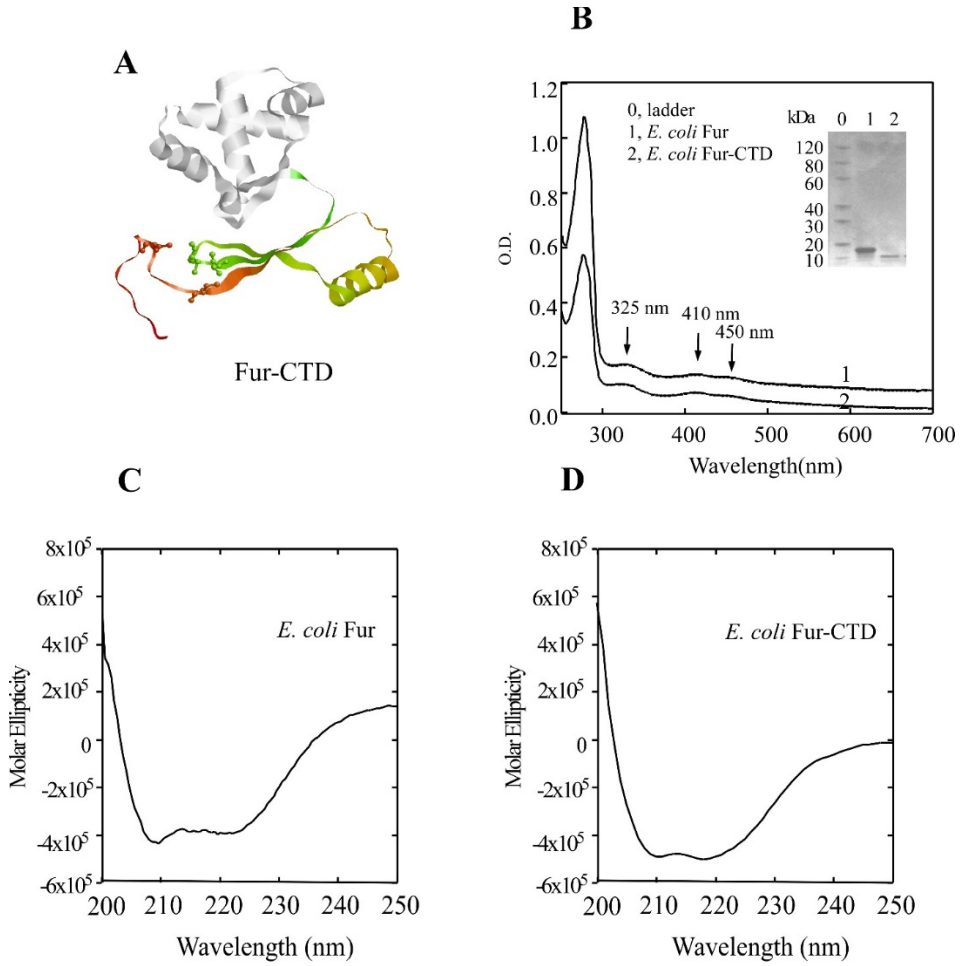


**Figure 1. The C-terminal domain of the *E. coli* Fur (Fur-CTD) purified from *E. coli* cells. A)**, structure model of *E. coli* Fur. The C-terminal domain (residues 83-148) of *E. coli* Fur is shown in color, and cysteine residues are shown in ball-and stick. **B**), purification of the C-terminal domain of *E. coli* Fur (Fur-CTD). The gene encoding the Fur-CTD was expressed in wild-type *E. coli* cells grown in LB medium under aerobic growth conditions. The Fur-CTD was purified from the *E. coli* cells. Spectrum 1, the full-length *E. coli* Fur purified from wild type *E. coli* cells. Spectrum 2, the Fur-CTD purified from wild-type *E. coli* cells. The protein concentrations of *E. coli* Fur and the Fur-CTD were 200 μM. **C)**, Circular dichroism spectrum of the full-length *E. coli* Fur purified from wild type *E. coli* cells. Fur (30 μM) was dissolved in buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0). **D)**, Circular dichroism spectrum of the Fur-CTD purified from wild type *E. coli* cells. The Fur-CTD (30 μM) was dissolved in buffer containing NaCl (500 mM) and Tris (20 mM, pH 8.0). The results are representative of three independent experiments.

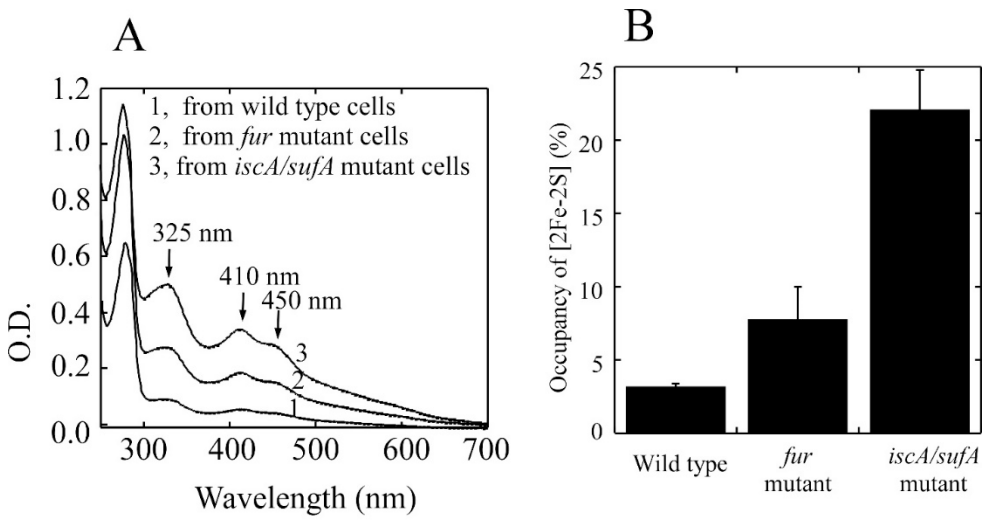


**Figure 2. Deletion of gene *fur* promotes the binding of a [2Fe-2S] cluster in the Fur-CTD in *E. coli* cells.** The gene encoding the Fur-CTD was expressed in wild type *E. coli*, the *fur* mutant cells, and the *iscA/sufA* mutant cells grown in LB medium under aerobic growth conditions. The Fur-CTD was purified from the cells and subjected to UV-Vis absorption measurements. **A**), UV-Vis absorption spectra of purified Fur-CTD. Spectrum 1, the Fur-CTD purified from wild type *E. coli* cells. Spectrum 2, the Fur-CTD purified from the *E. coli fur* mutant cells. Spectrum 3, the Fur-CTD purified from the *E. coli iscA/sufA* mutant cells. **B**), the occupancy of the [2Fe-2S] cluster in the Fur-CTD purified from wild type *E. coli*, the *fur* mutant, and *iscA/sufA* mutant cells grown in LB medium under aerobic conditions. The occupancy of the [2Fe-2S] cluster in the Fur-CTD was measured based on the amplitudes of the absorption peaks at 410 nm and 280 nm. The data are presented as the averages ± standard deviations from three independent experiments.

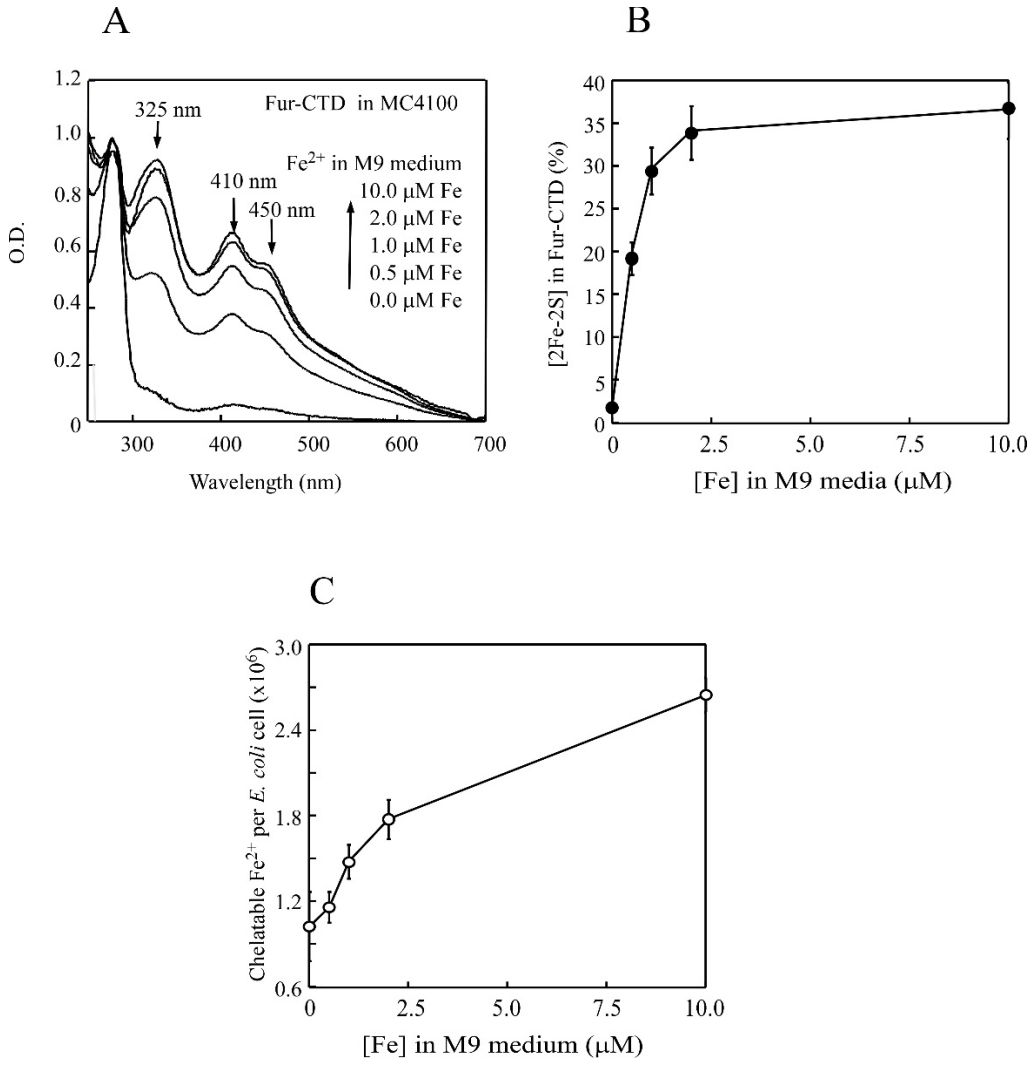


**Figure 3. The *E. coli* Fur-CTD progressively binds a [2Fe-2S] cluster in wild type *E. coli* cells grown in M9 medium supplemented with increasing concentrations of iron.** **A**), UV-vis absorption spectra of the Fur-CTD purified from wild type *E. coli* cells grown in M9 medium supplemented with 0, 0.5, 1.0, 2.0, and 10.0 µM $Fe(NH_4)_2(SO_4)_2$ at 37°C under aerobic conditions. **B**), the [2Fe-2S] cluster occupancies in the Fur-CTD purified from wild type *E. coli* cells grown in M9 medium supplemented with 0, 0.5, 1.0, 2.0, and 10.0 µM $Fe(NH_4)_2(SO_4)_2$ at 37°C under aerobic conditions. Data were collected from A). **C),** chelatable intracellular iron content in *E. coli* cells grown in M9 medium supplemented with increasing concentrations of iron. Chelatable intracellular iron content was measured using the membrane-permeable 2,2'-dipyridyl as described in Materials and Methods. The data are presented as the average ± standard deviations from three experiments.

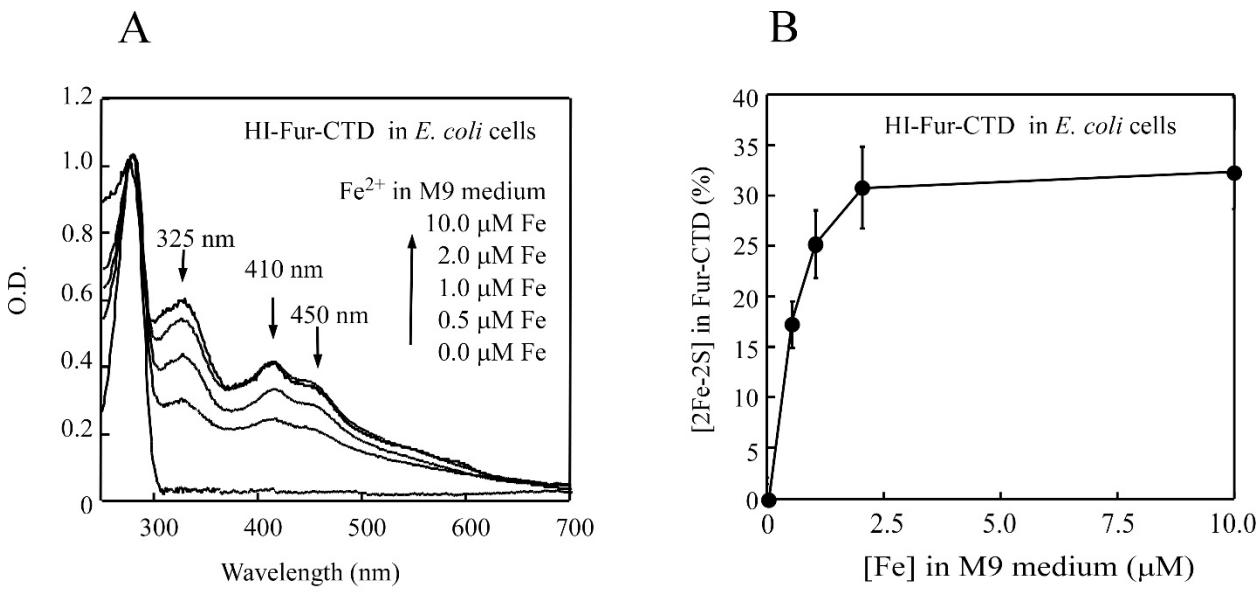


**Figure 4. The C-terminal domain of the *H. influenzae* Fur binds a [2Fe-2S] cluster in wild type *E. coli* cells in response to elevation of intracellular free iron content.** UV-vis absorption spectra of purified *H. influenzae* Fur-CTD (HI-Fur-CD). Wild type *E. coli* cells containing plasmid encoding HI-Fur-CTD were grown in M9 medium supplemented with 0, 0.5, 1.0, 2.0, and 10.0 μM $Fe(NH_4)_2(SO_4)_2$ at 37ºC under aerobic conditions. After three hours of cell growth to allow accumulation of intracellular free iron content, expression of the HI-Fur-CTD in *E. coli* cells was induced by adding L-arabinose (0.04%) for three more hours. The HI-Fur-CTD was purified from the cells and subjected to UV-Vis absorption measurements. Spectra were calibrated to O.D. at 280 nm of 1.0. The absorption peaks at 325 nm, 410 nm, and 450 nm represent binding of a [2Fe-2S] cluster in the HI-Fur-CTD. **B**), the occupancy of the [2Fe-2S] cluster in the HI-Fur-CTD in the wild type *E. coli* cells. The occupancies of the [2Fe-2S] cluster in the HI-Fur-CTD were calculated from the amplitude of the absorption at 410 nm to the protein concentration and plotted as a function of the iron concentrations in M9 medium. The data are presented as the averages ± standard deviations from three independent experiments.