Iron acquisition in aerobic habitats is complicated by the low solubility of ferric hydroxides. Siderophores that bind ferric iron with high affinity are used to mobilize iron. The reduction of ferric iron to the ferrous form can be coupled to the release of iron from siderophores. Iron is also stored intracellularly as a ferric mineral in proteins, such as ferritin, and must be reduced during release. In *Escherichia coli*, the *yqjH* gene encodes a putative ferric siderophore reductase that is also part of the Fur regulon. Here we show that *YqjH* has ferric reductase activity and is required for iron homeostasis in *E. coli*. Divergently transcribed from *yqjH* is the *yqjI* gene, which encodes a novel member of the winged-helix family of transcriptional regulators and also contains an N-terminal extension similar to the Ni²⁺-binding C-terminal tail of SlyD. Deletion of *yqjI* leads to constitutive high-level activity of the *yqjH* and *yqjI* promoters. Purified YqjI binds inverted repeat target sequences within the *yqjH* and *yqjI* promoters. We also observed that YqjI-dependent transcriptional repression is reduced when cells are exposed to elevated nickel levels, resulting in increased expression of *yqjH* and *yqjI*. YqjI binding to nickel or iron reduces YqjI DNA-binding activity *in vitro*. Furthermore, we found that elevated nickel stress levels disrupt iron homeostasis in *E. coli* and that deletion of *yqjH* increases nickel toxicity. Our results suggest that the YqjI protein controls expression of *yqjH* to help maintain iron homeostasis under conditions (such as elevated cellular nickel levels) that disrupt iron metabolism.

Iron is an essential transition metal required for critical cellular pathways, including respiration and photosynthesis. However, maintenance of iron homeostasis is a daunting task due to the low solubility of iron in aerobic environments and to the spurious redox chemistry catalyzed by iron in the presence of oxygen. It is also clear that other transition metals, such as copper and cobalt, can effectively displace and/or compete with iron during metalloenzyme assembly if they are present in excess in the intracellular environment (32, 33, 45, 50).

To circumvent the difficulties of iron acquisition and trafficking, complex iron homeostasis systems have evolved in most organisms. Bacterial iron homeostasis pathways include high-affinity extracellular chelators (siderophores) for extraction of ferric iron from the environment, membrane iron transporters for a variety of iron chelates, intracellular iron storage proteins, and dedicated iron metalloenzyme assembly systems (1). In *E. coli*, transcriptional expression of these various iron homeostasis pathways, collectively referred to as the iron stimulon, is largely controlled by the iron metalloregulatory protein Fur (20).

Iron coordination by the Fur protein controls its DNA-binding activity. The Fe²⁺-Fur homodimer binds to a 19-bp sequence (the “iron box” or “Fur box”) to repress transcription from target promoters, while apo-Fur dissociates from target promoters, leading to upregulation of iron acquisition systems to increase cellular iron levels (21). Fur also controls expression of the RyhB small RNA that acts as a posttranscriptional regulator of mRNA transcripts from the iron stimulon (35).

Despite extensive study of iron homeostasis in *E. coli*, the full picture of iron homeostasis is incomplete. For example, a recent DNA macroarray analysis of iron-dependent gene regulation in *E. coli* showed that approximately one-third of the 101 genes regulated by the Fe²⁺-Fur complex are hypothetical open reading frames with no known function (37). Establishing the biochemical roles of these uncharacterized genes is critical to gain a complete understanding of *in vivo* iron metabolism and homeostasis.

In this study, we examine the function and regulation of *yqjH*, which was shown to be part of the Fur regulon (37). We show here that YqjH is a NADPH-dependent ferric reductase that plays a role in iron homeostasis. Interestingly, we discovered that *yqjH* transcription is controlled primarily by a second regulator encoded by the divergent *yqjI* gene. YqjI represses *yqjH* transcription and is a nickel-binding protein. Our results suggest that YqjI may regulate *yqjH* and other target genes to protect iron homeostasis from disruption by environmental stresses, such as elevated intracellular nickel levels.

**MATERIALS AND METHODS**

*Bacterial strains and plasmids.* *Escherichia coli* wild-type strain MG1655 was the parent strain for all studies (Table 1). The gene deletion strains were constructed as described previously (14). Briefly, a kanamycin resistance (*Kan r*) cassette was amplified from pKD4 using primer pairs containing approximately 35 bp of sequence homologous to regions upstream and downstream of the target genes. The PCR products were transformed into NM400 expressing the λ Red recombinase system, resulting in replacement of the target gene with the Kan cassette. Mutations were then moved by P1 transduction into wild-type MG1655. In some cases, the Kan cassette was removed from a single mutant strain after transformation with the pCP20 plasmid so that double mutant strains could be constructed by P1 transduction (14). Promoter-*lacZ* fusions from the 287-bp intergenic region between *yqjH* and *yqjI* were constructed. The *yqjH*

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† Supplemental material for this article may be found at http://jb.asm.org/.

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TABLE 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype and/or characteristics*</th>
<th>Source or reference</th>
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<td>Laboratory strain</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>DJ480</td>
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</tr>
<tr>
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* Ts, temperature sensitive.

Apo-YqjI and Ni-YqjI were purified using the same protocol. Cell pellets were resuspended in 20 mM HEPES, pH 7.5, with 10 mM β-mercaptoethanol and 5% glycerol and lysed by sonication for 2 min. After centrifugation, cleared lysate was loaded onto a HiTrap heparin HP column (GE Healthcare) and eluted with a linear gradient of 0 to 1 M NaCl. The last two elution peaks were found by SDS-PAGE to contain YqjI. The fractions that correspond to YqjI peak 1 were pooled, concentrated, and loaded onto a Superdex 75 gel filtration column to purify the monomer form of YqjI, while the YqjI peak 2 fractions were further purified by a Superdex 200 gel filtration column (HiLoad 16/60; GE Healthcare) to obtain the oligomer form of YqjI. Both columns were run with 20 mM HEPES at pH 7.5, 10 mM β-mercaptoethanol, 50 mM NaCl, and 5% glycerol. Protein purity was estimated by SDS-PAGE to be ≥90%, and protein concentration was determined by the Bradford assay. In some cases, the oligomeric states of YqjI were confirmed by analytical gel filtration on a Superdex 200 column (Superdex 200 10/300 GL; GE Healthcare). The column was calibrated using cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), ovalbumin (44 kDa), bovine serum albumin (66 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa). The native oligomeric states were also confirmed by 16% native Bio-Trio PAGE (NativePAGE Novex; Invitrogen).

**Ferric reductase assay.** A ferric reductase assay was performed anaerobically in 25 mM Tris-HEPES buffer (pH 7.5), containing 50 μM apo-YqjI, 0.5 mM Tris-EDTA (0.1 M NaCl), and a ferric iron substrate, ferric EDTA (0 to 2 mM), ferric citrate (0 to 2 mM), or ferric enterobactin (0 to 0.7 mM), and 2 mM ferrozine to detect ferrous iron formation. Various concentrations of pure YqjH were added to initiate the reaction. The absorbance at 562 nm was recorded every 15 s for up to 20 min, and the extinction coefficient at 562 nm of the ferrous-ferrozine complex (ε_{562} = 27.9 M⁻¹ cm⁻¹) was used to quantify ferrous iron generation. To determine the Km for ferric-EDTA, NADPH concentration was fixed at 0.25 mM, and YqjH was added at a 0.73 μM final concentration. To determine the Km for NADPH, the ferric-EDTA concentration was fixed at 2 mM, and YqjH was added at a 0.73 μM final concentration. Ferric enterobactin was purified as previously described (42). Iron release from ferric enterobactin was monitored by the decrease of the absorbance at 495 nm for ferric enterobactin in the reaction.

**Ferric nickel reconstitution of the apo-YqjI oligomers.** Samples of the apo-YqjI oligomer reconstitution by ferric nickel reconstitution of the apo-YqjI oligomer was determined by Bradford assay and diluted with 20 mM HEPES at pH 7.5, 500 mM NaCl, 10 mM β-mercaptoethanol, and 5% glycerol to 20 μM. A total of 200 μM Nicl₂ (>99.99%) was prepared from 100 mM Nicl₂ solution by serial dilutions. Changes in the YqjI UV-visible absorption spectrum upon Ni²⁺ addition were monitored after stepwise addition of 2 μM of 0.1 M Nicl₂ to a quartz cuvette containing 20 μM of 20 mM apo-YqjI. Protein precipitation began to occur when the molar ratio of Ni²⁺ to YqjI monomers increased to above 4:1. For standard preparation of reconstituted Ni-YqjI, a 3-fold molar excess of Ni²⁺ was added to apo-YqjI, and the sample was incubated on ice for 1 h. Excess nickel was then removed by concentration and dilution three times using a YM-10 Microcon (Millipore). Use of this protocol typically resulted in a 2:1 molar ratio of Ni²⁺ to YqjI monomers.

**Metal analysis.** For protein metal analysis, apo-YqjI and holo-YqjI were diluted to ~0.5 to 1.0 mg/ml in 4 ml buffer. Samples were analyzed on a Varian Liberty Series II inductively coupled plasma atomic emission spectrometer (ICP-AES). Ni, Zn, and Fe standards were prepared as 0, 0.3, 0.6, 0.9, and 1.2 ppm concentrations in ultrapure water. A buffer-only control was also measured to control for background contamination of nickel and zinc in buffer components. Alternatively, the 4-(2-pyridylazo)resorcinol (PAR) assay was used to measure Ni content in apo- and holo-YqjI. The method was modified from a previously published protocol (24). YqjI was purified in 50 mM HEPES, pH 7.5, and 5% glycine-1 HCL and 5% guanidine hydrochloride. A total of 60 μM of PAR solution (1 mg/ml PAR dissolved in buffer) was added to 300 μl of standards or protein samples. Ni concentrations were measured after 20 min at 505 nm using a calibration calculated from Ni standards of 0, 3, 6, 9, 12, and 15 μM.
RESULTS

A key step in iron homeostasis is the mobilization of ferric iron from both extracellular and intracellular sources. Ferric iron reduction may help release iron from siderophores (47) and is required for mobilization of ferric iron from intracellular iron storage proteins like ferritin (26, 49). While there are a number of potential mechanisms to drive intracellular ferric iron reduction, only a few proteins in E. coli have been directly or indirectly linked to this process (36, 39).

YqjH is a homologue of ViuB that is required for siderophore utilization in Vibrio cholerae (7, 37). A recently published crystal structure of YqjH shows that it is structurally related to the NAD(P)H:flavodoxin reductase superfamily (4). Purified YqjH binds flavin adenine dinucleotide (FAD) and may utilize this cofactor to transfer electrons from NADH or NADPH to a ferric chelate to reduce iron to the ferrous form. However, the in vitro substrate for YqjH ferric reductase activity was freshly prepared in an anaerobic buffer under anaerobic conditions from ferrous ammonium sulfate immediately prior to use.

To further characterize the biochemical function of YqjH, we overexpressed and purified the recombinant protein. As previously reported, YqjH was purified in a bright yellow fraction, with UV-visible absorption maxima at 384 and 451 nm and shoulders at 428 and 475 nm, consistent with the presence of oxidized FAD (see Fig. S1A in the supplemental material) (4). We determined the flavin extinction coefficient at 451 nm by using published protocols and found it to be 10,482 M⁻¹ cm⁻¹ (31). Using this extinction coefficient, we conclude that purified YqjH contains 0.93 FAD per protein monomer.

Fluorescence measurement of YqjH after separation by SDS-PAGE and soaking in 7% acetic acid showed the presence of a fluorescent band at the same molecular weight as that of YqjH, demonstrating that FAD is covalently linked to and comigrates with YqjH (see Fig. S1B in the supplemental material). Pretreatment of the gel with 5% performic acid increased the YqjH-FAD fluorescence, indicating that the flavin is bound to YqjH via an S-cysteinyl flavin linkage consisting of a thioether bond between a cysteine side chain and the FAD (48). The UV-visible absorption spectrum of purified YqjH, with two absorption maxima at 384 and 451 nm (see Fig. S1A), indicates an 8α,6-3-cysteinyl flavin linkage rather than a 6-3-cysteinyl linkage, which typically shows only a single absorption peak around 437 nm (48). Based on our analysis, it appears that YqjH uses FADH₂/FAD as a covalently bound cofactor rather than a substrate. Thus, YqjH is distinct from other flavin reductases (such as Fru in E. coli) that release free reduced flavins as products that are then separately used to reduce ferric iron in vivo (47).

Next, we determined if purified YqjH can reduce ferric iron in vitro. Purified YqjH reduced ferric iron from the ferric-EDTA complex, with a specific activity of 22.0 nmol Fe²⁺/min/mg and a Kₘ of 33 μM. YqjH showed no ferric reductase activity toward ferric chloride. YqjH activity was dependent on the presence of NADPH, with a Kₘ for NADPH of 43 μM. No YqjH ferric reductase activity was detected using NADH as an electron donor, indicating that the enzyme is specific for NADPH. YqjH showed weak activity (1.0 nmol Fe²⁺/min/mg) toward purified ferric enterobactin. This in vitro analysis indicates that purified YqjH can function as a NADPH-dependent ferric reductase, although its in vivo substrate does not appear to be enterobactin due to its low activity toward that substrate. However, it is possible that a linear ferric enterobactin complex or iron bound to an enterobactin precursor, such as 2,3-dihydroxybenzoic acid, might be a substrate for YqjH ferric reductase activity. These possible substrates await further testing.

Based on its homology to viuB, we also tested if a yqiH deletion mutant is sensitive to iron starvation. A sufABCDSE deletion strain was monitored under the same conditions. The Suf pathway is a stress-responsive Fe-S cluster assembly system, and deletion of the suf operon renders E. coli sensitive to conditions that disrupt iron homeostasis, such as iron starvation and oxidative stress (41). However, on LB plates, a yqiH deletion strain was as resistant to the ferrous iron chelator 2,2'-dipyridyl as the wild-type strain (Fig. 1).

Next, we tested if a yqiH deletion strain demonstrates synthetic phenotypes when combined with mutations in siderophore utilization systems in E. coli. Release of ferric iron from the native E. coli siderophore enterobactin precedes through hydrolysis of the siderophore backbone by the Fes esterase, followed by an ill-defined iron reduction step and release of the iron (30). The FhuF ferric reductase is required for utilization of the nonnative iron source ferrioxamine B (36). We constructed the Δfes and ΔfhuF mutations alone and in combination with ΔyqiH and examined the resulting phenotypes.

Deletion of fes leads to a slow-growth phenotype even on rich media, such as LB (Fig. 1), and produces small pink colonies due to the accumulation of ferric enterobactin. This slow-growth phenotype increases in the ΔyqiH Δfes deletion strain, suggesting that YqjH and Fes work in parallel but separate pathways. Furthermore, both Δfes and ΔyqiH Δfes strains were extremely sensitive to 2,2'-dipyridyl (Fig. 1). In contrast, neither the ΔfhuF deletion strain nor the ΔyqiH ΔfhuF deletion strain showed any growth phenotypes on rich medium and were as resistant to 2,2'-dipyridyl as the wild-type control strain. We also attempted to repeat these experiments in chemically defined minimal medium. Unfortunately, the Δfes
single mutant strain grew poorly or not at all in M9 medium, making it difficult to compare relative growth phenotypes among the single and double deletion mutants of fes and yqjH (data not shown). The synthetic phenotypes observed in the ΔyqjH Δfes strain support a role for YqjH in adaptation to iron starvation but argue against a direct role for YqjH in ferric enterobactin utilization.

**YqjI and Fur regulate yqjH.** A putative binding site for the iron metalloregulatory protein Fur overlaps the predicted −10 RNA polymerase binding site upstream of yqjH (Fig. 2A) (11, 37). Two repetitive extragenic palindromic (REP) elements are also present in the yqjH-yqjI intergenic region (2). The functional roles of the REP elements are unclear, although they may be able to form DNA stem-loops or serve as novel protein binding sites. It was previously shown by DNA macroarray studies that yqjH is repressed by the iron-dependent regulator Fur and that Fur repression is lost under iron starvation conditions (37). In good agreement with previous studies, we observed that a yqjH-lacZ promoter fusion construct shows 2- to 3-fold induction in LB medium in the Δfur strain or upon addition of the ferrous iron chelator dipyridyl in the wild-type strain (data not shown).

The previously uncharacterized gene yqjI is divergently transcribed from yqjH. The N-terminal region 1 to 54 of YqjI includes a number of potential metal-binding amino acids (12 histidines, 7 cysteines, and 7 glutamates) and is similar to the SlyD C-terminal metal-binding tail (E value of less than 0.01). The SlyD peptidyl-prolyl cis/trans isomerase is required for [NiFe] hydrogenase maturation during anaerobic growth in *E. coli* (53). *E. coli* SlyD has a C-terminal metal-binding tail containing 15 histidines, 6 cysteines, and 7 aspartates or glutamates. The YqjI N terminus is also similar to residues 121 to 147 of the RenA Ni²⁺/Co²⁺ efflux transporter that are located on a loop between predicted transmembrane domains 3 and 4 and are part of the histidine-rich signature motif that characterizes major facilitator superfamily (MFS) transporters that eﬄux nickel (Fig. 2B) (46). At present, the exact functional role of this region in RcnA-mediated nickel efflux is unclear.

Residues 57 to 207 of YqjI are similar to those of the PadR family of winged helix-turn-helix (WHH) transcription factors (InterPro accession no. IPR005149) (34). PadR is a transcriptional repressor that controls genes involved in the phenolic acid stress response in some microorganisms (19). A model three-dimensional structure of YqjI was calculated using the crystal structure of AphA, a PadR homologue from *V. cholerae*, as a template (16, 44). The comparison to AphA suggests that residues 62 to 152 of YqjI constitute a winged helix fold DNA-binding domain, while residues 153 to 206 form an oligomerization and/or ligand-binding domain (Fig. 2B) (16).

Based on the close proximity of the potential YqjI regulator, we measured the transcriptional activity of the yqjH-lacZ promoter fusion in wild-type and ΔyqjI strains. Deletion of yqjI resulted in a 30-fold increase in the basal expression of yqjH over wild-type levels in LB (Fig. 2C). Introduction of a plasmid expressing a low basal level of yqjI (p′yqjI) (Fig. 2C) in the ΔyqjI strain reduced transcriptional expression of yqjH to much closer to wild-type levels, confirming that the YqjI protein is required to fully repress yqjH expression. Together, these results confirm that both Fur and YqjI repress transcription of yqjH.

Since many regulators can autoregulate their own expression, we also measured the activity of a yqjI-lacZ promoter fusion in the ΔyqjI strain. We found that basal expression of yqjI-lacZ in LB is increased by 130-fold in the ΔyqjI strain (Fig. 2D). YqjI provided in trans on a plasmid was able to reduce transcription from 130-fold to 12-fold over wild-type levels in the ΔyqjI strain (Fig. 2D). The residual induction of yqjI in the complementation experiment may be due to low expression of YqjI from the plasmid construct (which is noninducible in this strain). This result indicates that YqjI also represses its own transcription.

**Metal-dependent regulation of the yqjH promoter.** Based on the observed regulation by Fur, the presence of a potential nickel-binding region within the YqjI transcriptional regulator, and the observed regulation of yqjH and yqjI by YqjI, we tested if expression of yqjH is altered by transition metal ions in vivo. We conducted these experiments in chemically defined M9 minimal medium, with glucose as the carbon source. For cells grown in M9/glucose minimal medium, the basal expression of yqjH-lacZ is much higher (about 5 M) than in minimal medium (below 300 nM) (40), which leads to lower yqjH expression levels in cells grown in LB due to increased repression by Fe²⁺-Fur.

Addition of Fe²⁺ decreased yqjH expression starting at 100 nM, with a maximum repression of 4-fold occurring at 10 μM Fe²⁺ (Fig. 3A). Addition of CoCl₂ concentrations of more than 500 nM also repressed yqjH expression by about 50% (Fig. 3A). The iron- and cobalt-dependent repression of yqjH in M9/glucose medium was abolished in the Δfur strain (Fig. 3B), indicating that the Fur metalloregulatory protein mediates iron and cobalt regulation of yqjH. Although Fur primarily regulates iron homeostasis, Fur can respond to other divalent metals, including Co²⁺, in vivo and in vitro (3, 15).

Upon addition of NiCl₂, yqjH-lacZ expression increased...
starting at 100 nM NiCl$_2$, with maximum induction by 1 μM NiCl$_2$ (Fig. 3A). NiCl$_2$ induced $yqjH$ expression by 42% over basal levels. In contrast, CuCl$_2$ did not induce $yqjH$ expression even at levels of up to 100 μM (Fig. 3A). The nickel-dependent induction of $yqjH$ still occurred in the $\Delta$fur strain, demonstrating that Fur does not provide nickel-dependent regulation of $yqjH$ (Fig. 3B).

Deletion of $yqjI$ led to constitutive expression levels of $yqjH$ that were 30-fold higher than wild-type basal expression levels in M9/glucose medium (Fig. 4). Furthermore, deletion of $yqjI$ abolished nickel-responsive regulation of $yqjH$ (Fig. 4). In contrast, the iron-dependent repression of $yqjH$ still occurred in the $\Delta yqjI$ strain over the same concentration range of added iron (Fig. 4). Together, our results indicate that YqjI is re-

FIG. 2. Regulation of $yqjH$ by the YqjI transcription factor. (A) Diagram of the $yqjI$-$yqjH$ intergenic region showing the putative Fur binding site and REP elements (not to scale). (B) Diagram of the YqjI protein domain organization with predicted secondary structural elements shown for each domain (43). Below the diagram is an amino acid alignment of the Cys/His-rich YqjI N terminus with SlyD and RcnA Cys/His-rich regions. The shading indicates the degree of conservation, with darker colors indicating more conserved residues. (C) Activity of the $yqjH$-lacZ promoter fusion in various strains of E. coli grown in LB medium. “pYqjI” is the pET21a-yqjI plasmid expressing a low level of the YqjI protein.
quired for nickel-dependent regulation of yqjH, while Fur is required for iron- and cobalt-dependent regulation of yqjH. Since YqjI was seen to repress its own expression (Fig. 2D), we also tested to see if nickel addition alters expression of yqjI in vivo. Addition of nickel led to a maximum 6-fold increase in yqjI expression (see Fig. S2 in the supplemental material). This result further indicates that YqjI repression of target promoters is somehow responsive to elevated nickel in vivo.

Deletion of rcnA, which encodes the RcnA nickel efflux transporter, leads to increased nickel sensitivity in E. coli (46). To determine if disruption of nickel efflux alters yqjH nickel regulation, we monitored yqjH expression upon nickel addition in both wild-type and ΔrcnA strains (see Fig. S3 in the supplemental material). Basal expression of yqjH in the ΔrcnA strain in minimal medium did not change. However, yqjH expression was more highly induced at lower nickel concentrations in the ΔrcnA strain than that observed in the wild-type strain (see Fig. S3). This result confirms that yqjH expression is responsive to physiologically relevant changes in E. coli nickel homeostasis. Based on the altered regulation in the ΔrcnA strain, it seems likely that YqjI, which provides nickel-responsive regulation of yqjH, responds to the same intracellular pool of nickel transported by RcnA (25).

YqjI binds to target sequences in the yqjH-yqjI intergenic region. To characterize the DNA-binding activity of YqjI, we overexpressed and purified wild-type YqjI in E. coli. Recombinant YqjI protein, expressed in E. coli, eluted on a heparin column in two peaks (Fig. 5). The first peak to elute on the heparin column was separately retained and analyzed by gel filtration chromatography. Approximately 90% of YqjI from the first heparin peak eluted at an apparent molecular weight of 28,000. While this apparent molecular weight would be most consistent with a hexamer of YqjI, most PadR family members form dimer or tetramer species. The gel filtration data do not allow us to determine the exact stoichiometry of

![FIG. 3. Metal-responsive regulation of yqjH. Activity of the yqjH-lacZ promoter fusion was measured in triplicate in various strains of E. coli grown in M9/glucose medium at 37°C with increasing amounts of various divalent metals. (A) Ni^{2+}, Fe^{2+}, Cu^{2+}, or Co^{2+} was added to the wild-type strain. (B) Fe^{2+}, Co^{2+}, or Ni^{2+} was added to the Δfur strain. Lines are for emphasis only and are not fits.](http://jb.asm.org/)

![FIG. 4. Role of YqjI in yqjH nickel regulation. Activity of the yqjH-lacZ promoter fusion was measured in triplicate in the ΔyqjI strain grown in M9/glucose medium with increasing amounts of Ni^{2+} or Fe^{2+}. Lines are for emphasis only and are not fits.](http://jb.asm.org/)

![FIG. 5. Purification of YqjI. Overexpressed YqjI eluted as two peaks during heparin column chromatography (gray trace). The addition of 500 μM NiCl₂ to LB during cell growth and protein expression did not alter the subsequent YqjI elution profile (black trace). The first YqjI elution peak contained monomeric YqjI, while the second elution peak contained an oligomeric form of YqjI that is likely tetrameric or a higher quaternary structure (as subsequently determined by gel filtration chromatography). AU_{280}, absorbance at 280 nm.](http://jb.asm.org/)
the YqjI oligomer. Based on YqjI similarity to other PadR family members, we used the theoretical molecular weight of the YqjI tetramer for all molar calculations but refer to this complex as an YqjI oligomer.

Close examination of the yqjH-yqjI intergenic region revealed two highly similar inverted repeats located near the predicted −10 RNA polymerase binding sites for both the yqjH and yqjI promoters (referred to as PyqjH and PyqjI) (Fig. 6). Since both the yqjH and yqjI promoters are repressed by YqjI, we tested if these inverted repeats act as binding sites for YqjI. Both monomer and oligomer forms of apo-YqjI were incubated with fluorescent oligonucleotides matching the inverted repeat sequences, and the change in fluorescence anisotropy (FA) was measured (Fig. 6). The oligomer form of apo-YqjI bound the PyqjH and PyqjI oligonucleotides, with $K_d$ (dissociation constant) values of 68.9 nM for PyqjH (Fig. 6A) and 47.8 nM for PyqjI (Fig. 6B). In contrast, the monomer form of apo-YqjI showed only weak binding to the oligonucleotides (data not shown). As controls for nonspecific DNA binding, we also incubated oligomer apo-YqjI with a random oligonucleotide with the same length as PyqjH or with an oligonucleotide matching the Fur binding site upstream of yqjH. Apo-YqjI showed only weak interaction with these control oligonucleotides (Fig. 6C). Since the apo-YqjI oligomer binds PyqjH and PyqjI, these inverted repeat sequences are strong candidates for the apo-YqjI binding sites within the yqjH and yqjI promoters.

**Divalent metals can regulate YqjI DNA-binding activity in vitro.** The potential nickel-binding N terminus of YqjI and the *in vivo* nickel-responsive regulation of yqjH suggest that YqjI activity may be directly or indirectly regulated by divalent metals. The metal content of as-purified YqjI was measured to determine if YqjI is a metalloprotein. The Ni$^{2+}$/YqjI ratio was 0.06 for the monomer form and 0.15 for the oligomer form if YqjI was expressed in cells grown in standard LB, which contains approximately 120 nM Ni$^{2+}$ (40). If cells were grown in LB supplemented with 500 μM nickel chloride, the Ni$^{2+}$/YqjI ratio increased to 1.1 for the monomer form and 1.6 for the oligomer form of YqjI. All forms of YqjI (monomer and oligomer) also contained zinc at a Zn$^{2+}$/YqjI ratio of 0.5 ± 0.1, regardless of the nickel content of the medium. Attempts to remove all bound metals by overnight incubation of YqjI with EDTA resulted in protein precipitation. Addition of nickel to the LB medium during cell growth did not alter the separation of YqjI into monomer and oligomer peaks during purification on the heparin column (Fig. 5), and stoichiometric nickel addition to the apo-YqjI oligomer did not alter its oligomeric state, as monitored by gel filtration chromatography (data not shown).

To better define the maximum nickel stoichiometry of YqjI, 20 μM of the apo-YqjI oligomer (containing 0.15 Ni$^{2+}$ and 0.5 Zn$^{2+}$ per monomer) was titrated with increasing nickel concentrations. Upon nickel addition, the UV-visible absorption spectrum of YqjI showed peaks at 280 nm and 310 nm, consistent with $S \rightarrow Ni(II)$ or imidazole $\rightarrow Ni(II)$ charge transfer transitions from Cys-Ni or His-Ni coordination (Fig. 7A). The absorption intensity at 310 nm began to saturate as 2 to 3 molar equivalents of nickel were added (Fig. 7A). Similar spectra were observed for nickel titration of the apo-YqjI monomer (data not shown). After completion of the nickel titration, excess nickel was removed from YqjI by thorough buffer exchange. The buffer-exchanged, Ni-reconstituted YqjI retained 2.1 ± 0.02 Ni$^{2+}$ per YqjI monomer. Based on the UV-visible absorption spectra and the nickel content of buffer-exchanged YqjI, the YqjI protein can accommodate approximately 2 Ni$^{2+}$ per monomer *in vitro*. In comparison, the nickel stoichiometry for full-length SlyD (as measured by equilibrium dialysis) is 4.2 per monomer (27).

The UV-visible absorption spectra of Ni-YqjI in the 270- to 400-nm region could imply tetrahedral Ni(II) coordination (10, 24). Although the absence of significant absorbance in the far visible region (beyond 600 nm) would seem to argue against tetrahedral Ni coordination, this characteristic absorbance can be masked in some tetrahedral Ni complexes (51). In addition, the presence of a shoulder at around 420 nm is attributed to $d-d$ transitions that are similar to published square planar Ni(II) complexes (10). The complexity of the UV-visible absorption spectra for Ni-YqjI suggests multiple nickel-binding sites with different ligands and/or geometries. Recent X-ray absorption spectroscopy (XAS) and extended X-ray absorption fine-structure (EXAFS) spectroscopy of Ni-SlyD also show a mixture of nickel sites with different geometries and coordination numbers, indicating that the Ni-binding motifs of YqjI and SlyD are flexible in their ability to accommodate nickel (27).

We also tested if excess zinc is capable of displacing nickel bound to YqjI. Ni-reconstituted YqjI containing 2 Ni$^{2+}$ per monomer was titrated with increasing concentrations of zinc. We observed no change in the UV-visible spectrum of Ni-reconstituted YqjI, as zinc was added up to a 4-fold molar excess over YqjI protein (equivalent to a 2-fold molar excess over bound nickel), indicating excess zinc was unable to displace the bound nickel (data not shown). We were unable to continue the zinc titration above a 4-fold molar excess over the YqjI protein, as we observed protein precipitation at these levels of zinc.

Based on the *in vitro* nickel binding by apo-YqjI, we determined if the presence of nickel alters oligomer YqjI binding to PyqjH or PyqjI binding sites. As increasing levels of NiCl$_2$ were titrated into samples containing apo-YqjI and the PyqjI oligonucleotide, the FA decreased, indicating that nickel causes the dissociation of YqjI from PyqjH (Fig. 7B). A 50% decrease in FA occurs at a Ni$^{2+}$/YqjI ratio of 2:1, consistent with the nickel stoichiometry of 2:1 obtained after *in vitro* nickel reconstitution of apo-YqjI. We also observed that addition of ZnCl$_2$, had a weak effect on the FA, reducing it by approximately 20% (Fig. 7B). However, Fe$^{2+}$ addition (as anaerobically prepared ferrous ammonium sulfate) decreased the FA by nearly 80%, indicating it was more effective than nickel at reducing the YqjI affinity for DNA (Fig. 7B). These results indicate that divalent metals such as Ni$^{2+}$ and Fe$^{2+}$ have a similar negative effect on YqjI DNA-binding activity *in vitro*, although the observed nickel regulation parallels the *in vivo* nickel-specific YqjI-dependent regulation of the yqjH promoter.

**YqjI autoregulation may lead to dampening of yqjH nickel induction.** Although nickel can regulate YqjI DNA-binding activity *in vitro*, the nickel induction of the yqjH and yqjI promoters is rather mild *in vivo*, especially compared to the high constitutive expression observed in the ΔyqjI strain (Fig. 2D). However, the autoregulation of yqjI indicates that increased
FIG. 6. (Top) Alignment of inverted repeats found in the yqjH and yqjI promoters (indicated by arrows). The putative Fur box in the yqjH promoter is shown with asterisks. The previously mapped transcriptional start site of yqjI is indicated with a box (38). (A, B) Oligomer apo-YqjI binding to 10 nM PyqjH (A) or PyqjI (B) oligonucleotides, as measured by DNA fluorescence anisotropy. Boldface lines indicate fits to binding data calculated as described in Materials and Methods. (C) Binding of oligomer apo-YqjI to 10 nM PyqjH oligonucleotide (gray circle), random-sequence oligonucleotide (open triangle), or Pfur oligonucleotide (open square). Lines are for emphasis only and are not fits.
nickel levels block YqjI repression of yqjI transcription, thereby causing levels of the YqjI apoprotein to increase. Increased apo-YqjI then counteracts the loss of YqjI repression at the yqjH promoter (Fig. 8B). To test if this regulatory loop is occurring in vivo, one must uncouple expression of YqjI from its native promoter so that the addition of nickel will no longer lead to increased YqjI expression. Nickel induction of yqjH can then be measured without the added complication of YqjI autoregulation.

To accomplish this, we utilized the ΔyqjI strain carrying a plasmid that expresses a low basal level of yqjI (pYqjI) (Fig. 2). In this strain, yqjI expression is uncoupled from its native promoter and is driven only by leaky transcription from the plasmid-borne T7 promoter upstream of yqjI, which is adequate to repress yqjH and yqjI transcription nearly to the levels observed in the wild-type strain (Fig. 2). To match earlier complementation studies, these experiments were carried out in LB medium, which has an excess capacity to chelate divalent metals compared to that of minimal medium. Larger amounts of nickel were required to observe nickel induction of yqjH (see Fig. S4 in the supplemental material). Upon addition of 1 mM nickel to the wild-type strain (without the pYqjI plasmid), yqjH expression increased approximately 3-fold (Fig. 8A). In the ΔyqjI strain carrying the pYqjI plasmid, addition of 1 mM nickel increased yqjH expression to the same high level that was observed in the complete absence of yqjI (compare Fig. 8A to 2C). This result indicates that nickel addition is able to completely block YqjI repression of yqjH so long as YqjI protein levels are not allowed to increase due to the YqjI autoregulatory circuit (Fig. 8B) or differential regulation of YqjI by other factors.

YqjH helps maintain iron homeostasis under excess nickel conditions. To determine if yqjH is required for resistance to nickel toxicity, we analyzed the growth phenotype of a ΔyqjI strain of E. coli with or without pYqjI grown in LB medium at 37°C with or without 1 mM nickel. "pYqjI" is the pET21a-yqjI plasmid expressing a low level of the YqjI protein. (B) Proposed negative feedback regulation of yqjH under nickel stress.
strain, we encountered a highly variable phenotype. In approximately half of the independent growth assays, the ΔyqjH strain was as sensitive to elevated nickel as the ΔrcnA strain (Fig. 9A). However, in the other half of the assays, the ΔyqjH strain was more nickel resistant than the wild-type strain. We refer to this apparent suppressor phenotype as ΔyqjH\(^*\) (Fig. 9A). When ΔyqjH\(^*\) cells were isolated from the high-nickel medium and retested for nickel sensitivity, they uniformly grew the same or better than the wild-type control strain in response to elevated nickel, suggesting the selection of a stable suppressor mutation(s) (data not shown). We confirmed that both ΔyqjH and ΔyqjH\(^*\) still contained the actual yqjH deletion using colony PCR with primers flanking the site of the deletion (data not shown). The variable nickel sensitivity phenotype was always observed, even when we independently reconstructed and retested the ΔyqjH strain. The variable nickel sensitivity phenotype indicates a high level of suppressor mutations in the ΔyqjH strain in response to nickel toxicity in M9 gluconate minimal medium. At present, the exact nature of the suppressor mutation(s) is unclear and is the subject of ongoing studies.

We also examined the phenotypes of the various gene deletion strains under high-nickel conditions on LB medium. Although the nickel sensitivity phenotypes of all strains grown on LB were reduced compared to those of strains grown on gluconate minimal medium, we observed that the sufABCDEF deletion strain was sensitive to high-nickel concentrations, indicating the disruption of Fe-S cluster biosynthesis or increased turnover of Fe-S enzymes (Fig. 9B). A Δfes strain was also sensitive to high-nickel conditions (Fig. 9B). The yqjH deletion strain showed a degree of nickel sensitivity similar to that of ΔsufABCDEF. However, the ΔyqjH Δfes double mutant strain showed a higher sensitivity to high-nickel stress than either the ΔyqjH or fes single deletion strains (Fig. 9B). The severe phenotype of the ΔyqjH Δfes double mutant strain is consistent with parallel but separate physiological roles for YqjH and Fes during nickel stress. The yqjI gene deletion strain grew as well as the wild-type control strain.

**DISCUSSION**

**YqjI is the main regulator of yqjH and yqjI transcription.**

This study shows that YqjI is the primary regulator of yqjH transcription, since deletion of yqjI leads to a 40-fold increase in yqjH basal expression while deletion of fur derepresses yqjH by only 3-fold. A close regulatory connection between yqjH and yqjI is also supported by phylogenetic analysis. The yqjI and yqjH genes are found in close proximity in the *Gammaproteobacteria* and *Betaproteobacteria* subclasses of the *Proteobacteria* (see Fig. S5 in the supplemental material). In most *Enterobacteriaceae*, the genes are oriented divergently to each other, just as in *E. coli*. However, in *Pseudomonas* and *Xanthomonas Gammaproteobacteria* as well as in *Ralstonia* and *Burkholderia* spp., yqjI and yqjH are transcribed in the same direction rather than divergently (see Fig. S5). The N-terminal, SlyD-like region of *E. coli* YqjI is conserved mainly in species closely related to *E. coli*. However, *Ralstonia metallidurans* and *Pseudomonas syringae* each contain a YqjI homologue with a N-terminal extension that is histidine rich but lacks the cysteine residues present in *E. coli* YqjI (data not shown).
What are the key regulatory signals for YqjI in vivo? The nickel-dependent regulation of yqjH and yqjI by YqjI is physiologically consistent with the nickel homeostasis systems in E. coli that are regulated by the NikR and RcnR transcription factors. The nikABCDE nickel import system is repressed by Ni/NiK as nickel concentrations rise above 100 nM in M63/glucose minimal medium (25). Repression of the Ni²⁺/Cd²⁺ efflux transporter RcnA by apo-RcnR is relieved in M63/glucose minimal medium as nickel levels increase above 500 nM (25). Together, these previously published expression profiles indicate that nickel-responsive gene regulation in E. coli occurs over a concentration range of 100 to 500 nM nickel in minimal medium. Nickel-dependent regulation of yqjH by YqjI in minimal medium occurs beginning at nickel concentrations above 100 nM, with maximum induction between 500 nM and 1 μM. Since yqjH induction occurs at the high end of the regulatory concentration range, this suggests that YqjI could play a role in cellular adaptation to nickel toxicity.

The in vivo YqjI-dependent nickel-response regulation of yqjH and the in vitro nickel binding by YqjI suggest a model in which YqjI may regulate yqjH directly in response to nickel levels in order to integrate iron and nickel homeostasis. Such cross-regulation has been observed in species like Helicobacter pylori, which utilizes the NikR regulator to coordinate expression of both iron and nickel metal homeostasis systems (13). This cross-regulation may be important in H. pylori due to the high levels of nickel required to activate the highly abundant urease enzyme needed for cell survival at low pH (5). Previous studies in E. coli also have shown that rcnA may be regulated by Fur in response to iron and that the RcnR protein (that represses rcnA expression) is itself induced by iron via a Fur-independent pathway (29). Presumably, this cross-regulation of rcnA and rcnR by iron provides a careful balance between iron and nickel uptake and efflux to maintain enzyme maturation without poisoning metal homeostasis pathways.

However, full nickel induction of yqjH to the same expression levels observed in the ΔyqjI strain could be achieved only if YqjI expression was uncoupled from autoregulation (Fig. 8A). This autoregulatory circuit could indicate that nickel is not the only regulatory signal that controls YqjI DNA-binding activity in vivo. Multiple signal inputs may be required to fully regulate YqjI DNA-binding activity by directly binding the nickel or other ligands act as a molecular switch to negatively regulate YqjI DNA-binding activity by directly binding nickel homeostasis activity or interactions with substrate proteins (22, 52, 53).

One regulatory model, suggested by the in vivo regulation, in vitro phenotype, and in vitro metal-binding studies, shows that nickel or other ligands act as a molecular switch to negatively regulate YqjI DNA-binding activity by directly binding the N-terminal SlyD-like domain. However, at present we cannot conclude that nickel directly binds YqjI to regulate DNA-binding activity in vivo. It is possible that disruption of iron homeostasis by elevated nickel indirectly affects YqjI activity through an unknown mechanism.

Nickel disrupts iron homeostasis in E. coli. Strains lacking yqjH are sensitive to nickel toxicity (Fig. 9). In addition, we observed that a ΔufsABCDSE strain, lacking a stress-response Fe-S biosynthesis pathway, is also sensitive to elevated nickel levels (Fig. 9). Together, these results indicate that iron homeostasis can be disrupted by elevated nickel levels, providing a physiological rationale for cross-regulation of iron and nickel homeostasis in E. coli. It is not surprising that nickel can disrupt iron homeostasis since the Irving-Williams series (Mn < Fe < Co < Ni < Cu > Zn) predicts that nickel, cobalt, and copper should bind more strongly to potential cellular ligands than ferrous iron. Indeed, cobalt and copper have already been shown to disrupt iron metabolism in bacteria (23, 32, 45, 50).

Previous studies in higher eukaryotes have clearly shown that nickel competes directly with iron for cell entry through the DMT1 divalent metal transporter, leading to decreased cellular iron accumulation (8). More recent work indicates that nickel can compete directly with iron for incorporation into 2-oxoglutarate-dependent histone demethylases, suggesting that misincorporation of nickel into iron metalloproteins is another facet of nickel toxicity (9). The exact mechanism of nickel toxicity in E. coli remains to be elucidated and may involve disrupted iron transport and/or direct inhibition of iron metalloproteins by nickel.

Regardless of the mechanism of nickel toxicity, the YqjI ferric reductase appears to play a role in maintaining iron homeostasis under nickel stress. Combination of the yqjH deletion with a fes deletion leads to a synthetic phenotype in LB and under high nickel stress. The weak activity of YqjI toward purified enterobactin and the synthetic phenotype with Δfes argues against a direct role for YqjI in the enterobactin utilization pathway. YqjI may be used to reduce ferric iron from another iron siderophore or to release iron from ferric iron storage proteins, such as ferritin, under low-iron conditions. Further studies are needed to clarify the in vivo substrate for the YqjI ferric reductase.

These studies establish that the PadR family member YqjI represses the transcription of yqjH and yqjI in vivo. YqjI repression of these target promoters is weakly responsive to elevated nickel in vivo, although YqjI DNA-binding activity is clearly altered by transition metals in vitro. Future experiments...
REFERENCES


