Roles of the EnvZ/OmpR Two-Component System and Porins in Iron Acquisition in *Escherichia coli*

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ABSTRACT

Escherichia coli secretes high-affinity Fe$^{3+}$ chelators to solubilize and transport chelated Fe$^{3+}$ via specific outer membrane receptors. In microaerobic and anaerobic growth environments, where the reduced Fe$^{2+}$ form is predominant, ferrous transport systems fulfill the bacterial need for iron. Expression of genes coding for iron metabolism is controlled by Fur, which when bound to Fe$^{2+}$ acts as a repressor. Work carried out in this paper shows that the constitutively activated EnvZ/OmpR two-component system, which normally controls expression of the ompC and ompF porin genes, dramatically increases the intracellular pool of accessible iron, as determined by whole-cell electron paramagnetic resonance (EPR) spectroscopy, by inducing the OmpC/FeoB-mediated ferrous transport pathway. Elevated levels of intracellular iron in turn activated Fur, which inhibited the ferric but not the ferrous transport pathway. The data show that the positive effect of constitutively activated EnvZ/OmpR on feoB expression is sufficient to overcome the negative effect of activated Fur on feoB. In a tonB mutant, which lacks functional ferric transport systems, deletion ofompR severely impairs growth on rich medium not supplemented with iron, while the simultaneous deletion ofompC andompF is not viable. These data, together with the observation of de-repression of the Fur regulon in an OmpC mutant, show that the porins play an important role in iron homeostasis. The work presented here also resolves a long-standing paradoxical observation of the effect of certain mutant envZ alleles on iron regulon.

IMPORTANCE

The work presented here solved a long-standing paradox of the negative effects of certain missense alleles of envZ, which codes for kinase of the EnvZ/OmpR two component system, on the expression of ferric uptake genes. The data revealed that the constitutive envZ alleles activate the Feo- and OmpC-mediated ferrous uptake pathway to flood the cytoplasm with accessible ferrous iron. This activates the ferric uptake regulator, Fur, which inhibits ferric uptake system but cannot inhibit the feo operon due to the positive effect of activated EnvZ/OmpR. The data also revealed importance of porins in iron homeostasis.
INTRODUCTION

Iron, used as a redox center by many enzymes, is an essential trace metal required by almost all living organisms. The intracellular level of free catalytically active iron is typically kept low due to its toxic effects. Free ferrous iron reacts with hydrogen peroxide, a natural byproduct of aerobic respiration, to generate highly toxic hydroxyl radicals (OH•) via the Fenton reaction (1).

Due to this potentially damaging property of iron, there exists an intricate balance between iron transport, utilization and storage. Most bacteria possess mechanisms to import iron in its oxidized ferric state (Fe³⁺), reduced ferrous state (Fe²⁺) or both (for reviews, see 2, 3). The solubility of these two iron forms differs drastically at neutral pH: ferric iron has extremely low solubility at 10⁻¹⁸ M, whereas ferrous iron is readily soluble at 10⁻¹ M. To take up ferric iron, bacteria have developed high-affinity ferric iron chelators called siderophores to capture, solubilize, and deliver insoluble iron into the cell (4). Unlike the Fe³⁺ transport system, which requires a number of proteins involved in siderophore synthesis and Fe³⁺-siderophore acquisition, the Fe²⁺ transport system appears to consist of mainly one protein, FeoB (5). The FeoB protein is synthesized from the feoABC operon, whose expression is activated by Fnr, an anaerobic transcriptional regulator (5). FeoB is a highly conserved, 773-residue inner membrane protein that contains several GTP-binding motifs (6, 7, 8). In the absence of FeoB or FeoA, Fe²⁺ uptake is either virtually abolished (ΔfeoB) or mildly reduced (ΔfeoA) (5). The function of FeoC, which is present only in members of the Enterobacteriaceae family, is unknown (7, 8). FeoB and its homologs are required for full virulence in many bacteria, including E. coli (9), Salmonella Typhimurium (10, 11), and Helicobacter pylori (12).

Fur (ferric uptake regulator) in E. coli and its orthologs in many Gram-negative and Gram-positive bacteria are the master regulator of genes encoding both ferric and ferrous iron acquisition functions, as well as siderophore synthesis and uptake (13, 14). Cells lacking Fur experience iron overload that causes oxidative damage and mutagenesis (15). Fur-regulated genes contain one or more Fur-binding sites around the -35 and -10 regions of the promoter, often referred to as the Fur boxes (16, 17). Fur uses Fe²⁺ as a co-factor: when the level of available Fe²⁺ increases in the cell, it binds to Fur and enhances its affinity for DNA by almost 1,000-fold (2). The active Fur-Fe²⁺ complex then binds to a Fur box and represses transcription.
of the iron acquisition gene. RyhB is a small regulatory RNA whose transcription is also repressed by Fur-Fe^{2+} (18). Consequently, when Fur is active, the levels of RyhB are low, resulting in stabilization and translation of over a dozen mRNAs encoding non-essential iron-utilization proteins, including those that store iron (Bfr), detoxify superoxide (SodB), and catalyze steps of the TCA cycle (AcnA and SdhCDAB) (19). Thus, excess Fe^{2+} activates Fur to halt further iron uptake and at the same time, promotes the utilization of Fe^{2+}, and inversely, low intracellular iron level induces iron uptake and utilization (20). Recent genome-wide analyses revealed a more comprehensive profile of Fur and RyhB regulons (21, 22).

Whereas Fur and RyhB are the principal determinants of iron homeostasis in *E. coli*, evidence exists supporting the involvement of some two-component signal transduction systems (TCSs) in iron homeostasis. EnvZ and OmpR are the archetypal TCS in *E. coli*, where EnvZ serves as a sensor kinase and OmpR as a response regulator (23). They respond to medium osmolarity and influence the expression of OmpC and OmpF, the two major porins that facilitate the diffusion of small hydrophilic solutes (~600 Da) across the outer membrane (24). OmpC is preferentially expressed in high osmolarity, whereas OmpF expression is favored in low osmolarity (25). Microarray data from an ΔompR ΔenvZ background showed a significant increase in the expression of a number of Fur-regulated genes, particularly those involved in enterobactin siderophore synthesis and transport (26). Over three decades ago, Lundrigan and Earhart (27) reported that in a perA (envZ) mutant background, the levels of three iron-regulated proteins were significantly reduced. The authors suggested that this could be due to a posttranscriptional defect. Later, it was speculated that this inhibition could be due to the indirect effects of envZ/ompR, leading to alterations in the structure and diffusion properties of the outer membrane (28). While characterizing revertants of an *E. coli* mutant defective in outer membrane biogenesis, we discovered several pleiotropic envZ alleles conferring an OmpC^{+} OmpF^{-} LamB^{-} phenotype (29). These alleles were hypothesized to biochemically lock EnvZ into a conformation that causes increased OmpR phosphorylation. This activated EnvZ/OmpR state is thought to enable OmpR to bind to promoters with weak OmpR-binding affinities. One such pleiotropic envZ allele, *envZ_{R397L}* was characterized in detail (29). The preliminary whole genomic microarray analysis of the *envZ_{R397L}* mutant carried out in our laboratory found that the
largest group of genes (>50) affected by the activated EnvZ<sub>R397L</sub>/OmpR<sup>+</sup> background belonged to the Fur regulon (30; Table S1).

In this study, we show that EnvZ<sub>R397L</sub> exerts its effect on the Fur regulon in part by increasing the accessible intracellular pool of iron via the OmpC-FeoB-mediated Fe<sup>2+</sup> transport pathway. This, in turn, activates Fur and downregulates the Fe<sup>3+</sup> transport pathway. Our analyses also revealed the critical roles of EnvZ/OmpR and porins in iron homeostasis in the ΔtonB background where high-affinity iron transport systems are non-functional.
RESULTS

Effects of envZ<sub>R397L</sub> on the ferric transport system. We first set out to investigate the effects of envZ<sub>R397L</sub> on the Fur regulon. RNA isolated from mid-log phase grown cells was converted to cDNA and levels of various transcripts were analyzed by RT-qPCR. The data in Fig. 1 show relative transcript levels of four Fur-regulated genes: fecA, fepA, fhuA and fhuF. In the envZ<sub>R397L</sub> background, their transcript levels went down 10 (fecA), 3 (fhuA), and 2.5 (fepA and fhuF) folds relative to the wild type (EnvZ<sup>+</sup>) strain. As expected, in a Δfur background their expression was de-repressed, resulting in a dramatic increase in their transcripts (Fig. 1). In that background, the presence of envZ<sub>R397L</sub> was still able to reduce fecA and fepA transcript levels 3.6 and 9.5 folds, respectively, but not that of fhuA and fhuF, which experienced less than 20% reduction (Fig. 1). Using the chromosomally integrated fepA::lacZ and fhuA::lacZ gene fusion constructs, we were able to recapitulate the key RNA data shown in Fig. 1 (Fig. S1). This indicated that EnvZ/RompR or factors under the activated TCS’s control could also down-regulate fecA and fepA transcription in the absence of Fur. In contrast, the negative effect of envZ<sub>R397L</sub> on fhuA and fhuF expression requires Fur. Moreover, the repressive effect of envZ<sub>R397L</sub> on fecA and fepA in the fur<sup>+</sup> background was found to be independent of OmrA and OmrB (Fig. S2), the two EnvZ/RompR-dependent small regulatory RNAs whose overexpression from plasmids was previously shown to down-regulate fecA, fepA and other Fur-regulated genes (31). It is worth mentioning that the envZ<sub>R397L</sub> allele has been previously shown to increase omr::lacZ expression almost tenfold (29).

Expression of OmpC is activated constitutively in the envZ<sub>R397L</sub> background, while that of OmpF and LamB is severely inhibited (29). To ask whether OmpC is somehow involved in the envZ<sub>R397L</sub>-mediated down-regulation of fecA, fepA, fhuA and fhuF, we examined their transcript levels in the ΔompC and ΔompC envZ<sub>R397L</sub> backgrounds. Remarkably, without OmpC, envZ<sub>R397L</sub> was unable to exert any significantly negative effect on fepA, fhuA and fhuF expression, while the effect on fecA diminished from tenfold in the presence of OmpC to less than twofold without OmpC (Fig. 1). Interestingly, the levels of all four transcripts went up in ΔompC cells (Fig. 1). We theorize that without OmpC, diffusion of Fe<sup>2+</sup> into the cell is decreased and the less active Fur fails to fully repress fecA, fepA, fhuA and fhuF expression.
If the intake of Fe$^{2+}$ by OmpC porin increases active Fur-Fe$^{2+}$ levels, then the absence of FeoB, the Fe$^{2+}$-specific iron transporter, should also interfere with this activation and abrogate the Fur-mediated effects of envZ$_{R397L}$ on fecA, fepA, fhuA and fhuF. Indeed, just like in the ΔompC background, fecA, fepA, fhuA and fhuF transcript levels went up in the ΔfeoB background, and envZ$_{R397L}$ could either no longer impose a significant negative effect (fepA, fhuA and fhuF) or the effect was significantly reduced (fecA).

**Effects of envZ$_{R397L}$ on the ferrous transport system.** The data presented in Fig. 1 showed the involvement of the FeoB ferrous iron transporter and OmpC porin in envZ$_{R397L}$-mediated down-regulation of the ferric iron transport system. While ompC expression increases in the envZ$_{R397L}$ background (29), the status of the feo operon in this background is unknown. The feo operon is under the negative control of Fur (5). Consequently, if higher Fur-Fe$^{2+}$ activity is present in the envZ$_{R397L}$ background, as we have suggested above, then the expression of the feo operon, like that of fecA, fepA, fhuA and fhuF, should also be inhibited. This, however, will be incongruent with our data showing envZ$_{R397L}$’s dependence on feoB for its effects. We therefore hypothesized that feo expression, like that of ompC, is activated by envZ$_{R397L}$ to such a degree that it more than compensated for the feo down-regulation by increased Fur-Fe$^{2+}$ activity.

To test these possibilities, we analyzed feoA and feoB transcript levels in different genetic backgrounds by RT-qPCR (Fig. 2). Note that feoABC are part of a contiguous operon and therefore, likely expressed from a polycistronic message. Consequently, feoA and feoB transcript analysis probes their respective coding regions in a polycistronic message. In the EnvZ$_{R397L}$ background, feoA and feoB transcript levels went up dramatically over those in the envZ$^{+}$ control strain. As expected, their levels also went up in the Δfur background. Interestingly, in the envZ$_{R397L}$ Δfur background feoA and feoB transcript levels increased well above those in the individual mutation backgrounds, indicating that envZ$_{R397L}$ and Δfur act independently and synergistically to enhance feo expression. Again these observations were recapitulated using the chromosomally integrated feo::lacZ fusion (Fig. S1). These data support our hypothesis that envZ$_{R397L}$ activates feo expression in a fashion that counteracts repression by higher levels of Fur-Fe$^{2+}$. 
We then examined the effects of \textit{envZ}_{R397L} on \textit{feoA} and \textit{feoB} transcript levels in the absence of OmpC or FeoB. Without OmpC or FeoB, a modest twofold increase in \textit{feoA} and \textit{feoB} (\textit{\DeltaompC} or \textit{\DeltafeoA} (\textit{\DeltafeoB}) transcripts was observed (Fig. 2). We interpret this to reflect a modest relief in the Fur-mediated repression of the \textit{feo} operon, since we have already implicated OmpC and FeoB in the ferrous iron transport and increase in Fur-Fe$^{2+}$ levels (Fig. 1). The presence of \textit{envZ}_{R397L} in the \textit{\DeltaompC} or \textit{\DeltafeoB} background led to an increase in \textit{feoA} and \textit{feoB}, or \textit{feoA} transcripts, respectively, in a synergistic fashion, which is likely due to the simultaneous activation of \textit{feo} expression by \textit{envZ}_{R397L} and a modest decrease in the Fur-mediated repression of \textit{feo} from the absence of OmpC and FeoB. These data showed that \textit{envZ}_{R397L} inhibits ferric transport pathway but activates ferrous transport pathway.

**Intracellular iron levels in the \textit{envZ}_{R497L} mutant.** The OmpC/Feo-mediated increase in Fur-Fe$^{2+}$ activity in the \textit{envZ}_{R397L} background implies that the cytoplasm of the \textit{envZ}_{R397L} mutant contains higher levels of accessible iron than that in the cytoplasm of the EnvZ$^+$ cell. To test this directly, we measured the intracellular pool of accessible iron by whole-cell electron paramagnetic resonance (EPR) spectroscopy, a method established in the Imlay laboratory (32). The data presented in Fig. 3 show that the wild type (EnvZ$^+$) strain had 32 $\mu$M of accessible intracellular iron. Expectedly, this level rose fourfold to 120 $\mu$M in the \textit{\Deltafur} mutant. Remarkably, the level of accessible iron in the \textit{envZ}_{R397L} was also very high (135 $\mu$M) and remained high in the \textit{\Deltafur envZ}_{R397L} double mutant (105 $\mu$M), thus supporting the notion that a higher pool of accessible iron in the \textit{envZ}_{R397L} background is responsible for the higher levels of active Fur-Fe$^{2+}$.

Next, we tested whether the FeoB-mediated ferrous transport pathway is responsible for the elevated level of accessible iron in the \textit{envZ}_{R397L} mutant. The accessible iron level in the \textit{\DeltafeoB} mutant was 20 $\mu$M or 35\% less than the parental \textit{feoB}$^+$ strain (Fig. 3), explaining the observed de-regulation of the Fur regulon in the \textit{\DeltafeoB} mutant (Figs. 1 and 2). Strikingly, without \textit{feoB}, \textit{envZ}_{R397L} failed to increase intracellular iron levels (Fig. 3), thus confirming the involvement of the FeoB-mediated ferrous transport in elevating the intracellular pool of iron, which, in turn, would increase Fur-Fe$^{2+}$ levels and repress expression of \textit{fecA}, \textit{fepA}, \textit{fhuA} and \textit{fhuF}. As described
below, EnvZ/OmpR play a more direct role in activating feo expression to overcome the Fur-mediated downregulation.

**Effects of envZ<sub>R397L</sub> on fepA and feo requires phosphorylated OmpR.** Previously it was shown that the pleiotropic effects of the mutant envZ allele, envZ473 with its V241G substitution, is mediated through OmpR (33). In the paper, the authors did not analyze the iron regulon. In this work, we sought to test whether the effect of envZ<sub>R397L</sub> on iron regulon requires functional OmpR. We used a missense allele of ompR with a D55Y substitution, which confers a null phenotype with respect to ompC and ompF expression presumably due to the inability of the mutant OmpR to be phosphorylated. The conserved D55 residue of OmpR is the site of phosphorylation (34). The ompR<sub>D55Y</sub> allele was isolated in a fepA::lacZ envZ<sub>R397L</sub> background among Lac<sup>+</sup> revertants (Misra R., unpublished data). Using a linked Cm<sup>r</sup> marker, we transduced the ompR<sub>D55Y</sub> envZ<sub>R397L</sub> mutations into a feo::lacZ background so that the effects of the mutant ompR and envZ alleles on feo expression can be determined. It is worth noting that although ompR/envZ are highly linked to the feo operon, we were able to construct the above strain since feo::lacZ is marked by the Km<sup>r</sup> gene and the mutant ompR/envZ alleles produce a distinct porin phenotype.

Data presented in Fig. 4 show that envZ<sub>R397L</sub> reduced fepA::lacZ expression about fourfold, whereas ompR<sub>D55Y</sub> abolished this effect of envZ<sub>R397L</sub>. Likewise, the presence of envZ<sub>R397L</sub> elevated feo::lacZ expression fivefold and again ompR<sub>D55Y</sub> abolished this increase in feo expression. Curiously, feo::lacZ expression in the ompR<sub>D55Y</sub> envZ<sub>R397L</sub> background was slightly lower than that seen in the wild type background, suggesting a role for functional OmpR in the expression of the feo operon. Together, these data show unambiguously that the negative and positive effects of envZ<sub>R397L</sub> on fepA and feo, respectively, require functional OmpR.

**Direct regulation of feoABC operon by EnvZ/OmpR.** The data in Figs. 2 and 4 showed a dramatic increase in the feo transcript/transcription levels in the envZ<sub>R397L</sub>/omp<sup>R</sup> background. This could be due to the direct regulation of feo by OmpR or an effect of an OmpR-controlled factor on the feo promoter or feo transcript. We took cues from an earlier publication that showed overexpression of RstA, the response regulator of the RstB/RstA TCS, up-regulated feoB expression and repressed the Fur regulon in *Salmonella* Typhimurium (35). Electrophoretic
mobility shift assays (EMSA) showed direct binding of RstA to the *feo* promoter sequence (Jeon et al., 2008). Moreover, the authors identified the “RstA motif” (TACA-N$_6$-TACA) upstream of the *S. Typhimurium* *feoA* gene of the *feo* operon (35). Although OmpR recognition sequences are quite degenerate (36; 37), one of the motifs—GTTACANNNN—resembles that of RstA (Fig. 5A).

Indeed, both RstA and OmpR regulate some of the same genes by binding to overlapping promoter sequences (38). Our initial assessment detected two potential sequences (-294)-TTATCAtttcaTTAACA-(-278) and (-165)-CCAACAttcgCACACA-(-150) upstream of the *feoA* ATG codon that might contain both RstA and OmpR binding motifs (Fig. 5A).

EMSA was carried out to test whether OmpR can bind directly to the *feo* promoter region. The coding region of *ompR* was cloned into an expression vector, pET24d(+). To aid in protein purification, six consecutive histidine codons were included at the 3’ end of the gene during cloning and the protein was purified to near homogeneity by metal affinity chromatography (Fig. S3). The purified protein was used directly without *in vitro* phosphorylation. Using biotinylated primers, two DNA templates of the *feo* regulatory region, encompassing the predicted OmpR binding motifs, were amplified by PCR (Fig. 5A). As a positive control for OmpR binding, two *ompC* DNA fragments were also included for EMSA (Fig. 5B). No DNA gel shift occurred with the smaller *feoB* DNA fragment containing one of the predicted OmpR binding motifs (Fig. 5C). However, the larger *feo* promoter fragment, containing the upstream predicted OmpR binding motif, displayed shifts after incubation with purified OmpR$_{6\text{His}}$ (Fig. 5C). Consistent with these *in vitro* data, we found that overexpression of OmpR$_{\text{His}}$ from a pBAD24 replicon increased *feo::lacZ* expression twofold (from 140±8 Miller units in the pBAD24 vector containing strain to 296±25 Miller units in the strain containing pBAD24-*ompR$_{\text{His}}$*). OmpR bound to the *ompC* promoter fragment containing the high-affinity OmpR-binding motif C1 (39), but not with the one containing the partial C2 and the entire C3 motif (Fig. 5C). Incidentally, only the *ompC* fragment, containing all three OmpR motifs, expressed the promoter-less *lacZ* gene in an OmpR-dependent manner (Fig. S4), thus corroborating the EMSA data. Together these data indicated that OmpR positively regulates *feo* expression by directly binding to the *feo* promoter region.

**Role of porins in iron homeostasis.** The data in Figs. 1-4 revealed a possible mechanism by which a pleiotropic allele of *envZ* downregulates the ferric transport systems by employing the
OmpC/FeoB-mediated ferrous transport pathway. While these data implicated EnvZ/OmpR and OmpC in iron transport, the use of a pleiotropic envZ allele may have created an unnatural genetic environment in which EnvZ/OmpR and porins become involved in iron homeostasis. To eliminate this possibility, we determined the roles EnvZ/OmpR and porins in iron transport using the null alleles of ompR and the porin genes. Before testing their roles, we disabled the high-affinity ferric transport system, since porins likely mediate iron transport by simple diffusion of ferrous or small iron-chelated compounds and this passive activity of porins will likely be masked by the high-affinity iron transport system. In *E. coli*, the high-affinity iron transport principally involves a ferric chelator, enterobactin, and TonB that interacts with the outer membrane iron receptors for the release of chelator-Fe$^{3+}$ complexes bound to the receptor. Consequently, we disabled the ferric iron transport by deleting *aroB*, *tonB* or both. The *aroB* gene encodes 3-dehydroquinate synthase, which is required for the second step of the chorismate pathway in the synthesis of enterobactin, aromatic amino acids and other important compounds (40).

We first determined the iron dependency of wild type, Δ*aroB*, Δ*tonB*, and Δ*aroB* Δ*tonB* strains by growing them on LBA, LBA supplemented with 40 μM FeCl$_3$ and LBA containing 200 μM of 2,2'-dipyridyl (DP), a synthetic iron chelator (Fig. 6A-C). Bacterial growth in the absence of *aroB* was unaffected on LBA+FeCl$_3$ or LBA (Fig. 6A, B). However, significant growth impairment of the Δ*aroB* strain occurred on LBA+DP plates (Fig. 6C), reflecting the loss of a major, enterobactin-mediated iron transport system. In contrast to Δ*aroB*, the deletion of *tonB* impaired bacterial growth even on LBA (Fig. 6B), which contains around 6 μM of iron, and completely prevented growth on LBA+DP medium (Fig. 6C). The Δ*tonB* strain grew like WT on LBA+FeCl$_3$, showing that the growth impairment of this strain on LBA was due to low accessibility to iron. Interestingly, growth of the Δ*aroB* Δ*tonB* double mutant improved slightly on LBA compared to the Δ*tonB* strain (Fig. 6B), but ceased again on LBA+DP (Fig. 6C). An improvement in growth of the double mutant compared the Δ*tonB* strain on LBA may be due to the absence of extracellular enterobactin-Fe$^{3+}$ complexes, which, when allowed to accumulate outside the Δ*tonB* cells, would sequester iron from the medium and further exacerbate growth defects (41). Because of the greater growth dependence of the Δ*tonB* and Δ*tonB* Δ*aroB* strains on external iron sources than the Δ*aroB* strain, we selected the former two genetic backgrounds to
examine the effects of EnvZ/OmpR and porins in iron transport. It is worth noting that we did not determine bacterial growth rates by monitoring growth of liquid cultures because the ΔtonB strain frequently reverts without supplemented iron, and these faster growing revertants takeover the population to artificially display better than expected growth.

We employed two different null ompR alleles, ompR101 and ΔompR::Km, both of which produce the OmpC− OmpF− phenotype. The ompR101 allele was transduced into a ΔtonB background using a linked Tc marker, malPQ::Tn10, while ΔompR::Km was transduced directly using the Km gene that replaced the deleted ompR gene. Although both ompR alleles could be transduced in the ΔtonB strain when transductants were selected on LBA+FeCl₃ containing appropriate antibiotics, the resulting null ompR ΔtonB transductants grew poorly compared to the ompR+ ΔtonB strain (Fig. 7A; sectors 4 and 5). In contrast, ompR101 and ΔompR::Km severely compromised growth of the ΔtonB strain on LBA not supplemented with FeCl₃ (Fig. 7B; sectors 4 and 5). Similar to the ΔtonB ompR101 strain, we were able to construct the ΔaroB ΔtonB ompR101 strain on LBA+FeCl₃ medium, where it grew poorly (Fig. 7A; sector 8) but not as poorly as on LBA without FeCl₃, where the strain failed to form single colonies (Fig. 7B; sector 8). These observations pointed to a critical role for the EnvZ/OmpR TCS in iron transport in the absence of the high-affinity iron transport system.

Although the porin genes are the main targets of the EnvZ/OmpR regulatory system, transcription of other genes is also affected either directly or indirectly in the ompR null mutant (26). Therefore, to establish unambiguously the importance of porins in iron transport we attempted to delete the porin genes in a background devoid of the high-affinity transport system. In the ΔtonB background, the deletion of ompC or ompF individually did not significantly influence growth on LBA (Fig. 7C, D; compare sectors 3 and 4 with 2). Strikingly, however, we failed to delete ompC and ompF simultaneously, via P1 transduction of ΔompF::Km and ΔompC::Cm alleles, in the ΔtonB background even when transductants were selected on LBA+FeCl₃ plates carrying appropriate antibiotics. In contrast, when the ΔtonB ΔompC or ΔtonB ΔompF double mutant was first complemented with a plasmid expressing one of the porin genes, the un-complemented porin gene from the chromosome could be readily deleted by P1 transduction. The plasmid-complemented triple mutants displayed growth behavior similar to the
un-complemented ΔtonB ΔompC and ΔtonB ΔompF double mutants on LBA+FeCl₃ or LBA (Fig. 7C, D; compare sectors 3 and 4 with 7 and 8). It is worth noting that the ΔtonB ΔompC and ΔtonB ΔompF strains were not defective in P1 transduction, since drug resistant markers not associated with the porin genes or their regulators could be transduced readily into these strains. Moreover, unlike the ΔtonB strain, in the wild type and ΔaroB backgrounds the ompC and ompF genes could be deleted simultaneously without causing iron dependency or a significant growth defect (Fig. S5). These data indicated, for the first time, that the OmpC and OmpF porins play a critical role in iron intake when the high-affinity iron transport system is blocked.
DISCUSSION

Although the EnvZ/OmpR TCS is classically associated with the regulation of the OmpC and OmpF porins in response to medium osmolarity (23), recent transcriptomics and chromatin immunoprecipitation analyses showed that it is a global regulatory system (26, 37, 42). Indeed, missense alleles of \textit{envZ}, called \textit{perA} and \textit{tpo}, isolated over three decades ago, were shown to also influence non-porin regulons, including \textit{pho} and \textit{mal} (43, 44, 45). A separate study revealed that \textit{perA} lowered the expression of three iron-regulated proteins without an apparent reduction in the rate of enterobactin secretion (27). This led the authors to suggest that the effect of the \textit{perA (envZ)} allele on the expression of iron-regulated proteins is most likely post-transcriptional (27).

In this study, we sought to resolve the mechanism by which the activated EnvZ/OmpR TCS reduces expression of genes involved in iron homeostasis and determine the role of porins in iron acquisition. We used the \textit{envZ}_{R397L} allele, which is phenotypically similar to the pleotropic \textit{perA} and \textit{tpo} alleles of \textit{envZ}, i.e., in the \textit{envZ}_{R397L} background, OmpC levels go up, while those of OmpF and LamB go down dramatically (29). The RT-qPCR (this work) and the whole genome microarray data (30; Table S1) showed that in the presence of \textit{envZ}_{R397L}, transcript levels of several Fur-controlled genes, including \textit{fecA}, \textit{fepA}, \textit{fhuA} and \textit{fhuF}, went down significantly. In the case of \textit{fhuA} and \textit{fhuF}, the effects of \textit{envZ}_{R397L} required Fur, while expression of \textit{fecA} and \textit{fepA} was still reduced by \textit{envZ}_{R397L} in the absence of Fur. These observations indicated the involvement of at least two different mechanisms by which \textit{envZ}_{R397L} affected iron regulon. In support of the Fur-dependent mechanism, the whole-cell EPR data confirmed the presence of significantly elevated levels of accessible iron in the \textit{envZ}_{R397L} strain. Several observations supported the hypothesis that in the \textit{envZ}_{R397L} mutant, FeoB and OmpC are responsible for increased intracellular Fur-Fe$^{2+}$ level (Fig. 8). First, unlike the expression of genes involved in ferric iron transport or metabolism, expression of the \textit{feoAB} genes involved in ferrous iron transport went up dramatically in the \textit{envZ}_{R397L} background. This increase in the expression of the ferrous iron transport system had an adverse effect on the ferric iron transport system, since the absence of FeoB, the ferrous permease, abolishes or significantly reduces the negative effects of \textit{envZ}_{R397L} on ferric transport/metabolic genes. Second, like FeoB, the absence of OmpC
(envZ<sub>R397L</sub> already severely represses ompF expression; 29) largely negated the inhibitory effects of envZ<sub>R397L</sub> on ferric transport/metabolic genes. Because the single deletion of feoB or ompC and the simultaneous deletion of feoB and ompC reversed the effects of envZ<sub>R397L</sub> on fecA, fepA, flhA and fluF to the same extent, it indicated that FeoB and OmpC must act in the same pathway to transport ferrous iron into the cell and elevate Fur-Fe<sup>2+</sup> levels. Third, the absence of FeoB or OmpC in an EnvZ<sup>+</sup> background caused de-repression of six Fur-controlled genes, indicating that the ferrous iron transport pathway is active under our experimental conditions and that envZ<sub>R397L</sub> enhances this pathway to achieve its inhibitory effects on the ferric transport system. Lastly, we provided direct evidence of excessive iron inside the envZ<sub>R397L</sub> mutant by whole-cell EPR spectroscopy measurements, which showed that, as in the Δfur mutant, the level of accessible iron in the envZ mutant rose fourfold over that present in the parental strain. Moreover, this increase in the intracellular free pool of iron in the envZ<sub>R397L</sub> mutant was dependent on FeoB. From these observations, we conclude that the upregulation of the OmpC-FeoB ferrous iron transport pathway by envZ<sub>R397L</sub> elevates the intracellular Fur-Fe<sup>2+</sup> level, which, in turn, represses the expression of iron-regulated genes (Fig. 8). These effects of envZ<sub>R397L</sub> required functional OmpR since the presence of ompR<sub>D55Y</sub>, which confers a null phenotype, neutralized all envZ<sub>R397L</sub> phenotypes.

Whereas envZ<sub>R397L</sub>-mediated reduction in flhA and flhF transcript levels required Fur, the effects of envZ<sub>R397L</sub> on fepA and fecA transcripts did not. This suggested the existence of another regulatory mechanism responsible for the envZ<sub>R397L</sub>-mediated downregulation of fepA and fecA that did not involve Fur. Previous studies showed that the plasmid-mediated overexpression of OmrA and OmrB small RNAs, whose expression is under the EnvZ/OmpR control, can downregulate fepA and fecA transcript levels (31, 46). We have shown that envZ<sub>R397L</sub> increases OmrA expression almost tenfold (29). This increase in OmrA expression could contribute to the downregulation of fepA and fecA. However, the fact that deleting ompC or feoB in a Fur<sup>+</sup> background abolishes envZ<sub>R397L</sub>-mediated downregulation of the ferric iron transport genes suggests that envZ<sub>R397L</sub>-mediated increase in OmrA and OmrB levels contributes little, if any, to fepA and fecA repression. Consistent with this notion, we found that the deletion of ΔomrA and ΔomrB failed to reverse the negative effect of envZ<sub>R397L</sub> on fepA or fecA (Fig. S2). We conclude
that a mechanism independent of Fur and OmrA and OmrB must also exist for the \( envZ_{R397L} \)-mediated downregulation of \( fepA \) and \( fecA \). A direct role of EnvZ/OmpR has not been ruled out.

As stated above, unlike the ferric transport genes, expression of the ferrous transport genes \( feoAB \), which are also under the control of Fur, went up in the \( envZ_{R397L} \) background. At first glance, this appears inconsistent with the notion that an increase in the Fur-Fe\(^{2+} \) level by \( envZ_{R397L} \) should also decrease \( feoAB \) expression. Our data suggest that \( envZ_{R397L} \) overcomes the repressive effect of Fur-Fe\(^{2+} \) on \( feoAB \) by activating their expression. Moreover, because \( feoAB \) expression in the \( envZ_{R397L} \) background increases dramatically without Fur, it shows that Fur-Fe\(^{2+} \) does repress \( feoAB \) in the \( envZ_{R397L} \) background, but the positive effect of \( envZ_{R397L} \) on \( feoAB \) expression overwhelms the negative effect of Fur on these genes. The EMSA data showed that purified OmpR binds to the \( feo \) regulatory region containing a putative OmpR binding site, indicating that OmpR directly activates \( feoAB \) expression. Interestingly, the whole genome microarray data from an \( \Delta omr \Delta envZ \) (porin-minus) strain showed that \( feoB \) transcript levels decreased twofold, whereas those of \( fepA \) and \( fecA \) went up twofold (26). These observations are consistent with our proposal that the EnvZ/OmpR TCS directly stimulates the FeoB-OmpC pathway to increase intracellular Fe\(^{2+} \) levels and thus active Fur-Fe\(^{2+} \) complexes, which then downregulate the expression of the ferric iron transport genes.

The proposed role of EnvZ/OmpR in iron homeostasis is similar to that suggested for RstA in \( S. \) Typhimurium (35). The authors found that overexpression of RstA increased \( feoAB \) expression and repressed \( fhuA \) and \( fhuF \) expression. A RstA binding site was identified in the \( feo \) promoter and the EMSA data confirmed that RstA bound there (35). The RstA binding motif ‘TACAtntgtTACA’ resembles that of OmpR’s ‘GTTACAnnnnGTTACA’ and not surprisingly, both proteins regulate overlapping genes by binding to the similar sequences (38). Our EMSA data showed that OmpR binds to the \( feo \) promoter region. Specifically, it binds to a DNA fragment containing the sequence ‘ttATCAtttcattAACA’ located 278 bp upstream of the start codon of \( feoA \). The OmpR binding studies carried out here involved the purified protein not modified by \textit{in vitro} phosphorylation. Therefore, it is possible that stronger binding and/or additional binding sites may be discovered with phosphorylated OmpR. It is worth noting that in previous EMSA assays, unphosphorylated RstA from \( S. \) Typhimurium and \( E. \) coli was shown to
bind to their target promoter sequences (35, 47). Further work will be required to identify the
exact binding sequences and to determine whether OmpR and RstA bind to the same,
overlapping or distinct regulatory sequences of the \textit{feo} operon.

Our work also revealed, for the first time, the essential role of OmpC and OmpF porins in
ing acquisition when the TonB-dependent ferric transport pathways are inoperative. The
absence of OmpC or OmpF produced no growth defects in the $\Delta tonB$ background on LBA
supplemented with iron, but the construction of a triple knockout mutant ($\Delta tonB \Delta ompC \Delta ompF$)
required expression of at least one of the porin genes from a plasmid replicon. Interestingly,
unlike the porin-devoid triple knockout mutant, we were able to construct $\Delta tonB \Delta ompR$ and
$\Delta tonB \Delta aroB \Delta ompR$ mutants albeit, only on LBA supplemented with iron. In the $\Delta ompR$
background, $ompC$ and $ompF$ porin expression is extremely low but presumably not zero, which
is the case in the $\Delta tonB \Delta ompC \Delta ompF$ mutant. We think that this extremely low porin
expression permits the construction of the $\Delta tonB \Delta ompR$ strain, which can form single, albeit
very small colonies, but only on LBA supplemented with iron. Whereas the diffusion of ferrous
iron across the outer membrane occurs via OmpC or OmpF channels, at least three proteins—
FeoB, MntH and ZupT—can transport ferrous iron across the inner membrane of \textit{E. coli} cells
(48). Consistent with this, a $\Delta tonB \Delta feoB$ double mutant is viable and grows like the $\Delta tonB$
mutant (data not shown).

While the essential role of porins in iron acquisition becomes apparent without the TonB-
dependent, high-affinity ferric iron transport systems, their de-repression without OmpC or FeoB
indicate that the porin-mediated iron transport is active even in the presence of the TonB-
dependent high-affinity iron transport systems. The importance of the porin-FeoB pathway for
bacterial growth should further increase as \textit{E. coli} cells enter microaerobic or anaerobic
environments where the ferrous species predominates. The involvement of porin and FeoB in
iron-dependent growth and/or virulence has been reported for several bacteria, including \textit{E. coli}
(9), \textit{S. Typhimurium} (10; 11), \textit{Helicobacter pylori} (12), \textit{Vibrio cholerae} (49) and \textit{Mycobacterium
smegmatis} (50). Interestingly, \textit{M. smegmatis} porins increase ferric citrate uptake (50). Similarly,
a study reported liganded iron uptake via the OprF porin in \textit{Pseudomonas aeruginosa} (51). There
are no definitive reports in \textit{E. coli} showing the involvement of porins in liganded iron transport,
even for ferric citrate whose size is below the diffusion limits of the porins (52). Regardless of these ambiguities, published reports and the work carried out here highlight the importance of the porin/FeoB-mediated iron transport pathways in iron homeostasis.
MATERIALS AND METHODS

Bacterial strains, media and chemicals. Escherichia coli K-12 strains used in this study were constructed from MC4100 (53) and are listed in Table 1. Luria broth (LB) was prepared using LB Broth EZMix™ Powder (Lennox). LB agar (LBA) medium contained LB plus 1.5% agar (Becton Dickenson). ONPG (2-ortho-nitrophenyl-β-D-galactopyranoside) was purchased from ACROS. Diethylenetriaminepentaacetic acid (DTPA) and desferrioxamine were obtained from Sigma-Aldrich. All other chemicals were of analytical grade. The growth medium was supplemented with ampicillin (50 μg/ml), chloramphenicol (12.5 μg/ml), kanamycin (25 μg/ml) or tetracycline (10 μg/ml) when necessary. To induce plasmid-borne gene expression, L-arabinose (0.2%) or isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.4 mM) was added to the medium.

Genetic and DNA methods. Standard bacterial genetic methods, including P1 transduction and plasmid transformation, were carried out as described by Silhavy et al. (54). To clone the ompR and rstA genes into pBAD24 (55) and pET24 d(+) (Novagen), DNA corresponding to their open reading frames (ORFs) were amplified by PCR using primers that carried appropriate restriction enzyme sites for cloning. The reverse primers used for cloning into pBAD24 additionally contained nucleotides encoding six consecutive histidine codons. (Primer sequences are available upon request.) Deletion of the fepA, feoA, feoB and feoAB genes from their chromosomal locations and subsequent scarring of the antibiotic-resistant marker at the deletion sites were done using the λ-red-mediated gene recombination method (56). Deletions were confirmed by PCR and DNA sequence analyses. In some instances, promoter-less lacZY were recombined at the deletion scar site by the method of Ellermeier et al. (57).

RNA isolation, real-time quantitative PCR, and microarray analyses. Total RNA was extracted from 5 ml cells grown to log phase (OD600 ~0.6) at 37°C using TRIzol Max Bacterial RNA Isolation Kit (Invitrogen). RNA was further purified using the RNeasy kit (Qiagen), and quality of RNA was analyzed by Agilent 2100 BioAnalyzer (Agilent Technologies). The purified RNA was then converted to either single-stranded cDNA for use in RT-qPCR or double-stranded cDNA for use in DNA microarray analysis.
For RT-qPCR, single-stranded cDNA was synthesized from 10 µg of RNA using 100 pM random hexamer primer (Integrated DNA Technologies) and M-MuLV Reverse Transcriptase (New England Biolabs). After reverse transcription, cDNA was treated with 5 units of RNaseH (New England Biolabs) for 20 min at 37°C followed by purification with the QIAquick PCR purification kit (Qiagen). To quantify the RNA transcripts, 300 nM of primer specific to the gene of interest and 20 ng of cDNA was added to SYBR Green PCR Master Mix (Applied Biosystems) in a 20 µl reaction. Primers were designed per manufacturer’s protocol included with SYBR Green PCR Master Mix and RT-qPCR reagents. Critical threshold (Ct) values were determined using ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The relative quantification of target transcripts was calculated according to the $2^{-\Delta\Delta Ct}$ method (58) using ftsL and purC as the endogenous control genes. Briefly, changes in Ct value ($\Delta Ct$) for the gene of interest were calculated by subtracting that gene’s average Ct from the average Ct for the endogenous control gene. The $\Delta Ct$ for the mutant was then subtracted from the wild type strain’s $\Delta Ct$ value to give the $\Delta\Delta Ct$ value. Each PCR reaction was performed in triplicate and fold changes in transcript levels along with standard deviations were calculated from at least two experiments ($n \geq 2$).

For microarray analysis, Invitrogen Superscript Double-Stranded cDNA Synthesis Kit was used to generate double stranded cDNA per manufacturer’s instruction. Single-stranded cDNA was synthesized from 10 µg of RNA using 100 pM random hexamer primer (Integrated DNA Technologies) and Superscript II reverse transcriptase. Second strand synthesis was performed per manufacturer’s instructions and reaction was stopped with 0.5 M EDTA. RNA was then digested using RNaseA (25 µg/ml final concentration) followed by treatment with phenol:chloroform and precipitation with ethanol. Double-stranded cDNA was further purified with the QIAquick PCR purification kit (Qiagen) and quality tested by BioAanalyzer. Cy3 fluorescently labeled cDNA was used to probe array slides printed with 4,254 E. coli ORFs. Array slides contained 8 probes per gene (in duplicate) corresponding to roughly 72,000 probes per sample. Sample labeling with Cy3 fluorescent dye, hybridization to the 4-plex array (0771112 E. coli K-12 EXP X4, Catalog number A6697-00-01), washing, and one-color scanning was performed by Roche Nimblegen in accordance with their standard protocol. Analysis of gene expression profiles was performed using ArrayStar 2.0 software (DNASTAR)
with a focus on genes with ≥2-fold change in gene expression. P-values were generated with the Student’s t-Test and false positives were minimized with FDR (false discovery rate) analysis (59).

**Enzymatic assays.** The β-galactosidase assay was done following the Miller method (60). Assays were carried out with at least two independent cultures. The β-galactosidase activity was expressed as Miller Units (MU; 60). In some instances, kinetic analysis of enzyme activity was carried out using a VersaMax (Molecular Dynamics) microtiter plate reader in quadruplicate and activity was measured as the rate of ONPG cleavage divided by the cell density in each well.

**Electron paramagnetic resonance (EPR) spectroscopy.** Free iron concentration in whole cells was determined by EPR spectroscopic analysis (32) with some modifications. Briefly, overnight grown bacterial cultures were diluted 1:100 in 200 ml of LB and grown shaking at 37°C until \( \text{OD}_{600} \) of 0.8. Cells were pelleted by centrifugation in a GSA rotor (Sorvall) for 10 min at 6,000 x g. Pellets were resuspended in 10 ml of LB containing 10 mM DTPA (to chelate extracellular iron) and 20 mM desferrioxamine (to chelate intracellular free or accessible ferric iron) and incubated shaking for 37°C for 15 min. Cells were pelleted as before and washed twice with 5 ml of ice-cold 20 mM Tris-HCl, pH 7.4. The final cell pellet was resuspended in 0.3 ml of ice cold 20 mM Tris-HCl, pH 7.4, containing 30% glycerol. A 250 µl aliquot of this cell suspension was placed in a quartz EPR tube (length: 250 mm, external diameter: 4 mm; Wilmad-Labglass). Tubes were frozen in loosely packed dry ice and then transferred to -80°C until the EPR analysis. The remaining cells were diluted \( 10^3 \)-fold to determine \( \text{OD}_{600} \). Iron standards were prepared from a freshly prepared 10 mM FeCl\(_3\)·6H\(_2\)O stock in a buffer containing 20 mM Tris-HCl, pH 7.4, and 1 mM desferrioxamine. Theoretical concentrations of iron standards were 100, 50, 25, 10, 5, and 0 µM. The actual iron concentrations were determined by measuring \( \text{OD}_{420} \) of each standard and using the formula: molar concentration = \( A_{420}/\varepsilon \) where \([\text{Fe}]\varepsilon\) is 2.865 mM\(^{-1}\) Cm\(^{-1}\). A 250 µl aliquot of each standard was placed in separate EPR tubes that were then frozen. The standard curve was generated by plotting EPR signals against actual iron concentrations (Fig. S6). Free iron concentration for each strain was determined from the EPR data and the standard curve. Intracellular free iron concentration was then deduced by integrating intracellular volume of the cell (1 ml of 1.0 \( \text{OD}_{600} \) cells has an intracellular volume of 0.00052 ml; Jim Imlay, personal...
communication) and using the formula: intracellular free iron concentration = [Fe] from standard curve/cell paste OD_{600} x 0.00052 ml.

EPR measurements were carried out at the EPR Facility at Arizona State University. Continuous wave EPR spectra were recorded using an ELEXSYS E580 CW X-band spectrometer (Bruker, Rheinstetten, Germany) equipped with a Model 900 EPL liquid helium cryostat (Oxford Instruments, Oxfordshire, UK). For all measurements, the magnetic field modulation frequency was 100 kHz, the amplitude was 1.25 mT, the microwave power was 10 mW, the microwave frequency was 9.44 GHz, the sweep time was 42 s, and the temperature was 20 K.

**OmpR purification.** OmpR was purified from BL21(DE3) cultures carrying a pET24-ompR\textsubscript{6His} plasmid. Overnight cultures, grown without IPTG, were diluted 1:100 in 1 L LB and grown with vigorous shaking for 90 min and then supplemented with IPTG and grown for another 2 h. Cells were pelleted, washed with 10 mM Tris-HCl pH 7.5, resuspended in lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 µg/ml lysozyme) and incubated on ice for 30 min. MgCl\textsubscript{2} (10 mM final), PMSF (2 mM final) and DNase I (40 µg/ml final) were then added to the cell suspension. Cells were lysed by passing through the French pressure cell three times and the lysate was centrifuged at low speed to remove unlysed cells. Envelopes were removed from the lysate by ultracentrifugation at 105,000 x g for an hour at 4°C. Supernatant was filtered through a 0.45 µM syringe filter and the filtrate was subjected to nickel affinity column chromatography using buffers for protein binding (20 mM sodium phosphate [pH 7.4], 20 mM imidazole and 50 mM NaCl), washing (20 mM sodium phosphate [pH 7.4], 50 mM imidazole and 300 mM NaCl) and elution (20 mM sodium phosphate [pH 7.4], 300 mM imidazole and 300 mM NaCl). Samples from eluted fractions were analyzed by SDS-PAGE and protein bands were visualized after Coomassie blue staining (Fig. S3). Fractions representing OmpR\textsubscript{6His} peaks were pooled and dialyzed against a buffer containing 20 mM sodium phosphate (pH 7.4) and 300 mM NaCl. Purified proteins were stored at 4°C in the dialysis buffer supplemented with glycerol (5% final), EDTA (0.1 mM final) and DTT (0.1 mM final).

**Electrophoretic mobility gel shift assays (EMSA).** EMSA was carried out using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific). *ompC* and *feoABC* promoter fragments were
generated by PCR using primers specific to the region of interest, with one of the primers biotinylated. Biotin-labeled DNA probes (20 fmol), purified OmpR<sub>6His</sub> (100 pmol) and other relevant reagents provided with the kit were incubated for 20 min at room temperature, and the reaction was stopped by adding 5x loading buffer. The mixture was analyzed by 5% acrylamide gel, electro-blotted onto PVDF Immobilon-P membrane (Millipore) using a Mini Trans-Blot Cell (Bio-Rad). After transfer, DNA was cross-linked to the membrane using Hoefer Ultraviolet Crosslinker and incubated with Stabilized Streptavidin-HRP Conjugate for an hour. DNA was detected by the Molecular Imager ChemiDoc XRS system (Bio-Rad) after incubating the membrane for 5 min with freshly mixed Luminol/Enhancer and Stable Peroxide solutions.

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REFERENCES

1. Imlay JA. 2008. Cellular defenses against superoxide and hydrogen peroxide. *Annu Rev Biochem* **77**:755–776.

2. Andrews SC, Robinson A K, Rodriguez-Quinones, F. 2003. Bacterial iron homeostasis. *FEMS Microbiol Rev* **27**:215–237.

3. Grass G. 2006. Iron transport in *Escherichia coli*: all has not been said and done. *Biometals* **19**:159–172.

4. Pollack JR, Neilands JB. 1970. Enterobactin, an iron transport compound. *Biochem Biophys Res Commun* **38**:989–992.

5. Kammler M, Schon C, Hantke K. 1993. Characterization of the ferrous iron uptake system of *Escherichia coli*. *J Bacteriol* **175**:6212–6219.

6. Hantke K. 2003. Is the bacterial ferrous iron transporter FeoB a living fossil? *Trends Microbiol* **11**:192–195.

7. Cartron ML, Maddocks S, Gillingham P, Craven CJ, Andrews SC. 2006. Feo-transport of ferrous iron into bacteria. *Biometals* **19**:143–157.

8. Lau CKY, Krewulak KD, Vogel HJ. 2015. Bacterial ferrous iron transport: the Feo system. *FEMS Microbiol Rev* **40**:273–298.

9. Stojiljkovic I, Cobeljic M, Hantke K. 1993. *Escherichia coli* K-12 ferrous iron uptake mutants are impaired in their ability to colonize the mouse intestine. *FEMS Microbiol Lett* **108**:111–115.

10. Tsolis RM, Baumler AJ, Heffron F, Stojiljkovic I. 1996. Contribution of TonB- and Feo-mediated iron uptake to growth of *Salmonella typhimurium* in the mouse. *Infect Immun* **64**:4549–4556.

11. Boyer E, Bergevin I, Malo D, Gros P, Cellier MF. 2002. Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of *Salmonella enterica* serovar Typhimurium. *Infect Immun* **70**:6032–6042.

12. Velayudhan J, Hughes NJ, McCollm AA, Bagshaw J, Clayton CL, Andrews SC, Kelly DJ. 2000. Iron acquisition and virulence in *Helicobacter pylori*: a major role for FeoB, a high-affinity ferrous iron transporter. *Mol Microbiol* **37**:274–286.

13. Hantke K. 1981. Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a
14. Bagg A, Neilands JB. 1987. Ferric uptake regulation protein acts as a repressor, employing iron(II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. *Biochem* 26:5471–5477.

15. Touati D, Jacques M, Tardat B, Bouchard L, Despied S. 1995. Lethal oxidative damage and mutagenesis are generated by iron in delta fur mutants of *Escherichia coli*: protective role of superoxide dismutase. *J Bacteriol* 177:2305–2314.

16. de Lorenzo Wee S, Herrero M, Neilands JB. 1987. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (fur) repressor. *J Bacteriol* 169:2624–2630.

17. Chen Z, Lewis KA, Shultzaberger RK, Lyakhov IG, Zheng Z, Doan B, *et al*. 2007. Discovery of Fur binding site clusters in *Escherichia coli* by information theory models. *Nuc Acids Res* 35:6762–6777.

18. Masse E, Gottesman S. 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. *Proc Natl Acad Sci U S A* 99:4620–4625.

19. Masse E, Vanderpool CK, Gottesman S. 2005. Effect of RyhB small RNA on global iron use in *Escherichia coli*. *J Bacteriol* 187:6962–6971.

20. Masse E, Arguin M. 2005. Ironing out the problem: new mechanisms of iron homeostasis. *Trends Biochem Sci* 30:462–468.

21. Seo SW, Kim D, Latif H, O’Brien EJ, Szubin R, Palsson BO. 2014. Deciphering Fur transcriptional regulatory network highlights its complex role beyond iron metabolism in *Escherichia coli*. *Nature Comms* 5:4910.

22. Beauchene NA, Myers KS, Chung D, Park DM, Weisnicht AM, Keleş S, Kiley, PJ. 2015. Impact of anaerobiosis on the expression of the iron-responsive Fur and RyhB regulons. *mBio* 6:e01947.

23. Hall MN, Silhavy TJ. 1981. Genetic analysis of the *ompB* locus in *Escherichia coli* K-12. *J Mol Biol* 151:1–15.

24. Nikaido H. 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* 67:593–656.

25. van Alphen W, Lugtenberg B. 1977. Influence of osmolarity of the growth medium on the
outer membrane protein pattern of *Escherichia coli*. *J Bacteriol* **131**:623–630.

26. Oshima T, Aiba H, Masuda Y, Kanaya S, Sugiura M, Wanner BL, *et al*. 2002. Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli* K-12. *Mol Microbiol* **46**:281–291.

27. Lundrigan M, Earhart CF. 1981. Reduction in three iron-regulated outer membrane proteins and protein a by the *Escherichia coli* K-12 perA mutation. *J Bacteriol* **146**:804–807.

28. Earhart CF. 2004. Iron uptake via the enterobactin system. In *Iron Transport in Bacteria*. Crosa JH, Mey AR, Payne SM (eds): Washington, DC: ASM Press, pp. 133–146.

29. Gerken H, Charlson ES, Cicirelli EM, Kenney LJ, Misra R. 2009. MzrA: a novel modulator of the EnvZ/OmpR two-component regulon. *Mol Microbiol* **72**:1408–1422.

30. Gerken HG. 2009. Novel aspects of bacterial envelope response pathways. Ph.D. dissertation, Arizona State University. (Publication number 3391808).

31. Guillier M, Gottesman S. 2006. Remodeling of the *Escherichia coli* outer membrane by two small regulatory RNAs. *Mol Microbiol* **59**:231–247.

32. Woodmansee AN, Imlay JA. 2002. Quantitation of intracellular free iron by electron paramagnetic resonance spectroscopy. *Methods Enzymol* **349**:3–9.

33. Slauch JM, Garrett S, Jackson DE, Silhavy TJ. 1988. EnvZ functions through OmpR to control porin gene expression in *Escherichia coli* K-12. *J Bacteriol* **170**:439–441.

34. Delgado J, Forst S, Harlocker S, Inouye M. 1993. Identification of a phosphorylation site and functional analysis of conserved aspartic acid residues of OmpR, a transcriptional activator for *ompF* and *ompC* in *Escherichia coli*. *Mol Microbiol* **10**:1037–1047.

35. Jeon J, Kim H, Yun J, Ryu S, Groisman EA, Shin D. 2008. RstA-promoted expression of the ferrous iron transporter FeoB under iron-replete conditions enhances Fur activity in *Salmonella enterica*. *J Bacteriol* **190**:7326–7334.

36. Vuong P, Misra R. 2011. Guide to genome-wide bacterial transcription factor binding site prediction using OmpR as model. In *Selected Works in Bioinformatics*. Xia, X. (ed), InTech, pp. 41–56.

37. Quinn HJ, Cameron ADS, Dorman CJ. 2014. Bacterial regulon evolution: distinct responses and roles for the identical OmpR proteins of *Salmonella* Typhimurium and *Escherichia coli* in the acid stress response. *PLoS Genet* **10**:e1004125.
38. Ogasawara H, Yamada K, Kori A, Yamamoto K, Ishihama A. 2010. Regulation of *Escherichia coli* csgD promoter: interplay between five transcription factors. *Microbiol* **156**:2470–2483.

39. Head CG, Tardy A, Kenney LJ. 1998. Relative binding affinities of OmpR and OmpR-phosphate at the *ompF* and *ompC* regulatory sites. *J Mol Biol* **281**:857–870.

40. Bender SL, Mehdi S, Knowles JR. 1989. Dehydroquinate synthase: the role of divalent metal cations and of nicotinamide adenine dinucleotide in catalysis. *Biochem* **28**:7555–7560.

41. Qiu N, and Misra R. 2019. Overcoming iron deficiency of an *Escherichia coli* tonB mutant by increasing outer membrane permeability. *J Bacteriol* **201**:e00340-19.

42. Perkins TT, Davies MR, Klemm EJ, Rowley G, Wileman T, James K, *et al.* 2013. ChIP-seq and transcriptome analysis of the OmpR regulon of *Salmonella enterica* serovars Typhi and Typhimurium reveals accessory genes implicated in host colonization. *Mol Microbiol* **83**:526–538.

43. Wanner BL, Sarthy A, Beckwith J. 1979. *Escherichia coli* pleiotropic mutant that reduces amounts of several periplasmic and outer membrane proteins. *J Bacteriol* **140**:229–239.

44. Wandersman C, Moreno F, Schwartz M. 1980. Pleiotropic mutations rendering *Escherichia coli* K-12 resistant to bacteriophage TP1. *J Bacteriol* **143**:1374–1383.

45. Case CC, Bukau B, Granett S, Villarejo M R, Boos W. 1986. Contrasting mechanisms of *envZ* control of mal and pho regulon genes in *Escherichia coli*. *J Bacteriol* **166**:706–712.

46. Guillier M, Gottesman S. 2008. The 5' end of two redundant sRNAs is involved in the regulation of multiple targets, including their own regulator. *Nucleic Acids Res* **36**:6781–6794.

47. Ogasawara H, Hasegawa A, Kanda E, Miki T, Yamamoto K, Ishihama A. 2007. Genomic SELEX search for target promoters under the control of the PhoQP-RstBA signal relay cascade. *J Bacteriol* **189**:4791–4799.

48. Grass G, Franke S, Taudte N, Nies DH, Kucharski LM, Maguire ME, Rensing C. 2005. The metal permease ZupT from *Escherichia coli* is a transporter with a broad substrate spectrum. *J Bacteriol* **187**:1604–1611.

49. Wyckoff EE, Mey AR, Leimbach A, Fisher CF, Payne SM. 2006. Characterization of ferric and ferrous iron transport systems in *Vibrio cholerae*. *J Bacteriol* **188**:6515–6523.
50. Jones CM, Niederweis M. 2010. Role of orin in iron uptake in *Mycobacterium smegmatis*. *J Bacteriol* 192:6422-6417.

51. Meyer J-M. 1992. Exogenous siderophore-mediated iron uptake in *Pseudomonas aeruginosa*: possible involvement of porin in iron translocation. *J Gen Microbiol* 138:951–958.

52. Wagegg W, Braun V. 1981. Ferric citrate transport in *Escherichia coli* requires outer membrane protein receptor protein FecA. *J Bacteriol* 145:156–163.

53. Casadaban MJ. 1976. Transposition and fusion of the *lac* genes to select promoters in *Escherichia coli* using bacteriophage Lambda and Mu. *J Mol Biol* 141:541–555.

54. Silhavy TJ, Berman ML, Enquist LW. 1984. Experiments with gene fusions. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

55. Guzman LM, Belin D, Carson MJ, Beckwith J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_BAD_ promoter. *J Bacteriol* 177:4121–4130.

56. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97:6640–6645.

57. Ellermeier CD, Janakiraman A, Slauch JM. 2002. Construction of targeted single copy *lac* fusions using λ Red and FLP-mediated site-specific recombination in bacteria. *Gene* 290:153–161.

58. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^-ΔΔCT_ method. *Methods* 25:402–408.

59. Benjamin Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B* 57:289–300.

60. Miller JH. 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, pp. 71–74.

61. Werner J, Misra R. 2005. YaeT (Omp85) affects the assembly of lipid-dependent and lipid-independent outer membrane proteins of *Escherichia coli*. *Mol Microbiol* 57:1450–1459.
### Table 1. Bacterial strains used in this study.

| Strain   | Relevant genotype                                                                 | Source |
|----------|-----------------------------------------------------------------------------------|--------|
| RAM1292  | MC4100 (Δ[argF-lac]169 λ e14 flhD5301 Δ[fraK-yeR]725 relA1 rpsL150 (strR) rbsR22 Δ[jimB-fimE]632 deoC1) Δara714 | 61     |
| RAM1541  | RAM1292 envZ,R397L                                                                 | 29     |
| RAM2697  | RAM1292 Δfur::scar                                                                 | This study |
| RAM2698  | RAM1292 envZ,R397L Δfur::scar                                                     | This study |
| RAM2699  | RAM1292 ΔompC::scar                                                                | This study |
| RAM2700  | RAM1292 envZ,R397L ΔompC::scar                                                    | This study |
| RAM2701  | RAM1292 ΔfeoB::scar                                                                | This study |
| RAM2702  | RAM1292 envZ,R397L ΔfeoB::scar                                                   | This study |
| RAM2703  | RAM1292 envZ,R397L ΔfeoB::scar ΔompC::scar                                        | This study |
| RAM2704  | RAM1292 ΔompR::scar                                                                | This study |
| RAM2705  | RAM1292 ΔompR::scar pBAD24-ompR₆His                                              | This study |
| RAM2707  | RAM1292 ΔompR::scar pBAD24                                                         | This study |
| RAM2708  | RAM1292 pBAD24                                                                    | This study |
| RAM2709  | RAM1292 pBAD24-ompR₆His                                                           | This study |
| RAM2711  | RAM1292 ΔfeoA::lacZ::Km'                                                            | This study |
| RAM2712  | RAM1292 ΔfeoA::lacZ::Km' ΔompC::scar                                              | This study |
| RAM2713  | RAM1292 ΔfeoA::lacZ::Km' ΔompR::scar                                              | This study |
| RAM2714  | RAM1292 ΔfeoA::lacZ::Km' ΔompR::scar envZ,R397L                                    | This study |
| RAM2715  | BL21(DE3) pET24d-ompR₆His                                                           | This study |
| RAM2722  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2723  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2724  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2725  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2726  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2727  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2728  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2729  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2730  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2731  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2732  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2733  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2734  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2735  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2736  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2737  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2738  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2739  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2740  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2741  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2742  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2743  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2744  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2745  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2746  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2747  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2748  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2749  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2750  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2751  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2752  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2753  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2754  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2755  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2756  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2757  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2758  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2759  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2760  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2761  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2762  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2763  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2764  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2765  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2766  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2767  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2768  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2769  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2770  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2771  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2772  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2773  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2774  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2775  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2776  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2777  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2778  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2779  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2780  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2781  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2782  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2783  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2784  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2785  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2786  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2787  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2788  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2789  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2790  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2791  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2792  | RAM1292 ΔompC::Cm'                                                                  | This study |
| RAM2795 | RAM2790 ΔompC::Cm<sup>i</sup> | This study |
|---------|-------------------------------|------------|
| RAM2796 | RAM2792 ΔompF::Km<sup>i</sup> | This study |
| RAM2920 | RAM2469 Cm<sup>i</sup> linked to envZ<sup>r</sup> | This study |
| RAM2921 | RAM2470 Cm<sup>i</sup> linked to envZ<sub>R397L</sub> | This study |
| RAM2922 | RAM2799 Cm<sup>i</sup> linked to ompR<sub>DSSY</sub> envZ<sub>R397L</sub> | This study |
| RAM2924 | RAM2711 Cm<sup>i</sup> linked to envZ<sup>r</sup> | This study |
| RAM2925 | RAM2711 Cm<sup>i</sup> linked to envZ<sub>R397L</sub> | This study |
| RAM2926 | RAM2711 Cm<sup>i</sup> linked to ompR<sub>DSSY</sub> envZ<sub>R397L</sub> | This study |
| RAM2928 | RAM2711 pBAD24 (Ap<sup>r</sup>) | This study |
| RAM2932 | RAM2711 pBAD24-ompR<sub>His</sub> | This study |
**Figure Legends**

**Fig. 1.** Determination of fecA, fepA fhuA and fhuF expression in different genetic backgrounds by real-time quantitative PCR (RT-qPCR). RNA was isolated from bacterial cultures grown to mid-log. Relative quantification of transcripts was performed using the $2^{\Delta\Delta CT}$ method, with fisL and purC serving as the reference genes. Relative fold-changes in gene expression and error bars representing standard deviation are shown. Bacterial strains used are: RAM1292 (wild type), RAM1541 (envZ<sub>R397L</sub>), RAM2697 (∆fur), RAM2698 (envZ<sub>R397L</sub> ∆fur), RAM2699 (∆ompC), RAM2700 (envZ<sub>R397L</sub> ∆ompC), RAM2701 (∆feoB), and RAM2702 (envZ<sub>R397L</sub> ∆feoB).

**Fig. 2.** Determination of the relative gene expression of feoA and feoB by RT-qPCR. RNA was isolated from bacterial cultures grown to mid-log. Relative quantification of transcripts in various genetic backgrounds was performed using the $2^{\Delta\Delta CT}$ method, with fisL and purC serving as reference genes. Relative fold-changes in gene expression and error bars representing standard deviation are shown. Bacterial strains used are: RAM1292 (wild type), RAM1541 (envZ<sub>R397L</sub>), RAM2697 (∆fur), RAM2698 (envZ<sub>R397L</sub> ∆fur), RAM2699 (∆ompC), RAM2700 (envZ<sub>R397L</sub> ∆ompC), RAM2701 (∆feoB), and RAM2702 (envZ<sub>R397L</sub> ∆feoB).

**Fig. 3.** Determination of the intracellular free iron concentration. Shown are the averages of five ferric-chelate EPR scans per strain. All scans were normalized to the final culture OD<sub>600</sub> used in the measurements. EPR parameters were: microwave power, 10 mW; microwave frequency, 9.44 GHz; center field, 160 mT; sweep width, 80 mT; modulation amplitude, 1.25 mT; modulation frequency 100 kHz. Free intracellular iron concentrations, calculated as described in the Experimental procedure section, were: wild type, 32 μM; ∆fur, 120 μM; envZ<sub>R397L</sub>, 135 μM; ∆fur envZ<sub>R397L</sub>, 105 μM; ∆feoB, 20 μM; and ∆feoB envZ<sub>R397L</sub>, 29 μM. Bacterial strains used are: RAM1292 (wild type), RAM1541 (envZ<sub>R397L</sub>), RAM2697 (∆fur), RAM2698 (envZ<sub>R397L</sub> ∆fur), RAM2699 (∆ompC), RAM2700 (envZ<sub>R397L</sub> ∆ompC), RAM2701 (∆feoB), and RAM2702 (envZ<sub>R397L</sub> ∆feoB).

**Fig. 4.** Determination of fepA::lacZ and feo::lacZ activities in various genetic backgrounds. The β-galactosidase activities were measured from two independent overnight grown cultures. Error bars represent standard deviation. Bacterial strains used are: RAM2920 (ompR<sup>+</sup> envZ<sup>+</sup>...
fepA::lacZ), RAM2921 (ompR+ envZ<sub>R397L</sub> fepA::lacZ), RAM2922 (omp<sub>RDSY</sub> envZ<sub>R397L</sub>

fepA::lacZ), RAM2923 (ompR<sup>+</sup> envZ<sup>+</sup> feo::lacZ), RAM2924 (ompR<sup>+</sup> envZ<sub>R397L</sub>feo::lacZ), and

RAM2925 (ompR<sub>D55Y</sub> envZ<sub>R397L</sub>feo::lacZ).

**Fig. 5.** *In vitro* binding of purified OmpR<sub>6His</sub> to the *feoABC* and *ompC* promoter regions. DNA binding was examined by electrophoretic mobility shift assay (EMSA) using biotin-labeled DNA fragments of various lengths generated by PCR. (A) A cartoon showing the regulatory region of the *feoABC* operon (not drawn to scale). Grey and black boxes represent possible OmpR binding sequences. Nucleotide numberings are relative to the *feoA* start codon. Relative positions and lengths of the two DNA fragments used in EMSA are shown. Diamond marks the biotin-labeled end of the DNA probe. (B) A cartoon showing the regulatory region of the *ompC* gene (not drawn to scale). Three boxes represent known OmpR binding sites; DNA sequences of all three OmpR binding sites—C1, C2, and C3—are shown. Nucleotide numberings are relative the *ompC* start codon. Relative positions and lengths of the two DNA fragments used in EMSA are shown. Diamond indicates the biotin-labeled end of the DNA probe. (C) Polyacrylamide gels showing EMSA results. Plus and minus signs denote the presence and absence of OmpR in the reaction mixture prior to gel electrophoresis. Gels were electroblotted and DNA bands were detected by treating membranes with Stabilized Streptavidin-HRP Conjugate, followed by Luminol/Enhancer and Stable Peroxide. Arrows point to positions of un-shifted DNA fragments.

**Fig. 6.** Effects of ΔtonB and ΔaroB mutations on bacterial growth under iron replete and deplete conditions. Bacterial growth on LBA + 40 μM FeCl<sub>3</sub> (A), LBA (B), and LBA+200 μM 2,2’-dipyridyl (C) was recorded after incubating petri plates at 37°C for 24 h. Bacterial strains used are: 1, RAM1292 (wild type); 2, RAM2553 (ΔaroB); 3, RAM2572 (ΔtonB) and 4, RAM2574 (ΔaroB ΔtonB).

**Fig. 7.** Effects of *ompR* and the porin gene mutations on the growth of ΔtonB or ΔtonB ΔaroB mutants. Bacterial growth was monitored on LBA + 40 μM FeCl<sub>3</sub> (A and C) and LBA (B and D) after incubating petri plates at 37°C for 24 h. The relevant genotypes of strains used in A and B are: 1, RAM1292 (wild type); 2, RAM2572 (ΔtonB); 3, RAM2765 (ΔtonB...
malPQ::Tn10); 4, RAM2766 (ΔtonB malPQ::Tn10 ompR101); 5, RAM2767 (ΔtonB ΔompR::Km'); 6, RAM2754 (ΔtonB ΔaroB::Km'); 7, RAM2771 (ΔtonB ΔaroB::Km' malPQ::Tn10); and 8, RAM2772 (ΔtonB ΔaroB::Km' malPQ::Tn10 ompR101). The relevant genotypes of strains used in C and D are: 1, RAM1292 (wild type); 2, RAM2572 (ΔtonB); 3, RAM2769 (ΔtonB ΔompC::Cm'); 4, RAM2768 (ΔtonB ΔompF::Km'); 5 and 6, no bacteria; 7, RAM2792 (ΔtonB ΔompC::Cm' ΔompF::Km'/ompC); and 8, RAM2790 (ΔtonB ΔompF::Km' ΔompC::Cm'/ompF). pompF and pompC are pTrc99A plasmid clones expressing ompF and ompC, respectively. Expression of these plasmid-coded genes did not require induction by an inducer.

Fig. 8. A cartoon showing regulation of the ferric (Fe³⁺) and ferrous (Fe²⁺) uptake systems in E. coli. Fur-Fe²⁺ is the master regulator of transcription of genes involved in iron metabolism. Under aerobic growth conditions, where Fe³⁺ is the major source of iron, E. coli secretes enterobactin (Ent) in the medium to chelate Fe³⁺. The Fe³⁺-chelate complex is transported back into the cell through the outer membrane receptor protein, FepA. The TonB-ExbB-ExbD complex of the inner membrane facilitate FepA channel opening. In the periplasm, FepB interacts with the Fe³⁺-chelate and delivers it to the FepDGC complex for transport into the cytoplasm. Under micro-aerobic or anaerobic growth conditions, Fe²⁺ is the main source of iron. It is brought into the cell via porins OmpC and OmpF and FeoB. The EnvZ/OmpR two-component system, classically known for regulating the expression of the ompC and ompF porin genes, also induces feo expression when hyper-activated due to a specific mutation in envZ (envZR397L). This positive effect of EnvZR397L/OmpR on feo expression can overcome the negative effect of Fur-Fe²⁺ on feo expression, thus tipping the balance in favor of ferrous over ferric transport. Porins and EnvZ/OmpR play a crucial role in iron acquisition in a TonB-deficient background that lacks functional ferric transport systems. Abbreviation: OM, outer membrane; PS, periplasm; IM, inner membrane; and Cyt, cytoplasm; and P, promoter.
Supplementary Figure Legend (Gerken et al.)

**Figure S1.** Determination of *fepA*, *fhuA*, and *feo* expression using chromosomally integrated *lacZ* fusions. β-galactosidase activities (shown in Miller units) were measured from at least three independent biological replicates in various genetic backgrounds as shown. Error bars represent standard deviation.

**Figure S2.** Effects of OmrA and OmrB deletion on *fecA* and *fepA* in wild type and EnvZ<sub>R397L</sub> backgrounds. Determination of *fecA* and *fepA* expression was carried out by real-time quantitative PCR (RT-qPCR). Data were obtained from two independent biological samples and two technical replicates. Error bars represent standard deviation.

**Figure S3.** SDS-PAGE analysis of fractions containing OmpR<sub>Hi</sub>s obtained after nickel affinity chromatography. Protein samples were visualized after Coomassie Brilliant Blue staining. Fractions shown in the box were pooled and used for DNA binding studies. The position of OmpR<sub>Hi</sub>s is shown.

**Figure S4.** Determination of *ompC*: *lacZ* activities of constructs carrying two different lengths of the *ompC* promoter regions in front of a promoter-less *lacZ* gene. *ompC145*: *lacZ* and *ompC250*: *lacZ* contain 145 and 250 bp, respectively, of the *ompC* promoter region including ATG. The β-galactosidase activities, in *ompR<sup>+</sup>* or Δ*ompR* backgrounds, were measured from two independent cultures grown to late log phase. Error bars represent standard deviation.

**Figure S5.** Effects of Δ*ompC* and Δ*ompF* gene mutations on the growth of wild type and Δ*aroB* strains. Bacterial growth was monitored on an LBA plate incubated at 37°C for 24 h. The relevant genotypes are shown.

**Figure S6.** Standard curve of ferric chloride solutions of known concentrations. The actual concentrations of ferric chloride solutions were determined by measuring absorbance at 420 nm and then using the formula: molar concentration = A<sub>420</sub>/ε where [Fe]ε is 2.865 mM<sup>-1</sup> Cm<sup>-1</sup>. These values were plotted against the values obtained for the same solutions by electron paramagnetic resonance (EPR) spectroscopy. We used peak-to-peak EPR measurements instead of the double integration values due to a greater confidence of the former at lower ferric chloride concentrations. The standard curve was used to measure iron concentrations in the whole cell.
A

feoABC

(171 bp)

(253 bp)

TTATCAtttcaTTAACA (-294) CCAACAttcgCACACA (-165)

B

ompC

(145 bp)

(250 bp)

C1: TTTACAttttGAAACA; C2: AGCGATaatGAAACA; C3: AAAAGTtttaGTATCA

C1: (-179) C2: (-164) C3: (-158)

C

OmpR_{6His}

|    | -  | +  | -  | +  |
|----|----|----|----|----|
| (feoABC) |   |    |   |    |
| 253 bp   |   |    |   |    |
| 171 bp   |   |    |   |    |

OmpR_{6His}

|    | -  | +  | -  | +  |
|----|----|----|----|----|
| (ompC) |   |    |   |    |
| 250 bp   |   |    |   |    |
| 145 bp   |   |    |   |    |
