A small RNA promotes siderophore production through transcriptional and metabolic remodeling

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Siderophores are essential factors for iron (Fe) acquisition in bacteria during colonization and infection of eukaryotic hosts, which restrain iron access through iron-binding protein, such as lactoferrin and transferrin. The synthesis of siderophores by *Escherichia coli* is considered to be fully regulated at the transcriptional level by the Fe-responsive transcriptional repressor Fur. Here we characterized two different pathways that promote the production of the siderophore enterobactin via the action of the small RNA RyhB. First, RyhB is required for normal expression of an important enterobactin biosynthesis polycistron, entCEBAH. Second, RyhB directly represses the translation of cysE, which encodes a serine acetyltransferase that uses serine as a substrate for cysteine biosynthesis. Reduction of CysE activity by RyhB allows serine to be used as building blocks for enterobactin synthesis through the nonribosomal peptide synthesis pathway. Thus, RyhB plays an essential role in siderophore production and may modulate bacterial virulence through optimization of siderophore production.

enterobactin | iron | RyhB | sRNA

In a mammalian host at neutral pH, iron (Fe) is mostly inaccessible to bacteria, because it is either insoluble in its ferric (Fe^{3+}) form or is bound to host proteins such as serum transferrin, which comprise the first line of host defense against bacterial pathogens (1–3). Thus, to scavenge extracellular Fe^{3+}, many bacteria have developed uptake strategies using high-affinity molecules known as siderophores (4–6). Because they are often critical to survival within the host, many siderophores synthesized by pathogenic bacteria are virulence factors (7, 8).

The archetypal siderophore, called enterobactin, is produced by *Escherichia coli*, *Salmonella enterica*, *Shigella dysenteriae*, and *Klebsiella pneumoniae* species (5). Synthesis of enterobactin (see Fig. S1 for synthesis pathway) depends on 2,3-dihydroxybenzoic acid (DHB) and serine, which are assembled together by the nonribosomal peptide synthesis machinery (9). To synthesize DHB, the primary metabolite shikimate is first converted into chorismate (by AroK, AroA, and AroC), which is then converted into DHB through the action of EntC, EntB, and EntA (10). The final assembly of DHB and serine into enterobactin depends on the action of EntD, EntB, EntE, and EntF (11). Once enterobactin has been synthesized, it is transported through the inner membrane by EntS (12) and the outer membrane by TolC (13). Outside the cell, enterobactin will bind to ferric Fe (Fe^{3+}) with an extremely high affinity (14). Then, Fe-loaded enterobactin complexes are imported into the cell through the outer membrane receptor FepA protein and the TonB energy transducer system found in the cell envelope (15, 16).

When bacterial intracellular Fe levels become sufficiently high, transcription of Fe uptake genes is repressed by the Fur (*F*erric *u*ptake *r*egulator) protein (17–19). Fe-complexed Fur binds to the promoters of a number of genes involved in Fe uptake to repress transcription initiation (20). In contrast, at low Fe concentrations, Fur becomes inactive, which relieves the repression of genes involved in the biosynthesis, export, and import of siderophores.

Although the Fe^{3+}-siderophore import mechanisms are well defined, molecular mechanisms governing siderophore biosynthesis and export are much less characterized (reviewed in refs. 5, 21). The biosynthesis of catecholate siderophores in *E. coli* as well as other medically important bacterial species depends on chorismate, a metabolite produced as part of the shikimate pathway (22). The shikimate pathway is also responsible for synthesis of aromatic amino acids and folic acid, as well as ubiquinone (22). In bacteria, shikimate can either be synthesized de novo or imported from the extracellular environment through the inner membrane-bound permease ShiA (23). We recently reported that the activation of ShiA translation depends on the 90-nucleotide small regulatory RNA (sRNA) RyhB (24). RyhB sRNA is specifically expressed under low Fe conditions through Fur to help the cell adapt to depleted Fe conditions (24–27). As RyhB represses about 20 transcripts encoding abundant Fe-using proteins, an increase in free intracellular Fe level, namely Fe sparing, is observed (27, 28). Remarkably, RyhB also partially regulates *fur* translation (29). However, no physiological consequence of this regulation has ever been reported.

Here, we describe the essential role of RyhB sRNA in the normal production of the siderophore enterobactin through posttranscriptional mechanisms. RyhB expression allows normal levels of *entCEBAH*, which is a critical transcript encoding for part of the siderophore synthesis machinery. In Δ*ryhB* cells, the operon is reduced by a 3-fold factor, which correlates with reduced enterobactin production. In addition, we observed that the gene *cysE*, encoding the enzyme serine acetyltransferase that uses serine in the first step of the pathway necessary to synthesize cysteine, must be repressed through RyhB action to allow siderophore production. Inactivation of *cysE* in a Δ*ryhB* background permits recovery of the siderophore production to WT level. The *cysE* mRNA is the first RyhB target that is not encoding an Fe-using protein. With this work, we demonstrate two essential posttranscriptional mechanisms that were unsuspected in the mechanism of siderophore synthesis.

Results

The sRNA RyhB Is Essential for Siderophore Production in Fe-Limited Conditions. The production of siderophores from *E. coli* K-12 was monitored for WT and Δ*ryhB* cells growing in minimal M63 medium with or without addition of 1 μM $FeSO_4$. In absence of Fe, the WT strain produces a considerable amount of siderophores (Fig. 1*A*, lane 2), which migrates to the same level as

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the purified enterobactin control (lane 1). In contrast, we observed a dramatic decrease in siderophore production in the Δ*ryhB* mutant even in the absence of Fe (Fig. 1*A*, lane 4). This is unexpected because, to our knowledge, almost every gene involved in siderophore biosynthesis and export is known to be regulated at the promoter level by Fur (30). These data indicate that even in low Fe conditions, when Fur is inactive, RyhB has a critical role in biosynthesis and/or secretion of siderophores.

Because the expression of siderophore genes is strongly linked to the Fur regulon, we examined the effect of a Δ*fur* mutation in a Δ*ryhB* mutant. Our results indicate that inactivation of *fur* in a Δ*ryhB* background restores the production of siderophores to a level similar to that of the WT (Fig. 1*A*, compare lanes 2 and 8). Nevertheless, the double Δ*fur* Δ*ryhB* mutant produces significantly fewer siderophores than the Δ*fur* mutant (Fig. 1*A*, compare lanes 6 and 8). This suggests that even in the absence of *fur*, when siderophore biosynthesis genes are fully derepressed, RyhB expression still enhances the production of enterobactin. These results demonstrate a specific role for RyhB in the production of siderophores. Notably in the conditions we used, the growth curves for all cells were comparable with or without Fe supplementation as determined on a bioscreen (Fig. S2).

To investigate the role of RyhB in enterobactin production, we quantified the levels of enterobactin siderophores produced from cells growing in increasing amounts of Fe. To do this, we measured siderophores directly from culture supernatants using liquid chromatography coupled with mass spectrometry (LC–MS). The amounts of siderophores produced during growth without Fe (Fig. 1*B*) are consistent with the data observed by TLC (Fig. 1*A* lanes 2, 4, 6, and 8). When Fe was added at 1 μM in the culture, the WT strain shows reduced siderophore production (Fig. 1*B*), which is consistent with the conventional idea that more Fe enables Fur to shut down *ent* genes. As expected, when RyhB is expressed from a plasmid vector in Δ*ryhB* cells, the production of siderophores becomes similar to that of WT cells (Fig. 1*C*, pRS-c25).

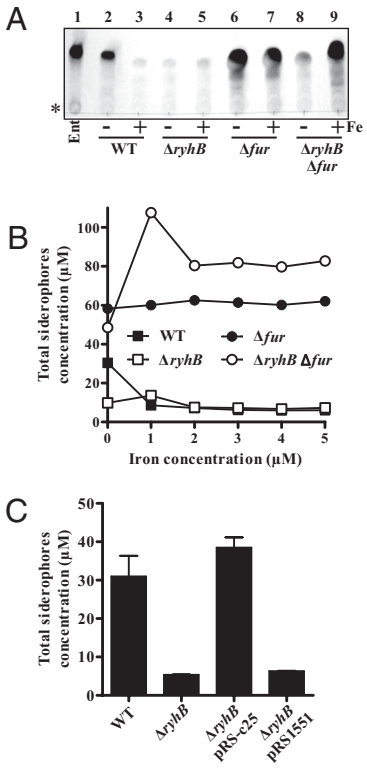

Fig. 1. The sRNA RyhB is essential for siderophore production in Fe-limited conditions. (*A*) Siderophore production as detected by TLC performed on *E. coli* WT, Δ*ryhB*, Δ*fur*, and Δ*ryhB* Δ*fur* strains growing in M63 minimal medium in the absence or presence of 1 μM of $FeSO_4$. The asterisk represents the loading spot on the TLC. (*B*) Determination of siderophore production by LC–MS on strains grown in the presence of increasing amounts of $FeSO_4$ (from 0 to 5 μM). Siderophore concentration at 0 μM Fe: WT (30.4 μM), Δ*ryhB* (9.8 μM), Δ*fur* (58.2 μM), and Δ*fur* Δ*ryhB* (48.5 μM). (*C*) Determination by LC–MS of siderophores produced in WT, Δ*ryhB*, and Δ*ryhB* overproducing RyhB (pRS–c25) or not (empty vector pRS1551).

RyhB Is Essential for Intracellular Fe Homeostasis and Fe Sparing Under Low Fe Conditions. The reduction of siderophores observed in a Δ*ryhB* mutant raises the possibility that, without RyhB, the free intracellular Fe becomes sufficiently elevated to activate the Fur repressor. Thus, we monitored the intracellular concentration of free Fe by using electron-paramagnetic resonance (EPR) on various cells (31). Our results demonstrate that, when Δ*ryhB* cells grow in M63 medium without Fe, they have about 3-fold less free intracellular Fe than WT cells (Fig. 2, compare WT and Δ*ryhB*, without Fe). Therefore, we cannot conclude that siderophore production decreases in the Δ*ryhB* mutant because of increased free Fe and Fur activation. However, this confirms that RyhB increases free intracellular Fe levels in the WT background growing under Fe starvation (Fig. 2, compare columns 1 and 3), demonstrating the Fe-sparing activity of RyhB.

In addition, although the Δ*fur* cells (column 5) have about 4-fold more free intracellular Fe than WT cells (column 1), the double Δ*fur* Δ*ryhB* cells (column 7) have as little free Fe as the Δ*ryhB* cells (column 3) when grown without Fe. This demonstrates the strong effect of RyhB on the levels of free intracellular Fe. However, when 1 μM Fe was added to the culture, both the Δ*ryhB* and the Δ*fur* Δ*ryhB* cells (columns 4 and 8) demonstrated a dramatic increase in free intracellular Fe. In contrast, the WT cells do not show any significant variation whether Fe is present or not (columns 1 and 2), demonstrating the robust mechanism for maintaining intracellular Fe homeostasis when both Fur and RyhB are functional.

RyhB Is Required for Normal Expression of Siderophore Synthesis Genes in Fe-Restricted Medium. As shown in Fig. 2, the free intracellular Fe is dramatically decreased in a Δ*ryhB* mutant as compared with WT under low Fe growth conditions. This low Fe availability leads one to expect that, in the Δ*ryhB* mutant, the Fur repressor must be inactive and Fur-regulated genes are fully derepressed. This was tested by monitoring the mRNA level of several genes involved in siderophore synthesis (*entC*, *entB*, and *entA*), secretion (*entS* and *tolC*), uptake (*fepA*), and transcriptional regulation (*fur*). In fact, the results in Fig. 3*A* clearly indicate that not only *entC*, *entB*, and *entA* genes are not

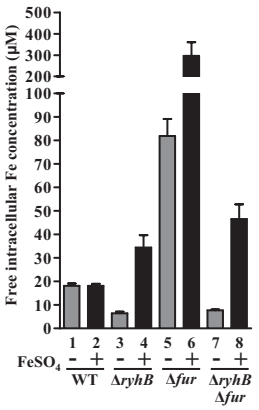

Fig. 2. RyhB is essential for intracellular Fe homeostasis and Fe-sparing under low Fe conditions as determined by EPR. Cells were grown in the absence or presence of 1 μM of $FeSO_4$ until an OD_{600} of 0.9, at which point they were assayed for free intracellular Fe (see *Materials and Methods* for details).

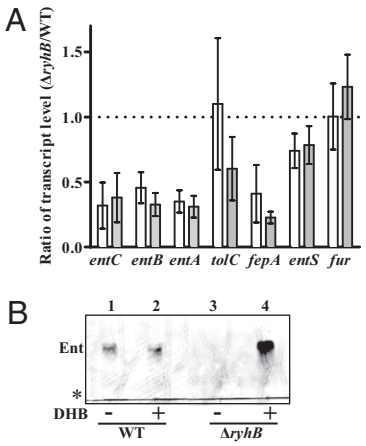

Fig. 3. RyhB is required for normal expression of siderophore synthesis genes in Fe-restricted medium. (*A*) Quantitative RT-PCR (qRT-PCR) showing the ratio of transcript level (Δ*ryhB*/WT) for several mRNAs involved in enterobactin synthesis (*entCEBAH*), enterobactin secretion (*tolC* and *entS*), enterobactin uptake (*fepA*), and transcriptional regulation (*fur*). The transcript levels were determined at OD_{600} of 0.9 (white bars) and 1.2 (gray bars). (*B*) The addition of DHB to the culture medium restores siderophore production specifically in Δ*ryhB* cells. Siderophore production as detected by TLC performed on *E. coli* WT and Δ*ryhB* strains growing in M63 minimal medium in the absence or presence of 5 μM of DHB. The asterisk represents the loading spot on the TLC.

derepressed but they are significantly reduced in the Δ*ryhB* background as compared with WT at OD_{600} of 0.9 and 1.2 (ratio Δ*ryhB*/WT <1). In contrast, both *entS* and *tolC* mRNAs remained equally expressed whether RyhB is present or not (ratio Δ*ryhB*/WT ~1). Although *entC*, *entB*, *entA*, *fepA*, *entS*, and *fur* are Fur-regulated genes, *tolC* expression is independent from Fur. Because *entS* and *fur* are not affected in these conditions, we cannot conclude that all Fur-regulated genes are repressed in Δ*ryhB* cells. Thus, even if intracellular Fe is low enough to inactivate Fur repression, we nevertheless observe significant repression of many Fur-regulated genes in Δ*ryhB* cells.

The previous results in Figs. 2 and 3*A* demonstrate that even though intracellular free Fe is low in a Δ*ryhB* mutant, it is not sufficient to induce Fur-regulated genes. This suggests that Fur is still active under low Fe in a Δ*ryhB* mutant. Indeed, RyhB was previously shown to partially repress *fur* translation (29). Thus, when growing under Fe starvation we expect Fur protein level to be higher in Δ*ryhB* cells as compared with WT cells. However, as shown in Fig. S3, a Western blot performed from cells expressing RyhB (WT) or not (Δ*ryhB*) indicates that the Fur protein levels are similar in Δ*ryhB* background as compared with WT. This demonstrates that RyhB does not affect *fur* translation in WT cells growing under Fe starvation. These data suggest that increased Fe, through the Fe-sparing action of RyhB, plays a key role in activating the expression of enterobactin synthesis genes. We tested this hypothesis by monitoring the effect of Fe on the levels of a number of transcripts in Δ*ryhB* Δ*fur* cells, which should not have any Fe-dependent effectors. As shown in Fig. S4, the mRNA levels of genes related to enterobactin, namely *entCEBAH*, *entS*, and *fepA* are significantly higher in Δ*ryhB* Δ*fur* cells in the presence of 1 μM $FeSO_4$. Thus, when growing under Fe starvation, RyhB-expressing cells will have increased intracellular Fe (Fig. 2, compare columns 1 and 3), which may contribute to the transcriptional activity or transcript stability of genes involved in synthesis of enterobactin.

The *entCEBAH* polycistron is required for the synthesis of DHB, which is one of the building blocks for enterobactin synthesis (Fig. S1). If *entCEBAH* expression is reduced in a Δ*ryhB* mutant, then DHB may not be sufficiently synthesized to allow siderophore production. To investigate this, we supplemented DHB in our Δ*ryhB* culture and monitored siderophore synthesis. As shown in Fig. 3*B*, the addition of 5 μM DHB to WT and Δ*ryhB* cells increases dramatically the production of siderophores specifically in Δ*ryhB* cells. This demonstrates that DHB is limiting in Δ*ryhB* cells, most likely due to reduced expression of siderophore synthesis genes (*entCEBAH*), as shown in Fig. 3*A*.

RyhB sRNA Pairs in Vitro with *cysE* mRNA to Reduce Translation Initiation. Although an explanation for the low *entCEBAH* expression in Δ*ryhB* cells remains elusive, another role for RyhB in regulation of siderophore synthesis was observed. In addition to DHB, the synthesis of enterobactin depends on the availability of the amino acid serine as a substrate. Serine is added to DHB through the nonribosomal peptide synthesis (9) in the final steps of the synthesis pathway to form enterobactin (Fig. S1). By using the bioinformatic tool TargetRNA (32), we observed that the gene *cysE*, encoding serine acetyltransferase, is a potential mRNA target of RyhB (see pairing in Fig. 4*A*). Serine acetyltransferase converts serine to *O*-acetyl-L-serine as the first step in the synthesis of cysteine. Because serine acetyltransferase (CysE) activity could limit the availability of serine for enterobactin synthesis, we reasoned that, under low Fe conditions, RyhB might reduce *cysE* expression to retain sufficient serine for enterobactin assembly. To address this, we used in vitro RNase T1 (cleaves unpaired guanines) and TA (cleaves unpaired adenines) and lead acetate (PbAc, cleaves any unpaired residues) assays for RyhB pairing with *cysE* mRNA. As demonstrated in Fig. 4*B*, RyhB clearly pairs at the ribosome-binding site of *cysE* mRNA. This result suggests that RyhB pairing with *cysE* mRNA inhibits translation initiation. We tested this hypothesis by using in vitro toeprint assays. As shown in Fig. 4*C*, the presence of RyhB (lane 11) clearly blocks the binding of the 30S ribosome subunit on the *cysE* mRNA as compared with *cysE* without RyhB (lane 7). These results strongly suggest that RyhB specifically binds to *cysE* mRNA to reduce translation initiation.

RyhB Directly Reduces CysE Expression in Vivo. We then monitored the *cysE* translation activity by using a protein fusion with the *lacZ* reporter gene in vivo. As shown in Fig. 5*A*, the expression of the *cysE′–′lacZ* translational fusion is significantly reduced in the WT background as compared with Δ*ryhB* cells. This demonstrates that RyhB efficiently reduces CysE protein level, and most likely serine acetyltransferase activity. To confirm pairing in vivo, we expressed either RyhB or the mutated RyhB6 construct from an arabinose-inducible vector and monitored the effect on WT *cysE′–′lacZ* and mutated *cysE6′–′lacZ* fusions (described in Fig. S5). As shown in Fig. 5*B*, the expression of the RyhB6 affects only the complementary *cysE6′–′lacZ* construct without affecting the WT *cysE′–′lacZ* fusion. This demonstrates that RyhB directly represses *cysE* translation in vivo.

Finally, we monitored by quantitative real-time PCR the *cysE* mRNA level after a 10-min pulse expression of RyhB. This pulse expression limits the possible indirect effect of expressing a sRNA. As shown in Fig. 5*C*, the mRNA level of *cysE* is significantly lower in the presence of the sRNA (pBAD–*ryhB*) as compared with a strain without RyhB (pNM12) or a control nontarget mRNA such as *icd* (25).

Reduced CysE Levels in Δ*ryhB* Cells Favor Siderophore Production. The previous results indicated that RyhB reduces *cysE* mRNA and protein levels. This suggests that a high level of CysE is incompatible with siderophore synthesis. To address this, we monitored the siderophore production from a strain carrying the *cysE* gene and endogenous promoter on a multicopy pBR322-derivative plasmid to overproduce CysE. As shown in Fig. 6*A*, the level of siderophore production is significantly less (30%) in the strain

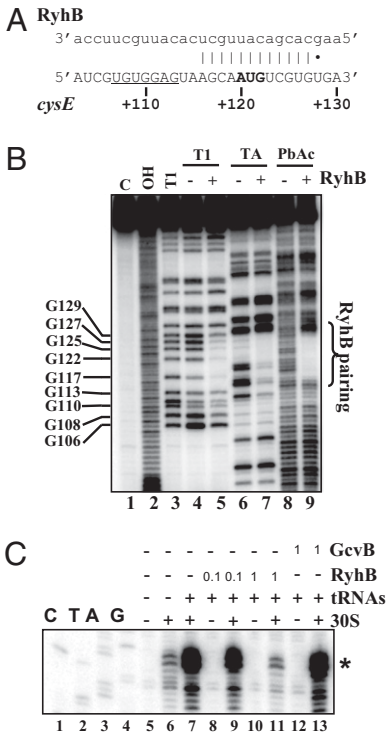

Fig. 4. RyhB sRNA pairs with *cysE* mRNA to reduce translation initiation. (*A*) Potential pairing between the sRNA RyhB and *cysE* mRNA. The ribosome-binding site of *cysE* is underlined and the first AUG codon is in bold. (*B*) In vitro pairing between the sRNA RyhB and 5′-end radiolabeled *cysE* mRNA as determined by RNase TI, RNase TA, and PbAc probing. Lane C is the control 5′-end radiolabeled *cysE* mRNA alone, lane OH is treated with NaOH, and TI is treated with RNase TI in denaturing conditions. The 5′-end radiolabeled *cysE* mRNA was incubated either with RNase TI (lanes 4, without RyhB and 5, with RyhB), RNase TA (lanes 6, without RyhB and 7, with RyhB), or PbAc (lanes 8, without RyhB and 9, with RyhB). The observed pairing between *cysE* and RyhB correlates with the potential pairing as shown in *A*. (*C*) Toeprint assay indicating that RyhB prevents *cysE* translation initiation by blocking the binding of ribosomal 30S subunit on the *cysE* mRNA. The GcvB sRNA was used as a negative control.

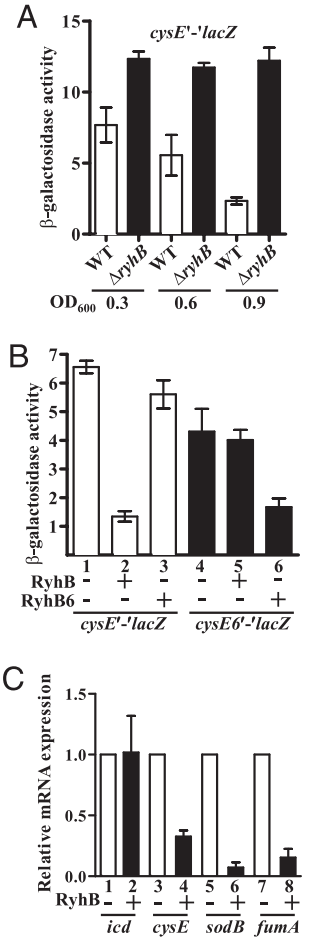

Fig. 5. RyhB directly reduces CysE expression in vivo. (*A*) β-Galactosidase assays of the translational *cysE′–′lacZ* reporter fusion in WT and Δ*ryhB* cells grown in minimal M63 medium without Fe at different OD_{600} (0.3, 0.6, and 0.9). (*B*) The effect of arabinose-induced WT RyhB and mutated RyhB6 on the translational WT *cysE′–′lacZ* and mutated *cysE6′–′lacZ* reporter fusions. See Fig. S5 for details. (*C*) The effect of arabinose-induced RyhB from pBAD-*ryhB* (as compared with the empty vector pNM12) on *cysE* mRNA, previously characterized target mRNAs (*sodB* and *fumA*), and a negative control mRNA (*icd*) as determined by qRT-PCR.

carrying the multicopy *cysE* (pFRΔ–*cysE*) gene as compared with the empty control vector (pFRΔ). We do not expect a full repression of siderophore production because the *ryhB* gene is present in this background. These data suggest that overexpression of CysE reduces the cell's ability to produce siderophores.

If *cysE* expression is too high in the Δ*ryhB* cells, then mutating the *cysE* gene should restore conditions in which siderophores are readily produced. Thus, we monitored siderophore production by a Δ*ryhB* Δ*cysE* double mutant. As expected, the production of siderophore by a Δ*ryhB* Δ*cysE* mutant is restored to normal WT levels (Fig. 6*B*). This shows the importance of keeping the expression of both RyhB and *cysE* in balance for normal siderophore production. Because both Δ*cysE* and Δ*ryhB* Δ*cysE* cells must be supplemented with cysteine to grow, we monitored the effect of the addition of cysteine (125 μM) on the production of siderophores from WT and Δ*ryhB* cells. As shown in Fig. S6, there is no significant effect of the addition of cysteine on the production of the siderophore enterobactin and derivatives.

Discussion

For more than 30 y, the transcriptional regulator Fur has been considered as the sole effector in the synthesis of siderophores (17). We show that posttranscriptional regulation through RyhB sRNA is equally important. RyhB takes part in two distinct pathways, both of which essential for siderophore production. One of these mechanisms relies on RyhB repression of the serine acetyltransferase CysE (see model in Fig. S7), which represents the first RyhB-repressed target mRNA that does not encode for an Fe-using protein. Repression of CysE potentially remodels the amino acids metabolism to increase the serine flux into the siderophore synthesis pathway to the detriment of the cysteine pathway (Figs. S1 and S7). Indeed, when serine is added to the medium, it partially suppresses the Δ*ryhB* phenotype and stimulates siderophore production (Fig. S6). Moreover, addition of high levels of cysteine in the medium also increases the siderophore production, probably by negative feedback (33), which results in reduction of CysE enzyme activity (Fig. S6).

The down-regulation of *cysE* transcript by RyhB is clearly not as strong as other target mRNAs (compare *cysE* mRNA levels with *sodB* or *fumA* in Fig. 5*C*). Also, although it is repressed by RyhB, the translational *cysE′–′lacZ* fusion in WT cells is still significantly active as compared with the Δ*ryhB* cells in midlog growth phase (Fig. 5*A*, OD_{600} of 0.3 and 0.6). This suggests that partial *cysE* repression by RyhB is preferred over classical full repression as observed with *sodB* and *fumA* target mRNAs (Fig. 5*C*). Because the CysE enzyme is essential for cysteine synthesis in minimal medium, we do not expect full repression of *cysE* by

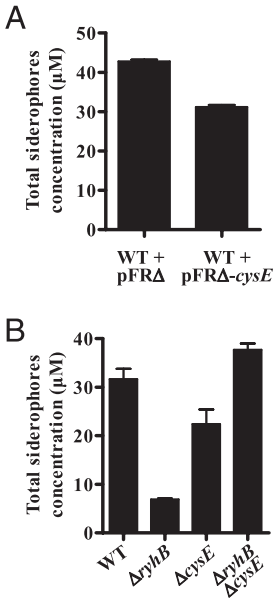

Fig. 6. RyhB reduces CysE levels to favor siderophore production. (*A*) Total siderophore production as measured by LC–MS in strains overproducing the CysE enzyme (pFRΔ–*cysE*) or not (empty vector pFRΔ). (*B*) Total siderophore production as measured by LC–MS in WT, Δ*ryhB*, Δ*cysE*, and Δ*ryhB* Δ*cysE* strains (see *Materials and Methods* for description).

RyhB. Additionally, because CysE does not encode an Fe-using protein, the RyhB-induced repression may not benefit from rapid full repression (<3–5 min) as observed with other target mRNAs encoding Fe-using proteins such as *sodB* and *fumA* (26). This type of repression prioritization of specific target mRNAs over other targets of the same sRNA reflects the powerful genetic modulation achieved by a sRNA.

An unexpected function of RyhB was suggested by our results in Fig. 3, which indicate that RyhB contributes to normal expression of transcripts involved in siderophores synthesis (*entCEBAH*) and uptake (*fepA*). This suggests a second mechanism in which RyhB contributes to siderophore production. Although the precise mechanism is unclear, our data indicate that RyhB-induced intracellular Fe level, namely Fe sparing, is central to this. Because many transcripts are affected independently from their cellular functions, this suggests a general effect of low intracellular Fe on gene expression. To analyze this further, we tested the effect of Fe alone on the double Δ*fur* Δ*ryhB* mutant, which should not have any Fe-dependent effector on mRNA expression or stability. As shown in Fig. S4, the presence of Fe greatly induces the expression of a number of genes, many of which involved in synthesis (*entB* and *entC*), secretion (*entS*), or uptake (*fepA*) of enterobactin. This result corroborates our siderophore analysis. Although the double Δ*fur* Δ*ryhB* mutant growing without Fe produces a fair amount of siderophore (Fig. 1 *A*, lane 8, and *B*), the addition of Fe stimulates by a 2-fold factor the production of siderophore (Fig. 1 *A*, lane 9, and *B*). These results indicate the essential role of RyhB in increasing intracellular Fe level to improve cellular function.

Indeed, the low intracellular Fe in Δ*ryhB* cells may reduce the activity of Fe-dependent enzymes involved in the shikimate–chorismate pathway (Fig. S1). However, we demonstrate that the activity of Fe-dependent aldolases (34), encoded by *aroFGH*, are not affected in our experimental growth conditions without Fe (Fig. S8) and remain fully active whether RyhB is present (WT) or not (Δ*ryhB*). It is therefore unlikely that the aldolases are involved in reduced expression of siderophores. Furthermore, despite the reduced intracellular Fe in Δ*ryhB* cells (Fig. 2, compare WT and Δ*ryhB* without Fe), this shows that not all Fe-dependent enzymes will be affected.

Remarkably, both Fur and RyhB regulate the siderophore production at different levels. Thus, one can question which gene is epistasic to the other in this system. Simply put, the *ryhB* gene expression depends on Fur, which depends on the Fe level in the medium. Our results, however, suggest that more factors than Fur and RyhB regulate the system. As shown in Fig. 1*A*, even in the absence of Fur and RyhB (Δ*ryhB* Δ*fur* background), Fe alone is sufficient to affect the system (lanes 8 and 9). These results add to the interpretation that siderophore production depends on more than just transcription activation in the absence of Fe. Three factors must be taken into account: (*i*) Fur inactivation, (*ii*) RyhB expression, and (*iii*) sufficient free intracellular Fe level (Fe sparing).

The measurements of free intracellular Fe by EPR (Fig. 2) demonstrate that both Fur and RyhB are needed to maintain a robust Fe homeostasis despite significant environmental Fe variation. The most significant results of this experiment are that Δ*ryhB* cells have 60% reduced free Fe [Fig. 2, lane 3 (6 μM) vs. lane 1 (18 μM)] as compared with WT. This is a unique demonstration that endogenous RyhB effectively generates free Fe in WT cells grown under Fe starvation. However, it is not clear why intracellular Fe becomes so high as compared with WT when Fe is available [Fig. 2, lane 4 (Δ*ryhB* + Fe) compared with lane 2 (WT + Fe)]. In addition, these results show that an important part of the free Fe levels in a Δ*fur* mutant depends on the action of RyhB [Fig. 2, compare lane 6 (Δ*fur* + Fe) with lane 8 (Δ*fur* Δ*ryhB* + Fe)]. Remarkably, as shown in Fig. S3 we did not reproduced previously published data suggesting that RyhB partly represses *fur* translation (29). We explain this by different experimental procedures used in our analysis (Fe-deprived medium) as compared with the previous analysis (addition of the Fe chelator 2,2′-dipyridyl in the medium).

This paper demonstrates that a single sRNA can act as a global regulator by adjusting simultaneously the cellular gene network and metabolism. Our study shows a unique role for RyhB extending beyond regulation of Fe-storage proteins and now includes modulation of metabolic pathways such as serine catabolism through down-regulation of *cysE* and modification of transcription of genes involved in enterobactin production (*entCEBAH*). By adjusting both gene expression and metabolic activity, the sRNA enables the cell to optimize to severe environmental changes. With these results in view, we should expect additional sRNAs conducting similar subtle metabolic adjustments that drive crucial cellular functions.

Materials and Methods

Analysis of Enterobactin Production by TLC. Enterobactin was extracted and visualized according to a previous report (12). Cells were grown in M63, 0.2% glucose, from a 10-fold or 100-fold dilution of an overnight culture in the same media. Depending on the experiment, 33 μM of DHB or 1 μM of $FeSO_4$ were added to the media. At an OD_{600} of 0.9, 4 mL of culture was pelleted. The supernatant was acidified with 25 μL of 10N HCl and extracted twice with a total of 4 mL of ethyl acetate. Aqueous phases were combined and dried in 874-μL aliquots in a SpeedVac Concentrator (Savant Instruments, SVC100). Extract residues were resuspended in 40 μL of methanol and 10 μL was spotted onto 250-μM layer-flexible (20 × 20 cm) PE SIL G/UV254 plates (Whatman). For some experiments, 25 μL of enterobactin (EMC Microcollections) was spotted as a control onto the plates. Plates were developed with benzene:glacial acetic acid:water (125:72:3 vol/vol/vol) in a closed chamber. Plates were then removed from the chamber and allowed to dry, then immersed briefly in 0.1% $FeCl_3$ to visualize Fe-binding compounds.

EPR Analysis of Whole Cells. Cells were grown overnight at 37 °C in M63 glucose medium, diluted 1:10 into 250 mL of freshly prepared M63 0.2% glucose ±1 μM of $FeSO_4$ and then grown at 37 °C in 1-L baffled flasks with vigorous shaking to an OD_{600} of 0.9. Cells were then harvested and prepared for EPR analysis as described in previous studies (28, 35), with the exception that M63 medium with 0.2% glucose was used in place of LB during the incubation of cells with DTPA and desferrioxamine.

Enzymatic and Chemical Probing of RyhB Interaction with *cysE* mRNA. Enzymatic and chemical probing experiments were performed as described earlier (36). Briefly, 50 pmol of *cysE* mRNA (transcribed from a PCR product—oligos EM1056–EM1153) was labeled using T4 polynucleotide kinase (New England Biolabs). Then, 0.1 μM of 5′-end radiolabeled *cysE* was incubated 15 min at 37 °C in the absence or in the presence of 1.6 μM RyhB RNA (transcribed from a PCR product—oligos EM88–EM89). Then, RNase T1 (0.05 U) (Ambion), or RNase TA (0.025 U) (Jena Bioscience), or PbAc (10 mM) (Sigma-Aldrich) were added to the reaction and the incubation continued for 2 min. Reactions were stopped by adding 10 μL of loading buffer II (Ambion). Samples were then separated on a 6% polyacrylamide/7 M urea gel.

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