Fe-S clusters are cofactors conserved through all domains of life. Once assembled by dedicated ISC and/or SUF scaffolds, Fe-S clusters are conveyed to their apo-targets via A-type carrier proteins (ATCs). Escherichia coli possesses four such ATCs. ErpA is the only ATC essential under aerobiosis. Recent studies reported a possible regulation of the erpA mRNA by the small RNA (sRNA) RyhB, which controls the expression of many genes under iron starvation. Surprisingly, erpA has not been identified in recent transcriptomic analysis of the iron starvation response, thus bringing into question the actual physiological significance of the putative regulation of erpA by RyhB. Using an sRNA library, we show that among 26 sRNAs, only RyhB expresses the presence of an erpA-lacZ translational fusion. We further demonstrate that this repression occurs during iron starvation. Using mutational analysis, we show that RyhB base pairs to the erpA mRNA, inducing its disappearance. In addition, IscR, the master regulator of Fe-S homeostasis, represses expression of erpA at the transcriptional level when iron is abundant, but depleting iron from the medium alleviates this repression. The conjunction of transcriptional derepression by IscR and post-transcriptional repression by RyhB under Fe-limiting conditions is best described as an incoherent regulatory circuit. This double regulation allows full expression of erpA at iron concentrations for which Fe-S biogenesis switches from the ISC to the SUF system. We further provide evidence that this regulatory circuit coordinates ATC usage to iron availability.

The ISC system functions under stress-inducing conditions, such as oxidative stress and iron scarcity (6, 7). The current model is that ATC diversity provides different transferral routes from the assembly machineries to the apoproteins as a function of environmental conditions and, possibly, targets to be matured. Remarkably, ErpA is the only essential ATC when E. coli grows under aerobic conditions because it transfers an Fe-S cluster to the IspG/H proteins that catalyze the synthesis of the essential isoprenoid precursors (10). However, the requirement of ErpA for maturation of IspG/H can be bypassed when the demand for Fe-S clusters is lowered. Thus, under anaerobiciosis, IscA can also mediate Fe-S transfer to IspG/H, making both ErpA and IscA functionally redundant under such conditions. Meanwhile, under iron limitation SufA was proposed to be the only ATC able to ensure Fe-S cluster transfer to IspG/H (11). Hence, starting from a set of biochemically related ATCs, the cell appears to use genetic regu-
lation likely to exploit functional ATC specialization that has not been revealed by biochemical studies. In particular, one might surmise that under iron limitation, it would be counterproductive to produce functionally redundant iron-using ATCs.

In *E. coli*, the major regulator of Fe-S homeostasis is the IscR transcription factor, which itself contains a [2Fe-2S] cluster (12–14). IscR is encoded by the first gene of the *iscRSUA* operon. Both apo and holo forms of IscR are active as transcriptional regulators; however, the IscR cluster ligation state modifies the DNA recognition properties (15, 16). Holo-IscR binds and represses expression at so-called type 1 promoters, which include the *isc* operon and the *erpA* and the *nfuA* promoters. Type 2 promoters can be bound by both apo- and holo-IscR. In particular, the *suf* promoter is a type 2 promoter at which IscR binds and activates transcription (7). Thus, IscR represses expression of the *iscRSUA* operon when Fe-S demand is fulfilled, while it activates expression of the *suf* operon under conditions that are detrimental for Fe-S formation, such as oxidative stress or iron scarcity.

The regulators Fur and RyhB also play an important role in controlling Fe-S biogenesis (17). Fur, the iron sensor regulator, represses transcription of several genes involved in iron import and metabolism when it is bound to Fe (18). Fur also represses expression of the noncoding RNA (small RNA [sRNA]) RyhB. Under iron limitation, Fur repression is alleviated, and RyhB is synthesized and regulated, mostly negatively, the expression of more than 50 genes, the majority of which encode iron-containing proteins (19). Thus, RyhB is thought to reallocate bioavailable iron to essential targets, helping the cell to cope with iron scarcity (20). RyhB base pairs to the *iscRSUA* mRNA upstream of the *iscS* gene, inducing the degradation of the 3′ part of the mRNA, while the 5′ part containing *iscR* remains stable (17). In this way, RyhB favors the use of the Suf system during iron starvation. Notably, Fur also represses the *suf* operon, encoding the Suf system, thus contributing to switching from ISC to Suf under those conditions (21).

RyhB was recently predicted to base pair to the *erpA* mRNA, and the *erpA* mRNA was copurified with MS2-tagged RyhB (22, 23). Surprisingly, while regulation of *erpA* by RyhB seemed very likely, global transcriptomic and deep sequencing approaches have failed to identify *erpA* as a target of RyhB (20, 24). Therefore, the question of the physiological regulation of *erpA* by RyhB remained to be addressed. We first here definitely established that RyhB indeed regulates *erpA* expression by base pairing to its mRNA and inducing its disappearance. We further show that IscR represses expression of *erpA* under opposite conditions than those of RyhB and that alleviation of repression by IscR masks the effect of RyhB under severe iron-limited conditions. Thus, these regulators form an incoherent circuit that controls *erpA* expression as a function of iron availability. The net outcome is a bimodal behavior, which culminates in the induction of *erpA* expression in a narrow range of iron concentrations. While establishing a case of a mixed circuit, including both an sRNA and a transcriptional regulator, this study provides a framework to describe how *E. coli* exploits the biochemical properties of ATCs while preventing redundancy to ensure Fe-S cluster delivery throughout a broad range of iron concentrations.

**RESULTS**

**RyhB represses *erpA* expression.** We first wanted to test if expression of *erpA* was regulated by RyhB and/or other sRNAs. To do so, we used a library of plasmids, containing 26 different *E. coli* sRNAs under the control of a *P lac* promoter, to test the effect of overexpressing these sRNAs on a *P Rad-erpA-lacZ* fusion (Fig. 1A) (25, 26). This fusion was placed under an inducible promoter to avoid the indirect effect of overexpressing the sRNAs on transcription of the fusion. Only the overexpression of RyhB had a significant effect by inducing a 2-fold repression of the activity compared to the empty vector (Fig. 1A). The same 2-fold-repressing effect of RyhB on the fusion could also be observed when cells were grown in culture flasks (Fig. 1B, left). In agreement with previous studies, these data indicated that RyhB was the only sRNA involved in *erpA* regulation.

Under physiological conditions, RyhB is normally expressed under iron-limiting conditions due to loss of Fur regulation (19). As regulation of *erpA* expression has been tested only through
overexpression of the sRNA (22), we tested if iron starvation repressed activity of the \( P_{\text{RAD}} \)-\( \text{erpA-lacZ} \) fusion in a RyhB-dependent manner. To do so, wild-type (WT) or \( \text{ryhB} \) mutant cells containing the fusion were grown in the presence or absence of 250 \( \mu M \) 2,2’-dipyridyl (DIP), a routinely used iron chelator, before measurement of \( \beta \)-galactosidase activity (Fig. 1B, right). No significant difference could be seen between the WT and the \( \text{ryhB} \) mutant in the absence of iron chelation. In contrast, the activity of the fusion was diminished by 2-fold when the WT strain was grown in the presence of 250 \( \mu M \) DIP but was unaltered in the \( \text{ryhB} \) mutant (Fig. 1B). We thus concluded that RyhB represses the activity of \( P_{\text{RAD}} \)-\( \text{erpA-lacZ} \) during iron starvation.

**RyhB base pairs to the \( \text{erpA mRNA} \).** If RyhB’s regulation of \( \text{erpA} \) expression is direct, base pairing between both \( \text{erpA} \) and RyhB transcripts is expected. We predicted a large base pairing involving almost all of the first 65 nucleotides (nt) of RyhB and 57 nt of the \( \text{erpA} \) mRNA using the Mfold program (Fig. 2A). Interestingly, this base-pairing region encompasses the ribosome binding site (RBS) and the AUG start codon of \( \text{erpA} \). A similar prediction had been proposed by Wright et al. using the target prediction program CopraRNA (22).

We introduced a series of five mutations in the \( \text{erpA-lacZ} \) fusion, named \( \text{mut1} \) to \( \text{mut5} \), predicted to disrupt the base pairing depicted above (Fig. 2A). Overexpression of RyhB failed to repress activity of \( \text{erpA}_{\text{mut2}} \) and \( \text{erpA}_{\text{mut4}} \) alleles but repressed expression of the 3 other fusion variants (Fig. 2B). This result indicated that while extensive base pairing can be predicted between the two RNAs, only the region of base pairing close to the translation initiation region seems required for effective regulation.

Next, we introduced a mutation in the plasmid carrying the \( \text{ryhB} \) gene such that it would disrupt base pairing with the WT \( \text{erpA} \) mRNA but restore complementarity to the \( \text{erpA}_{\text{mut3}} \) allele. The mutated allele of \( \text{ryhB} \) repressed (1.5-fold) the \( \text{erpA}_{\text{mut3-lacZ}} \) fusion, while it did not repress the activity of the WT \( \text{erpA-lacZ} \) fusion (Fig. 2C). We concluded that RyhB represses expression of \( \text{erpA} \) directly by RNA/RNA base pairing close to the \( \text{erpA} \) translation initiation region.

**Overexpression of RyhB induces the disappearance of the \( \text{erpA} \) mRNA.** In many cases, RyhB induces the degradation of its mRNA targets through the recruitment of RNase E (27). We thus performed Northern blotting experiments to test the effect of RyhB overexpression on the \( \text{erpA} \) mRNA. The \( \text{pRyhB} \) plasmid or the empty vector was transformed into a \( \text{ryhB} \)-deletion strain. Strains were grown to an optical density at 600 nm (OD\(_{600}\)) of 0.5, at which point synthesis of RyhB was induced with 100 \( \mu M \) isopropyl-\( \beta \)-D-thiogalactopyranoside (IPTG). The production of RyhB induced a rapid disappearance of the \( \text{erpA} \) mRNA (Fig. 3), while it remained stable in the strain transformed with the empty vector. Altogether, these data allowed us to conclude that RyhB binding induces a disappearance of the \( \text{erpA} \) mRNA, most likely by destabilization.

---

**FIG 2** RyhB base pairs to the \( \text{erpA mRNA} \). (A) Base pairing predicted between RyhB and the \( \text{erpA mRNA} \). Nucleotides belonging to RyhB are represented on top, and the ones corresponding to the \( \text{erpA mRNA} \) sequence are represented on the bottom; positions relative to the RyhB or \( \text{erpA} \) transscriptional start site are indicated above or below the sequences, respectively. The putative ribosome binding site and the AUG start codon of \( \text{erpA} \) are represented by the pink and blue boxes, respectively. Mutations introduced in the \( \text{erpA mRNA} \) used in panel B are denoted with arrows. (B) Cells containing either the WT \( P_{\text{RAD}} \)-\( \text{erpA-lacZ} \) fusion or the mutated versions of the fusion were transformed with the empty plac vector (dark gray bars) or the \( \text{pRyhB} \) plasmid (light gray bars) and grown in LB containing 100 \( \mu g/ml \) ampicillin, 0.02% arabinose, and 100 \( \mu M \) IPTG for 6 h, after which \( \beta \)-galactosidase activity was measured. (C) Cells containing either the WT \( P_{\text{RAD}} \)-\( \text{erpA-lacZ} \) fusion (left set of bars) or the \( \text{mut3} \) mutated version of the fusion (right set of bars) were transformed with the empty plac vector (light gray bars), the \( \text{pRyhB} \) plasmid (dark gray bars), or the \( \text{pRyhB-mut3} \) mutant (black bars), and \( \beta \)-galactosidase activity was measured as described for panel B. Each point represents the mean from 8 or more experiments; error bars represent standard deviations.
RyhB and IscR cancel each other’s effect during severe iron starvation. In our previous experiments, expression of the erpA-lacZ fusion was driven by an inducible P_BAD promoter. As the erpA promoter has previously been shown to be transcriptionally repressed by holo-IscR (15), we wanted to study erpA expression when both IscR and RyhB regulation can act concomitantly. Hence, we designed a new erpA-lacZ fusion, which contained the RyhB binding region and the endogenous erpA promoter, including the previously described IscR binding site. The resulting P_erpA-lacZ-containing strain was grown in LB with or without 250 μM DIP.

In striking contrast to the P_BAD-driven fusion that was repressed by RyhB when cells were treated with 250 μM DIP, the same treatment did not result in any significant change in activity of the P_erpA-lacZ fusion (Fig. 4A). However, deleting ryhB increased P_erpA-lacZ expression upon DIP treatment, confirming that RyhB repressed expression of erpA during iron starvation. Furthermore, deleting iscR from the chromosome increased the activity of the P_erpA-lacZ fusion-containing strain grown under iron-replete conditions (Fig. 4A), confirming repression of erpA by IscR. As expected, deleting both iscR and ryhB resulted in maximal expression of the fusion in the presence or absence of DIP. We thus hypothesized that, in the presence of DIP, IscR alleviation of repression at P_erpA was canceled by posttranscriptional repression by RyhB.

To test this hypothesis, we constructed two new mutated versions of the P_erpA-lacZ fusion by introducing cis-acting mutations in its control region. The first allele had an altered IscR binding site following modification of nucleotides −19 to −21 (GGG to CCC) (16). Activity of this fusion, named P_erpA(iscR<sup>ind</sup>) (for IscR independent), was only modestly augmented upon deletion of iscR (Fig. 4B), confirming that the mutation had severely affected the ability of IscR to repress the P_erpA-lacZ fusion promoter. Expression of the P<sub>_erpA(iscR<sup>ind</sup>)-lacZ</sub> fusion was decreased by 2-fold in WT or iscR mutant cells grown in the presence of 250 μM DIP. However, this effect was completely abolished when a ryhB mutation was introduced in both of these strains. Thus, a cis mutation preventing IscR repression clearly revealed RyhB-mediated regulation of erpA under iron-limiting growth conditions.

The second allele had its RyhB binding site containing the mut3 mutation described above and was named P<sub>_erpA(ryhB<sup>ind</sup>)</sub> (for RyhB independent) (Fig. 2A). Activity of this fusion was enhanced 2-fold upon addition of 250 μM DIP both in the wild type and the ryhB mutant (Fig. 4C). This indicated that P<sub>_erpA(ryhB<sup>ind</sup>)</sub> was not regulated by RyhB. However, expression of this fusion was still regulated by IscR, as deleting iscR yielded a 2-fold increase whether DIP was added or not.

Altogether, these results showed that under severely iron-limited conditions, RyhB-mediated repression is compensated by alleviation of IscR repression.
Coregulation by RyhB and IscR permits expression of erpA in a defined range of iron concentrations. The dual regulation by RyhB and IscR of erpA reminded us of an incoherent circuit in which a common signal gives rise to two antagonistic effects on one target gene (28). Such circuits have been shown to provoke bimodal expression of genes that peak at certain concentrations of inducer (29). We thus aimed at identifying if there was a given concentration of iron that would favor maximal expression of erpA by canceling both IscR and RyhB repression. To this end, cells containing the wild-type P\textsubscript{erpA}-lacZ fusion were grown in LB supplemented with increasing concentrations of DIP, from 0 to 300 \(\mu\)M. The ryhB, iscR, and ryhB iscR isogenic mutants were likewise assayed, and the results are presented in Fig. 5.

In the WT strain, P\textsubscript{erpA}-lacZ expression increased with DIP concentration from 0 to 150 \(\mu\)M, after which expression of the fusion went down. At 300 \(\mu\)M DIP, the activity of the fusion was similar to that of untreated cells (Fig. 5). The ryhB mutant profile was identical to that of the WT strain for concentrations of DIP of \(\leq 150\) \(\mu\)M. In contrast to the WT strain, though, in the ryhB mutant, expression of the P\textsubscript{erpA}-lacZ fusion remained high at elevated DIP concentrations (Fig. 5).

In the iscR mutant, P\textsubscript{erpA}-lacZ expression was maximal in the absence of DIP and decreased above 100 \(\mu\)M DIP to reach minimal WT-like levels (Fig. 5). In the double iscR ryhB mutant, P\textsubscript{erpA}-lacZ expression levels were at their maximum throughout all DIP concentrations tested.

To confirm these results, we performed Northern blotting experiments on the erpA mRNA by growing the cells either in the absence of DIP or with 150 \(\mu\)M or 300 \(\mu\)M DIP (Fig. 6). In a manner parallel to the P\textsubscript{erpA}-lacZ fusions described above, expression of erpA peaked in the presence of 150 \(\mu\)M DIP (1.5-fold compared to that in the absence of DIP) and remained minimal both in untreated cells and in cells treated with 300 \(\mu\)M DIP. In the ryhB mutant, erpA mRNA levels were similar to that of the WT strain at DIP concentrations of \(\leq 150\) \(\mu\)M but increased significantly at 300 \(\mu\)M DIP (2.5-fold higher than in untreated cells). In the iscR mutant strain, expression was maximal without DIP (2.5-fold higher than in the WT strain under the same conditions) and then decreased at 150 \(\mu\)M and 300 \(\mu\)M DIP. Of note, the regulatory effects seen on erpA mRNA levels are greater in Northern blotting experiments than with the translational fusion. This suggests that additional elements in the mRNA not present in the lacZ fusion may determine its degradation rate. An alternative explanation is that, in the \(\beta\)-galactosidase assays, the time after addition of DIP was too short to allow the preexisting pool of \(\beta\)-galactosidase to be diluted out by cell growth, masking the true extent of the effects.

Altogether, these results showed that the double IscR/RyhB coregulation allows maximal expression of erpA at concentrations of DIP ranging from 100 to 200 \(\mu\)M, with a peak at 150 \(\mu\)M DIP. At DIP concentrations lower than 100 \(\mu\)M, IscR represses erpA transcription, while at DIP concentrations above 200 \(\mu\)M, erpA expression is repressed by RyhB.

Coregulation by IscR and RyhB is linked to ATC functional redundancy. Previous work from our laboratory has shown that depending upon the growth conditions, defects in one ATC can be compensated by expression of another one (11). We thus hypothesized that the double RyhB/IscR regulation may serve to repress expression of erpA when other ATCs are present to sustain Fe-S delivery. Previous genetic analysis has revealed that SufA is used under iron-limiting conditions (21). Therefore, we predicted that allowing erpA expression under these conditions would allow cells to grow even in the absence of sufA.

To test this, we used erpA alleles containing the previously described \textit{cis} mutations that hampered either IscR or RyhB regulation, i.e., erpA (RyhB\textsuperscript{ind}) and erpA (IscR\textsuperscript{ind}). After overnight growth in LB, cells were inoculated in fresh medium containing increasing concentrations of DIP (from 0 to 300 \(\mu\)M), and growth was followed for 14 h. We first did a control experiment by testing growth of strains carrying the erpA(RyhB\textsuperscript{ind}) or the erpA(IscR\textsuperscript{ind}) allele. Introducing either of the erpA alleles had no significant effect on growth of the WT strain (see Fig. S1 in the supplemental material).

Introducing the sufA mutation in either the erpA\textsuperscript{ind} or erpA(IscR\textsuperscript{ind}) strain severely affected growth when DIP was present at concentrations higher than 150 \(\mu\)M (Fig. 7A and B). Strikingly, introducing the erpA(RyhB\textsuperscript{ind}) allele in the sufA mutant almost

FIG 5 RyhB and IscR allow full expression of erpA in a defined iron concentration range. The strains containing the P\textsubscript{erpA}-lacZ fusion and its ryhB, iscR, and ryhB iscR isogenic derivative mutants were grown for 6 h in various concentrations of DIP before cells were lysed and \(\beta\)-galactosidase activity was measured. Results are expressed in arbitrary units that were empirically determined to be approximately equivalent to Miller units; each point represents the mean from 8 or more experiments; error bars represent the 95% confidence intervals of the means.

FIG 6 Expression of the erpA mRNA peaks at intermediate iron concentration. The MG1655 WT strain and its ryhB and iscR mutant derivatives were grown in LB containing either 0, 150, or 300 \(\mu\)M DIP to an \(\text{OD}_{600}\) of 0.6 before samples were prepared. Total RNA was extracted and submitted to a Northern blotting experiment with probes directed against the erpA mRNA, RyhB. The numbers below the top panel represent the relative fold changes compared to the 0-h time point in the WT strain. Quantification was made using the 16S RNA as a loading control. The gel depicted here is representative of 3 independent experiments.
In the present study, we show that both the sRNA RyhB and the transcriptional regulator IscR repress expression of erpA, encoding the ATC transporter of Fe-S clusters essential under aerobic conditions. Interestingly we show that RyhB and IscR act under opposite conditions in regard to Fe concentration: IscR represses erpA when iron is abundant (a condition under which RyhB is not expressed), while RyhB represses erpA when iron is low (a condition under which IscR repression is alleviated). Under severely iron-depleted conditions (i.e., at high DIP concentrations), post-transcriptional repression by RyhB is compensated for by an increased transcription of the promoter that is no longer bound by holo-IscR. This phenomenon may provide an explanation of why regulation of erpA by RyhB was not found in previous, even recent, global transcriptomic approaches (20, 24). We propose that this mechanism enables expression of erpA when it is most needed and turns it down when other, functionally redundant ATCs are able to sustain Fe-S protein maturation.

The regulatory mechanism that we unraveled here for expression of erpA is best described as an “incoherent” circuit (30, 31). In such a circuit, a common signal generates two antagonistic effects (i.e., activation and repression) on the expression of a target gene (Fig. 8A) (32). Here, Fe concentration acts as a common signal perceived by both IscR and RyhB, with two antagonistic outcomes on the expression of erpA: Fe deprivation yields to RyhB repression and to induction by alleviation of holo-IscR repression (Fig. 8B).

Incoherent circuit motifs have been shown to drive nonmonotonic responses, also called biphasic responses, to an inducer (28). Consistently, erpA levels are not linearly correlated with Fe levels but instead peak within a narrow iron concentration range (Fig. 5 and 6). To our knowledge, only one other naturally occurring incoherent circuit giving rise to a biphasic response has been documented in E. coli: that of the galKETK operon, which is dually regulated by cyclic AMP (cAMP) receptor protein (CRP) and GalS (29). In this case, gal expression peaks at intermediate levels of the signal cAMP, but the physiological advantage of this peak remains elusive.

The biphasic regulation of erpA expression can be appreciated within the context of Fe-S cluster homeostasis throughout iron concentration fluctuation, as this occurs in natural settings. E. coli has two systems, ISC and SUF and a series of ATCs, which make and deliver Fe-S clusters to target proteins, respectively. Over 100 Fe-S cluster-containing proteins are predicted to arise in E. coli, making Fe-S cluster-based processes high Fe consumers. Previous work showed that ISC does not function at low iron concentrations while SUF is not synthesized at high iron concentrations (7). For instance, our work and that of Outten et al. (21) show that suf mutants are impaired in growth at DIP concentrations of ≥200 µM, strongly suggesting that this constitutes a turning point in the ISC-to-SUF utilization (Fig. 7C and reference 21). Strik-
ingly, erpA expression is maximized precisely at those same DIP concentrations (150 μM to 200 μM) where cells shift from ISC to Suf, as a result of the regulation that we here deciphered (Fig. 5). Thus, biphasic expression of erpA explains how E. coli coordinates ATC usage. Indeed, a cis mutation abrogating RyhB-mediated inhibition of ErpA was sufficient to compensate for the absence of SufA under severe iron limitation. This demonstrates that, under such iron limitation, both SufA and ErpA can be equally efficient ATCs but that the cell ought to use only one to optimize utilization of the scarce iron available. To do so, the cell relies on the IscR/ RyhB dual control here described.

We note that neither IscR nor RyhB repression of erpA expression is total and that, at any given iron concentration, production of erpA is maintained (Fig. 5). This is most likely because ErpA is essential under aerobic conditions to sustain Fe-S delivery to its essential targets IspG/H. Interestingly, a recent ribosome profiling study has shown that under iron-sufficient conditions ErpA is present at relatively high levels in the cell (33). We propose that this regulatory circuit results from an evolutionary tradeoff between the essentiality of ErpA and the need to limit its expression under conditions for which other ATCs are at least partially redundant for target maturation. ErpA being present at a high level in the cell, it is likewise a high Fe user. Therefore, it seems reasonable to propose that one of its negative regulators, presumably RyhB, appeared first during evolution to avoid unnecessary Fe utilization. However, as ErpA became essential under aerobic conditions, a second opposing regulator, IscR, was incorporated to counter the effect of the first, thus allowing the two constraints to be met: sparing iron at the cellular level and filling in the ErpA essential function.

Remarkably, the regulatory circuit behind erpA expression is a mixed regulatory circuit, comprising a transcriptional regulator and an sRNA. The mixed composition provides E. coli with a series of advantages. First, the recruitment of the sRNA RyhB is critical in providing “incoherence” to the circuit. Indeed, repression under low-iron conditions could not have been achieved by Fur and IscR, as they are active under high iron levels. The use of an sRNA (here RyhB) as an intermediate allows negative repression to arise at a low iron level as well. Second, use of both protein- and RNA-mediated regulation allows separation of their activities more effectively without much chance of cross-interference between the two. This was best shown here by the possibility of suppressing IscR regulation of erpA without affecting RyhB regulation (and vice versa). Uncoupling the regulatory levels very likely provides more flexibility on the tuning of the response, as affinity for one binding site of the target gene can be changed independently of the other. Third, using a mixed circuit may have effects on the dynamics of the response. For instance, RNA regulation is assumed to be faster than transcriptional regulation (34). Future studies will aim at studying the dynamic response of erpA expression when switching from high- to low-iron conditions.

The complex relationship between transcription factors and RNA regulators is an area of research that is in expansion (see references 26 and 35 for reviews). Examples of such relationships include sRNAs that are regulated by well-known two-component systems and that regulate these systems in return (e.g., OmrA/B and EnvZ-OmpR [36]) or major regulators that participate in so-called mixed circuit motifs with sRNAs (e.g., Spot42/CRP [37]). However, how mixed circuits differ from circuits composed only of transcriptional regulators is still only partially understood.

We believe that our study helps shed some light on the possible advantages of mixed regulatory circuits.

MATERIALS AND METHODS

Strains and culture. All strains used in this study are derivative of E. coli MG1655 and are listed in Table S1 in the supplemental material. Strains were grown in LB broth (Sigma), containing various concentrations of 2,2’-dipyridyl (DIP) (Sigma) when stated. Marked mutations were moved between strains using classical P1 phage transduction as previously described (38). The plasmid library and the plc and pRyhB plasmids used in this study are described in reference 25. Transformations were carried out as described previously (39). PCR amplifications were carried out using the GoTaq DNA polymerase from Promega.

For the growth assay presented in Fig. 7, overnight cultures of the different strains were diluted (1/1,000) in individual wells of a 96-well microtiter plate in 100 μL LB containing increasing concentrations of DIP. Cells were then grown at 37°C, with agitation, in a Tecan Infinite 200 microtiter plate reader. OD600 was measured every 15 min without removing the plate from the machine, and growth was followed for 14 h.

Genetic manipulations. The PBad-erpA-lacZ and PerpA-erpA-lacZ fusions were constructed using recombination in a specifically designed strain, as previously described (39). Briefly, sequences corresponding to the erpA gene starting from its +1 transcriptional start site or from −200 nt before the +1 site up to 30 nt downstream of the ATG codon were amplified using oligonucleotides PBad-erpA-F and LacZ-erpA-R or PerpA(-200)-F and LacZ-erpA-R, respectively. The purified PCR products were then electroporated into strain PM1205 for recombination. Recombinants carrying the desired fusion were selected on LB plates devoid of NaCl and containing 5% sucrose and 40 μg/mL X-Gal (5-bromo-4-chloro-3-indoly-β-D-galactopyranoside). Blue colonies were chosen, and the resulting fusions were sequenced using oligonucleotides lacI-F and Deep-lac.

Constructions of point mutations of the PBad-erpA-lacZ fusion or the PerpA-erpA-lacZ fusion were realized by an overlap PCR. For each mutant, two PCR products corresponding to the sequence upstream and downstream of the desired mutation were amplified by PCR with oligonucleotides containing the desired mutation and using the strains containing the WT fusions as the templates. The two PCR products were then joined by an overlap PCR using oligonucleotides lacI-F and Deep-lac. The resulting PCR products were purified and electroporated in strain PM1205 as described above.

The chromosomal point mutations on erpA designed to abrogate regulation by IscR or RyhB were constructed as follows. First, PCR products containing the desired point mutations were realized using overlap PCR with oligonucleotides yadQ-F and erpA-mut-1R and erpA-mut-1-F and erpA-R [for the erpA(yadQmut) mutation] and oligonucleotides yadQ-F and erpA-iscR-R and erpA-iscR-F and erpA-R [for the erpA(iscRmut) mutation], using MG1655 as a template. The PCR products were then electroporated in strain LL401, in which the erpA promoter had been previously replaced by a PBAD promoter. The LL401 strain had previously been transformed with mini-Lambda Red in order to allow recombination with PCR products. Recombinants were selected by plating the cells on LB plates containing 0.2% glucose. As erpA is essential, strain LL401 is unable to grow in the presence of glucose. Only clones that had lost the PBAD promoter by recombination with the PCR products were thus selected. The obtained clones were then sequenced using oligonucleotides yadQ-F and erpA-R.

Mutations in the pRyhB plasmid were obtained as follows. In a first step, the pRyhB plasmid, purified from a WT E. coli strain, was amplified by PCR with oligonucleotides RybB1.2-F and RybB1.2-R, containing the desired mutation. The resulting PCR product was digested with the DpnI enzyme for 1 h at 37°C to get rid of the native plasmid.

RNA extraction and Northern blotting experiments. Overnight cultures of the appropriate strains were diluted in fresh medium containing ampicillin when indicated and incubated at 37°C with agitation. At an OD600 of 0.5, 1-ml samples of the cultures were extracted and 2,2’-
dipryridyl and/or IPTG was immediately added to the culture before new samples were extracted at indicated time points. RNA was extracted from the samples using the hot-phenol method as previously described (27) and resuspended in 10 μl diethyl pyrocarbonate (DEPC)-treated water final. Total RNAs were run on 1.75% agarose denaturing gels. RNA was then transferred onto Zeta Probe (Bio-Rad) positively charged membranes by an overnight reversed capillary transfer. Transferred RNAs were cross-linked to the membrane using a UV cross-linker. Membranes were hybridized with specific biotinylated probes overnight at 42°C, and RNAs were detected using the North2South (Thermo Scientific) labeling kit according to the manufacturer’s instructions. Biotinylated oligonucleotide probes against the apR-mRNA, SsrA, and RyhB were ordered from Eurofins (see Table S2 in the supplemental material).

β-Galactosidase assays. For microtiter plate assays, overnight cultures of the specified strains were diluted 500-fold into 100 μl of fresh medium (containing ampicillin and IPTG or DIP when indicated) contained in a well of a microtiter plate. The microtiter plates were then incubated at 37°C with agitation. β-Galactosidase assays were performed as previously described (26) with slight modification. After 6 h of growth, samples were extracted at indicated time points. RNA was extracted from dipyridyl and/or IPTG was immediately added to the culture before new

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00966-16/-/DCSupplemental.

Figure S1, PDF file, 0.6 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.

ACKNOWLEDGMENTS
We thank members of the laboratory for comments on the manuscript and advices and discussions throughout the work. We also thank the reviewers for their kind remarks that no doubt helped strengthen this manuscript.

FUNDING INFORMATION
This work, including the efforts of Pierre Mandin, Sylvia Chareyre, and Frederic J Barras, was funded by Agence Nationale de la Recherche (ANR) (SPV05511). This work, including the efforts of Pierre Mandin, Sylvia Chareyre, and Frederic J Barras, was funded by Centre National de la Recherche Scientifique (CNRS). This work, including the efforts of Pierre Mandin, Sylvia Chareyre, and Frederic J Barras, was funded by Aix-Marseille Universitè (AMU).

The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union’s Seventh Framework Programme (FP7/2007-2013) under REA grant agreement no. 294027.
a sponge for small RNAs to prevent transcriptional noise. Mol Cell 58:393–405. http://dx.doi.org/10.1016/j.molcel.2015.03.013.

24. Beauchene NA, Myers KS, Chung D, Park DM, Weisnicht AM, Keleş S, Kiley PJ. 2015. Impact of anaerobiosis on expression of the iron-responsive Fur and RyhB regulons. mBio 6:e01947-15. http://dx.doi.org/10.1128/mBio.01947-15.

25. Mandin P, Gottesman S. 2010. Integrating anaerobic/aerobic sensing and the general stress response through the ArcZ small RNA. EMBO J 29:3094–3107. http://dx.doi.org/10.1038/emboj.2010.179.

26. Mandin P, Guillier M. 2013. Expanding control in bacteria: interplay between small RNAs and transcriptional regulators to control gene expression. Curr Opin Microbiol 16:125–132. http://dx.doi.org/10.1016/j.mib.2012.12.005.

27. Massé E, Escorcia FE, Gottesman S. 2003. Coupled degradation of a small regulatory RNA and its mRNA targets in Escherichia coli. Genes Dev 17:2374–2383. http://dx.doi.org/10.1101/gad.1127103.

28. Hart Y, Alon U. 2013. The utility of paradoxical components in biological circuits. Mol Cell 49:213–221. http://dx.doi.org/10.1016/j.molcel.2013.01.004.

29. Kaplan S, Ben A, Dekel E, Alon U. 2008. The incoherent feed-forward loop can generate non-montonic input functions for genes. Mol Syst Biol 4:203. http://dx.doi.org/10.1038/msb.2008.43.

30. Alon U. 2007. Network motifs: theory and experimental approaches. Nat Rev Genet 8:440–461. http://dx.doi.org/10.1038/nrg2102.

31. Hart Y, Antebi YE, Mayo AE, Friedman N, Alon U. 2012. Design principles of cell circuits with paradoxical components. Proc Natl Acad Sci U S A 109:8346–8351. http://dx.doi.org/10.1073/pnas.1117475109.

32. Burda Z, Krzywicki A, Martin OC, Zagorski M. 2011. Motifs emerge from function in model gene regulatory networks. Proc Natl Acad Sci U S A 108:17263–17268. http://dx.doi.org/10.1073/pnas.1109435108.

33. Li GW, Burkhart D, Gross C, Weissman JS. 2014. Quantifying absolute protein synthesis rates reveals principles underlying allocation of cellular resources. Cell 157:624–635. http://dx.doi.org/10.1016/j.cell.2014.02.033.

34. Shimon Y, Friedlander G, Hetzroni G, Niv G, Altvia S, Biham O, Margalit H. 2007. Regulation of gene expression by small non-coding RNAs: a quantitative view. Mol Syst Biol 3:138. http://dx.doi.org/10.1038/msb4100181.

35. Beisel CL, Storz G. 2010. Base pairing small RNAs and their roles in global regulatory networks. FEMS Microbiol Rev 34:866–882. http://dx.doi.org/10.1111/j.1574-6976.2010.00241.x.

36. 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. http://dx.doi.org/10.1093/nar/gkn742.

37. Beisel CL, Storz G. 2011. The base-pairing RNA spot 42 participates in a multioutput feedforward loop to help enact catabolite repression in Escherichia coli. Mol Cell 41:286–297. http://dx.doi.org/10.1016/j.molcel.2010.12.027.

38. Silhavy TJ, Berman ML, Enquist LW (ed). 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

39. Mandin P. 2012. Genetic screens to identify bacterial sRNA regulators. Methods Mol Biol 905:41–60. http://dx.doi.org/10.1007/978-1-61779-949-5_4.