Noncanonical repression of translation initiation through small RNA recruitment of the RNA chaperone Hfq

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The RNA chaperone Hfq is mostly known to help small regulatory RNAs (sRNAs) interact with target mRNAs to block initiating ribosomes. In this model, whereas the sRNA is directly competing with initiating 30S ribosomal subunits, Hfq plays only an indirect role, allowing optimal sRNA–mRNA pairing. Here we report that Hfq is recruited by a sRNA, Spot42, to bind to a precise AU-rich region in the vicinity of the translation initiation region (TIR) of *sdhC* mRNA and competes directly with 30S ribosomal subunits. We show that the sRNA Spot42 binds *sdhC* too far upstream of the TIR to directly repress translation initiation in vitro and in vivo. Contrary to the canonical model of sRNA regulation, this suggests a new mechanism where Hfq is directly involved in the translational repression of the target mRNA and where the sRNA acts only as a recruitment factor.

[**Keywords:** Spot42; Hfq; small RNA; translation initiation; noncanonical sRNA mechanism; translation block]

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Bacterial *trans*-encoded small RNAs (sRNAs) are a class of regulatory molecules that are widespread in *Enterobacteriaceae* species such as *Escherichia coli* and *Salmonella* sp. sRNAs are usually expressed in response to environmental stresses to maintain cell homeostasis (Waters and Storz 2009). A common feature among sRNAs is the requirement of the RNA chaperone Hfq for their activity (Vogel and Luisi 2011). This Sm-like RNA-binding protein was first discovered four decades ago as a host factor essential for bacteriophage Q β replication (Franze de Fernandez et al. 1968). In the last decade, elucidation of the role of Hfq in sRNA-mediated gene regulation has been an area of increasing interest. In most cases, sRNA-mediated regulation is decreased or abolished in a Δhfq deletion strain (Zhang et al. 1998; Massé and Gottesman 2002; Moller et al. 2002a; Valentin-Hansen et al. 2004). Although Hfq is not thought to be directly involved in the repression of translation (Maki et al. 2008), it is critical for sRNA stability and has been shown to facilitate the pairing between sRNAs and their target mRNAs (Moller et al. 2002a; Geissmann and Touati 2004). Hfq is also known to interact with and recruit RNase E to target mRNAs, allowing rapid and stoichiometric degradation of both sRNA and mRNA (Massé et al. 2003; Morita et al. 2005). The role of Hfq is not restricted to sRNA-mediated gene regulation.

Indeed, Hfq has been reported to be a translational repressor that competes in vitro with initiating 30S ribosomes for accessibility to the ribosome-binding site (RBS) on mRNA (Vytvytska et al. 2000). This behavior is explained by Hfq's preference to bind to AU-rich regions close to RBSs (Franze de Fernandez et al. 1972; Senear and Steitz 1976), which are known to act as translational enhancers (Zhang and Deutscher 1992; Hook-Barnard et al. 2007). In addition, Hfq is involved in RNA processing, as it regulates polyadenylation-dependent mRNA decay (Hajnsdorf and Regnier 2000; Mohanty et al. 2004).

One of the best-characterized Hfq-associated sRNAs is RyhB, which is expressed under conditions of iron (Fe) starvation (Massé and Gottesman 2002) and regulates many mRNAs encoding nonessential Fe-using proteins. This post-transcriptional regulation allows the cell to preserve sufficient intracellular Fe for essential cellular functions (Massé et al. 2005; Jacques et al. 2006; Salvail et al. 2010). One of the first described targets of RyhB was the polycistronic mRNA *sdhCDAB* (Massé and Gottesman 2002), which encodes the four subunits of Fe-dependent succinate dehydrogenase of the tricarboxylic acid (TCA) cycle. Although the negative effect of RyhB on *sdhCDAB* mRNA levels is clear, the direct interaction between both RNAs has not yet been demonstrated. Other examples of sRNAs interacting with Hfq include Spot42 (encoded by the *spf* gene), which responds to glucose availability (Moller et al. 2002b) and plays an important role in catabolic repression (Beisel and Storz 2011), and

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RybB, which is expressed following a membrane stress and is known to repress several outer membrane proteins (OMPs) (Johansen et al. 2006; Papenfort et al. 2006, 2010).

In general, sRNAs target the translation initiation region (TIR) of mRNAs by pairing with the Shine-Dalgarno (SD) sequence and/or the start codon, resulting in a direct competition with initiating 30S ribosomal subunits. Because 30S initiating ribosomes are known to cover the region spanning nucleotide –20 to nucleotide +19 relative to the initiation codon (Beyer et al. 1994; Huttenhofer and Noller 1994), it is believed that sRNAs must pair within this window to inhibit translation. In recent years, however, many cases of translation-repressing sRNAs binding outside of this canonical region have been reported, involving a different mechanism in each case. For example, IstR-1, a Hfq-independent antisense RNA, has been shown to pair to a putative ribosome standby site located ~100 nucleotides (nt) upstream of the start codon of the *tisB* mRNA to repress standby ribosomes (Darfeuille et al. 2007). Another example is GcvB sRNA, which targets C/A-rich regions in the 5' untranslated region (UTR) of many mRNAs (Sharma et al. 2007). These C/A-rich regions are thought to act as translation enhancers. More recently, OmrA and OmrB sRNAs have been shown to repress *csgD* mRNA by targeting its 5' UTR >60 nt upstream of the translation initiation codon. In this case, the mechanism is not fully understood but is known to involve a phylogenetically conserved RNA motif in the 5' UTR (Holmqvist et al. 2010). Furthermore, examples of sRNAs targeting the coding region to repress translation or destabilize mRNA have also been reported (Bouvier et al. 2008; Pfeiffer et al. 2009).

In this study, we addressed the regulation of the *sdhCDAB* mRNA by three sRNAs: Spot42, RyhB, and RybB. We present evidence that each one of the sRNAs paired at different regions within the 5' UTR of *sdhC* mRNA to repress the initiation of translation. Whereas both RyhB and RybB paired canonically at the TIR and recruited RNase E to actively degrade *sdhCDAB* mRNA, Spot42 paired far upstream of TIR without promoting the rapid degradation of the mRNA. Surprisingly, affinity purification data clearly showed that Spot42 pairing alone had no observable effect on mRNA in vivo, although it bound to *sdhC* in the absence of Hfq. This result, combined with additional in vitro data, suggested that Spot42 acted expressly to recruit Hfq at the TIR of *sdhC* mRNA, which inhibits the binding of 30S ribosomal subunits. We propose a novel mechanism where, contrary to the canonical model, the sRNA is not the main effector, but rather an intermediary factor for the recruitment of Hfq, which directly modulates translation initiation.

Results

Spot42, RyhB, and RybB regulate sdhC expression post-transcriptionally

We previously identified *sdhCDAB* mRNA as a target of sRNA RyhB in *E. coli* (Massé and Gottesman 2002). One of the physiological outcomes of this regulation is the inability of strains overproducing RyhB to grow on a

medium containing succinate as the sole carbon source. Notably, the same phenotype was also observed by another group in a bacterial strain overproducing sRNA Spot42 (Rice and Dahlberg 1982). In addition, a recent genomic study in *Salmonella* sp. has revealed that the expression of RybB sRNA led to a decrease in *sdhCDAB* mRNA level (Papenfort et al. 2006). This result suggested that, in addition to RyhB, sRNAs Spot42 and RybB could also be post-transcriptional regulators of the *sdhCDAB* transcript.

Although RyhB, Spot42, and RybB may regulate *sdhCDAB* mRNA expression, a direct pairing between these sRNAs and *sdhCDAB* mRNA has not yet been reported. Using the bioinformatics tool TargetRNA (Tjaden et al. 2006), we found putative pairing sites for the three sRNAs in the 5' UTR region of *sdhC* that corresponded to the first cistron of the mRNA. Whereas RyhB and RybB were predicted to pair at the SD sequence region, Spot42 was predicted to pair 47 nt upstream of the translational start codon of *sdhC* (Fig. 1). Of note, one additional putative RyhB pairing site has also been predicted in the TIR of *sdhD* (Massé and Gottesman 2002) but was not addressed in the present study.

To validate the regulation of *sdhC* by RyhB, Spot42, and RybB, we designed a translational reporter construct (termed SdhC₂₅₈-LacZ) harboring the complete 5' UTR of *sdhCDAB* mRNA (219 nt) and the first 39 nt of the *sdhC* ORF fused to the β -galactosidase-encoding *lacZ* gene (see the Materials and Methods for details). Strains were constructed carrying the SdhC₂₅₈-LacZ fusion (inserted as a single copy in the chromosome) and harboring plasmids producing each sRNA from an arabinose-inducible promoter (termed pBAD-*spf*, pBAD-*ryhB*, and pBAD-*rybB*). Next, each sRNA was expressed individually, and β -galactosidase activity was monitored for 2 h following addition of arabinose (Fig. 2A). We observed that each sRNA was able to repress SdhC₂₅₈-LacZ fusion expression, thus confirming that each one of the three sRNAs was a negative regulator of *sdhC*. We also tested the effect of the three sRNAs on a *sdhC* transcriptional fusion (termed *sdhC*₂₅₈-*lacZ*) (Fig. 2B) in which the β -galactosidase gene harbored its own TIR. In this case, sRNAs expression had no effect on β -galactosidase activity, indicating that the effects observed on SdhC₂₅₈-LacZ translational fusion were due to only translational repression and not a mRNA destabilization or a transcriptional repression. We also confirmed that none of the three sRNAs had an effect on the *sdhC* promoter (Supplemental Fig. S1A).

We next investigated the effect of the three sRNAs on SdhC₂₅₈-LacZ fusion regulation under physiological conditions. Because Spot42 is expressed in the presence of glucose (Moller et al. 2002b), we monitored SdhC₂₅₈-LacZ fusion in wild-type and Δ *spf* strains grown in M63-glucose medium. Results showed a twofold increase in SdhC₂₅₈-LacZ fusion activity in the Δ *spf* strain as compared with the wild-type strain (Fig. 2C). An experiment was then carried out to study RyhB regulation. Wild-type and Δ *ryhB* strains were grown in M63-glucose medium in the absence of Fe, as RyhB is highly expressed under these conditions (Desnoyers et al. 2009). Results showed a threefold increase in SdhC₂₅₈-LacZ fusion activity in the Δ *ryhB* strain as compared with wild-type (Fig. 2C). RybB

Figure 1. Putative pairing sites of Spot42, RyhB, and RybB sRNAs on the *sdhC* mRNA 5' UTR. (A) Schematic representation of the 5' UTR of *sdhC*. Nucleotides 1–148 are omitted from this representation. The secondary structure represented has been obtained by mfold (<http://snowwhite.wellesley.edu/targetRNA/index.html>). Putative pairing sites for Spot42 (○), RyhB (●), RybB (□), and the Hfq-binding site are indicated. The Hfq-binding site mutant (MH) is also indicated. (B) Predicted pairing sites for Spot42, RyhB, and RybB on the *sdhC* 5' UTR. Pairing of RyhB and RybB sRNAs overlap the TIR of *sdhC*, while pairing of Spot42 is situated 48 nt upstream of the start codon. Mutations used in Figure 4B are also indicated.

regulation was investigated with cells in the stationary phase, since RybB is mainly expressed under these conditions (Wassarman et al. 2001; Vogel et al. 2003; Johansen et al. 2006). Wild-type and Δ rybB strains were grown in Luria-Bertani (LB) medium and monitored for expression of SdhC₂₅₈-LacZ fusion during the late stationary phase. As expected, the RybB effect (1.8-fold) was observed only at OD₆₀₀ >2.0 (time >300 min). Also, as a control, we looked at the effect of knocking out the DsrA sRNA, which is not known to regulate *sdhC* expression. Accordingly, we compared wild-type and Δ dsrA strains grown at 30°C in LB medium, a condition in which DsrA is expressed (Sledjeski et al. 1996). As seen in Supplemental Figure S1B, there is no significant difference in the expression of the SdhC₂₅₈-LacZ fusion between the two strains. Taken together, these results indicated that RyhB, Spot42, and RybB were all translational repressors of *sdhC* mRNA.

Effects of Spot42, RyhB, and RybB on *sdhCDAB* mRNA levels in vivo

Translational repression by sRNAs is often associated with the recruitment of RNase E and a rapid degradation of the target mRNA (Massé et al. 2003). To determine whether RyhB, Spot42, and RybB promoted the rapid degradation of *sdhCDAB* mRNA, we determined the levels of *sdhCDAB* mRNA in a time-course manner following the expression of the three sRNAs. As shown in Figure 3A, the expression of both RyhB and RybB sRNA led to a very fast (<2 min to 5 min) decrease of *sdhCDAB* mRNA levels, suggesting an active degradation of *sdhCDAB*. In marked contrast, the effect of Spot42 on *sdhCDAB* mRNA was only observed 30 min after sRNA expression, suggesting that no RNase was actively recruited to promote mRNA degradation.

These differences in degradation kinetics could not be attributed to variation in sRNA induction kinetics (Supplemental Fig. S2), as all three sRNAs were expressed similarly.

To further investigate the potential role of RNase E and an RNA degradosome in sRNA-mediated degradation of *sdhCDAB* mRNA, we used a mutant (*rne131*) in which the scaffold region of RNase E required for the assembly of the RNA degradosome was absent (Leroy et al. 2002). This region of RNase E has also been shown to be essential for Hfq-dependent recruitment of RNase E for sRNA-induced mRNA decay (Ikeda et al. 2011). The results showed an absence of rapid degradation of *sdhCDAB* mRNA following RyhB and RybB expression in the *rne131* mutant (Fig. 3B) as compared with the wild-type strain (Fig. 3A). Remarkably, there was a striking similarity in mRNA turnover of *sdhCDAB* whether Spot42 was expressed in *rne131* or wild-type backgrounds. Taken together, these results reinforced the notion that RNase E was recruited following both RyhB and RybB expression, but not Spot42, to rapidly degrade *sdhCDAB* mRNA.

Spot42 pairs with *sdhC* far upstream of the TIR

We first carried out in-line probing using radiolabeled *sdhC* RNA in the presence or absence of Spot42, RyhB, or RybB to verify in vitro the putative pairing sites suggested by bioinformatics data (Fig. 1). In-line probing exploits the natural ability of unstructured and unpaired nucleotides in RNA molecules to spontaneously cleave in solution (Regulski and Breaker 2008). The results showed that cleavage in *sdhC* was significantly reduced from nucleotides A165 to C177 in the presence of Spot42 (Fig. 4A). A similar observation was made in the presence of RyhB, which afforded protection from nucleotides A205 to G213

Figure 2. Spot42, RyhB, and RybB sRNAs post-transcriptionally regulate *sdhC* mRNA. β -Galactosidase assays using *sdhC*₂₅₈-lacZ translational (A) and transcriptional (B) fusion were performed. Expression of each sRNA was individually induced at an OD₆₀₀ of 0.1, and specific β -galactosidase activity was measured 2 h later. The empty vector pNM12 was used as a control. (C) β -Galactosidase assays using SdhC₂₅₈-LacZ translational fusion under conditions of natural expression of Spot42 (M63 glucose), RyhB (M63 glucose without Fe), and RybB (LB). Wild-type strains are compared with the corresponding sRNA knockout strains.

and, RybB, which protected from nucleotides A196 to G208 (Fig. 4A). These results were consistent with in silico pairing data (Fig. 1).

In addition, we performed in vivo covariation mutagenesis using SdhC₂₅₈-LacZ translational fusion as a template. The three mutant constructs SdhC₂₅₈MF-LacZ, SdhC₂₅₈MJ-LacZ, and SdhC₂₅₈ML-LacZ (illustrated in Fig. 1B) were designed to weaken the pairing with Spot42, RyhB, and RybB, respectively. Each one of these mutations reduced the repression activity of the corresponding sRNA (Fig. 4B). We also constructed mutants of Spot42, RyhB, and RybB harboring the compensatory mutations that restored the pairing with the mutant constructs SdhC₂₅₈MF, SdhC₂₅₈MJ, and SdhC₂₅₈ML. These mutants, called Spot42MF', RyhBMJ', and RybBML', were all able to fully, or at least significantly, restore wild-type regulation (Fig. 4B). Taken together, in vitro and in vivo data confirmed the in silico prediction that both RyhB and RybB bind to the TIR of *sdhC* and that the Spot42-binding site was located far upstream of TIR.

Spot42, but not RyhB or RybB, fully depends on Hfq for repression of translation

Next, we addressed the question whether Spot42, RyhB, and RybB sRNAs were directly inhibiting *sdhC* translation initiation. We used toeprinting assays that detect the formation of the 30S translation initiation complex bound to mRNA. Results in Figure 5A showed that addition of purified 30S ribosomal subunits and initiator tRNA (lane 8) to *sdhC* mRNA created a strong block of reverse transcription 15 nt downstream from the AUG codon. These observations were consistent with the formation of a translation initiation complex on mRNA. When increasing amounts of sRNAs Spot42, RyhB, RybB, or DsrA (used as a negative control) (Majdalani et al. 1998) were used, we observed that only RyhB and RybB were able to repress formation of the initiation complex (sRNA:*sdhC* ratio of 15:1) (Fig. 5A, lane 11). However, the addition of Spot42 or DsrA at even a 15-fold excess over *sdhC* had no effect on the formation of the initiation complex (Fig. 5A,

Figure 3. RyhB and RybB, but not Spot42, induce an active degradation of *sdhCDAB* mRNA. Northern blots were performed on total RNA extracted from strain GD372 (A) or GD527 (*rne131* mutant) (B) harboring the indicated plasmids. Cells were grown on LB medium containing ampicillin to an OD₆₀₀ of 0.5, at which point arabinose was added at time 0 to induce sRNA expression. Total RNA was extracted at the indicated times. Probes complementary to *sdhC*, Spot42, RyhB, RybB, and 16S rRNA were used. The control plasmid (pNM12) panel was probed with all three sRNA probes.

lane 11). As a control, we confirmed by performing PbAc probing (Supplemental Fig. S3) that at the same concentrations and in the same buffer as the toeprint experiment, Spot42 is able to pair to the *sdhC* RNA in vitro. These results suggested that, contrary to RyhB and RybB, Spot42 alone could not repress the initiation of translation of *sdhC* even if it pairs to the region characterized in Figure 4.

We next examined the effect of Hfq under these experimental conditions. To address this, Hfq and *sdhC* were mixed in a 1:1 ratio, and then we monitored the capacity of Spot42, RyhB, RybB, or DsrA to repress the formation of the translation initiation complex. Results showed that the presence of Hfq barely affected the binding of 30S ribosomal subunits (Fig. 5A, cf. lanes 8 and 13). As expected, the presence of Hfq increased the efficiency of RyhB- and RybB-mediated repression (Fig. 5A, lanes 13–16). Surprisingly, the presence of Hfq allowed Spot42 to significantly repress 30S ribosomal subunit binding starting at a fivefold excess of Spot42 with respect to *sdhC* (Fig. 5A, lane 15). However, DsrA was still unable to repress the formation of the initiation complex in the presence of Hfq. Together, these results provided evidence that Spot42 absolutely required Hfq to repress the binding of the 30S ribosomal subunits to *sdhC*.

Next, we tested the effect of Hfq alone on the binding of 30S ribosomal subunits to *sdhC*. As shown in Figure 5B, the addition of increasing amounts of Hfq led to partial inhibition of the translation initiation complex at a 2.5-fold excess (lane 10) with respect to *sdhC* mRNA and to a complete inhibition at a fivefold excess (lane 11). These in vitro results suggested that a moderately high molar ratio of Hfq was able to directly repress the binding of 30S ribosomal subunits to *sdhC* mRNA. This finding was in marked contrast to results shown in Figure 5A, where even high molar ratio (15-fold) of Spot42 alone could not repress *sdhC*.

A Hfq-binding site on the 5' UTR of sdhC is essential for Spot42-induced repression of translation

The results reported above indicated that, although Spot42 could bind to *sdhC* (Fig. 4A, lane 6), it was not able to affect

30S ribosomal subunit binding to *sdhC* (Fig. 5A, lane 11), even at a high molar ratio (15:1). However, when Spot42 was in fivefold excess compared with *sdhC*, the presence of a stoichiometric amount of Hfq (5:1:1) efficiently repressed the binding of 30S ribosomal subunits (Fig. 5A, lane 15). These results suggested that Hfq, with the help of Spot42, could bind directly and repress the translation of *sdhC*. If this possibility were true, one should be able to map a binding site of Hfq at, or close to, the TIR of the *sdhC* 5' UTR. A series of experiments were therefore carried out to investigate this possibility. We used in vitro chemical (lead acetate, which cleaves ssRNA) footprinting assays using radiolabeled *sdhC* RNA and purified Hfq. The results showed that addition of Hfq at a 1:1 Hfq:*sdhC* ratio clearly protected *sdhC* against cleavage from nucleotides A192–A201 (Fig. 6A, left panel). This region is highly AU-rich, consistent with previously described Hfq-binding sites (Franze de Fernandez et al 1972; Senear and Steitz 1976). It also fits a previously identified A-R-N motif that was shown to be preferentially bound by Hfq (Link et al. 2009). To validate the importance of this AU-rich region for Hfq binding, we designed a *sdhC* mutant that would prevent Hfq binding (termed *sdhCMH*) by replacing two adenine residues (A194–A195) by two cytosine residues (Fig. 1A). The results showed that addition of Hfq to *sdhCMH* did not protect RNA from lead acetate cleavages, indicating that Hfq did not efficiently bind to *sdhCMH* (Fig. 6A).

We next tested the effect of Hfq binding to *sdhC* in vivo by introducing the MH (mutated Hfq-binding site) mutation into the SdhC₂₅₈-LacZ translational fusion, thus creating SdhC₂₅₈MH-LacZ fusion. The results showed the basal levels of translation for the SdhC₂₅₈MH-LacZ allele was decreased by a fourfold factor (Supplemental Fig. S4), suggesting that the AU-rich region recognized by Hfq had an important role in the translation activation of *sdhC*. We also tested the effect of each sRNA on SdhC₂₅₈MH-LacZ translational fusion. The results showed that RyhB and RybB were still able to repress SdhC₂₅₈MH-LacZ fusion expression by a factor of at least twofold (Fig. 6B). In addition, we found that, even if Spot42 could potentially pair with *sdhC*₂₅₈MH RNA (Supplemental

Translation repression through Hfq recruitment

Figure 4. Analysis of Spot42, RyhB, and RybB pairing sites on *sdhC* mRNA. (A) In-line probing of the 5'-end-labeled *sdhC* mRNA in the presence or absence of Spot42, RyhB, and RybB. The position of several G and A residues, relative to the transcriptional start, are indicated. (NR) Nonreacted control; (OH) alkaline ladder; (T1) RNase T1 ladder; (TA) RNase TA ladder. Except for the ladders, all experiments were carried out under native conditions. (B) Wild-type *SdhC*₂₅₈-LacZ translational fusion is specifically repressed by wild-type Spot42, RyhB, and RybB, whereas the mutated versions of the *SdhC*₂₅₈-LacZ translational fusion are specifically repressed by the corresponding mutated sRNAs. See Figure 1B for a drawing of the mutations used in this panel. Expression of each sRNA (Spot42MF', RyhBMJ', and RybBML') was individually induced by the addition of arabinose at an OD₆₀₀ of 0.1, and specific β -galactosidase activity was measured 2 h later. The empty pNM12 was used as a control.

Fig. S5A), Spot42 had no effect on *SdhC*₂₅₈MH-LacZ fusion in vivo. This observation strongly suggested that the Hfq-binding site in the vicinity of the *sdhC* TIR was essential for Spot42-mediated repression of translation. We also monitored the effect of all three sRNAs on the *SdhC*₂₅₈-LacZ fusion in a Δhfq background (Fig. 6B). In these experiments, RyhB was still able to strongly repress *sdhC* translation, whereas RybB had a slight but reproducible negative effect on translation. Spot42 did not repress translation of *sdhC* in the absence of Hfq. Of interest, basal expression levels of *SdhC*₂₅₈-LacZ fusion in the Δhfq background was slightly higher than the wild-type strain (Supplemental Fig. S4), further suggesting that Hfq acted as a translational repressor of *sdhC* mRNA in vivo.

We next monitored the effect of Spot42, RyhB, and RybB on *sdhCDAB* mRNA in the Δhfq background. The results of Northern blot analysis revealed that RyhB and RybB had a moderate effect on mRNA degradation, whereas Spot42 had no effect (Supplemental Fig. S6).

Because Hfq is thought to recruit RNase E to a mRNA targeted by a sRNA (Morita et al. 2005; Ikeda et al. 2011), it was expected that each one of the three sRNAs had a reduced effect on mRNA levels in a Δhfq -null strain. However, the fact that RyhB and RybB were still able to induce degradation of *sdhCDAB* mRNA, whereas Spot42 was not, suggested an additional role for Hfq in the context of sRNA Spot42. Taken together, these results suggested that, in contrast to RyhB and RybB, Spot42 was unable to inhibit *sdhC* expression in the absence of Hfq protein or the Hfq-binding site (nucleotides 191–200) on *sdhC*.

Interchanging sRNA pairings sites also interchanges Hfq dependency

Our data suggested that Spot42 binds to the 5' UTR too far upstream of the TIR to compete directly with 30S ribosomal subunits. Therefore, it appeared likely that Spot42 used Hfq as a bridge to compete with initiating

Figure 5. Repression of the *sdhC* translation initiation complex formation by Spot42, RyhB, RybB, and Hfq. (A) Toeprint assay indicating that RyhB and RybB were able to prevent translation initiation complex formation in the absence of Hfq, whereas Spot42 was able to perform repression only in the presence of Hfq. The DsrA sRNA was used as a negative control. (B) Toeprint assay indicating that Hfq was able to prevent translation initiation complex formation on the *sdhC* mRNA. Increasing amounts of Hfq were added (0 μM, 0.01 μM, 0.1 μM, 0.2 μM, 0.5 μM, and 1.0 μM). GATC refers to sequencing ladders generated with the same oligonucleotide (EM1262) used for toeprint.

ribosomes on the *sdhC* TIR. This interpretation suggests that recruitment of Hfq, regardless of the recruiting sRNA, should be sufficient to repress *sdhC*. To test this hypothesis, we interchanged the pairing sites of both Spot42 and RyhB sRNAs with *sdhC*. In this experimental design, the RyhB mutant (RyhBMS) would now pair at the original Spot42 pairing site far upstream of the RBS, and the Spot42 mutant (Spot42MR) would pair with the RBS of *sdhC* (Supplemental Fig. S7). We first confirmed that both mutant sRNAs paired with the *sdhC* 5' UTR (Supplemental Fig. S5B) and then monitored their effects in vivo on SdhC₂₅₈-LacZ fusion. The results showed that both Spot42MR and RyhBMS expressed from a pBAD promoter were able to repress SdhC₂₅₈-LacZ activity (Fig. 7A). Of significance, Spot42MR repressed translation to an extent similar to wild-type RyhB, and RyhBMS repressed as much as wild-type Spot42 (Fig. 7A). These data confirmed that direct pairing (by either Spot42MR or RyhB) to the TIR was more efficient than binding far upstream (such as RyhBMS and Spot42) to repress translation.

We next tested the importance of Hfq by analyzing the effect of Spot42MR and RyhBMS on mutant SdhC₂₅₈MH-LacZ fusion and wild-type SdhC₂₅₈-LacZ fusion in a Δhfq background. The results showed that Spot42MR was still able to repress β -galactosidase activity in both cases, whereas RyhBMS had no effect regardless of the cellular backgrounds (Fig. 7A). We interpreted these data to suggest that Hfq binding to *sdhC* was necessary for the RyhBMS mechanism, although this was not the case for the Spot42MR repression mechanism of *sdhC* translation.

We next studied the effect of both mutated sRNAs—Spot42MR and RyhBMS—on *sdhCDAB* mRNA levels in vivo. The results of Northern blot analysis showed that the expression of Spot42MR led to a rapid (<5 min) decrease in *sdhCDAB* mRNA levels; RyhBMS required >15 min to reduce the mRNA levels (Fig. 7B). Interestingly, a comparison of the kinetics of mRNA decay between wild-type and mutant sRNAs revealed that both sRNAs pairing directly to the TIR (RyhB and Spot42MR) were acting very fast (<5 min), whereas both sRNAs pairing upstream of the TIR (Spot42 and RyhBMS) acted at a much slower rate (>15 min). These observations suggested that the sRNA pairing site may also have an effect on the *sdhCDAB* mRNA's rate of decay. Furthermore, experiments using Spot42 and RyhB probes confirmed that the two mutant sRNAs were expressed at levels comparable with wild-type sRNAs (data not shown).

A recent study has shown that the 3'-end poly-U tail of RyhB is essential for Hfq binding (Otaka et al. 2011). We took the opportunity of this finding to test whether the Hfq protein involved in translation repression is recruited by the sRNA. Accordingly, we constructed RyhB-LS4U and RyhBMS-LS4U sRNAs (shorter poly-U tails) (see Supplemental Fig. S7), which should have lost their abilities to bind Hfq, and then tested the effect of these constructs on our translational SdhC₂₅₈-LacZ fusion. Whereas the RyhB-LS4U construct induced a 50% decrease in the expression of SdhC₂₅₈-LacZ, the RyhBMS-LS4U construct failed to repress the fusion (Fig. 7C). This suggested that, to repress translation of *sdhC*, the chaperone Hfq must be recruited by the poly-U tail of the sRNA binding at the upstream site of the TIR.

Next, we determined the in vivo capability of both the RyhB-LS4U and RyhBMS-LS4U constructs to bind to Hfq. To address this question, we developed an assay based on affinity purification of MS2 RNA stem-loop-tagged sRNA (see Supplemental Fig. S8A for a schematic view of the MS2-tagged construct). This approach has been used successfully to demonstrate Hfq binding on MS2-tagged sRNAs (Said et al. 2009). Each construct was cloned downstream from an arabinose-inducible promoter carried on a low-copy-number plasmid (see the Materials and Methods for details). After induction for 10 min with arabinose, total RNAs and proteins were extracted. As shown by Northern and Western blots (Fig. 7D, input), all RNAs of interest (RyhB, MS2-RyhB, MS2-RyhB-LS4U, and MS2-RyhBMS-LS4U) as well as Hfq were detected before loading on the MS2 affinity column. Although they were expressed at lower levels than MS2-RyhB and MS2-RyhBMS, both the MS2-RyhB-LS4U and MS2-RyhBMS-LS4U constructs were expressed

Figure 6. Hfq binding to *sdhC* is essential for Spot42-mediated repression. (A) Lead acetate probing of the 5'-end-labeled *sdhC* or *sdhCMH* mRNA in the presence of increasing amounts of Hfq (0 μM, 0.01 μM, 0.1 μM, 0.2 μM, 0.5 μM, and 1.0 μM). The *sdhCMH* mutation is pictured in Figure 1A. Nonreacted controls were performed in the absence (–) or presence (+) of 1 μM Hfq. The position of several G and A residues, relative to the transcriptional start, are given. (NR) Nonreacted control; (OH) alkaline ladder; (T1) RNase T1 ladder; (TA) RNase TA ladder; (PbAc) lead acetate. Except for the ladders, all experiments were carried out under native conditions. (B) β-Galactosidase assays with *SdhC₂₅₈MH-LacZ* or *SdhC₂₅₈-LacZ* translational fusion in wild-type or *Δhfq* strains. Expression of each sRNA (pBAD-*spot42*, pBAD-*ryhB*, or pBAD-*rybB*) was individually induced by the addition of arabinose at an OD₆₀₀ of 0.1, and specific β-galactosidase activity was measured 2 h later. The empty vector pNM12 was used as a control.

at comparable levels before purification (Fig. 7D, lanes 3,4).

After purification of the cellular extracts, the constructs MS2-RyhB and MS2-RyhBMS strongly bound to the Hfq chaperone, as expected (Fig. 7D, lane 7). In contrast, both the MS2-tagged RyhB-LS4U and RyhBMS-LS4U constructs have lost their ability to strongly bind Hfq in vivo (Fig. 7D, lanes 7,8). Together with the results in Figure 7C, this strongly suggests that sRNA constructs pairing upstream of the RBS region must bind directly to the Hfq protein for translation repression of *sdhC*.

Spot42 pairing on sdhC catalyzes the recruitment of Hfq in vivo

Our results indicated that Spot42 was fully dependent on Hfq to repress *sdhC* translation. Thus, we asked whether Spot42 was able to promote the recruitment of Hfq to the 5' UTR of *sdhC* mRNA in vivo. We designed an approach based on affinity purification of the *sdhC* transcript tagged with a MS2 RNA stem-loop (see Supplemental Fig. S8B for a schematic view of the construct). A plasmid (termed pFRA-*sdhC₂₅₈-MS2*) harboring the promoter, the 5' UTR of *sdhCDAB* mRNA, and the first 39 nt of the *sdhC* ORF fused to the MS2 RNA stem-loops (see the Materials and Methods for details) was engineered for that purpose. As controls, we included a vector expressing the MS2 tag only and a second vector expressing the *sdhC₂₅₈* transcript only, both under the control of the *sdhCDAB* promoter. Strains

carrying pFRA-*sdhC₂₅₈-MS2*, pFRA-MS2, or pFRA-*sdhC₂₅₈* plasmids and arabinose-inducible pGD3-*spf* or the empty pGD3 vector were treated with arabinose for 20 min before total RNAs and proteins were extracted. As shown by Northern and Western blots (Fig. 8A, input), all RNAs of interest (*sdhC₂₅₈-MS2*, MS2, and *sdhC₂₅₈*) as well as Hfq were detected before loading on the MS2 affinity column. Next, we performed affinity purification of *sdhC₂₅₈-MS2*, MS2, and *sdhC₂₅₈* by loading the cellular lysate on the MS2 affinity column. Total RNAs and proteins were collected and analyzed by Northern and Western blots. The results showed that equal amounts of the *sdhC₂₅₈-MS2* construct were retrieved whether Spot42 was absent or expressed (Fig. 8A, lanes 9,10, output). However, we noted that Spot42 was recovered exclusively from the strain expressing *sdhC₂₅₈-MS2* (Fig. 8A, lane 10) and not from strains expressing either MS2 (Fig. 8A, lane 14) or *sdhC₂₅₈* (Fig. 8A, lane 16) only. These experiments confirmed that sRNA Spot42 paired with *sdhC* 5' UTR RNA in vivo. Importantly, expression of Spot42 correlated with a 2.6-fold increase in the Hfq signal (Fig. 8A [cf. lanes 9 and 10, Western blots], C [densitometry]). There was no recovery of Hfq from strains expressing the MS2 or *sdhC₂₅₈* constructs (Fig. 8A, lanes 13–16).

We next inserted the MH mutation in the *sdhC₂₅₈-MS2* construct to generate the pFRA-*sdhC₂₅₈MH-MS2* plasmid. After performing affinity purification, we observed considerably less (50%) Hfq protein recovered from *sdhC₂₅₈MH-MS2* RNA as compared with the wild-type *sdhC₂₅₈-MS2*

Figure 7. Effect of interchanging Spot42 and RyhB pairing sites on the *sdhC* mRNA. (A) β -Galactosidase assays with SdhC₂₅₈-LacZ or SdhC₂₅₈MH-LacZ translational fusions in wild-type and Δhfq strains. Expression of each sRNA (pBAD-*spot42MR* or pBAD-*ryhBMS*) was individually induced by the addition of arabinose at an OD₆₀₀ of 0.1, and specific β -galactosidase activity was measured 2 h later. The plasmid pNM12 was used as a control. (B) Northern blot analysis of total RNA extracted from triple-knockout $\Delta spf/\Delta ryhB/\Delta rybB$ strains harboring the indicated plasmids, hybridized with *sdhC*- and sRNA-specific probes. Cells were grown on LB medium in the presence of ampicillin to an OD₆₀₀ of 0.5, at which point arabinose was added at time 0 to induce sRNA expression. Total RNA was extracted at indicated times. 16S rRNA was used as a loading control. (C) β -Galactosidase assay using SdhC₂₅₈-LacZ translational fusion. sRNA mutated for their Hfq-binding sites (LS4U; poly-U tail shortened) were individually induced by the addition of arabinose at an OD₆₀₀ of 0.1, and specific β -galactosidase activity was measured 2 h later. The empty pNM12 was used as a control. (D) Affinity purification from strains expressing different RyhB variants. Cells were grown in LB medium in the presence of ampicillin to an OD₆₀₀ of 0.5, at which point arabinose was added at time 0 to induce sRNA expression. Total RNA was extracted 10 min later. Northern blots (RyhB probe) and Western blots (Hfq antibody) were performed on samples taken before (input) and after (output) affinity purification.

construct (Fig. 8A, output, cf. lanes 9 and 11). These findings corroborated *in vitro* results (Fig. 6A) and strongly suggested that the Hfq-binding site on *sdhC* was functional *in vivo*. Interestingly, expression of Spot42 also led to a small but reproducible Hfq recovery in the case of *sdhC*₂₅₈MH-MS2 RNA (Fig. 8A, cf. lanes 11 and 12). In addition, Spot42 sRNA was also retrieved to a level comparable with the pFRA-*sdhC*₂₅₈-MS2 construct (Fig. 8A, cf. lanes 10 and 12), indicating that the Hfq-binding site mutation on the *sdhC* 5' UTR did not interfere with the pairing with Spot42 and that the results of Figure 6B were not due to a reduced sRNA binding.

We also inserted the MF (mutated Spot42 pairing site) mutation in the *sdhC*₂₅₈-MS2 construct and performed affinity purification with a pFRA-*sdhC*₂₅₈MF-MS2 plasmid. As shown in Figure 8C, disruption of Spot42 pairing with *sdhC*₂₅₈MF prevents Hfq recruitment. This confirms that pairing of Spot42 to *sdhC* mRNA is essential for the recruitment of Hfq. Finally, we performed affinity purification in Δhfq cells carrying the pFRA-*sdhC*₂₅₈-MS2 plasmid. As seen in Supplemental Figure S9, we were able

to affinity-purify Spot42 in the absence of Hfq, indicating that the chaperone is not necessary for the pairing of Spot42 to *sdhC*. Together, these results indicated that expression and pairing of Spot42 to the *sdhC* 5' UTR led to a recruitment of Hfq. They also suggested that the Hfq-binding site located in the TIR was essential for optimal Hfq recruitment.

Discussion

Our data indicate that sRNA Spot42 binds to target mRNA *sdhCDAB* only to recruit the RNA chaperone Hfq, which in turn binds to a precise region of the TIR to directly inhibit translation initiation by the 30S ribosomal subunits. This suggests a new mechanism that went against the traditional roles of the canonical model where Hfq recruits sRNA that blocks translation. In our model (Fig. 9), the function of the sRNA was only to recruit Hfq, the major contribution of which was to block translation. Indeed, except for the pairing sequence, the specific nature of the sRNA did not seem to be of critical

Figure 8. Spot42 binds to *sdhCDAB* in vivo and recruits Hfq to *sdhC* TIR. (A) Affinity purification was performed on strain EM1451 harboring pFRA-*sdhC₂₅₈-MS2*, pFRA-*sdhC₂₅₈MH-MS2*, pFRA-*MS2*, or pFRA-*sdhC₂₅₈* in combination with pGD3 (control plasmid) or pGD3-*spot42*. Northern blots (MS2, *sdhC*, and Spot42 probes) and Western blots (Hfq antibody) were performed on samples taken before (input) and after (output) affinity purification. (B) Densitometry from the “output” signal of the level of Hfq protein relative to the levels of *sdhC₂₅₈-MS2* or *sdhC₂₅₈MH-MS2* RNAs. Data are representative of three independent experiments. (C) Affinity purification was performed on strain EM1451 harboring pFRA-*sdhC₂₅₈MF-MS2* in combination with pGD3 or pGD3-*spot42*. Northern blots (MS2 and Spot42 probes) and Western blots (Hfq antibody) were performed on samples taken before (input) and after (output) affinity purification.

importance, as we showed that Spot42 and RyhB pairing sites could be functionally exchanged (Fig. 7). This observation suggested that sRNA pairing sites on the target mRNA, whether at the TIR (direct repression by sRNAs) or far upstream (direct repression by Hfq), were key to triggering the repression mechanism of *sdhCDAB*.

In addition to Spot42, RyhB and RybB were also involved in post-transcriptional regulation of *sdhCDAB* mRNA by directly pairing with the 5' UTR of *sdhC*. Whereas RyhB and RybB directly paired with the TIR of *sdhC*, Spot42 paired 48 nt upstream of the start codon. Furthermore, RyhB and RybB induced the rapid turnover of *sdhCDAB* mRNA by recruiting the RNA degradosome, but Spot42 did not. Finally, Spot42 absolutely required Hfq binding to an AU-rich region in the vicinity of the *sdhC* TIR to repress translation, but RyhB and RybB could function, at least partially, without Hfq.

Because RyhB and RybB paired directly with the SD sequence of *sdhC*, they were able to directly compete with initiating 30S ribosomes. Thus, Hfq seemed not to be essential for these sRNAs to repress translation. On the

other hand, because Spot42 base-paired far upstream of the TIR, it was unable to compete directly with initiating 30S ribosomes (Fig. 9A). Based on the fact that Hfq was able to compete in vitro with initiating ribosomes on *sdhC* (Fig. 5B) and that the AU-rich site that was bound by Hfq seemed to be important for normal *sdhC* translation in vivo (Fig. 6B), we hypothesized that the effect seen on *sdhC* translation when Spot42 is expressed was achieved through recruitment of Hfq to the *sdhC* TIR (Fig. 9B).

The observation that Hfq was able to repress translation by itself has been reported before (Vytvytska et al. 2000). However, to our knowledge, it is the first time that Hfq has been shown to be directly involved in 30S ribosomes competition in the context of sRNA-mediated gene regulation. Given the marked preference of Hfq for AU-rich regions and the fact that these regions are known to act as translational enhancers (Zhang and Deutscher 1992; Komarova et al. 2005; Hook-Barnard et al. 2007), we believe that Hfq may play a similar role in the action of many other sRNAs that are not pairing directly to the TIR and are therefore not able to directly compete with initiat-

Figure 9. Working model for sRNA-mediated repression of *sdhC* translation and the role of Hfq (see the text for details).

ing ribosomes. Even if it was not essential for RyhB- and RybB-mediated *sdhC* translational repression, Hfq remained involved in this regulation through roles of Hfq previously identified as being important for the stability of many sRNAs and for facilitating sRNA-mRNA pairing (Moller et al. 2002a; Zhang et al. 2002; Geissmann and Touati. 2004). Accordingly, all three sRNAs were less effective in repressing *sdhC* translation in the Δ hfq-null strain (Fig. 6B). In addition, the results (Supplemental Fig. S6) indicated that Hfq was essential for sRNA-mediated active mRNA degradation, as shown previously by other studies (Massé et al. 2003; Morita et al. 2005; Ikeda et al. 2011; Prévost et al. 2011).

The Hfq-binding site on *sdhC* not only acted to recruit Hfq, but also functioned as a translational enhancer. When *sdhCMH* was used, not only did Hfq not bind to the mRNA, but the 30S ribosomes could bind less efficiently. In the absence of Spot42, Hfq could still bind to *sdhC*, thereby slightly reducing the initiation of translation, but with decreased efficiency.

An intriguing finding of our study was the more rapid mRNA turnover induced by sRNAs binding directly to the TIR (RyhB, RybB, and Spot42MR) than sRNAs binding far upstream of the TIR (Spot42 and RyhBMS). This observation correlated with the observed efficiency of translational repression as measured with a *SdhC*₂₅₈-LacZ translational fusion. Indeed, whereas RyhB, RybB, and Spot42MR repressed *sdhC* translation by at least 3.5-fold (Figs. 2A, 7A), Spot42 and RyhBMS induced repression only twofold (Figs. 2A, 7A). It is possible that, contrary to RyhB or RybB, the twofold translational repression induced by Spot42 was insufficient to induce rapid cleavage by RNase E. Interestingly, *sdhCDAB* was not among the new Spot42 targets recently characterized by pulse expression of Spot42 for 15 min followed by microarray analysis (Beisel and Storz 2011). These data suggested that Spot42 as well as other well-characterized sRNAs may have more targets than those revealed solely by microarray-based data.

Whereas the *sdhC*₂₅₈-lacZ fusion was resistant to degradation by any of the sRNAs (Fig. 2B), the full-length mRNA was not (Fig. 3A). Thus, we hypothesized that the initial cleavage site was located downstream from the 39th nucleotide of the *sdhC* ORF. Indeed, our results suggested that the initial cleavage site on *sdhC* mRNA following sRNA expression was located downstream from the 39th nucleotide of the ORF (Fig. 2B). This finding was reminiscent of a recent study from our laboratory (Prévost et al. 2011) that showed that RyhB induced an initial cleavage into *sodB* target mRNA >350 nt downstream from the pairing site.

In terms of physiological significance, it is quite remarkable that three sRNAs, each expressed under different physiological conditions, repressed the same mRNA. Because succinate dehydrogenase is a Fe-dependent enzyme, regulation by RyhB was expected, since this sRNA is expressed when Fe is not available (Massé and Gottesman 2002). With respect to Spot42, the only known transcription regulator is the catabolic repressor protein (CRP), which represses Spot42 expression when cAMP levels are

high (Moller et al. 2002b). It has been known for some time that the expression of *sdhCDAB* mRNA is repressed in the presence of glucose (Park et al. 1995; Takeda et al. 1999). A role for the CRP-cAMP complex in the regulation of *sdhCDAB* transcription has been reported (Nam et al. 2005). In addition to transcription regulation, our results suggest that Spot42 adds an additional layer of regulation that may help to increase the strength and rapidity of catabolic repression in the presence of glucose, as it was recently shown for other targets of Spot42 (Beisel and Storz 2011). Finally, the RybB sRNA has been recently characterized as a regulator of mRNAs encoding OMPs (Johansen et al. 2006; Bouvier et al. 2008; Papenfort et al. 2006, 2010). In contrast, succinate dehydrogenase is an inner membrane-located protein. RybB transcription is controlled by the alternative σ factor σ^c , whose activity is modulated by a signal transduction pathway (Ades 2008) induced by the recognition of a conserved YxF peptide situated in the C terminus of OMPs (Walsh et al. 2003). However, because the SdhC protein is not an OMP, it is unclear why it is involved in the σ^c response. Thus, the variety of signals that affect the regulation of *sdhCDAB* mRNA—such as intracellular Fe, cAMP, and misfolded proteins—underline the importance of modulating succinate dehydrogenase according to extremely diverse environmental conditions.

Materials and methods

Strains and plasmids

Strains used in this study are listed in Supplemental Table S1. Their constructions are described in the Supplemental Material. Derivatives of EM1055 were used in all experiments. The DH5 α bacterial strain was used for routine cloning procedures. Cells were grown at 37°C in LB medium. Cells carrying pFRA, pRS1551, and pNM12 derivatives were grown in LB medium containing ampicillin at a final concentration of 50 μ g/mL. Cells carrying pGD3 derivatives were grown in the presence of chloramphenicol at a final concentration of 30 μ g/mL.

β -Galactosidase assays

Kinetic assays for β -galactosidase activity were performed as described (Prévost et al. 2007) using a SpectraMax 250 microtitre plate reader (Molecular Devices). Briefly, overnight bacterial cultures were incubated in LB medium at 37°C, diluted 1000-fold into 50 mL of fresh LB medium, and grown under mechanical shaking at 37°C. sRNA expression was induced when OD₆₀₀ reached a value of 0.1 by addition of arabinose to a final concentration of 0.1%. Specific β -galactosidase activity was calculated using the formula $V_{\max}/OD_{600}$. The results reported here correspond to data from a minimum of three independent experiments.

RNA extraction and Northern blot analysis

Total RNA was extracted using the hot-phenol procedure (Aiba et al. 1981). Cells were grown to an OD₆₀₀ of 0.5, and 0.1% arabinose was added. In the case of *sdhC* Northern blots, 20 μ g of total RNA was loaded on an agarose gel (1% agarose in MOPS buffer). In the case of sRNA Northern blots, 5 μ g of total RNA was loaded on polyacrylamide gel (5% acrylamide, 8 M urea). After migration, the RNA was transferred by capillarity (agarose gel) or electro-transferred (acrylamide gel) to a Hybond-XL

membrane (Amersham Biosciences) and UV-cross-linked. Prehybridization, hybridization, and washes were done as described (Desnoyers et al. 2009). To analyze RNA retrieved from affinity purification (below), RNA equivalent to 1 OD₆₀₀ (input) or 18 OD₆₀₀ (output) of culture were sized on polyacrylamide gels (10% bottom/5% top, 8 M urea) and electro-transferred on a Hybond-XL membrane. Prehybridization and hybridization were done in Church buffer (Church and Gilbert 1984). DNA probes were 5'-end-labeled with 50 µCi of [³²P]-γ-ATP by using T4 polynucleotide kinase (New England Biolabs) according to the manufacturer's protocol. Oligonucleotides used as probes were *sdhC* (EM1696), MS2 (EM1662), and *spot42* (EM1679). Washes were done in 2× SSC/0.1% SDS and 0.1× SSC/0.1% SDS. Membranes were exposed to phosphor storage screens and analyzed using a Typhoon Trio (GE Healthcare) instrument. Quantification was performed using the ImageQuant software (Molecular Dynamics).

In vitro RNA synthesis and radiolabeling

Oligonucleotides used to generate the DNA template for *in vitro* transcription are indicated in Supplemental Table S3. The radio-labeled probes used for Northern blot analysis were transcribed using T7 RNA polymerase (Roche) to generate the antisense transcript of the gene of interest and detection performed as described (Desnoyers et al. 2009). For the RNA used in secondary structure probing and toeprinting, transcription was performed in T7 transcription buffer, 5 mM NTP (A, C, G, and U), 40 U of RNaseOut (Invitrogen), 20 U of T7 RNA polymerase, and 0.5 µg of DNA templates. After 4 h of incubation at 37°C, the mixture was treated with 2 U of Turbo DNase (Ambion), extracted once with phenol-chloroform, and purified on denaturing acrylamide gel. To perform 5'-end labeling, transcripts were dephosphorylated with calf intestine phosphatase (New England Biolabs) and 5'-labeled with [³²P]-γ-ATP using T4 polynucleotide kinase (New England Biolabs) according to the manufacturer's protocol. Radiolabeled transcripts were purified on denaturing acrylamide gels before use.

RNA secondary structure probing

Secondary structure probing was performed on 5'-end-labeled *sdhC*₂₅₈ or *sdhC*₂₅₈MH RNA (as above). Hfq was purified as described (Prévost et al. 2007). In-line probing was performed as described (Regulski and Breaker 2008). Final concentrations of 0.2 µM *sdhC*₂₅₈ RNA and 1 µM Spot42, RyhB, or RybB were used. Lead acetate probing, ribonuclease T1/TA ladder, and alkaline (OH) ladder were performed as described (Desnoyers et al. 2009). A final concentration of 0.2 µM *sdhC*₂₅₈ or *sdhC*₂₅₈MH RNA was used. After reactions, samples were heated for 1 min to 90°C and separated on 8% polyacrylamide/7 M urea sequencing gel.

Toeprinting assays

Toeprinting assays and 30S ribosomal subunit purification were carried out as described (Fechter et al. 2009). In each condition, annealing mixture contained 2 pmol of unlabeled *sdhC*₂₅₈ RNA and 0.4 pmol of 5'-end-labeled EM1262 primer in toeprint buffer (20 mM Tris-HCl at pH 7.5, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT). Annealing mixtures were heated for 1 min at 90°C and chilled for 1 min on ice. MgCl₂ and dNTPs were added to final concentrations of 10 mM and 1 mM, respectively. When needed, Hfq was added, and the incubation was continued for 10 min at 37°C. Spot42, RyhB, RybB, or DsrA RNA was added afterward, and the incubation was continued for 10 min at 37°C. Two picomoles of 30S ribosomal subunits were then added, and incubation was

continued for 5 min. Thirteen picomoles of tRNA fmet (Sigma-Aldrich) were added, and, 15 min later, cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) for 15 min at 37°C. Reactions were stopped, and phenol-chloroform was extracted. cDNA was ethanol-precipitated and subsequently dissolved in water and loading buffer II (Ambion). cDNA products were analyzed on 8% polyacrylamide/7M urea sequencing gel. Toeprint signals were identified by comparison with sequences generated with the same 5'-end-labeled primer.

Affinity purification of MS2-tagged RNA

Affinity purification assays were performed as described (Said et al. 2009), with some modifications. The MS2-MBP protein was purified as described in the Supplemental Material. The bacterial strains were grown to an OD₆₀₀ of 0.5, at which point arabinose was added to a final concentration of 0.1% to induce the expression of pBAD-RyhB, pBAD-MS2-RyhB, pBAD-MS2-RyhB-LS4U, pBAD-MS2-RyhBMS-LS4U (10 min), or pGD3-*spot42* (20 min). Cells equivalent to 50 OD₆₀₀ were chilled for 20 min on ice. At this point, RNA was extracted (input) as described above from 600 µL of culture. The remaining cells were then centrifuged, resuspended in 1 mL of buffer A (20 mM Tris-HCl at pH 8.0, 150 mM KCl, 1 mM MgCl₂, 1 mM DTT), and centrifuged again. Cells were resuspended in 2 mL of buffer A and lysed using a French Press (8000 psi, three times). Lysate was then cleared by centrifugation (17,000g, 30 min, 4°C). At this step, 20 µL of the soluble fraction was mixed with 20 µL of protein sample buffer (input). The remaining soluble fraction was subjected to affinity chromatography (all steps performed at 4°C). The column was prepared by adding 75 µL of amylose resin (New England Biolabs) to Bio-Spin disposable chromatography columns (Bio-Rad). The column was washed with 3 mL of buffer A. Next, 100 pmol of MS2-MBP protein (diluted in 1 mL of buffer A) was immobilized on the amylose resin, and the column was washed with 1 mL of buffer A. The cleared lysate was then loaded onto the column, which was washed with 5 mL of buffer A. RNA and proteins were eluted from the column with 900 µL of buffer A containing 12 mM maltose. Eluted RNA was extracted with phenol-chloroform, followed by ethanol (3 vol) precipitation of the aqueous phase in the presence of 20 µg of glycogen. For protein isolation, the organic phase was subjected to acetone precipitation. RNA samples were analyzed by Northern blots as described above, and protein samples were analyzed by Western blots (below).

Western blot analysis

Proteins were resuspended in protein-loading gel electrophoresis buffer. An equivalent volume corresponding to 0.1 OD₆₀₀ (input) or 20 OD₆₀₀ (output) of cell cultures was separated on a 12% SDS-PAGE gel and transferred to nitrocellulose membrane. The anti-Hfq (kind gift of Gisela Storz, National Institutes of Health) was used at a dilution of 1:10,000. The IRDye 800CW-conjugated goat anti-rabbit secondary antibody (Li-Cor Biosciences) was used at a dilution of 1:15,000. Western blots were revealed on an Odyssey infrared imaging system (Li-Cor Biosciences), and quantification was performed using the Odyssey software.

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