Disruption of small RNA signaling caused by competition for Hfq

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Small RNAs (sRNAs) regulate diverse pathways, including stress responses, virulence, and metabolism in *Escherichia coli*. At the center of this large sRNA regulatory network is the Hfq protein. Hfq mediates the binding of sRNAs to their target mRNAs; without Hfq, most sRNAs cannot efficiently regulate target mRNA expression. Here, we show in vivo that Hfq can be a limiting factor for sRNA activity and that it can be easily depleted, causing disruption of the sRNA network. Depletion of the available Hfq can occur when sRNAs and target mRNAs are transcribed at high levels without their partners, resulting in the sequestration of Hfq into sRNA–Hfq and target mRNA–Hfq complexes. This can be avoided by coordinating the transcription of sRNAs with their target mRNAs so that they are turned on and off together to maximize duplex formation and minimize Hfq sequestration. Therefore, the limited availability of Hfq results in a highly interdependent sRNA network, wherein the activity of each sRNA depends on the activity of the other sRNAs and target mRNAs in the network.

silencing | microRNA | noncoding RNA | RNA chaperone

Small RNAs (sRNAs) have emerged as an important regulator in prokaryotes. In *Escherichia coli*, more than 80 sRNAs have been identified so far [reviewed in (1)], and they regulate pathways ranging from iron and sugar metabolism to oxidative stress [reviewed in (2–4)]. Most sRNAs act in *trans* by annealing to target mRNAs, typically at or near the ribosome-binding sequence to generate a sRNA-target mRNA duplex. The binding of sRNAs to target mRNAs is very specific; a single base substitution in the sRNA or target mRNA can be sufficient to disrupt duplex formation (5). Although sRNA binding is very specific, each sRNA can act on more than one target mRNA and each target mRNA can be regulated by multiple sRNAs [reviewed in (6)].

Duplex formation usually decreases translation and/or increases the degradation of target mRNAs, resulting in decreased target gene expression. Less commonly, it has the opposite effect on translation and mRNA degradation, causing increased target gene expression [reviewed in (6)]. For simplicity, we refer to sRNA-mediated decreases in expression as "silencing" and increases as "activation" irrespective of whether translation or mRNA degradation is altered. "sRNA activity" is used to refer to both silencing and activation.

Most *trans*-acting sRNAs require the Hfq protein to mediate the formation of the duplex [reviewed in (7)]. Hfq primarily exists as a cyclical homohexamer that has two RNA binding sites: a "proximal site" that binds sRNAs and target mRNAs and a "distal site" that binds poly(A) tails (8). Hfq hexamers provide a structure that promotes strand exchange, and/or they act as chaperones that alter the structure of sRNAs and target mRNAs to promote annealing (9, 10). In at least some cases, Hfq has an ongoing role after duplex formation in recruiting proteins that degrade the duplex and for translation (11, 12). Proteins that bind to Hfq include RNase E (13), polynucleotide phosphorylase (14), and ribosomal subunit S1 (15).

Estimates of the number of Hfq hexamers per cell range from ≈400 (16) to 5,000–10,000 (17, 18). The reason for the disparity is unclear. Even with the larger estimate, however, Hfq may be a limiting factor for sRNA activity under some conditions because (*i*) Hfq mediates duplex formation for more than 100 sRNAs and target mRNAs, some of which are present at high concentrations (19, 20); (*ii*) Hfq can bind to sRNAs and duplexes for an extended period to mediate their degradation or translation as mentioned above; and (*iii*) multiple Hfq hexamers may bind to each sRNA or target mRNA (21–23). Therefore, under some circumstances, there may be insufficient Hfq to mediate all these actions (24, 25), resulting in sRNAs and target mRNAs competing for Hfq.

The question of whether the activity of sRNAs is limited by Hfq availability has important ramifications for our understanding of the regulation of sRNA networks and their use in synthetic biology. If Hfq is limiting, it may constrain the number of sRNAs that can act concurrently and decrease their efficiency. In the first part of this study, we establish that the availability of Hfq is indeed a limiting factor for sRNA activity. In the second part, we show that transcribing sRNAs and target mRNAs without their partner can disrupt sRNA signaling, presumably by the formation of sRNA–Hfq and target mRNA–Hfq complexes that reduce the availability of free Hfq.

Results

Experimental System. To examine whether Hfq is a limiting factor in vivo for sRNA signaling, we first needed to identify sRNAs that primarily form duplexes via an Hfq-dependent mechanism. We selected four sRNAs (RyhB, DsrA, MicC, and OxyS) that have previously been shown to require Hfq for their action (25–29). Three of the sRNAs (RyhB, MicC, and OxyS) silence their target mRNA (*sodB*, *ompC*, and *fhlA*, respectively), and one (DsrA) activates its target mRNA (*rpoS*). The transcription of each sRNA was controlled by the pLlacO-1 promoter, which was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to the media (Fig. 1*A*). The part of the target mRNA that is necessary for sRNA regulation was fused to *gfp* (*sodB::gfp*, *ompC::gfp*, and *rpoS::gfp*) and constitutively transcribed from the pLtetO-1 promoter without TetR in the system (*Materials and Methods*). GFP fluorescence provides a quantitative measure of target gene expression and sRNA activity as previously reported (30, 31).

We examined whether Hfq was necessary for the activity of these sRNAs by measuring the expression of the *target mRNA::gfp* reporter fusions in cells with and without chromosomal *hfq* (Fig. 1 *B* and *C*, unshaded bars). These measurements confirmed that Hfq is necessary for their optimal function. Furthermore, we demonstrated that the transcription of these sRNAs did not alter the expression of a nontarget mRNA control sequence (Fig. 1 *B* and *C*, shaded bars); therefore, they appear to act specifically on their target sequence as opposed to generally altering gene expression.

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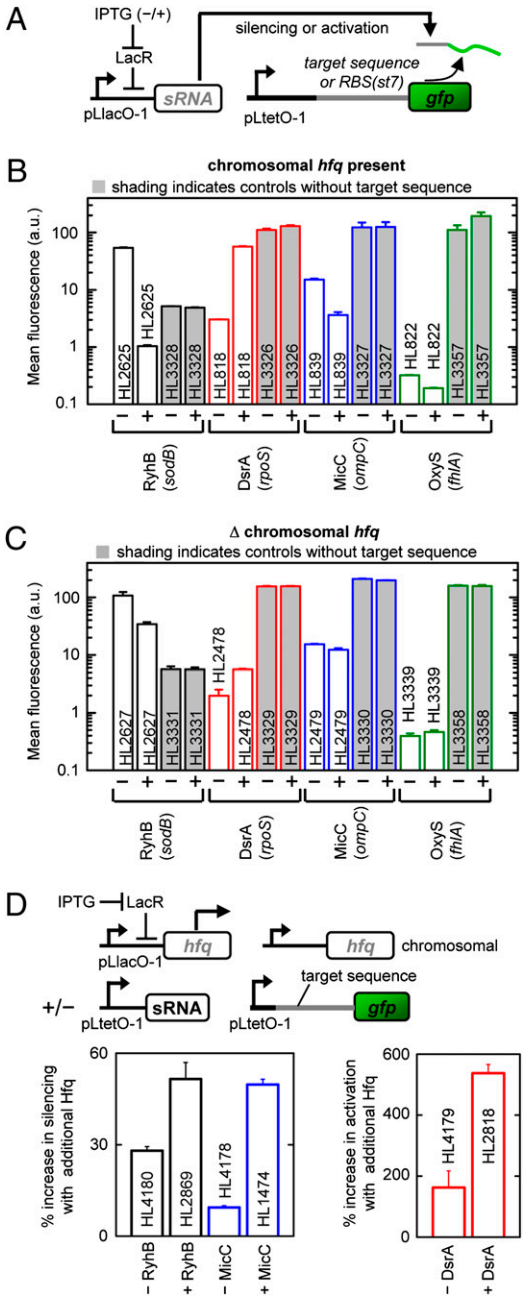

Fig. 1. Hfq is required for efficient sRNA silencing and activation. Error bars indicate the SEM. Gene fusions are abbreviated to the target mRNA name (e.g., *ompC::gfp* is abbreviated to *ompC*). (*A*) Experimental system as described in the main text. A weak promoter (pLtetO-1m9) was used for *sodB::gfp* because it was toxic at high rates of transcription. The RBS (st7) sequence was used in strains without the target mRNA sequence. (*B* and *C*) Target gene expression in strains with and without chromosomal *hfq*. sRNA transcription was uninduced (–) or induced (+) by the addition of IPTG to the media. The target mRNA for each sRNA is in parentheses. (*D*) Relative change (%) in the expression of target mRNAs with additional Hfq. The effect of the additional Hfq is compared between strains with and without sRNA (the latter measures the nonspecific effect of Hfq overexpression).

Hfq Is a Limiting Factor for sRNA Activity. Having established the necessity of Hfq for the optimal activity of RyhB, DsrA, and MicC, we examined the effect of additional Hfq. If Hfq is a limiting factor, increasing the Hfq concentration will increase sRNA activity. In contrast, if Hfq is in excess, increasing its concentration will have little effect. Therefore *hfq* was cloned onto a plasmid under control of the pLlacO-1 promoter and transformed into a strain with chromosomal *hfq* (Fig. 1*D*). We measured the expression of *sodB::gfp*, *ompC::gfp*, and *rpoS::gfp* in the presence of their complementary sRNA (RyhB, MicC, and DsrA, respectively) with and without extra Hfq. From these measurements, we calculated the percentage change in expression caused by the additional Hfq (Fig. 1*D*), which showed that silencing of *sodB::gfp* and *ompC::gfp* and activation of *rpoS::gfp* increased. The measurements were also performed without the sRNAs, and this showed a much smaller change in the expression of these target mRNAs (Fig. 1*D*).

To examine the relationship between the Hfq level and sRNA activity further, we placed the above circuits in a strain without chromosomal *hfq* and added IPTG to the media to vary Hfq production (Fig. 2*A*). To estimate the relative amount of Hfq produced at different IPTG concentrations, we placed *gfp* under the control of the pLlacO-1 promoter. We found that the amount of silencing and activation occurring with only chromosomal *hfq* (no induction of plasmid *hfq*) can be achieved at ≈10–20% of the maximal induction of plasmid *hfq* (Fig. 2 *B–D*, gray lines). Western blotting confirmed that the Hfq concentration increases with IPTG induction, and it showed that the level of Hfq produced by the chromosome is relatively low (Fig. 2 *E* and *F*).

Increasing the Hfq concentration above the level provided by the chromosomal copy produced an approximately linear increase in MicC silencing (i.e., decreased *ompC::gfp* expression) and DsrA activation (i.e., increased *rpoS::gfp* expression) (Fig. 2 *B* and *C*). This shows that the amount of Hfq was limiting the amount of silencing and activation. Increasing the Hfq concentration also increased RyhB silencing (Fig. 2*D*), suggesting that Hfq is also limiting in this system; however, it is not as clear in this case because the amount of silencing was also limited by the target mRNA concentration (i.e., *sodB::gfp* was transcribed from a weak promoter because of toxicity at high transcription levels).

Effect of Hfq on sRNA and Target mRNA Concentrations and Translation. Hfq binding has been shown to decrease the degradation of RyhB (32, 33), DsrA (27), and probably MicC (26) but not OxyS (34). We sought to confirm this in our system and also to examine the effect of Hfq on the stability of the target mRNAs and their translation (in the absence of sRNAs, and therefore duplex formation). The concentrations of the sRNAs and target mRNAs were measured by quantitative RT-PCR in strains with and without chromosomal *hfq* that expressed only the sRNA or only the target mRNA. The target mRNAs were measured in strains that also lacked the chromosomal copy of the partner sRNA. We found that RyhB, DsrA, and MicC had reduced concentrations and that the OxyS concentration was unchanged in the absence of Hfq (Fig. 3*A*). This is in agreement with previous studies (26, 27, 32, 33). In contrast, the stability and translation of the target mRNAs were not substantially different with and without Hfq (Fig. 3 *B–D*). The expression of *fhlA::gfp* was very low; therefore, the relative change in fluorescence was not considered to be accurate (Fig. 3 *C* and *D*).

The absence of any change in the expression of the target mRNA fusions with the deletion of Hfq is unlikely to be due to the target mRNAs not binding to Hfq because coimmunoprecipitation studies with Hfq indicate that *ompC* and *rpoS* bind to Hfq (35), in vitro studies show that Hfq binds to the *rpoS* (36) and *sodB* (37) mRNAs, and our competition experiments also suggest that target mRNAs bind to Hfq (see below). Instead, the results indicate that the binding of target mRNAs to Hfq (in the absence of sRNAs) does not alter their degradation and translation rates. This is an important point for the competition experiments below because it indicates that altering the free Hfq concentration does not directly affect the expression of the target mRNAs; therefore, any changes in expression are the result of altered sRNA activity.

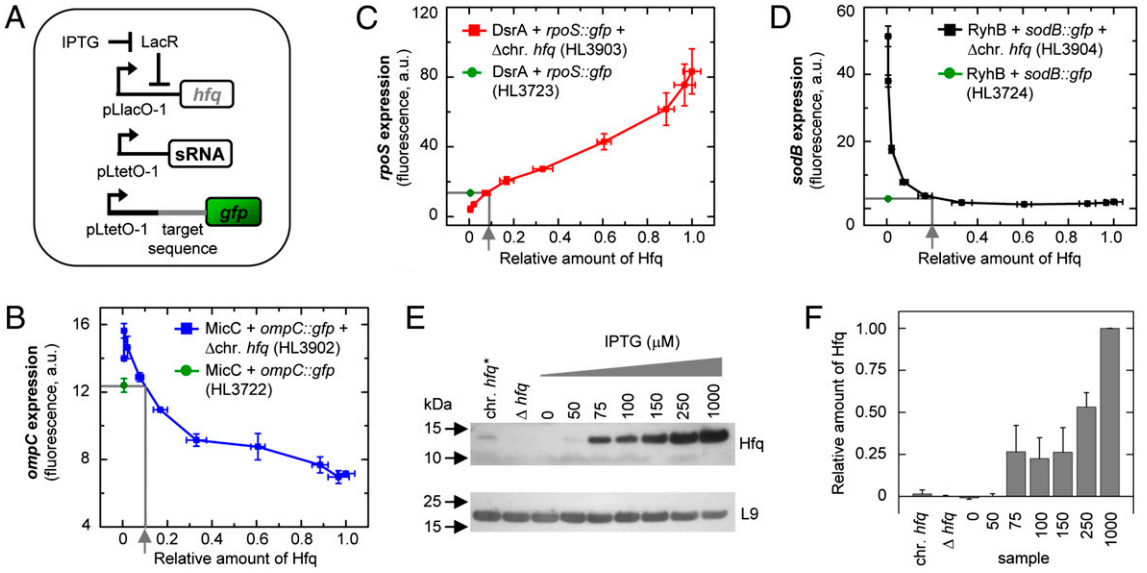

Fig. 2. Hfq is a limiting factor for sRNA silencing and activation. Error bars indicate the SEM. (*A*) Experimental system. (*B–D*) Target gene expression at varying Hfq levels in strains with the chromosomal *hfq* deleted. The gray lines indicate the level of Hfq production resulting in the same level of target gene expression as the strain with chromosomal *hfq* (green). (*E*) Hfq quantitation by Western blotting. Hfq and L9 (loading control) had multiple nonspecific bands that are not shown. The amount of Hfq produced by chromosomal *hfq* in *E. coli* MG1655 ("chr. *hfq*") was typically less than in this image. (*F*) Relative amount of Hfq measured by Western blotting normalized to the maximum level. Hfq quantification was performed in triplicate.

Loss of sRNA Activity Due to the Transcription of Unpartnered sRNAs and Target mRNAs. Given that Hfq availability can be limiting, we investigated whether the transcription of competing sRNAs and target mRNAs that bind to and form complexes with Hfq can decrease its availability, and consequently decrease sRNA activity.

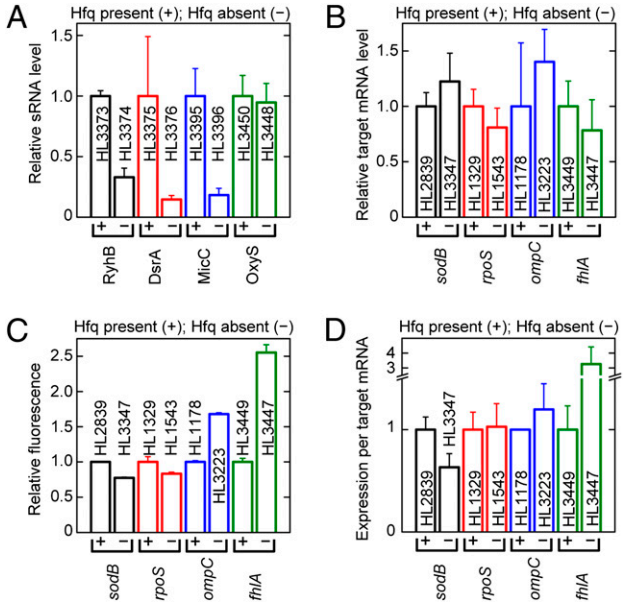

Fig. 3. Effect of Hfq availability on sRNA and target mRNA concentrations and translation. Error bars indicate the SEM. (*A* and *B*) Relative sRNA and target mRNA levels in strains without Hfq as measured by quantitative RT-PCR. The sRNA and target mRNA concentrations were normalized to the 5S RNA and to the concentration in the strain with Hfq. The complementary sRNA is deleted in all strains in which the *target mRNA::gfp* fusion was measured. (*C*) Relative fluorescence of the samples shown in *B*. (*D*) Translational efficiency of target mRNAs in the presence and absence of Hfq. The fluorescence level of each target mRNA (*C*) was divided by its mRNA concentration (*B*) to determine the average amount of translation per target mRNA.

To test this, we transcribed competing sRNAs and target mRNAs without their partner (i.e., "unpartnered") and measured their effect on RyhB and MicC silencing and DsrA activation (Fig. 4*A*). For the competing target mRNAs, only the sequence necessary for sRNA activity was fused to the *mCherry* gene. *mCherry* was found to interact with *rpoS::gfp*; therefore, the first one-third of the T7 RNA polymerase gene (T7RNAP) was used instead of *mCherry* for the competition experiments with *rpoS::gfp*.

We found that the activity of all sRNAs could be disrupted by at least one unpartnered sRNA or target mRNA. RyhB silencing of *sodB::gfp* was decreased (resulting in increased *sodB::gfp* expression) when unpartnered sRNAs (DsrA, MicC, and OxyS) and unpartnered target mRNAs (*ompC::mCherry* and *fhlA::mCherry*) were transcribed [Fig. 4*B*, compare level with unpartnered sRNAs (blue-shaded bars) and target mRNAs (pink-shaded bars) with the level without them (red dashed line)]. We demonstrated that this is due to competition and not to the unpartnered sRNAs and target mRNAs directly regulating *sodB::gfp* expression because expression does not increase in the absence of RyhB. Furthermore, nontarget mRNA sequences, which are not thought to bind to Hfq (mCherry and part of the T7RNAP gene) do not substantially effect RyhB silencing. Therefore, the observations are consistent with the unpartnered sRNAs and target mRNAs competing for Hfq.

MicC silencing also decreased (causing increased *ompC::gfp* expression) when an unpartnered sRNA (DsrA) or an unpartnered target mRNA (*sodB::mcherry*) was transcribed (Fig. 4*C*). Again, the unpartnered DsrA and *sodB::mCherry* did not directly increase *ompC::gfp* expression, and control sequences that are not thought to bind to Hfq did not interfere with MicC silencing. These results are also consistent with Hfq competition.

We observed that DsrA activation decreased when an unpartnered sRNA (MicC, OxyS, and perhaps RyhB) or target mRNA (*ompC::T7 RNAP*) was transcribed (Fig. 4*D*). Unfortunately, it is difficult to determine which of these unpartnered sRNAs and target mRNAs are competing for Hfq as opposed to acting directly on the *rpoS::gfp* target mRNA because the expression of *rpoS::gfp* is low without DsrA and further decreases in expression are difficult to measure. At least one of the sRNAs, OxyS, is

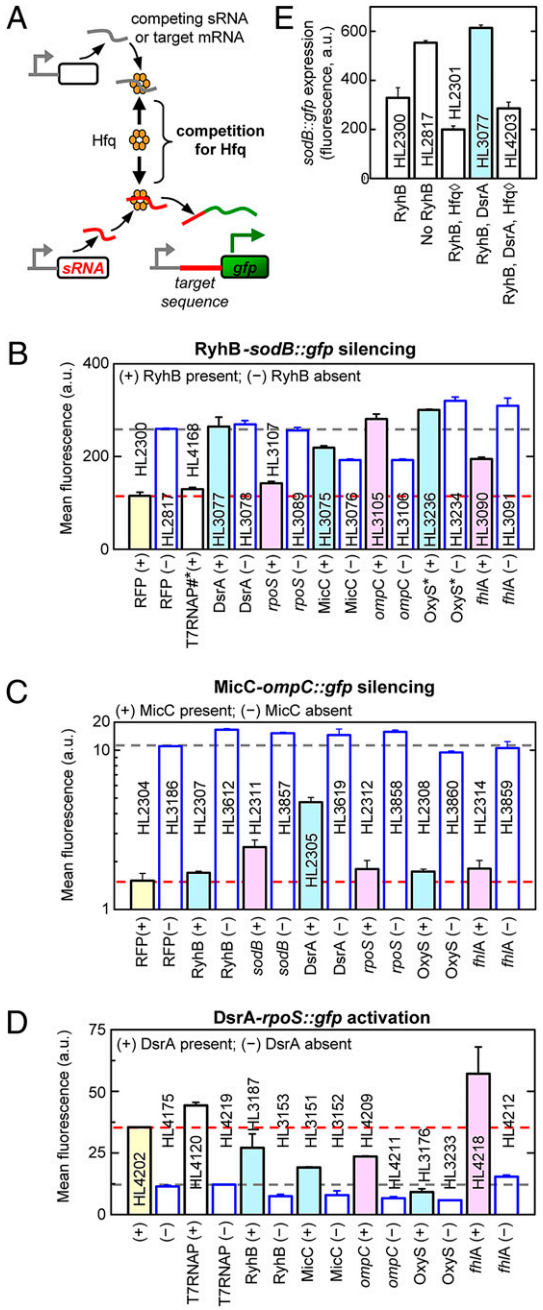

Fig. 4. Competition for Hfq by unpartnered sRNAs and unpartnered target mRNAs. Error bars indicate the SEM. Gene fusions are abbreviated to the target mRNA name. (*A*) The experimental system. (*B*) The effect of competing sRNAs and target mRNAs on RyhB silencing of *sodB::gfp*. The competitors were transcribed from the constitutive pcon promoter, and the competing target mRNAs were fused to mCherry (RFP). #As an additional control, part of the T7 RNA polymerase gene (T7RNAP) was fused to RFP (instead of the target mRNA), and this showed no effect on RyhB silencing. *Samples measured separately with controls that enabled the fluorescence values to be calibrated across experiments. (*C*) The effect of competing sRNAs and target mRNAs on MicC silencing of *ompC::gfp*. The competitors were transcribed using a weaker promoter (pLtetO-1), which enabled *sodB::mCherry* to be transcribed without toxicity. The competing target mRNAs were fused to RFP. (*D*) The effect of competing sRNAs and target mRNAs on DsrA activation of *rpoS::gfp*. The competitors were transcribed from the pcon promoter and the competing target mRNAs were fused to the first third of T7RNAP. (*E*) The expression of additional Hfq in the RyhB-*sodB::gfp* system. All strains have chromosomal *hfq*. ◊ indicates strains with additional *hfq* on a plasmid under pLtetO-1 control.

known to bind directly to *rpoS* (34). The transcription of the T7RNAP sequence, which is not thought to bind to Hfq, did not affect DsrA activation.

Expression of Additional Hfq Reverses the Effect of a Competing Unpartnered sRNA. If the observed loss of sRNA activity caused by the transcription of unpartnered sRNAs is due to Hfq competition, increasing Hfq should reverse the effect, causing sRNA activity to improve. In contrast, if the unpartnered sRNAs are causing loss of sRNA activity due to an off-target effect or by binding directly to the partnered sRNA or target mRNA, the loss of sRNA activity would be expected to be the same or worse with the additional Hfq. The hypothesis was tested in the RyhB-*sodB::gfp* system, where the transcription of unpartnered DsrA caused a profound loss of RyhB silencing. We found that the loss of RyhB silencing was largely reversed by increasing the production of Hfq, as predicted (Fig. 4*E*). This experiment provides compelling evidence to support the existence of competition for Hfq.

Dual Pathways for sRNA Activity. In some sRNA-target mRNA pairs, both the sRNA and the target mRNA appear to compete for Hfq and impair sRNA activity. For example, MicC and its target mRNA (*ompC*) can separately decrease RyhB silencing of *sodB::gfp* as well as DsrA's activation of *rpoS::gfp* (Fig. 4 *B* and *D*). The binding of both the sRNA and its target mRNA to Hfq indicates the potential for duplexes to form via dual pathways: one where the sRNA binds first to Hfq, followed by target mRNA binding, and another where the target mRNA binds first to Hfq, followed by sRNA binding. This is consistent with other studies that have found the sRNA and target mRNA in the DsrA-*rpoS* (36) and RyhB-*sodB* pairs (37) can separately bind to Hfq. For generality, all sRNAs are considered to act via a dual pathway, although it is clear that the rate constants substantially favor one pathway over the other in some cases.

Partnering sRNAs and Target mRNAs Reduces Hfq Competition. The above experiments support the hypothesis that unpartnered sRNAs and target mRNAs can sequester Hfq into sRNA–Hfq and target mRNA–Hfq complexes, resulting in a general loss of sRNA activity. These complexes should decrease if a competing sRNA is transcribed together with its target mRNA (i.e., the pair is "matched") because duplex formation can occur and Hfq will be released (Fig. 5*A*). In contrast, if a competing target mRNA is transcribed with a competing sRNA that is not its partner ("mismatched"), duplexes cannot form and Hfq availability will not improve and may worsen (Fig. 5*B*).

The predictions were tested using the RyhB-*sodB::gfp* pair as a reporter of sRNA activity. The silencing of *sodB::gfp* by RyhB was almost completely inhibited by unpartnered DsrA, resulting in high *sodB::gfp* expression (also shown earlier). When the matched target mRNA partner (*rpoS::mCherry*) for DsrA was cotranscribed, however, *sodB::gfp* expression decreased, indicating improved silencing (Fig. 5*C*, compare blue- and green-shaded bars). In contrast, the transcription of a mismatched target mRNA partner (*ompC::mCherry*) for DsrA did not improve silencing (Fig. 5*C*, compare blue- and pink-shaded bars). The same pattern was observed with unpartnered *ompC::mCherry* target mRNA, which inhibits RyhB silencing of *sodB::gfp*. The transcription of the matched partner (MicC) for *ompC::mCherry* improved silencing (Fig. 5*D*, compare gray- and green-shaded bars), whereas the transcription of a mismatched partner (DsrA) did not (Fig. 5*D*, compare gray- and pink-shaded bars).

The results show that competition for Hfq can be reduced if the transcription of sRNAs and target mRNAs is coordinated with their partner, such that duplexes can form. In our experiments, the partner did not completely ameliorate the effect of the competing sRNA or target mRNA. This is because the concentration of the sRNAs and target mRNAs is unlikely to be iden-

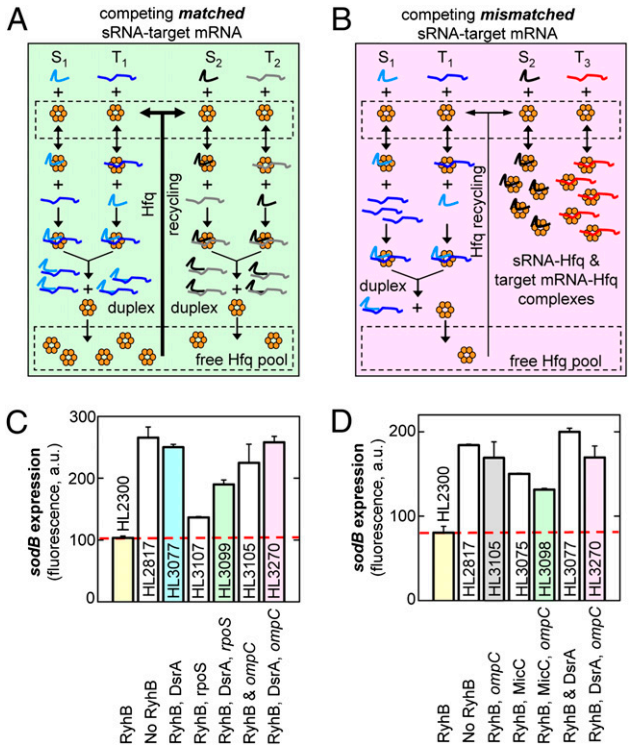

Fig. 5. Transcription of competing matched and mismatched sRNA-target mRNA pairs. Error bars indicate the SEM. Gene fusions are abbreviated to the target mRNA name. (*A* and *B*) Reaction schemes showing the effect of competing matched and mismatched sRNA-target mRNA pairs on duplex formation. S_1 and T_1 are the sRNA-target mRNA pair being measured. S_2 and T_2 are a competing matched sRNA-target mRNA pair, whereas S2 and T3 are an unmatched sRNA-target mRNA pair that cannot form duplexes. (*C* and *D*) RyhB silencing of *sodB::gfp* expression with competing sRNAs and target mRNAs that are matched and mismatched. In *D*, the sample with RyhB and *sodB::gfp* plus DsrA has higher expression than the control without RyhB. The reason for this is unclear, and it is not observed in *C*.

tical in the matched pairs; therefore, there is some residual amount of competing sRNA or target mRNA that is not incorporated into a duplex that sequesters Hfq.

Discussion

In this study, we have demonstrated that the availability of Hfq can be a limiting factor for sRNA activity. This was observed in three independent sRNA-target mRNA pairs (RyhB-*sodB*, DsrA-*rpoS*, and MicC-*ompC*), which suggests that it is a general feature of sRNA networks. One of the most important ramifications of this finding is that it gives rise to competition between different sRNAs and target mRNAs for Hfq. This means that the activity of an sRNA is dependent on the concentration and binding affinity for Hfq of the other sRNAs and target mRNAs being transcribed at the same time; therefore, the sRNA network is far more interdependent than if Hfq were present in excess. This was clearly demonstrated by our competition experiments, which showed that the transcription of unpartnered sRNAs and unpartnered target mRNAs can abolish the function of unrelated sRNAs.

We have demonstrated that Hfq competition can be reduced by ensuring that the transcription of sRNAs and their target mRNAs is coordinated, such that they turn on and off together. This enables duplex formation to occur, which prevents the accumulation of sRNA–Hfq and target mRNA–Hfq complexes that sequester Hfq. The need to coordinate the transcription of sRNAs and target mRNAs to prevent Hfq depletion does not necessarily conflict with the proposed regulatory roles for sRNAs, which include generating threshold-linear responses (30), filtering noise in gene regulation (38), and sharpening spatial patterns (39). Nevertheless, it does suggest that these roles are likely to be constrained to avoid Hfq overloading. This can be achieved by limiting the number of unpartnered sRNAs and target mRNAs that are concurrently transcribed, minimizing their concentrations, or increasing Hfq production (although there may be a fitness cost to overexpressing Hfq as we have observed).

It is possible that competition for Hfq is a desirable feature in sRNA networks because it can be used to prioritize signaling within the cell under stress conditions when many sRNAs are being transcribed. sRNA-target mRNA pairs that have greater affinity for Hfq and high rates of duplex formation and release will be preferentially processed. Furthermore, those sRNAs that are not able to compete for Hfq binding effectively will generally be rapidly degraded, thereby lowering their total concentration. This type of competition between different sRNA and target mRNA substrates is not unique to Hfq. For example, in human cells, there is in vivo and in vitro evidence that competition occurs between sRNAs for a key mediator of sRNA silencing (RNA-induced silencing complex) (40–42).

In summary, our study reveals that Hfq is a limiting factor for sRNA activity and that it can be easily depleted by transcribing a sufficient amount of unpartnered sRNAs or target mRNAs. Although this can lead to competition and decreased sRNA activity, it also provides a means for Hfq to tune sRNA activity and the amount of cross-talk between different sRNA-target mRNA pairs globally.

Materials and Methods

Bacterial Strains and Plasmids. Plasmids, strains, and oligonucleotide sequences are listed in Tables S1–S3. Plasmid structures are shown in Fig. S1. Further details are provided in *SI Materials and Methods*.

Data Collection and Analysis. Single-cell measurements of GFP expression were collected with a Beckman–Coulter EPICS XL-MCL, and the data were analyzed using Flow explorer 4.1 (R. Hoebe, University of Amsterdam, Amsterdam, The Netherlands) and custom programs written with Matlab (MathWorks) software. Measurements were performed on exponentially growing cultures grown at 37 °C in LB with the appropriate antibiotic and concentration of IPTG (*SI Materials and Methods*).

Western Blotting. Proteins were extracted from 4-mL aliquots of exponentially growing cells (≈0.3 OD_{600}) and then separated on 10% wt/vol Bis-Tris gel in Mes buffer. The gels were blotted onto PVDF membranes, blocked, and then incubated overnight with anti-Hfq using standard protocols (*SI Materials and Methods*). L9 was used as a loading control (43). Anti-Hfq and anti-L9 antibodies were kindly provided by Udo Blasi, Branislav Vecerek, and Isabella Moll (Max F. Perutz Laboratories, University of Vienna, Vienna). The secondary antibodies were donkey anti-rabbit IgG HRP and donkey anti-goat IgG-HRP. The antibodies were visualized with ECL Plus Western Blotting Detection Reagents and radiographic film (both from GE Healthcare Life Sciences). Digital images were captured by transillumination of the film using the Gel Doc XR imaging system, and the bands were quantified with Quantity One Analysis software (both from Bio-Rad). The Western blots were performed in triplicate.

Quantitative RT-PCR. Total RNA was extracted from five exponentially growing cell cultures using TRIzol (Invitrogen) and treated with DNase I. The cDNA was synthesized from DNase I-treated RNA using the iScript select cDNA synthesis kit and random primers (Bio-Rad). Quantitative PCR was performed to determine the concentration of cDNA using iQ SYBR Green Supermix with the iQ5 Real-Time PCR detection system (Bio-Rad). Samples without reverse transcriptase were measured in parallel to determine the concentration of any contaminating DNA. The sRNAs and target mRNAs were amplified with the oligonucleotides shown in Table S3. Differences in RNA extraction, loading, and efficiency of cDNA synthesis were normalized using the stable 5S ribosomal RNA (*rrfB*) (*SI Materials and Methods*). Triplicate (or more) measurements were performed.

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