Published in final edited form as:

*Mol Microbiol*. 2011 December ; 82(6): 1545–1562. doi:10.1111/j.1365-2958.2011.07907.x.

# Competition among Hfq-binding small RNAs in *Escherichia coli*

**Kyung Moon**[†], **Susan Gottesman**[*]

Laboratory of Molecular Biology, National Cancer Institute, Bethesda, MD 20892, USA.

## Summary

A major class of small bacterial RNAs (sRNAs) regulate translation and mRNA stability by pairing with target mRNAs, dependent upon the RNA chaperone Hfq. Hfq, related to the Lsm/Sm families of splicing proteins, binds the sRNAs and stabilizes them *in vivo* and stimulates pairing with mRNAs *in vitro.* Although Hfq is abundant, the sRNAs, when induced, are similarly abundant. Therefore, Hfq may be limiting for sRNA function. We find that, when overexpressed, a number of sRNAs competed with endogenous sRNAs for binding to Hfq. This correlated with lower accumulation of the sRNAs (presumably a reflection of the loss of Hfq binding), and lower activity of the sRNAs in regulating gene expression. Hfq was limiting for both positive and negative regulation by the sRNAs. In addition, deletion of the gene for an expressed and particularly effective competitor sRNA improved the regulation of genes by other sRNAs, suggesting that Hfq is limiting during normal growth conditions. These results support the existence of a hierarchy of sRNA competition for Hfq, modulating the function of some sRNAs.

## Introduction

Approximately 100 small RNAs (sRNAs) have been identified in *Escherichia coli* (*E. coli*) by genome-wide searches using a variety of approaches (reviewed in Sharma and Vogel, 2009; Waters and Storz, 2009; Backofen and Hess, 2010). Many of these sRNAs regulate gene expression either positively or negatively via partial base pairing with target genes (Gottesman, 2004; Waters and Storz, 2009). Hfq, an RNA-binding protein, is required for full action of this family of sRNAs in *E. coli* (reviewed in Brennan and Link, 2007). In most cases of positive regulation, sRNAs bind to and remodel secondary structures in the 5′ untranslated region (UTR) of a target gene to free the ribosome binding site (RBS), allowing translation. In negative regulation, on the other hand, sRNAs generally bind close to the RBS, resulting in decreased gene expression by blocking translation and/or decreasing the stability of the target mRNA.

Hfq, found in about 50% of bacterial species (Sun *et al.,* 2002), was originally described as a host factor for the replication of phage Qβ in *E. coli.* It forms a homohexameric doughnut-like structure with similarity to Sm/Lsm proteins in eukaryotes (reviewed in Brennan and Link, 2007). Hfq binds to both sRNAs and mRNAs *in vivo,* as judged by

[*]For correspondence. susang@helix.nih.gov; Tel. (+1) 301 496 3524; Fax (+1) 301 496 3875.
[†]Present address: J. Craig Venter Institute, Rockville, MD, USA.

Supporting information

Additional supporting information may be found in the online version of this article.

immunoprecipitation experiments (Zhang *et al.,* 2003; Sittka *et al.,* 2008). Many Hfq-binding sRNAs are stabilized by Hfq *in vivo;* without Hfq, at least some of these sRNAs are degraded by RNase E. In addition, Hfq helps to promote pairing of sRNA and target mRNA. Thus, in the absence of Hfq, the accumulation of many sRNAs is low, generally resulting in loss of regulation of the target genes.

While sRNAs can be induced to high levels under stress conditions, the amount of Hfq per cell does not increase when an sRNA is overproduced (Zhang *et al.,* 1998). This raises the possibility that Hfq may become limiting for sRNA action under some stress conditions. For instance, a single *E. coli* cell has been estimated to contain up to 10 000 Hfq hexamers, but one sRNA, OxyS, reaches a level of 4500 molecules per cell when induced by oxidative stress (Altuvia *et al.,* 1997). Given the number of sRNAs that can bind Hfq, as well as binding of Hfq to mRNAs, it seems likely that Hfq could be limiting under some conditions. If Hfq is limiting, sRNAs will compete for Hfq binding and an induced sRNA might interfere with regulation by other sRNAs. This mechanism was proposed for OxyS-negative regulation of the sigma factor RpoS (Zhang *et al.,* 1998). Overexpression of OxyS decreased the expression of RpoS; however, no base pairing between OxyS and the *rpoS* mRNA could be detected. Because Hfq and sRNAs are necessary for *rpoS* translation, Zhang *et al.* suggested that OxyS might compete for Hfq (Zhang *et al.,* 1998). We observed a similar phenomenon (Mandin and Gottesman, 2010). Using an sRNA library and a *PBAD–rpoS–lacZ* translational fusion, we found that a few sRNAs, including OxyS, negatively regulated *rpoS* expression, and this effect was Hfq-dependent. These observations led to the hypothesis that induced OxyS interferes with the action of DsrA, which is a positive regulator of RpoS, by sequestering Hfq. One expected consequence of sequestration of Hfq would be that DsrA is rapidly degraded, providing a reason why RpoS is negatively regulated. Consistent with this model, DsrA and OxyS compete with each other for binding to Hfq *in vitro* (Sledjeski *et al.,* 2001).

There are a number of expectations if competition is taking place: (i) if the stability and therefore the accumulation of an sRNA requires binding to Hfq, induction of one sRNA might reduce the accumulation of other sRNAs, (ii) induction of one sRNA should reduce the ability of other sRNAs to gain access to Hfq, as measured by lower levels of immunoprecipitation with Hfq, (iii) induction of one sRNA might interfere with the ability of other sRNAs to carry out regulation, while competing with them for Hfq binding, and (iv) deletion of the genes for a competing sRNA might improve the regulation by other sRNAs.

In this study, we investigated sRNA competition for Hfq. We have focused our attention on those sRNAs that are relatively abundant under normal growth conditions to allow monitoring of these sRNAs without specific induction treatments. We monitored total accumulation and Hfq binding to these sRNAs, as well as the activity of target genes for these sRNAs after induction of alternative sRNAs. We find that sRNAs can compete with each other for Hfq, limiting the extent of gene regulation by some sRNAs. Individual sRNAs show significant differences in their ability to compete. Thus, Hfq competition provides another regulatory mechanism that Hfq-dependent sRNAs can use in addition to regulation by base pairing. This conclusion is consistent with recent studies also showing competition between selected sRNAs for Hfq (Hussein and Lim, 2011; Olejniczak, 2011).

## Results

### Accumulation of sRNAs depends on Hfq

Many sRNAs in *E. coli* require Hfq for their function. Many research groups have shown that sRNAs are less stable and do not accumulate in the absence of Hfq (for example, see Massé *et al.,* 2003; Basineni *et al.,* 2009; Figueroa-Bossi *et al.,* 2009; Moon and Gottesman, 2009; Papenfort *et al.,* 2009). We have confirmed this with a set of seven sRNAs to be used in this work, under our growth conditions. The level of a given sRNA in an *hfq* mutant represents the expectation if Hfq were totally unavailable due to competition. RNA samples were collected from a wild type (NM22540, a derivative of MG1655) and an *hfq* deletion mutant at both exponential phase ($OD_{600}$ = 0.5) and early stationary phase ($OD_{600}$ = 1.5) at three different temperatures (32°C, 37°C and 42°C), and the levels of each sRNA were then compared (Fig. S1, Table 1). The levels of these sRNAs in $hfq^+$ cells varied with temperature and growth phase (Fig. S1). As expected, DsrA was more abundant at low temperature than at 42°C (Repoila and Gottesman, 2001). Spot42 was also more abundant at lower temperatures than at 42°C; CyaR was more abundant at higher temperatures. These differences were most evident during exponential growth.

As expected, the levels of the sRNAs were significantly lower when *hfq* was deleted (Fig. S1; Table 1). However, the extent of the loss in the *hfq* mutant varied with different sRNAs. For instance, the accumulation of MgrR and the long form of DsrA decreased three- to fivefold in the absence of Hfq compared with the wild type during exponential growth at all temperatures. CyaR and Spot42 decreased approximately 10-fold under the conditions at which they were best expressed (Fig. S1, Table 1). ChiX showed the most dramatic change, more than 15-fold under some conditions, while there was little change in the level of GcvB throughout all growth conditions. ArcZ is processed from a 120 nt form to an abundant 56 nt form, and, as previously observed, none of the processed form was detected in an *hfq* mutant (Sittka *et al.,* 2009; Mandin and Gottesman, 2010). The loss of the processed form in the *hfq* mutant might reflect Hfq-dependent processing of ArcZ, instability of the processed form, or both. DsrA, in which a proportion of the sRNA is in a shorter form, showed a similar pattern; the long form decreased only modestly in the *hfq* mutant, but essentially no short form was detected.

These results suggest very different degrees of dependence upon Hfq for stability of different sRNAs. There is some correlation between the effect of deleting *hfq* and the half-lives measured in the presence of Hfq. For instance, GcvB has been reported to be unstable (half-life of 2 min) and its accumulation is not much changed in an *hfq* mutant, while ChiX, with a long half-life, is dramatically decreased in accumulation in the *hfq* mutant (compare Table 1 and Vogel *et al.,* 2003). Why Hfq does not stabilize some Hfq-binding sRNAs remains to be determined.

### Less Hfq lowers sRNA accumulation

The accumulation of many Hfq-binding sRNAs was dramatically changed in the absence of Hfq (Fig. S1, Table 1). However, competition for Hfq will not lead to total absence of Hfq but instead may lower the available pool. Therefore, we examined the effect of a mutation

known to decrease Hfq levels on the levels of Hfq-binding sRNAs. Sharma *et al.* reported that DksA positively regulates Hfq expression in *Shigella* (Sharma and Payne, 2006). In the *Shigella dksA* mutant, the levels of both the *hfq* mRNA and protein decreased by 50% compared with the wild type. We therefore examined the effect of the *dksA* mutation on sRNA accumulation and function as well as competition for Hfq in *E. coli.*

The levels of Hfq in both the wild type and the *dksA* mutant were measured to confirm the phenotype in *E. coli*; protein samples were collected at mid-log phase ($OD_{600}$ = 0.5) and stationary phase ($OD_{600}$ = 1.5) at 30°C, and Western blots were performed with Hfq antiserum. As observed in *Shigella,* in the absence of DksA, the level of Hfq decreased to approximately 50% of that in wild-type cells (Fig. 1, bottom panel); a similar reduction was seen at 37°C.

sRNA samples were analysed from the wild-type and *dksA* mutant strains (Fig. 1; a ratio of > 1 indicates less sRNA in the Δ*dksA* mutant). Accumulation of both ChiX and MgrR was decreased in the *dksA* mutant, compared with wild-type cells (Fig. 1). In the *dksA* mutant, CyaR levels were significantly less only in stationary phase (when it is most abundant), while Spot42, more abundant in exponential phase than in stationary (Fig. S1), showed moderately lower accumulation only in exponential phase (Fig. 1). ArcZ and DsrA long and short forms were analysed separately. In both cases, the effects of the *dksA* mutant were moderate. Note that there is enough Hfq, at least in stationary-phase cells, for significant accumulation of the short form of ArcZ in the *dksA* mutant (Fig. 1) in contrast to the situation in an *hfq* mutant (Fig. S1).

## Competition by OxyS overexpression and rescue by increased Hfq

The results with a *dksA* mutant suggested that if a highly induced sRNA competes with other sRNAs for Hfq, we would expect a decrease in the total accumulation of these other sRNAs. We revisited the negative regulation of RpoS by OxyS as an initial test for competition (Zhang *et al.,* 1998). RpoS is positively regulated by three sRNAs and Hfq (Majdalani *et al.,* 1998; 2001; Sledjeski *et al.,* 2001; Mandin and Gottesman, 2010). If Hfq is limiting for sRNA action when OxyS is overexpressed, we would expect a decrease in the accumulation of RpoS, as previously seen, as well as a decrease in levels of DsrA and other sRNAs. In addition, overexpression of Hfq should reduce or reverse sRNA competition.

This was examined in the experiments shown in Fig. 2. A plasmid carrying OxyS under the control of the $P_{bad}$ promoter was used; cells also carried either a compatible low-copy vector control or a compatible low-copy plasmid expressing Hfq, also from a $P_{BAD}$-inducible promoter. Hfq and/or OxyS expression was induced, and samples analysed for OxyS RNA and Hfq protein levels (Fig. S2). In addition to OxyS, the levels of five different Hfq-dependent sRNAs, including DsrA, were examined; all were chosen because their levels were high enough to be easily detected in cells growing under these conditions (LB, mid-log, 30°C; see Fig. S1). RpoS levels were also monitored.

Approximately 1800 OxyS molecules per cell were present after 60 min of induction (Fig. 2B; OxyS panel, lane 2; Fig. S2); this increased to 2700 molecules when excess Hfq was present (OxyS panel, lane 4; Fig. S2). This increase suggests that OxyS stability is itself

limited by availability of Hfq. Hfq levels were measured (Fig. S2) to be about 4000 Hfq hexamers per cell in the wild-type situation, increased to 6000–8000 hexamers per cell when the $P_{BAD}$–*hfq*$^{+}$ plasmid was induced, a modest overproduction of Hfq. Higher levels of Hfq from the plasmid were seen in the presence of the OxyS plasmid (Fig. 2B, Hfq panel, compare lanes 3 and 4); the reason for this is not known. The level of Hfq in the wild type (lane 1) was comparable to that seen by Kajitani *et al.* (1994) (5000–10 000 hexamers). RpoS levels during exponential phase growth are relatively low, and induction of OxyS had only a modest negative effect (Fig. 2B, RpoS panel, lane 2 versus lane 1). However, a twofold increase in Hfq resulted in a fourfold increase in RpoS levels in the absence of OxyS induction (RpoS panel, compare lane 3 with lane 1). Thus, Hfq is apparently limiting for RpoS translation, even in the absence of an overexpressed RNA. Overexpressing OxyS as well as Hfq modestly reduced this effect, suggesting that OxyS can still compete to some extent even with extra Hfq.

For the six endogenously encoded sRNAs examined, five decreased in levels as OxyS was induced (Fig. 2B: DsrA, ChiX, ArcZ, CyaR and MgrR, lanes 1 versus 2). In all cases, excess Hfq overcame this decrease (compare lanes 3 and 4), and, in fact, the sRNAs accumulated to a somewhat higher level than in wild-type cells (compare lane 3 to lane 1 for these panels). Thus, under normal growth conditions, Hfq levels were not sufficient to fully bind all sRNAs, and competition was greater under conditions of OxyS induction. Extra Hfq increased sRNA accumulation, with or without OxyS induction, presumably by binding more of the sRNAs and stabilizing them.

The only sRNA not significantly affected by OxyS overexpression was Spot 42. In parallel with this observation, Hfq overexpression did not significantly affect Spot 42 levels (Fig. 2B).

## Competition for Hfq binding to sRNAs

The studies above measured accumulation of sRNAs as a proxy for the effect of Hfq binding on sRNA stability. From the results in Fig. 2, we would predict that induction of OxyS should also reduce the binding of other sRNAs to Hfq. This was examined by inducing OxyS, performing Hfq co-immunoprecipitation and measuring the sRNAs in the immunoprecipitate. To mimic the level of induction of OxyS by hydrogen peroxide, without the other complications of hydrogen peroxide treatment, we induced an arabinose-inducible promoter in place of the native *oxyS* promoter with arabinose for 1 h. Levels of OxyS were determined by comparison with an *in vitro* transcribed OxyS control. Induction of chromosomal $P_{BAD}$–*oxyS* gave a level of OxyS within a factor of two of that seen after hydrogen peroxide induction, 600–1000 molecules per cell, less than that found previously (Zhang *et al.,* 1998) and two- to fivefold less than that seen from the plasmid in Fig. 2 (Fig. S3). These induced cells were used for Hfq co-immunoprecipitation, and seven sRNAs were monitored by Northern blot (Fig. 3). The amounts of sRNA in the immunoprecipitate were normalized to levels without arabinose at the same time point. At 0 min (no accumulation of OxyS), this ratio is close to 1 for all the sRNAs (Fig. 3). When OxyS was induced for 60 min, the level of each of the sRNAs in the immunoprecipitate was significantly reduced. The least drastic effect was for immunoprecipitation of ChiX, possibly suggesting that OxyS is

less effective in competing with ChiX for Hfq, compared with competition with other sRNAs (Fig. 3B). Because less OxyS was expressed here than in Fig. 2, there was less of a decrease in total RNA (Fig. 3A). However, the measurement of immunoprecipitated sRNAs was clearly a more sensitive measure of competition than accumulation of the sRNA. Immunoprecipitation also enriched for some sRNA species not detected in the total sRNA Northern blot (Fig. 3A); these may be degradation products forming during the immunoprecipitation procedure. These species were also less abundant after OxyS induction.

Taken together, induction of OxyS, whether from a plasmid or from the chromosome at physiologically relevant levels, was sufficient to reduce levels of DsrA and other sRNAs, reduce binding of these sRNAs to Hfq, and this reduction in levels was overcome by increasing Hfq, consistent with competition for Hfq *in vivo.*

## Competition for RpoS regulation by other sRNAs

OxyS was chosen for competition experiments here because it had previously been shown to negatively regulate *rpoS* without apparent base-pairing (Zhang *et al.*, 1998). However, we would expect that other sRNAs, if overproduced, should also compete. In a previous study, downregulation of an *rpoS–lacZ* translational fusion by multiple sRNAs was observed and attributed to competition for Hfq (Mandin and Gottesman, 2010). The method used in that work may thus be appropriate for further investigating competition. In these experiments, each of 26 Hfq-binding sRNAs was expressed from a $P_{lac}$ promoter on a plasmid, and the activity of a translational fusion to a given target of interest was measured. We found that two of the plasmids, expressing DicF or IS118 (now known to encode a short ORF; K. Moon, unpublished), downregulated a $P_{bad}$–*lacZ* control fusion (Fig. S4). These plasmids were therefore omitted from further screening, leaving 24 Hfq-binding sRNAs that were screened in each experiment.

We first confirmed the previous results with an *rpoS–lacZ* fusion. Stimulation of expression by the three known positive regulators of *rpoS* translation was clearly seen (Fig. 4A). OxyS and five other sRNAs decreased expression of the $P_{BAD}$–*rpoS–lacZ* fusion by at least twofold. Our results parallel those of Mandin and Gottesman; three sRNAs, MgrR, RydC, RyeB were just below the twofold cut-off in their work but just above it in this experiment (Mandin and Gottesman, 2010). The chromosome in these cells is wild type for all three of the positively acting sRNAs; previous work suggests that chromosomally encoded ArcZ and DsrA contribute the most to expression of the fusion under our growth conditions (Mandin and Gottesman, 2010). Thus, any competition seen presumably reflects competition with ArcZ and DsrA.

If sRNAs compete for Hfq, we would expect anything else that limits Hfq levels to exacerbate competition. As shown earlier, a *dksA* mutant reduced the level of Hfq by 50%. We introduced the sRNA library into a *dksA* deletion mutant derivative of the $P_{BAD}$–*rpoS–lacZ* fusion strain and compared the profile for sRNA competition to that for the wild-type strain. The basal level of *rpoS* was unchanged (40 units of β-galactosidase activity in WT versus 38 units in the Δ*dksA* strain), and ArcZ, DsrA and RprA were again detected as positive regulators (Fig. 4B). Negative regulation by all the sRNAs observed in the wild-type

cells was seen, but the extent of negative regulation varied. Four additional sRNAs, FnrS, GcvB, RyhB and GlmZ, downregulated the fusion by twofold or more (Fig. 4B). These results are consistent with these sRNAs successfully competing when Hfq levels are lower, due to the *dksA* mutation, while they do not compete as well when Hfq is more abundant (wild-type cells). This may in part reflect the lower levels of DsrAandArcZ in the *dksA* mutant (Fig. 1). It is also possible that deletion of *dksA* affects the abundance of the competing sRNAs by affecting synthesis of either the sRNAs or their targets. Note that not all sRNAs competed; Spot42 and RybB had very little effect on *rpoS* translation in either the wild-type or *dksA* mutant cells. When *hfq* was deleted, the basal level of *rpoS* was twofold lower, and positive regulation by RprA and ArcZ was lost (Fig. S5A); multicopy DsrA was still able to act, as previously observed (Mandin and Gottesman, 2010). The negative regulation by other sRNAs was also reduced in the *hfq* mutant (Fig. S5A), consistent with the negative regulation by these sRNAs (Fig. 4) reflecting competition for Hfq.

To confirm these results, two of the sRNAs were examined further, testing effects on RpoS itself rather than the fusion, and directly measuring DsrA and ArcZ. ChiX was an effective competitor for *rpoS,* while Spot 42 did not compete in the microtitre assays (Fig. 4). These two sRNAs were each induced for 180 min from a plasmid under control of a $P_{bad}$ promoter, to be more parallel to the tests with OxyS, and samples were analysed for DsrA, the short (processed) form of ArcZ (ArcZ56), and RpoS protein (Fig. 5; Fig. S6). Consistent with the results shown in Fig. 4, induction of ChiX significantly reduced the amount of RpoS (Fig. 5A), while Spot 42 did not (Fig. 5B). The levels of both DsrA and ArcZ were also affected by ChiX induction, with a somewhat greater effect on ArcZ (Fig. 5A). On the other hand, the induction of Spot42 had a much less dramatic effect on these two sRNAs. We note that less Spot42 accumulates (Fig. 5B; 1500 molecules of Spot 42 compared with 3000 molecules of ChiX at 120 min), but even at 30 min, when the level of ChiX is significantly less than the final level of Spot 42, there is a dramatic decrease in RpoS levels by ChiX. These observations suggest that different sRNAs have different abilities to compete for Hfq and consequently to affect Hfq titration, and imply tight binding of ChiX to Hfq.

## Negatively regulated genes are also targets for Hfq competition

The studies discussed above confirmed that competition for Hfq can have significant effects on the positive regulation of *rpoS.* While positive regulation provides a particularly sensitive target for studying competition, if the model of Hfq titration is correct, we would expect a similar competition for sRNAs that negatively regulate targets. We would again expect competition to mimic the effect of an *hfq* mutant, in this case increasing expression.

To test this, four known negative targets of sRNAs were selected for study. In each case, the target gene is regulated by a relatively abundant sRNA that is expressed under normal growth conditions, allowing us to see regulation without special growth conditions. For each target, a translational fusion, under the control of the $P_{bad}$ promoter, was constructed and tested with the library of sRNAs.

The first target tested was *eptB,* negatively regulated by MgrR, an sRNA that was an effective competitor for *rpoS* regulation (Moon and Gottesman, 2009 and Fig. 4). The sRNA library screening of the $P_{BAD}$–*eptB–lacZ* fusion confirmed negative regulation by MgrR and

demonstrated that *eptB* was also negatively regulated by ArcZ, even in the absence of MgrR (Fig. 6A and B). ArcZ acts directly on numerous targets, including positively regulating RpoS (Papenfort *et al.,* 2009; Mandin and Gottesman, 2010); it can directly pair with *eptB* (Fig. S7 and K. Moon, unpubl. obs.). sRNAs ChiX and OmrA moderately upregulated *eptB* expression in the presence or absence of MgrR (Fig. 6). Two more sRNAs, MicC and RydC, acted as positive regulators only in a strain deleted for MgrR, suggesting that these sRNAs win the competition with ArcZ, but not with MgrR (Fig. 6B). In a strain deleted for *hfq,* both positive and negative regulation was lost (Fig. S5B), as expected.

*dppA* is a known target of GcvB. In a $P_{BAD}$–*dppA*–*lacZ* strain, the negative regulation by GcvB was easily seen (Fig. 7A). When we screened the sRNA library in a strain carrying a $P_{BAD}$–*dppA*–*lacZ*strain, only ChiX upregulated the expression of the *dppA* mRNA. This upregulation was reduced but was still retained if GcvB was deleted, suggesting that other sRNAs or Hfq itself may also downregulate *dppA* and be subject to competition (Fig. 7B). Candidates for other sRNA regulators are Spot42 and RyeB, which show negative regulation of *dppA* in the absence, but not in the presence of GcvB (Fig. 7B). In the strain deleted for GcvB, the expression of the fusion is higher, but two additional sRNAs, RyhB and MicC, can act as positive regulators for *dppA* (Fig. 7B); again, these may effectively compete with the weak regulation by Spot42 and RyeB but are not able to out-compete GcvB. In the absence of Hfq, the basal level of the fusion is significantly higher (154 units versus 74 units in the wild-type control), and all positive regulation is lost, consistent with positive regulation being due to Hfq titration (Fig. S5C). However, a number of sRNAs were still able to negatively regulate *dppA,* including GcvB. The other sRNAs may act indirectly (regulating GcvB) or act at some other level of *dppA* regulation.

The third target examined, *ompX,* is negatively regulated by CyaR and MicA (Johansen *et al.,* 2008; Papenfort *et al.,* 2008; De Lay and Gottesman, 2009; Gogol *et al.,* 2011) (Fig. 8). In a wild-type strain, no sRNAs showed positive regulation of this fusion above the twofold cut-off (Fig. 8A). Deleting either *micA* or *cyaR* from the chromosome did not uncover a better competitor (Fig. 8B and C). When either CyaR or MicA was deleted, negative regulation by Spot 42 was seen, although it is not known if this is direct (Fig. 8B and C).

Another CyaR target, *yqaE,* was also tested. As for *ompX,* no strong positive regulators were detected, and, in the absence of CyaR, Spot 42 was able to negatively regulate this gene (Fig. S8). The finding that two different CyaR targets do not show competition may suggest that CyaR is not easily competed, or that these two targets have particularly strong pairing (see Discussion).

## ChiX acts as a competitor at chromosomally encoded levels

In the experiments above, ChiX was consistently observed as a strong Hfq competitor (Figs 4–7), and levels of ChiX are significant in cells under our growth conditions (Fig. S1). In fact, chromosomally encoded ChiX has been shown to strongly negatively regulate the gene for an outer membrane porin, *ybfM* (*chiM*) (Figueroa-Bossi *et al.,* 2009; Overgaard *et al.,* 2009; Rasmussen *et al.,* 2009). A reporter for *ybfM* was tested; although the basal level of β-galactosidase in a $chiX^{+}$ strain was too low to measure, overexpression of three sRNAs, MicC, OmrA and RydC, reproducibly increased expression (Table S1), consistent with some

competition for Hfq and therefore relief of ChiX repression. When the chromosomal copy of ChiX was deleted, the basal level of *ybfM* was significantly higher, and negative regulation by two sRNAs, ChiX, sRNAs, ChiX, as expected, and RprA, not previously described, was seen (Table S1). However, no positive regulators were detected. Because RprA is not well expressed under these growth conditions, and *chiX* is deleted, the lack of positive regulators is consistent with the likely absence of any direct pairing sRNAs for *ybfM* under these growth conditions, and therefore the absence of an sRNA to compete with.

It seemed possible that the chromosomally encoded levels of ChiX would be sufficient for it to compete for Hfq in a physiologically significant fashion. This was examined in a number of ways. The effect of the sRNA library on *dppA* was tested in the absence of ChiX. In this strain, multiple sRNAs were now able to positively regulate (compete), including those seen before (ChiX, MicC and RyhB), as well as FnrS, CyaR, RydC, RybB and DsrA (Fig. S9; compare with Fig. 7A). One interpretation of this is that chromosomally encoded ChiX competes for Hfq as effectively as some of the overproduced sRNAs. In the absence of both GcvB and ChiX, the pattern of regulation was similar to that seen in the absence of GcvB (compare Fig. S9B with Fig. 7B).

The ability of ChiX to compete was also evaluated by a somewhat more sensitive assay, quantitative RT-PCR, for both $P_{BAD}$–*eptB–lacZ* and $P_{bad}$–*dppA–lacZ* fusions, in strains carrying deletions of *gcvB, mgrR* or *chiX* and appropriate combinations of deletions. RNA samples were isolated and analysed from cells growing under the same conditions as for the sRNA library screening. Cells harbouring a $P_{BAD}$–*lacZ–lacZ* fusion were a negative control and did not show any changes in the different backgrounds, as expected (Fig. 9A). *eptB* is directly regulated by MgrR, but not by GcvB. As expected, the level of *eptB–lacZ* did not change in the absence of GcvB, while it increased dramatically in the absence of MgrR (Fig. 9B). The level of *dppA–lacZ* increased significantly in the absence of GcvB, again as expected. However, an increase was also seen in the *mgrR* deletion strain (Fig. 9C). Multicopy MgrR had no effect on the *dppA–lacZ* fusion (Fig. 7), making it unlikely that MgrR is a direct regulator of *dppA–lacZ;* this effect might be indirect (for instance, changing the levels of other sRNA regulators). Deletion of ChiX decreased the levels of *eptB–* and *dppA–lacZ* fusions by 50%. For both *eptB* and *dppA,* a double mutant of *chiX* and the regulating sRNA showed expression similar to that for the deletion of the direct regulator (Δ*mgrR* Δ*chiX* for Fig. 9B, Δ*gcvB* Δ*chiX* for Fig. 9C). This result strongly suggests that ChiX, under normal growth conditions, titrates Hfq, limiting the ability of MgrR and GcvB to negatively regulate their targets and thus modulating Hfq-dependent regulation of many genes.

## Discussion

Trans-encoded Hfq-dependent sRNAs regulate various genes via base pairing. Our results and the results of others (Hussein and Lim, 2011) suggest that Hfq is a limiting factor for sRNA regulation. We have shown that the limitation of the available Hfq pool for sRNAs causes competition among them, affecting the stability and activity of the sRNAs and, consequently, changing the outcome of gene regulation. Under conditions of overexpression of one sRNA, accumulation of other sRNAs decreases, binding of these other sRNAs to Hfq

is reduced, and the regulation of target genes is perturbed. Even without overexpression, we find that an abundant sRNA affects the regulation of targets, apparently by limiting Hfq availability.

The idea of competition for Hfq is not new. Zhang and co-workers suggested in 1998 that OxyS negatively regulates RpoS by competing for Hfq (Zhang *et al.,* 1998). Our experiments confirm this, demonstrating that both the accumulation of sRNAs important for RpoS translation (DsrA and ArcZ) and binding of these sRNAs to Hfq is reduced when OxyS is overexpressed, and the competition is overcome by increasing levels of Hfq (Figs 2 and 3). Thus in this physiologically relevant example, OxyS is able to downregulate RpoS by interfering with positive regulation. Overexpression of ArcZ has been observed to lead to widespread changes in mRNA profiles, consistent with titration of Hfq (Papenfort *et al,* 2009).

## Hfq is a limiting factor for sRNA accumulation

Hfq plays multiple roles in stimulating sRNA-dependent function (Brennan and Link, 2007). One role that is relatively easy to monitor is that binding to Hfq stabilizes sRNAs, protecting them from RNase E. If Hfq becomes limiting, we expected this to be reflected in lower levels of sRNA accumulation. In an initial test, the effect of deleting Hfq on sRNA accumulation was found to differ significantly for different sRNAs. Those not significantly affected by deletion of *hfq* may be stable even in the absence of Hfq, or may be unstable even when bound to Hfq. ChiX is very stable in wild-type strains (Vogel *et al.,* 2003), and its accumulation is drastically reduced in an *hfq* mutant (Fig. S1, Table 1). GcvB is unstable even in the presence of Hfq (Vogel *et al.,* 2003) and deletion of *hfq* had minimal effects on its accumulation (Fig. S1, Table 1), consistent with GcvB either binding Hfq poorly, transiently, or in a manner that does not stabilize the sRNA.

This experiment, carried out at three temperatures and in both exponential and stationary phase, leads to additional conclusions. ArcZ has previously been reported to be processed; the appearance of the processed form is Hfq-dependent (Sittka *et al.,* 2009; Mandin and Gottesman, 2010). At low temperature (32°C), the full-length RNA was more abundant in the absence of Hfq than in the $hfq^+$ strain (Fig. S1; Table 1). This is consistent with Hfq-dependent processing, although it does not rule out instability of the processed form in the absence of Hfq as well. Possibly at 32°C the pathway for degradation of the full-length RNA is slow enough to see accumulation in the *hfq* mutant. The *dsrA* promoter is known to be less active at high temperature (42°C) (Repoila and Gottesman, 2001; 2003); this temperature effect was less striking in stationary phase (Fig. S1). CyaR is positively regulated by cyclic AMP and CRP, while Spot 42 is negatively regulated (Møller *et al.,* 2002; Johansen *et al.,* 2008; Papenfort *et al.,* 2008; De Lay and Gottesman, 2009). In our experiments, the accumulation of CyaR and Spot 42 was reciprocal, with CyaR most abundant at high temperature or in stationary phase and Spot 42 low under those conditions. MgrR was highly dependent upon Hfq for accumulation in exponential phase, but not in stationary-phase cells, particularly at 37°C. Possibly the degradation machinery is limiting under these conditions.

### Different sRNAs compete to different extents

We have used a library of plasmids, each expressing an sRNA, to examine the hierarchy of sRNAs for competition, for both positive and negative regulation. The results support rather different behaviours for different sRNAs and/or sRNA/mRNA pairs. Some of these differences may reflect differences in accumulation and/or stability of the sRNAs, but others seem likely to reflect differences in Hfq binding affinity and possibly in mode of Hfq binding (see, for instance, Olejniczak, 2011).

ChiX represents an example of an sRNA that competes particularly well. ChiX overexpression disrupted positive regulation of RpoS (Figs 4 and 5), and negative regulation of *eptB* and *dppA* (Figs 6 and 7). Overproduction of OxyS had much less effect on binding of ChiX to Hfq, compared with other sRNAs (Fig. 3). Finally, deletion of *chiX* was sufficient to improve GcvB-dependent regulation of *dppA* and MgrR-dependent regulation of *eptB* (Fig. 9), strongly suggesting that ChiX helps to modulate sRNA regulation under normal growth conditions. It seems likely this reflects strong binding of ChiX to Hfq. This is consistent with evidence from others suggesting that ChiX may be unusual in its interaction with Hfq (Overgaard *et al.,* 2009). ChiX is reported to act catalytically, stimulating the degradation of multiple mRNA target molecules/sRNA (Overgaard *et al.,* 2009). If pairing usually leads to dissociation of Hfq and the destruction of both sRNA and mRNA (Massé *et al.,* 2003; De Lay and Gottesman, 2011; Hussein and Lim, 2011), possibly ChiX binding by Hfq is qualitatively or quantitatively different. It may not be displaced from Hfq after pairing (or by competition). Spot 42, on the other hand, represents an sRNA that seems to be unable to compete effectively (Figs 4, 5 and 7). Levels of Spot 42 did not decrease significantly when OxyS was overproduced (Fig. 2), and decreased only modestly in a *dksA* mutant (Fig. 1). Spot 42 clearly binds to Hfq, and immunoprecipitation with Hfq was significantly reduced when OxyS was overproduced (Fig. 3). The simplest conclusion is that Spot 42, even when overproduced, does not bind tightly enough to compete with DsrA, ArcZ and MgrR, for instance. We also note that Spot 42 has many mRNA targets (Beisel and Storz, 2011). If pairing with a mRNA helps to remove an sRNA from Hfq (Massé *et al.,* 2003; Hussein and Lim, 2011 and see below), it is possible that Spot 42 binding to Hfq is particularly transient. In fact, for two of the substrates we tested, Spot 42 proved to have some ability to negatively regulate them, possibly directly (Figs 7 and 8), although it is striking that this regulation could only be detected when the primary regulators were deleted from the chromosome, again suggesting that Spot 42 competes poorly with other sRNAs.

Other sRNAs fell between these two extremes. MicC, RydC and OmrA were able to compete modestly with ChiX (Table S1), and also competed for regulation of *eptB,* but only in the absence of MgrR, when ArcZ may be regulating it (Fig. 6B). Similarly, MicC and RyhB competed for regulation of *dppA,* but again only when the primary regulator, GcvB is deleted; direct regulation in this case may be by Spot 42 or RyeB (Fig. 7). Others (RprA, DsrA, MicA, MicF, among others) were never able to compete sufficiently to cause a twofold change in expression.

Hfq competition affected the downregulation of both *eptB* and *dppA* but was not seen for *ompX* regulation (Fig. 8). *ompX* is negatively regulated by CyaR and MicA; it is the chromosomally encoded levels of these sRNAs that would be subject to competition.

Whether the failure to see competition reflects something about *ompX* mRNA itself, about the sRNAs pairing with it and how they bind Hfq as compared with ChiX, for instance, or whether the interaction of CyaR and MicA with this target is particularly effective is not yet known (see Fig. S7 for predicted pairing). We speculate that each sRNA's abundance and Hfq binding affinity plays a crucial role in determining the hierarchy of sRNAs in the system, although competitive affinity studies will be needed to establish some of the relevant values.

Hfq is an abundant protein. Nonetheless, our work and work reported by others suggest that it is normally limiting for many of its functions. Thus, increasing Hfq levels modestly increased accumulation of sRNAs, significantly increased RpoS levels, and decreased competition (Fig. 2). Reducing Hfq levels by twofold, via a deletion of *dksA,* increased the ability of sRNAs to compete, suggesting more stringent competition for Hfq (Fig. 4B). Hussein and Lim (2011) found that increasing Hfq levels increased both positive regulation (RpoS synthesis) and negative regulation (silencing of target genes), consistent with our findings.

### What is the mechanism of competition for Hfq?

For sRNAs to compete for Hfq, Hfq must be limiting, and binding to one sRNA must be transient enough to allow another sRNA to displace it before it is able to act. In a recent study, Fender *et al.* propose a model for Hfq binding and exchange of sRNAs in which an incoming sRNA competes with an sRNA bound to Hfq in a concentration-dependent fashion (Fender *et al.,* 2010). *In vitro* experiments suggested a slow off-rate for sRNAs bound to Hfq in the absence of such a second competing RNA. However, their data suggest that *in vivo* one would expect sRNAs to move on and off Hfq rapidly. The competition we see is consistent with this. Hfq binding to sRNAs were dramatically reduced when another sRNA was induced (Fig. 3).

In another study, Hussein and Lim also reported that Hfq is a limiting factor for sRNA-dependent regulation (Hussein and Lim, 2011). In their experiments, the ability of an overproduced sRNA to regulate a direct target mRNA, also overproduced, was improved by increasing the level of Hfq, and overproducing either an sRNA or a mRNA interfered with regulation of other sRNA : mRNA pairs. They observed that this competition was lessened if the sRNA and its partner mRNA were both overproduced, suggesting that after pairing, Hfq sequestration is lost; we did not investigate this in our system. They suggest that the availability of the target mRNA for a bound sRNA eases competition, by allowing pairing and subsequent release from Hfq and degradation of the two RNAs. In this model, an excess of sRNA over target, probably the case in our overproduction experiments, would be expected to exacerbate competition. This would also assume that each sRNA is used for pairing once, a condition that may not hold for tight binding sRNAs such as ChiX.

Our results agree with and extend these observations, establishing evidence for differential competition by different sRNAs and evidence for a role for competition under physiologically relevant conditions. Some sRNAs, such as ChiX, may compete so well that they are never induced to very high levels, but instead are regulated in a unique fashion, by regulated destruction (Figueroa-Bossi *et al.,* 2009; Overgaard *et al.,* 2009). Others may

compete only when induced. Others are apparently unable to compete effectively, although they still bind Hfq sufficiently well to regulate their targets.

This discussion assumes similar requirements for binding of the sRNAs we have investigated. While most sRNAs are believed to bind to the distal face of Hfq, others may use the proximal face (reviewed in Vogel and Luisi, 2011), and competition presumably would not occur between sRNAs binding at different sites (Olejniczak, 2011). Similarly, mRNAs may also compete for binding, certainly with other mRNAs and possibly with sRNAs. We have not investigated these issues here.

Overall, our study provides evidence that Hfq levels are such that competition for Hfq modulates the effectiveness of the sRNA. Under specific induction conditions, many, but not all, sRNAs will shut down or diminish the effects of other, uninduced sRNAs, by competing for Hfq, thus providing an additional and novel level of connectivity to the various sRNA regulons in the cell. While this work and others cited here have been carried out in *E. coli,* competition for Hfq will certainly occur in other bacteria. In addition, competition among RNAs in eukaryotes has recently been proposed, in which the microRNAs serve as connections and modulators (Salmena *et al.,* 2011); the work in *E. coli* may serve as a model for what might be expected in eukaryotic systems.

## Experimental procedures

### Bacterial strains and plasmids

Strains and plasmids used in this study are listed in Table S2. All *E. coli* strains in this work are derived from MG1655. The recipient strain for standard cloning procedures was DH5α. Strains carrying *lacZ* fusions were constructed in PM1205 (Mandin and Gottesman, 2009), which contains the $P_{BAD}$–*catsacB* segment upstream of *lacZ* at the chromosomal *lacZ* site. To construct a *lacZ* translational fusion of a gene of interest, primers were designed to give a PCR product with 40 nt homologous to the $P_{BAD}$ promoter followed by the specific gene from +1 to codon 9 followed by lac homology (40 nt). Primers are listed in Table S3. The PCR products were introduced into the PM1205 chromosome using the red recombination system (Yu *et al.,* 2000; Mandin and Gottesman, 2009). For the sRNA library, the plasmids were those described by Mandin and Gottesman (2010). The Δ*dksA* mutants were provided by Dr M. Cashel (NICHD, NIH).

### Media and growth conditions

All strains were grown in LB. Antibiotic concentrations (in micrograms per millilitre) were as follows: ampicillin on plates, 50; ampicillin in liquid cultures, 100; kanamycin, 25; chloramphenicol, 25, tetracycline, 25. Each plasmid was freshly transformed into the strain of interest, and a colony was cultured in LB with the appropriate antibiotic overnight at 30°C. Overnight cultures were subcultured into fresh medium at $OD_{600}$ 0.05 (about 1:500 dilution) and grown to $OD_{600} \approx 0.3$. Cells were induced with arabinose for the designated time. For co-immnunoprecipitation experiments, 2 $OD_{600}$ of cells were collected before and after arabinose induction. Cells were washed with cold DEPC-PBS and then frozen at −80°C

for the next steps. Unless otherwise indicated, cells were grown at 30°C to increase the expression of DsrA.

### RNA isolation and Northern blot analysis

To examine the effect of OxyS expressed from an arabinose-inducible promoter, cells were treated with 0.2% arabinose at $OD_{600}$ = 0.3. Cells were collected and total RNA extracted at different time points by the hot-phenol method as previously described (Massé *et al.,* 2003) and detailed in Moon and Gottesman (2009). Detection was performed with the corresponding biotinylated probes in Table S3 ('Biotinylated probes used for Northern blots'). Bands were quantified by imaging using a LAS-4000 mini camera (FujiFilm Medical Systems USA) before the blots were saturated, and analysed with MultiGauge software (Fuji); all samples were normalized to SsrA loading controls.

For quantification of sRNA molecules per cell, we used RNAs transcribed *in vitro* to establish a standard curve (see Fig. S2). *In vitro* transcribed RNA was eluted from the gel, precipitated with ethanol, resuspended in buffer and quantified using a Nanodrop (Thermo-Fisher) in triplicate; the average value was used for the standard curve.

### In vitro transcription

To produce specific sRNAs *in vitro,* we used a template harbouring the T7 promoter followed by the sRNA sequence. For OxyS *in vitro* transcription, the plasmid template pSP64-OxyS was linearized with a restriction enzyme and purified using a QIAGEN PCR purification kit. The primer templates used for DsrA, ChiX and Spot42 are listed in Table S3. sRNAs were synthesized using MEGAscript T7 (ambion) Kit followed by TBE 5%-urea gel purification to remove remaining NTPs and other non-specific fragments.

### Protein sample preparation and Western blot analysis

Protein samples were collected in parallel with RNA samples. One millilitre of cells were collected at each time point and processed as described in Ranquet and Gottesman (2007), with details described in Supporting information.

### Co-immunoprecipitation

Cell samples were resuspended with 400 μl of lysis buffer and were sonicated for 5 min at medium intensity followed by centrifugation at 1400 r.p.m. for 10 min at 4°C. The supernatant of each sample was processed based on the method described by Sun *et al.* (2006) and adapted as described in Supporting information.

### sRNA library screening

The library was introduced into a specific background strain by TSS transformation and spotted on an LB plate containing ampicillin as described previously (Mandin and Gottesman, 2010); details are given in Supporting information.

### β-Galactosidase assay

The β-galactosidase activity of strains carrying *lacZ* fusions was assayed on a SpectraMax 250 (Molecular Devices) microtitre plate reader as described previously (Majdalani *et al.,* 1998). Specific activities are represented as the $V_{max}$ divided by the $OD_{600}$.

### Real-time RT-PCR analysis

To determine more quantitatively the expression of each *lacZ* fusion gene in various background strains, real-time RT-PCR was performed on RNA obtained from wild-type and mutant strains carrying the control $P_{BAD}$–*lacZ*–*lacZ* fusion or either $P_{BAD}$–*eptB*–*lacZ* or $P_{BAD}$–*dppA*–*lacZ* fusions grown in LB at 37°C as described above (see sRNA library screening). Real-time PCR was performed using an iQcycler (Bio-Rad) as described in Moon and Gottesman (2009). Expression of the SsrA gene was used as an internal standard, and SYBR Green Supermix was used as a signal reporter. Each sample was tested in quadruplicate and analysed as described in Moon and Gottesman (2009).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

We thank members of the laboratory and G. Storz and Kumaran Ramamurthi for comments on the manuscript and discussions throughout this work. We thank R. Gourse for pointing out the effect of *dksA* on Hfq levels, M. Cashel for *dksA* mutants and A. Zhang for OxyS plasmids. J.H. Kim (LMP, NCI) is thanked for use of the iQcycler. This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

## References

Altuvia S, Weinstein-Fischer D, Zhang A, Postow L, and Storz G (1997) A small stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator. Cell 90: 43–53. [PubMed: 9230301]

Backofen R, and Hess WR (2010) Computational predictions of sRNAs and their targets in bacteria. RNA Biol 7: 33–42. [PubMed: 20061798]

Basineni SR, Madhugiri R, Kolmsee T, Hengge R, and Klug G (2009) The influence of Hfq and ribonucleases on the stability of the small non-coding RNA OxyS and its target *rpoS* in *E. coli* is growth phase dependent. RNA Biol 6: 584–594. [PubMed: 20016254]

Beisel CL, and 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. [PubMed: 21292161]

Brennan RG, and Link TM (2007) Hfq structure, function and ligand binding. Curr Opin Microbiol 10: 125–133. [PubMed: 17395525]

De Lay N, and Gottesman S (2009) The Crp-activated small noncoding regulatory RNA CyaR (RyeE) links nutritional status to group behavior. J Bacteriol 191: 461–476. [PubMed: 18978044]

De Lay N, and Gottesman S (2011) Role of polynucleotide phosphorylase in sRNA function in *Escherichia coli*. RNA 17: 1172–1189. [PubMed: 21527671]

Fender A, Elf J, Hampel K, Zimmermann B, and Wagner EG (2010) RNAs actively cycle on the Sm-like protein Hfq. Genes Dev 24: 2621–2626. [PubMed: 21123649]

Figueroa-Bossi N, Valentini M, Malleret L, and Bossi L (2009) Caught at its own game: regulatory small RNA inactivated by an inducible transcript mimicking its target. Genes Dev 23: 1981–1985. [PubMed: 19723760]

Gogol EB, Rhodius VA, Papenfort K, Vogel J, and Gross CA (2011) Small RNAs endow a transcriptional activator with essential repressor functions for single-tier control of the global stress regulon. Proc Natl Acad Sci USA 108: 12875–12880. [PubMed: 21768388]

Gottesman S (2004) The small RNA regulators of *Escherichia coli*: roles and mechanisms. Annu Rev Microbiol 58: 273–301. [PubMed: 15487939]

Hussein R, and Lim HL (2011) Disruption of small RNA signaling caused by competition for Hfq. Proc Natl Acad Sci USA 108: 1110–1115. [PubMed: 21189298]

Johansen J, Eriksen M, Kallipolitis B, and Valentin-Hansen P (2008) Down-regulation of outer membrane proteins by noncoding RNAs: unraveling the cAMP-CRP- and $\sigma^E$-dependent CyaR-*ompX* regulatory case. J Mol Biol 383: 1–9. [PubMed: 18619465]

Kajitani M, Kato A, Wada A, Inokuchi Y, and Ishihama A (1994) Regulation of the *Escherichia coli hfq* gene encoding the host factorfor phage Q beta. J Bacteriol 176: 531–534. [PubMed: 8288550]

Majdalani N, Cunning C, Sledjeski D, Elliott T, and Gottesman S (1998) DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription. Proc Natl Acad Sci USA 95: 12462–12467. [PubMed: 9770508]

Majdalani N, Chen S, Murrow J, St. John K, and Gottesman S (2001) Regulation of RpoS by a novel small RNA: the characterization of RprA. Mol Microbiol 39: 1382–1394. [PubMed: 11251852]

Mandin P, and Gottesman S (2009) A genetic approach for finding small RNA regulators of genes of interest identifies RybC as regulating the DpiA/DpiB two-component system. Mol Microbiol 72: 551–565. [PubMed: 19426207]

Mandin P, and Gottesman S (2010) Integrating anaerobic/aerobic sensing and the general stress response through the ArcZ small RNA. EMBO J 29: 3094–3107. [PubMed: 20683441]

Massé E, Escorcia FE, and Gottesman S (2003) Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. Genes Dev 17: 2374–2383. [PubMed: 12975324]

Møller T, Franch T, Udesen C, Gerdes K, and Valentin-Hansen P (2002) Spot 42 RNA mediates discoordinate expression of the *E. coli* galactose operon. Genes Dev 16: 1696–1706. [PubMed: 12101127]

Moon K, and Gottesman S (2009) A PhoQ/P-regulated small RNA regulates sensitivity of *Escherichia coli* to antimicrobial peptides. Mol Microbiol 74: 1314–1330. [PubMed: 19889087]

Olejniczak M (2011) Despite similar binding to the Hfq protein regulatory RNAs widely differ in their competition performance. Biochemistry 50: 4427–4440. [PubMed: 21510661]

Overgaard M, Johansen J, Moller-Jensen J, and Valentin-Hansen P (2009) Switching off small RNA regulation with trap-mRNA. Mol Microbiol 73: 790–800. [PubMed: 19682266]

Papenfort K, Pfeiffer V, Lucchini S, Sonawane A, Hinton JC, and Vogel J (2008) Systematic deletion of *Salmonella* small RNA genes identifies CyaR, a conserved CRP-dependent riboregulator of OmpX synthesis. Mol Microbiol 68: 890–906. [PubMed: 18399940]

Papenfort K, Said N, Welsink T, Lucchini S, Hinton JC, and Vogel J (2009) Specific and pleiotropic patterns of mRNA regulation by ArcZ, a conserved, Hfq-dependent small RNA. Mol Microbiol 74: 139–158. [PubMed: 19732340]

Ranquet C, and Gottesman S (2007) Translational regulation of the *Escherichia coli* stress factor RpoS: a role for SsrA and Lon. J Bacteriol 189: 4872–4879. [PubMed: 17449615]

Rasmussen AA, Johansen J, Nielsen JS, Overgaard M, Kallipolitis B, and Valentin-Hansen P (2009) A conserved small RNA promotes silencing of the outer membrane protein YbfM. Mol Microbiol 72: 566–577. [PubMed: 19400782]

Repoila F, and Gottesman S (2001) Signal transduction cascade for regulation of RpoS: temperature regulation of DsrA. J Bacteriol 183: 4012–4023. [PubMed: 11395466]

Repoila F, and Gottesman S (2003) Temperature sensing by the *dsrA* promoter. J Bacteriol 185: 6609–6614. [PubMed: 14594834]

Salmena L, Poliseno L, Tay Y, Kats L, and Pandolfi PP (2011) A ceRNA hypothesis: the rosetta stone of a hidden RNA language? Cell 146: 353–358. [PubMed: 21802130]

Sharma AK, and Payne SM (2006) Induction of expression of *hfq* by DksA is essential for *Shigella flexneri* virulence. Mol Microbiol. 62: 469–479. [PubMed: 17020583]

Sharma CM, and Vogel J (2009) Experimental approaches for the discovery and characterization of regulatory small RNAs. Curr Opin Microbiol 12: 536–546. [PubMed: 19758836]

Sittka A, Lucchini S, Papenfort K, Sharma CM, Rolle K, Binnewies TT, et al. (2008) Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq. PLoS Genet 4: e1000163. [PubMed: 18725932]

Sittka A, Sharma CM, Rolle K, and Vogel J (2009) Deep sequencing of *Salmonella* RNA associated with heterologous Hfq proteins *in vivo* reveals small RNAs as a major target class and identifies RNA processing phenotypes. RNA Biol 6: 266–275. [PubMed: 19333007]

Sledjeski DD, Whitman C, and Zhang A (2001) Hfq is necessary for regulation by the untranslated RNA DsrA. J Bacteriol 183: 1997–2005. [PubMed: 11222598]

Sun BK, Deaton AM, and Lee JT (2006) A transient heterochromatic state in Xist preempts × inactivation choice without RNA stabilization. Mol Cell 21: 617–628. [PubMed: 16507360]

Sun X, Zhulin I, and Wartell RM (2002) Predicted structure and phyletic distribution of the RNA-binding protein Hfq. Nucleic Acids Res 30: 3663–3671.

Vogel J, and Luisi BF (2011) Hfq and its constellation of RNA. Nat Rev Microbiol 9: 578–589. [PubMed: 21760622]

Vogel J, Bartels V, Tang HH, Churakov G, Slagter-Jager JG, Huttenhofer A, and Wagner EGH (2003) RNomics in *Escherichia coli* detects new sRNA species and indicates parallel transcriptional output in bacteria. Nucleic Acids Res 31: 6435–6443. [PubMed: 14602901]

Waters LS, and Storz G (2009) Regulatory RNAs in bacteria. Cell 136: 615–628. [PubMed: 19239884]

Yu DG, Ellis HM, Lee EC, Jenkins NA, Copeland NG, and Court DL (2000) An efficient recombination system for chromosome engineering in *Escherichia coli*. Proc Natl Acad Sci USA 97: 5978–5983. [PubMed: 10811905]

Zhang A, Altuvia S, Tiwari A, Argaman L, Hengge-Aronis R, and Storz G (1998) The oxyS regulatory RNA represses rpoS translation by binding Hfq (HF-1) protein. EMBOJ 17: 6061–6068.

Zhang A, Wassarman KM, Rosenow C, Tjaden BC, Storz G, and Gottesman S (2003) Global analysis of small RNA and mRNA targets of Hfq. Mol Microbiol 50: 1111–1124. [PubMed: 14622403]

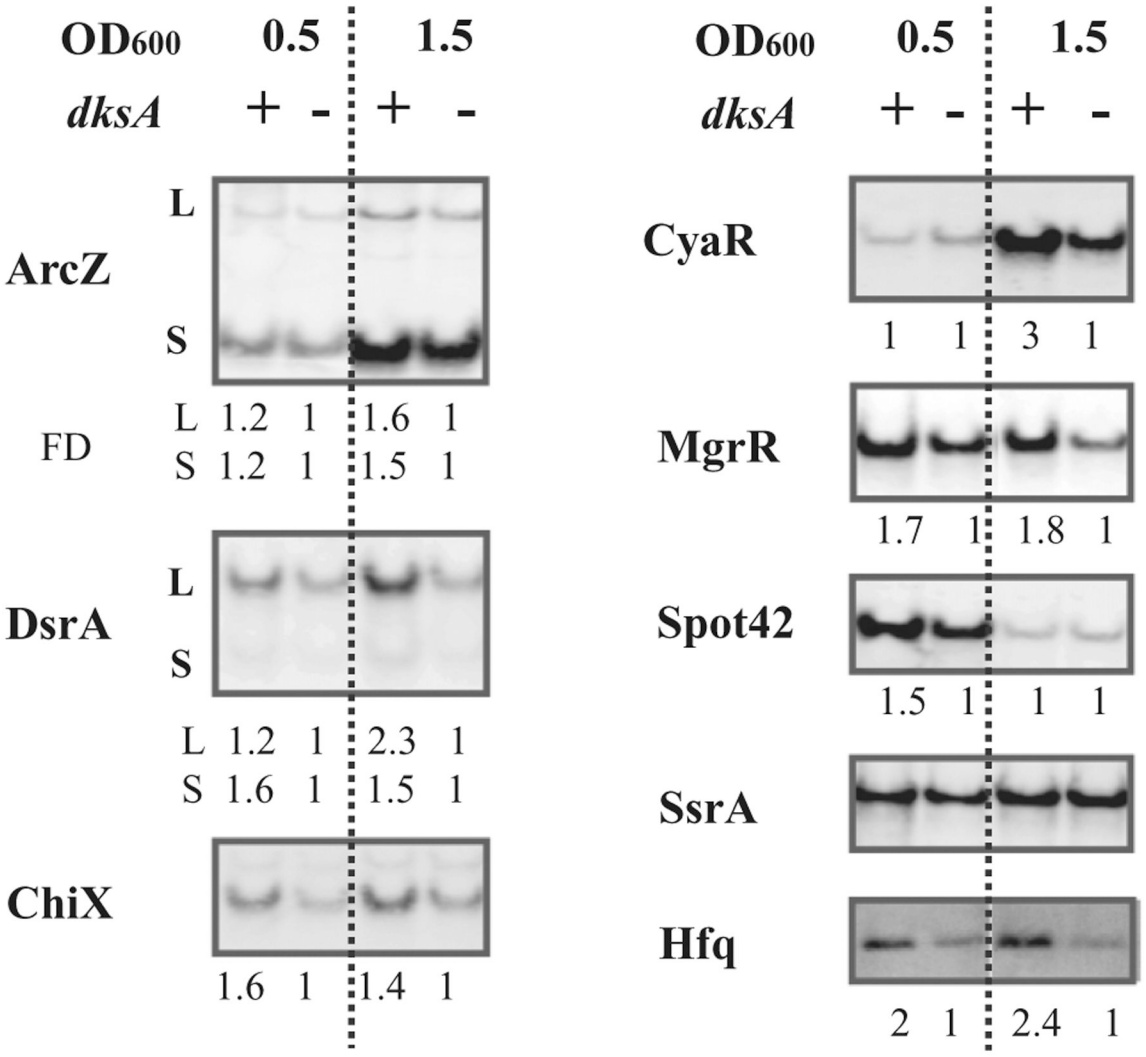


**Fig. 1.**

Effect of a *dksA* mutant on levels of sRNAs. Wild type (MG1655) and an isogenic *dksA* deletion derivative (CF9240) were grown at 30°C and samples were taken for Western blot for Hfq and Northern analysis for the same sRNAs as in Fig. S1. The levels of the sRNAs were compared between the wild type and *dksA* mutant for the three experiments and the fold difference (FD) was obtained as the ratio of wild type to Δ*dksA;* these values are listed below each blot. For ArcZ and DsrA, the levels of the full-length long (L) and processed short (S) transcripts were analysed separately. Normalization was to levels of SsrA.

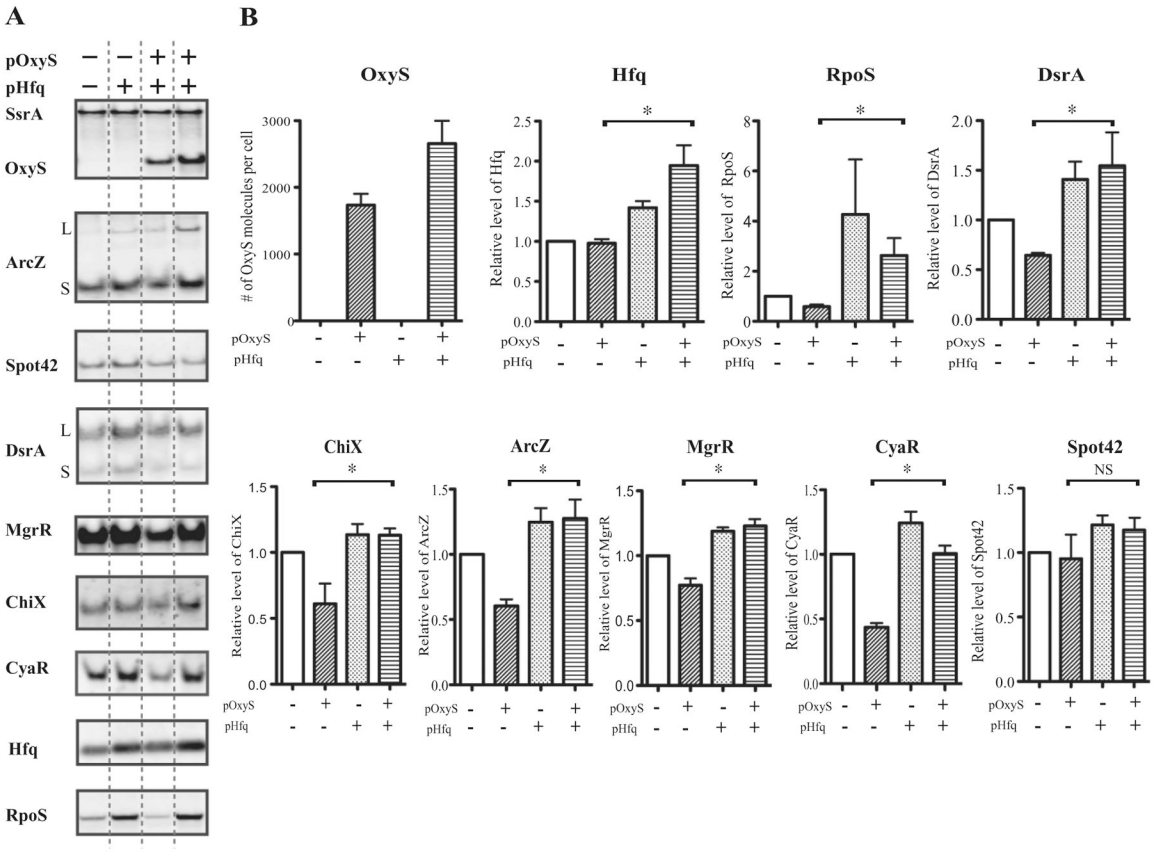


**Fig. 2.**

OxyS induction effect on sRNA levels and rescue by extra Hfq. Strain KM153 (*hfq+* Δ*oxyS*) containing either the vector pNM12 or pGFK1014, a plasmid expressing OxyS, and either the vector pBAD33 or pKMT4, a plasmid expressing Hfq, was grown in LB containing ampicillin and chloramphenicol at 30°C. Cells were induced with 0.2% arabinose at $OD_{600}$ = 0.2–0.3 for 1 h.

A. Samples were taken at $OD_{600}$ = 0.5, and processed as previously for Western blots for Hfq or RpoS, or Northern blots probed for the sRNAs.

B. The graphs represent the averages from three independent experiments as for (A), quantified as described in *Experimental procedures* and as shown in Fig. S2 for Hfq and OxyS. For OxyS, a standard curve was generated with a known amount of purified OxyS from *in vitro* transcription (Fig. S2). If the cell extracts undergo loss of sRNAs during preparation of samples, our estimates for the number of molecules may be low, but relative numbers in different samples should be similarly decreased. For all other graphs, the level of the protein (Hfq or RpoS) or sRNA in cells carrying the two vectors was set to 1, and the relative amounts expressed by comparison to this number. Note that for ArcZ the short form of the sRNA was plotted; for DsrA the short form and long form were combined and plotted. Normalization was to levels of SsrA (not shown). Brackets marked with an asterisk (*) indicate numbers considered significantly different ($P < 0.05$; NS: not significant, determined by *t*-est).

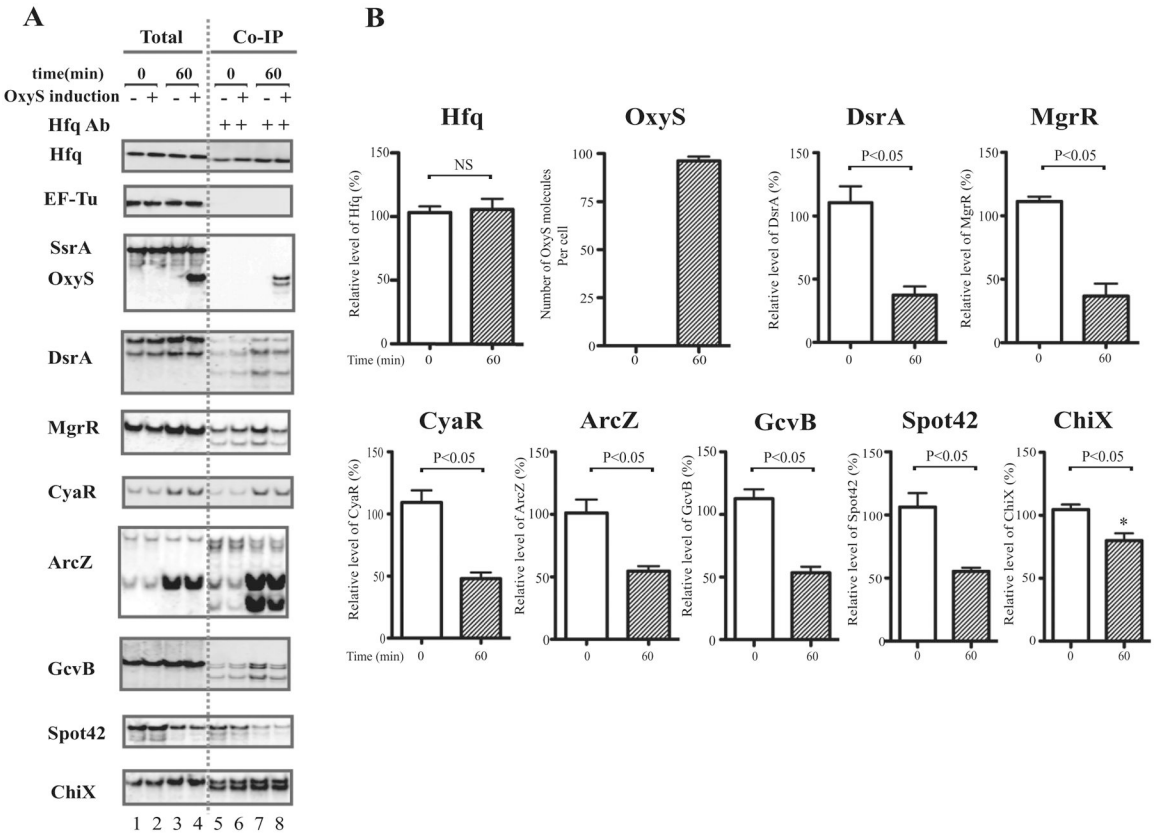


**Fig. 3.**

OxyS induction lowers sRNA binding to Hfq.

A. Strain KM94, harbouring $P_{BAD}$–*oxyS* inserted into the host chromosome, was grown in LB at 30°C, induced at $OD_{600}$ = 0.3 for 60 min with 0.2% arabinose, and samples processed as described in *Experimental procedures.* Control samples (lanes 1, 3, 5 and 7) were grown without arabinose. Co-IP: co-immunoprecipitate samples

B. Relative levels are expressed as the value in the immunoprecipitate after arabinose induction compared with levels without arabinose induction at 0 min and 60 min. Total input was normalized to levels of Elongation Factor TU (Ef-Tu). For immunoprecipitated samples, levels were normalized to levels of Ig visible on the gels. Graphs are the average of three experiments. The number of OxyS molecules was determined by comparison to the standard curve (Fig. S2). No significant difference was observed for the amount of Hfq with and without OxyS induction (NS: not significant). After OxyS induction, the amount of sRNAs in the immunoprecipitate was significantly lower than that observed without induction ($P < 0.05$). Asterisk indicates that the relative amount of ChiX in the immunoprecipitate after OxyS induction, while lower than without induction, was significantly higher than that for the other sRNAs ($P < 0.05$; determined by *t*-test). The values for both ArcZ and DsrA represent the levels of the short (processed) forms of these sRNAs.

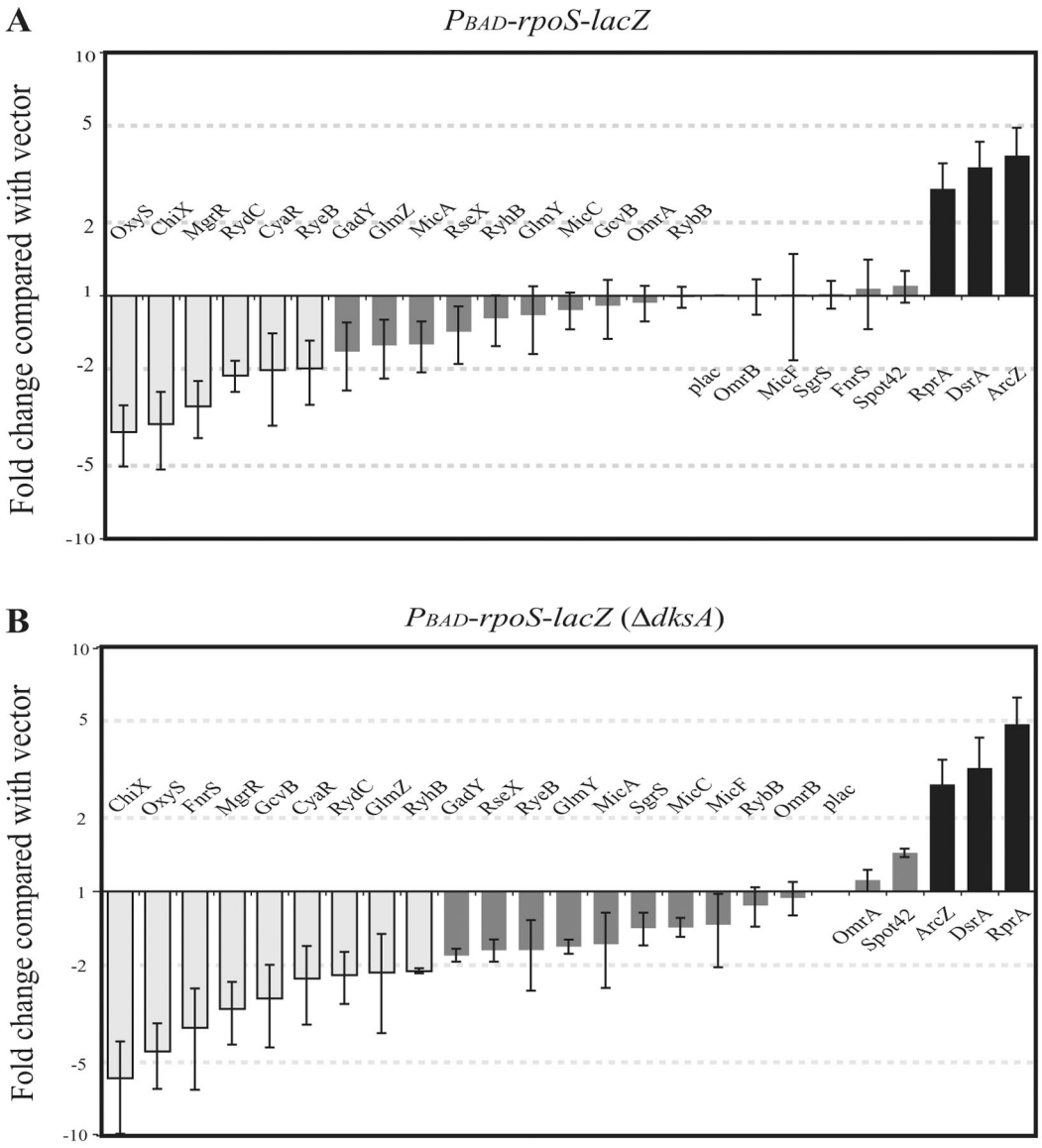


**Fig. 4.**

Competition for RpoS regulation by other sRNAs. Screening of the sRNA library with the *$P_{BAD}$–rpoS–lacZ* translation fusion in (A) WT (PM1409), (B) Δ*dksA* (KM341). The cells were grown in LB containing ampicillin, 0.02% arabinose and 100 μM IPTG in microtitre plates at 37°C for 6 h. The effect of the overexpression of each sRNA on the *rpoS–lacZ* fusion was plotted as a function of the fold change compared with the basal activity of each strain containing a pBR-plac control vector (A: 40 units; B: 38 units). Fold changes greater than two were considered significant. Dark grey bars represent sRNAs for which effects were not considered significant; black and light grey bars indicate sRNAs having an activating or a repressing effect respectively.

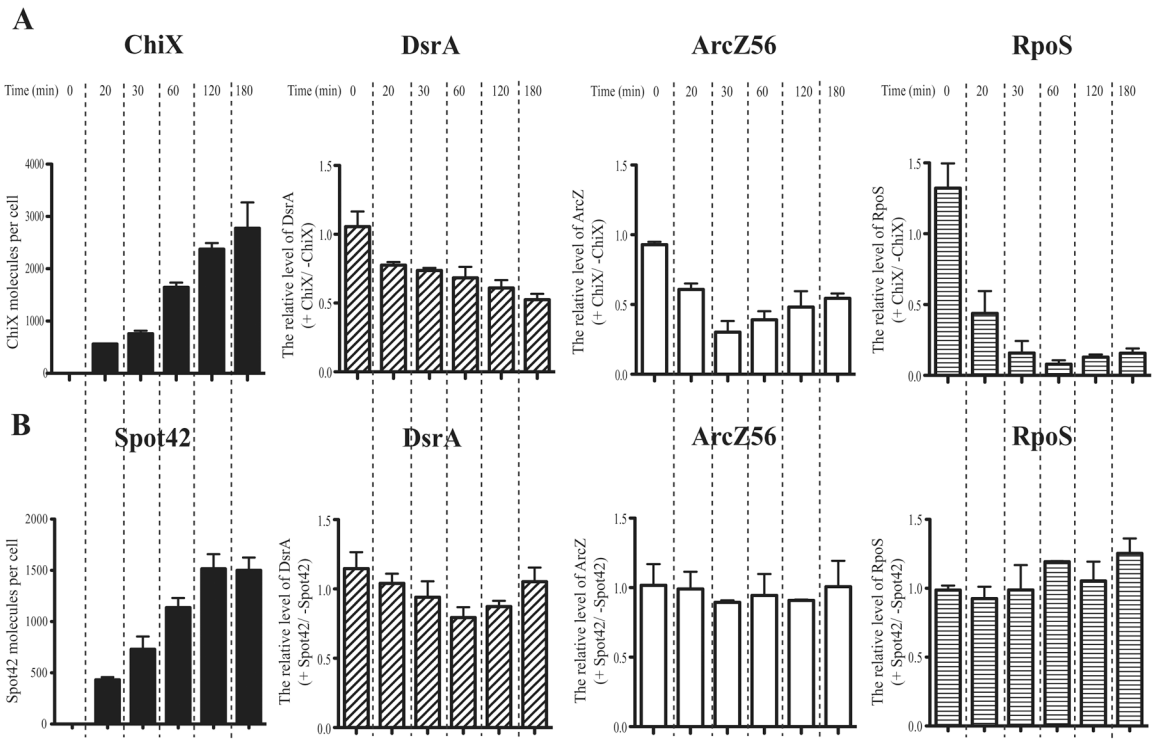


**Fig. 5.**

ChiX but not Spot 42 interferes with RpoS and sRNA accumulation. Cells carrying vector (pNM12) or plasmids expressing ChiX (pGFK1035) (A) or Spot 42 (pGFK1034) (B) were grown in LB ampicillin at 30°C and induced with 0.2% arabinose at $OD_{600}$ 0.3. Both RNA and protein samples were taken at each time point as indicated for Northern blot for ChiX, DsrA, ArcZ and Western blot analysis of RpoS. The $OD_{600}$ at 30 min and 60 min induction were approximately 0.5 and 0.8 respectively. SsrA levels were used as an internal control for the Northern blot. An example of a gel from this experiment is shown in Fig. S6. A standard curve was generated for ChiX and Spot42 by using *in vitro* transcribed RNA as described in *Experimental procedures* (see example for OxyS in Fig. S2). Values were determined by comparison to those curves. Averages from three experiments are expressed as the ratio of samples after sRNA induction to the same time points in the absence of sRNA induction (in cells carrying vector plasmid). Levels of the short (processed) form of ArcZ were used to generate the graph. The level of Hfq was consistent throughout the experiment with or without ChiX or Spot42 induction.

A. Strain KM255 (Δ*chiX)* containing either a vector control (pNM12) or a plasmid expressing ChiX (pGFK1035).

B. Strain KM349 (Δ*spf)* containing either a vector control (pNM12) or a plasmid expressing Spot42 (pGFK1034).

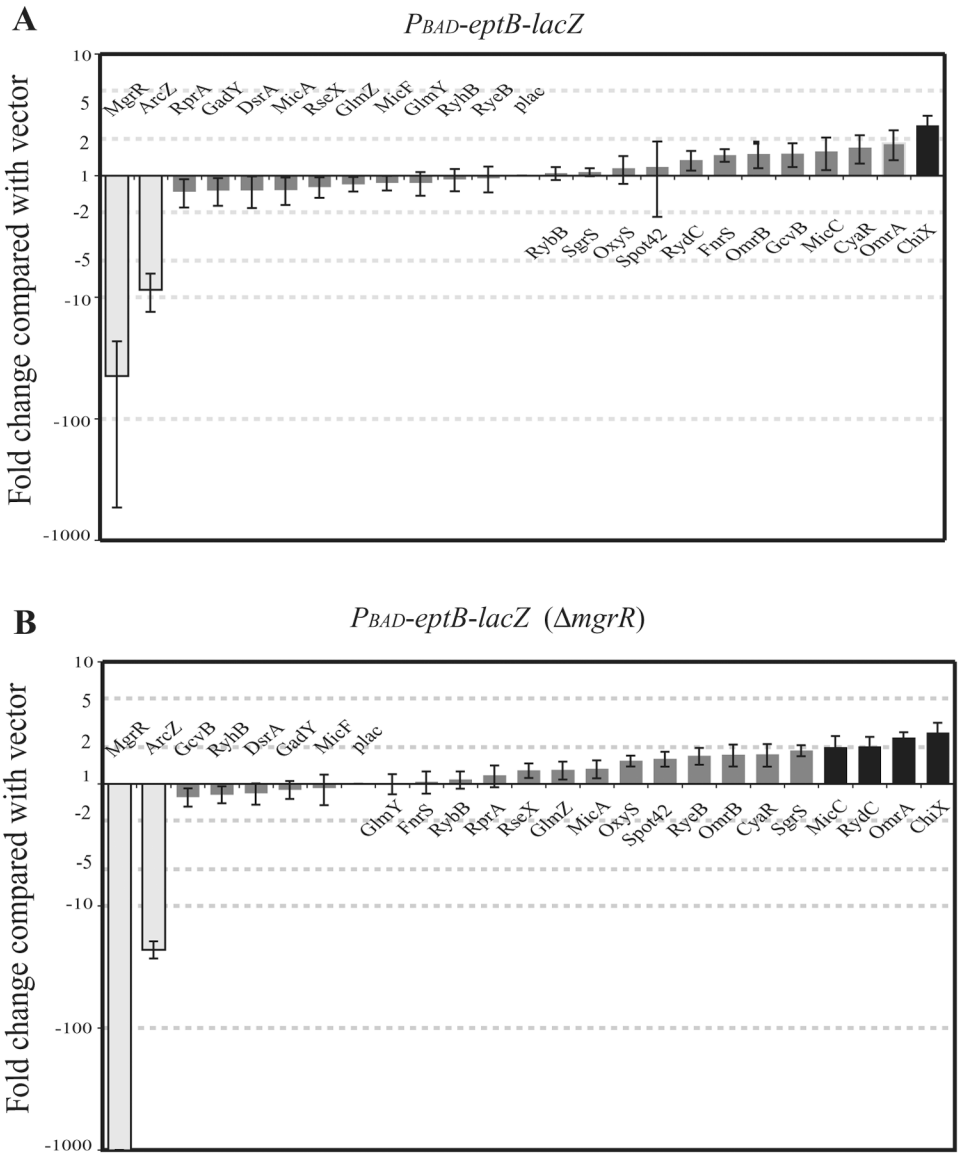


**Fig. 6.**

sRNA competition for negative regulation of *eptB.* Cells harbouring a *$P_{BAD}$–eptB–lacZ* translational fusion, which is negatively regulated by MgrR, were used for examining Hfq competition on a negatively regulated target. The cells were grown in LB containing ampicillin, 0.02% arabinose and 100 μM IPTG. The results were plotted as described in Fig. 4. The basal activity was that seen with the *plac* vector control plasmid for a given strain. Strains used were (A) WT (KM125); basal activity was 3.3 units; (B) Δ*mgrR* mutant (KM225); basal activity was 2.7 units.

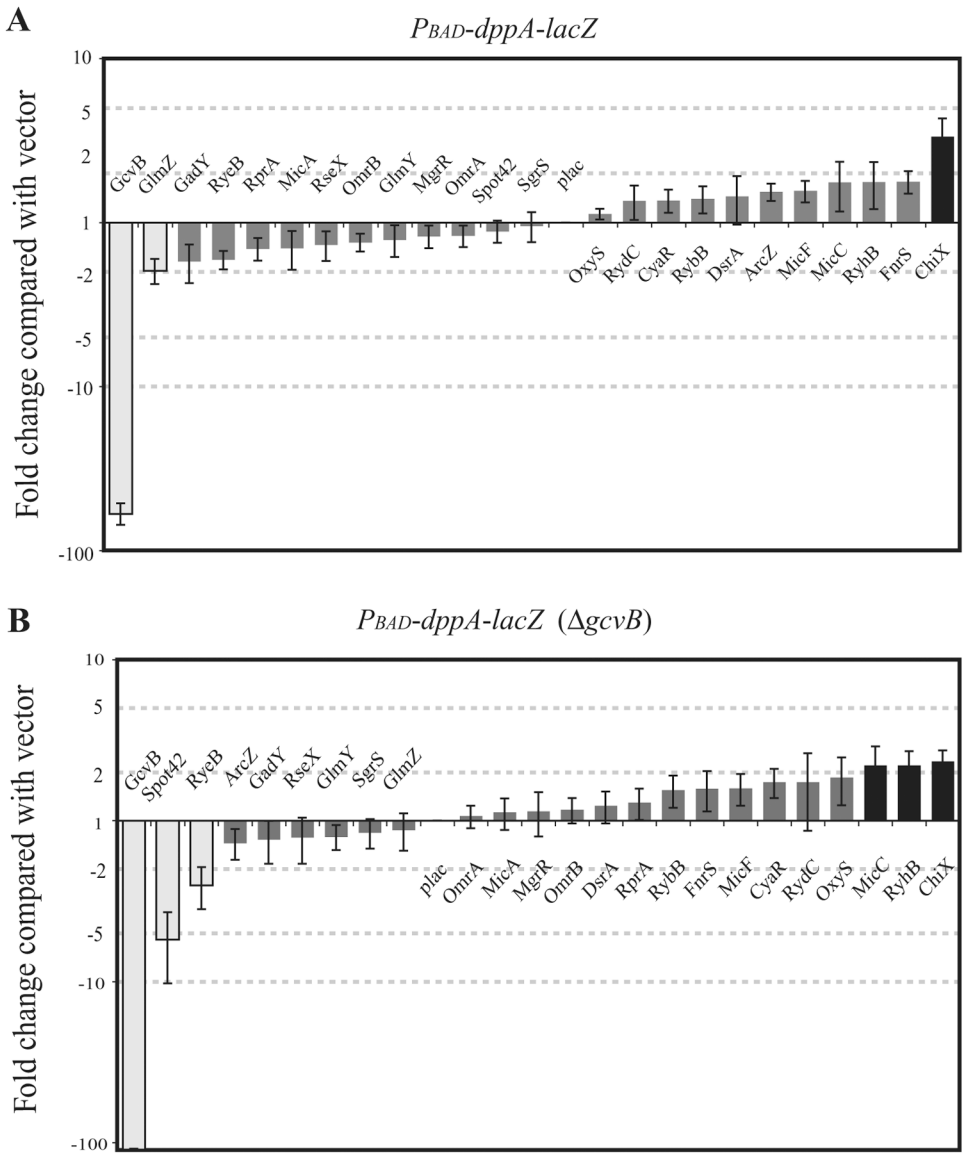


**Fig. 7.**

*dppA,* a negative target of GcvB, is subject to sRNA competition. Isogenic strains carrying a *$P_{BAD}$–dppA–lacZ* fusion were used to observe sRNA competition, as for Fig. 4. Cells were grown and assayed as for Fig. 6, but 0.0005% arabinose was used to induce the fusion; for each strain, the basal activity was that seen with the plac control plasmid.

A. WT (KM 86); basal activity was 74 units.

B. Δ*gcvB* (KM339); basal activity was 116 units.

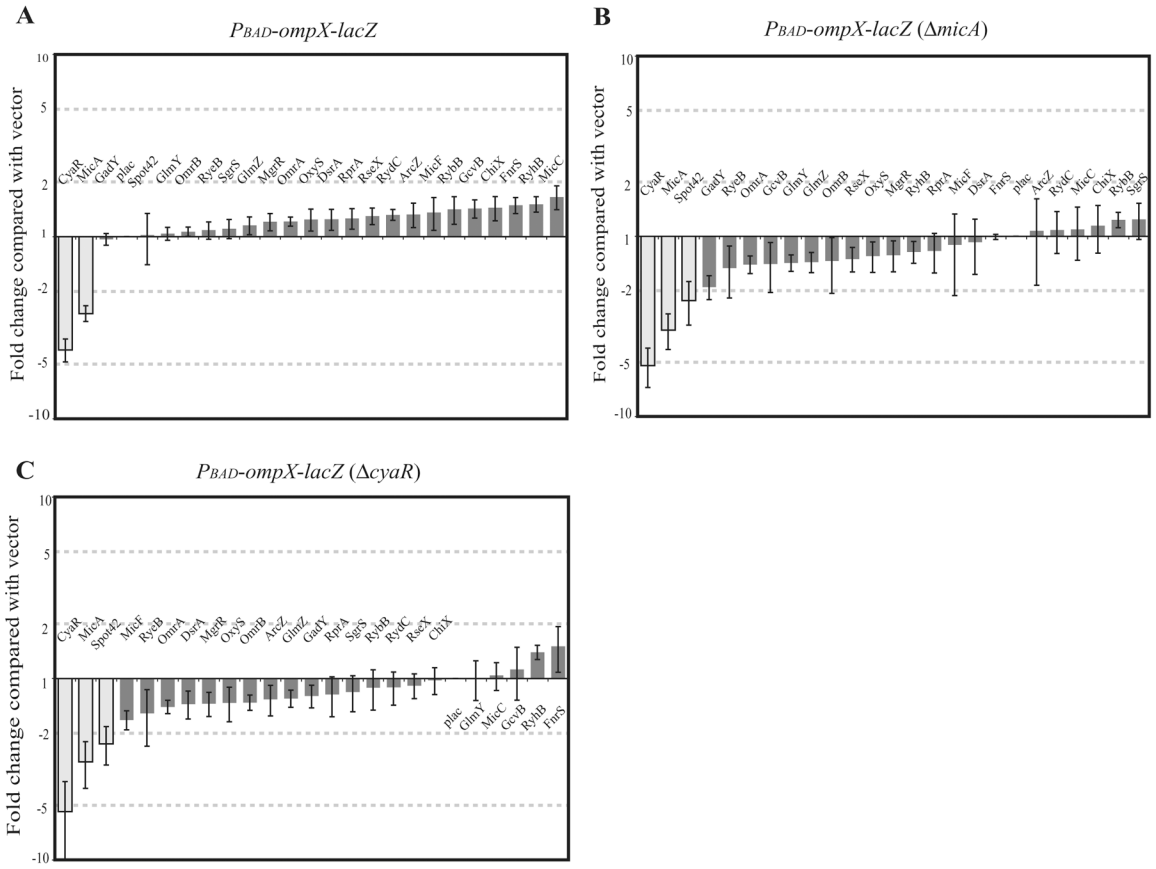


**Fig. 8.**

*ompX,* a negative target of CyaR, was not subject to sRNA competition. Isogenic strains carrying a translational fusion from the second transcriptional start site of *ompX* fused to the *araBAD* promoter were examined for competition by overexpressed sRNAs. The *ompX–lacZ* fusion was induced with 0.0005% arabinose in LB and specific activity was obtained as for Fig. 4. (A) Wild type (KM331); basal activity was 149 units; (B) Δ*micA* (KM342); basal activity was 165 units; (C) Δ*cyaR* (KM340); basal activity was 130 units.

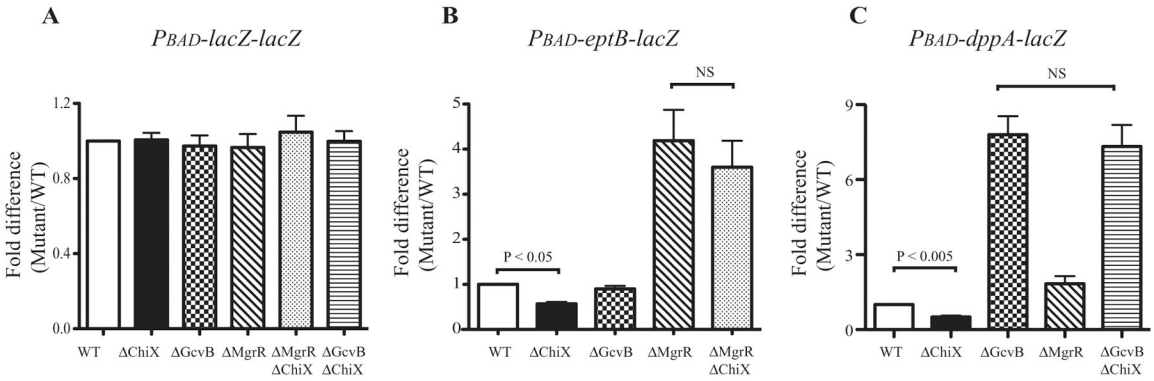


**Fig. 9.**

Chromosomally encoded ChiX affects the level of *eptB* and *dppA*. Isogenic derivatives of cells harbouring different *lac* fusions were grown in LB with arabinose (0.02% for both *lacZ–lacZ* and *eptB–lacZ* and 0.0005% for *dppA–lacZ)* at 37°C for 6 h, RNA isolated and cDNAs prepared for qRT-PCR as described in *Experimental procedures.* The normalized mRNA level of the WT strain for each set was designated as 1.0, and mutants compared with this value. For each set, a Δ*gcvB* derivative, an Δ*mgrR* derivative and a Δ*chiX* derivative were used.

A. $P_{BAD}$*–lacZ–lacZ* derivatives grown in 0.02% arabinose: WT: (PM1410); Δ*chiX* (KM359); Δ*gcvB* (KM360), Δ*mgrR* (KM361), Δ*mgrR* Δ*chiX* (KM390), Δ*gcvB* Δ*chiX* (KM391).

B. $P_{BAD}$*–eptB–lacZ* derivatives grown in 0.02% arabinose; WT: KM125; Δ*chiX* (KM363); Δ*gcvB* (KM364), Δ*mgrR* (KM225), Δ*mgrR* Δ*chiX* (KM381).

C. $P_{BAD}$*–dppA–lacZ* derivatives grown in 0.0005% arabinose; WT: KM86; Δ*chiX* (KM352); Δ*gcvB* (KM339), Δ*mgrR* (KM366), Δ*gcvB* Δ*chiX* (KM353).

Error bar shows standard deviation among four different trials. NS indicates no significant difference observed among samples; all the samples in (A) showed no significant differences.

**Table 1.**

Comparison of the amount of sRNAs in the wild type and the *hfq*-deleted strain.

| | **Growth phase** | **Exponential phase ($OD_{600}$ = 0.5)** | | | **Stationary phase ($OD_{600}$ = 1.5)** | | |
|---|---|---|---|---|---|---|---|
| | **Temperature (°C)** | **32** | **37** | **42** | **32** | **37** | **42** |
| ArcZ | Long form | 0.2 (± 0) | 1.0 (± 0.2) | 2.8 (± 2.3) | 0.3 (± 0.2) | 0.7 (± 0.2) | 1.7 (± 0.7) |
| | Short form[a] | > 100 | > 100 | > 100 | > 100 | > 100 | > 100 |
| DsrA | Long form | 3.9 (± 1.8) | 4.7 (± 2.1) | 4.2 (± 2.3) | 4.1 (± 2.3) | 1.8 (± 1.1) | 2.1 (± 0.7) |
| | Short form | 27 (± 19) | 22 (± 7.1) | 38 (± 14) | 243 (± 102) | 30 (± 14) | 24 (± 9) |
| ChiX | | 11 (± 3.5) | 15 (± 4.7) | 19 (± 7.8) | 11 (± 4.4) | 18 (± 8.4) | 23 (± 9.3) |
| CyaR | | 2.0 (± 1.3) | 1.9 (± 0.6) | 6.4 (± 3.4) | 5.8 (± 2.0) | 4.5 (± 1.8) | 11.3 (± 2.5) |
| GcvB | | 1.4 (± 0.6) | 1.6 (± 0.2) | 2.5 (± 0.9) | 1.5 (± 0.9) | 1.1 (± 0.5) | 2.0 (± 0.6) |
| MgrR | | 3.3 (± 0.7) | 3.5 (± 0.7) | 5.5 (± 3) | 3.5 (± 0.7) | 4.3 (± 2) | 5.4 (± 0.6) |
| Spot 42 | | 9.5 (± 4.8) | 3.8 (± 2) | 1.2 (± 0.2) | 1.6 (± 0.6) | 2.1 (± 1.2) | 1.5 (± 0.3) |

[a]For ArcZ, no short-form RNA was detectable above background in the Δ*hfq* strain at any temperature; this has been arbitrarily shown here as > 100.

Numbers are ratio between WT (NM22540) versus *hfq*$^{-}$ (NM22562). Northern blot signals were determined using biotinylated probes and quantified as described in *Experimental procedures*. Numbers represent the averages of three independent experiments; Fig. S1 shows a representative experiment.