Many small RNAs (sRNAs) regulate gene expression by base pairing to their target messenger RNAs (mRNAs) with the help of Hfq in *Escherichia coli*. The sRNA DsrA activates translation of the rpoS mRNA in an Hfq-dependent manner, but this activation ability was found to partially bypass Hfq when DsrA is overproduced. The precise mechanism by which DsrA bypasses Hfq is unknown. In this study, we constructed strains lacking all three rpoS-activating sRNAs (i.e., ArcZ, DsrA, and RprA) in hfq+ and Hfq- backgrounds, and then artificially regulated the cellular DsrA concentration in these strains by controlling its ectopic expression. We then examined how the expression level of rpoS was altered by a change in the concentration of DsrA. We found that the translation and stability of the rpoS mRNA are both enhanced by physiological concentrations of DsrA regardless of Hfq, but that depletion of Hfq causes a rapid degradation of DsrA and thereby decreases rpoS mRNA stability. These results suggest that the observed Hfq dependency of DsrA-mediated rpoS activation mainly results from the destabilization of DsrA in the absence of Hfq, and that DsrA itself contributes to the translational activation and stability of the rpoS mRNA in an Hfq-independent manner.

**Keywords:** DsrA, *Escherichia coli*, Hfq, rpoS, small RNAs

**INTRODUCTION**

There are about 100 small noncoding RNA (sRNA) in *Escherichia coli*. Many sRNAs are involved in fine tuning gene regulation for different growth environments, thereby helping the cell survive under various stress conditions (Bobrovskyy and Vanderpool, 2013; De Lay et al., 2013; Gottesman and Storz, 2011; Majdalani et al., 2005; Murina and Nikulin, 2015; Storz et al., 2011; Wassarman et al., 1999; Waters and Storz, 2009). Base pairing between an sRNA and a messenger RNA (mRNA) can regulate gene expression by changing the accessibility of the ribosome-binding site or altering the RNA-turnover rate (Majdalani et al., 2005; Santiago-Frangos et al., 2016). In most cases, sRNA-mediated regulation requires the presence of Hfq, a host protein that is required for Qβ bacteriophage replication (Vogel and Luisi, 2011). Hfq is an Sm-like protein that forms a homoheptameric ring-like structure (Brennan and Link, 2007; Link et al., 2009; Sauter et al., 2003). A uridine-rich RNA sequence in an sRNA can bind to the proximal face (Brennan and Luisi, 2007; Link et al., 2009; Sauter et al., 2003). A uridine-rich RNA sequence in an sRNA can bind to the proximal face (Lorenz et al., 2010; Panja et al., 2015; Sauer and Weichenrieder, 2011; Updegrove et al., 2016; Wang et al., 2011; Zhang et al., 2013) and outer rim (Panja et al., 2015; Sauer et al., 2012; Zhang et al., 2013) of Hfq, whereas an (ARN)n sequence motif of an mRNA can bind to its distal face (Malecka et al., 2015; Mikulecky et al., 2004; Schu et al., 2015; Updegrove et al., 2016). Hfq participates in sRNA-dependent translational regulation in various ways. First, Hfq can accelerate the base pairing between sRNAs and their mRNA targets (Hopkins et al., 2011; Panja and Woodson, 2012; Ross et al., 2013; Schu et al., 2015). While the binding of Hfq to sRNAs can prevent them from being degraded (Ikeda et al., 2011; Möller et
The rpoS mRNA usually forms a large stem–loop structure and its activity is not limited to cold shock stress conditions. DsrA is also induced by acid stress at 37°C (Bak et al., 2014), activation at 37°C (Mandin and Gottesman, 2010). Since 1996). The increases of both the dsrA promoter activity and antisense mechanism (Lease and Belfort, 2000; Majdalani et al., 1998; McCullen et al., 2010; Sledjeski et al., 1996). DsrA synthesis is increased at low temperatures, contributing to high levels of RpoS under these conditions (Hämmerle et al., 2013; Repola and Gottesman, 2001; Sledjeski et al., 1996). The increases of both the dsrA promoter activity and the DsrA stability at low temperatures are responsible for the enhanced DsrA expression (Hämmerle et al., 2013; Repola and Gottesman, 2001; Sledjeski et al., 1996). Therefore, it was thought that DsrA may be functional only under cold shock conditions. Nevertheless, DsrA can act on rpoS activation at 37°C (Mandin and Gottesman, 2010). Since DsrA is also induced by acid stress at 37°C (Bak et al., 2014), its activity is not limited to cold shock stress conditions. The rpoS mRNA usually forms a large stem-loop structure upstream of the start codon, which inhibits ribosome binding (Lease and Woodson, 2004; Soper et al., 2010; Wang et al., 2011). When DsrA binds to an upstream region in the 5’-UTR of rpoS, this stem-loop is disrupted, the ribosome binding site (RBS) is revealed, and translation of the rpoS mRNA is efficiently activated (Lease and Woodson, 2004). The DsrA-mediated translation of rpoS translation is Hfq-dependent at 30°C (Sledjeski et al., 2001) as well as at 25°C and 37°C (Supplementary Fig. S1). Hfq forms a stable ternary complex with DsrA and the rpoS mRNA, and this complexation increases the annealing rate of DsrA to the rpoS mRNA in vitro (Resch et al., 2008). However, overexpressed DsrA has also been shown to partially bypass the requirement of Hfq for rpoS activation (Soper et al., 2010; Večerek et al., 2010). In this respect, DsrA differs from two other rpoS-activating sRNAs, RprA and ArcZ, which stringently require Hfq for rpoS activation (McCullen et al., 2010). It has been proposed that the ability of overexpressed DsrA to partially bypass the requirement of Hfq for rpoS activation may be related to the ability of DsrA to tightly bind the 5’-UTR of the rpoS mRNA even in the absence of Hfq (Soper et al., 2010). However, the precise mechanism underlying the ability of overexpressed DsrA to bypass the requirement for Hfq remains unknown.

In the present work, we investigated the detailed mechanism underlying the requirement for Hfq in DsrA-mediated rpoS activation. For this purpose, we constructed strains lacking all three rpoS-activating sRNAs (i.e., ArcZ, DsrA, and RprA) in hfq+ and hfq- backgrounds, and controlled the cellular DsrA concentrations in these cells by ectopic expression. We then examined how the expression level of rpoS changed according to alterations in the concentration of DsrA. We found that the DsrA-mediated translational activation of rpoS occurred at similar levels in hfq+ and hfq- cells, but that DsrA and the rpoS mRNA both showed instability in hfq- cells. Our results suggest that the in vivo Hfq dependency of DsrA-mediated rpoS activation mainly results from the destabilization of DsrA in the absence of Hfq, but that DsrA itself contributes to the translational activation and stabilization of the rpoS mRNA in an Hfq-independent manner.

**MATERIALS AND METHODS**

**Strains and plasmids**
The bacterial strains and plasmids used in this study are listed in Table 1. Strain PM1409 carrying a chromosomal rpoS-

| Name     | Description                                                                 | Source                  |
|----------|------------------------------------------------------------------------------|-------------------------|
| Strains  |                                                                              |                         |
| PM1409   | *Escherichia coli* PM120S lacI-::PBAD-rpoS-lacZ                              | (Soper et al., 2010)    |
| PM1409Δhfq| PM1409 Δhfq::kanA                                                            | This study              |
| PM1409Δ3  | PM1409Δ3 text:kanA ΔprRA ΔdsrA                                               | This study              |
| PM1409Δ3Δhfq| PM1409Δ3Δhfq::kanA ΔprRA ΔdsrA                                               | This study              |
| PM1409ΔΔΔ  | PM1409ΔΔΔ::kanA ΔprRA ΔdsrA Δhfq                                             | This study              |
| PM1409ΔΔΔ  | PM1409ΔΔΔ::kanA ΔprRA ΔdsrA Δhfq                                             | This study              |
| PM1409ΔΔΔ  | PM1409ΔΔΔ::kanA ΔprRA ΔdsrA Δhfq                                             | This study              |
| PM1409ΔΔΔΔhfq| PM1409ΔΔΔΔhfq::kanA ΔprRA ΔdsrA Δhfq                                         | This study              |
| Plasmids  |                                                                              |                         |
| pPM150    | A derivative of pHM1 (54), AmpR, IPTG-inducible transcription from immediately after the EcoRI site, modified mPB terminator (GAUUU to GGAGU) next to the XbaI site. | (Bak et al., 2014)      |
| pVIAZ     | pHM1 derivative expressing ArcZ                                             | (Bak et al., 2014)      |
| pVIAZ     | pHM1 derivative expressing RprA                                              | (Bak et al., 2014)      |
| pVIAZ     | pHM1 derivative expressing DsrA                                              | (Bak et al., 2014)      |
| pVIAZ     | FLP<sup>+</sup>, λ cI857<sup>−</sup>, λ Pr<sup>+</sup>, AmpR, Cm<sup>+</sup>, expression of site-specific FLP recombinase under control of a heat inducible promoter, temperature sensitive replication. | (Cherepanov and Wackernagel, 1995) |
lacZ translational fusion was gifted by Dr. S. Gottesman and referred to WT. The PM1409 Δhfq mutant was obtained by P1 transduction (Moore, 2011; Thomason et al., 2007) using the deletion strain, which was obtained from the *E. coli* Keio strain collection (Baba et al., 2006). PM1409Δ3 (a mutant strain having deletion of dsrA and rprA, and an arcZ promoter mutation) was obtained by P1 transduction using the relevant deletion strains (Bak et al., 2014). Briefly, kanamycin-marked mutations were transferred into the desired strain background using P1 transduction. The FRT-flanked kanamycin cassette introduced into the first dsrA deletion strain was removed using the Flp recombinase from pCP20 plasmid (Cherepanov and Wackernagel, 1995). The second rprA deletion was introduced by P1 transduction (Müller-Hill, 1985), and the kanamycin cassette was once again removed. To construct PM1409Δ3Δhfq, an additional hfq deletion was introduced. The arcZ promoter mutation was finally introduced by P1 transduction. PM1409Δ3ΔaΔdΔpr was constructed by the first rprA deletion and the second arcZ promoter mutation through P1 transduction. PM1409Δ3ΔdΔaΔdΔpr were constructed by the first dsrA deletion and the second arcZ promoter mutation or rprA deletion. PM1409Δ3ΔaΔdΔhfq was constructed by the first rprA deletion, the second hfq deletion, and the final arcZ promoter mutation.

**LacZ activity assay**

Three colonies for each strain were cultured in LB medium containing ampicillin (100 μg/ml) at 37°C or 25°C when necessary, and the overnight culture was diluted to 1:100 and cultured with the fresh medium. Arabinose (0.02%) and IPTG were added at 2 h and 3.5 h, respectively, and the culture was incubated further for 0.5 h. LacZ activity was assayed as described previously (Zhang and Bremer, 1995). At least three independent measurements were performed for each strain.

**RNA purification**

Three colonies for each strain were cultured in LB medium containing ampicillin (100 μg/ml) at 37°C, and the overnight culture was diluted to 1:100 and cultured with the fresh medium. Arabinose (0.02%) and IPTG were added at 2 h and 3.5 h, respectively, and the culture was incubated further for 0.5 h. Total cellular RNAs were extracted using the acidic hot-phenol method, as described previously (Kim et al., 1996).

**In vitro transcription**

To prepare DsrA and LacZ200 (a transcript consisting of 200 nt of the lacZ mRNA), DNA templates were obtained via polymerase chain reaction (PCR) using appropriate primer pairs (Supplementary Table S1) and *in vitro* transcription was carried out using T7 RNA polymerase (Promega, USA).

**Northern blot analysis**

For sRNA analysis, 0.5 to 20 μg of total RNAs were fractionated on a 7 M urea, 5% polyacrylamide gel, and electrotransferred onto a Hybond-XL membrane (Amersham Biosciences, UK), as previously described (Park et al., 2013). Known amounts of *in vitro*-transcribed DsrA were loaded along with RNA samples for quantification standards. For mRNA analysis, total RNAs (10 μg) were loaded on an agarose gel (1%, 1× MOPS) and transferred onto a Hybond-XL membrane through capillary diffusin (Streit et al., 2009). The membrane was hybridized with 32P-labeled DNA probes in PerfectHyb Plus Hybridization Buffer (Sigma-Aldrich, USA) according to the manufacturer’s instructions. Hybridization signals were analyzed using an Image Analyzer FLA7000 (Fuji, Japan). The utilized probes are listed in Supplementary Table S1.

**Quantitative real-time PCR**

To measure the levels of transcripts, 5 μg of total RNA were DNase treated using a TURBO DNA-free Kit (Ambion, USA).
Complementary DNAs (cDNAs) were synthesized from 0.5 μg of DNase-treated RNA using a SuPrimeScript RT-PCR premix (Genet Bio, Korea). cDNAs were amplified with SuPrimeScript qRT-PCR Premix (Genet Bio) using a Bioneer Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer, Korea). Primer pairs specific to the lacZ ORF, rpoS ORF, rpoS 5′ ORF, or rrsA mRNA were used for quantitative real-time reverse transcription-PCR (qRT-PCR). The used primers are listed in Supplementary Table S1. Cycle threshold (Ct) data were normalized to rrsA (16S rRNA gene) expression. To generate quantification standards of rpoS-lacZ mRNA, total cellular RNAs isolated from non-induced (without arabinose) PM1409Δ3 cells and PM1409Δ3Δhfq cells were mixed with known amounts of in vitro-transcribed LacZ200 and used for qRT-PCR, as described previously (Park et al., 2013). The abundance of rpoS-lacZ mRNA was estimated using the standard curves.

**RNA stability assay**
RNA stability was assessed as described previously (Kim et al., 1996). Briefly, three colonies for each strain were cultured in LB medium containing ampicillin (100 μg/ml) at 37°C, and the overnight culture was diluted to 1:100 and cultured with the fresh medium. Arabinose (0.02%) and IPTG were added at 2 h and 3.5 h, respectively, and the culture was incubated further for 0.5 h. For DsrA and rpoS transcription were halted by the addition of rifampicin (Milligan and Uhlenbeck, 1989) at a final concentration of 500 μg/ml. For rpoS-lacZ mRNA, the cultured cells were washed with LB medium lacking arabinose and then cultured for different time periods in LB medium containing ampicillin (100 μg/ml) and 0.1 mM IPTG. Total cellular RNAs were prepared and subjected to Northern blot analysis or qRT-PCR.

**RESULTS**

**Activation of rpoS by ectopically expressed sRNAs**

*E. coli* expresses three rpoS-activating sRNAs: ArcZ, DsrA, and RprA. It was previously shown that rpoS activation occurs in *arcZ* ΔrprA cells but not in *arcZ* ΔrprA Δhfq cells, suggesting that the activation of *rpoS* by DsrA is Hfq-dependent (Majdalani et al., 1998; McCullen et al., 2010; Repoila and Gottesman, 2001; Sledjeski et al., 1996). However, it is not known whether this dependency on Hfq reflects an impact on DsrA stability, translational activation, or both due possible coincident effects of Hfq and DsrA on rpoS activation. To clarify the role of DsrA on rpoS activation, we first constructed *arcZ* ΔdsrA ΔrprA strains in *hfq+* and *hfq−* backgrounds carrying a *rpoS-lacZ* translational fusion; this generated PM1409Δ3 and PM1409Δ3Δhfq cells. RNA expression plasmids expressing each of the three sRNAs under IPTG induction were introduced into the generated strains, and the expression of the LacZ fusion was measured (Fig. 1). The lack of all three *rpoS*-activating sRNAs in *hfq+* cells (PM1409Δ3 cells) decreased the LacZ activity arising from the *rpoS-lacZ* translational fusion to less than 20% of the level in sRNA-expressing cells (PM1409 cells). Ectopic overexpression of any one of the sRNAs restored LacZ activity and even further stimulated *rpoS-lacZ* translation (Fig. 1B). In contrast, *hfq−* cells (PM1409Δ3Δhfq or PM1409Δ3ΔhfqΔhfq cells) exhibited sharply decreased LacZ activity regardless of sRNA gene knockout (Fig. 1C). Then we examined overexpression effects of three sRNAs on *rpoS-lacZ* translation in *hfq−* cells. Ectopic overexpression of ArcZ and RprA in these cells had relatively minor effects on *rpoS-lacZ* expression, regardless of sRNA gene knockout: about 2-fold decrease and increase by ArcZ and RprA, respectively. However, overexpression of DsrA in *hfq−* cells highly activated rpoS expression, increasing it by ~7-fold although it is approximately 50% of the level activated by DsrA overexpression in *hfq+* cells (Fig. 1C).

**Protection from degradation of DsrA by Hfq**

Ectopic expression of DsrA from pDsra by induction with 0.1 mM IPTG was capable of stimulating *rpoS* translation in *hfq−* cells (PM1409Δ3Δhfq or PM1409Δ3ΔhfqΔhfq cells), but the expression level achieved in these cells was significantly lower than that obtained in *hfq+* cells (PM1409 or PM1409Δ3 cells) (Figs. 1B and 1C). The level of *rpoS* activation seen in *hfq−* cells was consistent with that described in the previous report showing that overexpression of DsrA could bypass the requirement of Hfq for *rpoS* activation (Soper et al., 2010).
The observed weaker *rpoS* activation in *hfq*- cells might be in some part due to the low level of DsrA. Since endogenous DsrA was shown to be rapidly decayed in *hfq*- cells (Sledjeski et al., 2001), we speculated that overexpressed DsrA might be also rapidly degraded in *hfq*- cells. We found that the half-life of ectopically overexpressed DsrA was 1.5 min in *hfq* cells (PM1409Δ3Δhfq cells), compared to 14 min in *hfq*+ cells (PM1409Δ3 cells) (Fig. 2). These data indicated that Hfq helps protect DsrA against degradation *in vivo* even when DsrA is overexpressed. This is contrast with the previous results that ectopically overexpressed DsrA had comparable stability to endogenous one (Sledjeski et al., 2001).

**Fig. 3.** Cellular levels of DsrA in *hfq*+ and *hfq*- cells. (A) Total cellular RNA was prepared from 0.02% arabinose- and IPTG-treated cells grown at 37°C, and subjected to Northern blot analysis as in Figure 2B. In *vivo* DsrA transcripts were used as standards for the quantitation of *in vivo* DsrA levels. Cells containing pDsrA were treated with IPTG at increasing concentrations from 0 to 0.1 mM. Representative blots are shown. The spliced image from the same Northern membrane was shown with the insertion of a dividing line between spliced lanes. (B) Standard curves for quantification of cellular DsrA. For the standard curve, the data with *in vitro* transcribed DsrA transcripts were used. Relative northern signals of DsrA of 0.0008 to 0.2 pmol were measured and graphs of relative northern signals vs. DsrA amounts were drawn. The standard curve equations for Northern membranes of PM1409Δ3 (Δ3) and PM1409Δ3Δhfq (Δ3Δhfq) cells and of PM1409ΔaΔr (ΔaΔr) cells from panel (A) were represented on the left and right graphs, respectively. R-squared means coefficient of determination. (C) The quantity of DsrA in a cell was estimated using the standard curve shown in (B). Three Northern experiments were conducted and the mean DsrA concentrations ± SD were calculated. Δ3, arcZ dsrA rprA hfq+; Δ3Δhfq, arcZ dsrA rprA hfq- ΔΔΔr, arcZ dsrA’ rprA’ hfq-; V, vector control.
Effects of Hfq on the activation of \( rpoS \) by different cellular levels of DsrA

Next, we used different IPTG concentrations to change the cellular levels of ectopic DsrA expressed from pDsrA in PM1409Δ3 and PM1409Δ3Δhfq cells, which were referred to hfq\(^+\) (or Δ3) and hfq\(^−\) (or Δ3Δhfq) cells in the subsequent studies, respectively, unless specified, and monitored the activation of \( rpoS \). We found that pDsrA was a bit leaky so that it could produce a small amount of DsrA without the IPTG treatment. The steady-state concentration of DsrA increased with the concentration of IPTG in both hfq\(^+\) and hfq\(^−\) cells, but the saturation level of DsrA was about 5-fold lower in hfq\(^−\) cells. This might be due to the rapid decay of DsrA in hfq\(^−\) cells. Interestingly we found that the level of DsrA was much lower in hfq\(^+\) cells exposed to no IPTG or to 0.0001 mM IPTG, compared to equivalently treated hfq\(^−\) cells.

Fig. 4. Up-regulation of \( rpoS \) translation by DsrA in hfq\(^+\) and hfq\(^−\) cells. Cells containing pDsrA, which were grown at 37°C, were induced with 0.02% arabinose and IPTG at increasing concentrations from 0 to 0.1 mM and LacZ activity was measured. The indicated values were calculated from at least three independent experiments. Error bars represent SD. Δ3, arcZ \( dsrA \) \( rprA \) hfq\(^+\); Δ3Δhfq, arcZ \( dsrA \) \( rprA \) hfq\(^−\); V, vector control.

Fig. 5. Northern analysis of effects of DsrA on \( rpoS\)-lacZ mRNA accumulation in Δ3 and Δ3Δhfq cells. (A) Total cellular RNA was prepared from 0.02% arabinose- and IPTG-treated cells grown at 37°C, and subjected to Northern blot analysis. Cells containing pDsrA were treated with IPTG at increasing concentrations from 0 to 0.1 mM. The \( rpoS\)-lacZ mRNA was probed with an anti-lacZ oligonucleotide and the 23S ribosomal RNA was detected as a loading control. Representative blots are shown. (B) Northern signals were presented in a bar graph. Δ3, arcZ \( dsrA \) \( rprA \) hfq\(^+\); Δ3Δhfq, arcZ \( dsrA \) \( rprA \) hfq\(^−\); ΔaΔr, arcZ \( dsrA \) \( rprA \) hfq\(^−\); V, vector control. Three Northern experiments were conducted and the mean \( rpoS\)-lacZ concentrations ± SD were calculated.
whereas DsrA was highly accumulated in hfq+ cells exposed to 0.001 mM or higher IPTG concentrations. This may imply that Hfq uses some DsrA RNAs to bind other target mRNAs (e.g., mreB, hns, and/or rbsD), which could lead to the rapid decay of DsrA in the presence of Hfq (Lalouuna and Massé, 2016). The steady-state concentration of DsrA in hfq+ cells exposed to 0.001 mM IPTG was equivalent to that in hfq- cells exposed to 0.01 mM IPTG (Fig. 3). The LacZ activity in hfq+ cells exposed to 0.001 mM IPTG was 2-fold higher than that in hfq- cells exposed to 0.01 mM IPTG (Fig. 4). Moreover, Northern blot analysis (Fig. 5) and qRT-PCR (Fig. 6) revealed that the mRNA level of rpoS-lacZ was 2- to 3-fold higher in hfq+ cells than in hfq- cells at the above-listed IPTG concentrations. Since we also found that the endogenous level of DsrA in PM1409ΔΔsdh (arcZ dsrA+ rprA- hfq-) cells was comparable to the ectopic DsrA level resulting from basal expression in PM1409ΔΔsdh (arcZ dsrA+ rprA- hfq-). cells without IPTG induction (Fig. 3), we compared the LacZ activity and the level of rpoS-lacZ mRNA between these two cells. Both the LacZ activity and the rpoS-lacZ mRNA level in PM1409ΔΔsdh (hfq+) cells were about 2.5-fold higher than those in PM1409ΔΔsdh (hfq-) cells (Figs. 4 and 6). Therefore, it is likely that the LacZ activity was correlated to the rpoS-lacZ mRNA levels regardless of the presence of Hfq, implying that the effects of Hfq on the translatability of rpoS-lacZ mRNA would be rather slight. Altogether, these data suggest that the higher-level activation of rpoS by DsrA in hfq+ cells is mainly due to the presence of higher rpoS mRNA levels.

The level of rpoS-lacZ in hfq- cells that lacked any DsrA expression was about 2-fold lower than that in hfq+ cells (Figs. 5 and 6C), suggesting that Hfq alone could protect the rpoS-lacZ mRNA from degradation or translation enhanced by Hfq could lead to a stabilization of rpoS-lacZ mRNA. We also examined how the ectopic expression of DsrA affected the endogenous rpoS mRNA level (Fig. 6D and Supplementary Fig. S2). Our results indicated that the endogenous rpoS mRNA level showed an increasing pattern similar to that of the rpoS-lacZ mRNA under DsrA overexpression, suggesting that the 5' leader sequence of the rpoS mRNA is responsible for the ability of DsrA to increase the rpoS mRNA level. We also found that the level of endogenous rpoS mRNA increased with the level of DsrA, regardless of the presence of Hfq.

**Effects of DsrA on the premature transcription termination of rpoS in the absence of Hfq**

DsrA, ArcZ, and RprA have all been shown to suppress premature Rho-dependent transcription termination by binding the 5’ leader sequence of the rpoS mRNA (Sedlyarova et al., 2016), suggesting that the ability of DsrA to increase the rpoS mRNA level might result from an inhibition of Rho-dependent transcriptional termination. We therefore examined the effect of Hfq on this DsrA-mediated antitermination. We selected two rpoS regions that had been amplified in previous studies (Sedlyarova et al., 2016), and used them as amplicons for qRT-PCR to assess the amounts of rpoS mRNA carrying the 5’ region and the internal region. The selected regions comprised the 5’ proximal sequence of +37 to +134 of the rpoS ORF (“5’ORF” amplicon) and the internal ORF sequence of +484 to +593 relative to the +1 translation start site (“ORF” amplicon) (Figs. 6A and 7). The final product ratio of the two amplicons was taken as representing the Rho-dependent termination efficiency. The [5’ORF]/[ORF] ratio was not significantly altered by the deletion of hfq in the absence of all three sRNAs, but DsrA overexpression decreased it by 20% in both hfq+ and hfq- cells. This suggests that DsrA-mediated antitermination appears to involve the absence of Hfq and contributes to increasing the rpoS mRNA level. The antitermination effect first appeared at a low concentration of DsrA, but did not increase further as the concentration of DsrA increased (Fig. 7). Although future work may be warranted to examine why this effect does not increase with the concentration of DsrA, our present results suggest that DsrA-mediated antitermination seems to have only a minor contribution to increasing rpoS mRNA levels.

**Translational activation of rpoS by DsrA**

Finally, we examined whether DsrA activates the translation of rpoS in the absence of Hfq. To determine how DsrA affected the translation of LacZ from the rpoS-lacZ mRNA (Fig. 9), we defined translation efficiency as the ratio of LacZ activity to the amount of rpoS-lacZ mRNA. The relative translational efficiencies obtained in hfq- and hfq+ cells expressing various amounts of DsrA were calculated relative to that obtained in the absence of DsrA, which was given an arbitrary value of 1. Ectopic expression of DsrA increased the relative translation efficiency to about 1.5 regardless of Hfq unless the rpoS-lacZ mRNA was abundant (Fig. 9). Higher translation efficiencies were observed at very low concentrations of DsrA, but these increased efficiencies were reduced as the DsrA concentration.

[Reference Text]
Fig. 6. qRT-PCR analysis of effects of DsrA on rpoS mRNA accumulation in hfq+ and hfq- cells. (A) Schematic diagrams of the rpoS::lacZ chromosomal reporter fusion and the rpoS gene structure. The P
BAD promoter is indicated by PBAD, while the rpoS promoter is located within the nlpD gene. TSS, transcription start site; ATG, translation start codon. The locations of the qRT-PCR amplicons are indicated by ellipse below each diagram. (B) To generate the standard curve for quantitation of the rpoS::lacZ mRNA, total cellular RNA prepared from PM1409Δ3 and PM1409Δ3Δhfq cells grown at 37°C with no arabinose was mixed with known amounts of LacZ200, an in vitro transcript consisting of 200 nucleotides from lacZ mRNA, and also subjected to qRT-PCR using the lacZ amplicons. Cycle threshold (Ct) data of lacZ mRNA were normalized to rrsA expression. Graphs of relative Ct values vs. amounts of the lacZ transcript were drawn. The standard curve equations, y1 and y2, shown on the graph, represent equations for hfq+ and hfq- respectively. R-squared means coefficient of determination. (C) Total cellular RNA was prepared from arabinose-and IPTG-treated cells, which were grown at 37°C, and subjected to qRT-PCR. After normalization to rrsA expression the amount of rpoS-lacZ transcript per μg of total cellular RNA was estimated using the standard curve of (B). Values are means ± SD; n = 3; **P < 0.01, *P < 0.05; ns, non-significant (Student’s t-test, equal variance with the V/Δ3 value for hfq+ cells and with the V/Δ3Δhfq value for hfq- cells). (D) Levels of the rpoS transcript in PM1409ΔΔdr, PM1409Δ3, and PM1409ΔΔ3Δhfq cells were determined by qRT-PCR, which was performed as described in (C) using the ORF amplicon. Δ3, arcZ - dsrA - rprA--hfq+; Δ3Δhfq, arcZ - dsrA - rprA- hfq-; ΔΔdr, arcZ - dsrA+ - rprA+ - hfq+; V, vector control.
increased. This contrasts with our observation that the rpoS-lacZ mRNA level increased with the DsrA level until a plateau was reached at 7-fold and 4-fold increases in rpoS-lacZ mRNA at DsrA concentration of about 20 fmol/μg of total RNA in hfq+ and hfq- cells, respectively (Fig. 9B). Therefore, it seems likely that a small amount of DsrA can activate translation of the rpoS mRNA, but that more DsrA is required to stabilize the rpoS mRNA. Translational activation of the rpoS-lacZ mRNA by DsrA was observed at up to rpoS-lacZ mRNA concentrations of 0.55 fmol/μg of total RNA in hfq+ cells and at up to 0.65 fmol/μg in hfq- cells, but was not observed at 1.5 fmol/μg in hfq+ cells (Figs. 6C and 9). Therefore, DsrA-mediated translational activation may not be effective at more than rpoS-lacZ mRNA concentration of 1.5 fmol/μg of total RNA. Endogenous DsrA activated the translation of the rpoS-lacZ mRNA with a relative translation efficiency of 1.34 at rpoS-lacZ mRNA concentration of 0.55 fmol/μg of total RNA.

**DISCUSSION**

To determine the precise mechanism underlying the Hfq-independent DsrA-mediated regulation of rpoS translation at 37°C, we herein expressed ectopic DsrA in hfq+ and hfq- strains lacking all three rpoS-activating sRNAs (i.e., ArcZ, DsrA, and RprA). We then examined the translational regulation of rpoS mostly using an rpoS-lacZ translational fusion, as the translation of the 5’ leader sequence of the rpoS mRNA fused to lacZ can be taken as representing the regulatory characteristics of rpoS translation (McCullen et al., 2010; Peng et al., 2014; Resch et al., 2008; Soper et al., 2010). First, we found that ectopically expressed DsrA was very unstable in cells lacking Hfq. This is consistent with a previous report that the stability of endogenous DsrA is markedly decreased in the absence of Hfq at 30°C (Sledjeski et al., 2001). However, the previous authors reported that
plasmid-expressed DsrA did not show a significant decrease of stability in the hfq- background (Sledjeski et al., 2001), which contrasts with our present findings. Although future work is needed to resolve this discrepancy, it is likely that ectopically expressed DsrA in our system mimics endogenous DsrA. Second, we found that the absence of Hfq was associated with a decrease in rpoS mRNA stability, which should contribute to the observed decrease in its translation. Binding of Hfq to rpoS mRNA may contribute to increasing rpoS mRNA stability because its 5' leader sequence has Hfq-binding sites (Hämmerle et al., 2013; Lease and Woodson, 2004; Soper et al., 2010; Updegrove and Wartell, 2011). Alternatively, the reduction of rpoS translation by altered ribosome biogenesis in the absence of Hfq (Andrade et al., 2018) could also contribute to the decrease in rpoS mRNA stability because a lower abundance of translating ribosomes would mean that fewer mRNAs would be undergoing translation at a given moment, and more non-translating mRNAs would be vulnerable to RNases. In addition, since the Hfq binding to the 5' leader sequence of rpoS mRNA can remodel the RBS structure of rpoS mRNA for efficient translation (Hämmerle et al., 2013), this binding can in turn enhance rpoS mRNA stability by increasing translation. Third, we showed that rpoS mRNA stability is enhanced by DsrA regardless of the presence of Hfq. The DsrA-mediated increase of rpoS mRNA stability resulted in accumulation of the rpoS mRNA. The DsrA-mediated accumulation of rpoS mRNA could be achieved through protection from RNase E.

Table 2. Half-lives of the rpoS-lacZ mRNA

| Strain                  | Vector (min) | pDsrA (min) |
|-------------------------|--------------|-------------|
| hfq^a                   | 1.76 ± 0.08  | 2.64 ± 0.49 |
| hfq                     | 1.22 ± 0.33  | 2.35 ± 0.22 |

Values are means ± SD for three independent experiments. ^Half-lives were determined by linear regression analysis from the data presented in Figure 7. We assumed that the disappearance of rpoS-lacZ mRNA after arabinose washing followed a first-order decay.
Fig. 9. Effects of DsrA on the translational activation of rpoS in hfq<sup>+</sup> and hfq<sup>-</sup> cells. Translational efficiency was defined as the ratio of LacZ activity to the amount of rpoS-lacZ messenger RNA (mRNA) and calculated from the data presented in Figures 3, 4, and 6. Translational efficiencies (A) and rpoS-lacZ mRNA concentrations (B) in hfq<sup>+</sup> and hfq<sup>-</sup> cells are plotted against the concentration of DsrA fmol/μg of total RNA. (C) Translational efficiencies in hfq<sup>+</sup> and hfq<sup>-</sup> cells are plotted against the concentration of rpoS-lacZ mRNA fmol/μg of total RNA.

Fig. 10. A model for DsrA-mediated rpoS activation and the role of Hfq. Hfq stabilizes rpoS messenger RNA (mRNA) and is required for efficient translation of rpoS mRNA, while it inhibits degradation DsrA. The efficient translation can cause an increase of the stability of rpoS mRNA. Binding of DsrA to the rpoS mRNA enhances the stabilization and translation of the rpoS mRNA. Translational activation of rpoS mRNA occurs in the presence of a small amount of DsrA, while stabilization of rpoS mRNA requires more DsrA. The translational activation can further contribute to stabilization of rpoS mRNA.
Hfq-Independent Activation Mechanisms of rpoS by DsrA
Wonkyong Kim et al.

It is possible that the impact of DsrA on rpoS mRNA stability, to some extent, can result from the DsrA-mediated translation activation. However, the DsrA-mediated translation activation in both hfqΔ and hfqΔ cells appears not to make a major contribution to rpoS mRNA stability because we showed here that the amount of rpoS mRNA was not correlated to translation efficiency but to the amount of DsrA in each strain. Rather, base-pairing between DsrA and rpoS mRNA to a large extent contributes to the stability of rpoS mRNA, leading to the increased levels of rpoS mRNA.

We found that the increased levels of rpoS mRNA by the same amount of DsrA was lower in hfqΔ cells than in hfqΔ cells. The similar reduction of rpoS mRNA with its decreased half-life was also observed in the absence of DsrA, suggesting that Hfq affects rpoS mRNA stability regardless of the presence of Hfq.

Furthermore, we found that suppression of Rho-dependent transcription termination by DsrA can occur in the absence of Hfq, also resulting in rpoS activation. Finally, we found that the translational activation of the rpoS mRNA by DsrA is Hfq-independent. Although it has been reported that a ternary complex of DsrA-rpoS mRNA-Hfq forms well in vitro (Hämmerle et al., 2013; McCullen et al., 2010; Peng et al., 2014; Soper and Woodson, 2008; Updegrove and Wartell, 2011), the complex, even if formed in vivo, may not be required for translational activation. Instead, it may be related to the stabilization of the rpoS mRNA. Interestingly, translational activation of rpoS mRNA occurs in the presence of a small amount of DsrA, while stabilization of rpoS mRNA requires more DsrA, suggesting that DsrA may have the concentration-dependent dual actions. Another interesting finding of the present work is that translational activation was effective only at low concentrations of the rpoS mRNA. Although we do not yet know why translational activation by Hfq does not occur at high levels of the rpoS mRNA, we speculate that this activation could be coupled with ribosome loading. If an mRNA is relatively abundant, the ribosome-loading rate would be a rate-limiting step due to competition among available mRNAs.

Our results that DsrA itself can contribute to the translational activation and stabilization of the rpoS mRNA in an Hfq-independent manner in vivo may be contradictory to previous in vitro findings: Hfq interacts specifically with the 5' leader sequence of rpoS mRNA to accelerate annealing of DsrA and rpoS mRNA (Soper and Woodson, 2008), and induces conformational changes of DsrA, potentially allowing for efficient base-pairing with rpoS mRNA (Večerek et al., 2008). The relatively high stability of DsrA-rpoS mRNA complex in the absence of Hfq (Soper et al., 2010) may allow DsrA to stimulate rpoS activation without Hfq in vivo even though Hfq is essential for activating the annealing process between DsrA and rpoS mRNA in vitro. In this regard, it is noteworthy that we cannot exclude additional roles of Hfq in DsrA-mediated rpoS activation through enhancement of rpoS mRNA stability or facilitation of ribosome loading on the mRNA in vivo.

A previous study (Hämmerle et al., 2013) reported that RpoS synthesis was sharply reduced at early exponential phase at 24°C in the absence of Hfq despite DsrA-rpoS mRNA duplex formation by overexpressed DsrA and that this sharp reduction is due to the lack of Hfq that is required to re-structure the RBS of the rpoS mRNA for efficient ribosome loading at low temperatures. However, data from other study (Soper et al., 2010) as well as ours (Supplementary Fig. S4) showed that rpoS activation by DsrA overexpression in the absence of Hfq (as assayed using rpoS-lacZ translational fusions) was almost half of that seen in the presence of Hfq at 25°C. Although the basis of the difference in levels of DsrA-mediated RpoS synthesis at low temperatures remains to be clarified, it seems likely that DsrA-rpoS mRNA base-pairing without Hfq still can contribute to a large extent (at least at specific growth phases) to rpoS activation at the low temperatures.

It was reported that RpoS synthesis is rather independent of Hfq and DsrA at 37°C because synthesis of RpoS in hfqΔ cells was found to be moderately reduced compared to that in hfqΔ cells at the early exponential phase (Hämmerle et al., 2013). Nevertheless, since there was still a reduction of RpoS synthesis in hfqΔ cells at this specific growth phase, the reduction should be due to the absence of Hfq and the absence of rpoS activation by DsrA itself and possibly by other Hfq-dependent RpoS-activating sRNAs ArcZ and RprA. We found that the basal level of DsrA among three rpoS-activating sRNAs had the largest positive effects on the rpoS-lacZ translational fusion in hfqΔ cells at the late exponential phase at 37°C (Supplementary Fig. S5) and similar results were also previously reported by Mandin and Gottesman (2010). Cells expressing only DsrA (ΔaΔr cells) synthesized LacZ from the rpoS-lacZ fusion 3-fold higher than Δ3 cells (Supplementary Fig. S5). When the ΔaΔr cells were shifted from 37°C to 25°C for 1.5 h, the rpoS-lacZ expression was slightly lowered at 25°C although a larger fold increase (about 4-fold) was observed in cells kept growing at 37°C (Supplementary Fig. S5D). Furthermore, DsrA is induced following acid challenge during the exponential phase at 37°C (Bak et al., 2014). Therefore, it is likely that DsrA-mediated rpoS activation plays an important role in RpoS synthesis at 37°C as well as at low temperatures.

While DsrA activates rpoS expression by binding to the 5'-UTR of its mRNA, it negatively regulates the hns mRNA by binding to the translation initiation region to inhibit translation. When DsrA represses hns and rbsd expression, Hfq is essential even if DsrA is overexpressed (Lalouna et al., 2015; Lease and Belfort, 2000). This difference may reflect the presence of a repression mechanism in which the pairing of an sRNA with its mRNA targets most often results in degradation of those mRNAs. Since Hfq is believed to be involved in recruiting the RNA degradation machinery, it would be essential for the DsrA-mediated repressions of hns or rbsd. Alternatively, Hfq may play a critical role in facilitating DsrA-hns or rbsd mRNA interactions. In this regard, we note that while DsrA binds well to the rpoS mRNA in the absence of Hfq, the other two rpoS-activating sRNAs, ArcZ and RprA, which absolutely require Hfq for rpoS mRNA binding (McCullen et al., 2010).

To summarize, we herein dissected the coincident effects
of Hfq and DsrA on rpoS activation to gain novel insights into the mechanisms underlying the DsrA-mediated translational activation of the rpoS mRNAs. As shown in a proposed model (Fig. 10), we reveal that the translation and stability of the rpoS mRNAs are enhanced by DsrA regardless of the presence of Hfq, although Hfq depletion causes a rapid degradation of DsrA and decreases the stability of the rpoS mRNAs. This Hfq-independent DsrA-mediated rpoS activation occurs not only at the overexpression levels but also at the endogenous levels. These results suggest that the observed Hfq dependency of DsrA-mediated rpoS activation mainly results from the destabilization of DsrA in the absence of Hfq, but that DsrA itself can contribute to the translational activation and stability of the rpoS mRNAs in an Hfq-independent manner. We further found that the proper concentrations of DsrA and rpoS mRNA can modulate the levels of the translational activation and of stability of rpoS mRNA. This work expands our understanding of the functions of sRNAs and their relationships with those of Hfq.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This study was supported by grants from the National Research Foundation of Korea (NRF) Grant by the Korean government (MSIT) (2017R1A2B4010713; 2019R1H1A2039730) and the Intelligent Synthetic Biology Center of Global Frontier Project funded by MSIT (2013M3A6B073557). The authors would like to thank NBRP-E. coli at NIG for providing E. coli strain containing the Keio knockout library and Dr. S. Gottesman for providing strain PM1409. We also would like to thank Dr. D. Lalaouna for giving some information useful for rpoS and for providing strain TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene 158, 9-14.

De Lay, N., Schu, D.J., and Gottesman, S. (2013). Bacterial small RNA-based negative regulation: Hfq and its accomplices. J. Biol. Chem. 288, 7996-8003.

Desnoyers, G., and Massé, E. (2012). Noncanonical repression of translation initiation through small RNA recruitment of the RNA chaperone Hfq. Genes Dev. 26, 726-739.

Bobrovskyy, M., and Vanderpool, C.K. (2013). Duplex formation between the sRNA DsrA and rpoS mRNA is not sufficient for efficient RpoS synthesis at low temperature. RNA Biol. 10, 1834-1841.

Hämmerle, H., Veeček, B., Resch, A., and Bläsi, U. (2013). Duplex formation between the sRNA DsrA and rpoS mRNA is not sufficient for efficient RpoS synthesis at low temperature. RNA Biol. 10, 1834-1841.

Hoppins, J.F., Panja, S., and Woodson, S.A. (2011). Rapid binding and release of Hfq from ternary complexes during RNA annealing. Nucleic Acids Res. 39, 5193-5202.

Ikeda, Y., Yagi, M., Morita, T., and Alba, H. (2011). Hfq binding at RhfR-recognition region of RNase E is crucial for the rapid degradation of target mRNAs mediated by sRNAs in Escherichia coli. Mol. Microbiol. 79, 419-432.

Kim, S., Kim, H., Park, I., and Lee, Y. (1996). Mutational analysis of RNA structures and sequences postulated to affect 3' processing of M1 RNA, the RNA component of Escherichia coli RNase P. J. Biol. Chem. 271, 19330-19337.

Lalaouna, D., and Massé, E. (2016). The spectrum of activity of the small RNA DsrA: not so narrow after all. Curr. Genet. 62, 261-264.

Lalaouna, D., Morissette, A., Carrier, M.C., and Massé, E. (2015). DsrA regulatory RNA represses both hns and rbsD mRNAs through distinct mechanisms in Escherichia coli. Mol. Microbiol. 98, 357-369.

Lease, R.A., and Belfort, M. (2000). Riboregulation by DsrA RNA: trans-actions for global economy. Mol. Microbiol. 38, 667-672.

Lease, R.A., Cusick, M.E., and Belfort, M. (1998). Riboregulation in Escherichia coli: DsrA RNA acts by RNA:RNA interactions at multiple loci. Proc. Natl. Acad. Sci. U. S. A. 95, 12456-12461.

Lease, R.A., and Woodson, S.A. (2004). Cycling of the Sm-like protein Hfq on the DsrA small regulatory RNA. J. Mol. Biol. 344, 1211-1223.

Link, T.M., Valentin-Hansen, P., and Brenneman, R.G. (2009). Structure of Escherichia coli Hfq bound to polyriboadenylate RNA. Proc. Natl. Acad. Sci. U. S. A. 106, 19292-19297.

Lorenz, C., Gesell, T., Zimmermann, B., Schoeberl, U., Bilusic, I., Rajkowitsch, L., Waldisch, C., von Haezeler, A., and Schroeder, R. (2010). Genomic SELEX for Hfq-binding RNAs identifies genomic aptamers predominantly in antisense transcripts. Nucleic Acids Res. 38, 3794-3808.

Majdalani, N., Cuning, 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. U. S. A. 95, 12462-12467.

Majdalani, N., Vanderpool, C.K., and Gottesman, S. (2005). Bacterial small RNA regulators. Crit. Rev. Biochem. Mol. Biol. 40, 93-113.
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.

McCullen, C.A., Benhammou, J.N., Majdalani, N., and Gottesman, S. (2010). Mechanism of positive regulation by DsrA and RprA small noncoding RNAs: pairing increases translation and protects rpoS mRNA from degradation. J. Bacteriol. 192, 5559-5571.

Mikulecky, P.J., Kaw, M.K., Brescia, C.C., Takach, J.C., Sledjeski, D.D., and Feig, A.L. (2004). *Escherichia coli* Hfq has distinct interaction surfaces for DsrA, rpoS and poly(A) RNAs. Nat. Struct. Mol. Biol. 11, 1206-1214.

Milligan, J.F., and Uhlenbeck, O.C. (1989). Synthesis of small RNAs using T7 RNA polymerase. Methods Enzymol. 180, 51-62.

Meller, T., Franch, T., Hojrup, P., Keene, D.R., Bächinger, H.P., Brennan, R.G., and Valentin-Hansen, P. (2002). Hfq: a bacterial Sm-like protein that mediates RNA-RNA interaction. Mol. Cell 9, 23-30.

Moore, S.D. (2011). Assembling new *Escherichia coli* strains by transduction using phage P1. Methods Mol. Biol. 765, 155-169.

Müller-Hill, B. (1985). Experiments with gene fusions. Trends Genet. 1, 61.

Murina, V.N., and Nikulin, A.D. (2015). Bacterial small regulatory RNAs and Hfq protein. Biochemistry (Mosc) 80, 1647-1654.

Panja, S., Santiago-Frangos, A., Schu, D.J., Gottesman, S., and Woodson, S.A. (2015). Acidic residues in the Hfq chaperone increase the selectivity of sRNA binding and annealing. J. Mol. Biol. 427, 3491-3500.

Peng, Y., Soper, T.J., and Woodson, S.A. (2014). Positional effects of AAN motifs in rpoS regulation by sRNAs and Hfq. J. Mol. Biol. 426, 275-285.

Repolla, F., and Gottesman, S. (2001). Signal transduction cascade for regulation of *rpoS*: temperature regulation of DsrA. J. Bacteriol. 183, 4012-4023.

Resch, A., Afonjushkin, T., Lomba, T.B., McDowell, K.J., Bläsi, U., and Kaberdin, V.R. (2008). Translational activation by the noncoding RNA DsrA involves alternative RNA3 III processing in the rpoS 5' leader. RNA 14, 454-459.

Ross, J.A., Ellis, M.J., Hossain, S., and Haniford, D.B. (2013). Hfq interacts with RNA-A and RNA-OUT and facilitates antisense pairing in the Tn10/IS10 system. RNA 19, 670-684.

Salvail, H., Caron, M.P., Bélanger, J., and Massé, E. (2013). Antagonistic functions between the RNA chaperone Hfq and an mRNA repressor. J. Bacteriol. 195, 6728-6736.

Santiago-Frangos, A., Kavita, K., Schu, D.J., Gottesman, S., and Woodson, S.A. (2016). C-terminal domain of the RNA chaperone Hfq drives sRNA competition and release of target RNA. Proc. Natl. Acad. Sci. U. S. A. 113, E6089-E6096.

Sauer, E., Schmidt, S., and Weichenrieder, O. (2012). Small RNA binding to the lateral surface of Hfq hexamers and structural rearrangements upon mRNA target recognition. Proc. Natl. Acad. Sci. U. S. A. 109, 9396-9401.

Sauer, E., and Weichenrieder, O. (2011). Structural basis for RNA 3' end recognition by Hfq. Proc. Natl. Acad. Sci. U. S. A. 108, 13065-13070.

Sauter, C., Basquin, J., and Suck, D. (2003). Sm-like proteins in Eubacteria: the crystal structure of the Hfq protein from *Escherichia coli*. Nucleic Acids Res. 31, 4091-4098.

Shu, D.J., Zhang, A., Gottesman, S., and Storz, G. (2015). Alternative Hfq-sRNA interaction modes dictate alternative mRNA recognition. EMBO J. 34, 2557-2573.

Sedlayeva, N., Shamovsky, I., Bharati, B.K., Epstein, V., Chen, J., Gottesman, S., Schroeder, R., and Nudler, E. (2016). RNA-mediated control of transcription termination in E. coli. Cell 167, 111-121.e13.

Sledjeski, D.D., Gupta, A., and Gottesman, S. (1996). The small RNA, DsrA, is essential for the low temperature expression of *RpoS* during exponential growth in *Escherichia coli*. EMBO J. 15, 3993-4000.

Sledjeski, D.D., Whitman, C., and Zhang, A. (2001). Hfq is necessary for regulation by the untranslated RNA DsrA. J. Bacteriol. 183, 1997-2005.

Soper, T., Mandin, P., Majdalani, N., Gottesman, S., and Woodson, S.A. (2010). Positive regulation by small RNAs and the role of Hfq. Proc. Natl. Acad. Sci. U. S. A. 107, 9602-9607.

Soper, T.J., and Woodson, S.A. (2008). The rpoS mRNA leader recruits Hfq to facilitate annealing with DsrA sRNA. RNA 14, 1907-1917.

Storz, G., Vogel, J., and Wassarman, K.M. (2011). Regulation by small RNAs in bacteria: expanding frontiers. Mol. Cell 43, 880-891.

Streit, S., Michalski, C.W., Erkan, M., Kleeff, J., and Fries, H. (2009). Northern blot analysis for detection and quantification of RNA in pancreatic cancer cells and tissues. Nat. Protoc. 4, 37-43.

Thomason, L.C., Costantino, N., and Court, D.L. (2007). *E. coli* genome manipulation by P1 transduction. Curr. Protoc. Mol. Biol. Chapter 1, Unit 1.17.

Updegrove, T.B., and Wartell, R.M. (2011). The influence of *Escherichia coli* Hfq mutations on RNA binding and sRNA • mRNA duplex formation in rpoS riboregulation. Biochim. Biophys. Acta 1809, 532-540.

Updegrove, T.B., Zhang, A., and Storz, G. (2016). Hfq: the flexible RNA matchmaker. Curr. Opin. Microbiol. 30, 133-138.

Vecerek, B., Beich-Frandsen, M., Resch, A., and Bläsi, U. (2010). Translational activation of rpoS mRNA by the non-coding RNA DsrA and Hfq does not require ribosome binding. Nucleic Acids Res. 38, 1284-1293.

Vecerek, B., Rajkowitsch, L., Sonnelethner, E., Schroeder, R., and Bläsi, U. (2008). The C-terminal domain of *Escherichia coli* Hfq is required for regulation. Nucleic Acids Res. 36, 133-143.

Vogel, J., and Luisi, B.F. (2011). Hfq and its constellation of RNA. Nat. Rev. Microbiol. 9, 578-589.

Wang, W., Wang, L., Zou, Y., Zhang, J., Gong, Q., Wu, J., and Shi, Y. (2011). Cooperation of *Escherichia coli* Hfq hexamers in DsrA binding. Genes Dev. 25, 2106-2117.

Wassarman, K.M., Zhang, A., and Storz, G. (1999). Small RNAs in *Escherichia coli*. Trends Microbiol. 7, 37-45.

Waters, L.S., and Storz, G. (2009). Regulatory RNAs in bacteria. Cell 136, 615-628.

Zhang, A., Schu, D.J., Tjaden, B.C., Storz, G., and Gottesman, S. (2013). Mutations in interaction surfaces differentially impact *E. coli* Hfq association with small RNAs and their mRNA targets. J. Mol. Biol. 425, 3678-3697.

Zhang, X., and Bremer, H. (1995). Control of the *Escherichia coli* rrnB P1 promoter strength by ppGpp. J. Biol. Chem. 270, 11181-11189.
Supplementary Table S1. Oligonucleotides used in this study

| Name                  | Sequence (5' to 3')                        |
|-----------------------|--------------------------------------------|
| **For Northern blotting** |                                           |
| DsrA_NP               | GTTACACCAGGAAATCTGATGTGT                 |
| lacZsdR1939           | TATTCGCTGGTCATTTCGATGG                  |
| rpoS_NP               | CTTCATTTAATCATGAACATTTCGCTATTCTGACTCAT   |
| **For in vitro transcription** |                                       |
| DsrA_F_T7             | TAATACGACTCTATAGGAACACACAGATTTCTGTT      |
| DsrA_R                | AAATCCCGACCCTGAGGG                      |
| lacZsdF1713_T7        | GTGTAATACGACTCTATAGGTTGTCTGGGAATTGGTGGGTGATCAG |
| lacZsdR1978           | AAATCCCGACCCTGAGGG                      |
| **For qRT-PCR**       |                                           |
| rpoS:5'ORF_FW         | GAAGATGCGGAAATTTGATGAGAAC              |
| rpoS:5'ORF_RV         | AGTTCTCTCTCGGCAAAATC                   |
| rpoS:ORF_FW           | ACCCGTACTATTCGTTTGG                    |
| rpoS:ORF_RV           | ATCTCTTCGCACTTGGTTC                    |
| lacZsdF1713           | GTCTGGGACTGGGTGGATCAG                  |
| lacZsdR1939           | TATTCGCTGGTCACTTGGATGG                 |
| rrsA_968F             | AACGCGAAGAACCTTAC                      |
| rrsA_1387R            | CGGTGTGTACAAGGCCCC                   |
Supplementary Table S2. Half-lives of the rpoS mRNA

| strain | Half-lives (min)\(^a\) |
|--------|-------------------------|
|        | Vector | pDsra |
| hfq\(^+\) | 1.26 ± 0.42 | 2.08 ± 0.58 |
| hfq\(^-\) | 0.83 ± 0.16 | 1.69 ± 0.52 |

\(^a\)Half-lives were determined by linear regression analysis from the data presented in Supplementary Figure S3. We assumed that the disappearance of rpoS mRNA after rifampicin treat followed a first-order decay. Values are means ± SD for three independent experiments.
Supplementary Figure S1

Figure S1. Up-regulation of rpoS translation by endogenous DsrA in hfq+ and hfq- cells. (A) Schematic diagram of experimental conditions. Three colonies for each strain were cultured in LB medium containing ampicillin (100 μg mL⁻¹) and the overnight culture was diluted to 1:100 and cultured with the fresh medium. 0.02% arabinose at 37°C and IPTG were added at 2 h and 3.5 h, respectively, and the culture was incubated further for 30 min and 60 min at 37°C or 25°C. Cells grown at 37°C (B) and 25°C (C) were assayed for LacZ activity (Miller unit). The OD₆₀₀ of cell cultures was also measured. The indicated values were calculated from at least three independent experiments (error bars represent standard deviation). ΔaΔr, arcZ dsrA⁺ rprA⁺ hfq⁺; ΔaΔrΔhfq, arcZ dsrA⁺ rprA⁺ hfq⁻.
Supplementary Figure S2

Figure S2. Northern analysis of effects of DsrA on rpoS mRNA accumulation in Δ3 and Δ3Δhfq cells. Total cellular RNA was prepared from IPTG-treated cells grown at 37°C, and subjected to Northern blot analysis. Cells containing pDsra were treated with IPTG at increasing concentrations from 0 to 0.1 mM. The rpoS mRNA was probed with an anti-rpoS ORF oligonucleotide and the 23S rRNA was detected as a loading control. Δ3, arcZ′ dsrA′ rprA′ hfq+; Δ3Δhfq, arcZ′ dsrA′ rprA′ hfq−; ΔaΔr, arcZ′ dsrA+ rprA′ hfq+; V, vector control.
Supplementary Figure S3

Fig. S3. Effects of DsrA on the stability of the rpoS mRNA in hfq⁺ and hfq⁻ cells. Total cellular RNA was prepared from 0.1 mM IPTG-induced DsrA-expressing cells grown at 37°C, at the indicated times after rifampicin treatment. (A) Cellular levels of rpoS mRNA were measured using Northern blot analysis. rpoS mRNA was probed with an anti-rpoS oligonucleotide and the 23S rRNA was detected as a loading control. (B to E) The % rpoS mRNA remaining was plotted on a semi-log scale as a function of time. Values are means ± SD; n = 3. PM1409Δ3 cells containing control vector and pDsrA (B), PM1409Δ3 and PM1409Δ3Δhfq cells containing control vector (C), PM1409Δ3Δhfq cells containing control vector and pDsrA (D), and PM1409Δ3 and PM1409Δ3Δhfq cells containing pDsrA (E). Δ3, arcZ⁻ dsrA⁻ rprA⁻ hfq⁺; Δ3Δhfq, arcZ⁻ dsrA⁻ rprA⁻ hfq⁻; V, vector control. At least three independent measurements were performed for each strain (error bars represent standard deviation).
Fig. S4. Stimulation of rpoS translation by DsrA overexpression in the absence of Hfq at 37°C and 25°C. Cells were cultured with following condition and LacZ activity was measured. Three colonies for each strain were cultured in LB medium containing ampicillin (100 μg mL\(^{-1}\)) and the overnight culture was diluted to 1:100 and cultured with the fresh medium. 0.02% arabinose at 37°C and IPTG were added at 2 h and 3.5 h, respectively, and the culture was incubated further for 30 min and 60 min at 37°C or 25°C. rpoS activation by overexpression of sRNAs in WT, Δhfq, Δ3 and Δ3Δhfq (lacking all three rpoS-activating sRNAs) was assayed by LacZ expression. Cells grown at 37°C (A), and 25°C (B). Control vector, pHMB1 (V). Plasmids pArcZ, pRprA, and pDsrA overexpress DsrA, RprA, and ArcZ, respectively. WT, arcZ\(^+\) dsrA\(^+\) rprA\(^+\) hfq\(^+\); Δhfq, arcZ\(^+\) dsrA\(^+\) rprA\(^+\) hfq\(^-\); Δ3, arcZ\(^-\) dsrA\(^-\) rprA\(^-\) hfq\(^+\); Δ3Δhfq, arcZ\(^-\) dsrA\(^-\) rprA\(^-\) hfq\(^-\).
Figure S5. Up-regulation of rpoS translation by endogenous rpoS-activating sRNAs at 37°C and 25°C.

(A) Schematic diagram of experimental conditions. Three colonies for each strain were cultured in LB medium containing ampicillin (100 μg mL⁻¹) and the overnight culture was diluted to 1:100 and cultured with the fresh medium. 0.02% arabinose at 37°C and IPTG were added at 2 h and 3.5 h, respectively, and the culture was incubated further for 30 min and 60 min at 37°C or 25°C. Cells grown at 37°C (B) and 25°C (C) were assayed for LacZ activity (Miller unit). The OD₆₀₀ of cell cultures was also measured. OD₆₀₀ values (B), LacZ activity (C), and fold changes in LacZ activities of cells expressing only one rpoS-activating sRNA relative to Δ3 cells (D). The indicated values were calculated from at least three independent experiments (error bars represent standard deviation). Δ3, arcZ dsrA⁻ rprA⁻ hfq⁺; ΔaΔr, arcZ dsrA⁺ rprA⁺ hfq⁺; ΔaΔd, arcZ dsrA⁺ rprA⁺ hfq⁺; ΔdΔr, arcZ⁺ dsrA⁻ rprA⁻ hfq⁺.