Molecules and Cells

Mechanisms for Hfq-Independent Activation of *rpoS* by DsrA, a Small RNA, in *Escherichia coli*

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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 homohexameric 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 (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 (Małecka 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; Møller et

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al., 2002; Sledjeski et al., 2001; Vogel and Luisi, 2011), Hfq can also accelerate degradation of both sRNA and mRNA by recruiting the degradosome to sRNA-mRNA complexes (Andrade et al., 2012; Folichon et al., 2003; Ikeda et al., 2011). Moreover, Hfq reportedly plays more sophisticated roles in the sRNA-mediated translational regulation of the mRNAs for Spot42, SgrS, and RyhB (Bandyra et al., 2012; Desnoyers and Massé, 2012; Salvail et al., 2013).

DsrA, which is an 84-nucleotide Hfq-dependent RNA that can regulate multiple mRNAs (Lalaouna and Massé, 2016; Lalaouna et al., 2015; Lease et al., 1998; Sledjeski et al., 2001; Soper and Woodson, 2008), has been shown to activate the expression of the *rpoS* mRNA by an anti-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; Repoila 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; Repoila 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 activation 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*-

Table 1. Strains and plasmids used in this study

Name	Description	Source
Strains		
PM1409	*Escherichia coli* PM1205 *lacI*^{q}::PBAD-*rpoS-lacZ*	(Soper et al., 2010)
PM1409Δ*hfq*	PM1409 Δ*hfq*::*kan*^{R}	This study
PM1409Δ*3*	PM1409 *arcZ*$^{-}$::*kan*^{R} Δ*rprA* Δ*dsrA*	This study
PM1409Δ*3*Δ*hfq*	PM1409 *arcZ*$^{-}$::*kan*^{R} Δ*rprA* Δ*dsrA* Δ*hfq*	This study
PM1409Δ*a*Δ*r*	PM1409 *arcZ*$^{-}$::*kan*^{R} Δ*rprA*	This study
PM1409Δ*a*Δ*d*	PM1409 *arcZ*$^{-}$::*kan*^{R} Δ*dsrA*	This study
PM1409Δ*d*Δ*r*	PM1409 Δ*dsrA*::*kan*^{R} Δ*rprA*	This study
PM1409Δ*a*Δ*r*Δ*hfq*	PM1409 *arcZ*$^{-}$::*kan*^{R} Δ*dsrA* Δ*hfq*	This study
Plasmids		
pHMB1	A derivative of pHM1 (54), Amp^{R}, IPTG-inducible transcription from immediately after the *Eco*RI site, modified *rnp*B terminator (GAUUU to GGAGU) next to the *Xba*I site.	(Bak et al., 2014)
pArcZ	pHMB1 derivative expressing ArcZ	(Bak et al., 2014)
pRprA	pHMB1 derivative expressing RprA	(Bak et al., 2014)
pDsrA	pHMB1 derivative expressing DsrA	(Bak et al., 2014)
pCP20	FLP^{+}, λ cI857^{+}, λ P_{R} Rep^{ts}, Amp^{R}, Cm^{R}, 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). PM1409*Δ3* (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 PM1409*Δ3Δhfq*, an additional *hfq* deletion was introduced. The *arcZ* promoter mutation was finally introduced by P1 transduction. PM1409*ΔaΔr* was constructed by the first *rprA* deletion and the second *arcZ* promoter mutation through P1 transduction. PM1409*ΔaΔd* and PM1409*ΔdΔr* were constructed by the first *dsrA* deletion and the second *arcZ* promoter mutation or *rprA* deletion. PM1409*ΔaΔrΔhfq* 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 isopropyl β-D-1-thiogalactopyranoside (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 diffusion (Streit et al., 2009). The membrane was hybridized with ^{32}P-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).

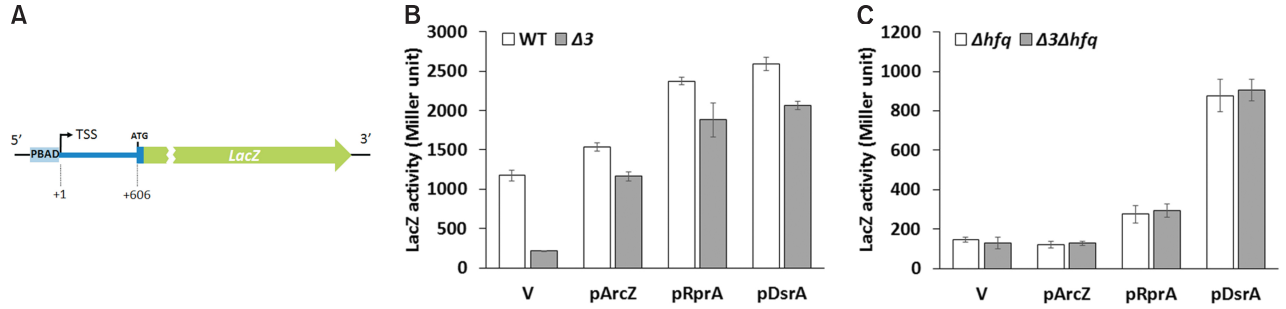

Fig. 1. Stimulation of *rpoS* translation by DsrA overexpression in the absence of Hfq. (A) The *rpoS*::*lacZ* chromosomal reporter fusion in strain PM1409. PBAD, the arabinose-inducible pBAD promoter; Position +1, the transcription start site (TSS) of *rpoS*; ATG, the translation start codon of *rpoS*. The sequence encoding the 5'-terminal 606 nt of the *rpoS* messenger RNA was fused to *lacZ*. (B) *rpoS* activation by overexpression of small RNAs (sRNAs) in PM1409 (WT) and PM1409*Δ3* (*Δ3*) lacking all three *rpoS*-activating sRNAs. Cells containing sRNA-overexpressing plasmids, which were grown at 37°C, were induced with 0.02% arabinose and 0.1 mM IPTG, and LacZ activity was measured. Control vector (V), pHMB1. Plasmids pArcZ, pRprA, and pDsrA overexpress DsrA, RprA, and ArcZ, respectively. (C) *rpoS* activation by overexpression of sRNAs in PM1409*Δhfq* (*Δhfq*) and PM1409*Δ3Δhfq* (*Δ3Δhfq*). LacZ activity was measured as described in (B). WT, *arcZ*$^+$ *dsrA*$^+$ *rprA*$^+$ *hfq*$^+$; *Δhfq*, *arcZ*$^+$ *dsrA*$^+$ *rprA*$^+$ *hfq*$^-$; *Δ3*, *arcZ*$^-$ *dsrA*$^-$ *rprA*$^-$ *hfq*$^+$; *Δ3Δhfq*, *arcZ*$^-$ *dsrA*$^-$ *rprA*$^-$ *hfq*$^-$. At least three independent measurements were performed for each strain. Error bars represent SD.

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) PM1409*Δ3* cells and PM1409*Δ3Δhfq* 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.

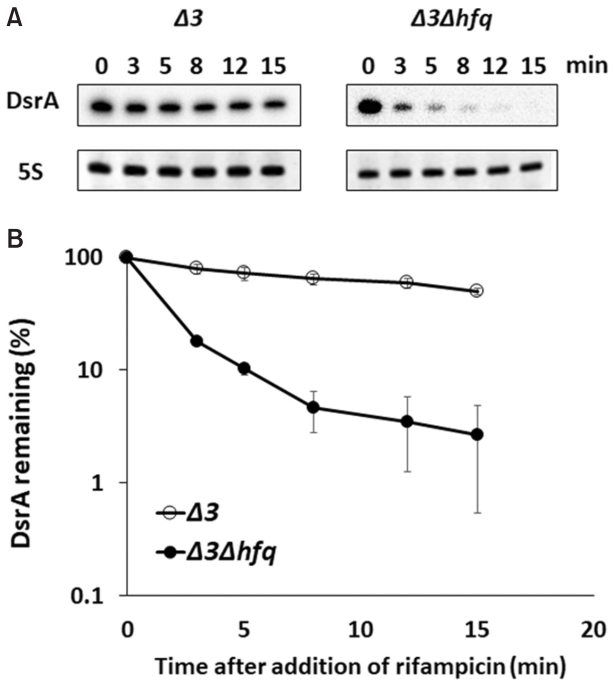

Fig. 2. Half-lives of DsrA in *Δ3* and *Δ3Δhfq* cells. (A) Total cellular RNA was prepared from 0.02% arabinose- and 0.1 mM IPTG-induced cells containing pDsrA, which were grown at 37°C, at the indicated times after rifampicin treatment. Cellular levels of DsrA were measured using Northern blot analysis. DsrA was probed with an anti-DsrA oligonucleotide and the 5S ribosomal RNA was detected as a loading control. Representative blots are shown. *Δ3*, *arcZ*$^-$ *dsrA*$^-$ *rprA*$^-$ *hfq*$^+$; *Δ3Δhfq*, *arcZ*$^-$ *dsrA*$^-$ *rprA*$^-$ *hfq*$^-$.(B) The % RNA remaining against time are presented relative to that in cells before rifampicin treatment on a semi-log scale. Three Northern experiments were conducted and the mean DsrA concentrations ± SD were calculated.

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*$^-$ *rprA*$^-$ cells but not in *arcZ*$^-$ *rprA*$^-$ *hfq*$^-$ 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*$^-$ *dsrA*$^-$ *rprA*$^-$ strains in *hfq*$^+$ and *hfq*$^-$ backgrounds carrying a *rpoS-lacZ* translational fusion; this generated PM1409*Δ3* and PM1409*Δ3Δhfq*. 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 (PM1409*Δ3* 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 (PM1409*Δhfq* or PM1409*Δ3Δhfq* 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 (PM1409*Δhfq* or PM1409*Δ3Δhfq* cells), but the expression level achieved in these cells was significantly lower than that obtained in *hfq*$^+$ cells (PM1409 or PM1409*Δ3* 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 (PM1409*Δ3Δhfq* cells), compared to 14 min in *hfq*⁺ cells (PM1409*Δ3* 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).

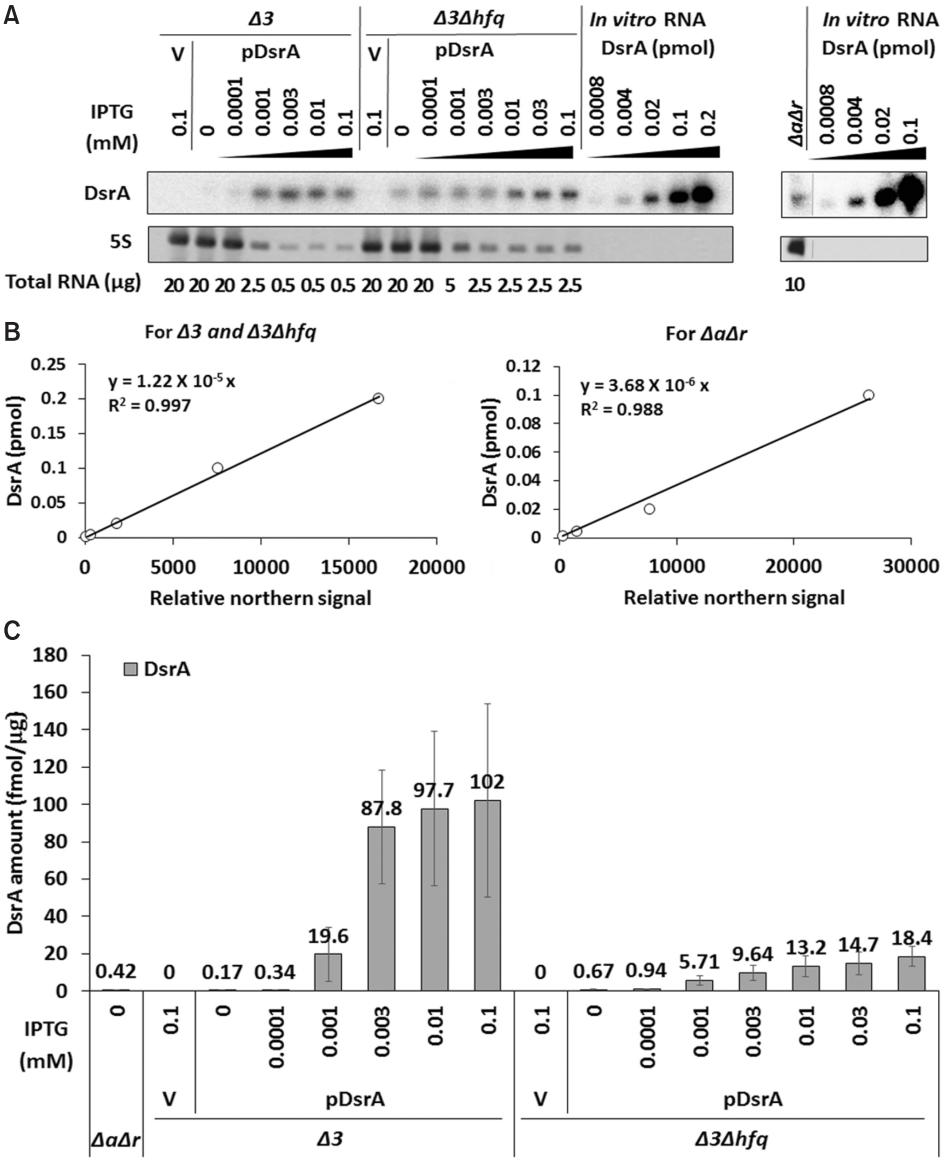

Fig. 3. Cellular levels of DsrA in *hfq*⁺ and *hfq*⁻ cells. (A) Total cellular RNA was prepared from 0.02% arabinose- and IPTG-treated cells grown at 37°C, and subjected to Northern blot analysis as in Figure 2B. *In vitro* DsrA transcripts were used as standards for the quantitation of *in vivo* DsrA 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 PM1409*Δ3* (*Δ3*) and PM1409*Δ3Δhfq* (*Δ3Δhfq*) cells and of PM1409*ΔaΔr* (*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*⁻; *ΔaΔr*, *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 PM1409*Δ3* and PM1409*Δ3Δhfq* 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,

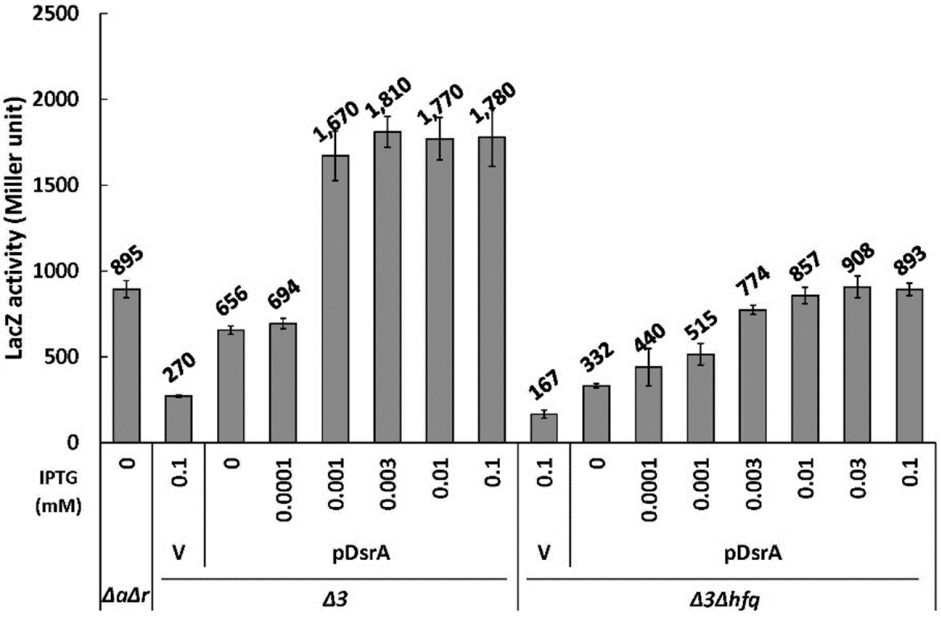

Fig. 4. Up-regulation of *rpoS* translation by DsrA in *hfq*$^+$ and *hfq*$^-$ cells. Cells containing pDsrA, which were grown at 37°C, were induced with 0.02% arabinose and IPTG at increasing concentrations from 0 to 0.1 mM and LacZ activity was measured. The indicated values were calculated from at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

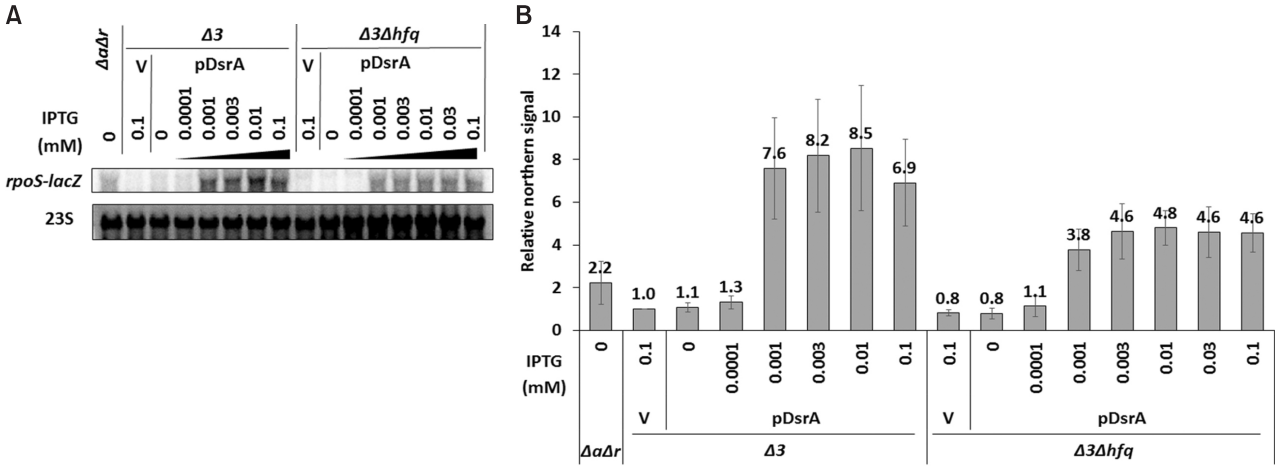

Fig. 5. Northern analysis of effects of DsrA on *rpoS-lacZ* mRNA accumulation in *Δ3* and *Δ3Δhfq* cells. (A) Total cellular RNA was prepared from 0.02% arabinose- and IPTG-treated cells grown at 37°C, and subjected to Northern blot analysis. Cells containing pDsrA were treated with IPTG at increasing 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 (Lalaouna 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 PM1409*ΔaΔr* (*arcZ*$^-$ *dsrA*$^+$ *rprA*$^-$ *hfq*$^+$) cells was comparable to the ectopic DsrA level resulting from basal expression in PM1409*Δ3Δhfq* (*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 PM1409*ΔaΔr* (*hfq*$^+$) cells were about 2.5-fold higher than those in PM1409*Δ3Δhfq* (*hfq*$^-$) cells (Figs. 4 and 6). Therefore, it is likely that the LacZ activities were 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 thus 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 occurs in 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.

Effects of Hfq and DsrA on *rpoS-lacZ* decay

We also examined how Hfq and DsrA might increase the *rpoS-lacZ* mRNA level. To examine whether this effect reflected a simple increase in the half-life of the *rpoS-lacZ* mRNA, we determined the half-life of *rpoS-lacZ* mRNA produced under the control of pBAD by monitoring its disappearance after the removal of arabinose. Our results revealed that the half-life of the *rpoS-lacZ* mRNA was slightly increased in the presence of Hfq and also by overexpression of DsrA, regardless of Hfq (Fig. 8 and Table 2). Although the more common rifampicin chase-experiment could potentially mask the precise effects of DsrA because rifampicin might also inhibit the transcription of DsrA (Milligan and Uhlenbeck, 1989), we performed rifampicin chase experiments to see any effects of DsrA on stability of *rpoS* mRNA (Supplementary Fig. S3 and Supplementary Table S2). We found that DsrA also increased the half-live of *rpoS* mRNA. These results altogether suggest that the binding of DsrA to the *rpoS* mRNA inhibits the decay of the *rpoS* mRNA regardless of the presence of Hfq although Hfq may inhibit the decay of the *rpoS* mRNA. However, it should be noted that the inhibitory effect of DsrA or Hfq on the *rpoS* mRNA decay could be indirectly achieved through the increased translation in the presence of DsrA or the decreased translation in the absence of Hfq because the alteration of translation efficiency can affect mRNA stability.

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

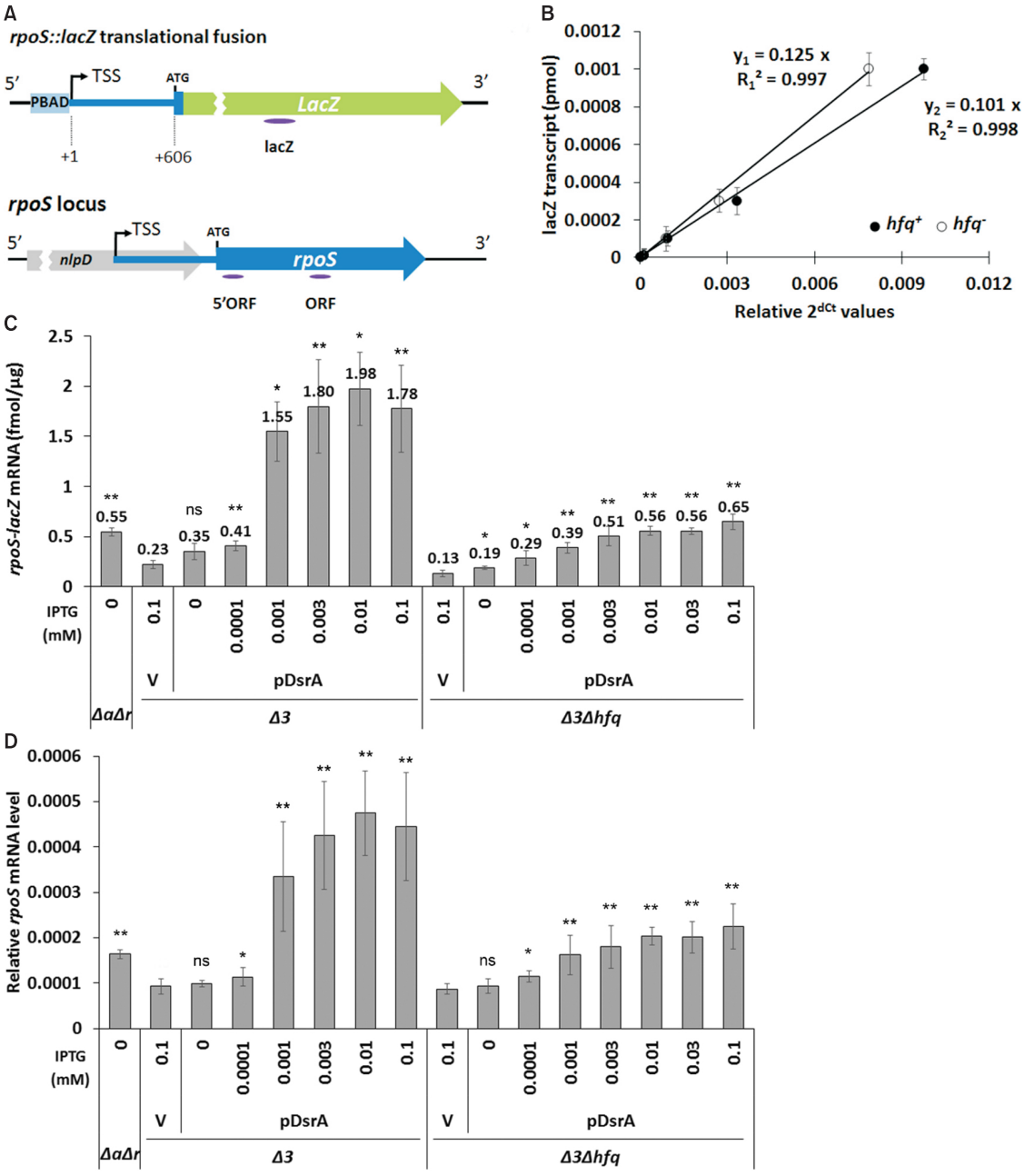

Fig. 6. qRT-PCR analysis of effects of DsrA on *rpoS* mRNA accumulation in *hfq*$^+$ and *hfq*$^-$ cells. (A) Schematic diagrams of the *rpoS*::*lacZ* chromosomal reporter fusion and the *rpoS* gene structure. The P_{BAD} promoter is indicated by PBAD, while the *rpoS* promoter is located within the *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 PM1409*Δ3* and PM1409*Δ3Δhfq* 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 PM1409*ΔaΔr*, PM1409*Δ3*, and PM1409*Δ3Δhfq* 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*$^-$; *ΔaΔr, arcZ*$^-$ *dsrA*$^+$ *rprA*$^-$ *hfq*$^+$; V, vector control.

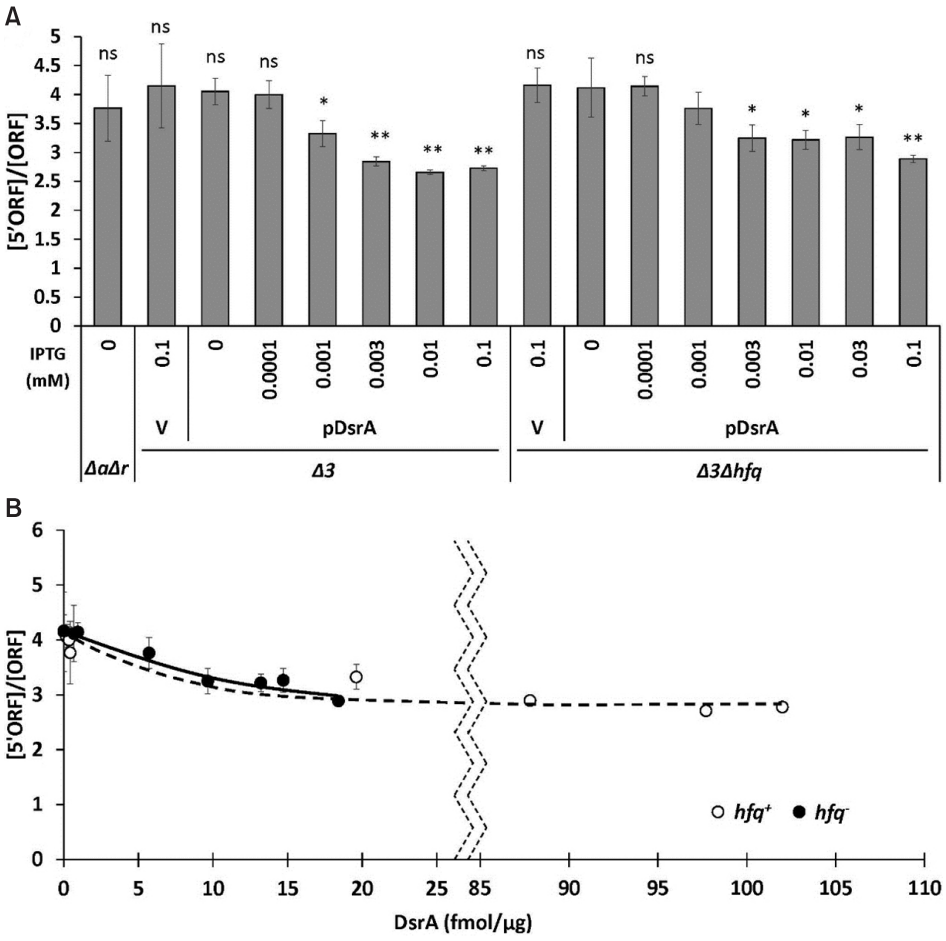

Fig. 7. Effects of DsrA on premature termination of *rpoS* transcription in *hfq*$^+$ and *hfq*$^-$ cells. (A) Levels of *rpoS* transcripts in PM1409*Δ3* and PM1409*Δ3Δhfq* cells grown at 37°C, were determined by performing qRT-PCR of the 5' ORF amplicon, as described in Figure 6A. After cycle threshold (Ct) data were normalized to *rrsA* expression, the normalized values were divided by those of the *rpoS* ORF amplicon. An increase in the [5'ORF]/[ORF] ratio corresponds to an increase in the Rho-dependent termination efficiency, whereas a decrease in the [5'ORF]/[ORF] ratio corresponds to a decrease in the Rho-dependent termination efficiency. Values are means ± SD; n = 3; $^{**}P < 0.01$, $^{*}P < 0.05$; ns, non-significant (Student's *t*-test, equal variance with V/*Δ3Δhfq* value). (B) The [5'ORF]/[ORF] ratio was plotted against the concentration of DsrA fmol/μg of total RNA. *Δ3, arcZ*$^-$ *dsrA*$^-$ *rprA*$^-$ *hfq*$^+$; *Δ3Δhfq, arcZ*$^-$ *dsrA*$^-$ *rprA*$^-$ *hfq*$^-$; *ΔaΔr, 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

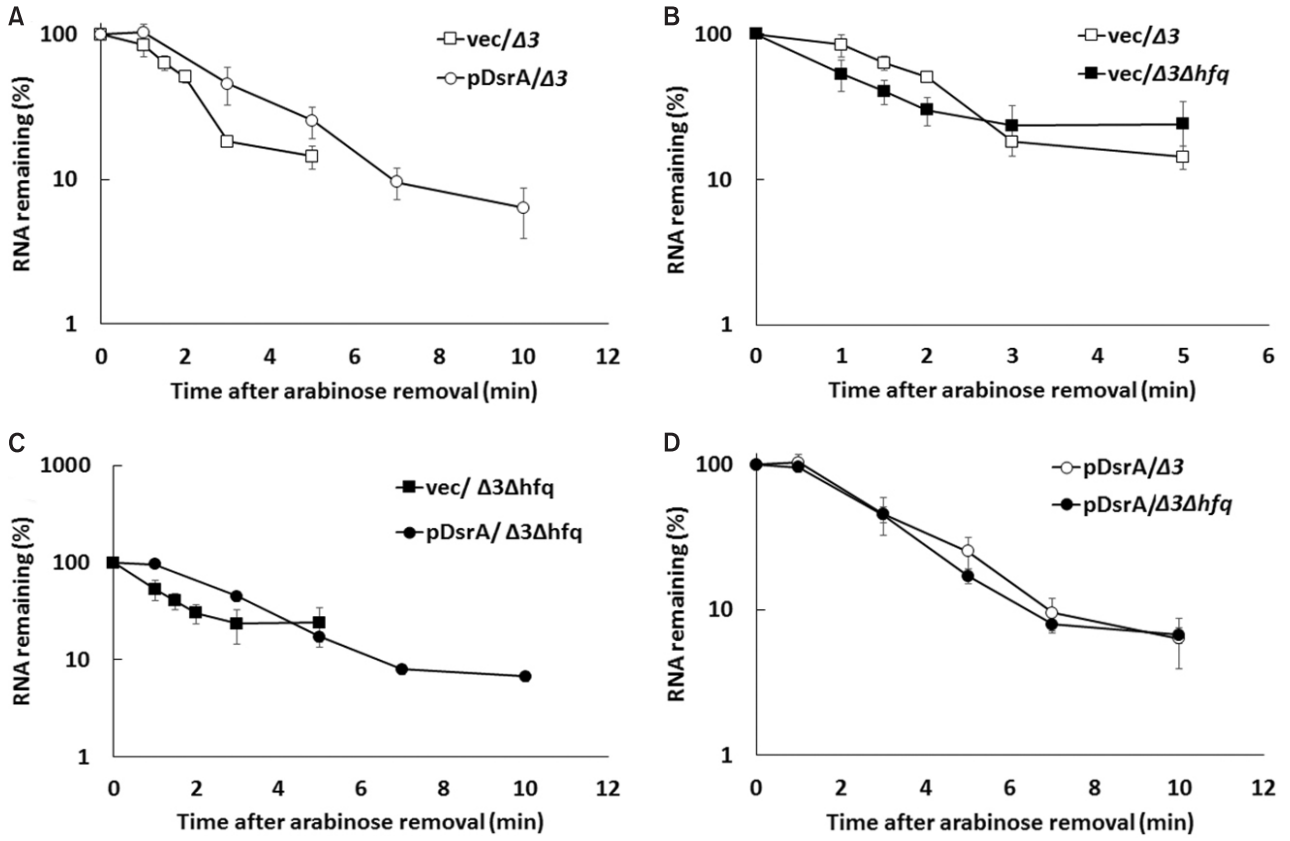

Fig. 8. Effects of DsrA on the stability of the *rpoS-lacZ* mRNA in *hfq*$^+$ and *hfq*$^-$ cells. Total cellular RNA was prepared from 0.02% arabinose- and 0.1 mM IPTG-induced DsrA-expressing cells grown at 37°C, at the indicated times after arabinose was washed. Cellular levels of *rpoS-lacZ* mRNA were analyzed by qRT-PCR of PM1409*Δ3* cells containing control vector and pDsrA (A), PM1409*Δ3* and PM1409*Δ3Δhfq* cells containing control vector (B), PM1409*Δ3Δhfq* cells containing control vector and pDsrA (C), and PM1409*Δ3* and PM1409*Δ3Δhfq* cells containing pDsrA (D). Cycle threshold (Ct) values were normalized to *rrsA* expression. The normalized values are used to calculate the fraction (%) of RNA remaining. The % RNA remaining was plotted on a semi-log scale as a function of time. Values are means ± SD; n = 3. *Δ3, arcZ*$^-$ *dsrA*$^-$ *rprA*$^-$ *hfq*$^+$; *Δ3Δhfq, arcZ*$^-$ *dsrA*$^-$ *rprA*$^-$ *hfq*$^-$; V, vector control.

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

Strain	Half-lives (min)[a]	
	Vector	pDsrA
hfq$^+$	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.
[a]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.

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

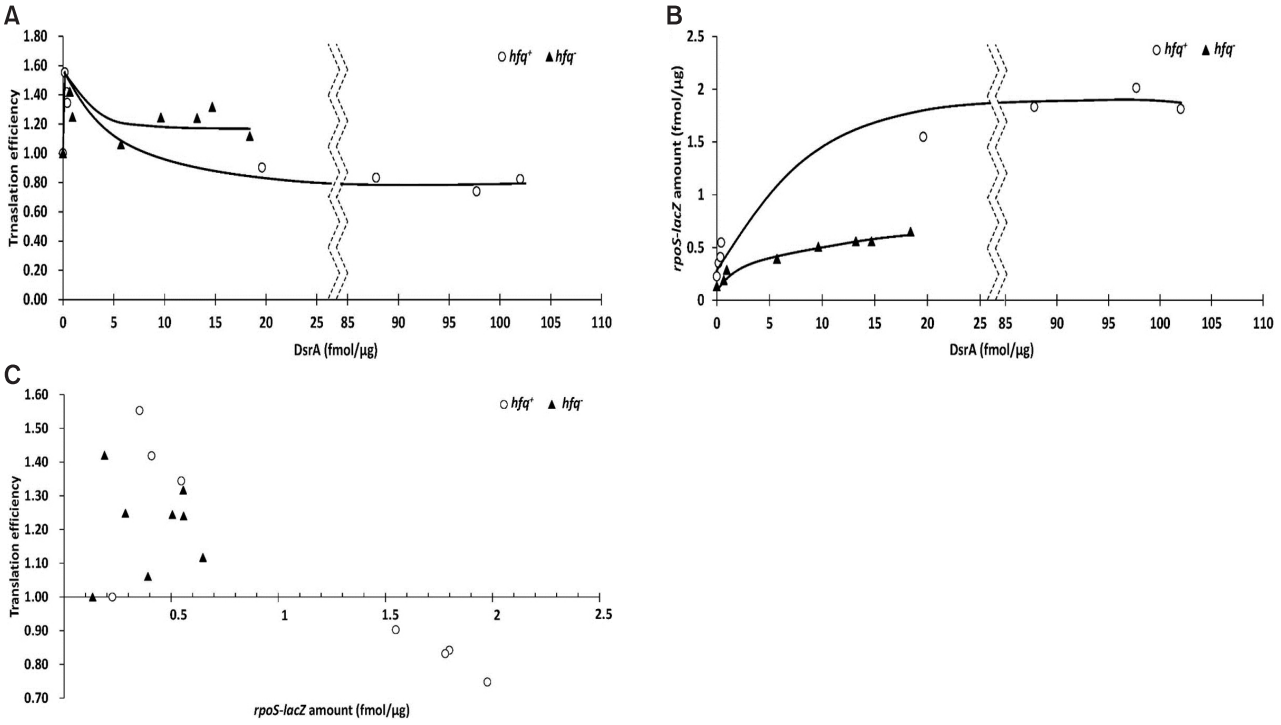

Fig. 9. Effects of DsrA on the translational activation of *rpoS* in *hfq*$^+$ and *hfq*$^-$ cells. Translational efficiency was defined as the ratio of LacZ activity to the amount of *rpoS-lacZ* messenger RNA (mRNA) and calculated from the data presented in Figures 3, 4, and 6. Translational efficiencies (A) and *rpoS-lacZ* mRNA concentrations (B) in *hfq*$^+$ and *hfq*$^-$ cells are plotted against the concentration of DsrA fmol/μg of total RNA. (C) Translational efficiencies in *hfq*$^+$ and *hfq*$^-$ cells are plotted against the concentration of *rpoS-lacZ* mRNA fmol/μg of total RNA.

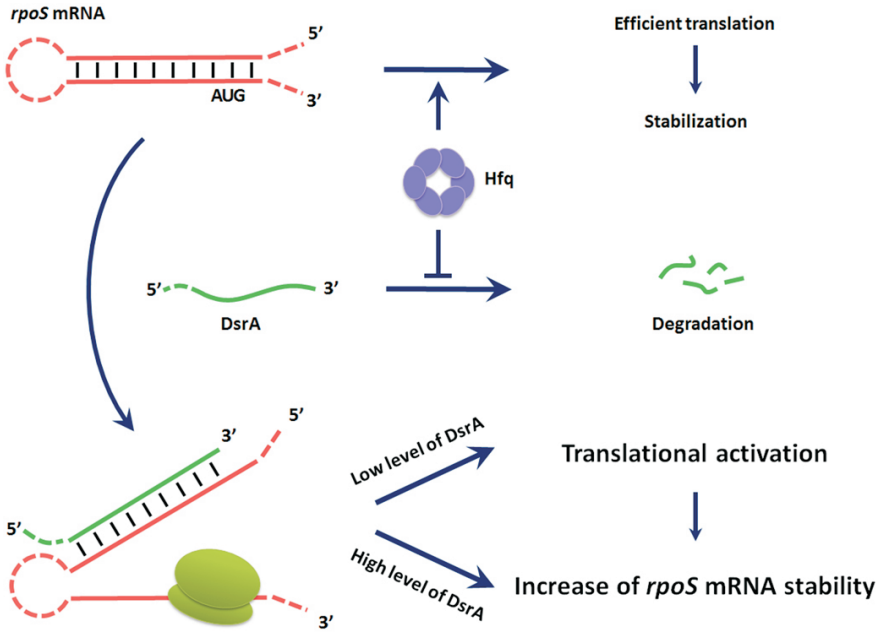

Fig. 10. A model for DsrA-mediated *rpoS* activation and the role of Hfq. Hfq stabilizes *rpoS* messenger RNA (mRNA) and is required for efficient translation of *rpoS* mRNA, while it inhibits degradation DsrA. The efficient translation can cause an increase of the stability of *rpoS* mRNA. Binding of 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.

degradation (McCullen et al., 2010) or the alternative RNase III processing (Resch et al., 2008). 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 *hfq*$^{+}$ and *hfq*$^{-}$ 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 *hfq*$^{-}$ cells than in *hfq*$^{+}$ 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 DsrA.

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 *hfq*$^{-}$ cells was found to be moderately reduced compared to that in *hfq*$^{+}$ cells at the early exponential phase (Hämmerle et al., 2013). Nevertheless, since there was still a reduction of RpoS synthesis in *hfq*$^{-}$ 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 AcrZ 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 *hfq*$^{+}$ 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 (Lalaouna 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* mRNA. As shown in a proposed model (Fig. 10), we reveal that the translation and stability of the *rpoS* mRNA 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* mRNA. 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* mRNA in an Hfq-independent manner. We further found that the proper concentrations of DsrA and *rpoS* mRNA can modulates 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).

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Molecules and Cells

Supplementary Table S1. Oligonucleotides used in this study

Name	Sequence (5' to 3')
For Northern blotting	
DsrA_NP	GTTACACCAGGAAATCTGATGTGTT
lacZsdR1939	TATTCGCTGGTCACTTCGATGG
rpoS_NP	CTTCATTTA AATCATGAACTTTCAGCGTATTCTGACTCAT
For in vitro transcription	
DsrA_F_T7	TAATACGACTCACTATAGGAACACATCAGATTTCCTGGTG
DsrA_R	AAATCCCGACCCTGAGGGGG
lacZsdF1713_T7	GTGTAATACGACTCACTATAGGGGTCTGGGACTGGGTGGATCAG
lacZsdR1978	AAATCCCGACCCTGAGGGGG
For qRT-PCR	
rpoS:5'ORF_FW	GAAGATGCGGAATTTGATGAGAAC
rpoS:5'ORF_RV	AGTTCCTCTTCGGCCAAATC
rpoS:ORF_FW	ACCCGTACTATTCGTTTGCC
rpoS:ORF_RV	ATCTCTTCCGCACTTGGTTC
lacZsdF1713	GTCTGGGACTGGGTGGATCAG
lacZsdR1939	TATTCGCTGGTCACTTCGATGG
rrsA_968F	AACGCGAAGAACCTTAC
rrsA_1387R	CGGTGTGTACAAGGCCCGGG

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

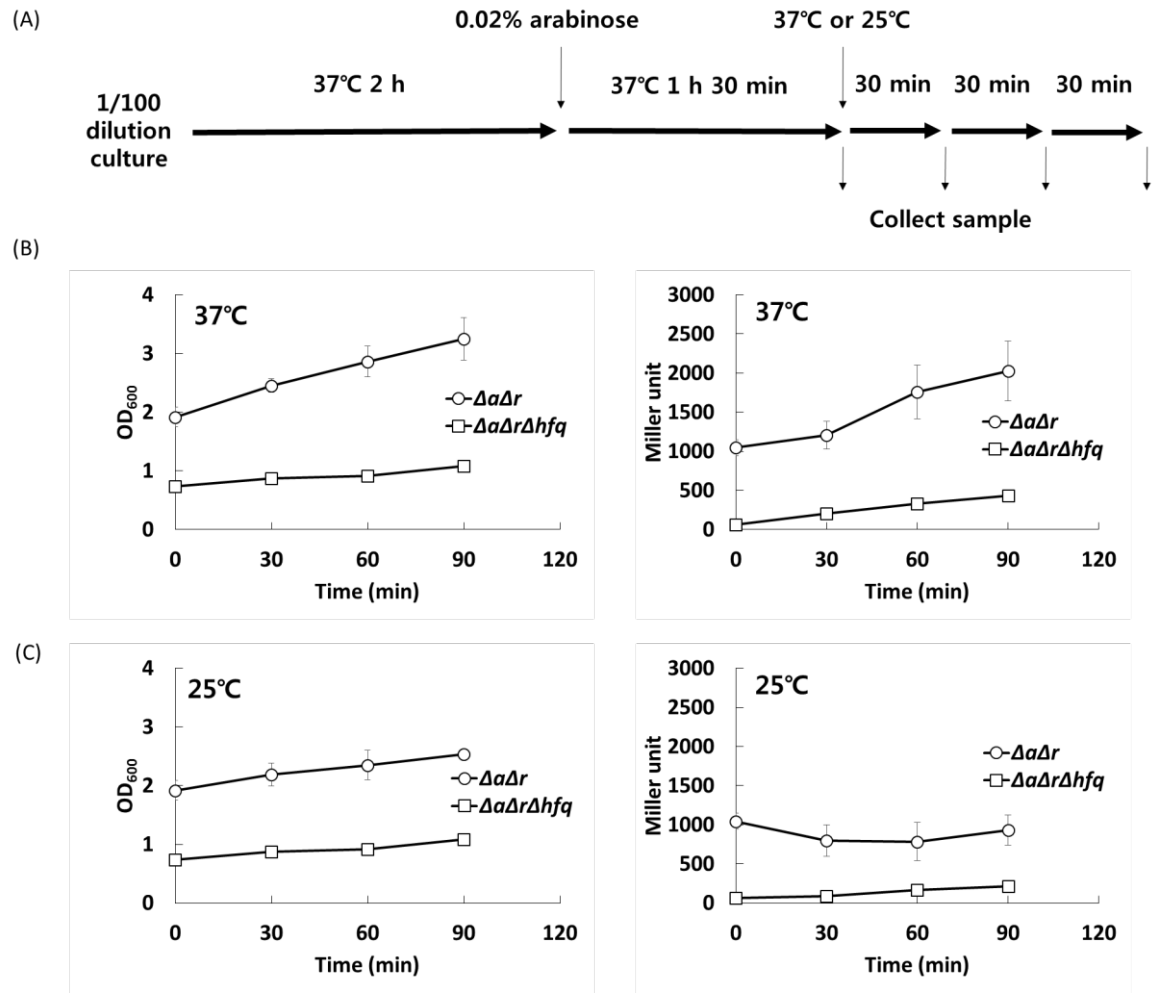

Figure S1. Up-regulation of *rpoS* translation by endogenous DsrA in *hfq*$^{+}$ and *hfq*$^{-}$ cells. (A) Schematic diagram of experimental conditions. Three colonies for each strain were cultured in LB medium containing ampicillin (100 μg mL^{-1}) and the overnight culture was diluted to 1:100 and 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 OD_{600} 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*$^{-}$ d*srA*$^{+}$ *rprA*$^{-}$ *hfq*$^{-}$.

Supplementary Figure S2

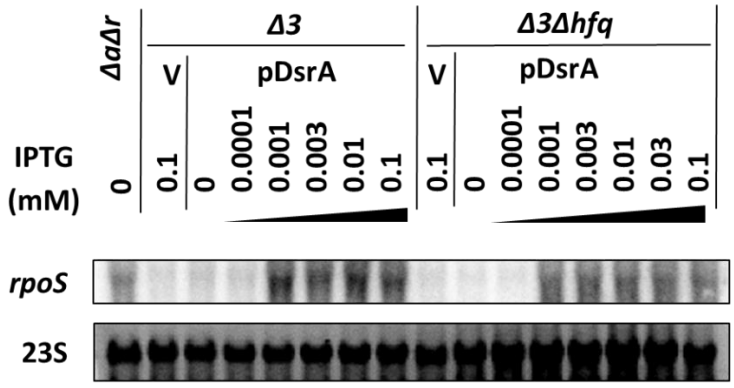

Figure S2. Northern analysis of effects of DsrA on *rpoS* mRNA accumulation in *Δ3* and *Δ3Δhfq* cells. Total cellular RNA was prepared from IPTG-treated cells grown at 37°C, and subjected to Northern blot analysis. Cells containing pDsrA were treated with IPTG at increasing concentrations from 0 to 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

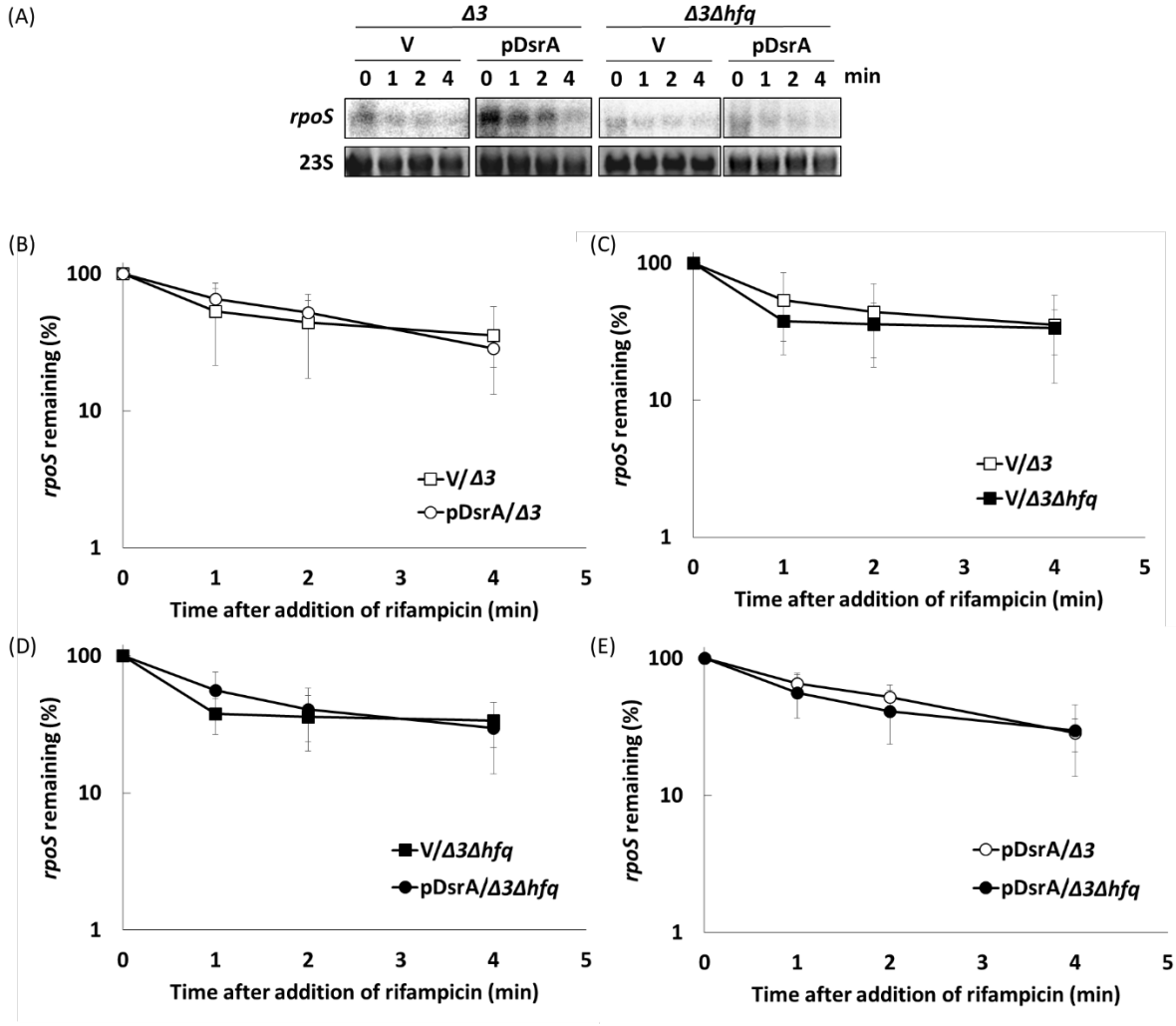

Fig. S3. Effects of DsrA on the stability of the *rpoS* mRNA in *hfq*$^{+}$ and *hfq*$^{-}$ cells. Total cellular RNA was prepared from 0.1 mM IPTG-induced DsrA-expressing cells grown at 37°C, at the indicated times after rifampicin treatment. (A) Cellular levels of *rpoS* mRNA were measured using Northern 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. PM1409*Δ3* cells containing control vector and pDsrA (B), PM1409*Δ3* and PM1409*Δ3Δhfq* cells containing control vector (C), PM1409*Δ3Δhfq* cells containing control vector and pDsrA (D), and PM1409*Δ3* and PM1409*Δ3Δhfq* 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).

Supplementary Figure S4

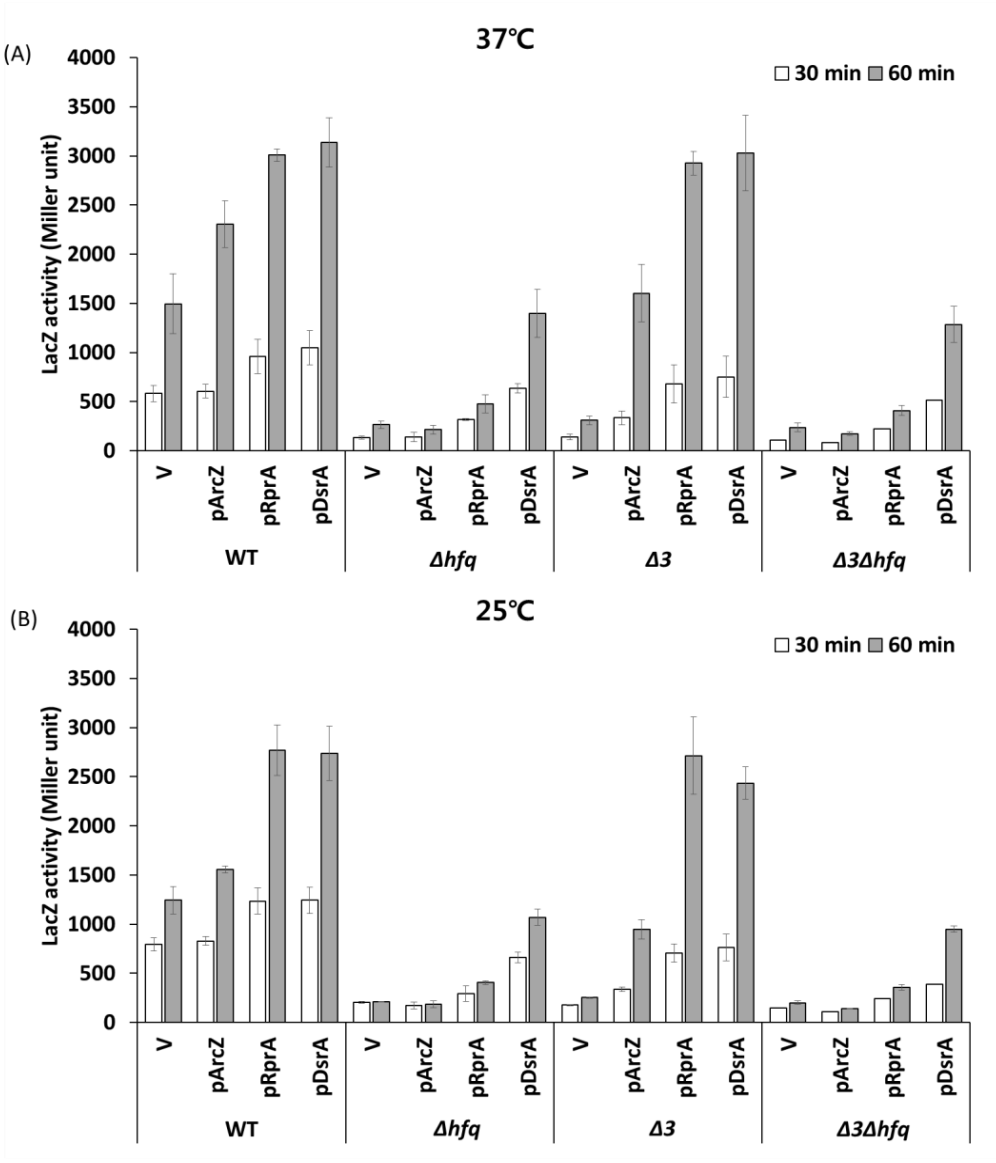

Fig. S4. Stimulation of *rpoS* translation by DsrA overexpression in the absence of Hfq at 37°C and 25°C. Cells were cultured with following condition and LacZ activity was measured. Three colonies for each strain were cultured in LB medium containing ampicillin (100 μg mL^{-1}) and the overnight culture 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*$^{-}$.

Supplementary Figure S5

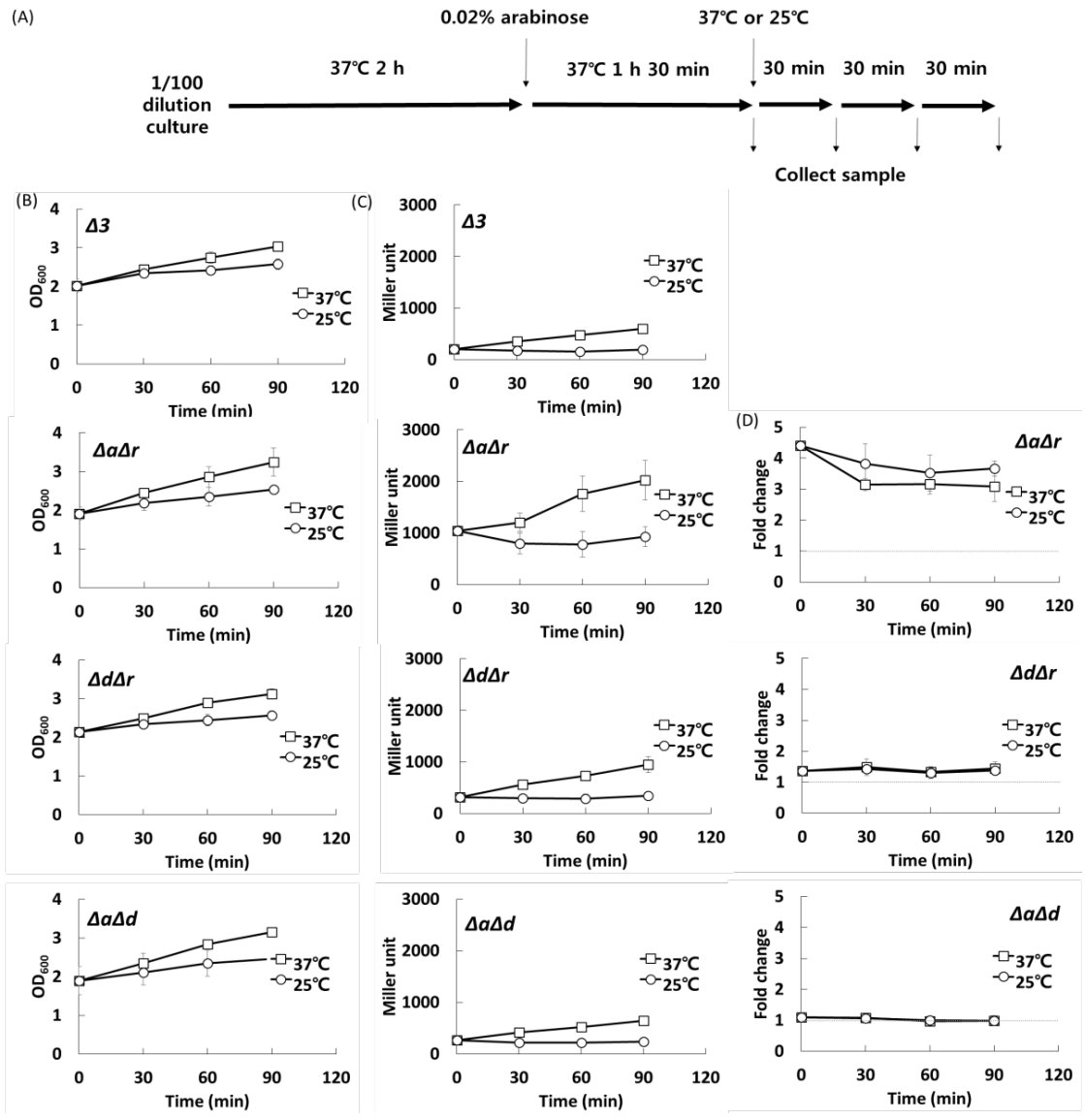

Figure S5. Up-regulation of *rpoS* translation by endogenous *rpoS*-activating sRNAs at 37°C and 25°C. (A) Schematic diagram of experimental conditions. Three colonies for each strain were cultured in LB medium containing ampicillin (100 μg mL^{-1}) and the overnight culture was diluted to 1:100 and 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 OD_{600} of cell cultures was also measured. OD_{600} 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⁻* d*srA⁻ rprA⁺ hfq⁺*; *ΔdΔr, arcZ⁺ dsrA⁻ rprA⁻ hfq⁺*.