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Duplex formation between the sRNA DsrA and *rpoS* mRNA is not sufficient for efficient RpoS synthesis at low temperature

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At low temperatures the *Escherichia coli rpoS* mRNA, encoding the stationary phase sigma factor RpoS, forms an intramolecular secondary structure (iss) that impedes translation initiation. Under these conditions the small RNA DsrA, which is stabilzed by Hfq, forms a duplex with *rpoS* mRNA sequences opposite of the ribosome-binding site (rbs). Both the DEAD box helicase CsdA and Hfq have been implicated in DsrA•*rpoS* duplex formation. Hfq binding to A-rich sequences in the *rpoS* leader has been suggested to restructure the mRNA, and thereby to accelerate DsrA•*rpoS* duplex formation, which, in turn, was deemed to free the *rpoS* rbs and to permit ribosome loading on the mRNA. Several experiments designed to elucidate the role of Hfq in DsrA-mediated translational activation of *rpoS* mRNA have been conducted in vitro. Here, we assessed RpoS synthesis in vivo to further study the role of Hfq in *rpoS* regulation. We show that RpoS synthesis was reduced when DsrA was ectopically overexpressed at 24 °C in the absence of Hfq despite of DsrA•*rpoS* duplex formation. This observation indicated that DsrA•*rpoS* annealing may not be sufficient for efficient ribosome loading on *rpoS* mRNA. In addition, a Hfq_{G29A} mutant protein was employed, which is deficient in binding to A-rich sequences present in the *rpoS* leader but proficient in DsrA binding. We show that DsrA•*rpoS* duplex formation occurs in the presence of the Hfq_{G29A} mutant protein at low temperature, whereas synthesis of RpoS was greatly diminished. RNase T1 footprinting studies of DsrA•*rpoS* duplexes in the absence and presence of Hfq or Hfq_{G29A} indicated that Hfq is required to resolve a stem-loop structure in the immediate coding region of *rpoS* mRNA. These in vivo studies corroborate the importance of the A-rich sequences in the *rpoS* leader and strongly suggest that Hfq, besides stabilizing DsrA and accelerating DsrA•*rpoS* duplex formation, is also required to convert the *rpoS* mRNA into a translationally competent form.

Introduction

In bacteria, small regulatory trans-acting RNAs (sRNAs) can modulate different stress responses through post-transcriptional regulation.[1,2] These sRNAs either prevent ribosome loading onto the mRNA by base-pairing with or in the vicinity of the ribosome binding site (rbs) or activate translation by abrogating intramolecular inhibitory stem-loop structures (iss) that block ribosome binding. The RNA chaperone Hfq has been shown to be crucial for riboregulation in Enterobacteriaceae, which results on the one hand from binding to and protection of sRNAs from nucleolytic decay,[3-5] and on the other hand from accelerating annealing of sRNAs with their target mRNAs.[3] *E. coli* Hfq hexamers have dedicated RNA binding sites, preferably binding uridine-rich stretches of sRNAs around the central pore of the proximal surface[6-10] and A-rich sequences on the distal surface.[11] Moreover, binding of the sRNA RybB to Hfq not only involves contacts via 3′ terminal U-rich stretches but also with the lateral surface of the hexamer.[12] The dedicated sRNA and mRNA binding surfaces on either site of the Hfq-hexamer may serve to transiently increase the local concentration of two RNA substrates. In addition, the inherent capacity of Hfq to induce conformational changes in RNAs,[13-15] together with the observed structural flexibility of RNA ligands bound to Hfq,[8,10] may stochastically facilitate base-pairing.

A paradigm for Hfq-mediated translational activation by a sRNA is the *E. coli rpoS* mRNA, encoding the stationary phase sigma factor RpoS. The sRNA DsrA, which is mainly transcribed at low-growth temperature[16,17] (≤ 25 °C), is necessary for translational activation of *rpoS* mRNA[18] under these conditions. The DsrA-*rpoS* mRNA interaction counteracts an iss that impedes ribosomal access to the rbs of *rpoS*.[19] In vivo DsrA•*rpoS* duplex formation at low temperature further requires the CsdA helicase,[20] and creates RNase III cleavage sites within the duplex that prevents reuse of DsrA.[21] DsrA was shown to bind with a 1:1 stoichiometry on the proximal face of Hfq,[8,22] whereas *rpoS* mRNA is recruited to

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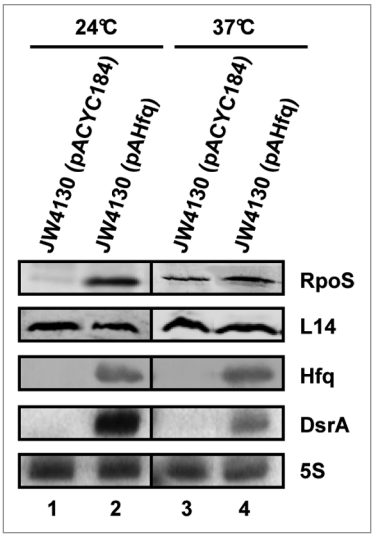

Figure 1. Hfq/DsrA requirement at 24 °C and 37 °C. The steady-state levels of RpoS were determined by quantitative western blotting in the *E. coli hfq*⁻ strain JW4130 harboring the control plasmid pACYC184 (lanes 1 and 3) and plasmid pAHfq, encoding Hfq (lanes 2 and 4), respectively. The strains were grown to early log phase (OD_{600} of 0.4) at 24 °C (lanes 1 and 2) or 37 °C (lanes 3 and 4). Equal amounts of cellular protein were loaded onto the SDS-polyacrylamide gel. Immunodetection of RpoS, ribosomal protein L14 (loading control), and Hfq as well as the detection of DsrA and 5S rRNA (loading control) by northern blot analysis was performed as described in the Materials and Methods. Only the relevant parts of the immunoblots and the autoradiographs are shown.

the distal side of Hfq via A-rich elements in the mRNA leader.[23,24] The interaction of Hfq with both RNAs, albeit accelerating annealing, appears to be transient.[23,25] The need for Hfq to cycle off its binding site on DsrA prior to or during annealing with *rpoS*[26] could result from the fact that at least part of the Hfq binding site on DsrA base-pairs with *rpoS* mRNA.[27,28] Moreover, Hfq binding to an $(AAN)_4$ repeat element in the *rpoS* leader has been shown to be critical for regulation by sRNAs,[24] and for DsrA·*rpoS* duplex formation.[29] Binding to these A-rich motifs has been suggested to result in restructuring of the *rpoS* leader, priming the mRNA for productive interactions with DsrA.[29]

Despite intensive research on the DsrA-*rpoS* model system, the Hfq-dependent steps toward ribosome loading on *rpoS* mRNA at low temperature are not completely understood. Here, we have addressed in vivo whether DsrA·*rpoS* annealing is sufficient for efficient RpoS synthesis at low temperature. Although DsrA·*rpoS* duplex formation occurred in the absence of Hfq as well as in the presence of a Hfq_{G29A} mutant protein deficient in binding to the $(AAN)_4$ motifs, RpoS synthesis was diminished. RNase T1 footprinting studies of DsrA·*rpoS* duplexes in the absence and presence of Hfq or Hfq_{G29A} indicated that Hfq is required to resolve a stem-loop structure in the immediate coding region of *rpoS* mRNA. These in vivo studies corroborate the importance of the A-rich sequences in the *rpoS* leader and show that Hfq, besides accelerating DsrA·*rpoS* duplex formation, is apparently additionally required to convert the *rpoS* mRNA into a translationally competent form.

Results and Discussion

Hfq- and DsrA-independent synthesis of RpoS at 37 °C

The design of in vivo studies to assess the requirement for Hfq in *rpoS* translation is complicated as Hfq performs several interdependent functions; (1) it affects the stability of sRNAs involved in *rpoS* translational regulation, e.g., DsrA,[4,18,24] (2) it accelerates the interaction between DsrA and *rpoS* mRNA,[25] and (3) it has been implicated in restructuring the *rpoS* leader, which, in turn, is deemed to be required for DsrA·*rpoS* duplex formation.[29] In addition, some studies addressing the requirement for sRNAs and/or Hfq were performed at 37 °C,[24] where DsrA is less abundant than at 24 °C[16,17] (**Fig. 1**, lanes 2 and 4). In this study, we aimed at dissecting Hfq and/or DsrA-dependent steps that lead to in vivo RpoS synthesis. First, we tested whether the temperature affects RpoS synthesis in a Hfq-dependent manner. The *hfq*⁻ strain JW4130 was transformed with plasmids pACYC184 (control) or pAHfq (encodes Hfq_{wt}) and cultivated at 24 °C and 37 °C, respectively. As expected, no RpoS synthesis was observed in the absence of DsrA and Hfq at 24 °C in strain JW4130(pACYC184), whereas RpoS synthesis occurred in strain JW4130(pAHfq) in the presence of DsrA and Hfq, respectively (**Fig. 1**, lanes 1 and 2). Most likely, the absence of DsrA in strain JW4130(pACYC184) is attributable to a lack of stabilization by Hfq.[4,18,24] In contrast, RpoS synthesis was rather independent of Hfq and DsrA at 37 °C. The RpoS levels in strain JW4130(pACYC184) equalled ~66% of that observed in strain JW4130(pAHfq) (**Fig. 1**, lanes 3 and 4). Although the presence of Hfq and DsrA stimulated translation of *rpoS* mRNA in strain JW4130(pAHfq) at 37 °C, both are apparently not essential, i.e., the iss occluding the ribosome binding site of *rpoS* appears to be not stable enough to efficiently counteract initiating ribosomes at this temperature. It seems worth noting that this can lead to false-positive results when Hfq mutant proteins are tested for their capacity to stimulate RpoS translation at 37 °C.

Similarly, Soper et al.[24] showed that a *rpoS::lacZ* translational fusion gene is translated at 37 °C with an approximately 8-fold higher rate in a *hfq*⁺ strain when compared with a *hfq*⁻ strain. In addition to DsrA, two other sRNAs, RprA[30] and ArcZ,[31] are involved in translational activation of *rpoS* mRNA. As these sRNAs are likewise destabilized in a *hfq*⁻ background,[24] and as the activity of a *rpoS::lacZ* fusion was higher in a triple *dsrA*, *rprA*, and *arcZ* mutant background than in a *hfq* deletion mutant at 37 °C,[31] the observed difference in RpoS synthesis between the *hfq*⁻ strain and the *hfq*⁺ strain at 37 °C (**Fig. 1**, lanes 3 and 4) could be attributable to the function of Hfq rather than to that of the sRNAs. Clearly, we cannot disregard the possibility that the observed differences in RpoS/RpoS-LacZ synthesis result from a reduced stability of the mRNA at 37 °C in the absence of Hfq. However, this seems less likely, as no differences in the steady-state levels of full-length *rpoS* mRNA were previously observed in the presence and absence of Hfq at 37 °C.[21]

DsrA·*rpoS* duplex formation occurs in the absence of Hfq at 24 °C

To test whether DsrA·*rpoS* duplex formation is the decisive event or whether the RNA chaperone function of Hfq stimulates,

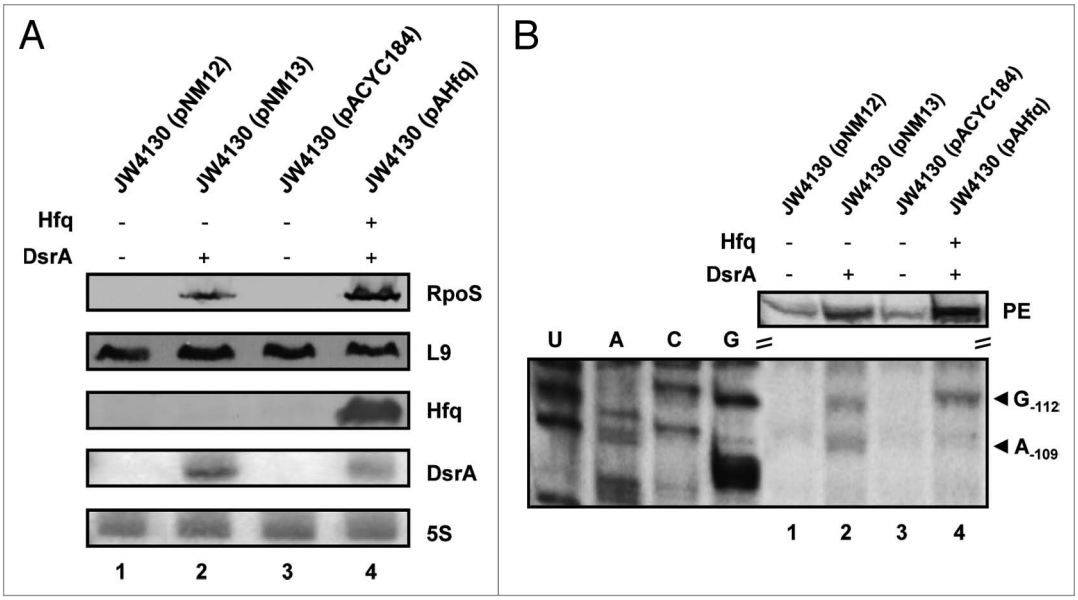

Figure 2. DsrA•*rpoS* duplex formation does not lead to efficient translation of *rpoS* mRNA in the absence of Hfq. (**A**) Immunodetection of RpoS, L9 ribosomal protein (loading control), and Hfq protein and detection of DsrA and 5S rRNA in the *hfq*⁻ strain JW4130 harboring plasmid pNM12 (control; lane 1), pNM13 (encoding DsrA; lane 2), pACYC184 (control; lane 3), and pAHfq (encoding *hfq*; lane 4), respectively. The proteins and RNAs were visualized as described in the legend to **Figure 1**. Only the relevant sections of the immunoblots and autoradiographs are shown. (**B**) Lanes 1–4, primer extension analysis of total RNA isolated from strains JW4130(pNM12), JW4130(pNM13), JW4130(pACYC184), and JW4130(pAHfq), respectively. The primer extension (PE) signals for *rpoS* mRNA isolated from the different strains are shown on top. The RNase III-mediated cleavage signals in *rpoS* mRNA are marked by arrows (G_{-112} and A_{-109}). U, A, C, G, sequencing ladder.

besides its effects on DsrA stability and DsrA•*rpoS* pairing, efficient translation of *rpoS* at low temperature, we next asked whether over-production of DsrA at 24 °C can compensate for the absence of Hfq. Overexpression of *dsrA* resulted in increased levels of DsrA in the *hfq*⁻ when compared with the *hfq*⁺ background, wherein *hfq* was ectopically expressed from plasmid pAHfq and DsrA originated from the endogenous gene, respectively (**Fig. 2A**, lanes 2 and 4). When compared with the *hfq*⁻ and DsrA-overexpressing strain JW4130(pNM13), RpoS synthesis was ~7-fold increased in the *hfq*⁺ strain JW4130(pAHfq) (**Fig. 2A**, lanes 2 and 4).

To test whether the reduced synthesis of RpoS could result from diminished DsrA•*rpoS* duplex formation in the *hfq*⁻ strain JW4130(pNM13), we utilized the reported RNase III specific cleavages of *rpoS* mRNA upon duplex formation with DsrA[21,32] (**Fig. S1**) as a diagnostic marker. The strains JW4130(pMN12), JW4130(pMN13), JW4130(pACYC184), and JW4130(pAHfq) were grown to an OD_{600} of 0.4 at 24 °C, when total RNA was purified from either strain. RNase III cleavage of *rpoS* mRNA was assessed by primer extension using a [^{32}P]-5′end-labeled *rpoS*-specific primer. As anticipated, no RNase III specific cleavage signals were detected at positions -112[21] and -109[32] of *rpoS* mRNA in the absence of DsrA and Hfq (**Fig. 2B**, lanes 1 and 3). Notably, the levels of full-length *rpoS* mRNA were reduced in the absence of Hfq and DsrA in strains JW4130(pMN12) and JW4130(pACYC184), respectively (**Fig. 2B**, lanes 1 and 3; PE). This can be reconciled with a faster turnover of un-translated *rpoS* mRNA in the absence of DsrA, which has been attributed to initial RNase III clevages at positions -94 and -15 in the 5′UTR of *rpoS* mRNA.[21] In support, mutations in *dsrA* that disrupt pairing between DsrA and *rpoS* also destabilize the mRNA.[32] In contrast, alternative cleavage sites at positions -112[21] and -109[32] of *rpoS* mRNA are created upon DsrA·*rpoS* pairing, which contributes to translation and furthermore to stabilization of *rpoS* mRNA.[21]

RNase III dependent cleavage signals at positions -112 and -109 in *rpoS* mRNA were observed in both, the *hfq*⁻ strain as well as in the *hfq*⁺ strain in the presence of DsrA (**Fig. 2B**, lanes 2 and 4), showing that Hfq is not essential for DsrA·*rpoS* duplex formation at low temperatures. When compared with strain JW4130(pNM13), RpoS synthesis was increased in the *hfq*⁺ background JW4130(pAHfq) (**Fig. 2A**, lanes 2 and 4). However, as apparent from the full-length primer extension signals (**Fig. 2B**, lanes 2 and 4), the steady-state levels of *rpoS* mRNA were as well somewhat increased in JW4130(pAHfq) when compared with the *hfq*⁻ and DsrA-overexpressing strain JW4130(pNM13). We interpreted this as showing that the increased synthesis of RpoS in strain JW4130(pAHHfq) leads to stabilization of *rpoS* mRNA.[21,32] Nevertheless, it should be noted that the mRNA levels in JW4130(pAHfq) were only ~1.5-fold higher than in JW4130(pNM13), whereas the RpoS levels were ~7-fold higher in JW4130(pAHfq) than in JW4130(pNM13). In addition, the intensity of the RNase III-dependent cleavage signals at positions -112 and -109 of *rpoS* mRNA indicated more *rpoS* mRNA in complex with DsrA in strain JW4130(pNM13) than in strain JW4130(pAHfq). Hence, these studies suggested that DsrA•*rpoS* duplex formation may not suffice for efficient translation of *rpoS* mRNA, and

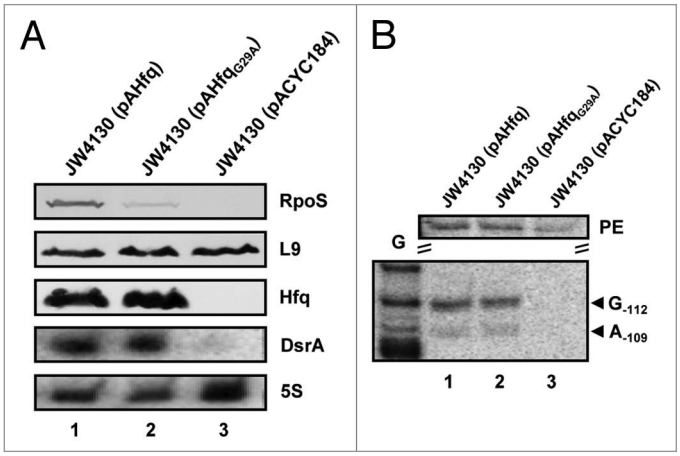

Figure 3. DsrA·*rpoS* duplex formation in the presence of Hfq_{G29A} does not result in efficient translation of *rpoS* mRNA at low temperature. (**A**) Immunodetection of RpoS, ribosomal protein L9, and Hfq in strains JW4130(pAHfq) (lane 1), JW4130(pAHfq$_{G29A}$) (lane 2), and JW4130(pACYC184) (lane 3). DsrA and 5S rRNA were detected as described in the legend to **Fig. 1**. Only the relevant parts of the immunoblots and autoradiographs are shown. (**B**) Lanes 1–3, primer extension analysis of total RNA isolated from strains JW4130(pAHfq), JW4130(pAHfq$_{G29A}$), and JW4130(pACYC184), respectively. The primer extension (PE) signals for *rpoS* mRNA isolated from the different strains are shown on top. The RNase III-mediated cleavage signals in *rpoS* mRNA are marked by arrows (G_{-112} and A_{-109}). G, G sequencing ladder.

that Hfq, besides stabilizing DsrA[4,18,24] and facilitating pairing between both RNAs,[25] positively affects the translatability of *rpoS* mRNA at low temperature.

DsrA·*rpoS* duplex formation is not sufficient for translation initiation of *rpoS* mRNA

To further study whether Hfq is per se stimulating efficient translation at low temperatures, we made use of a Hfq mutant deficient in binding to the A-rich motifs present in the *rpoS* leader, but proficient in DsrA binding. The distal face of the Hfq hexamer harbors six tripartite binding motifs that consist of an adenosine-specific site (A-site), a purine nucleotide selective site (R-site), and a sequence-non-discriminating RNA entrance/exit site (E-site).[11] Common to all binding sites is the coordination of the adenosine ribose 2'-OH by the carbonyl-group of Gly-29.[33] This coordination was reported to likely convey selectivity for RNA over DNA for this binding site.[11] Gly-29 adopts main-chain conformation allowing only for residues, which do not bare a side-chain. This suggested that any substitution of Gly-29 will dramatically reduce the capacity of Hfq to bind an adenosine ligand in the R-site. We therefore constructed a hfq_{G29A} mutant allele with the aim to reduce binding of Hfq_{G29A} to the A-rich motifs present in the *rpoS* leader but capable to bind to DsrA. As anticipated, purified Hfq_{G29A} did not bind to the $(AAN)_4$ repeat (**Fig. S2**), but retained the ability to bind to DsrA (**Fig. S3**). Accordingly, Hfq_{G29A} stabilized DsrA in vivo as the levels of the sRNA were comparable in the presence of Hfq_{wt} and Hfq_{G29A} (**Fig. 3A**, lanes 1 and 2).

Next, we tested whether the apparent deficiency of Hfq_{G29A} in binding to the $(AAN)_4$ motif affects DsrA·*rpoS* duplex formation at 24 °C by assessing RNase III processing of *rpoS* mRNA in strains JW4130(pAHfq), JW4130(pAHfq$_{G29A}$), and JW4130(pACYC184), respectively. In the absence of Hfq/DsrA, the *rpoS* leader was not cleaved at positions -112/-109 in strain JW4130(pACYC184) (**Fig. 3B**, lane 3). The intensities of the RNase III-dependent cleavage signals obtained in strains JW4130(pAHfq) and JW4130(pAHfq$_{G29A}$) were comparable (**Fig. 3B**, lanes 1 and 2), showing that DsrA·*rpoS* duplex formation occurs in the presence of Hfq_{G29A} under these conditions at low temperature. Nevertheless, despite almost equal steady-state levels of full-length *rpoS* mRNA (**Fig. 3B**, lanes 1 and 2; PE) in JW4130(pAHfq) and JW4130(pAHfq$_{G29A}$), RpoS was hardly synthesized in the presence of Hfq_{G29A} in strain JW4130(pAHfq$_{G29A}$) (**Fig. 3A**, lane 2). This observation suggested that binding of Hfq to the *rpoS* leader may, in addition to accelerating DsrA·*rpoS* duplex formation,[23,25] restructure the *rpoS* mRNA into a translationally competent form.

To support this hypothesis, structural probing with RNase T1 was performed in vitro on DsrA·*rpoS* duplexes in the absence of Hfq and in the presence of Hfq_{wt} or Hfq_{G29A} protein. In vitro synthesized full-length *rpoS* mRNA was first annealed to full-length DsrA and a [^{32}P]-5′-end labeled primer. Upon cooling to 24 °C, the annealing mix was incubated for 10 min in the absence of Hfq and in the presence of Hfq_{wt} or Hfq_{G29A}, followed by incubation with RNase T1. Next, reverse transcriptase was added to map the T1-dependent cleavage signals. Most obvious was the difference in the cleavage pattern observed in the presence and absence of Hfq_{wt} within and adjacent to the *rpoS* start codon. In the absence of Hfq_{wt} (**Fig. 4**, lane 2) cleavage at position G+3, G+5, and G+9 was hardly obvious. In addition, pronounced stop signals appeared at position A+1 and A+8. In contrast, in the presence of $Hfq_{wt,}$ G+3, G+5, and G+9 were accessible for RNase T1 and the stop signals were not observed (**Fig. 4**, lane 3). Therefore, the reduced accessibility of the start codon for productive interactions with initiator tRNA could explain why *rpoS* mRNA is not efficiently translated at 24 °C in the absence of Hfq but presence of DsrA (**Fig. 2A**, lane 2). Moreover, as G+3, G+5, and G+9 were protected in the absence of Hfq_{wt}, whereas the G+15, G+18, and G+22 were accessible for RNase T1, the cleavage pattern indicated the presence of a local secondary structure including the G+3 of the start codon and sequences immediately downstream (**Fig. S4**). Thus, in addition to the reduced accessibility of the start codon, the local secondary structure could as well interfere with ribosome binding in the absence of Hfq. At present, we can also not exclude Hfq-induced conformational changes further upstream, which might impact on *rpoS* translation. Although RNase III cleavage of the DsrA·*rpoS* duplex was omitted in these structural probing experiments, it seems less likely that cleavage at positions -112[21] and -109[32] in *rpoS* mRNA would affect this local secondary structure. Taken together, these results would support the notion that DsrA·*rpoS* duplex formation is not sufficient for efficient ribosome loading on *rpoS* mRNA, and that restructuring by Hfq is also required to convert *rpoS* mRNA into a translationally competent form. Even though the accessibility of G+3, G+5, and G+9 for RNase T1 increased as well in the presence of Hfq_{G29A}, deprotection was not as pronounced as with Hfq_{wt}. In addition, in contrast to Hfq_{wt}, the stop signal at A+8 was maintained in the presence of Hfq_{G29A}

(**Fig. 4**, lane 4). Thus, the lack of Hfq_{G29A} binding to the $(AAN)_4$ motif in the *rpoS* leader could lead to translationally incompetent populations of *rpoS* mRNA, which, in turn, could explain its deficiency in *rpoS* translation (**Fig. 3A**, lane 2).

CsdA does not rescue the deficiency of Hfq_{G29A}

We have previously shown that the CsdA helicase is required for in vivo DsrA·*rpoS* duplex formation at 24 °C, and have hypothesized that opening of the iss in *rpoS* by CsdA at low temperature is required for DsrA·*rpoS* duplex formation.[20] We next asked whether overexpression of *csdA* could also rescue the defect of Hfq_{G29A} in translation initiation of *rpoS* at low temperature. A plasmid borne copy of *csdA* was co-expressed in strain JW4130 in the presence of Hfq_{G29A} and of Hfq_{wt}, respectively. The cells were grown at 24 °C to an OD_{600} of 0.4 when *csdA* expression was co-induced with the respective *hfq* gene/allele. Immunodetection of RpoS revealed no increase of translation in the presence of Hfq_{G29A}, whereas in the presence of Hfq_{wt}, synthesis of RpoS slightly increased (**Fig. 5**). Thus, while CsdA is required for DsrA·*rpoS* duplex formation,[20] it apparently cannot rescue the defect of Hfq_{G29A} in terms of converting *rpoS* mRNA into a translationally compentent form, which assigns distinct roles to CsdA and Hfq toward *rpoS* translation.

Conclusions

In the context of all work performed on the DsrA/*rpoS* system, these studies add another facet to Hfq function in translational activation of *rpoS* mRNA. They suggest that DsrA·*rpoS* duplex formation, albeit clearly stimulating, is not the ultimate event that results in efficient translation initiation of *rpoS* mRNA. Besides its function in stabilization of DsrA,[4,18] and in accelerating annealing between DsrA and *rpoS*,[23,25] Hfq is apparently also required to restructure the rbs of *rpoS* mRNA before or upon DsrA·*rpoS* duplex formation to permit efficient ribosome loading (**Fig. 6**). Soper et al.[23] have put forward a model wherein Hfq remains bound to the *rpoS* leader after DsrA·*rpoS* annealing. In light of our results, it seems feasible that lingering of Hfq at the A-rich sites in the *rpoS* leader brings about efficient ribosome loading on *rpoS* mRNA by inducing these structural changes. Although some studies indicated an association of Hfq with the translational machinery,[34,35] recent studies failed to reveal an interaction of Hfq with ribosomes or with ribosomal protein S1,[36] the latter of which is required for translation initiation of structured mRNAs.[37,38] Although other interaction partners of Hfq can at present not be excluded, the RNA chaperone function of Hfq might suffice to present the mRNA in a conformation readily accessible for the 30S ribosome.

Materials and Methods

Bacterial strains and plasmids

The *E. coli hfq*⁻ strain JW4130 has been described.[39] The cells were grown in Luria-Bertani (LB) medium[40] at 24 °C supplemented with ampicillin (100 μg/ml), kanamycin (25 μg/ml), or chloramphenicol (20 μg/ml) where appropriate to maintain plasmids.

The plasmid pAHfq[13] encoding Hfq_{wt} protein, plasmids pNM12 (empty vector) and pNM13[28] used for *dsrA* overexpression,

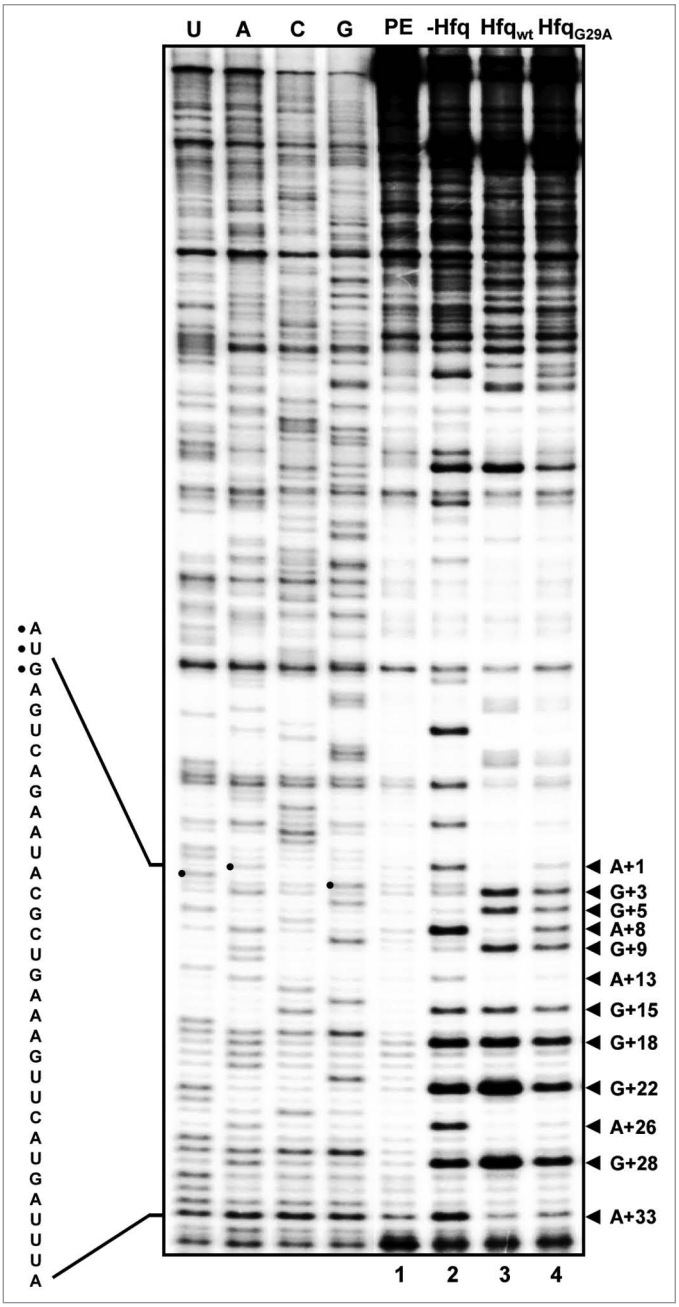

Figure 4. Structural probing of the translation initiation region of *rpoS* mRNA in complex with DsrA in the absence of Hfq and in the presence of Hfq_{wt} or Hfq_{G29A}. First, *rpoS* mRNA was annealed to DsrA and to the [^{32}P]-5′-end labeled primer. The annealing mix was cooled to 24 °C and divided into three parts followed by incubation for 10 min in the absence and presence of Hfq_{wt} and Hfq_{G29A}, respectively. Then RNase T1 was added for 10 min followed by primer extension with AMV reverse transcriptase at 45 °C. The primer extension products were separated on a 8% polyacrylamide-8M urea gel and visualized by a PhosphorImager (Molecular Dynamics). The sequence of the immediate coding region is shown at the left. The start codon is marked by dots. RNase T1 cleavage 3′ of G residues and stop signals of the reverse transcriptase at A residues are indicated at the right. The numbering is given with regard to the A of the start codon (+1). Lane 1, primer extension without RNase T1 digestion. Lane 2–4, structural probing with RNase T1 in the absence of Hfq, in the presence of Hfq_{wt} and in the presence of Hfq_{G29A}, respectively. U, A, C, G, sequencing ladder.

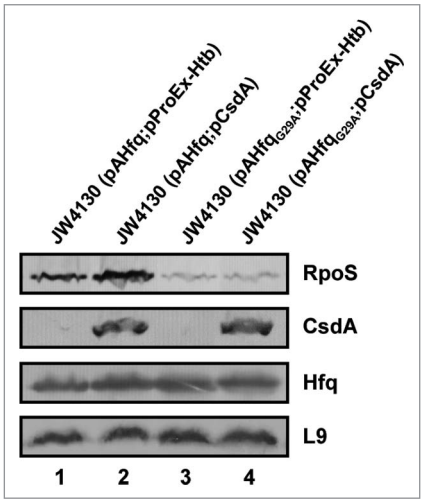

Figure 5. Overexpression of *csdA* does not rescue *rpoS* translation in the presence of Hfq_{G29A}. Immunodetection of RpoS, CsdA, Hfq, and ribosomal protein L9 in strains JW4130(pAHfq;pProEx-Htb) (lane 1), JW4130(pAHfq;pCsdA) (lane 2), JW4130(pAHfq$_{G29A}$; pProEx-Htb) (lane 3), and JW4130(pAHfq$_{G29A}$;pCsdA) (lane 4), respectively. The proteins were visualized as described in the legend to **Figure 1**. Only the relevant sections of the immunoblots are shown.

and plasmids pProEx-Htb (empty vector; Invitrogen) and pCsdA[41] for overexpression of *csdA* have been described.

Construction of pAHfq$_{G29A}$

To introduce the G29A mutation into the *hfq* sequence, a DNA fragment was generated by means of PCR using the *hfq* forward primer (5′-GC*TCTAGA*AA TATAATAGTT TAACTTTAAG AAGGAGATAT ACATATGGCT AAGGGGCAAT CTTTACAAGA TCCGTTCCT-3′), containing an *Xba*I site (italics), and the reverse primer (5′-AGACTCGATT TGCCCTTGCA GCTTAATAGC ATTCACCAAA-3′). The DNA fragment was used in a second PCR as forward primer together with the *hfq* reverse primer (5′-G*GAATTC*CCG TGTAAAAAAA CAGCCCGAAA C-3′), containing an *EcoRI* site (italics), to generate the full hfq_{G29A} gene. The PCR product was cleaved with *Xba*I and *EcoRI* and ligated into the corresponding sites of plasmid pUC19 (New England Biolabs). From the resulting plasmid pUHfq$_{G29A}$, the hfq_{G29A} allele was re-isolated as a *Pvu*II fragment, and ligated into the *Eco*RV/*Nru*I sites of plasmid pACYC184 (New England Biolabs), yielding plasmid pAHfq$_{G29A}$.

Western blot analyses

The cellular levels of Hfq_{wt}, Hfq_{G29A}, L9/L14 ribosomal protein(s), RpoS, and CsdA were determined by quantitative immunoblotting. For the experiments shown in **Fig. 1 and Fig. 3A**, the *E. coli* strains JW4130(pACYC184), JW4130(pAHfq), and JW4130(pAHfq$_{G29A}$) were grown at 24 °C or 37 °C in LB medium supplemented with appropriate antibiotics to maintain plasmids. For the experiment shown in **Fig. 2A**, the *E. coli* strains JW4130(pNM12), JW4130(pNM13), JW4130(pACYC184), and JW4130(pAHfq) were grown at 24 °C in LB medium supplemented with 0.4% arabinose to induce expression of *dsrA* from the arabinose promoter of plasmid pNM13. All cells were grown to an OD_{600} of ~0.4 when equal amounts of cells were withdrawn and boiled in protein sample buffer. For *csdA* overexpression (**Fig. 5**), the *E. coli hfq*$^-$ strain JW4130-bearing plasmid pAHfq or pAHfq$_{G29A}$ was co-transformed with either the control plasmid pProEx-HTb (Invitrogen) or with plasmid pCsdA. The strains were grown in LB medium at 24 °C to an OD_{600} of 0.4 when expression of *csdA* was induced by addition of 1 mM IPTG (final concentration). After 1 h of growth equal amounts of the cells were withdrawn and boiled in protein sample buffer. Equal amounts of total protein were separated on 12% SDS–polyacrylamide gels and blotted onto a nitrocellulose membrane (GE-healthcare). The blots were blocked with 5% dry milk in TBS buffer, and then probed with anti-Hfq (lab. stock) anti-L9/-L14 (lab. stock), anti-His-tag (Sigma), or anti-RpoS (Santa Cruz Biotech) antibodies. The antibody–antigen complexes were visualized with alkaline–phosphatase conjugated secondary antibodies (Sigma) using the chromogenic substrates nitro blue tetrazolium chloride (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP).

Northern blot analyses

The steady-state levels of DsrA were determined by northern blot analysis using 10 µg of total RNA. RNA extraction was performed by the Trizol method (Ambion). The RNA samples were denatured for 5 min at 85 °C in RNA loading dye, separated on 8% polyacrylamide-8 M urea gels, and then transferred to a nylon membrane (Amersham Hybond-N) by electroblotting. The RNA was cross-linked to the membrane by exposure to UV light. The membrane was hybridized with DsrA-specific [γ-^{32}P]-5′-end-labeled (Amersham Pharmacia Biotech) oligonucleotide (5′-TCGTTACACC AGGAAATCTG ATGT-3′) or 5S-rRNA-specific [^{32}P]-5′-end labeled oligonucleotide (5′-GGTGGGACCA CCGCGCTACG GCCGCCAGGC-3′). Hybridization signals were visualized using a PhosphorImager (Molecular Dynamics).

Primer extension assays

For the experiment shown in **Fig. 2B**, the *E. coli* strains JW4130(pNM12), JW4130(pNM13), JW4130(pACYC184),and JW4130(pAHfq) were grown at 24 °C in LB medium supplemented with 0.4% arabinose to induce expression of *dsrA* from the arabinose promoter of plasmid pNM13. For the experiment shown in **Fig. 3B**, the *E. coli hfq*$^-$ strain JW4130 bearing plasmids pACYC184, pAHfq$_{G29A}$, and pAHfq, respectively, was grown at 24 °C. At an OD_{600} of 0.4, samples for RNA isolation and for western blot analysis were withdrawn. Isolation of total RNA was performed using the Trizol method (Ambion). The primer extension analysis, used to detect the RNase III-specific cleavage at position G_{-112}/A_{-109} in *rpoS* mRNA, was performed as described.[21] In brief, two units of AMV reverse transcriptase (Promega) were used for 20 µg of purified total RNA primed with the *rpoS*-specific [^{32}P]-5′-end labeled oligonucleotide (5′-TCCGTTCTCA TCAAATTCCG CATC-3′). The extension products along with a sequencing ladder, which was prepared using the 5′-segment (nt -564 to +188) of *rpoS* mRNA as a template, were resolved on a 8% polyacrylamide-8M urea gel. The resulting signals were visualized by a PhosphorImager (Molecular Dynamics).

RNA preparation for in vitro studies

To prepare full-length *rpoS* mRNA, plasmid pUrpoS16[13] digested with *EcoRI* was used as template for in vitro transcription

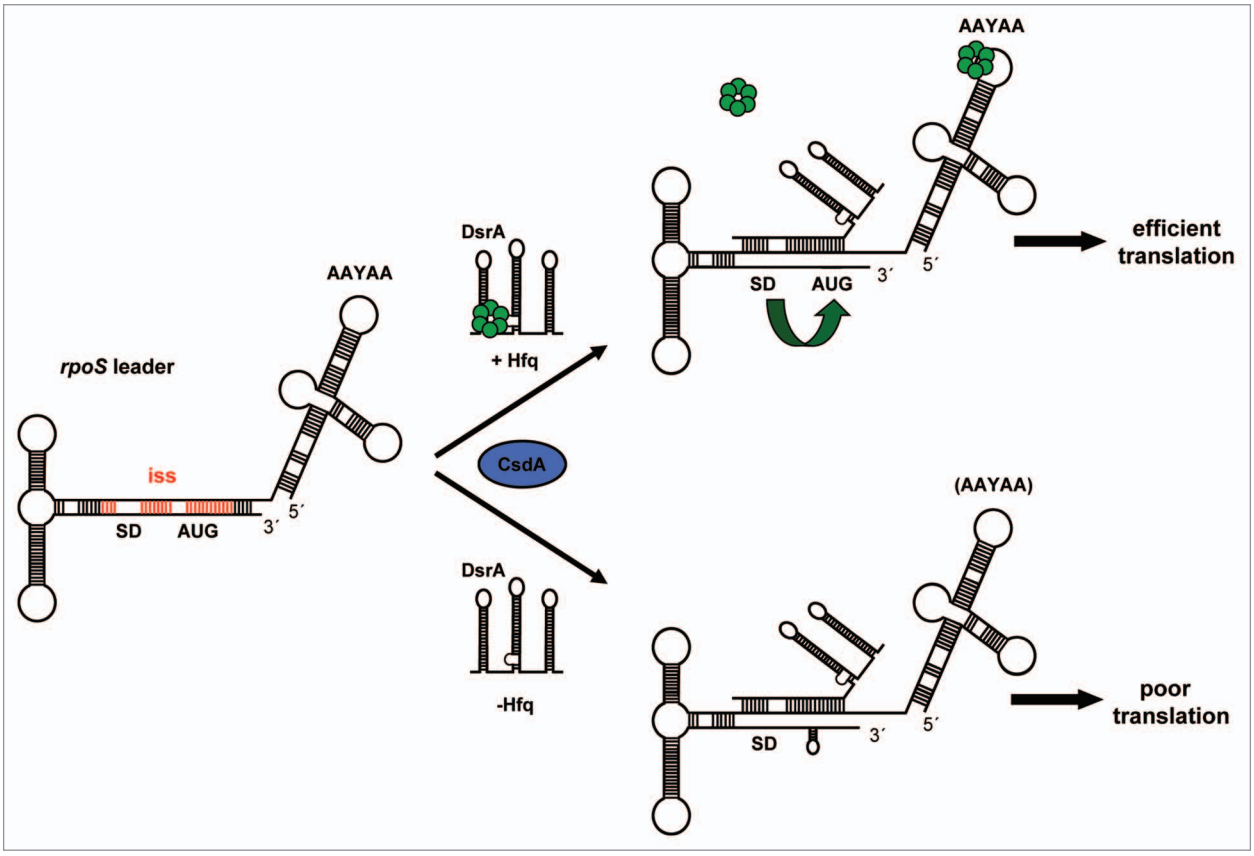

Figure 6. Working model for translational activation of *rpoS* mRNA at low temperature. The rbs of *rpoS* mRNA is masked by an iss (in red). In the presence of Hfq (upper scheme) the sRNA DsrA is bound and stabilized by Hfq.[4,18,24] CsdA (blue oval) is required for DsrA·*rpoS* duplex formation,[20] which leads to opening of the iss and in displacement of Hfq from DsrA.[26] Hfq bound to A-rich segments in the *rpoS* leader (AAYAA) promotes DsrA·*rpoS* annealing[24] and restructuring of *rpoS* mRNA into a translationally competent conformer (green bent arrow), which, in turn, permits efficient *rpoS* translation. In the absence of Hfq (lower scheme), DsrA·*rpoS* duplex formation occurs when *dsrA* is overexpressed. However, in the absence of Hfq, *rpoS* is poorly translated because the local secondary structure within the immediate coding region is not efficiently resolved. The Shine-Dalgarno (SD) sequence and start codon (AUG) in *rpoS* mRNA are highlighted, Hfq is shown in green.

with T7 RNA polymerase (Promega). Preparation of DsrA RNA was performed as described.[21] The run-off transcripts were purified on 8% polyacrylamide-8M urea gels following standard procedures. The RNA concentration was determined by measuring the A_{260}.

RNaseT1 probing

Hfq_{wt} and Hfq_{G29A} proteins were purified from strain AM111F′[42] harboring plasmids pUH5[43] (Hfq_{wt}) and pUHfq_{G29A} (Hfq_{G29A}), respectively, as described.[44] 1.8 pmol of full-length *rpoS* mRNA, 3.6 pmol of DsrA RNA, and 2 pmol [^{32}P]-5′-end labeled primer (5′-TTTTCGTCAA AAACCTCAAC TCCG-3′; corresponds to nucleotides +59 to +83 in the *rpoS* gene) were annealed by incubation at 85 °C for 3 min in ddH_20. Then, the complexes were cooled to 24 °C and concentrated AMV buffer (Promega) was added. The annealing mix was divided into three parts. One aliquot was further incubated in the absence of Hfq, whereas the other two aliquots were incubated in the presence of 5 pmol Hfq_{wt} and 5 pmol Hfq_{G29A}, respectively for 10 min at 24 °C. Then, 2 U of RNase T1 dilution (Ambion) was added to the reactions. The samples were further incubated for 5 min at 24 °C, before they were heated to 45 °C. Then, dNTPs and reverse transcriptase (2 U AMV) were added. The primer extension products along with a sequencing ladder, which was prepared using the 5′-segment (nt -564 to +188) of *rpoS* mRNA as a template, were resolved on 8% polyacrylamide-8M urea gels. The resulting signals were visualized by a PhosphorImager (Molecular Dynamics).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/rnabiology/article/27100/

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