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Requirement of the CsdA DEAD-box helicase for low temperature riboregulation of *rpoS* mRNA

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The ribosome binding site of *Escherichia coli rpoS* mRNA, encoding the stationary sigma-factor RpoS, is sequestered by an inhibitory stem-loop structure (iss). Translational activation of *rpoS* mRNA at low temperature and during exponential growth includes Hfq-facilitated duplex formation between *rpoS* and the small regulatory RNA DsrA as well as a concomitant re-direction of RNase III cleavage in the 5′-untranslated region of *rpoS* upon DsrA·*rpoS* annealing. In this way, DsrA-mediated regulation does not only activate *rpoS* translation by disrupting the inhibitory secondary structure but also stabilizes the *rpoS* transcript. Although minor structural changes by Hfq have been observed in *rpoS* mRNA, a prevailing question concerns unfolding of the *iss* in *rpoS* at low growth temperature. Here, we have identified the DEAD-box helicase CsdA as an ancillary factor required for low temperature activation of RpoS synthesis by DsrA. The lack of RpoS synthesis observed in the *csdA* mutant strain at low growth temperature could be attributed to a lack of duplex formation between *rpoS* and DsrA, showing that at low temperature the sole action of Hfq is not sufficient to permit DsrA·*rpoS* annealing. An interactome study has previously indicated an association between Hfq and CsdA. However, immunological assays did not reveal a physical interaction between Hfq and CsdA. These findings add to a model, wherein Hfq binds upstream of the *rpoS iss* and presents DsrA in a conformation receptive to annealing. Melting of the *iss* by CsdA may then permit DsrA·*rpoS* duplex formation, and consequently *rpoS* translation.

Introduction

Bacterial trans-acting small regulatory RNAs (sRNA) are generally synthesized in response to certain growth or stress conditions.[1] For instance, *E. coli* sRNAs are known to be synthesized during iron depletion, oxidative stress, outer membrane stress, elevated glycine levels, changes in glucose concentration and elevated glucose-phosphate levels.[2-6]

The majority of the hitherto characterized sRNAs in *E. coli* and several other Bacteria act as negative regulators by preventing ribosome loading onto the mRNA through base-pairing with, or in the vicinity of the ribosome binding site (rbs). As a result, the respective mRNA is prone to rapid decay.[7-9] Variations of negative regulation by sRNAs exerted distantly from the rbs can further include binding to and obstructing of an upstream ribosome loading site,[10] obstruction of a C/A rich element[11] as well as binding to the coding region.[12] In addition, indirect translational silencing by preventing translation of an upstream reading frame to which the target gene is translationally coupled has also been reported.[13]

Positive regulation by sRNAs appears to be less frequent. In some cases the sRNAs act by an "anti-antisense" mechanism and open up intramolecular inhibitory stem-loop structures (iss), which block ribosome binding.[14-16] Examples include translation activation of (1) *hla* mRNA by RNAIII in *S. aureus*,[16] (2) *vca0939* mRNA by Qrr1 in *V. cholerae*,[17] (3) *glmS* mRNA by GlmZ,[18] (4) *shiA* mRNA by RyhB[19] and (5) *rpoS* mRNA by DsrA,[20] RprA[21] or ArcZ,[22] with the three latter regulatory events occurring in *E. coli.*

A well studied model system is the translational activation of *rpoS* mRNA, encoding the stationary phase sigma-factor, RpoS, by the sRNA DsrA. DsrA is predominantly transcribed at low growth temperature (25°C) and its stability decreases with increasing temperature.[23] At low growth temperature, the DsrA·*rpoS* interaction was shown to relieve an iss that impedes ribosome access to the *rpoS* rbs.[20] Later on it became apparent that the hexameric RNA chaperone Hfq stimulates DsrA-mediated activation of *rpoS* translation.[24] More recently, the double strand-specific nuclease RNase III was shown to cleave *rpoS* mRNA upstream of the rbs, which in turn leads to a rapid inactivation of the mRNA. In contrast, base-pairing of DsrA with the *rpoS* leader creates an alternative RNase III cleavage site within the DsrA·*rpoS* duplex that prevents reuse of DsrA.[25]

The sRNA DsrA binds to Hfq on the positively charged proximal site,[26] whereas poly(A) oligonucleotides bind with high affinity to the distal site.[26] A structural study by Link et al.[27] suggested that the distal binding site can accommodate $(A\text{-}R\text{-}N)_i$ or $(A\text{-}R\text{-}N\text{-}N)_i$ tracts, with R being a purine nucleotide and N being any nucleotide. Binding of different RNA sequences to the distal site could thus explain why Hfq can interact with many

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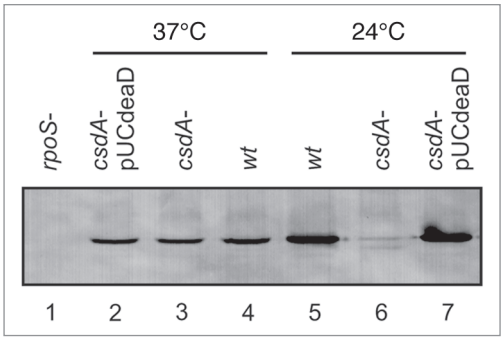

Figure 1. Steady-state levels of RpoS determined by quantitative western-blotting in the *wt* strain and the isogenic *csdA* mutant. The *E. coli* strains WJW45 (*wt*), the isogenic *csdA* deficient strain WJW45ΔcsdA (*csdA*-) as well as WJW45ΔcsdA harbouring plasmid pUCdeaD[38] (*csdA*-/pUCdeaD) were grown to early log phase (OD_{600} of 0.4) either at 37°C (lane 2–4) or at 24°C (lane 5–7), respectively. Cell extracts of the *rpoS* mutant strain RH90,[53] served as a negative control (lane 1). Equal amounts of total cellular proteins were loaded in each lane of the SDS-polyacrylamide gel. The RpoS protein was detected by immunological means as described in Materials and Methods. Only the relevant section of the immunoblot is shown.

different RNA ligands.[27] On the other hand, the dedicated RNA binding surfaces on either site of the Hfq-hexamer could allow to bring two RNAs together in a pair-wise fashion, which can be reconciled with several studies wherein Hfq was shown to stimulate annealing between two RNA ligands.[28-33] It was shown that high affinity binding of Hfq to the *rpoS* leader depends on an A_6 segment and on $AAN_{(4)}$ repeats situated upstream of the DsrA·*rpoS* base-pairing site.[30] *RpoS* leaders containing these sequences formed stable ternary complexes with Hfq and DsrA. In a recent study Soper et al.[22] provided evidence that Hfq in fact increases the stability of the DsrA-*rpoS* complex by binding to this upstream A-rich regions in the leader rather than the kinetics of DsrA·*rpoS* duplex formation. Even though Hfq was shown to induce structural changes in mRNAs targeted by sRNAs,[34] footprinting studies[30,35] did not strongly support the idea that Hfq is sufficient to open up the iss in *rpoS*. In addition, the requirement for Hfq in *rpoS* translation could be bypassed when DsrA was over-produced.[22] Although the spatial positioning of the *rpoS*-Hfq-DsrA complex upstream of the annealing site could bring the interaction sites in close proximity and thereby accelerate DsrA·*rpoS* annealing,[30] it remained puzzling how the *rpoS* iss opens at low temperature to allow anti-antisense annealing between DsrA and *rpoS*.

The *E. coli* CsdA DEAD-box helicase is induced upon a temperature downshift.[36] The CsdA protein has been implicated in ribosome biogenesis,[37-40] in mRNA turnover upon cold-adaptation[41,42] as well as in translation initiation.[43] Most likely, these functions are rooted in the ATP-dependent helicase activity of CsdA, which has been shown to resolve secondary structures in RNA.[36,44,45] Interestingly, an interactome study[46] identified CsdA as one of the ~70 proteins associated with Hfq.

Using the DsrA/*rpoS* model system, we addressed the question, whether CsdA might represent an auxiliary factor involved in translational activation. We show that at low growth temperature synthesis of RpoS requires CsdA and that DsrA·*rpoS* duplex formation does not occur in a *csdA* mutant strain. As no evidence was obtained for a physical interaction between Hfq and CsdA we infer that the CsdA-mediated destabilization of the iss in *rpoS* precedes DsrA·*rpoS* annealing.

Results

CsdA is required for low temperature synthesis of *rpoS* in vivo and stimulates *rpoS* synthesis in vitro. Given that Hfq was found to be associated with the dead box helicase CsdA[46] and that Hfq did not significantly perturb the iss in *rpoS* mRNA,[30,35] we asked whether CsdA might represent an ancillary factor involved in Hfq-mediated translational activation of *rpoS* mRNA by DsrA at low temperature. As shown previously,[23] during exponential growth RpoS synthesis was increased at 24°C when compared to 37°C (**Fig. 1**, lanes 4 and 5). Whereas no significant differences in the RpoS levels were observed at 37°C in both, the *csdA* mutant strain and the wild-type strain (**Fig. 1**, lanes 3 and 4), hardly any RpoS synthesis was observed in the *csdA*- strain at 24°C (**Fig. 1**, lane 6). Ectopic expression of *csdA* in the *csdA*- background did not affect RpoS synthesis at 37°C but restored it at 24°C (**Fig. 1**, lanes 2 and 7).

Next, we tested whether the stimulatory effect of CsdA on DsrA-mediated *rpoS* synthesis at low temperature could be recapitulated in an in vitro translation system (PURESYSTEM) at 24°C. However, at this temperature the in vitro translation system was hardly active and effects of CsdA on *rpoS* mRNA translation were difficult to discern (not shown) despite the presence of Hfq in the PURESYSTEM (**Suppl. Fig. S1**). As CsdA is active at 37°C,[45] we tested whether addition of CsdA would stimulate *rpoS* synthesis in the presence of DsrA at the elevated temperature. As shown in **Figure 2**, the addition of CsdA at a molecular ratio of 1:1 to *rpoS* mRNA led to an ~2-fold increase of RpoS synthesis (lane 6), whereas the same amount of heat-inactivated CsdA (lane 7) resulted in RpoS levels comparable to that obtained in the absence of CsdA (lane 4).

CsdA is required for DsrA·*rpoS* duplex formation. We next asked whether the absence of RpoS synthesis in the *CsdA*- strain at low temperature is attributable to a lack of annealing between *rpoS* mRNA and DsrA. We addressed this question by utilizing RNase III clevage upon *rpoS*·DsrA duplex formation[25] as a diagnostic marker. The *E. coli* strains WJW45 and WJW45Δ*csdA* were grown to an OD_{600} of 0.4 at 24°C, as this temperature leads to a strong induction of DsrA synthesis.[23] Total RNA obtained from either strain was purified and RNase III-dependent cleavage of *rpoS* was assessed by primer extension using a [^{32}P] 5'end-labeled *rpoS*-specific primer. Compared with the wild-type strain, the primer extension signal for the 5'-end of *rpoS* mRNA (i.e., the steady state levels of *rpoS* mRNA) was approximately 40–50% reduced in the *csdA*- strain. Nevertheless, RNase III dependent cleavage in *rpoS* mRNA was not observed in the *csdA*- strain (**Fig. 3**, lane 6) but, as previously shown, occurred in the wild-type strain at G_{-112}[25] (**Fig. 3**,

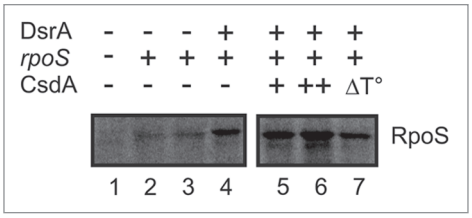

Figure 2. CsdA stimulates *rpoS* synthesis in vitro. 10 pmol of in vitro synthesized full length *rpoS* mRNA were translated using the PURESYSTEM. Translation of *rpoS* mRNA was performed in the absence (lanes 2–3) or in the presence of equimolar amounts of DsrA (lanes 4–7). Lane 1, no mRNA was added to the reaction mixture. Lane 2, equimolar amounts of the sRNA RyhB, which does not stimulate *rpoS* translation, was added. Lane 5 and 6, translation in the presence of 5 (+) and 10 pmol (++) CsdA, respectively. Lane 7, 10 pmol of heat-inactivated CsdA protein (ΔT°) was added to the reaction mixture. The protein samples were resolved on a 12% SDS-polyacrylamide gel. The signals were visualized by a PhosporImager (Molecular Dynamics). The experiment was performed in triplicate. Only the relevant section of one representative autoradiogram is shown. The position of the RpoS is indicated.

lane 5). As the levels of both, DsrA and Hfq, were not grossly altered at 24°C in either strain (**Fig. 3**), we conclude that CsdA is required for DsrA·*rpoS* duplex formation.

Hfq and CsdA do not physically interact. Next, we asked whether Hfq and CsdA interact directly with each other or whether the observed association between both proteins is RNA mediated. To distinguish between these possibilities we used Hfq and CsdA-His_6 proteins purified to homogeneity with and without micrococcal nuclease (MN) treatment and performed Far-western-blot analyses and co-immunoprecipitation studies. For Far-western analysis, the CsdA-His_6 protein treated or left untreated with MN was loaded on a 10% SDS polyacrylamide gel and then transferred onto a nitrocellulose membrane. After denaturation and renaturation, membrane bound CsdA-His_6 was incubated with Hfq, which was treated with MN. Then anti-Hfq-antibodies were used to detect Hfq protein eventually bound to CsdA-His_6. Hfq binding to CsdA-His_6 was only observed when CsdA-His_6 was not treated with MN (**Fig. 4A**, lane 1). However, Hfq binding to CsdA-His_6 was not detected after treatment of both proteins with nuclease (**Fig. 4A**, lane 2). As the anti-Hfq antibody did not recognize CsdA-His_6 (not shown), the Far-western studies suggested that the observed CsdA-His_6-Hfq association is RNA-mediated.

The Far-western studies were verified by co-immunoprecipitation experiments using purified Hfq and CsdA-His_6 proteins either treated or untreated with MN. The proteins were mixed together (see Materials and Methods) and anti-Hfq antibodies bound to "Dynabeads protein G" were used for immunoprecipitation. The protein immunoprecipitates were electrophoresed on a 10% SDS-polyacrylamide gel. Then, a western-blot was performed with anti-His antibodies with the aim to detect CsdA-His_6 eventually co-immunoprecipitated with Hfq. As shown in **Figure 4B**, lane 2, when both, Hfq and CsdA-His_6 were not treated with MN, CsdA-His_6 co-immunoprecipitated with Hfq. As CsdA-His_6 was not immunoprecipitated with the anti-Hfq antibodies bound "Dynabeads protein G" alone (not shown), Hfq was apparently

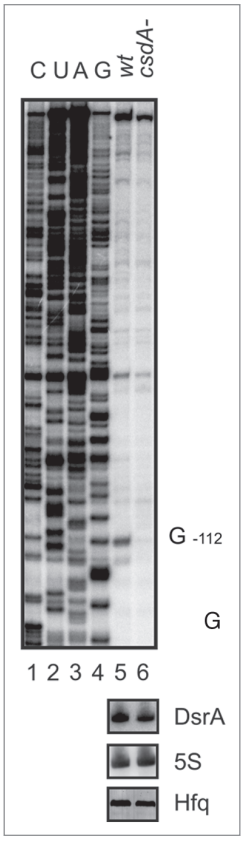

Figure 3. Primer extension analysis of total RNA isolated from the *E. coli* strain WJW45 (*wt*) and the *csdA* deficient strain WJW45ΔcsdA (*csdA*-) grown to an OD_{600} of 0.4 at 24°C. Top: The product of the RNase III cleavage within the *rpoS* leader (position G_{-112}) accumulated only in the wild-type strain (lane 5) and was hardly detectable in the *csdA* deficient strain (lane 6). Lanes 1–4, sequencing ladder. The experiment was performed in triplicate. One representative autoradiograph is shown. Bottom: The DsrA RNA levels and 5S rRNA levels (loading control) of the *wt* and *csdA*-strain grown at 24°C were determined by northern-blot analysis as described in Materials and Methods. The Hfq protein levels in both strains were determined by western-blotting using equal amounts of total protein of the two strains at the time the cells were harvested for preparation of total RNA.

in complex with CsdA-His_6. However, when both proteins were treated with MN, no co-immunoprecipitation of CsdA with Hfq was observed (**Fig. 4B**, lane 3). A reciprocal co-immunoprecipitation experiment (not shown) where anti-His-antibodies bound to Dynabeads protein G were used for immunoprecpitation and anti-Hfq antibodies were used to detect on a western-blot Hfq possibly co-immunoprecipitated with CsdA-His_6 resulted in the same outcome. These studies corroborated the Far-western studies in that the association between Hfq and CsdA does not result from direct protein-protein contacts but that is most likely brought about by RNA(s) associated with the proteins after purification.

Discussion

This study implicates the dead box helicase CsdA in Hfq-mediated low temperature activation of *rpoS* mRNA by DsrA. Soper et al.[22] have recently put forward a model, in which the

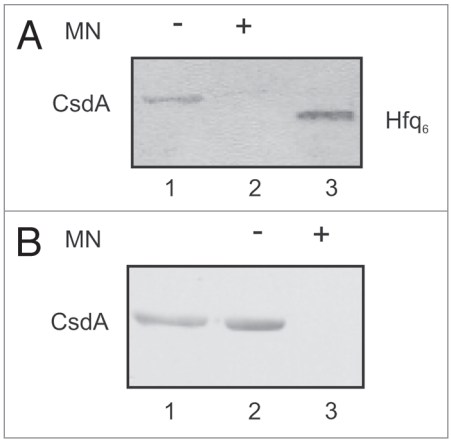

Figure 4. The interaction between CsdA and Hfq is RNA mediated. (A) Far western-blotting does not reveal an interaction between CsdA and Hfq. 25 pmol of CsdA-His_6 (lane 1) or 25 pmol of CsdA-His_6 (lanes 2) treated with micrococcal nuclease (MN) and 25 pmol of Hfq (lane 3, control) were separated by SDS-polyacrylamide gel electrophoresis and then electroblotted onto nitrocellulose membranes. After re-naturation of the proteins the membranes were incubated with Hfq (150 pmol Hfq as hexamer) as described in Materials and Methods. The CsdAHis_6-Hfq complex was visualized by immuno-detection using anti-Hfq antibodies (lane 1), whereas no CsdA-Hfq complex could be detected with anti-Hfq antibodies after treatment with MN (lane 2). Lane 3, Hfq protein (control) was detected with anti-Hfq antibodies. (B) Co-immunoprecipitation assay with protein CsdA-His_6 and Hfq using anti-Hfq antibodies. Lane 1, CsdA-His_6 (10 pmol) was loaded as a positive control for the anti-His antibodies. 25 pmol of purified hexameric Hfq were incubated together with 50 pmol CsdA-His_6 (lane 2) or 50 pmol CsdA-His_6 treated with MN (lane 3) followed by immunoprecipitation using anti-Hfq antibodies bound to the Dynabeads (see Materials and Methods). The co-immunoprecipitated CsdA-His_6 was detected on the western-blot with anti-His antibodies. Only the relevant sections of the immunoblots are shown.

Hfq-DsrA complex binds to A-rich segments in the *rpoS* leader, i.e., upstream of the DsrA·*rpoS* annealing site (**Fig. 5**). Although structural probing of *rpoS* mRNA upon incubation with Hfq suggested a less tightly folded iss,[30] it remained uncertain whether these perturbations are sufficient for relieving the iss, which is in turn required for DsrA·*rpoS* annealing. Based on our observations, we hypothesize that CsdA is involved in opening the iss at low temperature and that DsrA bound by Hfq at the A-rich segment(s) in the *rpoS* leader can then base-pair with *rpoS* opposite of the rbs (**Fig. 5**). Clearly, as opening of stem-loop 1 of DsrA appears to be required for DsrA·*rpoS* duplex formation,[35] it cannot be excluded that CsdA also acts by unfolding DsrA. Nevertheless, Hfq was shown to induce conformational changes in DsrA[31] and Hfq at least partially destabilized stem-loop 1 in DsrA.[35] In addition, NMR studies of a complex between a C-terminally truncated Hfq protein (aa 1–65) and a 34 nucleotide DsrA RNA revealed no imino-shifts arising from nucleotide base-pairing, also indicating that stem-loop 2 is unfolded upon Hfq binding (Beich-Frandsen M, et al. unpublished). Thus, it is conceivable the *rpoS*-Hfq-DsrA complex situated in the leader presents DsrA in a manner that allows partial duplex formation opposite of the rbs of *rpoS*. It is interesting to note that the minimal base-pairing region required for the interaction between the sRNA SgrS with its target mRNA *ptgS* was recently determined with 14 nucleotides.[47] Thus, the proximal base-pairing nucleotides upstream of the Hfq binding site in DsrA[35] could suffice to initiate duplex formation opposite of the *rpoS* rbs. This event may dislodge DsrA from Hfq (**Fig. 5**). In addition, it seems plausible that the action of CsdA contributes to recycling of Hfq from the leader region upon DsrA is released (**Fig. 5**). As CsdA is known to function as a bidirectional helicase[44] this might occur through unwinding of the 5'leader. Alternatively, it is also possible that CsdA acts by displacement of Hfq without unwinding the leader, a function that has been attributed to other helicases.[48] In any case, the involvement of CsdA in low temperature activation of *rpoS* adds a new actor to riboregulation. This observation could even have implications in understanding the mode of action of sRNAs in bacteria that lack apparent Hfq homologues but posses helicases.

Several proteins including RNA polymerase, ribosomal protein S1,[49] RNase E,[50] polyA-polymerase (PAP I) and polynucleotide-phosphorylase (PNPase)[51] have been found in complex with Hfq. However, follow up studies did neither reveal a physical interaction between Hfq and ribosomal protein S1,[52] nor with RNase E.[53] Most likely, these complexes are as infered for the CsdA-Hfq association (**Fig. 4**) RNA-mediated and result from the spatial association of the transcriptional, translational and RNA decay machineries.

Materials and Methods

Bacterial strains and growth conditions. The *E. coli* strains WJW45, the isogenic *csdA* deletion strain WJW45Δ*csdA*[37] the *rpoS* mutant strain RH 90,[54] as well as plasmid pUCdeaD[38] have been previously described. The bacterial strains were grown in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml) or tetracycline (20 μg/ml) where appropriate.

Western-blot analysis. Samples of the bacterial cultures grown in LB medium at 24°C or at 37°C were harvested in early logarithmic growth phase (OD_{600} of 0.4), pelleted and boiled in protein sample buffer. Equal amounts of total protein were separated on 12% SDS-polyacrylamide gels, blotted onto a nitrocellulose membrane and probed with anti-RpoS antibodies (provided by F. Norel, Pasteur Institute, Paris), or specific antibodies against Hfq (Pineda, Berlin). The antibody-antigen complexes were visualized with alkaline-phosphatase conjugated secondary antibodies using the chromogenic substrates NBT and BCIP as described.[31]

RNA preparation for in vitro studies. For full length *rpoS* mRNA synthesis, the plasmid pUrpos16,[31] cleaved with *Eco*RI was used as template for in vitro transcription with T7 RNA polymerase (Fermentas). Synthesis of DsrA and RhyB RNA was performed as described.[25,31] The run-off transcripts were further gel-purified following standard procedures and the RNA concentration was determined by measuring the A_{260}.

In vitro translation assay. The PURESYSTEM classic II (PURE2030C; Wako Chemicals GmbH, Germany) was used for the in vitro translation assays according to the manufacturer's instructions. The 25 μl reaction contained 10 pmol of in vitro transcribed full length *rpoS* mRNA template, 10 pmol

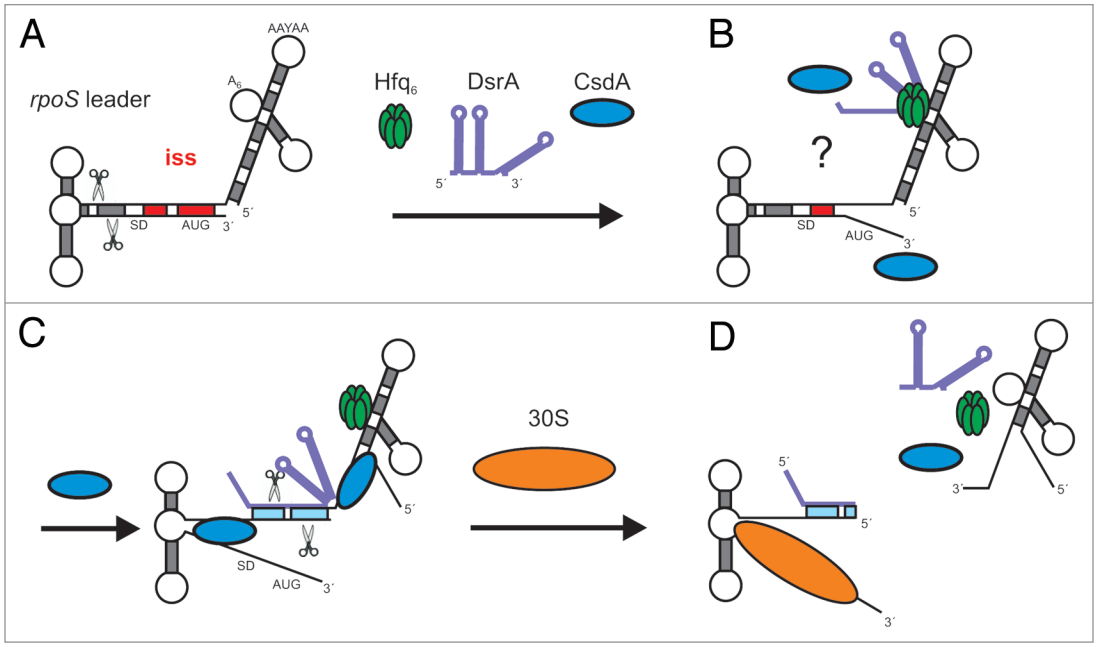

Figure 5. Model for translational activation of *rpoS* mRNA by DsrA, Hfq and CsdA. (A) At low temperature and during exponential growth RNase III cleavage occurs in the *rpoS* leader, which primes rapid decay of the mRNA.[25] As DsrA·*rpoS* annealing (Fig. 3) and consequently *rpoS* translation (Fig. 1) did not occur in the *csdA*- strain, intramolecular cleavage in the *rpoS* leader most likely accounts for the reduced steady state levels of *rpoS* mRNA in the latter when compared to the wild-type strain (see Fig. 3). (B) Hfq-DsrA binding to A-rich segments (A6 and/or $AAN_{(4)}$[30]) in the *rpoS* leader occurs at low temperature when DsrA is expressed[30] (for clarity only one possible Hfq binding site is occupied by the Hfq-DsrA complex). CsdA aids in melting the iss and perhaps in unfolding of DsrA. (C) Annealing between DsrA and the *rpoS* mRNA occurs opposite of the rbs. (D) Translation of *rpoS* ensues and intermolecular RNase III cleavage of the DsrA·*rpoS* duplex (at G_{-112} in *rpoS* mRNA) prevents reuse of DsrA. Hfq is eventually recycled as a consequence of DsrA·*rpoS* base-pairing and/or by CsdA action. The segment of the iss is in red. Scissors signify RNase III cleavage. All other components are denoted.

DsrA or RyhB (internal control; **Fig. 2**, lane 2), 10 pmol L-[^{35}S]-Methionine (Hartmann Analytic, Germany) and two different concentrations of CsdA as indicated in the legend to **Figure 2**. As an additional mock control CsdA was heat-inactivated (**Fig. 2**, lane 7) by boiling for 10 min at 100°C and subsequent cooling on ice before use. The components were mixed together on ice and incubated for 2 hour at 37°C. The in vitro translation reactions were terminated with four volumes of acetone, kept on ice and the proteins were precipitated by centrifugation (16,000 g, 15 min, 4°C), and then separated on 12% SDS-polyacrylamide gel. The signals were visualized by a PhosporImager (Molecular Dynamics) and quantified by ImageQuant software.

RNA isolation and primer extension analysis. The *E. coli* strains WJW45 and WJW45ΔcsdA were grown to an OD_{600} of 0.4 at 24°C to induce low temperature DsrA synthesis.[23] Isolation of total RNA and primer extension analysis to detect the RNase III specific cleavage signals at position G_{-112} in *rpoS* mRNA were performed as recently described.[25,52] In brief, total RNA was purified from culture aliquots using the hot phenol method.[55] Primer extension analysis was performed using AMV reverse transcriptase (Promega) together with 15 μg of purified total RNA primed with the *rpoS*-specific [^{32}P] 5'-end labeled oligonucleotide (5'-TCC GTT CTC ATC AAA TTC CGC ATC-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 6% sequencing gel. The resulting signals were visualized by a PhosphorImager (Molecular Dynamics).

Northern-blot analysis. The steady-state levels of DsrA in strains WJW45 and WJW45ΔcsdA were determined by northern-blot analysis using 15 μg RNA of the same RNA preparations used for the primer extension analysis (see above). The RNA samples were denatured for 5 min at 82°C in RNA-loading buffer containing 50% formamide, separated on 8% polyacrylamide/8 M urea gels, and then transferred to nylon membranes by electroblotting. The RNA was crosslinked to the membrane by exposure to UV light. The membrane was hybridized with DsrA-specific [^{32}P] 5'-end labeled oligonucleotide (5'-TCG TTA CAC CAG GAA ATC TGA TGT-3'), and as a loading control with 5S rRNA-specific oligonucleotide R25 (5'-GGT GGG ACC ACC GCG CTA CGG CCG CCA GGC-3'). The hybridization signals were visualized using a PhosphorImager (Molecular Dynamics).

Purification of CsdA and Hfq. The purification of CsdA was performed as described by Bizzebard et al.[44] with some modifications. The plasmid pCsdA[44] was transformed into *E. coli hfq*- strain JW4130 (Keio gene knockout collection, NBRP, Japan) and incubated at 28°C. At an OD_{600} of 0.6, the synthesis of CsdA was induced by addition of IPTG to a final concentration of 1 mM. After an additional hour of incubation at 28°C, the cells were harvested by centrifugation at 4,000x g for 10 minutes at 4°C. All subsequent procedures were performed at 4°C. Approximately 7 g of cells were resuspended in 20 ml lysis buffer (500 mM NaCl, 50 mM NaH_2PO_4, pH 8.0, 200 μg/ml PMSF, 5 μg/ml lysozyme, 20 μg/ml DNaseI, 20 mM imidazole) and then

lysed using a French press (SimAminco) at 10,000 psi. The lysate was centrifuged for 30 minutes at 10,000, 15,000 and 30,000x g respectively, to remove cellular debris. The supernatant (16 ml) was incubated over-night at 4°C with 2 ml Ni-NTA agarose (QIAGEN). Then, the lysate-Ni-NTA mixture was loaded on a column according to the protocol of the manufacturer and washed with washing buffer (500 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) containing 20 mM imidazole. The proteins were eluted with elution buffer, consisting of washing buffer with increasing concentrations (60–500 mM) of imidazole. The fractions obtained from step-wise elution were analysed by SDS-PAGE. The purest fractions were pooled, dialysed against CsdA storage buffer (1 M NaCl, 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 1 mM DTT), and subsequently stored in the same buffer. The activity of CsdA was tested as specified by Turner et al.[45] Hfq was purified as described by Vecerek et al.[51]

Micrococcal nuclease treatment of Hfq and CsdA. To remove remaining RNA, the purified CsdA (CsdA-His_6) and Hfq proteins were treated with micrococcal nuclease (Fermentas) and re-purified on a Ni-NTA agarose column. Briefly, 700 pmol of protein in storage buffer were treated in the presence of 10 mM $CaCl_2$ with 100 U micrococcal nuclease for one hour at 37°C. The protein fractions were then incubated with 200 µl Ni-NTA overnight at four 4°C or for two hours on ice. The protein-Ni-NTA mixture was then loaded on a Spin-X column (Costar), washed with lysis buffer (500 mM NaCl, 50 mM NaH_2PO_4, pH 8.0, 20 mM imidazole) and eluted with lysis buffer containing 100 mM imidazol. The eluted proteins were further dialysed against the corresponding storage buffers and used for the experiments.

Far-western analysis. Far-western analysis was performed according to the protocol of Wu et al.[56] with some modifications described by Vecerek et al.[51] Briefly, CsdA (25 pmol) treated or left untreated with micrococcal nuclease was separated on a 10% SDS-polyacrylamide gel and then electroblotted onto a nitrocellulose membrane at 15V for 30 minutes. De-naturation and re-naturation of the proteins bound to the membrane was performed three times in AC buffer (100 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.5 mM EDTA, 1 mM DTT, 10% glycerol, 0.1% Tween-20 and 2% milk powder) with decreasing concentrations of Guanidine-HCl.[56] After the final wash with AC buffer the nitrocellulose membrane was incubated for three hours at RT with Hfq (150 pmol as hexamer) in 10 ml AC buffer, followed by several washing steps. Bound Hfq protein was detected following a standard western-blot protocol with anti-Hfq antibodies and visualized as described above.

Co-immunoprecipitation. 25 pmol of purified hexameric Hfq protein was incubated in 100 µl AC buffer (10% glycerol, 100 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.5 mM EDTA, 0.1% Tween-20, 1 mM DTT) at room temperature together with 50 pmol CsdA protein for one hour. Anti-Hfq antibodies were bound to Dynabeads according to the manufacture's instructions. The Dynabeads-anti-Hfq antibody complex was added to the protein mixture and incubated for an additional hour at room temperature. Non-specifically bound proteins were removed from the beads by magnetic separation and by washing the pellets three times with AC buffer. The washed Dynabeads-Ab-Ag complex and the co-immunoprecipitated proteins were resuspended in Laemmli buffer and analysed by immunoblotting with anti-His antibodies directed against CsdA-His_6.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/ReschRNA7-6-Sup.pdf

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