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Protein-protein Interaction Studies with Hfq

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1 Abstract

Hfq, a Sm-like protein of *Escherichia coli* has many essential and overlapping functions in cellular processes. It is involved in expression of the *rpoS* gene that encodes the stationary sigma factor, σ^S, in iron metabolism, in stability control of numerous messenger RNAs (mRNAs) and small non-coding RNAs (ncRNAs), in riboregulation of target mRNAs by ncRNAs. In addition it acts as a virulence factor in some bacterial pathogens. Hfq was found to be associated with components of the ribosome, degradosome and other cellular machines that are involved in RNA metabolism.

The major aim of this study was to determine whether Hfq interacts physically with candidate protein partners, like ribosomal protein S1 and the DEAD-box helicase CsdA. This study revealed that the interaction between S1, CsdA and Hfq is indirect and probably mediated by RNA. Moreover the C-terminal part of CsdA is necessary for indirect association with Hfq.

2 Zusammenfassung

Hfq, ein Sm-like Protein von *Escherichia coli* hat vielfache Funktionen in zellulären Prozessen. Es ist involviert in die Expression des *rpoS* Gens, das für den Sigma Faktor σ^S kodiert, in den Eisen Metabolismus, in die Stabilitätskontrolle zahlreicher mRNAs und kleiner nicht-kodierender RNAs (ncRNAs), als auch in der Regulierung von mRNAs durch ncRNAs. Außerdem ist Hfq ein bedeutender Faktor in der Virulez von einigen bakteriellen Pathogenen. Es ist bekannt, dass Hfq mit Komponenten des Ribosoms, des Degradosoms und anderen zellulären Maschinen, die im RNA Metabolismus involviert sind, assoziiert ist.

Das Ziel dieser Studie war zu ergründen, ob Hfq direkt mit den vermeintlichen Partnern S1 und der DEAD-box Helikase CsdA interagiert. Es konnte gezeigt werden, dass Hfq indirekt mit dem ribosomalen Protein S1 und CsdA interagiert und dass diese Interaktionen mit hoher Wahrscheinlichkeit durch RNA mediiert sind. Es konnte weiters gezeigt werden, dass der C-terminale Teil von CsdA für die indirekte Assoziation mit Hfq notwendig ist.

3 Introduction

3.1 Protein-protein interactions

Protein-protein interactions are involved in many cellular processes, like DNA replication, transcription, secretion, cell growth, cell cycle control, metabolic pathways, signal transduction and translation. Therefore, they have evoked a lot of interest in pharmacy, medicine as well as in bioscience in the last years (Phizicky and Fields, 1995). Interactions between proteins can have numerous effects. One result of protein-protein interactions can be the change of the kinetic properties. Furthermore, protein-protein interactions can form new binding sides for other proteins or substrates. Proteins can also be activated or inactivated as a result of protein interactions. Another outcome of interacting proteins is the change of the specificity of a protein for its substrate (Phizicky and Fields, 1995). These are only some of many effects resulting from protein-protein interactions. Protein-protein interactions may be mediated by a small region of one protein fitting into a cleft of another protein or by two surfaces interacting over a large area. Interactions between proteins can be tight or weak (Phizicky and Fields, 1995).

3.2 Hfq

3.2.1 Hfq structure and function

The *Escherichia coli* Hfq protein was first described as a host factor required for phage Qβ RNA replication. Together with ribosomal protein S1, Hfq is required for the initiation of translation of the plus strand of Qβ RNA (Fernandez *et al*., 1968). Electron microscopy studies of the *E. coli* Hfq protein (Møller *et al*., 2002; Zhang *et al*., 2002) as well as X-ray crystallography of the *Staphylococcus auerus* Hfq homologue (Schumacher *et al*., 2002) (Fig. 1) demonstrated that Hfq belongs to the eukaryotic and archaeal family of Sm and Sm-like (Lsm) proteins. These proteins are involved in RNA metabolism in Eukaryotes and

bind to RNAs by recognizing short U-rich stretches, which are known as SM sites (Achsel *et al*., 2001). While eukaryotic Sm proteins form ring-shaped hetero-heptamers (Kambach *et al*., 1999), bacterial Hfq forms homo-hexameric rings like the archaeal Sm proteins (Toro *et al*., 2001). The Sm proteins consist of two conserved segments, the Sm1 and Sm2 motifs, that are separated by a region of variable length and sequence. The Sm motif provides a common folding domain that is required for RNA-binding and oligomerization (Valentin-Hansen *et al*., 2004).

Hfq forms a toroidal core with an outer diameter of ~70 Å and a thickness of ~25 Å. The central pore is 8 Å to 12 Å wide. Hfq contains a N-terminal α-helix followed by five β-strands. The first three β-strands belong to the Sm1 motif, whereas the Sm2 motif encompasses β-strands 4 and 5. The hexamer is formed by interactions of β4 from one monomer with β5 from its neighbouring subunit (Brennan and Link, 2007).

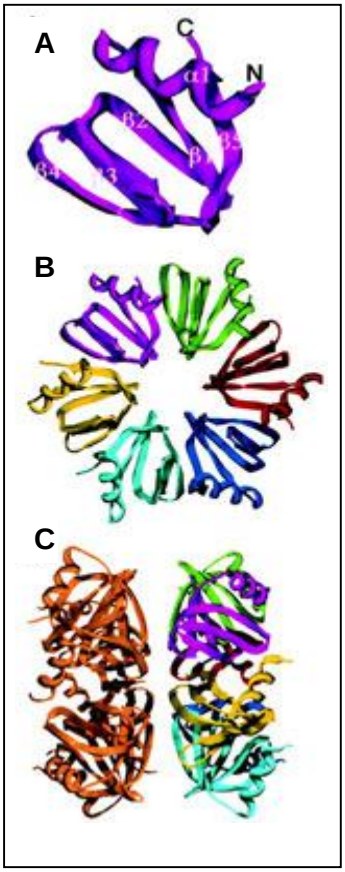

Figure 1: Structure of Hfq from *Escherichia coli*. (**A**) Structure of the Hfq monomer. Secondary structural elements are labelled. (**B**) Structure of the Hfq hexamer with each subunit in a different colour. (**C**) The two Hfq hexamers in the crystallographic asymmetric unit. This view is rotated 90° to (B) along the vertical axis in the plane of the paper (Schumacher *et al.*, 2002).

The heat stable Hfq protein of *E. coli* is small in size (11.2 kDa, 102 aa), basic and forms a hexamer in solution (Arluison *et al*., 2002; Vassilieva *et al*., 2002). Hfq binds preferentially to AU-rich single stranded RNAs (Moll *et al*., 2003; Brescia *et al*., 2003; Mikulecky *et al*., 2004). One binding site is located on the proximal site of the hexamer including the inner rim made up of six potential nucleotide binding pockets. This binding site is used by poly(U) and presumably by ncRNAs. The second binding site, which is required for poly(A) binding is located on the distal surface of the hexamer (Mikulecky *et al*., 2004).

E. coli Hfq homologues have been found in many Gram-negative and Gram-positive bacteria (Møller *et al*., 2002; Zhang *et al*., 2002). The N-terminus of Hfq, encompassing amino acid residues 1-66 contains the Sm1 motif. It is highly conserved in different organisms, whereas considerable variations exist at the C-terminal end (Sonnleitner *et al*., 2002). The first 65 N-terminal amino acid residues of *E. coli* Hfq are sufficient for hexamer formation as well as for Qβ replication (Sonnleitner *et al*., 2004). Recent studies from our laboratory showed that a Hfq variant containing only the first N-terminal amino acids (Hfq_{65}) is non-functional in Hfq-auto-regulation, RyhB-mediated repression of *sodB* mRNA, as well as in DsrA-mediated stimulation of *rpoS* mRNA translation (Večerek *et al*., 2008). In contrast to the full-length Hfq (Hfq_{wt}), Hfq_{65} does not stimulate annealing of complementary RNA oligonucleotides. In addition, two non-complementary RNA oligonucleotides were not able to bind Hfq_{65} at the same time on the surface. Moreover, it was shown that amino acid residues following the first N-terminal 66 amino acids of Hfq are involved in mRNA binding (Večerek *et al*., 2008).

Hfq expression is controlled at both, the transcriptional and translational level. The *hfq* gene is part of a superoperon. The internal “heat shock” promoters of this operon ensure that high Hfq levels are ensured during stress conditions. It autoregulates its own expression at the translational level through binding to the *hfq* mRNA, and by inhibiting the formation of the translation initiation complex (Večerek *et al*., 2005). Hfq seems not to require any post-transcriptional modifications or cofactors for binding to RNA (Valentin-Hansen *et al*., 2004). The number of molecules per cell in *E. coli* is 50.000 to 60.000 (~10.000 hexamers). The majority (80% to 90%) of Hfq is found in the

cytoplasmic fraction in association with the translational machinery (Kajitani *et al*., 1994). It was demonstrated early on that Hfq co-purifies with 30S ribosomal subunits (DuBow *et al*., 1977). The remaining intracellular Hfq is associated with the chromosome and was shown to bind to DNA (Azam *et al*., 2000).

3.2.2 Hfq, a RNA chaperone

A characteristic feature of RNA-chaperones is to prevent either misfolding of RNAs or to resolve misfolded RNA structures (Herschlag, 1995). The stimulation of annealing between complementary nucleic acids is another property of RNA-chaperones (Tsuchihashi and Brown, 1994). Moll *et al*. (2003) showed using an *in vivo* RNA-chaperone assay and an *in vitro* assay with *ompA* mRNA that Hfq has RNA-chaperone activity. In the *in vivo* RNA-chaperone assay Hfq was able to rescue a “folding trap” in the intron of the phage T4 *td* gene (Semrad and Schroeder, 1998, Clodi *et al.*, 1999).

More recently the RNA chaperone activity of Hfq was examined by real-time fluorescence resonance energy transfer (FRET). The effect of Hfq on intermolecular base pairing between DsrA and *rpoS* was studied. Strand exchange results from the Hfq-mediated association of DsrA and *rpoS*. This was followed by melting of the *rpoS* stem region and slow annealing of the two RNAs. It was also shown that Hfq disrupts pre-formed DsrA-*rpoS* complexes. It is also important to note that the chaperone activities of Hfq are independent of ATP hydrolysis (Arluison *et al*., 2007, Brennan *et al*., 2007, Reikowitsch and Schroeder, 2007).

3.2.3 Hfq, a post-transcriptional regulator

Hfq appears to have different functions in post-transcriptional regulation. The importance of Hfq for riboregulation was first recognized in studies on OxyS RNA, a regulator of oxidative stress response (Zhang *et all*., 1998). Since then

Hfq was shown to bind to DsrA, RprA and Spot42 RNAs as well as to other small RNAs (Sledjedki *et al*., 2001; Wasserman *et al*., 2001; Møller *et al*., 2002). Several sRNAs are involved in translational regulation of mRNAs and Hfq seams to increase their interaction with these targets.

Hfq was identified as a factor that associates with the 5' UTR of *ompA* mRNA (Vytvytska *et al*., 1998). Hfq has also been implicated in negative post-transcriptional regulation. Hfq represses translation initiation of *ompA* mRNA, which encodes the outer membrane protein A, by interfering with ribosome binding, which results in degradation of the mRNA (Vytvytska *et al*., 2000). More recently, it was shown that Hfq regulation of *ompA* expression requires the sRNA MicA (Udekwu *et al*., 2005), which regulates the stability of *ompA* mRNA by competing with 30S subunits for binding to the *ompA* 5' UTR.

3.2.4 Hfq – interaction with candidate proteins

Sukhodolets and Garges (2003) suggested that the ribosomal protein S1 mediates Hfq binding to RNA polymerase, and thereby modulates the transcriptional activity of the enzyme. However, a direct protein-protein interaction between S1 and Hfq has not been demonstrated (Sukhodolets and Garges, 2003).

Similarly, Hfq has been purified together with PNP (polynucleotide phosphorylase) and PAP I (poly(A) polymerase I). PNP of *E. coli* has two functions *in vitro*; it can synthesize RNA by using nucleotide phosphates as precursors and it can exonucleolytically degrade RNA when inorganic phosphate is present. *In vivo* this enzyme works mainly as an exonuclease because of the high concentrations of inorganic phosphate in growing cells (Mohanty and Kushner, 2000). PNP is part of the degradosome like RNase E, the DEAD helicase and enolase. The function of PAP I is to add adenine residues to the 3' end of pre-mRNAs to form a poly(A) tail (August et al., 1962). The polyadenylation of RNA molecules is part of the RNA degradation pathway

in Bacteria (Cohen, 1995). Further, it was suggested that Hfq interacts with PNP and PAP I (Mohanty *et al.*, 2004).

During studies of the mechanism of RNase E in the degradation of target mRNAs, Hfq was found to form together with SgrS and RyhB ribonucleoprotein complexes (Morita *et al*., 2005). Furthermore, the RNase E-Hfq-sRNA complex results in translational inhibition and rapid degradation of target mRNA. Interestingly, Hfq binding to these sRNAs without RNase E still results in translational repression without mRNA degradation. The Hfq-RNase E complex seemed also to be formed without sRNA, and therefore it was assumed that Hfq binds directly to RNase E (Morita *et al*., 2006).

In a large-scale analysis of protein complexes in *E. coli*, Hfq was found to be associated to a large number of proteins. Over 50 different proteins were detected by liquid chromatography-tandem mass spectrometry (LC-MS) or MALDI-TOF (gel-based peptide mass fingerprinting using matrix assisted laser desorption/ionization-time-of-flight mass spectrometry) as interaction partners of Hfq, including CsdA and S1 (Butland *et al*., 2005). However, a direct protein-protein interaction between Hfq and CsdA or S1 was not demonstrated.

3.3 CsdA

3.3.1 The DEAD-box protein family of Escherichia coli

The DEAD-box family of ATP-dependent RNA helicases consists of about 500 eukaryotic and prokaryotic proteins, and is thus the largest member of the super-family 2 (SF2) (Cordin *et al*., 2006). These proteins share a core of nine conserved sequence motifs that are essential for ATPase and/or RNA unwinding activity of numerous helicases (Turner *et al*., 2007). *Escherichia coli* has five ATP-dependent DEAD-box RNA helicases, which play essential roles in many cellular processes involving RNA splicing, transport, degradation, translation initiation, ribosome biogenesis and cell division. These proteins are DbpA, SrmB, RhlB, RhlE and CsdA (Kalman *et al*., 1991).

DbpA is the best characterized member of the DEAD-box family and functions as an RNA-specific ATPase. It is proposed to play a role in the biogenesis of the large ribosomal subunit (Prud'homme-Généreux *et al*., 2004). SrmB was identified as a gene copy-dependent suppressor of a temperature sensitive mutation in *rplX*, which encodes L24, a protein that is required for 50S subunit assembly (Nishi *et al*., 1988). Recently it was demonstrated that SrmB is important for the biogenesis of the 50S subunit at low temperature (Charollais *et al*., 2003). It has also been implicated in the interaction with poly(A) polymerase (Iost and Dreyfus, 1994). The DEAD-box protein RhlB is a component of the RNA degradosome (Miczka *et al*., 1996) where its activity is stimulated by RNase E and RNA. *In vitro* it functions to unwind RNA secondary structures (Prud'homme-Généreux *et al*., 2004).

The cold-shock DEAD-box protein A (Fig. 2), termed CsdA or DeaD, a 70 kDa protein, is different from the other four *E. coli* RNA helicases in that it contains a C-terminal domain (CTD) of ~20 kDa that is unique to this sub-family. The *csdA* gene (*deaD*) was originally identified as a multicopy suppressor of a temperature-sensitive mutation in the *rpsB* gene, which encodes ribosomal protein S2 (Toone *et al*., 1991). Overexpression of *csdA* in a *rpsB* mutant resulted in re-incorporation of ribosomal proteins S1 and S2 into 30S ribosomal subunits (Moll *et al*., 2002). However, CsdA is also involved in the biogenesis of the 50S ribosomal subunit (Charollais *et al*., 2004), and may also be required for the initiation of translation of mRNAs with extensive secondary structures at lower temperatures (Lu *et al*., 1999). In cells grown at 37°C, CsdA is present in small amounts but is induced significantly upon a shift to lower temperatures. Mutational analysis on the *csdA* gene by Jones and co-workers showed that CsdA knockout mutants have little effects on cell growth at 37°C, but show a growth defect at 15°C (Jones *et al*., 1996). The ATPase and helicase activities of a C-terminally truncated CsdA protein (named CsdAΔ; aa 1 - 444) (Fig. 2) were observed at lower temperatures (Bizebard *et al*., 2004, Turner *et al*., 2007). However, it was shown that the CTD of CsdA is required for *in vivo* function at lower temperatures, while the DEAD-box motif is essential for both, the *in vivo* function at lower temperatures as well as for efficient *in vitro* ATPase and helicase activities. *In vivo* and *in vitro* studies demonstrated that CsdA

associates with components of the RNA degradosome (Prud'homme-Généreux *et al*., 2004).

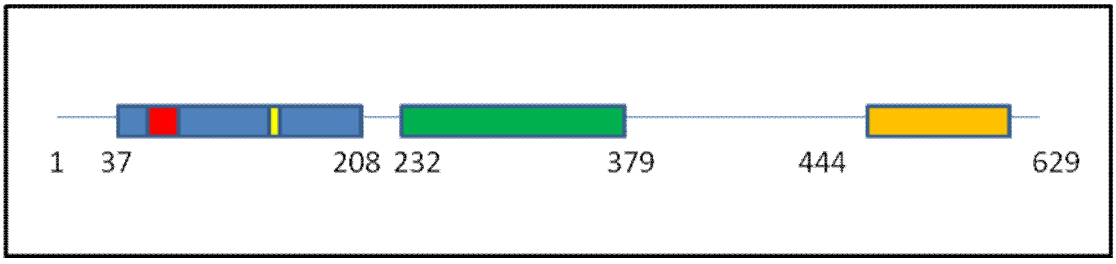

Figure 2: The domains of the DEAD-box CsdA protein.
The full length CsdA protein is depicted (aa 1 – 629). The helicase ATP-binding domain is shown in blue from position 37-208 aa which includes the ATP-binding region (red) as well as the DEAD box motif (yellow). The helicase domain is depicted in green from aa 232 – 379. The C-terminal domain of CsdA contains RNA binding sites (orange). The CsdAΔ protein used in this study comprises aa 1 – 444. (http://www.uniprot.org/uniprot/P0A9P6).

3.3.2 CsdA is involved in ribosome biogenesis

Non-ribosomal factors such as RNA helicases or chaperones are essential for the assembly of ribosomal subunits *in vivo* (Nierhaus *et al*., 1991; Williamson *et al*., 2003). CsdA is involved in the biogenesis of the large ribosomal subunit. The deletion of the *csdA* gene leads to a depletion of free 50S ribosomes and to the accumulation of 40S particles. The comparison of ribosomal profiles of a Δ*csdA* and a wt strain at 37°C and 20°C indicated that CsdA is critical for ribosomal biogenesis only at low temperatures. Nevertheless, the protein composition of the CsdAΔ 40S particle was shown to be different from the ΔSrmB 40S particle and indicates that the defect in ribosomal assembly occurs at a later step. All 50S proteins were detected in the 40S-like particle, but in different concentrations. It was also demonstrated that CsdA associates with 50S precursors at low temperatures (Charollais *et al*., 2004).

3.3.3 The "cold shock" degradosome

In addition to the above mentioned functions of CsdA, the protein associates with the RNA degradosome after cold shock. Puls-labeling experiments demonstrated that CsdA associates with RNase E upon a shift to low temperature. CsdA is required for normal RNase E activity at lower temperatures and *in vitro* studies revealed that CsdA interacts directly with RNase E. Moreover, CsdA can fully replace RhlB in the degradosome at low temperatures (Prud'homme-Généreux, 2004). RhlB and CsdA bind to different sites of RNase E (Khemici *et al*., 2004). The primary role of CsdA in cold acclimation of cells appears to be in mRNA decay, and its helicase activity seems to be essential for promoting degradation of mRNAs, which are stabilized at low temperatures (Awano *et al*., 2007). CsdA was also suggested to interact with other proteins. A large number of different proteins, including Hfq, were found to be associated with CsdA (Butland *et al*., 2005). However, a physical interaction between CsdA and Hfq has not been demonstrated.

3.4 Ribosomal protein S1

3.4.1 Structure of S1 and association with the ribosome

S1 is the largest ribosomal protein of the *E. coli* 30S ribosomal subunit. It is encoded by the *rpsA* gene and consists of 557 amino acid residues. S1 is an acidic protein, whereas all other ribosomal proteins, except L7 and L12, are basic (Sengupta *et al*., 2001). It is the only ribosomal protein that binds to the *E. coli* 30S subunit by means of protein-protein interactions (Boni *et al*., 1982). S1 is found in nearly all Gram-negative and in some Gram-positive bacteria (Muralikrishna and Suryanarayana, 1985; Farwell *et al*, 1992), but it is not present in Eukaryotes. The N-terminal domain of S1 binds to the platform region of the 30S subunit and is responsible for protein-protein interactions (Giorginis and Subramanian, 1980). The C-terminal domain, located on the solvent side and facing ribosomal protein S2, serves to facilitate the initial interaction

between mRNA and the 30S subunit (Sillers and Moore, 1981). The two domains are separated by a central globular domain (Sengupta *et al*., 2001).

Sequence analyses of the *E. coli* S1 protein demonstrated that S1 consists of six conserved structural domains (D1-D6), the so called S1 domains. This motif is found in many other proteins that are involved in RNA metabolism. It was demonstrated that the first two domains bind to the ribosome, whereas the last four bind to mRNA (Subramanian *et al*., 1983). The association of S1 with the ribosome is weak and reversible, while most other ribosomal proteins are strongly bound (Subramanian and van Duin, 1977). S1 has an elongated structure that makes several contacts with components of the head, platform and main body of the small ribosomal subunit (Sengupta *et al*., 2001).

The localization of S1 on the small ribosomal subunit is on the platform, near the junction of head and main body (Fig. 3). S1 has a complex shape, with a central globular (CG) portion including two holes of unequal size and two stretched arm-like extensions of unequal lengths, a short arm (SA) and a long arm (LA). The LA is bound to the head of the 30S subunit, while the SA is located on the solvent side, facing ribosomal protein S2 (Fig. 3). The LA represents the N-terminal ribosomal binding site of S1 that is located on the ribosome near protein S9 and SA represents the C-terminal portion, an mRNA interaction site which is located near protein S6. The larger hole in the CG portion is located on the platform side and allows a direct association of the cleft of the 30S subunit with the 50S subunit. The small hole is partly blocked by protein S18 from the platform. The CG part contains a portion of the N-terminal half and a major portion of the C-terminal half (Sengupta *et al*., 2001).

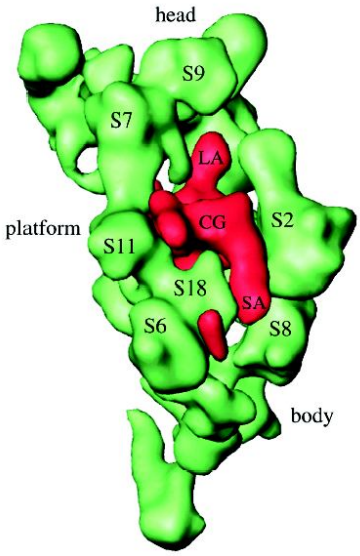

Figure 3: Neighbourhood and structure of the ribosomal protein S1.
Position of S1 with respect to other small-subunit ribosomal proteins analysed by cryo-electron microscopy. The protein S1 with its long arm (LA), short arm (SA) and its central globular (CG) portion is shown in red, whereas all other proteins of the 30S ribosome are shown in green (Sengupta *et al*., 2001).

S1 faces proteins from head, platform and main-body regions on the solvent side of the 30S subunit. Ribosomal proteins S11 and S18 interact directly with S1. Protein S6 faces S1 with a surface of four β-sheets and an unstructured end that reaches near S1. The long arm of S1 is surrounded by proteins S7 and S9 from the head side and by protein S5 from the shoulder side (Sengupta *et al*. 2001). The solvent side of S1 interacts with the large surface of protein S2, which is essential for binding of protein S1 to the 30S ribosomal subunit (Fig. 3) (Moll *et al*., 2002).

3.4.2 Functions of S1

Ribosomal protein S1 is essential for translation of highly structured mRNAs (Szer *et al*., 1975) as well as for the formation of the translation initiation complex at internal ribosome binding sites (RBS) (Tedin *et al*., 1997). The S1 protein binds poly(U), poly(A) and poly(C) with similar affinities (Subramanian, 1983). During translation initiation, the ribosomal protein S1 recognizes single-stranded U-rich regions. It was also shown that U-rich regions are often found 5' of the Shine-Dalgarno sequence in *E. coli* mRNAs, and these regions were found to be targets for S1 (Boni *et al*., 1991).

S1 stimulates the T4 endoribonuclease RegB, which inactivates phage mRNAs by cleaving them in the middle of their Shine-Dalgarno sequence. S1 is also one of the four subunits of the fd and Qβ RNA bacteriophage replicases (Wahba *et al*., 1974). It forms a complex with the protein β of phage λ (Muniyappa *et al*., 1993). In the case of Qβ replicase, S1 recognizes the S and M sites on the phage RNA (Boni *et al*., 1991, Aliprandi et al., 2008). Thus, the role of S1 in the formation of the replication complex is to recognize the same U-riche region as in the formation of the translation initiation complex (Boni *et al*., 1991).

Recently it was suggested that the *E. coli* S1 protein plays a role in the coupling between transcription and translation (Sukhodolets *et al*., 2006). S1 was also demonstrated to regulate and control its own synthesis *in vivo* as an autogenous repressor (Boni et al., 2001). The S1 protein is essential for growth of *E. coli* and for all Gram-negative bacteria which posses a S1 homologue (Sørensen *et al*., 1998).

3.4.2.1 S1 and translation initiation

The initiation of translation is a complex process which requires many proteins and factors. The efficiency by which 30S particles recognize and bind translation initiation regions of mRNAs determines the level of protein synthesis (Gold *et al*., 1981). There are two ways by which a ribosome is able to recognize a binding site on mRNA while searching for a translational start: RNA-RNA and RNA-protein interactions. Both kinds of interactions are involved in initiation of translation (Gold *et al*., 1988). A large number of bacterial and phage mRNAs use the Shine-Dalgarno sequence during the initiation step of translation to interact with the ribosome, which results in a RNA-RNA interaction.

The ribosomal protein S1 is involved in the second type of ribosome-messenger interactions. Several observations suggest that S1 is directly involved in the process of mRNA recognition and binding (Subramanian *et al*.,

1983). As mentioned before, S1 is associated with the 30S subunit through its N-terminal domain by means of protein-protein interactions. Sengupta and co-workers reported that the CG part of S1 may interact with an 11-nucleotide-long fragment of the 5' region of the mRNA (Fig. 4). The 5' end of the mRNA also interacts with the anti-Shine-Dalgarno sequence which is located on helix 45 of the 16S rRNA. Thus, the 5' stretch of mRNA can bind S1 on the upstream side and the anti-SD on the downstream side (Sengupta *et al*., 2001).

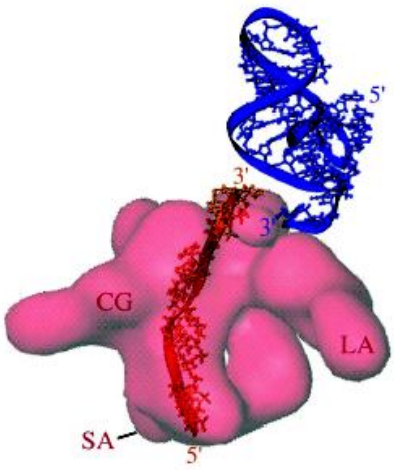

Figure 4: Stereo presentation of the relative positions of S1, helix 45 of 16S rRNA and mRNA segment.
Protein S1 is shown in red. The mRNA segment (orange) corresponding to the 11-nucleotide stretch is embedded on the surface of the CG region of the S1 protein. The 3' end of the mRNA lies near the 3' end of the helix 45 (blue) with the anti-SD sequence. (Sengupta *et al*., 2001).

3.4.3 S1 - interaction with proteins

S1 was demonstrated to interact by means of direct protein-protein interactions with ribosomal protein S2 (Boni *et al*., 1982). Later S1 and nucleic acid-binding protein Hfq were suggested to form a complex with RNA polymerase (Sukhodolets and Garges, 2003). Recently, S1 was reported to interact with many other proteins, including Hfq. Using LC-MS and MALDI-TOF methods over 30 different proteins were found to be associated with S1 (Butland *et al*., 2005). However, a direct protein-protein interaction between S1 and Hfq has not been demonstrated.

4 Aim oft the study

Hfq, an *Escherichia coli* RNA chaperone has pleiotropic functions. It has been suggested to be associated with components of the ribosome, degradosome and other cellular machines that are involved in RNA metabolism. In particular, Hfq has been suggested to interact with proteins, S1 (Sukhodolets *et al*., 2003), PNP, PAP I (Mohanty *et al*., 2004), and RNase E (Morita *et al*., 2005). The major aim of this study was to investigate whether the interaction between Hfq and ribosomal protein S1 and the DEAD-box RNA helicase CsdA is based on direct protein-protein interactions or whether it is mediated by RNA.

5 Materials and methods

5.1 Microbiological Methods

5.1.1 Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All strains were routinely grown at 37°C or 28°C in Luria-Bertani (LB) medium (Miller, 1972) supplemented with the appropriate antibiotics to maintain selection of plasmids. The final concentration of antibiotics used was: 100 µg/ml ampicillin, 50 µg/ml kanamycin, 10 µg/ml tetracycline. The final concentration of glucose was 1%.

Table 1: Bacterial strains and plasmids used in this study

E. coli strains	Gentype	Source
BL21(DE3)p-LysS	*F^- ompT gal dcm lon $hsdS_B(r_B^-\ m_B^-)$ λ(DE3) pLysS(cm^R)*	Stratagene
BL21(DE3)	*F^-, dcm, ompT, $hsdS(r_B^-\ m_B^-)$, galλ(DE3)*	Stratagene
XL1-Blue	*endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac gln V44 F' [::Tn10 $proAB^+lacI^q$Δ (lacZ)M15]*	Stratagene
TOP10	*F^- mcrAΔ(mrr-hsdRMS-mcrBC) φ80lacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R)*	Invitrogen
MC4100	*F^- araD139 Δ(argF-lac)U169 rspL150 relA1 flbB5301 fruA25 deoC1 ptsF25 e14-*	
Plasmids		
pUC19	*Amp^R, lacZ`*	New England Biolabs
pAHfq	pACYC184 encoding the Hfq_{wt} protein	Večerek *et al*., 2003
pACYC184	*cat (Cm^R), Tc^R, p15A-ori*	Fermentas
pAHfq65	pACYC184 containing	Večerek *et al*., 2007

	the codons 1 to 195 of the *hfq* gene	
pProEX-HTb	*colE1 ori, Amp^R,* N-terminal His_6-tag, TEV cleavage site*, trc promoter, lacI,*	Invitrogen
pCsdA	pProEX-HTb encodes CsdA, with a 5' His_6-tag*, Ap^R*	Bizebard *et al*., 2004
pCsdAΔ	pProEX-HTb containing codons 1 to 444 of the *csdA* gene with a 5' His_6-tag, *, Ap^R*	Bizebard *et al*., 2004
pS1	pProEX-HTb encodes S1 with a 5' His_6-tag*, Ap^R*	This study

5.2 Recombinant DNA techniques

5.2.1 Construction of pS1

The pProEX-HTb plasmid encoding the S1 protein was constructed in the following manner: the *rpsA* gene encoding protein S1 was amplified from genomic DNA of *E. coli* MC4100 by PCR using the forward primer C1 (5'-AAAA**GGCGCC**ATGACTGAATCTTTTGCTCAACTC-3') containing an *EheI* (bold) restriction side and the reverse primer C2 (5'-GGGAG**CTCGAG**AATTAC TCGCCTTTAGCTG C-3') containing *XhoI* (bold) and *SacI* restriction sites. The obtained PCR product was subsequently cleaved with *EheI* and *XhoI* and ligated into the corresponding sites of the pProEX-HTb expression vector (Table 1). The resulting plasmid encodes an N-terminal His-tagged S1 protein (Fig. 5). The His_6-tag can be removed by TEV protease cleavage.

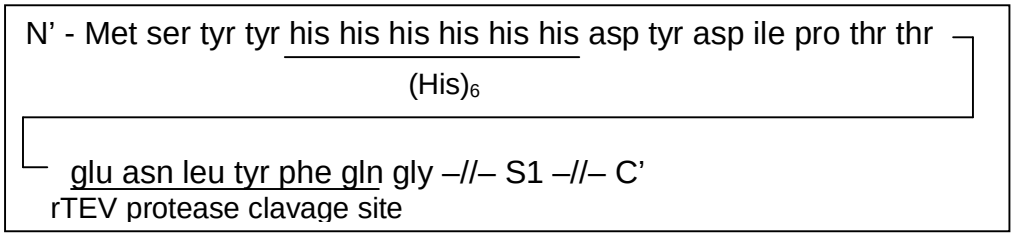

Figure 5: The Sequence of the S1 protein construct.
The S1 protein is shown with the sequence of the His_6-tag and the TEV protease cleavage site.

5.3 Protein Purification

5.3.1 Purification of Hfq_{wt} and Hfq_{65}

Escherichia coli strain BL21(DE3) harbouring plasmid pAHfq or plasmid pAHfq65 (Table 1) was incubated at 37°C in LB medium. At an OD_{600} of 0.6 the synthesis of Hfq_{wt} and Hfq_{65} was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and the cells were harvested by centrifugation after 3 h. The cells were resuspended in lysis buffer (50 mM Tris/HCl, pH 8.0, 250 mM $MgCl_2$, 1.5 mM EDTA, 5 mM DTT and 0.5 mM PMSF), and then lysed using a French press (SimAminco). The lysate was heated for 20 minutes at 85°C, and than centrifuged at 18 000 r.p.m. for 40 minutes. After addition of 1.5 M ammonium sulphate, the lysate was loaded on a Butyl-sepharose column (Sigma). The column was washed with buffer containing 1.5 M NaCl, 1.5 M ammonium sulphate and 50 mM Tris/HCl, pH 8.0. Then, the protein was eluted with 50 mM Tris/HCl, pH 8.0, and dialysed against and then stored in Hfq buffer containing 50 mM Tris/HCl, pH 8.0 and 200 mM NaCl (Sonnleitner *et al*., 2006).

5.3.2 Purification of S1 under native conditions

For synthesis of the S1 protein, the *E. coli* BL21(DE3)pLysS strain (Tabe 1) harbouring the pS1 plasmid (Table 1) was used. For that purpose four litres LB

medium were inoculated with *E. coli* BL21(DE3)pLysS(pS1) and incubated at 28°C. The synthesis of S1 was induced by addition of IPTG (final concentration 1 mM) at an OD_{600} of ~0.6. After an additional hour of incubation at 28°C the cells were harvested by centrifugation at 4000 x g for 15 minutes at 4°C. All subsequent procedures were performed at 4°C. Approximately 6 g of the cells (wet weight) were resuspended in 20 ml lysis buffer (500 mM NaCl, 50 mM NaH_2PO_4, pH 8.0, 200 µg/ml Phenyl-methylflourid (PMSF), 5 µg/ml lysozyme, 20 µg/ml DNaseI, 20 mM imidazole) and then lysed using a French press (SimAminco) at 10.000 psi. Cellular membranes and debris were removed by three centrifugation steps at 10.000, 15.000 and 30.000 x g for 30 minutes. The supernatant (16 ml) was incubated over night at 4°C with 2 ml Ni-NTA agarose (QIAGEN). Subsequently the lysate-Ni-NTA mixture was loaded on a column according to the manufacturer's protocol and washed with two column volumes of 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 pure fractions were pooled, dialysed against and stored in VD buffer (10 mM Tris-HCl, pH 7.4, 6 mM NH_4Cl, 10 mM MgOAc) containing 0.1 mM EDTA and 1 mM DTT. The protein concentration was determined using the BCA™ Protein Assay Kit (Pierce) according to the manufacturer's instructions.

5.3.3 Purification of CsdA and CsdAΔ under native conditions

The purification of the CsdA and CsdAΔ proteins was done according to the protocol of Bizzebard *et al.* (2004) with some modifications. The plasmids pCsdA or pCsdAΔ (Table 1) were transformed into *E. coli* BL21(DE3). Four litres LB medium were inoculated with *E. coli* BL21(DE3)/pCsdA or *E. coli* BL21(DE3)/pCsdAΔ and incubated at 28°C. At an OD_{600} of 0.6, the production of CsdA and CsdAΔ proteins 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 4000 x g for 10-15 minutes at 4°C. All

subsequent procedures were performed at 4°C. Approximately 7 g of the 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 30.000 x g respectively, to remove cellular membranes and other debris. Upon judgement by SDS-PAGE (samples of the pellet and the supernatant were used) whether most of the protein is in solution, the supernatant (16 ml) was incubated over-night at 4°C with 2 ml Ni-NTA agarose (QIAGEN). Subsequently 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 the CsdA-storage buffer (1 M NaCl, 20 mM Tris-HCl, pH 7.5, 0.1mM EDTA, 1 mM DTT), and subsequently stored in the same buffer.

5.4 Biochemical and immunological Methods

5.4.1 Co-immunoprecipitation

Co-immunoprecipitation provides a means to study protein-protein interactions. In this study purified proteins were used to analyse protein-protein interactions. Briefly, 25 pmol of purified hexameric Hfq_{wt}/Hfq_{65} proteins were incubated in AC buffer (10% glycerol, 100 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.5mM EDTA, 0.1% Tween-20, 1 mM DTT) at room temperature with 50 pmol CsdA, CsdAΔ or S1 protein for one hour. The Dynabeads-antibody complex (see 5.4.2) was subsequently added to the protein mixture and incubated for an additional hour at room temperature. Non-specifically bound proteins were removed from the beads by washing them with AC buffer. The washed complex, which included the beads, the antibody and proteins associated with the antibody were resuspended in SDS-PAGE buffer and analysed by immunoblotting.

5.4.2 Binding of Antibodies to Dynabeads

"Dynabeads – Protein G" (Invitrogen) were used for the co-immunoprecipitation experiments. The antibodies were bound to Dynabeads. 20 µl Dynabeads were washed three times with 100 µl 0.1 M Na-acetate pH 5.0. 100 µl of a 1:1000 diluted antibody (anti-Hfq antibody or anti-His_6-tag antibody) were incubated for 40 minutes at room temperature with gentle mixing with washed "Dynabeads – Protein G". During the incubation the Fc part of the antibodies binds to the "Dynabeads – Protein G". Then, the Dynabeads with bound antibodies were washed three times with 500 µl 0.1 M Na-acetate pH 5.0 and resuspended in 100 µl of the same buffer. 10 µl of the antibody-"Dynabead – Protein G" complex were used in the co-immunoprecipitation experiments.

5.4.3 Far Western Blot

Far western blotting is a technique to identify protein-protein interactions. To regain the native conformation of the proteins they were denaturated and renaturated on a nitrocellulose membrane by washing with AC buffer (10% glycerol, 100 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.5mM EDTA, 0.1% Tween-20, 1 mM DTT) containing decreasing concentrations of Guanidine-HCl (Schwarz *et al*., 2001). Far western blotting was performed according to the protocol of Schwarz *et al*. (2001) with some modifications. Briefly, the proteins, CsdA, CsdAΔ and S1 (25 pmol each) were electrophoresed on a denaturing 10% SDS-polyacrylamide gel at 20 mA for approximately 40 minutes. The proteins were then transferred from the gel onto a nitrocellulose membrane using a semi-dry blotting device (Biorad) at 15 V for 30 minutes. Then, the membrane was washed with 6 M Guanidine-HCl in AC buffer containing 2% milk powder for 30 minutes at room temperature. The second time the membrane was washed with 3 M Guanidine-HCl in AC buffer (plus 2% milk powder) for 30 minutes at room temperature. The last two washing steps were performed with 1 M and 0.1 M Guanidine-HCl in AC buffer (plus 2% milk powder), respectively, for 30 minutes at 4°C. Finally, the nitrocellulose membrane was incubated in 2%

milk powder solution in AC buffer over night at 4°C to block the unspecific binding sites on the membrane. After the blocking step, the nitrocellulose was washed 2x with AC buffer, and then incubated with 150 pmol of hexameric Hfq_{wt} or Hfq_{65} protein in 10 ml AC buffer. After the incubation time of three hours at room temperature the membrane was washed 4 x 10 minutes with AC buffer. The nitrocellulose was then incubated with the first antibody (anti-Hfq-AB from rabbit) and afterwards with the second antibody (anti-rabbit-AP-AB). The visualization of the proteins on the membrane was performed by alkaline phosphatase (AP) staining. For that purpose the membrane was incubated with a staining solution containing 0.33 mg/ml NBT and 0.165 mg/ml BCIP in development buffer (100mM Tris-HCl, pH 9.5, 100mM NaCl, 5 mM $MgCl_2$).

5.4.4 TEV Protease Cleavage

To remove the His_6-tag from the purified proteins a TEV-protease cleavage was performed. 1 mg protein was incubated with 200 U TEV-protease (Invitrogen) for three hours at room temperature. Then, the protein fraction was incubated with 500 µl Ni-NTA over-night at 4°C to eliminate the His_6-tag. The protein Ni-NTA mixture was loaded on a spin-column and the purified His_6-tag free protein was eluted by centrifugation.

5.4.5 Micrococcal Nuclease Digestion

To remove RNAs from the protein fractions, the purified proteins were treated with micrococcal nuclease (Fermentas) as follows: 700 pmol of protein in storage buffer was incubated with 10 mM $CaCl_2$ and 100 U micrococcal nuclease for one hour at 37°C. After digestion, the proteins were again purified over Ni-NTA. The protein fractions were first incubated with 200 µl Ni-NTA over-night at four 4°C or for two hours on ice. Then, the protein-Ni-NTA mixture was loaded on a column washed and eluted with lysis buffer containing IMM. The

eluted proteins were dialysed against the corresponding storage buffer and used for the experiments.

6 Results

6.1 Protein Purification and activity assay (FRET)

For purification of the proteins CsdA and S1 the *csdA* and *rpsA* genes were cloned into the pPROEX-HTb expression vector, which permits IPTG-inducible high level synthesis of the proteins with an N-terminal tag. The tag consists of a $(His)_6$ peptide followed by a cleavage site for TEV protease. The proteins were purified by nickel affinity chromatography (Fig. 6).

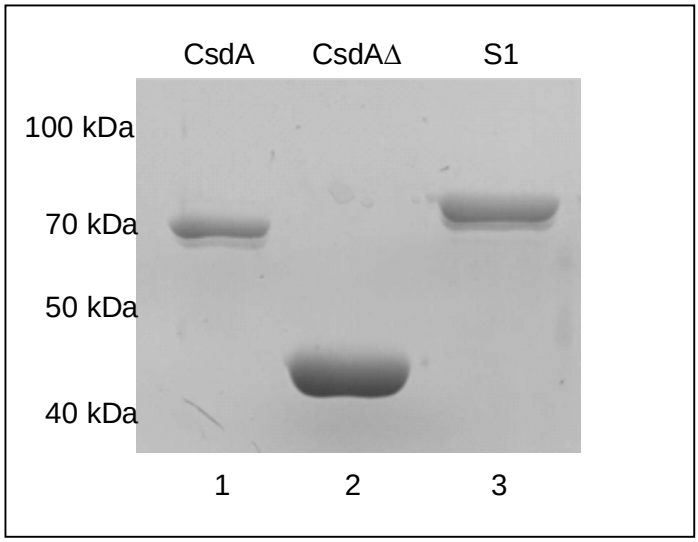

Figure 6: Purification of CsdA, CsdAΔ and S1 proteins via His_6-tag.
The purified N-terminal His-tagged CsdA, CsdAΔ and S1 proteins were separated on a 10% SDS-polyacrylamide gel and stained with Coomassie brilliant blue. Lanes 1-3, 10 pmol, 25 pmol and 15 pmol of protein CsdA, CsdAΔ and S1, respectively, were loaded.

After protein purification the specificity of the antibodies used in this study was tested by western blotting. The proteins were separated on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Then, the membrane was incubated either with anti-Hfq or anti-His_6 antibodies. The anti-His_6 antibody recognized the His_6-tagged CsdA, CsdAΔ and S1 proteins but not Hfq_{wt} protein (Fig. 7A). Figure 7B shows that the anti-Hfq antibody recognizes Hfq_{wt} but does not bind unspecifically to CsdA, CsdAΔ or S1.

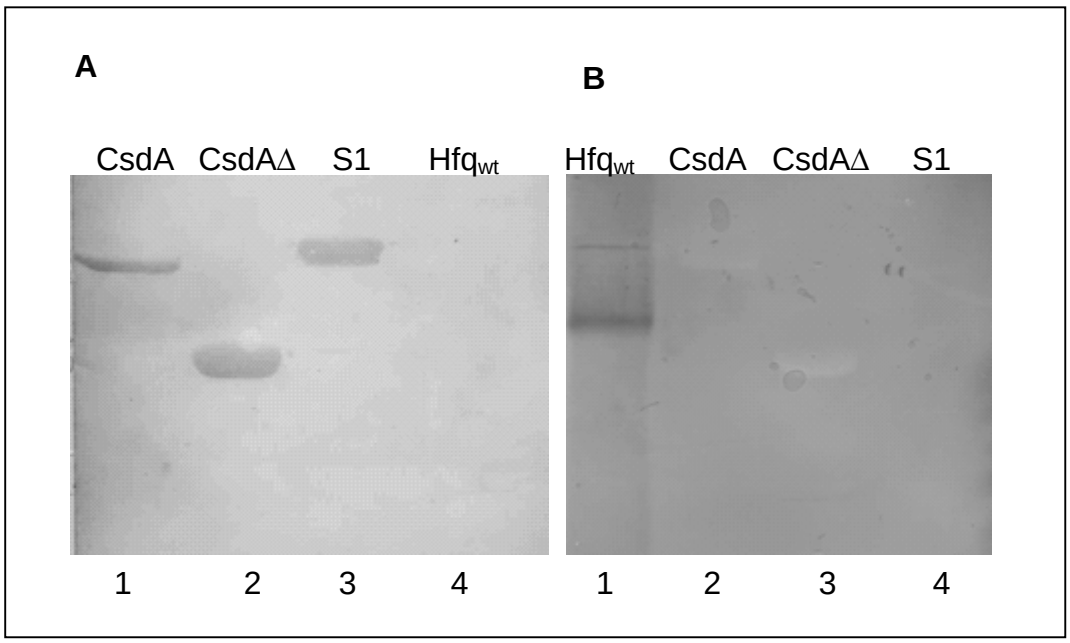

Figure 7: Specificity of the anti-His_6 and anti-Hfq antibodies.
(**A**) The specificity of the anti-His_6 antibody was tested for CsdA (lane1), CsdAΔ (lane 2), S1 (ane 3) and Hfq_{wt} (lane 4). (**B**) The specificity of the anti-Hfq antibody was tested for Hfq_{wt} (lane 1), CsdA (lane 2), CsdAΔ (lane 3) and S1 (lane 4).

Prior to the use of the Hfq, CsdA, CsdAΔ and S1 proteins for the protein-protein interaction studies the activity of the proteins was tested by FRET (fluorescence resonance energy transfer). The activity assay was done in collaboration with L. Rajkowitsch. Hfq protein has RNA annealing activity, S1 protein has strand displacement activity and CsdA has RNA helicase activities which can be measured. A recently developed assay that combines the RNA annealing and strand displacement activities in a single set-up and detects double-stranded RNA by FRET (Rajkowitsch and Schroeder, 2007) was used to analyse the activity of the proteins.

In the first phase of the assay, two fluorophore-tagged RNAs Cy5-5'-UUAAUUUAAUGUUUUAUUUAUUAGGGUUUAUGGCUGUUCGCCAUUU-3' (named JM1heli, Fig. 8) and Cy3-5'-UAAUAAAUAAAACAUUAAAUU-3' (named J12, Fig. 8) were incubated at 30°C in a buffer containing 50 mM Tris-HCl pH 7.5, 3 mM $MgCl_2$ and 1 mM DTT in the absence or presence of the tested CsdA protein (Rajkowitsch and Schroeder, 2007). The RNAs for CsdA were constructed based on the results of Turner *et al*. (2007), where it was shown that CsdA exhibits ATPase and helicase activities in the presence of short RNA

duplexes with either 3' or 5' extentions at low temperature. The Cy5-JM1heli-RNA that was used for CsdA testing has a 3' hairpin structure (Fig. 8).

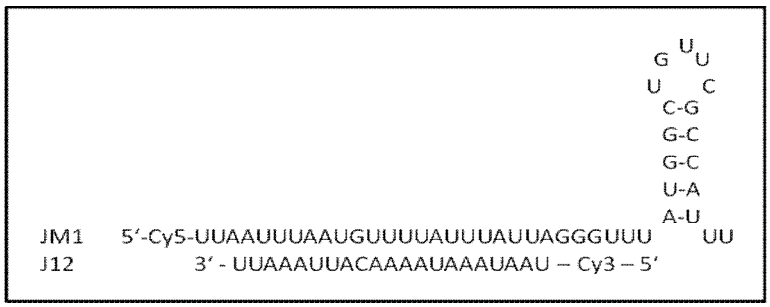

Figure 8: RNA substrates used in FRET assay.
The JM1heli and J12 RNAs were either Cy3 or Cy5 labeled. The 3' hairpin structure of the JM1heli-RNA is shown. The J12-RNA is complementary to the linear part of the JM1heli-RNA.

The tagged RNA oligonucleotides were purchased from VBC-Biotech (Vienna, Austria). A Tecan GENios Pro microplate was used for the experiment. Annealing was started by injection of 10 nM Cy5-JM1heli into a well (96-well black microtiter plate, Greiner Bio-One) containing an equal volume of 10 nM Cy3-J12 and different concentrations of the CsdA protein (Rajkowitsch and Schroeder, 2007). The reaction was allowed to proceed for 180 seconds while donor and acceptor dye fluorescence emissions were measured once every second. The second phase was started by injection of an excess of non-labeled competitor RNA (J12). Readings were taken for another 180 seconds. The time-resolved ratio of the fluorescence emission (FRET index F_{Cy5}/F_{Cy3}) was normalized to 1 at t_{180s} and least-square fitted with Prism 4.03 (GraphPad Software Inc.). For phase I, the second-order reaction equation for equimolar initial reactant concentration was used: $y = A[1-1/(k_{ann,1}t+1)]$. $k_{ann,1}$ is the observed annealing reaction constant and A is the maximum reaction amplitude. Phase II was fitted with a single exponential function for signal increase $y = y_0+A[1-\exp(-k_{ann,2}t)]$ or signal decay $y = y_0+A\exp(-k_{SD}t)$. While strand annealing activities result in an increase of the FRET signal, strand displacement causes a decrease in fluorescence emissions (Rajkowitsch and Schroeder *et al*., 2007).

The activities of the Hfq, S1, CsdA and CsdAΔ proteins were measured by FRET method. The RNA helicase activity of CsdA is demonstrated in Figure 9. For this assay different concentrations of the CsdA protein were used. The CsdA activity assay was performed with and without ATP. The results verified that the helicase activity of CsdA is ATP-dependent. The strand annealing activity of Hfq and the strand displacement activity of S1 were also demonstrated (Rajkowitsch and Schroeder *et al*., 2007).

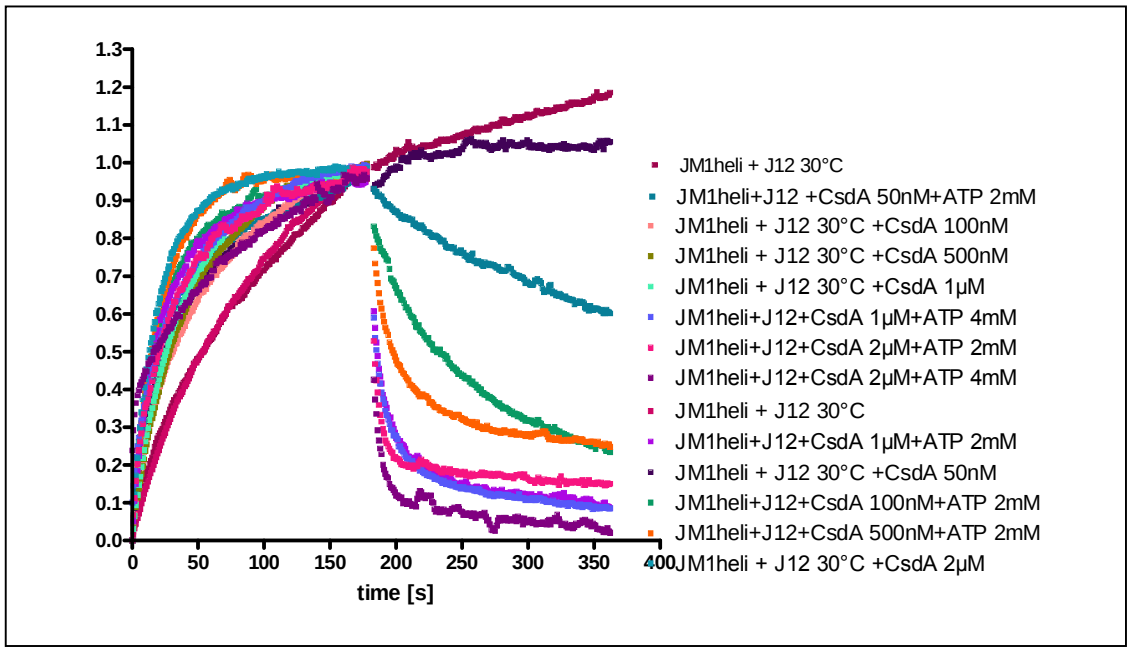

Figure 9: RNA unwinding activity of CsdA
The FRET assay was performed at 30°C, with and without ATP. In phase I, the two complementary JM1heli and J12 RNAs were annealed in a microplate reader. The donor (Cy3) and acceptor (Cy5) fluorescence emission were quantified every second. The FRET index was calculated as F_{Cy5}/F_{Cy3} and normalized at t_{180s}. The added CsdA protein induced strand displacement and its activity was ATP-dependent. The different curves are recorded as a function of different concentrations of CsdA and/or ATP.

6.2 Hfq does not associate directly with S1

Sukhodolets and Garges suggested that ribosomal protein S1 mediates Hfq binding to RNA polymerase, and thereby modulates the transcription activity of the enzyme. However, a direct interaction between Hfq and S1 has not been shown (Sukhodolets *et al*., 2003). Here, I analysed whether purified Hfq interacts directly or indirectly with purified S1 using Far western-blotting and co-immunoprecipitation assays. For the Far western-blot and co-immunoprecipitation experiments purified S1 (Fig. 6) and Hfq_{wt} proteins were used.

For the Far western-blotting experiment 2.5 pmol (Fig. 10, lane 2) and 5 pmol (Fig. 10, lane 3) of ribosomal protein S1 were electrophoresed on a 10% SDS-polyacrylamide gel and then transferred onto a nitrocellulose membrane. After denaturation and renaturation of protein S1, the membrane was incubated with 300 pmol Hfq_{wt} (in 10 ml AC-buffer containing 1 mM DTT) and binding was assayed with an anti-Hfq-antibody. Hfq_{wt} served as a positive control for the anti-Hfq-antibody (Fig. 10A, lane 1). The S1 protein was detected on the blot after de- and renaturation using the anti-His_6 antibody (Data not shown). As Hfq protein could not be detected on the Far western-blot (Fig. 10A, lane 2 and 3), our data suggest that Hfq_{wt} does not bind to S1 in a direct manner through protein-protein interactions. The S1-S2 interaction (Moll *et al*., 2002) served as an additional control for the Far western-blot. As shown in Figure 10B, S2 binding to S1 was verified by this method.

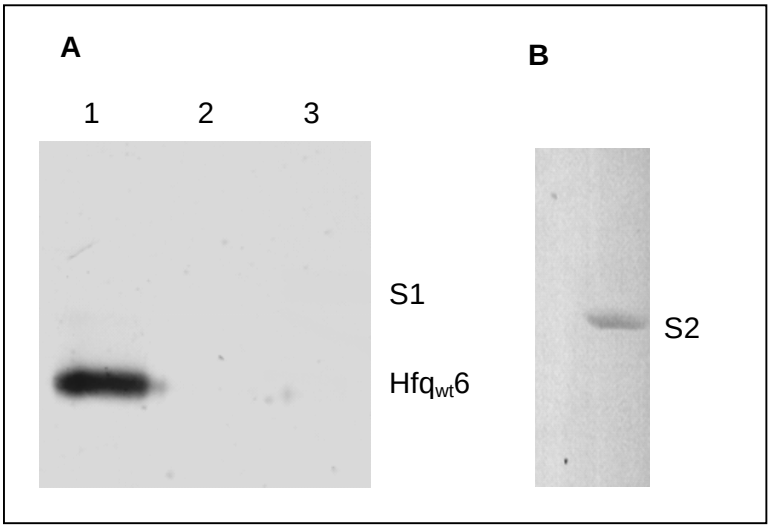

Figure 10: Hfq-S1 interaction analysis by Far western-blotting.
(**A**) 5 pmol of Hfq$_{wt}$ (hexamer) was loaded in lane 1. Lane 2 (25 pmol) and 3 (50 pmol) contained different concentrations of S1. The membrane was incubated with 300 pmol Hfq$_{wt}$6 (in 10 ml AC-buffer containing 1 mM DTT). For detection of Hfq an anti-Hfq antibody was used. (**B**) Positive control. Interaction of ribosomal protein S1 (25 pmol) and S2 (25 pmol).

Next, a co-immunoprecipitation experiment with N-terminally His-tagged S1 protein and Hfq was performed. As for the Far western-blot, for the co-IP purified proteins (Fig. 6) were used. The co-IP experiments were performed with either anti-Hfq-antibody or anti-His$_6$-antibody. The co-IP experiment was performed as described in Materials and Methods (5.4.1). The Dynabeads-antibody-protein complex was resuspended in SDS-PAGE buffer and analysed by immunoblotting. Figure 11 shows that S1 did not co-immunoprecipitate with Hfq$_{wt}$ (lane 2). To exclude that S1 protein does not precipitate with “Dynabeads protein G” only or the anti-Hfq antibody alone, two additional controls were performed. In the first control S1 was incubated only with “Dynabeads protein G” (Fig. 11, lane 4) and in the second control S1 was incubated with “Dynabeads protein G”, anti-Hfq antibody but without Hfq protein (Fig. 11, lane 5). Neither the “Dynabeads protein G” nor the anti-Hfq-antibody recognized S1 unspecifically. Thus, the co-immunoprecipitation results indicated that Hfq$_{wt}$ does not interact with S1 through direct protein-protein interactions.

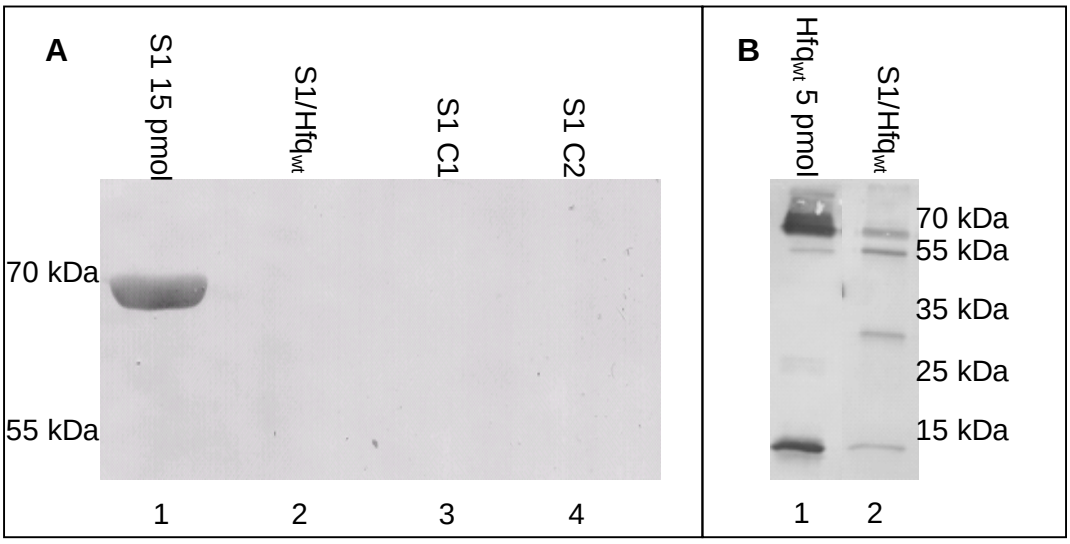

Figure 11: Hfq-S1 interaction analysis by co-immunoprecipitation with anti-Hfq-antibody.
For the co-immunoprecipitation an anti-Hfq antibody was used. **(A)** For the western-blot an anti-His_6 antibody was used for the detection of S1-$(His)_6$. The proteins were loaded and separated on a 10 % SDS-polyacrylamide gel. 15 pmol of S1-$(His)_6$ were loaded as positive control for the anti-His_6 antibody (lane 1). Immonoprecipitates obtaind after incubation of Hfq_{wt} with S1-$(His)_6$ were loaded in lane 2, respectively. Two negative controls, C1 and C2 were also performed (see text). **(B)** For the western-blot an anti-Hfq antibody was used for the detection of Hfq. The proteins were loaded and separated on a 15 % SDS-polyacrylamide gel. 5 pmol of Hfq were loaded as positive control for the anti-Hfq antibody (lane 1). Immonoprecipitates obtaind after incubation of Hfq_{wt} with S1-$(His)_6$ were loaded in lane 2, respectively.

In addition the same experiment was done in the reverse manner using a His_6-specific antibody. Hfq did not co-immunoprecipitate with S1 (Fig. 12, lane 2). Also for this experiment a negative control was performed. In the control (C) Hfq was incubated with “Dynabeads protein G” and anti-His_6 antibody but without S1 (Fig. 12, lane 3). The results showed that Hfq_{wt} did not bind unspecifically neither to anti-His_6-antibody nor to “Dynabeads protein G”. Thus, the results from both co-immunoprecipitation experiments suggested that Hfq and S1 do not interact via direct protein-protein interactions.

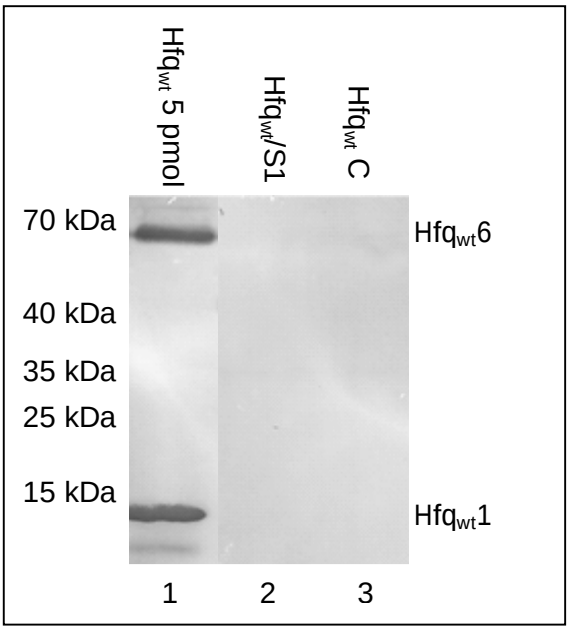

Figure 12: Co-immunoprecipitation of Hfq with S1-(His)$_6$.
For the Co-Immunoprecipitation the anti-His$_6$-anti body was used. For the western blot the anti-Hfq-antibody was used to detect whether Hfq$_{wt}$ co-immunoprecipitates with S1. 5 pmol (hexamer) Hfq$_{wt}$ (lane 1) was loaded as positive control for the anti-Hfq-antibody. Hfq$_{wt}$-hexamer migrates at ~66 kDa and the monomer at ~10 kDa. The reaction of S1 with Hfq$_{wt}$ was loaded in lane 2. Hfq with Dynabeads and anti-His$_6$ antibody was loaded in lane 3 (negative control).

6.3 Hfq$_{wt}$ does not interact directly with CsdA

Recently an *E. coli* interaction study suggested an association of Hfq with more than 50 proteins (Butland *et al*., 2005). One of these potential interaction partners was the DEAD-box ATP dependent RNA helicase CsdA. Therefore, the possible interaction between Hfq and CsdA was scrutinized in this study. Both, Hfq and CsdA, are known to bind RNA. It was therefore considered that the interaction between Hfq and CsdA could be mediated by RNA. To analyse whether the interaction between Hfq and CsdA is based on direct protein-protein interactions or RNA-dependent the Hfq and CsdA protein fractions were treated with micrococcal nuclease (Fermentas). The nuclease treatment was performed as described in 5.4.5. The Far western-blot as well as the co-immunoprecipitation experiments were carried out with (+MN) and without (-MN) micrococcal nuclease digested proteins. After MN treatment both proteins, Hfq$_{wt}$ and CsdA, were analysed on a 15% SDS-polyacrylamide gel, which revealed that the nuclease treatment did not apparently affect the integrity of the proteins (Fig. 13).

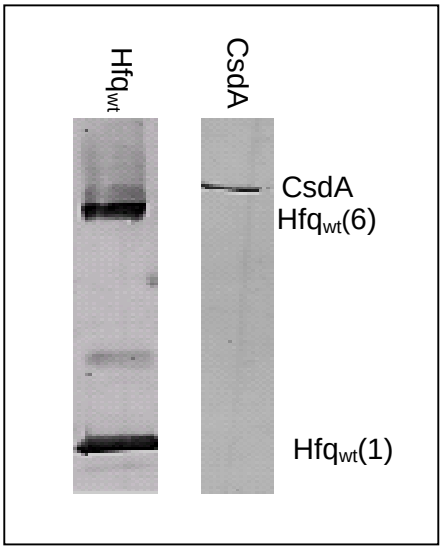

Figure 13: Integrity of CsdA and Hfq_{wt}.
The Intergrity of CsdA and Hfq_{wt} were analyzed by western blotting after MN digestion. For Hfq an anti-Hfq antibody was used, and for CsdA-$(His)_6$ an anti-His_6-tag antibody.

The results of the Far western-blot experiments are shown in Fig. 14. CsdA protein pre-treated with (+) or without (-) treatment with micrococcal nuclease was loaded on a 15% SDS polyacrylamide gel and then transferred onto a nitrocellulose membrane. After denaturation and renaturation, the membrane bound proteins were incubated with Hfq_{wt} pre-treated with (+) MN, followed by incubation with anti-Hfq-antibodies that were used to detect Hfq protein bound to CsdA. Hfq_{wt} was loaded as positive control for the anti-Hfq-antibody (Fig. 14, lane 3). Hfq binding to CsdA could be detected when CsdA was not digested with micrococcal nuclease (Fig. 14, lane 1). However, it was not possible to detect Hfq binding to CsdA after both proteins were treated with MN (Fig. 14, lane 2). Thus, the interaction between Hfq_{wt} and CsdA seems not to be direct, but the proteins seemed to associate through RNA.

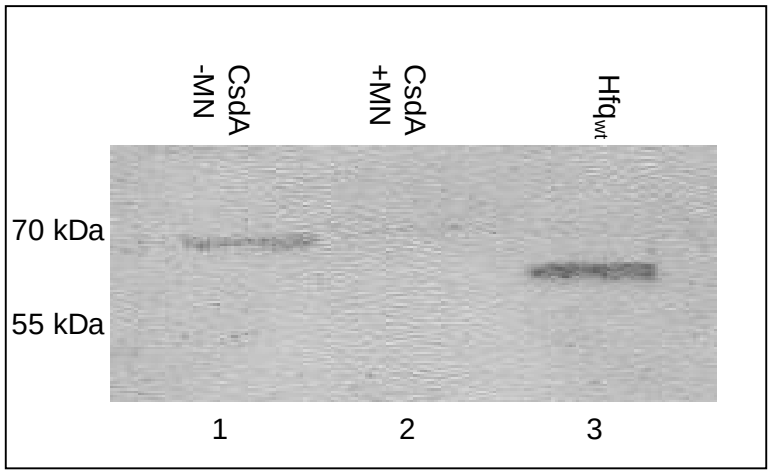

Figure 14: Hfq_{wt}-CsdA interaction analysis by Far western-blotting.
25 pmol untreated (lane 1) and micrococcal nuclease (Fermentas) treated CsdA protein (lane 2) were loaded on a 15% SDS polyacrylamide gel and incubated with MN treated Hfq_{wt} protein after transfer onto a nitrocellulose membrane. Lane 3, Hfq_{wt} was loaded as positive control for the anti-Hfq-antibody.

In addition, a co-immunoprecipitation experiment was carried out to verify the Far western-blotting results shown in Fig. 14. The co-IP experiments were performed with both anti-Hfq-antibody and anti-His_6-antibody. The proteins used for the co-IP were either treated with micrococcal nuclease (MN+) or left untreated (MN-). To detect whether there were any physical interactions among CsdA and Hfq the immunoprecipitates were electrophorased on a 10% SDS-polyacrylamide gel and probed for CsdA with anti-His_6-tag antibody antibody. Fig. 15 shows that CsdA co-immunoprecipitated with Hfq before MN digestion (Fig. 15, lane 2) but not after treatment with micrococcal nuclease (Fig. 15, lane 4). To exclude that CsdA protein does precipitate with “Dynabeads protein G” or the anti-Hfq antibody alone, two additional controls were done. In the first control CsdA was incubated only with “Dynabeads protein G” (Fig. 15, lane 6) and in the second control CsdA was incubated with “Dynabeads protein G”, anti-Hfq antibody but without Hfq protein (Fig. 15, lane 7). Neither the “Dynabeads protein G” nor the anti-Hfq-antibody recognized CsdA unspecifically.

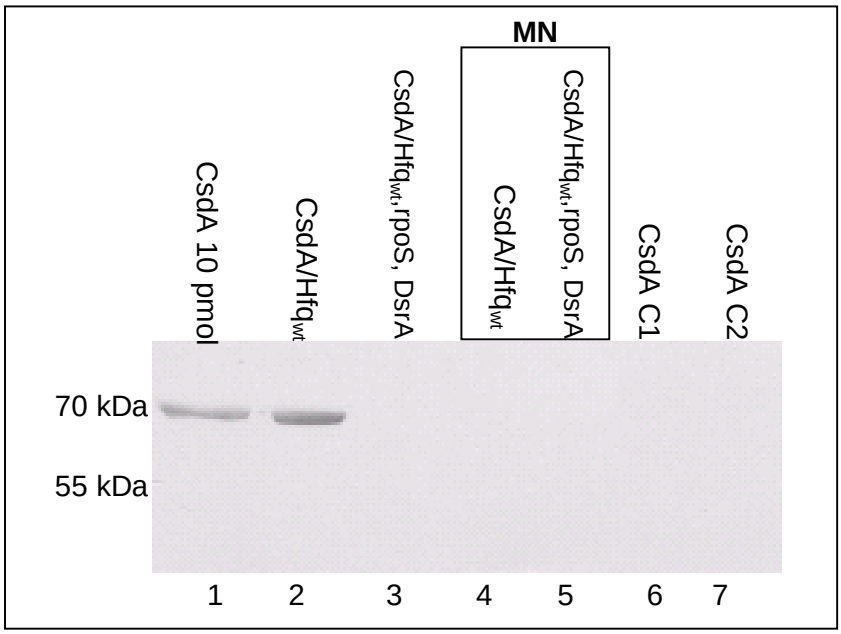

Figure 15A: Hfq-CsdA interaction analysis by co-immunoprecipitation with anti-Hfq antibody.
For the co-immunoprecipitation an anti-Hfq antibody was used. For the western-blot an anti-His_6 antibody was used to detect whether CsdA co-immunoprecipitates with Hfq. 10 pmol of His_6-CsdA were loaded as positive control for the antibody (lane 1). The co-immunoprecipitate of CsdA with Hfq_{wt} without nuclease treatment was loaded in lane 2. Lane 4, CsdA-Hfq_{wt} treated with micrococcal nuclease. The complexes of Hfq_{wt}, CsdA, DsrA and RpoS were loaded in lane 3 (-MN) and lane 5 (+MN). Two controls were loaded in lane 6 (C1) and 7 (C2) (see text).

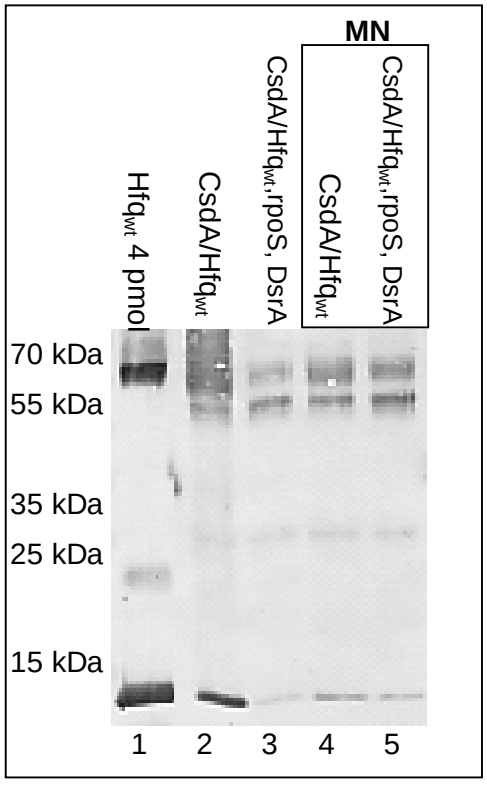

Figure 15B: Hfq-CsdA interaction analysis by co-immunoprecipitation with anti-Hfq antibody.
For the co-immunoprecipitation an anti-Hfq antibody was used. For the western-blot an anti-Hfq antibody was used to detect the pull down of Hfq. 4 pmol of Hfq_{wt} were loaded as positive control for the antibody (lane 1). The co-immunoprecipitate of CsdA with Hfq_{wt} without nuclease treatment was loaded in lane 2. Lane 4, CsdA-Hfq_{wt} treated with micrococcal nuclease. The complexes of Hfq_{wt}, CsdA, DsrA and RpoS were loaded in lane 3 (-MN) and lane 5 (+MN).

In the reciprocal co-IP experiment using a His_6-specific antibody, Hfq was detected to co-immunoprecipitate with CsdA before MN treatment (Fig. 16, lane 2). In contrast, after nuclease treatment no Hfq_{wt} could be detected to co-immunoprecipitate with CsdA (Fig. 16, lane 4). Taken together, the Far western-blot and co-IP experiments showed that the association of Hfq and CsdA does not result from direct protein-protein interactions but that the proteins might associate via RNAs. Also for this experiment two controls were performed to exclude unspecific binding of Hfq to "Dynabeads protein G" or the anti-His_6 antibody. In the first control (C1) Hfq was incubated with "Dynabeads protein G" and anti-His_6 antibody but without CsdA (Fig. 16, lane 6). In the second control (C2) Hfq was only incubated with "Dynabeads protein G" (Fig. 16, lane 7). The results showed that Hfq_{wt} did not bind unspecifically to the anti-His_6-antibody or to "Dynabeads protein G".

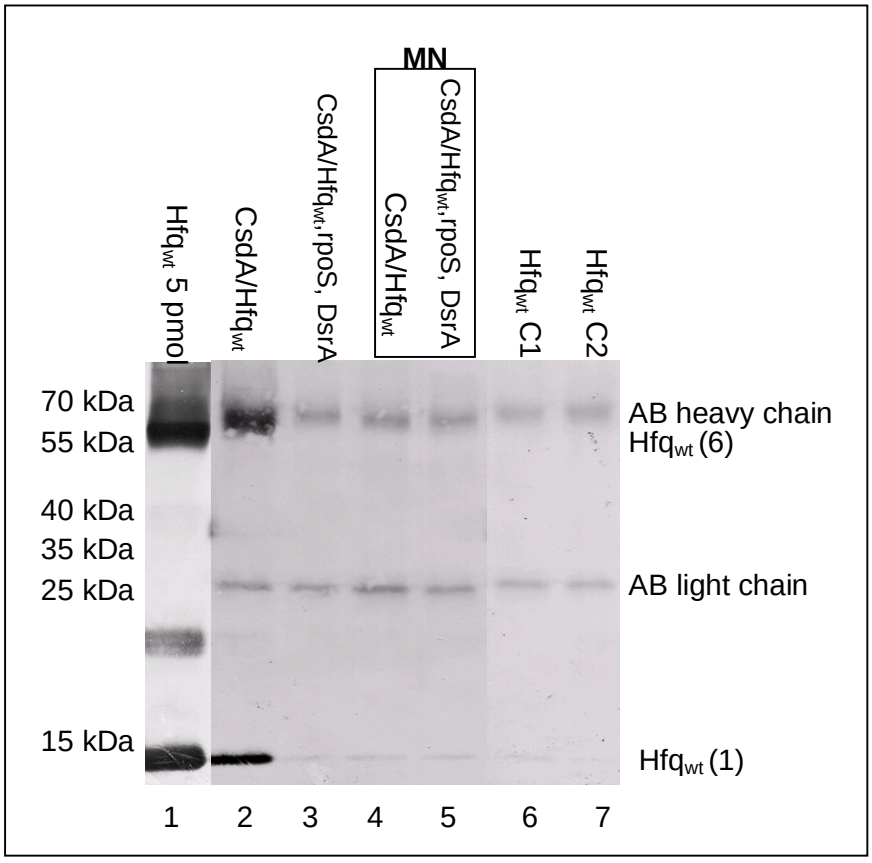

Figure 16: Hfq-CsdA interaction analysis by Co-IP with anti-His_6 antibody.
For the co-immunoprecipitation an anti-His_6 antibody was used. For the western blot an anti-Hfq antibody was used to detect whether Hfq_{wt} co-immunoprecipitates with CsdA. 5 pmol (hexamer) Hfq_{wt} (lane 1) was loaded as positive control for the anti-Hfq antibody. The complex of CsdA with Hfq_{wt} without nuclease treatment was loaded in

lane 2. CsdA-Hfq_{wt} (lane 4) was treated with micrococcal nuclease. The complex of Hfq_{wt}, CsdA, DsrA and RpoS were loaded in lane 3 (-MN) and lane 5 (+MN).Two controls were done, C1 (lane 6) and C2 (lane 7) (see text).

Hfq is known to be involved in translation regulation of several RNAs. It was shown that Hfq binds DsrA, an 85-nucleotide untranslated RNA, and regulates rpoS translation (Sledjeski *et al*. 2001). Our results suggested that Hfq and CsdA interact only when RNA is present. Therefore, it was tested whether Hfq and CsdA could interact via DsrA and/or rpoS RNAs. For that purpose the co-immunoprecipitation method was used. MN treated and untreated proteins were used as described before. Surprisingly the results showed that Hfq_{wt} and CsdA are not associated even when DsrA and RpoS were added, neither when the untreated (Fig. 15, lane 3) (Fig. 16, lane 3) nor when the MN treated (Fig. 15, lane 5) (Fig. 16, lane 5) protein fractions were used.

6.4 The C-terminal part of CsdA is necessary for association with Hfq

We could show that the proteins CsdA and Hfq do not interact through direct protein-protein interactions. As these results suggested that the association between CsdA and Hfq is mediated by RNA, we were interested to elucidate which region of the CsdA protein is essential for the association with Hfq. A protein-ligand interaction site analysis of the CsdA protein was done by Professor R. Konrat, University of Vienna. For that purpose the binding domains of CsdA-related proteins were compared and potential ligand binding domains for CsdA were calculated. Ligand binding sites were predicted in the N-terminal as well as in the C-terminal region of CsdA (Fig. 17). Due to these results we assumed that the C-terminal part of CsdA could be necessary for the interaction with Hfq. Therefore, a CsdAΔ protein (45 kDa), which is a truncated version of the full length CsdA protein and lacks most of the C-terminal extension, was used. It was shown that the CsdAΔ protein possesses ATPase activity but reduced RNA helicase activity when compared to full-length CsdA (Turner *et al*., 2007).

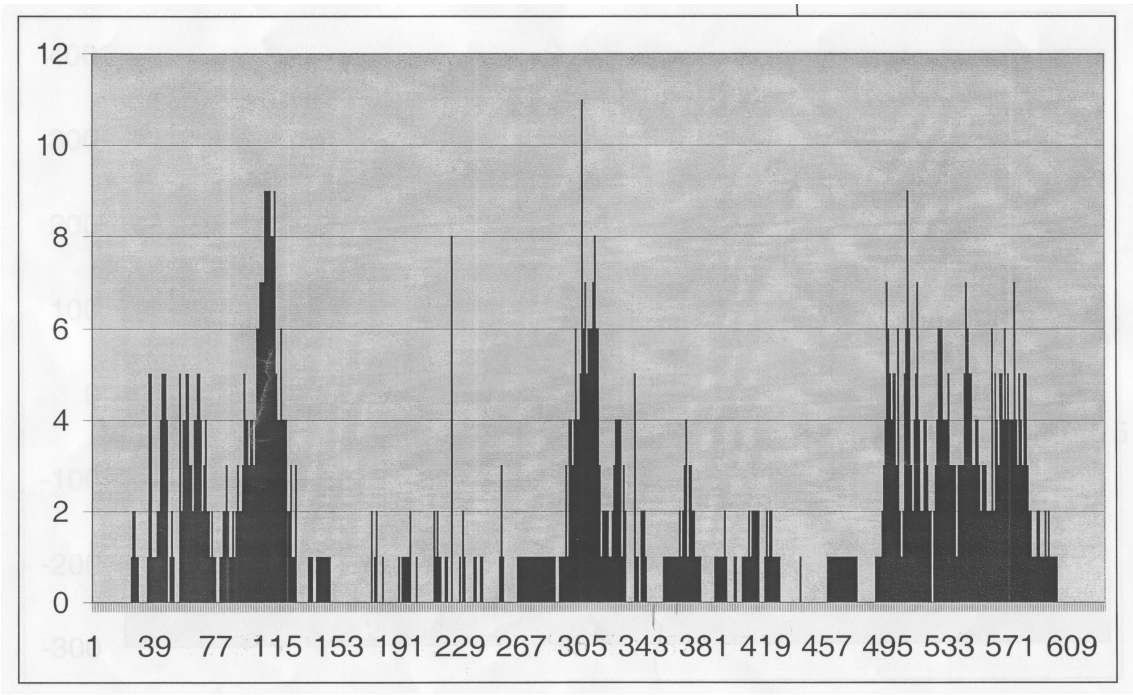

Figure 17: Meta structure based prediction of protein-ligand binding sites for CsdA.
The ligand interaction site analysis for CsdA was done by R. Konrat, University of Vienna. The program provides a protein-ligand interaction probability on a per residue basis. Ligand binding sites are predicted at the N-terminus, the C-terminus and between aa residues 280-340. The probability for ligand binding sites is mediated by arbitrary units.

For the *in vitro* protein interaction analysis between Hfq and CsdA or CsdAΔ the Far western-blotting and co-immunoprecipitation were used. The results from the Far western-blot experiment are shown in Figure 18. In the absence of nuclease treatment Hfq interacted with CsdA (Fig. 18, lane 1 and 2) but not with CsdAΔ (Fig. 18, lane 3 and 4). Thus, our results indicated that the RNA mediated interaction between Hfq and CsdA requires the C-terminus of CsdA, i.e. the C-terminal RNA binding sites of CsdA (Cordin *et al*., 2006).

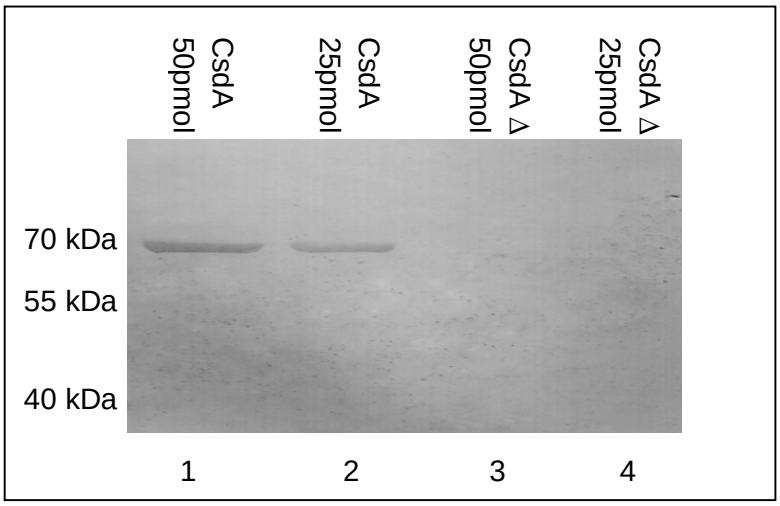

Figure 18: Hfq-CsdAΔ interaction analysis by Far western-blotting.
50 pmol CsdA (lane 1) and 25 pmol CsdA (lane 2) were loaded. CsdAΔ was loaded in lane 3 (50 pmol) and 4 (25 pmol). The membrane was incubated with purified 300 pmol Hfq. For detection of Hfq the anti-Hfq-antibody was used.

To verify the results from Far western-blotting showed in Fig. 18 a co-immunoprecipitation experiment was done with N-terminally His_6-tagged CsdA or CsdAΔ proteins and untagged Hfq. The co-IP experiments were performed with both anti-Hfq-antibody and anti-His_6-antibody as described in Materials and Methods (5.4.1). The immunoprecipitates were resuspended in SDS-PAGE buffer and analysed by western-blotting. Figure 19 shows that CsdA did co-immunoprecipitate with Hfq_{wt} (lane 2) but CsdAΔ did not (lane 5). This indicated that CsdA interacts with Hfq and that the C-terminal domain of CsdA is essential for this interaction. A negative control was performed where CsdA or CsdAΔ were incubated with Dynabeads (Fig. 19, lane 3), anti-Hfq antibody (Fig. 19, lane 6) but without Hfq protein. Neither the Dynabeads nor the anti-Hfq-antibody bind CsdA or CsdAΔ unspecifically.

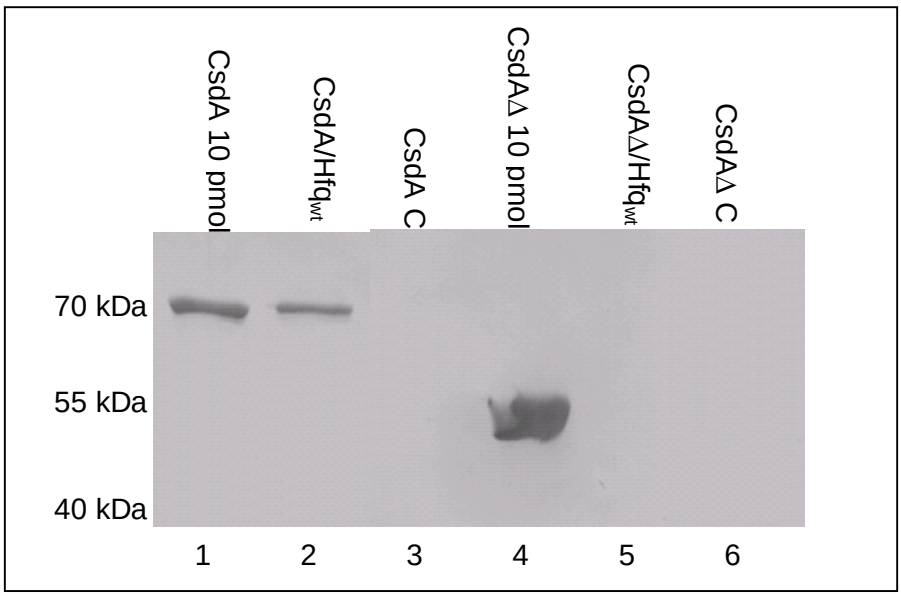

Figure 19: Hfq-CsdA/CsdAΔ interaction analysis by co-immunoprecipitation.
For the co-immunoprecipitation an anti-Hfq antibody was used. An anti-His_6 antibody was used for the detection of CsdA or CsdAΔ. The co-immunoprecipitates were separated on a 10 % SDS-polyacrylamide gel. 15 pmol of His_6-CsdA (lane 1) or His_6-CsdAΔ (lane 4) were loaded as a positive control for the antibody. The complex of Hfq with CsdA was loaded in lane 2 and Hfq with CsdAΔ in lane 5. Lane 3 and 6, negative controls (see text).

In addition the same experiment was done in the reverse manner. In the reciprocal co-IP experiment using a His_6-specific antibody, Hfq was shown to co-immunoprecipitate with CsdA (data not shown) but not with CsdAΔ (data not shown).

7 Discussion

Recently, Hfq was reported to be associated with a large number of proteins. Over 50 different proteins were detected by liquid chromatography-tandem mass spectrometry (LC-MS) or MALDI-TOF (gel-based peptide mass fingerprinting using matrix assisted laser desorption/ionization-time-of-flight mass spectrometry) as interaction partners of Hfq, including CsdA and ribosomal protein S1 (Butland *et al*., 2005). However, a direct protein-protein interaction between Hfq and CsdA or S1 was not demonstrated.

In addition, other studies suggested an interaction of Hfq with components of the ribosome, degradosome and other cellular machines that are involved in RNA metabolism. In particular, Hfq was suggested to interact with S1 (Sukhodolets *et al*., 2003), CsdA (Butland *et al*., 2005), PNP and PAP I (Mohanty *et al*., 2004) and RNase E (Morita *et al*., 2006). In contrast to the in vitro experiments performed in this study, Mohanty and colleagues analysed proteins which co-purified with PNP and PAP I, and one of the co-purified proteins was Hfq. These experiments have been performed in the presence of nucleases but it remains ambiguous whether the RNA was indeed removed. Thus, the co-purified proteins could be part of large RNA-protein complexes. Therefore it remains unclear whether Hfq interacts by direct protein-protein interactions with PNP or PAP I, or whether other unknown proteins and/or RNA are involved in the interaction between Hfq and PNP or PAP I. Recently the interaction of RNase E and Hfq was challenged. Highly purified Hfq did not interact with RNase E, whereas Hfq samples containing RNAs where found to interact with RNase E. In summary these results showed that the interaction between Hfq and RNase E is RNA depedent (Worrall *et al*., 2008).

In contrast to the results of Butland *et al*. (2005) the results of this work suggest that Hfq does not physically interact with S1 (Fig. 10A, 11, 12) nor with CsdA (Fig. 14, 15, 16). In contrast to Butland *et al*. (2005), purified proteins were used for the protein-protein interaction studies. The purified proteins were then treated with micrococcal nuclease (MN) to remove residual RNA. The co-immunoprecipitation studies showed that Hfq bound to CsdA in the presence of

RNAs (Fig. 16, lane 2). However, after micrococcal nuclease digestion and thus removal of ribonucleotides this interaction was lost (Fig. 16, lane 4). These results were confirmed by far western-blotting (Fig. 14). Thus, the interaction between Hfq_{wt} and CsdA is not a direct protein-protein interaction, but the proteins seem to be associated via RNAs.

Computer algorithms predicted ligand binding sites at the C-terminus of CsdA (Fig. 17). One known RNA binding site of CsdA resides in the C-terminal region (Cordin *et al*., 2006). Therefore we were interested to elucidate whether the C-terminal part of CsdA is necessary for the RNA-mediated interaction with Hfq. For that purpose a truncated version of the full length CsdA protein (CsdAΔ, aa residues 1-444), which lacks most of the C-terminus was used. The ATPase and helicase activities of the full length and truncated CsdA proteins were indistinguishable (Bizebard *et al*., 2004). In comparison to the interaction between CsdA and Hfq (Fig. 19, lane 2), no interaction between the truncated CsdA protein and Hfq was detected, even though the proteins were not treated with nucleases (Fig. 19, lane 6). Based on these results it can be assumed that the C-terminal region of CsdA is necessary for the RNA mediated interaction with Hfq.

8 Abbreviations

aa	Amino Acid
AP	Alkaline Phosphatase
ATP	Adenosine Tri-Phosphate
DNA	Deoxyribonucleic Acid
C	Carbon
$CaCl_2$	Calcium Chloride
CTD	C-terminal Domain
CG	Central Globular
Co-IP	Co-Immunoprecipitation
CsdA	Cold shock DEAD-box protein A
Da	Dalton
DTT	Dithiotreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
FRET	Fluorescence Resonance Energy Transfer
g	Gravitational Acceleration, g ~ 9.78 m/s^2
h	Hour
HCl	Hydrochloric Acid
$Hfq_{wt65}1$	$Hfq_{wt/65}$ Monomer
$Hfq_{wt/65}6$	$Hfq_{wt/65}$ Hexamer
IPTG	Isopropyl-β-D-thiogalactopyranoside
IMM	Imidazole
k	Kilo
LA	Long Arm
LB	Luria-Bertani Medium
MgOAc	Magnesium Acetate
$MgCl_2$	Magnesium Chloride
ml	Milliliter
mM	Millimolar – Millimol / Liter
M	Molar – mol / Liter
MN	Micrococcal Nuclease
m-RNA	Messenger RNA

N	Nitrogen
NH_4Cl	Ammonium Chloride
NaCl	Sodium Chloride
NaH_2PO_4	Sodium-dihydrogen-phosphate
nc-RNA	non coding RNA
µg	Micro Gramm
µl	Micro Liter
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Potential Hydrogenii
PM	Protein Marker
PMSF	Phenylmethylsulfonylflourid
r-RNA	ribosomal RNA
RBS	Ribosomal binding site
RNA	Ribonucleic Acid
rpm	Revolutions per Minute
RT	Room Temperature
SA	Short Arm
S. aureus	*Staphylococcus aureus*
SD	Shine Dalgarno
SDS-PAGE	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
SF2	Super Family 2
U	Unit
UTR	Untranslated Region

9 Appendix

9.1 Buffers

Lysis Buffer (Hfq)	50 mM Tris/HCl, pH = 8.0, 250 mM $MgCl_2$, 1.5 mM EDTA, 5mM DTT, 0.5 mM PMSF
Wash Buffer (Hfq)	50 mM Tris/HCl, pH = 8.0, 1.5 M NaCl, 1.5 M ammonium sulphate
Elution Buffer (Hfq)	50 mM Tris/HCl, pH = 8.0
Hfq Storage Buffer	50 mM Tris/HCl, pH = 8.0, 200 mM NaCl
Lysis Buffer (CsdA/S1)	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 IMM
Wash Buffer (CsdA/S1)	500 mM NaCl, 50 mM NaH_2PO_4 pH = 8.0, 20 mM IMM
Elution Buffer (CsdA/S1)	500 mM NaCl, 50 mM NaH_2PO_4 pH = 8.0, 60-500 mM IMM
CsdA Storage Buffer	1 M NaCl, 20 mM Tris-HCl pH = 7.5, 0.1 mM EDTA, 1 mM DTT
VD Buffer	10 mM Tris-HCl pH = 7.4, 6 mM NH_4Cl, 10 mM MgOAc, 0.1 mM EDTA, 1 mM DTT
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

9.2 Antibodies

Antibody	Source	Concentration used
Anti-Hfq antibody		1:10.000
Anti-His_6-tag antibody		1:5.000
Anti-mouse antibody	Sigma	1:10.000
Anti-rabbit antibody	Sigma	1:10.000

10 References

Achsel, T., Stark, H., and Lührmann, R. (2001). The Sm domain is an ancient RNA-binding motif with oligo(U) specifity. *Proc. Natl Acad. Sic. USA* **98**: 3685-3689

Aliprandi, P., Sizun, C., Perez, J., Mareuil, F., Caputo, S., Leroy, J.L., Odaert, B., Laalami, S., Uzan, M. and Bontems, F. (2008). S1 ribosomal protein functions in translation initiation and ribonuclease RegB activation are mediated by similar RNA/protein interactions. An NMR and SAXS analysis. *J. Biol. Chem*. **283**: 13289-13301

Arluison, V., Derreumaux, P., Allemand, F., Folichon, M., Hajnsdorf, E., and Regnier, P. (2002). Structural modelling oft the Sm-like protein Hfq from Escherichia coli. *J Mol Biol* **320**: 705-712

Arluison, V., Hong, S., Roy, R., Pellegrini, O., Gingery, M., Régnier, P., and Ha, T. (2007). Spectroscopic observation of RNA chaperone activities of Hfq in post-transcriptional regulation by a small non-coding RNA. *Nucleic Acid Research* **35**(3): 999-1006

August, J.T., Ortiz, P.J., and Hurwitz, J. (1962). Ribonucleic Acid-dependent Ribonucleotide Incorporatio. *The Journal of Biological Chemistry* **237**(12): 3786-3793

Awano, N., Xu, C., Ke, H., Inoue, K., Inouye, M., and Phadtare S. (2007). Complementation analysis of the cold-sensitive phenotype of the *Escherichia coli csdA* deletion strain. *J. Bacteriol.* **189**(16): 5808-5815

Azam, T.A., Hiraga, S., and Ishihama, A. (2000). Two types of localization of the DNA-binding proteins within the *Eschrichia coli* nucleoid. *Genes Cells* **5**: 613-626

Bizebard, T., Ferlenghi, I., Iost, I., and Dreyfus, M. (2004). Studies on three *E. coli* DEAD-Box helicases point to an unwinding Mechanism different from that of model DNA helicases. *Biochemistry* **43**: 7857-786

Boni, I.V., and Budowsky, E.I. (1982). Ribosomal protein S1 associates with the *Escherichia coli* ribosomal 30S subunit by means of protein-protein interactions. *Eur J Biochem.* **121**: 371-176

Boni, I.V., Isaeva, D.M., Musychenko, M.L., and Tzareva, N.V. (1991). Ribosome-messenger recognition: mRNA target sites for ribosomal protein S1. *Nucleic Acid Res* **19**(1): 155-162

Boni,I.V., Artamonova, V.S. and Dreyfus M. (2000). The last RNA-Binding Repeat of the *Escherichia coli* Ribosomal Protein S1 is Specifically Involved in Autogenous Control. *J. Bacteriol* **182**(20): 5872-5879

Boni, I.V., Artamonova, V.S., Tzareva, N.V., and Dreyfus, M. (2001). Non-canonical mechanism for translational control in bacteria: synthesis of ribosomal protein S1. *EMBO J.* **20**(15): 4222-4232

Brennan, R.G., and Link, T.M. (2007). Hfq structure, function and ligand binding *Sience Direct* **10**: 1-9

Brescia, C.C., Mikulecky, P.J., Feig, A.L., and Sledjeski, D.D. (2003). Identification of the Hfq-binding site on DsrA RNA: Hfq binds without altering DsrA secondary structure. *RNA* **9**: 33-43

Butland, G., Peregrin-Alvarez, J.M., Li, J., Yang, W., Yang, X., Ganadlen, V., Starostine, A., Richards, D., Beattie, B., Krogan, N., Davey, M.,Parkinson, J., Greenblatt, J., and Emili, A. (2005). Interaction network containing conserved and essential protein complexes in *Escherichia coli*. *Nature* **433**: 531-537

Charollais, J., Pflieger, D., Vinh, J., Dreyfus, M., and Iost, I. (2003). The DEAD-box RNA helicase SrmB is involved in the assembly of 50S ribosomal subunit in *Escherichia coli*. *Mol. Microbiol.* **48**: 1253-1265

Charollais, J., Dreyfus, M., Iost, I. (2004). CsdA, a cold-shock RNA helicase from *Escherichia coli*, is involved in the biogenesis of 50S ribosomal subunit. *Nucleic Acids Res*. **32**: 2751–2759

Clodi, E., Semrad, K. and Schroeder, R. (1999). Assaying RNA chaperone activity *in vivo* using a novel RNA folding trap. *EMBO J*. **13**: 3776-3782

Cohen, S.N. (1995). Surprises at the 3' end of prokaryotic RNA. *Cell* **80**(6): 829-832

Cordin, O., Banroques, J., Tanner, N.K., and Linder, P. (2006). The DEAD-box protein family of RNA helicases. *Gene* **367**: 17-37

DuBow, M.S., Ryan, T., Young, R.A., and Blumenthal, T. (1977). Host factor for coliphage Q beta RNA replication: presence in prokaryotes and association with the 30S ribosomal subunit in Escherichia coli. *Mol General Genet* **153**: 39-43

Farwell, M.A., Roberts, M.W., and Rabinowitz, J.C. (1992). The effect of ribosomal protein S1 from *Escherichia coli* and *Micrococcus luteus* on protein synthesis *in vitro* by *E. coli* and *Bacillus subtilis*. *Mol Microbiol* **6**(22): 3375-3383

De Fernandez, F.MT., Eoyang, L., and August, J.T. (1968). Factor fraction required fort he synthesis of bacteriophage Qβ-RNA. *Nature* **219**: 588-590.

Giorginis, S., and Subramanian, A.R. (1980). The major ribosome binding site of *Escherichia coli* ribosomal protein S1 is located in its N-terminal segment. *J. Mol. Biol.* **141**: 393-408

Gold, L.,Pribnow, D., Schneider, T., Shinedling, S., Singer, B.S., and Stormo, G. (1981). Translational initiation in prokaryotes. *Annu Rev Microbiol.* **35**: 365-403

Gold, L. (1988). Posttranscriptional regulatory mechanisms in *Escherichia coli*. *Annu Rev Biochem.* **57**: 199-233

Herschlag, D. (1995). RNA chaperones and the RNA folding problem. *J. Biol. Chem.* **270**: 20871-20874

Iost, I., and Dreyfus, M. (1994). mRNAs can be stabilized by DEAD-box proteins. *Nature* **372**: 193-196

Jones, P.G., Mitta, M., Kim, Y., Jiang, W., and Inouye, M. (1996). Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **93**: 76-80

Kajitani, M., Kato, A., Wada, A., Inokuchi; Y., and Ishihama, A., (1994). Regulation of the Eschrichia coli *hfq* gene encoding the host factor for phage Q-**Beta**. *J Bacteriol* **176**: 531-534

Kalman, M., D.R. Gentry, M. Cashel (1991). Characterization of the *Escherichia coli* K12 gltS glutamate permease gene. *Mol.Gen.Genet.* **225**:379-386

Kambach, C. *et al.* (1999). Cristal structure of two Sm protein complexes and their implication for the assembly of the spliceosomal snRNPs. *Cell* **96**: 375-387

Khemici, V., Toesca, I., Poljak, L., Vanzo, N.F., Carpousis, A.J. (2004). The RNase E of *Escherichia coli* has at least two binding sites for DEAD-box RNA helicases: Functional replacement of RhlB by RhlE. *Mol. Microbiol.* **54**:1422–1430.

Lu, J., Aoki, H., and Ganoza, M.C. (1999). Molecular characterization of a prokaryotic translation factor homologous to the eukaryotic initiation factor eIF4A. *Int. J. Biochem. Cell. Biol.* **31**: 215-229

Miczak, A., V.R. Kaberdin, C.L. Wei, S. Lin-Chao (1996). Proteins associated with RNase E in a multicomponent ribonucleolytic complex. *Proc.Natl.Acad.Sci.USA* **93**:3865-3869

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

Miller, J.H. (1972). Experiments in molecular Genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Mohanty, B.K and Kushner, S.R. (2000). Polynucleotide phosphorylase functions both as a 3′ → 5′ exonuclease and a poly(A) polymerase in *Escherichia coli. PNAS* **97**(22): 11966-11971

Mohanty, B.K., Maples, V.F., and Kushner, S.R. (2004). The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*. *Mol Microbiol* **54**: 905-920

Moll, I., Grill, S., Grundling, A., and Bläsi, U. (2002). Effects of ribosomal protein S1, S2 and the DeaD/sdA DEAD-box helicase on translation of leaderless and canonical mRNAs in *Escherichia coli*. *Mol. Microbiol.* **44**: 1387-1396

Moll, I., Leitsch, D., Steinchauser, T., and Bläsi, U. (2003). RNA chaperon activity oft he Sm-like Hfq protein. *EMBO Rep* **4**: 284- 289

Morita, T., Maki, K., and Aiba, H. (2005). RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial non coding RNAs. *Genes Dev* **19**: 2176-2186

Morita, T., Mochizuki, Y., and Aibat, H. (2006). Translational repression is sufficient for gene silencing by bacterial small non coding RNAs in the absence of mRNA destruction. *Proc Natl Acad Sci USA* **103**: 4858-4863

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

Muniyappa, K., and Mythili, E. (1993). Phage lambda beta protein, a component of general recombination, is associated with host ribosomal S1 protein. *Biochem Mol Biol Int* **31**(1): 1-11

Muralikrishna, P., and Suryanarayana, T. (1985). *Biochem Int* **11**: 691-699

Nierhaus, K.H. (1991). The assembly of prokaryotic ribosomes. *Biochemie* **73**: 739-755

Nishi, K., F. Morel-Deville, J.W.B. Hershey, T. Leighton, J. Schnier (1988). An eIF-4A-like protein is a suppressor of an *Escherichia coli* mutant defective in 50S ribosomal subunit assembly. *Nature* 336:496-498

Eric M. Phizicky and Stanley Fields (1995). Protein-Protein Interactions: Methods for Detection and Analysis. *Microbiol. Rev.* **59**

Prud'homme-Genereux, A., Beran, R.K., Iost, I., Ramey, C. S., Mackie, G. A., and Simons, R.W. (2004). Physical and functional interactions among RNase E, polynucleotide phosphorylase and the cold-shock protein, CsdA: evidence for a 'cold shock degradosome'. *Mol. Microbiol.* **54**:1409-1421

Rajkowitsch, L. and Schroeder, R. (2007). Dissecting RNA chaperone activity. *RNA* **13**: 1-8

Schumacher, M.A., Pearson, R.F., Møller, T., Valentin-Hansen, P., and Brennan, R.G. (2002). Structures of the pleitropic translational regulator Hfq and Hfq-RNA complex: a bacterial Sm-like protein. *EMBO J.* **21**: 3546-3556.

Schwartz, C.J.E., Sampson, H.M., Hlousek, D., Percival-Smith, A., Copeland, J.W.R. and Krause, H.M. (2001) FTZ-Factor1 and Fushi tarazu interact via conserved nuclear receptor and coactivator interaction motifs. *EMBO J.,* **20**: 510-519*.*

Semrad, K. and Schroeder, R. (1998) A ribosomal function is necessary for efficient splicing of the T4 phage thymidylate synthase intron *in vivo*. *Genes Dev*., **12**: 1327-1337.

Sengupta, J., Agrawal, R.K., and Frank, J. (2001). Visualization of protein S1 within the 30S ribosomal subunit and its interaction with messenger RNA. *PNAS* **98**(21): 11991-11996

Sillers, I.Y., and Moor, P.B. (1982). Position of protein S1 in the 30S ribosomal subunit of *Escherichia coli*. *J. Mol. Biol.* **153**: 761-780

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

Sonnleitner, E., Moll, I., and Bläsi, U. (2002). Functional replacement of the *Escherichia coli hfq* gene by the homologue of *Pseudomonas aeruginosa*. *Microbiology* **148**: 883-891

Sonnleitner, E., Napetsching, J., Afonyushkin, T., Ecker, K., Večerek, B., Moll, I., Kaberdin, V.R., and Bläsi, U. (2004). Functional effects of variants of the RNA chaperone Hfq. *Biochem. Biophys. Res. Commun.* **323**: 1017-1023

Sonnleitner, E., Schuster, M., Sorger-Dmenigg, T., Greenberg, E.P., and Bläsi, U. (2006). Hfq-dependent alterations oft he transcriptome profile and effects on quorum sensing in *Pseudomonas aeruginosa*. *Molecular Microbiology* **59**(5): 1542-1558

Sørensen, M.A., Fricke, J., and Pedersen, S. (1998). Ribosomal protein S1 is required for translation of most, if not all, natural mRNAs in *Escherichia coli*. *J. Mol. Biol.* **280**: 561-569

Subramanian, A.R., and van Duin, J. (1977). Exchange of individual ribosomal proteins between ribosomes as studied by heavy isotope-transfer experiments. *Mol. Gen. Genet.* **258**: 1-9

Subramanian, A.R. (1983). Structure and functions of ribosomal protein S1. *Prog Nucleic Acid Res Mol Biol.* **28**: 101–142.

Sukhodolets, M.V., and Garges, S. (2003). Interaction of *Escherichia coli* RNA polymerase with the ribosomal protein S1 and the Sm-like ATPase Hfq. *Biochemistry* **42**: 8022-8034

Sukhodolets, M.V., Garges, S., and Adhya, S. (2006) Ribosomal protein S1 promotes transcriptional cycling. *Rna* **12**(8): 1505-1513

Szer, W., Hermoso, J.M., and Leffler, S. (1975). Ribosomal protein S1 and polypeptide chain initiation in bacteria. *Proc. Natl. Acad. USA* **72**: 2325-2329

Tedin, K., Resch, A., and Bläsi, U. (1997). Requirements for ribosomal protein S1 for translation initiation of mRNAs with and without a 5'-leader sequence. *Mol. Microbiol.* **25**: 189-199

Toone, W.M., Rudd, K.E., and Friesen, J.D. (1991). deaD, a new *Escherichia coli* gene encoding a presumed ATP-dependent RNA helicase, can suppress a mutation in *rpsB*, the gene encoding ribosomal protein S2. *J. Bacteriol.* **173**: 3291-3302

Toro, I. *et al*. (2001). RNA binding in an Sm core domain: X-ray structure and function analysis of an archeal Sm protein complex. *EMBO J.* **20**: 2293-2303

Tsuchihashi, Z. and Brown, P.O. (1994). DNa strand exchange and selective DNA annealing promoted by the human immunodeficiency virus type 1 nucleocapside protein. *J. Virol.* **68**: 5863-5870

Turner, A.M.W., Love, C.F., Alexander, R.W., and Jones, P.G. (2007). Mutational analysis of the *Escherichia coli* DEAD box protein CsdA. *J Bcteriol* **189**(7): 2769-2776

Udekwu, K.I., Darfeuille, F., Vogel, J., Reimegård, J., Holmqvist, E., and Wagner, E.G.H. (2005). Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Genes Dev.* **19**: 2355-2366

Valenti-Hansen, P., Eriksen, M., and Udesen, C. (2004). The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Molecular Microbiology* **51**: 1525-1533

Vassilieva, I.M., Rouzanov, M.V., Zelinskaya, N.V., Moll, I., Bläsi, U., and Garber, M.B. (2002). Cloning, purification, and crystallization of a bacterial gene expression regulator Hfq protein from Escherichia coli. *Biochemistry (Moscow)* **67**: 1293-1297

Večerek, B., Moll, I., Afonyushkin, T., Kaberdin, V., and Bläsi, U. (2003). Interaction of the RNA chaperon Hfq with mRNAs: direct and indirect roles of Hfq in iron metabolism of *Escherichia coli*. *Mol. Microbiol.* **50**(3): 897-909

Večerek, B., Moll, I., and Bläsi, U. (2005). Translational autocontrol of the *Escherichia coli hfq* RNA chaperone gene. *RNA* **11**: 976-984

Večerek, B., Rajkowitsch, L., Sonnleitner, E., Schröder, R., and Bläsi, U. (2007). The C-terminal domain of Eschrichia coli Hfq is required for regulation. *Nucleic Acid Research* **36**(1): 133-143

Vytvytska, O., Jakobsen, J.S., Balcunaite, G., Andersen, J.S., Baccarini, M., and Gabain, A. (1998). Host factor I, Hfq, binds to *Escherichia coli ompA* mRNA in a growth rate-dependent fashion and regulates its stability. *Proc Natl Acad Sci USA* **95**: 14118-14123

Vytvytska, O., Moll, I., Kaberdin, V.R., Gabain, A.V., and Blasi, U. (2000). Hfq (HF1) stimulates *ompA* mRNA decay by interfering with ribosome binding. *Genes & Dev.* **14:** 1109–1118.

Wahba, A.J., Miller, M.J., Niveleau, A., Landers, T.A., Carmichael, G.G., Weber, K., Hawley, D.A., and Slobin, L.I. (1974). Inhibition of synthetic and natural messenger translation. I. Purification and properties of a protein isolated from *Escherichia coli* MRE 600 ribosomes. *J. Biol Chem* **249**(10): 3314-3316

Wasserman, K.M., Repoila, F., Rosenow, C., Storz, G., and Gottesman, S. (2001). Identification of novel small RNAs using comparative genomics and microarrays. *Genes Dev* **15**: 1637-1651

Williamson, J.R. (2003). After the ribosome structure: how are the subunits assembled?. *RNA* **9**: 165-167

Worrall, J.A.R., Górna, M., Crump, N.T., Phillips, L.G., Tuck, A.C., Price, A.J., Bavro, V.N. and Luisi, B.F. (2008). Reconstitution and analysis of the multienzyme *Escherichia coli* RNA degradosome. *J. Mol. Biol*. **382**: 870-883

Zhang, A., Altuvia, S., Tiwari, A., Argaman, L., Hengge-Aronis, R., and Storz, G. (1998). The OxyS regulatory RNA represses *rpoS* translation and bind the Hfq (HF-I) protein. *EMBO J* **17**: 6061-6068

Zhang, A., Wasserman, K.M., Ortega, J., Steven, A.C., and Storz, G. (2002). The Sm-like Hfq protein increases OxyS RNA interaction with target mRNAs. *Mol Cell* **181**: 4639-4643

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