# Coincident Hfq binding and RNase E cleavage sites on mRNA and small regulatory RNAs

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## ABSTRACT

**The *Escherichia coli* RNA chaperone Hfq was discovered originally as an accessory factor of the phage Qβ replicase. More recent work suggested a role of Hfq in cellular physiology through its interaction with *ompA* mRNA and small RNAs (sRNAs), some of which are involved in translational regulation. Despite their stability under certain conditions, *E. coli* sRNAs contain putative RNase E recognition sites, that is, A/U-rich sequences and adjacent stem–loop structures. We show herein that an RNase E cleavage site coincides with the Hfq-binding site in the 5′-untranslated region of *E. coli ompA* mRNA as well as with that in the sRNA, DsrA. Likewise, Hfq protects RyhB RNA from in vitro cleavage by RNase E. These in vitro data are supported by the increased abundance of DsrA and RyhB sRNAs in an RNase E mutant strain as well as by their decreased stability in a *hfq*$^-$ strain. It is commonly believed that the RNA chaperone Hfq facilitates or promotes the interaction between sRNAs and their mRNA targets. This study reveals another role for Hfq, that is, protection of sRNAs from endonucleolytic attack.**

**Keywords: Hfq; *ompA* mRNA; RNase E; RNA stability; small regulatory RNAs**

## INTRODUCTION

Although known for more than 30 yr as being required for *Escherichia coli* phage Qβ replication (Franze de Fernandez et al. 1968), the Hfq protein has recently received renewed attention owing to its multiple functions in cellular physiology. Most data on Hfq–RNA interactions stem from studies on small *E. coli* RNAs (sRNAs). Hfq binds to OxyS, DsrA, RprA, RyhB, and Spot42 RNAs, as well as to other sRNAs, the function of which is as yet unknown (Zhang et al. 1998, 2002; Sledjeski et al. 2001; Wassarman et al. 2001; Møller et al. 2002). Some sRNAs are involved in translational regulation, and Hfq has been suggested to facilitate their interaction with target mRNAs. Hfq stimulates both the interaction of spot42 RNA with *galK* mRNA (Møller et al. 2002) and that of OxyS with *fhlA* mRNA (Zhang et al. 2002). RyhB RNA, which is negatively controlled by Fur, seems to down-regulate iron storage as well as iron-containing proteins (Massé and Gottesman 2002), and thereby has an important function in establishing priorities in iron usage. DsrA stimulates and represses translation of the *E. coli rpoS* and *hns* mRNAs (Lease et al. 1998; Majdalani et al. 1998), respectively, and Hfq has been shown to be necessary for DsrA-mediated regulation of both *rpoS* and *hns* (Sledjeski et al. 2001). It seemed possible that the molecular mechanism by which Hfq brings about these interactions entails unfolding of DsrA. However, a recent study revealed that Hfq does not alter the secondary structure of DsrA (Brescia et al. 2003).

In contrast to several sRNAs, only one Hfq–mRNA interaction has been studied in more detail. Hfq was shown to destabilize the *ompA* mRNA encoding the outer membrane protein A (Vytvytska et al. 1998), and a closer examination revealed that Hfq exerts this destabilizing effect on *ompA* mRNA by counteracting the protective role of initiating ribosomes in blocking the decay initiating RNase E cleavages in the 5′-untranslated region (UTR) (Vytvytska et al. 2000; Fig. 1A). Thus, the Hfq-mediated *ompA* mRNA decay results from a lack of translation. We have recently reported that Hfq induces structural changes in and in the vicinity of the *ompA* ribosome binding site (RBS), explaining its negative effect on 30S ribosome binding (Moll et al. 2003). Moreover, these structural changes were shown to prevail upon proteolytic removal of Hfq, demonstrating that the

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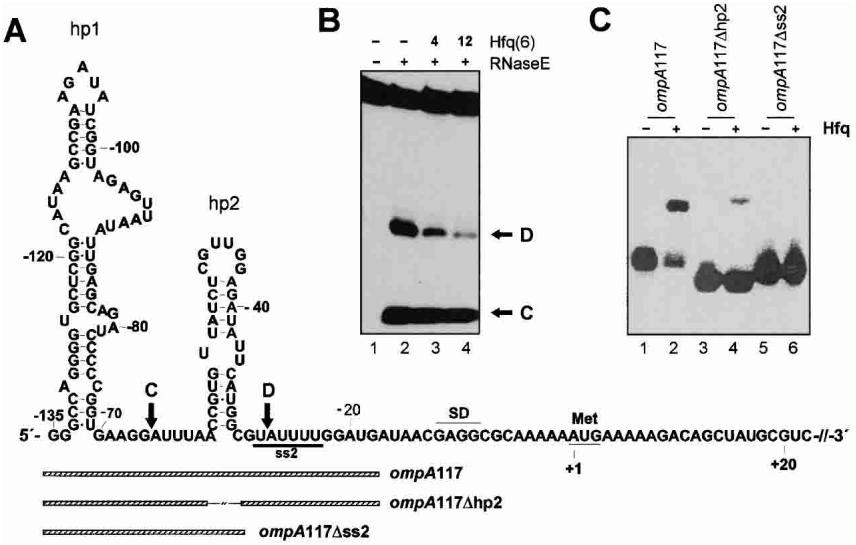


**FIGURE 1.** Hfq protects *ompA* mRNA from RNase E cleavage at site D. (*A*) Primary structure of the 5′UTR of *ompA*180 mRNA with the 5′-terminal stem–loop structures (hp1 and hp2) and the 5′-initial coding region (structure III, in Rosenbaum et al. 1993). The mRNA contains the 5′UTR and the first 45 nucleotides of the *ompA* gene. The A/U-rich sequence ss2 is depicted by a bar. The SD sequence, as well as the start codon (Met), are indicated by bars and the positions of the RNase E cleavage sites C and D are depicted by arrows. The *ompA*117, *ompA*117Δhp2, and *ompA* 117Δss2 mRNAs used in the gel mobility-shift assays (see C) are depicted by hatched bars. (*B*) In vitro cleavage of *ompA*180 mRNA in the presence or absence of Hfq. [$^{32}$P]-5′-end labeled *ompA*180 mRNA was incubated in RNase E cleavage buffer at 37°C without (lane *1*) or in the presence of a degradosome preparation (lanes *2–4*). Hfq-hexamer [Hfq(6)] was added in a fourfold (lane *3*) and 12-fold (lane *4*) molar excess over *ompA*180 mRNA prior to addition of the RNase E preparation. The RNase E cleavage reaction was carried out for 30 min at 37°C. Cleavage at sites C and D (see *A*) by the degradosome preparation is indicated at *right*. (*C*) Binding of Hfq to *ompA*117, *ompA*117Δhp2, and *ompA*117Δss2 mRNAs. The 5′end-labeled mRNAs were incubated with purified Hfq protein to allow formation of the RNA–protein complex as specified in Materials and Methods, and then resolved on a 4% native polyacrylamide gel. (Lanes *1,3,5*) Electrophoretic mobility of *ompA*117, *ompA*117Δhp2, and *ompA*117Δss2 mRNAs, respectively; (lanes *2,4,6*) electrophoretic mobility of *ompA*117, *ompA*117Δhp2, and *ompA*117Δss2 mRNAs, respectively, in the presence of a fourfold molar excess of Hfq-hexamer.

protein functions as a genuine RNA chaperone on *ompA* mRNA.

Electron microscopic studies of the *E. coli* Hfq protein (Møller et al. 2002; Zhang et al. 2002), as well as X-ray crystallography of the *Staphylococcus aureus* Hfq homolog (Schumacher et al. 2002) showed that it has a hexameric ring-shaped structure, and that it belongs to the large family of Sm-like proteins which are involved in RNA processing in eukaryotic cells. These proteins bind to various RNAs, primarily recognizing short U-rich stretches, known as SM sites (Achsel et al. 2001). Hfq interacts with A/U-rich regions in OxyS RNA (Zhang et al. 2002), and an A/U-rich region in the 5′UTR of *ompA* mRNA has been implicated in Hfq binding (Vytvytska et al. 2000). Moreover, adjacent secondary structures seem to be as important for Hfq binding as a linear A/U-rich sequence (Zhang et al. 2002; Brescia et al. 2003). A/U-rich sequences together with adjacent stem–loop structures can likewise comprise recognition sites for RNase E (Mackie 1998; Kaberdin et al. 2000), which plays important roles in RNA metabolism in *E. coli*. In addition to its role in processing rRNA precursors, the enzyme introduces endonucleolytic cleavages in mRNAs, which in concert with other degradosome components, lead to rapid degradation of the transcripts (for review, see Cohen and McDowall 1997).

Here, we report that (1) Hfq binding to an A/U-rich motif in the 5′UTR of *ompA* mRNA protects from RNase E cleavage, (2) that the Hfq-binding site and the RNase E cleavage site overlap on DsrA RNA, and that (3) another sRNA, RyhB, is protected from RNase E cleavage by Hfq. We further show that the half-life of RyhB, like that of DsrA (Sledjeski et al. 2001), is decreased in a *hfq*$^{-}$ strain, and that the abundance of both, DsrA and RyhB, is increased in an *rne*$^{ts}$ mutant. These data suggest that Hfq, beside its possible role in modulating the function of sRNAs, protects them from endonucleolytic attack, which may in part account for the stability of some of the sRNAs studied so far.

## RESULTS AND DISCUSSION

### The Hfq-binding site within the 5′UTR of *ompA* mRNA overlaps with an RNase E cleavage site

The Hfq-binding sites have only been mapped on three sRNAs, DsrA (Brescia et al. 2003), OxyS (Zhang et al. 2002), and Spot42 RNA (Møller et al. 2002), and to some extent on *ompA* mRNA (Vytvytska et al. 2000; Moll et al. 2003). As shown in Figure 1A, two RNase E cleavage sites, denoted as C and D have been mapped previously in the 5′UTR of *ompA* (Melefors and von Gabain 1988). Both sites are A/U rich and are preceded or followed by a stem–loop structure. With the reasoning that RNase E cleavage sites and Hfq-binding sites may overlap, we tested whether Hfq binding to either site would affect RNase E cleavage. As shown in Figure 1B, lane 2, in the absence of Hfq, RNase E cleavage occurred at both sites C and D in *ompA*180 mRNA. Increasing amounts of Hfq added to this mRNA impeded cleavage at site D, whereas cleavage at site C was almost unaffected (Fig. 1B, lanes 3,4). This result was in agreement with a recent enzymatic footprinting study (Moll et al. 2003), wherein we showed that the A at position −27, corresponding to the 3′ nucleotide of the scissile bond at site D (Fig. 1A) is protected from RNase CV1 cleavage, which, in turn, was in accordance with an oligonucleotide protection assay that

implicated the ss2 region (Fig. 1A) in Hfq binding (Vytvytska et al. 2000). In addition, Hfq protected the G at position −44 from RNase T1 attack (Moll et al. 2003). Thus, both the ss2 sequence (Fig. 1A) and hp2 are apparently contacted by Hfq. In contrast, the two consecutive G's at positions −66 and −67, which are immediately 5′ of the RNase E cleavage site C, were not protected by Hfq from RNase T1 attack (Moll et al. 2003). This, in turn, explains why Hfq did not protect from RNase E cleavage at site C.

Protection of site D by Hfq in vitro seems, at first glance, difficult to reconcile with the decreased stability of *ompA* mRNA in a *hfq*$^+$ strain when compared with a *hfq*$^-$ strain (Vytvytska et al. 1998; 2000). In other words, Hfq binding to site D would be expected to result in stabilization rather than in the reported destabilization of the mRNA. However, we have shown that binding of either Hfq or 30S ribosomes to the *ompA* 5′UTR is mutually exclusive, and that binding of the 30S subunit protects both sites D and C from RNase E cleavage (Vytvytska et al. 2000). In contrast to the 30S subunit, binding of Hfq to site D would still leave site C vulnerable to RNase E. Therefore, these data do not affect our proposed model for the destabilizing effect of Hfq exerted on *ompA* mRNA (Vytvytska et al. 2000).

It has been reported that a primary A/U-rich sequence, albeit relevant, is not sufficient for Hfq binding (Zhang et al. 2002; Brescia et al. 2003). Given that Hfq seemed to contact at least parts of hp2, we next used different deletion constructs to test whether both the ss2 region and hp2 are required for Hfq binding. As shown before, preincubation of [$^{32}$P]-5′-end labeled *ompA*117 mRNA (Fig. 1A), comprising hp1 and hp2 as well as the 12 nucleotides downstream of hp2 (A/U-rich Sm motif of the ss2 region) with Hfq resulted in a mobility shift (Fig. 1C, lane 2) (Vytvystka et al. 2000). In contrast, Hfq bound to *ompA*117Δhp2 mRNA, (Fig. 1A) lacking hp2 and comprising the ss2 region, with a strongly reduced efficiency (Fig. 1C, lane 4). *Omp*A117Δss2, containing the *ompA* 5′UTR up to only nucleotide −28, was not sufficient to be shifted by Hfq (Fig. 1C, lane 6). Thus, both hp2 and the A/U-rich sequence of the ss2 region are apparently required for efficient recognition of the *ompA* 5′UTR. These results support the notion that both an A/U-rich sequence and adjacent stem–loop structures are important structural features for Hfq binding (Brescia et al. 2003).

## Hfq protects the small RNAs DsrA and RyhB from RNase E cleavage

As reported by Sledjeski et al. (2001) and verified in this study (data not shown), DsrA RNA has a half-life of ~30 min when chromosomally encoded, and of ~60 min when the gene is plasmid encoded. In a *hfq*$^-$ strain the half-life of chromosomally encoded DsrA RNA decreased to 1 min, and that of plasmid encoded RNA to 36 min (Sledjeski et al. 2001). We observed that the same applies to chromosomally encoded RyhB sRNA, which has a half-life of >30 min in a wild-type strain, whereas its stability is significantly decreased (15 min) in the *hfq*$^-$ strain (Fig. 2A). To examine whether protection from RNase E cleavage by Hfq can account for these observations, we determined the steady-state levels of the DsrA and RyhB RNAs in the *rne*$^{ts}$ strain N3438 (Miczak and Apirion 1993). As shown in Figure 2B, lanes 2 and 4, when compared with the *rne*$^+$ strain N3433, the steady-state levels of both DsrA and RyhB were greatly increased in the *rne*$^{ts}$ strain upon shift to the nonpermissive temperature. Moreover, the ratio between the reported full-length (F) and the truncated forms (T) of DsrA (Repoila and Gottesman 2001) is changed in the *rne*$^{ts}$ strain upon shift to the nonpermissive temperature from 1:1 to 5:1 in favor of the full-length molecule (Fig. 2B, lane 2). In fact, the in

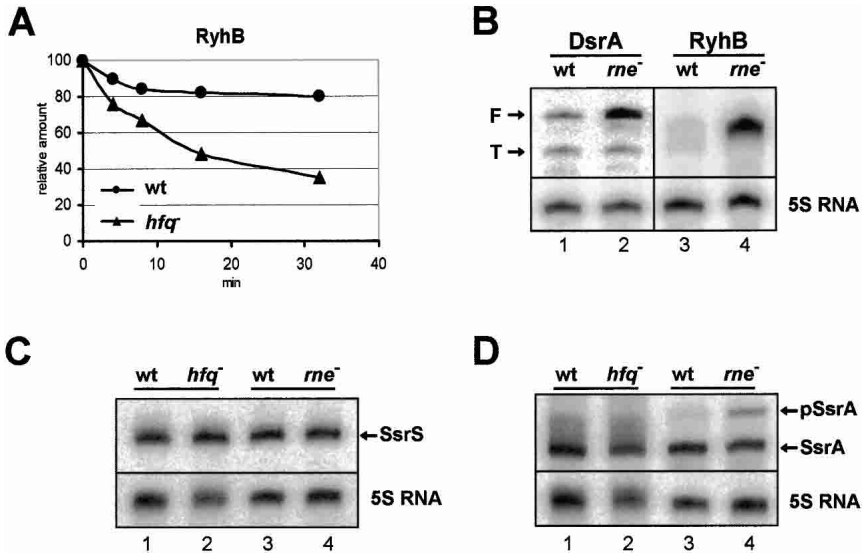


**FIGURE 2.** Effects of Hfq and RNAse E on the steady-state levels of DsrA, RyhB, SsrS, and SsrA RNAs and on the half-life of RyhB. (*A*) Hfq affects the half-life of RyhB sRNA. Graphical representation of RyhB RNA decay in an *hfq*$^+$ (●) and an *hfq*$^-$strain (▲). The Northern-blot analysis was carried out as described in Materials and Methods using a labeled riboprobe comprising nucleotides 19–60 of RyhB RNA. (*B*) The steady-state levels of both DsrA and RyhB sRNA are increased in an*rne*$^{ts}$ strain at the nonpermissive temperature. The full-length (F) and the truncated (T) forms of DsrA are indicated by arrows on the *left*. (*C*) Equal steady-state levels of SsrS RNA in the *hfq*$^-$ and *rne*$^{ts}$ strains (lanes *2,4*) when compared with the respective wild-type strains (lanes *1,3*). (*D*) The steady-state levels of SsrA RNA in the *hfq*$^-$ strain and in the *hfq*$^+$ strain are equivalent (lanes *1,2*), whereas SsrA precursor RNA accumulates upon inactivation of RNase E (cf. lanes *3,4*). (*B,C,D*, *bottom*) Detection of 5S rRNA (loading control). The Northern-blot analysis and detection of sRNAs in total RNA was performed as described in Materials and Methods.

vitro cleavage of DsrA by RNase E at nucleotide 28 (see Fig. 3A,B) agrees with the hypothesis of Repoila and Gottesman (2001), in that DsrA form T results from endonucleolytic processing.

To verify the observed effects of Hfq and RNase E on DsrA and RyhB, a variation control experiment was performed. The *hfq*$^-$ and *rne*$^{ts}$ strains and their corresponding wild-type strains were additionally probed for SsrS (6S) RNA and SsrA RNA (tm-RNA) (Brownlee 1971; Chauhan and Apirion 1989), both of which do not bind Hfq (Wassarman et al. 2001). As expected, the steady-state levels of these sRNAs were equivalent in the *hfq*$^-$ strain and in the isogenic *hfq*$^+$ strain (Fig. 2C,D, lanes 1,2). SsrA is known to be processed by RNase E (Lin-Chao et al. 1999), whereas the SsrS RNA is not (Li et al. 1998). In agreement, the levels of SsrS RNA were the same in the *rne*$^+$ and *rne*$^{ts}$ strains (Fig. 2D, lanes 3,4). In contrast, upon temperature upshift, SsrA RNA precursors accumulate in the *rne*$^{ts}$ strain (Fig. 2D, lane 4). These data show that the effects of Hfq and RNase E on DsrA and RyhB RNA are specific.

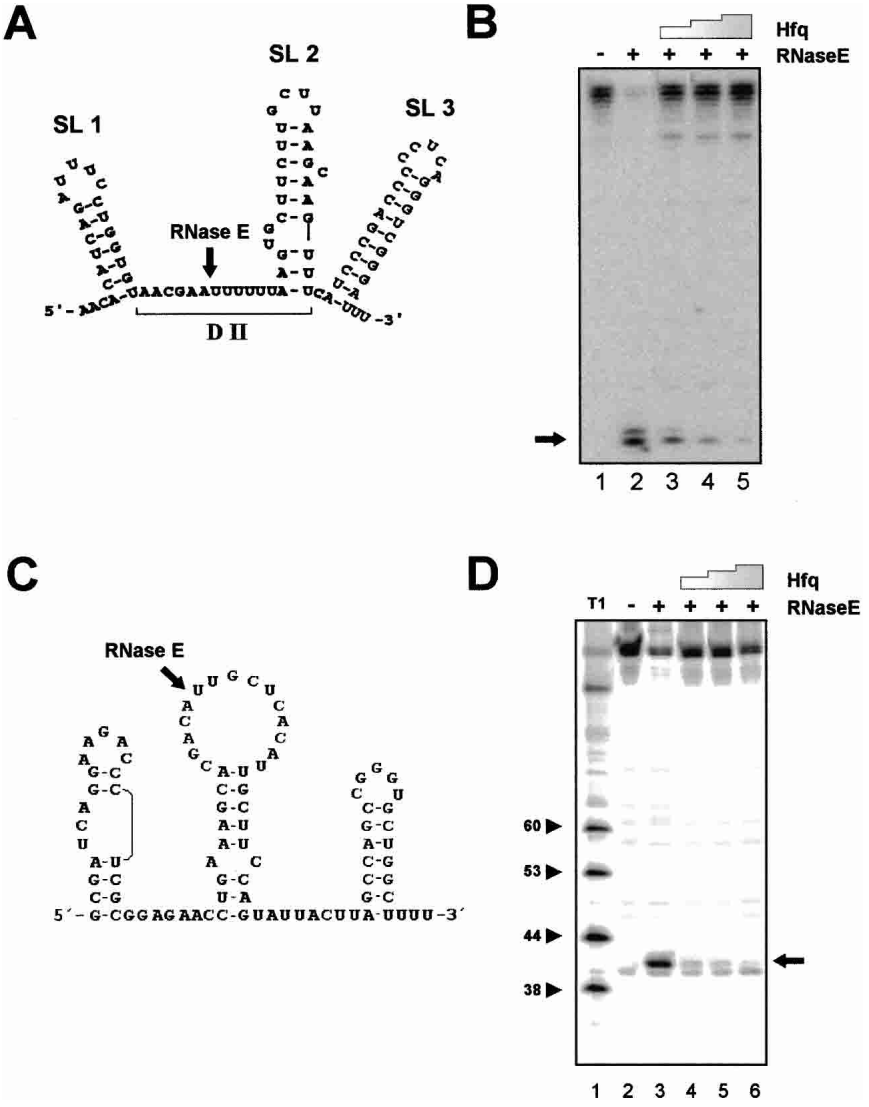


**FIGURE 3.** Hfq protects DsrA and RyhB sRNAs from RNase E cleavage. (*A*) The secondary structure of DsrA RNA was determined by Brescia et al. (2003). A ribo-oligonucleotide corresponding to Domain II (DII) inhibited binding of Hfq to DsrA (Brescia et al. 2003). The position of the RNase E cleavage site is indicated by an arrow. SL1, SL2, and SL3 denote the three stem–loop structures. (*B*) In vitro cleavage of DsrA RNA in the presence and absence of Hfq. [$^{32}$P]-5′-end labeled DsrA was incubated in RNase E cleavage buffer at 37°C without (lane *1*) or in the presence of a degradosome preparation (lanes *2–5*). Hfq-hexamer was added in a fourfold (lane *3*), 12-fold (lane *4*), and 20-fold (lane *5*) molar excess over DsrA prior to addition of the RNase E preparation. The RNase E cleavage reaction was carried out for 3 min at 37°C. (*C*) Secondary structure of RyhB RNA as revealed by Massé and Gottesman (2002) using bioinformatics. The RNase E cleavage site is indicated by an arrow. (*D*) In vitro cleavage of RyhB RNA in the presence and absence of Hfq. [$^{32}$P]-5′-end labeled RyhB was incubated in RNase E cleavage buffer at 37°C without (lane *2*) or in the presence of a degradosome preparation (lanes *3–6*). Hfq-hexamer was added in a fourfold (lane *4*), 12-fold (lane *5*), and 20-fold (lane *6*) molar excess over RyhB prior to addition of the RNase E preparation. The RNase E cleavage reaction was carried out for 3 min at 37°C. (Lane *1*) RNase T1 ladder.

As shown by Brescia et al. (2003), DsrA domain II (Fig. 3A) competes with full-length DsrA for binding to Hfq, suggesting that it comprises the Hfq-binding site. To test whether the stability of DsrA can be attributed to an overlap of the Hfq binding and RNase E cleavage site, DsrA was synthesized in vitro and subjected to in vitro RNase E cleavage in the presence or absence of Hfq. As shown in Figure 3B, RNase E cleavage occurred at nucleotide 28 in DsrA RNA (Fig. 3A). The same position along with other nucleotides between stem–loop 1 and 2 of DsrA has been shown to be protected from RNase I attack by Hfq (Brescia et al. 2003). Likewise, addition of Hfq prior to addition of RNase E protected from cleavage at nucleotide position 28 in a concentration-dependent manner (Fig. 3B, lanes 3–5). Because RNase E cleavage was reduced only at site D but not at site C of *ompA* mRNA upon addition of Hfq (Fig. 1B, lanes 3,4), the Hfq-dependent protection of DsrA from RNase E cleavage is unlikely attributable to an inhibitory effect of Hfq on the enzyme.

Sledjeski et al. (2001) have reported that overexpression of DsrA can partially compensate for the reduced translation of the *rpoS* mRNA in a *hfq*$^-$ strain. Taking these results together with that shown in Figure 3, A and B, there appear to be two possibilities to explain

the modulation of the DsrA function by Hfq. First, Hfq stimulates the interaction between DsrA and its target *rpoS*. However, as shown by Brescia et al. (2003), Hfq binding to DsrA does not affect the secondary structure of the RNA. Therefore, these authors suggested that Hfq might act by binding to the 5′UTR of *rpoS*, unfold it, and prime it for DsrA interactions. DsrA binding, in turn, would trap the *rpoS* mRNA in a translatable conformer. For the following reasons, we consider this possibility of Hfq action less likely. Hfq is not essential for *rpoS* translation (Sledjeski et al. 2001), and we have not observed a significant difference in the in vitro translation rate of *rpoS* mRNA in a wild-type S30 extract when compared with an extract prepared from an isogenic *hfq*$^-$ strain (K. Ecker and U. Bläsi, unpubl.). Moreover, we have shown by in vitro toeprinting that 30S ternary complex formation on *rpoS* mRNA strictly requires DsrA but not Hfq (T. Steinhauser and U. Bläsi, unpubl.). Second, the stimulatory effect of Hfq on the DsrA–*rpoS* interaction is indirect, and results mainly from the protection of DsrA by Hfq from endonucleolytic attack. As mentioned above, the second possibility is underscored by the considerably reduced stability of DsrA in the *hfq*$^-$ strain, as well as by its increased abundance in the *rne*$^{ts}$ strain.

The Hfq-binding site on RyhB RNA has not been examined, and its predicted structure (Fig. 3C; Massé and Gottesman 2002) has not been experimentally verified. Because its stability was increased in the *hfq*$^-$ mutant and its levels were higher in the *rne*$^{ts}$ mutant, we tested whether it is likewise a target of RNase E. As shown in Figure 3D, RNase E cleavage in this RNA was observed in the loop, 3′ of the A nucleotide at position 41. Although RNase E cleavage sites are usually found in single-stranded regions, cleavage by RNase E can occur in loops as shown previously for RNAI (Kaberdin et al. 1996). Hfq protected RyhB from RNase E cleavage when added prior to enzyme (Fig. 3D). Again, these experiments demonstrated that the Hfq-binding site and the RNase E cleavage site overlap on this RNA. Most likely, the 3′-terminal stem–loop structures (for review, see Wassarman et al. 1999) present in all sRNAs characterized so far protect them from exonucleolytic attack. As shown in this study, another reason for the long half-life of sRNAs seems to be their Hfq-mediated protection from endonucleolytic cleavage by RNase E. However, these data do not exclude that endonucleolytic cleavage by a RNase other than RNase E could also initiate the decay of sRNAs in the absence of Hfq.

Our data seem to be at variance with the observation that the stability of OxyS RNA was unchanged in a *hfq*$^-$ background when compared with that in a *hfq*$^+$ strain (Zhang et al. 2002). However, as for DsrA and RyhB, a putative RNase E cleavage site in OxyS RNA was found to coincide with the reported (Zhang et al. 2002) Hfq-binding site, and Hfq bound to OxyS protected from RNase E cleavage in vitro (I. Moll, unpubl.). How can we reconcile this result with the unaltered stability of OxyS in the *hfq*$^-$ strain? In the studies described above, neither DsrA nor RyhB RNA was induced by an environmental stimulus. DsrA RNA was constitutively expressed from a plasmid, whereas RyhB was present during cell growth in sufficient amounts to be detected by labeled probes. In contrast, in the experiments performed by Zhang et al. (2002), OxyS RNA was induced by oxidative stress, that is, by addition of hydrogen peroxide. It seems possible that OxyS targets are likewise induced, and that newly synthesized OxyS is protected from RNase E through binding to its target mRNAs. This possibility is currently being investigated.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

*E. coli* strain MC4100 [F Δ(*argF lac*) U169 *araD139 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*] (Casadaban 1976) and its derivative AM111(*hfq1*) [*hfq*::Ω, *Bcl*I] (Tsui et al. 1994), as well as strain N3433 [*rne*$^+$] (Goldblum and Apirion 1981) and the *rne*$^{ts}$ strain N3438 [*rne*-3071, *recA*] (Miczak and Apirion 1993) were used in this study. Cells were grown in LB medium (Miller 1972) supplemented with 100 μg/mL ampicillin when plasmid pNM13 (Majdalani et al. 1998) was present.

### RNAs used in this study

Plasmid pT7OMPA+5* (Chen et al. 1991) linearized with *Hin*dIII served as a template for in vitro transcription of *ompA*180 mRNA by T7 RNA polymerase. *OmpA*180 mRNA (see Fig. 1A) encompasses nucleotides −135 to +45 of wild-type *ompA* mRNA. The *ompA*117 mRNA (nucleotides −135 to −18) was obtained after linearization of plasmid pT7OMPA+5* with *Mnl*I. *OmpA*117Δss2 mRNA (nucleotides −135 to −30) and *ompA*117Δhp2 mRNA (nucleotides −135 to −18, except for nucleotides −59 to −31) were prepared after *Mnl*I cleavage of plasmids p132 and p131, respectively. Plasmids p132 and p131 (Lundberg 1991) contain the *Bcl*I–*Nru*I fragment of plasmid pOMPAΔ104-114 and pOMPAΔ74-103, respectively (Emory et al. 1992), cloned into the *Bam*HI and *Sal*I (fill-in) sites of plasmid SE18 (Chen et al. 1991).

DsrA and RyhB RNA were in vitro transcribed with T7 RNA polymerase from PCR templates generated with the following oligonucleotides (nucleotides comprising the T7 promoter region are underlined): RyhBfw, 5′-GGGTCTAGACGTAATACGACTCACTATAGGCGATCAGGAAGACCCTCGCGGAGAACC-3′; RyhBrev, 5′-TTTTAAGCTTAAAAGCCAGCACCCGGCTGGC-3′; DsrAfw, 5′-GGGTCTAGACGTAATACGACTCACTATAGAACACATCAGATTTCCTGGTGTAACGAATTTTTTAAG TG-3′; DsrArev, 5′-GAGAATTCTTTAAATCCCGACCCTGAGG-3′.

### In vitro RNase E assay

The [$^{32}$P]-5′-end labeled *ompA*180 mRNA as well as the DsrA and RyhB sRNAs were used as substrates for in vitro RNase E cleavage. RNA cleavage was carried out as described previously (Kaberdin et al. 1996) using the *E. coli* degradosome as a source for RNase E. The degradosome was purified according to Miczak et al. (1996)

including micrococcal nuclease treatment, which prevents the co-purification of proteins dependent on the presence of RNA. These preparations did not contain Hfq as judged by Western-blot analysis. The [$^{32}$P]-5′-end labeled RNAs were incubated with an equimolar amount of the degradosome in RNase E cleavage buffer (Kaberdin et al. 1996). The cleavage reaction was started by addition of $MgSO_4$ to a final concentration of 5 mM, and was carried out for 3 or 30 min at 37°C. When Hfq was present in the reaction, it was incubated with the RNA in the hexamer-ratios specified in the figure legends for 5 min at 37°C, prior to the addition of RNase E. The reaction was stopped by addition of EDTA to a final concentration of 10 mM. The samples were extracted with phenol and analyzed on a 10% polyacrylamide-8 M urea gel. The cleavage sites for RNase E were mapped using a T1 digest of the respective RNA as well and a nucleotide (OH-) ladder of the respective RNA.

### Gel mobility shift assays

The mRNAs were synthesized in vitro using T7 RNA polymerase and a Stratagene transcription kit. Purified Hfq-hexamer was added to the [$^{32}$P]-5′-end labeled *ompA* mRNAs at a molar ratio of 4:1, and incubated for 10 min on ice. The RNA–protein complexes were then resolved on 4% native polyacrylamide gels.

### Determination of the steady-state levels of DsrA, RyhB, SsrS, and SsrA sRNAs, and of the half-life of RyhB

For determination of the steady-state levels of the sRNAs the *E. coli* strains N3433 (*rne*$^+$) and N3438 (*rne*$^{ts}$) harboring either no plasmid or plasmid pNM13, encoding DsrA RNA (Majdalani et al. 1998), were used. The cells were grown in LB medium at 30°C. When pNM13 was present, 0.02% arabinose was added at an $OD_{600}$ of 0.2. When the $OD_{600}$ reached 0.35, the cultures were shifted to 44°C. After 15 min of incubation, 8 mL aliquots were withdrawn and total RNA was extracted by the hot phenol method (Lin-Chao and Bremer 1986). The samples were further treated with 10 U of RNase-free DNaseI (MBI Fermentas) and precipitated twice. After the second precipitation, the RNA pellet was dissolved in RNA loading dye [A 1:1 aliquot of 50% deionized formamide, 6% formaldehyde, 5 mM MOPS (pH 7.0) and 2 mM sodium acetate and 0.25 mM EDTA (pH 8.0), were mixed]. The concentration of total RNA was determined by $A_{260}$ measurement. To determine the half-life of RyhB RNA, the *E. coli* strains MC4100 (*hfq*$^+$) and AM111 (*hfq*$^-$) were grown at 37°C in LB medium to early logarithmic phase ($OD_{600}$ = 0.4). Then, rifampicin (0,25 mg/mL) was added, and 8 mL aliquots were withdrawn at 0, 4, 8, 16, and 32 min thereafter for isolation of total RNA as described above.

For detection of the respective sRNAs, 5 μg of total RNA were separated on a 6% polyacrylamide gel containing 8 M urea and blotted using the Trans-Blot SD DNA/RNA Blotting Kit (Bio-Rad) according to the manufacturer's instructions. Zeta-Probe blotting membranes (Bio-Rad) were used for the experiments according to the manufacturer's instructions, except that Background Quencher (Molecular Research Center) was added to minimize nonspecific binding of the labeled probe. For Northern hybridization, $^{32}$P internally labeled RNA probes were synthesized by T7 polymerase Transcription Kit (MBI Fermentas), according to the manufacturer's instructions. The labeled riboprobes contained the nucleotide sequences complementary to nucleotides 37–84 of DsrA RNA, nucleotides 19–60 of RyhB RNA, nucleotides 129–180 of SsrS RNA, and nucleotides 118–320 of SsrA RNA. The 5′-labeled DNA oligonucleotide 5′-GGTGGGACCACCGCGCTAC GGCCGCCAGGC-3′ served as a probe for 5S rRNA that was used as loading control. The signals obtained with the labeled probes were visualized by a PhosphorImager (Molecular Dynamics) and quantified by ImageQuant software.

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