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The C-terminal domain of *Escherichia coli* Hfq is required for regulation

Branislav Večerek, Lukas Rajkowitsch, Elisabeth Sonnleitner, Renée Schroeder and Udo Bläsi*

Max F. Perutz Laboratories, University of Vienna, Dr. Bohrgasse 9, 1030 Vienna, Austria

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ABSTRACT

The *Escherichia coli* RNA chaperone Hfq is involved in riboregulation of target mRNAs by small *trans*-encoded non-coding (ncRNAs). Previous structural and genetic studies revealed a RNA-binding surface on either site of the Hfq-hexamer, which suggested that one hexamer can bring together two RNAs in a pairwise fashion. The Hfq proteins of different bacteria consist of an evolutionarily conserved core, whereas there is considerable variation at the C-terminus, with the γ- and β-proteobacteria possessing the longest C-terminal extension. Using different model systems, we show that a C-terminally truncated variant of Hfq (Hfq_{65}), comprising the conserved hexameric core of Hfq, is defective in auto- and riboregulation. Although Hfq_{65} retained the capacity to bind ncRNAs, and, as evidenced by fluorescence resonance energy transfer assays, to induce structural changes in the ncRNA DsrA, the truncated variant was unable to accommodate two non-complementary RNA oligonucleotides, and was defective in mRNA binding. These studies indicate that the C-terminal extension of *E. coli* Hfq constitutes a hitherto unrecognized RNA interaction surface with specificity for mRNAs.

INTRODUCTION

The *Escherichia coli* host factor I/Q (Hfq) was first described as an accessory factor of the phage Qβ replicase (1) and its importance in cellular physiology became evident when the broadly pleiotropic phenotypes of an *E. coli hfq* mutant were characterized (2). The observations that Hfq is involved in expression of the *rpoS* gene encoding the stationary sigma factor, σ^S (3,4), in iron metabolism (5,6), in stability control of several mRNAs (7–9) and small non-coding regulatory RNAs (ncRNAs) (10–13), in riboregulation of target mRNAs by ncRNAs (5,10,14–17) and that it acts as a virulence factor in several bacterial pathogens (18–21) has recently sparked great interest in this highly conserved bacterial protein (22).

Most data on Hfq–RNA interactions stem from studies on ncRNAs. Hfq-binding sites on ncRNAs have been demonstrated to coincide with cleavage sites of the *E. coli* major riboendonuclease RNase E, and Hfq was shown to protect ncRNAs from degradation (11,12). Hfq binds to OxyS, DsrA, RprA, RyhB, Spot42 and SgrS RNAs as well as to other ncRNAs identified in *E. coli* (10,13,14,16,17,23–27). These ncRNAs are involved in translational regulation of their cognate mRNAs. By forming RNA duplexes in the close vicinity or within the translational initiation region of target mRNAs, ncRNAs can either activate or silence translation, whereby the latter mode of action appears to be predominant (28). Hfq has been shown to stimulate *in vitro* annealing of Spot42 RNA with *galK* mRNA (16), of OxyS with *fhlA* mRNA (14), of RyhB with *sodB* mRNA (13,27) as well as that of SgrS with *ptsG* mRNA (17). It has been demonstrated in some cases that Hfq is dispensable once the interaction between the ncRNA and the target mRNA has taken place (13,14,29). It appears possible that the molecular mechanism by which Hfq brings about these interactions entails unfolding of ncRNAs. However, recent *in vitro* studies did not reveal significant changes in the secondary structure of the ncRNAs DsrA (29,30) and RyhB (27). In contrast, the Hfq RNA chaperone activity induced structural changes in the 5′untranslated regions of the RyhB target *sodB* (27) and of the MicA target *ompA* (31,32), which could facilitate the interaction of these ncRNAs with their target mRNAs. At least for *ompA* mRNA it was shown that the structural changes induced by Hfq prevailed upon proteolysis of the protein, another criterion, which classified Hfq as an RNA chaperone (31).

The 3D structures of the *Staphylococcus aureus* Hfq homologue (33), the N-terminal 72 amino acid (aa) of *E. coli* Hfq (34) and of *Pseudomonas aeruginosa* Hfq (35) revealed that it has a hexameric ring-shaped structure,

*To whom correspondence should be addressed. Tel: ++43-1-4277-54609; Fax: ++43-1-4277-9546; Email: Udo.Blaesi@univie.ac.at

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

and corroborated the idea that it belongs to the large family of Sm- and Sm-like proteins. These proteins are involved in RNA processing in eukaryotes and bind to various RNAs, primarily recognizing short U-rich stretches, known as SM sites (36). An A/U-rich region preceded or followed by a stem-loop structure has likewise emerged as a common RNA-binding motif for Hfq (27,30,31,37)

A prevailing question concerns the interaction of a Hfq-hexamer with RNA substrates. The X-ray structure of *S. aureus* Hfq complexed with a single-stranded oligoribonucleotide showed that it binds to the protein in a circular conformation around a central basic cleft of the hexameric ring (33). Mutational studies suggested that one *E. coli* Hfq-hexamer provides two binding surfaces. These studies implicated the known binding cavity along the inner rim made up of six potential nucleotide-binding pockets (38) as well as the proximal amino acid residues R16 and F39 (39) in the interaction with the ncRNA DsrA. In addition, in the same studies the amino acid residues Y25, I30 and K31, located on the distal site of the hexamer have been shown to be required for poly(A) binding, which culminated in the hypothesis that one Hfq-hexamer can bring together two RNAs in a pairwise fashion (38).

Escherichia coli Hfq homologues have been found in a number of Gram-negative and Gram-positive bacteria. The Hfq proteins of different organisms display an evolutionarily conserved common core consisting of amino acid residues 7–66, whereas there is considerable variation at the C-terminal end (14,16,34,40,41). The removal of 19 aa (42) had no significant effect on binding of the truncated Hfq_{83} protein to polyadenylated RNA.

In addition, we have previously shown that the first 65 N-terminal amino acid residues of *E. coli* Hfq are sufficient for hexamer formation, retain the capacity for binding of the ncRNA DsrA as well as for phage Qß replication (43).

Studies on Hfq-mediated riboregulation have only been performed in *E. coli* and some close relatives, the Hfq protein of which contains an extended C-terminus. Here, we addressed the question whether the C-terminus of *E. coli* Hfq contributes to ncRNA-mediated riboregulation. Using different test systems, we show that a Hfq variant, comprising the first 65 N-terminal amino acid (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. Hfq_{65} displayed no gross defect in binding to the sRNAs RyhB and DsrA, and as evident from real-time fluorescence energy transfer (FRET) assays, Hfq_{65} retained the capacity to induce structural changes in DsrA. Using FRET, we further show that in contrast to full-length Hfq (Hfq_{wt}), Hfq_{65} is impaired in annealing of complementary RNA oligonucleotides. In addition, while Hfq_{wt} was able to bind two non-complementary RNA oligonucleotides on the surface, Hfq_{65} lacked this capacity. Moreover, Hfq_{65} was defective in binding to all tested mRNAs. In summary, this study showed that amino acid residues following the conserved core of Hfq are involved in mRNA binding and thereby identifies a third interaction surface with specificity for mRNAs.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The *E. coli strains* MC4100 (44), AM111 (MC4100 hfq1::Ω) (4) and the corresponding F′ (*lacI*^{q}) variants (41,44) have been described. They were grown in Luria–Bertani (LB) medium (45) or in M9 medium supplemented with 0.2% glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 10 μg/ml thiamine. Where indicated, glucose was substituted by 0.2% succinate and the iron chelator 2,2′-dipyridyl (50 μM final concentration) was added to the medium. Ampicillin (100 μg/ml), kanamycin (25 μg/ml), tetracycline (30 μg/ml) or chloramphenicol (15 μg/ml) were added to the medium where appropriate to maintain plasmids.

Construction of plasmids

The plasmid pRB381 (46) derivatives pRhfq131 (37) and pRsodB-lacZ (15), which bear inducible *hfq-lacZ* and *sodB-lacZ* gene fusions, respectively, have been described. The plasmid pAHfq used for the synthesis of Hfq_{wt} protein was constructed as follows. The *hfq* gene along with the *lac* promoter was recovered on a PvuII fragment from plasmid pUH5 (6), and ligated into the EcoRV–NruI sites of pACYC184 (New England Biolabs Ltd., UK). Plasmid pAHfq65, encoding the Hfq_{65} protein was constructed as follows: the fragment containing the hfq_{65} gene was obtained by means of PCR using the *hfq* forward primer (5′-GC*TCTAGA*AATATAATAGTTTAACTTTA AGAAGGAGATATACATATGGCTAAGGGGCAAT CTTTACAAGATCCGTTCCT-3′), containing a XbaI site (italics), and the reverse primer (5′-TTTTTT*GAATT C*TTACTA**AGA**CGGGACAACAGTAGAAATCG-3′), which contains two stop codons (underlined) after the triplet encoding Ser65 (bold) as well as an EcoRI site (italics). The PCR product was cleaved with XbaI and EcoRI and ligated into the corresponding sites of plasmid pUC19 (New England Biolabs Ltd., UK). From the resulting plasmid pUHfq65, the hfq_{65} gene was re-isolated by cleavage with PvuII and then ligated into the EcoRV and NruI sites of pACYC184, yielding plasmid pAHfq65.

The construction of the plasmids used for purification and *in vivo* synthesis of *S. aureus* Hfq (Hfq_{Sa}) and *Bacillus subtilis* Hfq (Hfq_{Bs}), respectively, is described in Supplementary Data.

The plasmids pUhfqwt, pUsod and pURyhB, which served as templates for *in vitro* synthesis of *hfq*126, *sodB*192 and RyhB RNAs, respectively, have been described (6,13,37).

Plasmid pUrpoS16 used for *in vitro* synthesis of *rpoS652* mRNA was constructed using primer C16 (5′-GGGC*TCTAGA*GTAATACGACTCACTATAGTC GGGTGAACA-GAGTGCTAACAAAATGTTGC CG-3′), which contained a XbaI site (italics) and the T7 promoter sequence (underlined) followed by the 5′-terminal part of the 5′untranslated region of the *rpoS* gene (begins at the transcriptional start of *rpoS* mRNA

when transcribed from the main P2 promoter) (47) and the reverse primer P21 (5′-AAA*GAATTC*CTGACAGATGC TTACTTACTCGCGGAACAG-3′), which contains an EcoRI site (italics) and is complementary to the sequence following the stop codon of the *rpoS* gene. The PCR product obtained with primers C16/P21 was cleaved with XbaI and EcoRI, and ligated into the corresponding sites of plasmid pUC18, resulting in plasmid pUrpos16.

Relative translational efficiency of *hfq-lacZ* and *sodB-lacZ* fusions

Cultures of AM111F′ (pRhfq131) and AM111F′ (pRsodB-lacZ) co-transformed with the compatible plasmids pACYC184 (control), pAHfq and pAHfq65, respectively, were cultivated in LB or M9 medium (Figure 2) at 37°C. At an OD_{600} of 0.5, the plasmid-encoded genes were induced by addition of IPTG (1 mM). After 60 min, triplicate aliquots were taken for the β-galactosidase assays and for western blot analysis to verify Hfq_{wt} or Hfq_{65} production. In parallel, samples were withdrawn for isolation of total RNA to determine the respective *hfq-lacZ* and *sodB-lacZ* mRNA levels. The β-galactosidase activity was determined from triplicate samples as described (45) and the total RNA was purified by the hot phenol method (48). The averaged β-galactosidase activities obtained with the pRhfq131 and pRsodB-lacZ constructs were normalized to the corresponding *hfq-lacZ* and *sodB-lacZ* mRNA levels (=relative translational efficiencies in Figure 2A and B). The corresponding *lacZ* mRNA levels were determined by primer extension with AMV reverse transcriptase (Promega GmbH, Germany) using 5 μg of total RNA primed with the *lacZ*-specific 5′-end labelled probe (5′-GGGAAGGGCGATCGGT-3′) and normalized to the 5S rRNA levels (internal control), which were determined using primer R25 (5′-GGTGGGACCACCGCGCTACGGCCGCCAGGC-3′). The signals were visualized by a PhosphoImager (Molecular Dynamics) and quantified by ImageQuant software. Two independent sets of experiments were performed.

Western blot analysis

The cellular levels of Hfq_{wt}, Hfq_{65} and σ^S were determined by quantitative immunoblotting. The σ^S levels were determined in strain AM111F′ harbouring plasmids pUC18 (control), pUH5 (Hfq_{wt}) and pUHfq65 (Hfq_{65}), respectively. The strains were grown at 28°C in LB medium until they reached an OD_{600} of 0.3, at which time IPTG was added to a final concentration of 1 mM. At an OD_{600} of 1.0 equal amounts of cells were withdrawn and boiled in protein sample buffer. The Hfq_{wt} or Hfq_{65} levels were determined at the times indicated in Figure 1A, concomitantly with the determination of the relative β-galactosidase values (Figure 2A and B), and the quantification of the σ^S levels (Figure 2C), respectively. Equal amounts of total protein were separated on 12% SDS–polyacrylamide gels and blotted to a nitrocellulose membrane. The blots were blocked with 5% dry milk in TBS buffer, and then probed with anti-Hfq (49) or anti-σ^S (kindly provided by Dr F. Norel, Pasteur Institute, Paris) antibodies. The antibody–antigen complexes were visualized as described previously (49). The quantification of the Hfq- or RpoS-specific bands was performed with ImageQuant software.

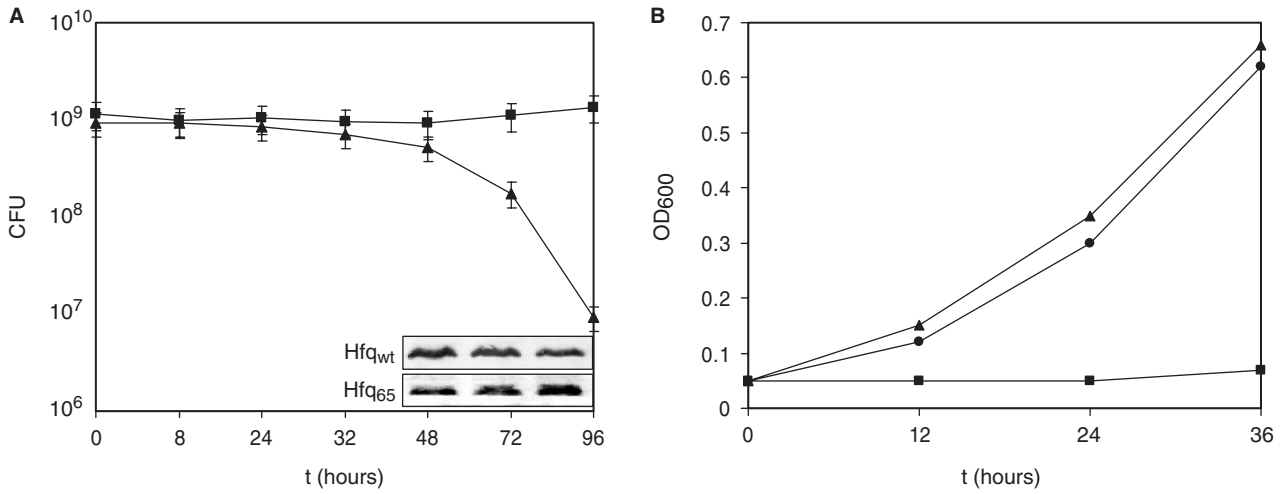

Figure 1. Effect of Hfq_{65} on survival under nutrient limitation and on growth on succinate. (**A**) *Escherichia coli* strains AM111(pAHfq) (closed square) and AM111(pAHfq65) (closed triangle) were grown in LB-medium, washed with M9-minimal medium, and then resuspended in M9-minimal medium containing 0.2% glucose to 1×10^9 cells ml^{-1}. The CFU at the indicated times was determined by plating of serial dilutions on LB plates containing kanamycin and chloramphenicol. The experiment was performed in triplicate. The error bars represent standard deviations. The inset shows the concentrations of Hfq_{wt} and Hfq_{65} at different times after incubation from one representative experiment. At times 48, 72 and 96h aliquots of both strains were withdrawn, and equal amounts of total cellular protein was subjected to western blot analysis using Hfq-specific antibodies as described in Materials and Methods section. Only the Hfq-specific bands are shown. (**B**) *Escherichia coli* strains AM111(pAHfq) (closed square) and AM111(pAHfq65) (closed triangle) and AM111(pACYC184) (closed circle) were grown overnight in LB-broth, washed with M9-minimal medium and then resuspended to an OD_{600} of 0.05 in M9-minimal medium containing 0.2% succinate and 2,2′-dipyridyl (50 μM final concentration). Growth was followed by measuring the OD_{600} at the times indicated. The result of one representative experiment is shown.

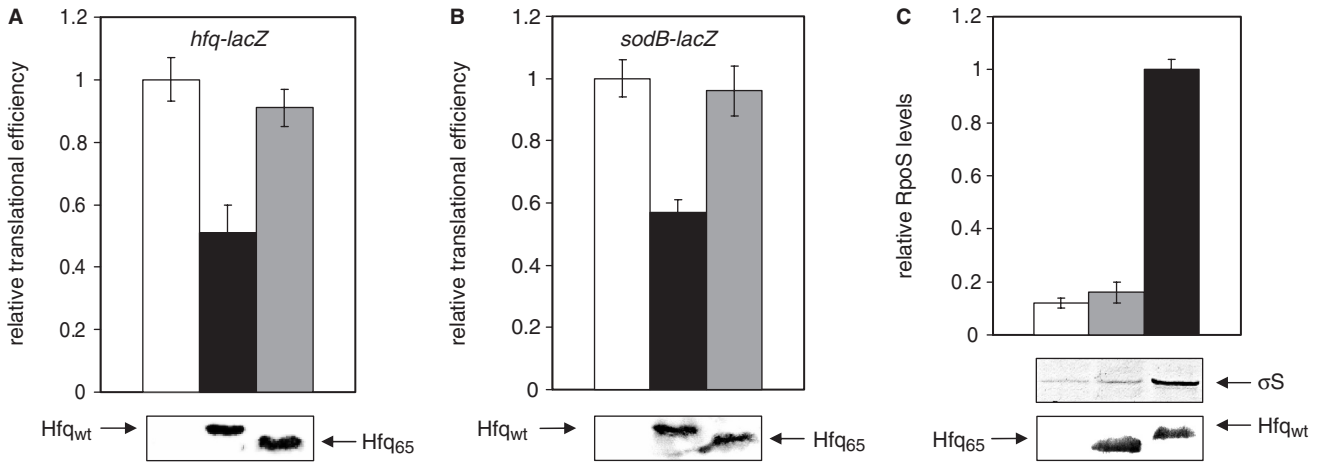

Figure 2. Hfq_{65} is defective in auto- and riboregulation. (**A**) Relative translational efficiency of *hfq*131-*lacZ* mRNA in the absence of Hfq (white bar), in the presence of Hfq_{wt} (black bar) and Hfq_{65} (grey bar) in strains AM111F′ (pRhfq131; pACYC184), AM111F′ (pRhfq131; pAHfq) and AMF′111(pRhfq131; pAHfq65) grown in LB medium, respectively. (**B**) Relative translational efficiency of *sodB-lacZ* mRNA in the absence of Hfq (white bar), in the presence of Hfq_{wt} (black bar) and Hfq_{65} (grey bar) in strains AM111F′ (pRsodB; pACYC184), AM111F′ (pRsodB; pAHfq) and AM111F′ (pRsodB; pAHfq65) grown in M9 medium, respectively. The averaged β-galactosidase values normalized to mRNA levels obtained in the absence of Hfq was set to 1 (white bar). The values obtained in the presence of Hfq_{wt} (black bar) and Hfq_{65} were normalized to the control. The experiment was performed in duplicate. The error bars represent standard deviations. Bottom: determination of the levels of Hfq_{wt} and Hfq_{65} in the respective strains by quantitative immunoblotting (see Materials and Methods section). (**C**) Graphical representation of the σ^S levels in strain AM111F′ harbouring plasmid pUC18 (lane 1; control), pUHfq65 (lane 2; Hfq_{65}) and pUH5 (lane 3; Hfq_{wt}), respectively. The western blot analysis was carried with equal amounts of total cellular protein as described in Materials and Methods section. Only the relevant sections of the immunoblots (lower panels) showing the σ^S-and Hfq-specific bands are depicted. Quantification of the western blot was done with ImageQuant software. Values were normalized to the σ^S signal obtained in the presence of Hfq_{wt} in strain AM111F′ (pUH5), which was set to 1. The results represent data from duplicate experiments. The error bars represent standard deviations.

RNA preparation for *in vitro* studies

For *hfq*126 mRNA synthesis, the plasmid pUhfqwt digested with AflIII was used as a template for *in vitro* transcription with T7 RNA polymerase (Promega). To prepare *sodB*192 mRNA, *rpoS*652 mRNA and RyhB RNA, the pUsod, pUrpoS16 and pURyhB plasmids digested with Asp718I, StuI and DraI, respectively, were used as templates. The run-off transcripts were purified on 6% polyacrylamide–8M urea gels following standard procedures. The mRNA concentration was determined by measuring the A_{260}.

Electrophoretic mobility shift assays

Hfq_{wt} and Hfq_{65} proteins used in gel mobility assays were purified from AM111(pUH5) and AM111(pUH65) cells, respectively, as described (43). Gel-purified mRNAs were 5′-end labelled with [γ-^{32}P]-ATP (Amersham Pharmacia Biotech) and again purified on 6% polyacrylamide–8M urea gels. Labelled mRNAs (5 nM) were incubated without or with increasing amounts of purified Hfq_{wt}, Hfq_{65}, Hfq_{Sa}, or Hfq_{Bs} proteins (as indicated in the legends to Figures 3C and 5 and in Supplementary Figure S3C) in a 10 µl reaction in binding buffer (10 mM Tris, pH 7.5, 60 mM NH_4Cl, 5 mM β-mercaptoethanol, 2 mM MgOAc, 100 ng of yeast tRNA) for 5 min at 37°C and then for 10 min at 0°C. The samples were mixed with 40% glycerol to a final concentration of 10% and loaded on a native 5% polyacrylamide gel. Electrophoresis was performed in TAE buffer at 60 V for 12 h. Radioactive bands were visualized using a PhosphoImager.

FRET assays

This method is described in more detail in Ref. (50). Two complementary, fluorophore-tagged RNA 21-mers (Cy5-5′-AUGUGGAAAAUCUCUAGCAGU-3′ (Cy5-21R$^+$) and Cy3-5′-ACUGCUAGAGAUUUUCCACAU-3′ (Cy3-21R$^-$) were used in the annealing experiment shown in Figure 4A. For binding of non-complementary RNA oligonucleotides (Figure 4B), Cy5-21R$^+$ and the RNA oligonucleotide Cy3-21R$^+$ (Cy-3-duplex) (5′-Cy3-CUUUCAUUGGUCGGUCUCUCC-3′) were employed. The tagged RNA oligonucleotides were purchased from VBC-Biotech (Vienna, Austria). Using a Tecan GENios Pro microplate reader, the first oligoribonucleotide was injected into wells with or without Hfq protein (1 µM final Hfq–hexamer concentration), and the measurement was started with the injection of the second oligoribonucleotide. The reaction was performed in annealing buffer (50 mM Tris–HCl pH 7.5, 3 mM $MgCl_2$ and 1 mM DTT) at 37°C. The final concentration of the RNAs was 5 nM in a volume of 40 µl. The reaction was allowed to proceed for 180 sec, and with Cy3 excited, donor and acceptor dye fluorescence emissions were measured once every second. The time-resolved ratio of the fluorescence emissions (FRET index F_{Cy5}/F_{Cy3}) was normalized to 1 at t_{180s} and least-square fitted with Prism 4.03 (GraphPad Software Inc., San Diego, CA, USA) with the second-order reaction equation for equimolar initial reactant concentrations $y = A\ [1 - 1/(k_{ann}\, t + 1)]$; k_{ann} = observed annealing reaction constant, A = maximum reaction amplitude. The reaction curves were fitted with $y = A\ [1 - 1/(k_{db}\, t + 1)]$,

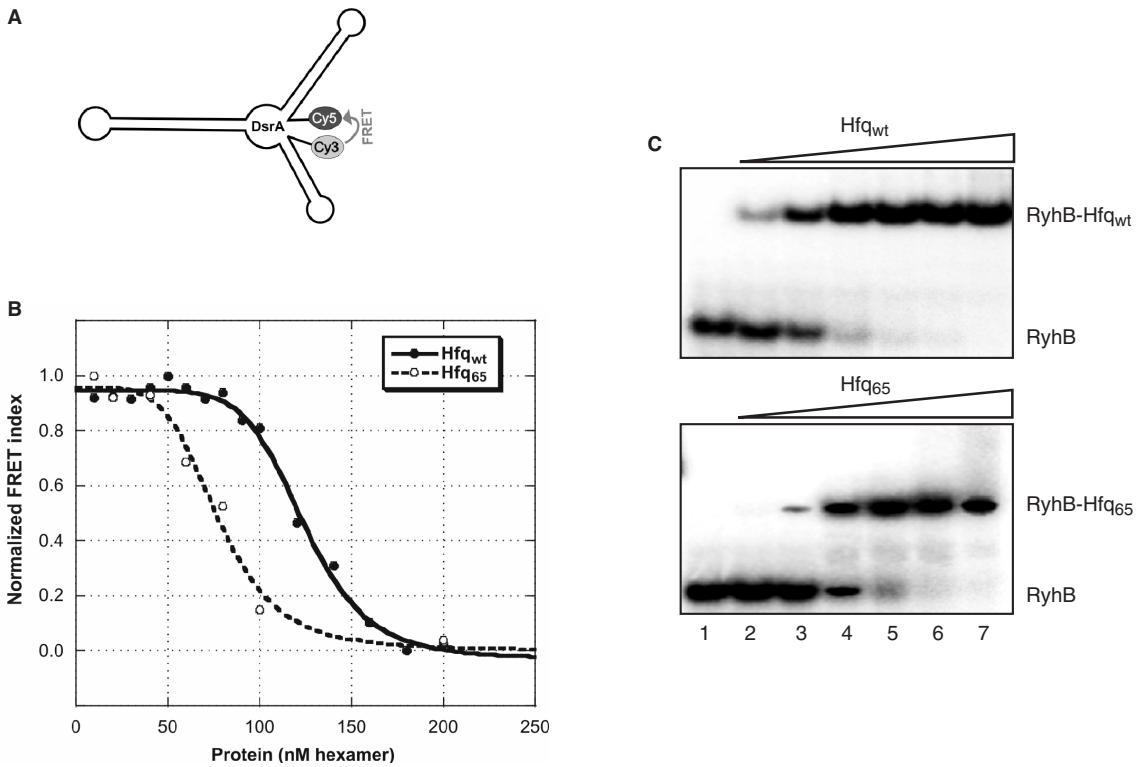

Figure 3. Hfq_{65} induces structural changes in DsrA and binds to RyhB. (**A**) Schematic depiction of the secondary structure of DsrA as predicted by mfold (56). (**B**) Titration of 5 nM dual-labelled DsrA with Hfq_{wt} or Hfq_{65}. Binding of the protein to the RNA results in a reduction of the FRET index (calculated as F_{Cy5}/F_{Cy3} ratio) indicating a spatial separation of the fluorophores. The data was fitted with the equilibrium binding equation $y = y_0 + A/(1-(K_{1/2}/c)^{nH})$ yielding a $K_d = K_{1/2}{}^{nH}$ for Hfq of $124^{7.2}$ nM and $77^{4.8}$ nM for Hfq_{65}. (**C**) Affinity of Hfq_{wt} and Hfq_{65} for RyhB as revealed by gel mobility shift assays. 5′ End-labelled RyhB (5 nM) was incubated in the absence (lane 1), in the presence of 10 nM (lane 2), 20 nM (lane 3), 40 nM (lane 4), 80 nM (lane 5), 160 nM (lane 6) and 320 nM Hfq_{wt}-hexamer or Hfq_{65}-hexamer, respectively.

k_{db} = observed double binding reaction constant. The curves shown are representative; the observed reaction constants k_{ann} and k_{db} were calculated as the average of three individually fitted reactions.

DsrA RNA labelled at the 5′- and 3′-ends with the fluorophores Cy3 and Cy5, respectively, was obtained from Dharmacon (USA). Five nanometre of this RNA was incubated with indicated concentrations of Hfq or Hfq_{65} (Figure 3B) in a buffer containing 50 mM Tris–HCl pH 7.5, 3 mM $MgCl_2$, 1 mM DTT. After incubation at 25°C for 30 min, donor (Cy3) and acceptor (Cy5) emissions were measured, and the FRET index was calculated as F_{Cy5}/F_{Cy3}. The data were fitted in Kaleidagraph 3.51 (Synergy Software, Reading, PA, USA) with the equilibrium binding equation $y = y_0 + A/(1-(K_{1/2}/c)^{nH})$; A = maximum amplitude, $K_{1/2}$ = dissociation constant, n_H = Hill coefficient.

RESULTS

Hfq_{65} confers a reduced viability under nutrient limitation and does not support RyhB-mediated repression of growth on succinate

Although *E. coli hfq*− mutants are viable under laboratory conditions they show pronounced pleiotropic phenotypes (2). In an initial survival study, the *hfq*− strain AM111F′ was co-cultivated with the isogenic *hfq*+ strain MC4100F′ in minimal medium, and growth of both strains was followed over 4 days by scoring the total colony forming units (CFU). In contrast to MC4100F′ the CFU of AM111F′ declined rapidly from day one to day four, when the strain was close to extinction (data not shown). These data clearly indicated that Hfq is pivotal for survival of cells under nutrient limitation that can be reconciled with its requirement for riboregulation under adverse conditions (51). To test whether the C-terminus of Hfq is required for function, we therefore asked whether a truncated variant of Hfq, comprising the first N-terminal 65 aa, can sustain viability under nutrient limiting conditions. The growth and survival rates of the *hfq*− strain AM111, bearing the plasmid borne hfq_{65} allele [AM111(pAHfq65)] were compared with that of AM111 bearing the plasmid borne hfq_{wt} gene [AM111(pAHfq)]. Both strains were grown in LB-medium, washed several times with minimal medium, and then resuspended to 1×10^9 cells/ml in M9 minimal medium supplemented with 0.2% glucose. As shown in Figure 1A, the CFU of strain AM111(pAHfq65) started to decrease ~24 h after inoculation. While the CFU of AM111(pAHfq65) was reduced to <1% over the 4 day observation period that of AM111(pAHfq) remained constant. As the CFU was determined in the presence of the selective antibiotic, it is unlikely that the reduced viability of AM111(pAHfq65) can be attributed to plasmid loss. In addition, quantitative

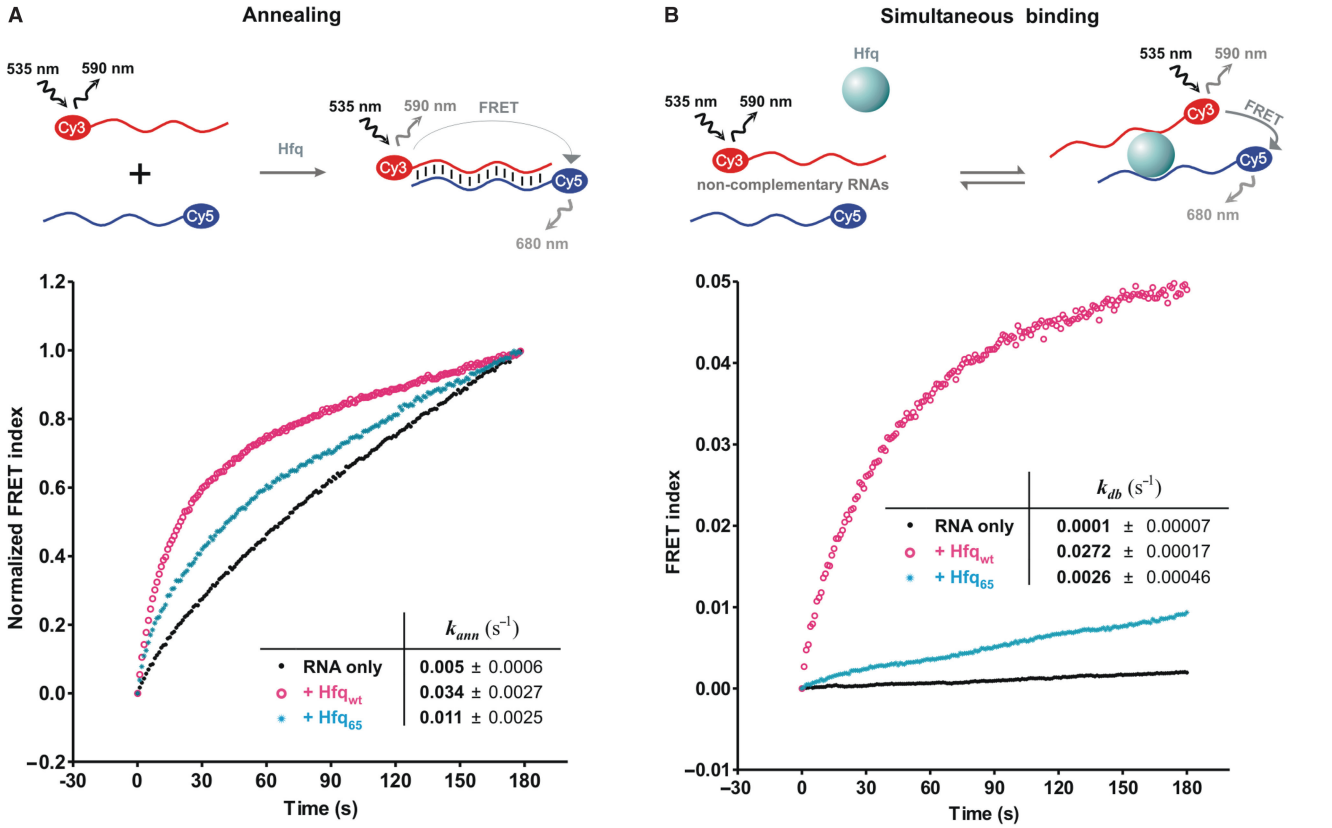

Figure 4. Deletion of the Hfq C-terminus results in a decreased RNA annealing activity and abolishes the ability to bind two RNAs simultaneously. (**A**) Annealing of two fluorophore-labelled RNA 21-mers can be accelerated by RNA chaperones (top; 50) that can be monitored by FRET. Bottom, 5 nM of each RNA oligonucleotide Cy5-21R$^+$ and Cy3-21R$^-$ were annealed at 37°C either in the absence or in the presence of 1 μM Hfq$_{wt}$ or Hfq$_{65}$ hexamer. FRET was calculated as ratio of acceptor/donor emission (F_{Cy5}/F_{Cy3}), normalized to 1 at t_{180s}, and the data was fitted with the second-order reaction equation for equimolar initial reactant concentrations $y = A\ [1 - 1/(k_{ann}\ t + 1)]$. Hfq$_{wt}$ and Hfq$_{65}$ increased the observed annealing reaction constant k_{ann} 7- and 2-fold, respectively. (**B**) In a set-up with the non-complementary Cy5-21R$^+$ and Cy3-21R$^+$ (Cy3-duplex) RNA oligonucleotides, simultaneous binding of the two RNAs to a protein can be measured (top). Bottom, the time-resolved FRET index curves were fitted with $y = A\ [1 - 1/(k_{db}\ t + 1)]$ to yield the dual RNA oligonucleotide binding rate constant k_{db}. The RNA oligonucleotides by themselves do not anneal, whereas Hfq$_{wt}$ can bind both RNAs simultaneously. Incubation with Hfq$_{65}$ resulted in a k_{db} 10-fold lower than with Hfq$_{wt}$, indicating that the C-terminal truncation coincides with a severely reduced capacity for simultaneous binding of the two non-complementary RNA oligonucleotides. The FRET index in this graph was not normalized to indicate the reaction amplitudes.

immunoblotting experiments after 48, 72 and 96 h did not reveal a significant difference in the level of Hfq$_{wt}$ and Hfq$_{65}$ in the respective strains (Figure 1A).

We next sought for more direct *in vivo* evidence for the apparent dysfunction of Hfq$_{65}$. Massé and Gottesman (5) have shown that both Hfq and the ncRNA RyhB are required for translational repression of the *sdhCDAB* operon, resulting in the inability to grow on succinate or fumarate as the sole carbon source. To test whether RyhB-mediated negative regulation of the *sdhCDAB* genes is impaired in the presence of Hfq$_{65}$, the strains AM111(pAHfq65) and AM111(pAHfq) were cultured in succinate-minimal medium in the presence of the iron chelator 2,2′-dipyridyl, which is known to induce RyhB synthesis (5). As expected, the strain AM111(pAHfq) did hardly grow in the presence of succinate, whereas similar growth rates were observed for AM111(pAHfq65) and the *hfq*− strain AM111(pACYC184) (Figure 1B), indicating that Hfq$_{65}$ is non-functional in supporting RyhB-mediated negative regulation of the *sdhCDAB* operon.

Hfq$_{65}$ is non-functional in *hfq*-autoregulation, in RyhB-mediated repression of *sodB* mRNA and in DsrA-mediated stimulation of *rpoS* mRNA

Three model systems were used to further corroborate the idea that the C-terminus of Hfq is required for post-transcriptional regulation. First, we tested whether Hfq$_{65}$ is affected in autogenous regulation (37). When compared to strain AM111*hfq*− (pACYC184; pRhfq131), co-expression of the *hfq* gene from plasmid pAHfq and the *hfq*131-*lacZ* reporter gene from the compatible plasmid pRhfq131 in AM111 *hfq*− (37) resulted in ∼50% decrease of the relative translational efficiency of the *hfq131-lacZ* mRNA. In contrast, Hfq$_{65}$ was defective in translational autocontrol, despite comparable intracellular levels of Hfq$_{wt}$ and Hfq$_{65}$ (Figure 2A).

Second, we employed the well-studied RyhB/*sodB* model system, wherein the ncRNA RyhB has been shown to repress translation initiation of *sodB* mRNA (encodes an iron containing superoxide-dismutase) in a Hfq-dependent manner (5,6,27,52). As shown

in Figure 2B, Hfq_{65} was likewise defective in RyhB-mediated translational repression of a *sodB-lacZ* reporter gene despite comparable intracellular levels of Hfq_{wt} and Hfq_{65}.

Third, we asked whether Hfq_{65} can support DsrA-mediated stimulation of *rpoS* mRNA, encoding the stationary sigma factor, σ^S. The ncRNA DsrA has been shown to bind to the 5-untranslated region of *rpoS* mRNA in a Hfq-dependent manner, which leads to an exposure of the *rpoS* translation initiation determinants, and thereby facilitates translation (53–55). σ^S synthesis was assessed in the presence of either Hfq or Hfq_{65} at 28°C, when the level of DsrA is known to be elevated (55). In contrast to Hfq_{wt}, Hfq_{65} did not stimulate σ^S synthesis, again suggesting that the C-terminus of Hfq is involved in regulation (Figure 2C).

Hfq_{65} binds ncRNAs

We have previously shown that Hfq_{65} binds with almost unaltered affinity to the ncRNA DsrA (43). Recent studies (30,38) suggested that DsrA binds to the inner core of the Hfq-hexamer that would explain why binding to DsrA is retained by Hfq_{65}. It appeared possible that the molecular mechanism by which Hfq brings about ncRNA–mRNA interactions entails unfolding of ncRNAs. However, recent *in vitro* studies did not reveal significant changes in the secondary structure of DsrA (29,30) and RyhB (27). Nevertheless, using enzymatic probing and biophysical methods, Brescia *et al.* (30) suggested that Hfq might affect the tertiary structure of DsrA. Structural mapping of DsrA placed its 5′ and 3′ end at a distance of 24 consecutive nucleotides (30), which prompted us to employ a FRET assay to verify, (i) whether Hfq alters the structure of DsrA, and if so, (ii) whether this function is retained by Hfq_{65}. Five nanomol full-length DsrA labelled with Cy3 and Cy5 at the 5′ and 3′ end (Figure 3A), respectively, was incubated at 37°C with increasing amounts of Hfq. The decrease in FRET (Figure 3B) upon binding of Hfq indicated a change in the DsrA tertiary (and possibly secondary) structure with the two 5′ and 3′-end positioned fluorophores (Figure 3A) moving apart. A similar picture was observed with Hfq_{65}, indicating that the conserved core region of Hfq is not only capable of DsrA binding (43) but also sufficient to induce conformational changes in the ncRNA.

Hfq_{65} was likewise demonstrated to bind to the ncRNA RyhB. As calculated from the band-shift assays (Figure 3C), Hfq_{65} bound to RyhB with only a ~2-fold lower affinity (16 nM and 35 nM for Hfq_{wt}- and Hfq_{65}-hexamers, respectively) when compared with Hfq_{wt}. Thus, the C-terminus of Hfq does not contribute significantly to binding of the studied ncRNAs.

The C-terminus of Hfq provides a RNA-binding surface

We next asked whether the defects observed in Hfq_{65}-mediated regulation (Figure 2) could be attributed to the absence of a RNA-binding surface. To test this possibility, we first used the two complementary RNA oligonucleotides, Cy5-21R^+ and Cy3-21R^-, which were labelled at their 5′-ends with Cy5 and Cy3, respectively. Hfq stimulated annealing of these RNA oligonucleotides with a rate constant k_{ann} of 0.034 s^{-1}, which was ~7-fold higher than observed for self-annealing of the two RNA oligonucleotides (Figure 4A). In contrast, the observed annealing constant for Hfq_{65} was ~3-fold lower than that determined for Hfq_{wt}, and with 0.011 the k_{ann} for Hfq_{65} was only approximately twice of that seen for self-annealing of the two RNA oligonucleotides (Figure 4A). Next, we used two non-complementary RNA oligonucleotides Cy5-21R^+ and Cy3-21R^+ (Cy3-duplex) in the FRET assay (Figure 4B). Under these conditions no significant increase in the FRET signal was observed in the presence of Hfq_{65} (Figure 4B), whereas an increase in FRET was observed for Hfq_{wt}. Given the spatial considerations required for FRET under these conditions this result corroborated other data in that a Hfq-hexamer can bind two RNAs simultaneously (38,39,57). In contrast to Hfq_{wt}, the dual rate binding constant k_{db} for Hfq_{65} was ~10-fold lower, suggesting that Hfq_{65} is severely impaired in simultaneous binding of the two non-complementary oligonucleotides.

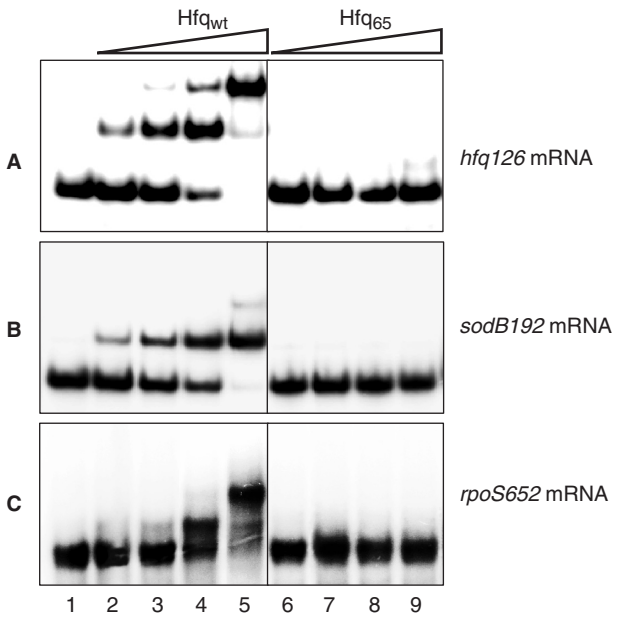

Figure 5. Hfq_{65} fails to bind to *hfq*, *sodB* and *rpoS* mRNAs. 5′ End-labelled *hfq*126 mRNA (**A**), *sodB*192 mRNA (**B**) and *rpoS*652 mRNA (**C**) was incubated in the absence (lane 1), in the presence of 2.5 nM (lanes 2 and 6), 5 nM (lanes 3 and 7), 10 nM (lanes 4 and 8) and 20 nM (lanes 5 and 9) Hfq_{wt}- and Hfq_{65}-hexamer, respectively. The molar concentration of either mRNA fragment used was 5 nM.

Finally, we tested whether Hfq_{65} is defective in binding to *hfq*126 mRNA, *sodB*192 mRNA and *rpoS*652 mRNA to either of which Hfq_{wt} was shown to bind (Figure 5). As shown in Figure 5A–C, Hfq_{65} did not bind to either mRNA fragment, which readily explained why Hfq_{65} was defective in auto- as well as riboregulation.

DISCUSSION

Although an A/U-rich region adjacent to a stem-loop structure has emerged as a RNA-binding motif for Hfq

(12,27,30), it remains poorly understood which sites of the Hfq-hexamer are involved in binding different RNA substrates. So far, mutational studies (38,39) revealed two independent RNA-binding surfaces, one of which is located on the proximal site of the hexamer and includes RNA interactions with the central cavity. As a poly(U) oligonucleotide competed with binding of the ncRNA DsrA (30), this binding site is apparently used by poly(U) (33) as well as by ncRNAs (30,38). Hence, this work corroborates mutational analyses (30,38), and verifies through studies on RyhB that the C-terminus is not instrumental for binding of ncRNAs. Moreover, as shown in Figure 3B, the C-terminus of Hfq is dispensable for Hfq to induce structural changes in DsrA. Taken together, these findings suggest that the evolutionary conserved common core of Hfq (34), i.e. the proximal surface of the Hfq-hexamer, specifies the 'business surface' for ncRNAs.

A second binding surface with a specificity for poly(A) has been located on the distal site of the hexamer with amino acid residue Y25, I30 and K31 being instrumental for binding to poly(A) stretches. In this case, poly(A) binding did not compete with DsrA binding, showing that both RNAs use indeed different binding surfaces (38). Binding of poly(A) to the distal site can also be reconciled with the unaltered binding affinity of a C-terminal Hfq deletion mutant, lacking the last 19 aa, and of Hfq_{65} for a polyadenylated mRNA (42) and for poly(A_{27}) (Večerek,B., unpublished data), respectively. In addition, it has been shown that Hfq_{65} is proficient in phage Qβ replication and that mutations in K31 severely affect this function (43). Thus, it is possible that the distal site of Hfq is also pivotal for its function in Qβ replication. Moreover, the distal site could be involved in binding of Hfq to DNA A-tracts (58) that could explain the presence of Hfq in the nucleoid (59).

Equilibrium unfolding studies (42) indicated that the C-terminus contributes to the stability of the Hfq-hexamer. However, as Hfq_{65} forms stable hexamers in solution (43) and binds to ncRNAs (Figure 3) it seems less likely that the failure of Hfq_{65} to bind to *hfq, sodB* and *rpoS* mRNA (Figure 5) results from an altered stability. Therefore, we suggest that the C-terminus of Hfq provides a third RNA interaction surface with specificity for mRNAs, which can readily explain why negative translational autoregulation of *hfq* mRNA as well as Hfq-mediated riboregulation by the ncRNAs RyhB and DsrA was abolished in the presence of Hfq_{65} (Figure 2). Zambrano *et al.* (60) demonstrated that *rpoS* null strains died rapidly when mixed with *rpoS*+ strains. Therefore, the loss of viability of AM111(pHfq65) upon entry into stationary phase (Figure 1A) can at least partially be attributed to the inability of Hfq_{65} to mediate translational activation of *rpoS* by DsrA. Similarly, as Hfq_{65} failed to bind to *sodB* mRNA (Figure 5), and thus to mediate translational repression of this mRNA by RyhB (Figure 2), it is reasonable to assume that the observed growth on succinate in the presence of Hfq_{65} and RyhB (Figure 1B) results likewise from the failure of the truncated protein to interact with the *sdh*CDAB mRNA.

Both, Hfq_{wt} and Hfq_{65}, bound with comparable affinities to all three RNA oligonucleotides ($K_{1/2}$ of ~50–230 nM; Supplementary Figure S1), whereby the $K_{1/2}$ of Hfq_{wt} for the RNA oligonucleotides was somewhat reduced when compared with Hfq_{65}. Assuming two independent binding sites on Hfq_{wt} for the RNA oligonucleotides, the $K_{1/2}$ for Hfq_{wt} would represent a mean value, which could explain the reduced affinity of Hfq_{wt} for these substrates when compared with Hfq_{65}. In any case, these results (Supplementary Figure S1) showed that neither Hfq_{wt} nor Hfq_{65} discriminate against any of the RNA oligonucleotides used, and can therefore be readily reconciled with the idea that Hfq_{65} fails to accommodate two RNAs. Hfq_{65} stimulated annealing of the two complementary RNA oligonucleotides 2-fold (Figure 4A). We speculate that this moderate acceleration of self-annealing results from a reduction of Brownian molecular motion through immobilization of one RNA oligonucleotide. Obviously, the same effect cannot stimulate FRET with non-complementary RNA oligonucleotides, as they need to be fixed in close proximity. Recent studies (38), showed that amino acid substitutions at the proximal face including alterations within the Sm2 motif, i.e. at the 'business surface' for ncRNAs, had a minor effect on *rpoS* binding, and that poly(A) did not compete with *rpoS* mRNA binding. Taken together with our observations that the C-terminus of Hfq is required for *rpoS* mRNA binding (Figure 5), and that Hfq_{wt} stimulated FRET with non-complementary RNA oligonucleotides (Figure 4B), the available data collectively suggest that the C-terminus of Hfq forms a major and perhaps independent interaction surface for (m)RNA. A truncated version of *E. coli* Hfq, consisting of the first 75 aa was functional in stimulating *rpoS* translation (41). Therefore, the sequence between amino acid 65 and 75 of Hfq will be of prime interest to tackle the C-terminal sub-domain required for binding of mRNAs. As the binding motif for Hfq appears to be similar in ncRNAs and mRNAs (12,27,30), the molecular features underlying the preference of the C-terminus for mRNA remains to be elucidated. As yet, structural data on the C-terminus of Hfq are lacking. The PONDR (predictor of natural disordered regions) algorithm (www.pondr.com) predicts that the C-terminus of Hfq is structurally disordered (Supplementary Figure S2) that is a hallmark of RNA chaperones (61). Such flexible regions are believed to provide conformational fluctuations that facilitate intermolecular interactions. The flexibility of the C-terminus could therefore provide the molecular basis for the interaction of Hfq with many mRNA substrates or proteins. Indeed, several proteins including RNA polymerase, ribosomal protein S1 (62), RNase E (63), polyA-polymerase (PAP I) and polynucleotidephosphorylase (PNPase) (64) have been found in complex with Hfq. However, it remains to be elucidated in either case whether Hfq is in direct physical contact with these candidate proteins or whether these findings result from the spatial association of the transcriptional, translational and RNA decay machineries in bacteria.

As obvious from Figure 4B, Hfq_{wt} can bind two non-complementary RNAs, whereas Hfq_{65} lacked this capacity. This seems somewhat at variance with band shift assays showing that Hfq only facilitates the interaction

between RNAs able to base pair (14). However, the RNA oligonucleotides used here may differ in their binding requirements from the natural substrates used by Zhang *et al*. (14), and monitoring binding in real time in solution most likely puts less constrains on the stability of RNA–protein complexes than gel electrophoresis. Nevertheless, as Hfq_{65} was defective in binding two RNA oligonucleotides one binding site for these substrates appears to be provided by the C-terminus.

An extended C-terminus is only found in Hfq proteins of γ- and β-proteobacteria (42). Interestingly, Hfq-mediated riboregulation of mRNAs by ncRNAs has unequivocally been demonstrated only in *E. coli* (51) and *Salmonella enterica* (65), and was implicated in *Shigella* (66) and *Vibrio* (67) species, all of which belong to the γ-proteobacteria. In contrast, with 73 aa and 77 aa, respectively, the firmicutes *B. subtilis* and *S. aureus* possess Hfq proteins with short C-terminal extensions (40). Ectopic expression of the *S. aureus* or the *B. subtilis hfq* gene in *E. coli* AM111F′*hfq*− did not (i) inhibit growth on succinate in the presence of RyhB (Supplementary Figure S3A), was unable (ii) to support RyhB-mediated repression of *sodB* translation and did not (iii) result in translational auto-repression of *E. coli hfq* mRNA (Supplementary Figure S3B). Consistent with these observations, neither the *S. aureus* nor the *B. subtilis* Hfq protein bound to *hfq*126 or *sodB*192 mRNA (Supplementary Figure S3C). Thus, phenotypically both proteins behaved like Hfq_{65}. Despite of the heterologous *E. coli* system used, these experiments could imply that there are no other inherent features in these shorter Hfq variants that would compensate for the C-terminal extension present in the Hfq proteins of γ-proteobacteria. In addition, they lend further support to the notion that an extended C-terminus is required for riboregulation. In fact, no Hfq requirement has as yet been reported for ncRNA–mRNA interactions in *B. subtilis* (68,69) and in *S. aureus* (70,71). Moreover, in contrast to a reduced resistance towards several stress conditions and an attenuated virulence phenotype reported for *hfq* deletion mutants of several γ-proteobacteria including *Vibrio cholerae* (21), *P. aeruginosa* (43) or *Salmonella typhimurium* (72), a *S. aureus hfq*− mutant showed no detectable defects (73). This contrasts the somewhat reduced stress tolerance and virulence reported for a *hfq*− mutant of *Listeria monocytogenes* (20) that contains likewise a Hfq protein with a short C-terminal extension. In the latter bacterium, Hfq has been shown to confer stability to at least one ncRNA (74). Taken these reports, it remains to be seen whether Hfq-mediated riboregulation, i.e. translational silencing or activation of mRNAs by ncRNAs, is confined to γ- and perhaps β-proteobacteria. On the other hand, the elucidation of the molecular function of Hfq homologues with short C-terminal tails in firmicutes remains a further challenge.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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