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Arginine patch predicts the RNA annealing activity of Hfq from Gram negative and Gram positive bacteria

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Abstract

The Sm-protein Hfq facilitates interactions between small non-coding RNA (sRNA) and target mRNAs. In enteric Gram negative bacteria, Hfq is required for sRNA regulation, and *hfq* deletion results in stress intolerance and reduced virulence. By contrast, the role of Hfq in Gram positive is less established and varies among species. The RNA binding and RNA annealing activity of Hfq from *Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Bacillus subtilis* and *Staphylococcus aureus* were compared using minimal RNAs fluorescence spectroscopy. The results show that RNA annealing activity increases with the number of arginines in a semi-conserved patch on the rim of the Hfq hexamer and correlates with the previously reported requirement for Hfq in sRNA regulation. Thus, the amino acid sequence of the arginine patch can predict the chaperone function of Hfq in sRNA regulation in different organisms.

Graphical Abstract

Keywords

Hfq; RNA chaperone; bacterial sRNA; molecular beacon; fluorescence anisotropy; small non-coding RNA; Sm protein

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Hfq is an abundant bacterial member of the Sm-superfamily that was originally discovered as an essential host factor for replication of *E. coli* bacteriophage Q β (1, 2). More recently, the role of Hfq in small RNA (sRNA) mediated gene regulation has received considerable attention (3). sRNAs regulate diverse physiological behaviors such as stationary phase, stress responses, sugar metabolism, iron utilization and quorum sensing (4, 5, 6).

The Sm core of Hfq forms a ring-shaped homo-hexamer that binds the U-rich terminator at the 3' end of most sRNAs and stabilizes the sRNA against turnover (7, 8). In most Gram negative bacteria, the opposite (distal) face also binds single-stranded A-rich RNA sequences found in mRNA targets and some sRNAs (9, 10, 11, 12). Importantly, complementary sequences in the sRNA and mRNA target also interact with an arginine patch (R16, R17 and R19) on the rim of the *E. coli* Hfq hexamer (13, 14, 15, 16, 17) that is essential for Hfq's RNA annealing activity (15). In vitro annealing assays showed that *E. coli* Hfq accelerates sRNA-mRNA base pairing 30 to 100 times by nucleating the double helix between two complementary RNA strands (18, 19). This annealing activity is eliminated when all three arginines are replaced with alanine, and even an arginine to lysine substitution reduces *E. coli* Hfq's chaperone activity (15).

Although Hfq is needed for sRNA regulation in Gram negative bacteria such as *Escherichia coli* and *Salmonella* (20), the requirement for Hfq in post-transcriptional regulation by Gram positive bacteria remains ambiguous (21). Hfq contributes to stress tolerance and pathogenicity in *Listeria monocytogenes* (22), *Clostridium difficile* (23) and other Gram positive bacteria (24). By contrast, deletion of Hfq from *Bacillus subtilis* and *Staphylococcus aureus* has almost no detectable phenotype (25, 26).

We noticed that the reported role of Hfq in sRNA-mediated gene regulation correlates with variations in the amino acid sequence of the basic patch on the Hfq rim (15). Although the potent *E. coli* Hfq chaperone has three arginines between residues 16–19 (RRER), the *B. subtilis* protein has only one arginine (RKEN) and the *S. aureus* protein has none (KANQ) (Fig. 1). Here, we use a well-established fluorescent RNA annealing assay to measure the chaperone activity of Hfq homologs from two Gram negative bacteria (*E. coli*, *Pseudomonas aeruginosa*) and three Gram positive bacteria (*L. monocytogenes*, *B. subtilis* and *S. aureus*). We show that RNA annealing activity increases with the number of arginine residues on the rim, with Gram positive Hfq proteins having little or no activity in our assay. Thus, the amino acid sequence of the arginine patch predicts Hfq's chaperone activity and the degree to which Hfq facilitates interactions between sRNA and mRNA pairs in different bacteria.

RNA annealing activity of Hfq from different bacteria

An alignment of Hfq sequences from different bacteria revealed a partial conservation of amino acid residues that lie on the outer rim of the Hfq hexamer (15). Nearly all Hfq sequences contain an arginine at position 16 (*E. coli* numbering) on the proximal side of the rim. Position 17 is usually R or K, position 18 is neutral polar or acidic, and position 19, which lies closest to the distal face, is the most variable (15). In order to understand how this sequence variation alters the chaperone activity of Hfq, we over-expressed and purified five

Hfq homologs (*E. coli*, *P. aeruginosa*, *L. monocytogenes*, *B. subtilis* and *S. aureus*) with different numbers of arginines in this motif (Fig. 1b).

The annealing activity was measured using a fluorescent molecular beacon that base pairs with target RNAs that end with 3' U₆ (target-U6) or 3' A₁₈ (target-A18) (Fig. 2a) (27). These target RNAs specifically bind the proximal face or distal face of *E. coli* Hfq, respectively, with $K_d = 0.45$ nM and 0.1 nM Hfq₆ (27). The complementary region of the target and the beacon interacts with the rim of Hfq.

The molecular beacon (50 nM) and target RNA (100 nM) were rapidly mixed in a stopped-flow spectrometer as described previously (28). The increase in beacon fluorescence over time was fit to a double exponential rate equation and the rate constants of the fast phase were plotted against Hfq₆ concentration (Fig. 2b, c). As previously observed (19), the rate of RNA annealing increased with added *E. coli* Hfq, reaching a maximum value in 50 nM Hfq₆, which equals the amount of molecular beacon. For target-U6 RNA, annealing was 15 times faster with 50 nM Hfq, compared to the no Hfq background (0.08 s⁻¹ to 1.3 s⁻¹; Fig. 2b). When the target RNA specifically binds to the distal face of Hfq (target-A18), 50 nM Hfq increased the annealing rate 100 times over the background (0.08 s⁻¹ to 8 s⁻¹; Fig. 2c).

We next compared the activity of *E. coli* Hfq, which has a rim motif of RRER, with that of *P. aeruginosa* Hfq (RKER). *P. aeruginosa* Hfq was noticeably less active in this assay than *E. coli* Hfq, increasing the maximum annealing rates only 4-fold and 10-fold for target-U6 and target-A18 RNAs, respectively, in saturating Hfq concentrations (Fig. 2b,c). This is presumably due to replacement of R17 by lysine in *P. aeruginosa*. *L. monocytogenes* Hfq, which has lysines at positions 17 and 19 (RKEK), was even less active, raising the maximum annealing rates only two- to three-fold (Fig. 2b,c). Hfq proteins from *B. subtilis* or *S. aureus* had no measurable activity in this assay above the no protein control (Fig. 2b,c). *S. aureus* Hfq is an extreme example in which even the highly conserved R16 is replaced by lysine (KANQ), and the other side chains on the rim are neutral.

sRNA binding to Hfq

Although the proximal face of Hfq is well conserved between different bacteria, differences in sRNA binding might also explain the range of observed annealing rates. We measured equilibrium binding between Hfq and a Cy3-labeled mini-sRNA using fluorescence anisotropy. The 3' end of the mini-sRNA contains the important recognition elements of class I sRNAs (12), such as a 3' U₆ tail which binds to the proximal pore and a RydC sequence that binds to the rim of Hfq (Fig. 3a).

E. coli Hfq formed a tight complex with Cy3-sRNA (2.7 ± 0.8 nM; Fig. 3), but the other bacterial Hfq proteins bound the Cy3-sRNA less tightly than *E. coli* Hfq (18 to 100 nM). This decrease in sRNA affinity may arise from the loss of arginine residues on the rim. Nevertheless, even *S. aureus* Hfq, which has no arginines on its rim, binds to Cy3-sRNA with $K_d = 100$ nM, well within the range of concentrations tested in our annealing assays. Because we were unable to recover RNA annealing activity at higher Hfq concentration, the lack of annealing activity cannot be explained by poor sRNA binding.

RNA binding to the distal face

The distal face of *E. coli* Hfq preferentially interacts with a repeated (AAN) triplet motif (9, 31), but this protein surface varies among Hfq homologs, resulting in altered binding specificities (32). For example, the *Bacillus* protein is reported to bind an AG dinucleotide (33). Fluorescence anisotropy binding assays (Fig. 4a) showed that target-A18 RNA interacts strongly with *E. coli* Hfq ($K_d = 2.8 \pm 0.1$ nM), but weakly or not at all with *L. monocytogenes* and *S. aureus* Hfq (> 2 μ M; Fig. 4b). The inability of Gram positive Hfq proteins to recognize an A-rich RNA target may contribute to their poor chaperone function, but does not explain their inability to act on sRNAs.

Conclusion

Our results show that the ability of Hfq to facilitate RNA interactions increases with the number of arginines in the rim motif, and that the rim motif also correlates with the reported role of Hfq in sRNA-mediated regulation in the respective host organism (Fig. 1). Hfq from *E. coli* and other γ -proteobacteria have a strong arginine patch (RRER or RKER) and are highly active in our RNA annealing assay, while *hfq* variants with low arginine content (RKEN or KANQ) have no measurable activity.

The strong RNA annealing activity of *E. coli* and *P. aeruginosa* Hfq agrees with the integral role of Hfq in sRNA regulation in enteric γ -proteobacteria. For example, *E. coli*, *Salmonella*, *Pseudomonas* and *Vibrio* sRNAs are known to control stress response, pathogenicity, quorum sensing and biofilm formation (4, 34, 35, 36). In contrast, although many Gram positive bacteria encode sRNAs, the requirement for Hfq in these organisms varies considerably. For example, deletion of *hfq* has little effect on stress response and pathogenesis in *B. subtilis* and *S. aureus*, suggesting that Hfq is dispensable in these species. Other bacteria lack Hfq entirely (37). Yet others, such as *Burkholderia cenocepacia* and *Bacillus anthracis*, encode two or more *hfq* homologs that appear to have different functions (38, 39).

Jousselin et al. (21) proposed that the requirement for Hfq lessens as the free energy of sRNA-mRNA pairing becomes more favorable, suggesting that Hfq is needed to stabilize sRNA-mRNA interactions. This concept agrees with the observed correlation between RNA chaperone activity and genetic function (Fig. 1b). On the other hand, *C. difficile hfq* (40), *B. subtilis* Hfq (25), and *B. anthracis hfq2* and *hfq3* (39) partially complement an *hfq* null in *E. coli*, indicating that they have some ability to support sRNA regulation. These proteins retain the proximal face residues involved in sRNA recognition. Other sequence variations, such as the capacity to recognize A-rich sequence motifs in mRNA targets or the size of the C-terminal extension, likely contribute to the function of Hfq in different types of bacteria. For example, recognition of A-rich RNA motifs is important for the function of *E. coli* Hfq (28), yet the distal face residues that form the A-specific binding site are missing in many Gram positive *hfq* sequences (24), eliminating this RNA interaction (Fig. 4). More work is needed to understand how each of these interactions contributes to the varied roles of Hfq and other Sm proteins. Nevertheless, our results show that the arginine content of the rim motif is useful for predicting the chaperone activity of Hfq in different organisms.

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Highlights

- *E. coli* Hfq facilitates sRNA-mRNA base pairing via a semi-conserved arginine patch.
- sRNA binding and RNA annealing by Hfq from different bacteria were compared.
- The RNA annealing activity of Hfq correlates with the size of the arginine patch.
- The arginine patch motif predicts the genetic requirement for Hfq in a bacterium.

Fig. 1. Basic patch on the rim of Hfq

(a) *E. coli* Hfq (*1HK9*) rendered as cyan surface, superimposed with the structure of RydC sRNA in complex with Hfq (*4V2S*). Red sticks, 3' U₆ in the proximal pore; yellow sticks, internal 5' CUUC bound to the rim of Hfq. The remaining RydC residues are omitted for clarity. Blue surface, arginine patch with R16, R17 and R19 side chains shown for one subunit. The rim sequences of Hfq proteins used in this study are shown on the right. (b) Summary of Hfq activity from different organisms. The RNA annealing rates relative to no

Hfq and sRNA binding are from this work; sRNA regulation is based on published work as described in the text.

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Fig. 2. RNA annealing activity depends on the rim sequence

E. coli, *B. subtilis* and *S. aureus* Hfq proteins were purified using a Ni-affinity column as described previously (29). *P. aeruginosa* and *L. monocytogenes* Hfq proteins were purified using the IMPACT-CN system (30). All proteins were further purified using a cation exchange column (GE) (29) and verified by SDS-PAGE. The ratio $A_{260}/A_{280} \sim 0.7$ for all of the preparations. (a) Molecular beacon assay for RNA annealing. The loop of the molecular beacon (5'-FAM-GGUCCCCACUCGACUCACCACCGGACC-3'-DABCYL) is complementary to target-U6 (5'-GUGGUCAGUCGAGUGGU₆) and target-A18 (5'-

GUGGUCAGUCGAGUGGA₁₈). The increase in fluorescence intensity due to base pairing between molecular beacon and target RNAs was monitored by an Applied Photophysics SX 18MV stopped-flow spectrometer as described previously (27). 50 nM molecular beacon and 100 nM target RNAs were rapidly mixed in 0 – 250 nM Hfq in TNK buffer (10 mM Tris-HCl, pH 7.5; 50 mM NaCl and 50 mM KCl) at 30 °C. Individual kinetic traces were fitted to a double exponential rate equation (18). (b) Initial rate constants of annealing target-U6 at different concentrations of Hfq hexamer. The rate constants are the average of 5 technical replicates with standard deviations less than 5%. (c) Rate constants of annealing target-A18 versus Hfq concentration, as in (b).

Fig. 3. Effects of rim arginines on sRNA binding

(a) Hfq binding to synthetic Cy3-labeled mini-sRNA (5' Cy3 – GUGGUCAGUCGAGUGGCUUCCGUCCAUUUCGACGUUUUUU; IDT) increases the fluorescence anisotropy of Cy3. The mini-sRNA contains 3' U₆ (red), a loop from RybB, and a CUUC motif from RydC (yellow). (b) Fractional binding from the relative change in anisotropy versus [Hfq₆]. The anisotropy of 5 nM Cy3-sRNA in TNK buffer at 30 °C was measured using a Fluorolog-3 spectrofluorometer (Horiba) with single excitation and emission monochromators at 540 nm and 565 nm, respectively. Individual data sets were

fitted to $f = \Delta A [\text{Hfq}]^n / ([\text{Hfq}]^n + K_d^n)$, in which ΔA is the difference in the anisotropy of the sRNA-Hfq complex and the free sRNA, and K_d is the dissociation constant. For titrations with *E. coli* Hfq, the data were iteratively fit by using the initial estimate of K_d to adjust the concentration of free Hfq, $[\text{Hfq}] = [\text{Hfq}]_{\text{total}} - 6f_B[\text{RNA}]_{\text{total}}$, which was used to obtain a new estimate of K_d . The uncertainty in K_d was less than 25% based on the error in the fits and the difference between two independent trials.

Fig. 4. Gram-positive Hfq proteins do not recognize A-rich target RNA

(a) Binding of FAM-labeled rA₁₈ RNA to the distal face of Hfq was measured by fluorescence anisotropy, as previously described (15). (b) Fractional binding versus [Hfq₆] was measured and fitted to a two-state Hill equation as described in Fig. 3b.