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Structure and RNA-binding properties of the bacterial LSm protein Hfq

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Abbreviations: ncRNA, non-coding RNA; sRNA, small RNA; Hfq, host factor for Qβ replication; (L)Sm, like-Sm; poly-(A), poly-adenosine; mRNA, messenger RNA; PAPI, poly-(A) polymerase I; aLSM protein, archeal (L)Sm protein; A, adenosine; U, uridine; Y, pyrimidine; R, purine; SELEX, systematic evolution of ligands by exponential enrichment

Over the past years, small non-coding RNAs (sRNAs) emerged as important modulators of gene expression in bacteria. Guided by partial sequence complementarity, these sRNAs interact with target mRNAs and eventually affect transcript stability and translation. The physiological function of sRNAs depends on the protein Hfq, which binds sRNAs in the cell and promotes the interaction with their mRNA targets. This important physiological function of Hfq as a central hub of sRNA-mediated regulation made it one of the most intensely studied proteins in bacteria. Recently, a new model for sRNA binding by Hfq has been proposed that involves the direct recognition of the sRNA 3' end and interactions of the sRNA body with the lateral RNA-binding surface of Hfq. This review summarizes the current understanding of the RNA binding properties of Hfq and its (s)RNA complexes. Moreover, the implications of the new binding model for sRNA-mediated regulation are discussed.

Introduction

Post-transcriptional regulation of gene expression by non-coding RNAs (ncRNAs) is a crucial mechanism for the cell to impose complex and rapid control over its proteome and, hence, its physiological state. In bacteria, a large variety of ncRNAs regulate genes that are responsible for the specific adaptation to constantly changing metabolite and environmental conditions (for review, see refs. 1 and 2). A special group of bacterial ncRNAs are the so-called Hfq-binding small RNAs (sRNAs) that specifically interact with mRNA targets based on partial sequence complementarity. Ultimately, sRNAs affect bacterial gene expression by regulating the stability and translation of the respective transcripts (for review, see refs. 3 and 4). The physiological function of these sRNAs depends on the homohexameric (L)Sm protein Hfq, which is now established as a central mediator of sRNA-based gene regulation in bacteria. Hfq specifically recognizes the structurally diverse sRNAs and facilitates the interaction with their target mRNAs (for review, see ref. 5). The specificity of Hfq for sRNAs, however, could not be explained by previously described RNA-binding modes of this protein.[6-8] Therefore, one of the central aims in the field is to understand how Hfq recognizes and binds RNA in general and sRNAs in particular. Recently, several studies provided new insights into the RNA binding properties of Hfq, ultimately suggesting a new model for sRNA recognition.[9-12] This review therefore summarizes the current knowledge of the atomic structure of Hfq and its interactions with RNA. Furthermore, the new sRNA binding model and its implications for sRNA-mediated regulation are discussed in the context of current and previously published results. A detailed discussion of the (L)Sm protein superfamily as well as a revision of the role of Hfq in the context of sRNA-mediated regulation and mRNA degradation is provided in the accompanying articles within this Special Focus.

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Hfq - The Bacterial (L)Sm Protein

Discovery and cellular functions. Hfq is the only bacterial LSm homolog and one of the first characterized RNA binding proteins. The first known and also eponymous function of Hfq coincided with its original identification as a host factor for the replication of the Qβ-phage in *Escherichia coli*.[13] In this context, Hfq was shown to bind at the cytosine-rich 3' end of the plus-strand viral RNA and Hfq binding was required for the initiation of minus-strand RNA synthesis by the Qβ-replicase.[14-16] Moreover, Hfq has been implicated in various processes of bacterial RNA metabolism and several studies suggested a function of Hfq in polyadenylation-mediated mRNA degradation.[17-20] It was shown that Hfq binds oligo-adenosine stretches of degradation intermediates and that stoichiometric Hfq binding stimulates the processive polyadenylation by poly-(A) polymerase I (PAPI).[21,22] The resulting poly-$(A)_n$ termini represent a bacterial degradation signal and ultimately promote RNA decay by the degradosome and/or serve as a toehold for exonucleases.[23,24]

Inactivation of the *hfq* gene in *Escherichia coli* and *Salmonella typhimurium* resulted in severe phenotypes especially under adaptive growth conditions. The observed pleiotropic effects included changes in cell viability and morphology

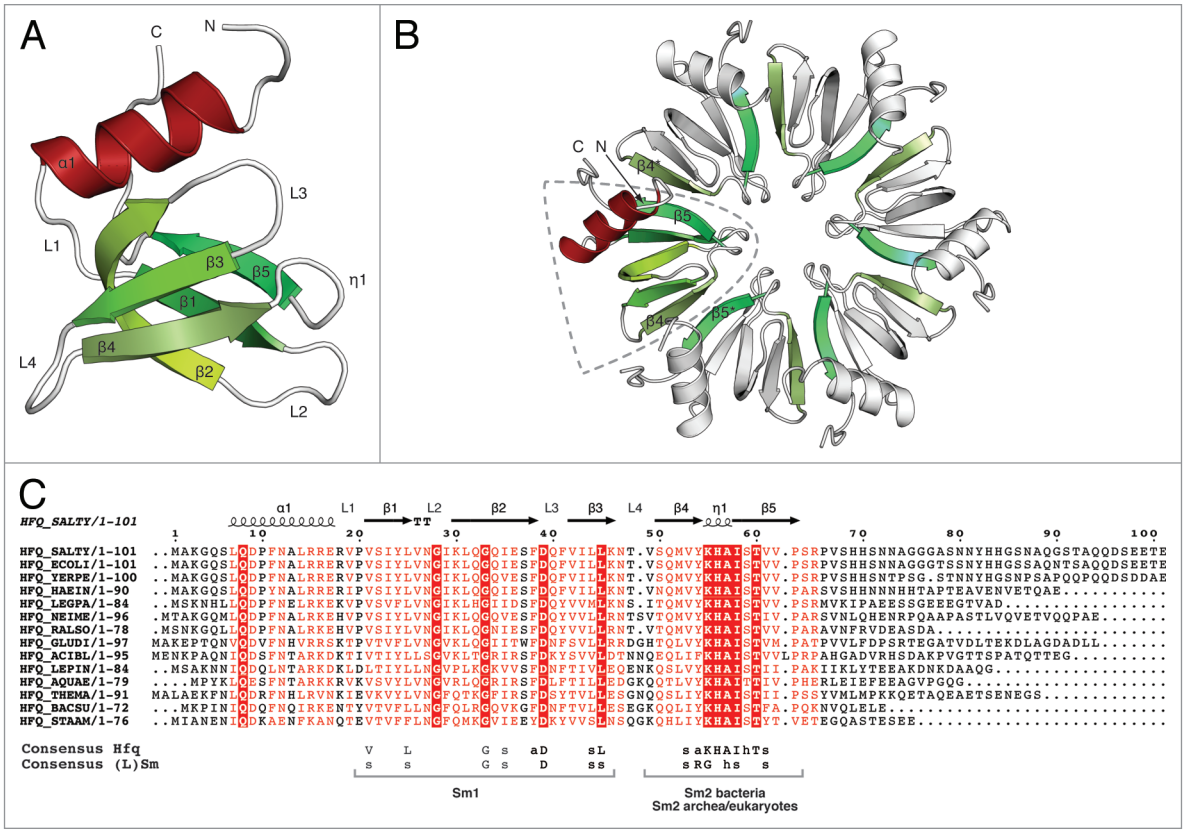

Figure 1. Fold and oligomerization of the LSm domain. (**A**) Cartoon representation of the LSm domain of *Salmonella typhimurium* Hfq (PDB-ID: 2YLB[9]). Secondary structure elements (α-helix 1, red; β-sheets 1–5, green; loops 1–4, white) as well as the N- and C-termini are indicated. The five β-strands form a half-open barrel with the N-terminal α-helix stacked on top. (**B**) Cartoon representation of the *Salmonella typhimurium* Hfq_6 ring. A single LSm domain is highlighted and colored as in (**A**); secondary structure elements involved in intersubunit interactions are colored green. Six LSm domains assemble into a homohexameric ring resulting in an extended β-sheet spanning the entire hexamer. Intersubunit contacts are provided by backbone interactions between strands β4 and β5 to strands β5* and β4* in the neighboring (indicated by *) monomers, respectively. All (L)Sm rings assemble in a polar way with the N-terminal α-helices located on the same side of the oligomer. (**C**) Multiple sequence alignment of bacterial Hfq proteins. The secondary structure of *Salmonella typhimurium* Hfq (PDB-ID: 2YLB[9]) is superimposed on the primary sequence. The Sm consensus sequences are shown below the alignment (the nature of the amino acid side-chains is: s = small hydrophobic, I, L, V; h = hydrophilic, S, T; a = aromatic, Y, F). Highly conserved residues are red (> 70% conservation) or white in red boxes (100% conservation). While the Sm1 signature is conserved in all domains of life, Sm2 is divergent in bacteria. The species abbreviations and UniProt-IDs are: γ-Proteobacteria**:** SALTY, *Salmonella typhimurium* (P0A1R0); ECOLI *Escherichia coli* (P0A6X3); YERPE, *Yersinia pestis* (A4TRN9); HAEIN, *Hemophilus influenza*, (P44437); LEGPA *Legionella pneumophila* (Q5X982). β-Proteobacteria: NEIME, *Neisseiriameningitides* (B9VV05); RALSO, *Ralstonia solanacearum* (Q8Y025). α-Proteobacteria: GLUDI, *Gluconacetobacter diazotrophicus* (Q8RMG6); Acidobacteria: ACIBL, *Acidobacteria bacterium* (Q1IIF9). Spirochaetales: LEPIN, *Leptospira interogans* (Q8F5Z7). Aquafecales: AQUAE *Aquifex aeolicus*, (O66512). Thermotogales: THEMA, *Thermotoga maritime* (Q9WYZ6). Fermicutes: BACSU, *Bacillus subtilis* (O31796); STAAM, *Staphylococcus aureus* (Q99UG9).

accompanied by decreased growth rates and virulence.[25,26] The bacteria showed general defects in transcription and/or translation: in *Salmonella*, for example, transcriptomic profiling and deep-sequencing of Hfq-associated RNAs revealed that Hfq, as a global regulator, (in)directly affects approximately one-fifth of the *Salmonella* genome.[27] Furthermore, the cells were strongly impaired in their adaptability to stress conditions like stationary growth, oxidation or UV light.[28-30] The strong influence of Hfq on bacterial gene expression is now attributed to its central role in sRNA-mediated regulation, where Hfq was shown to (1) bind and stabilize sRNAs in the cell, (2) facilitate base pairing between sRNAs and their targets and (3) trigger subsequent steps like translational repression and decay (for review, see ref. 5).

The (L)Sm fold and oligomerization. Hfq is a member of the (L)Sm protein superfamily. The founding members of this large group are the classical Sm and like-Sm (LSm) proteins, which are conjointly referred to as (L)Sm proteins (for review, see refs. 31 and 32, as well as the reviews by C. and J. Wilusz and C. Mura et al. in this Special Focus). (L)Sm proteins are found in all three domains of life and are characterized by the presence of a conserved protein fold, the so-called LSm-domain.[33] Topologically, the LSm fold consists of an N-terminal α-helix (α1) followed by five β-strands (β1-5) and the secondary structure elements are separated by five loops (L1-5) of variable length (**Fig. 1A**). The β-strands form an antiparallel, strongly bent β-sheet with the α-helix stacked on top of the open barrel. On the primary sequence level, the LSm fold is characterized by

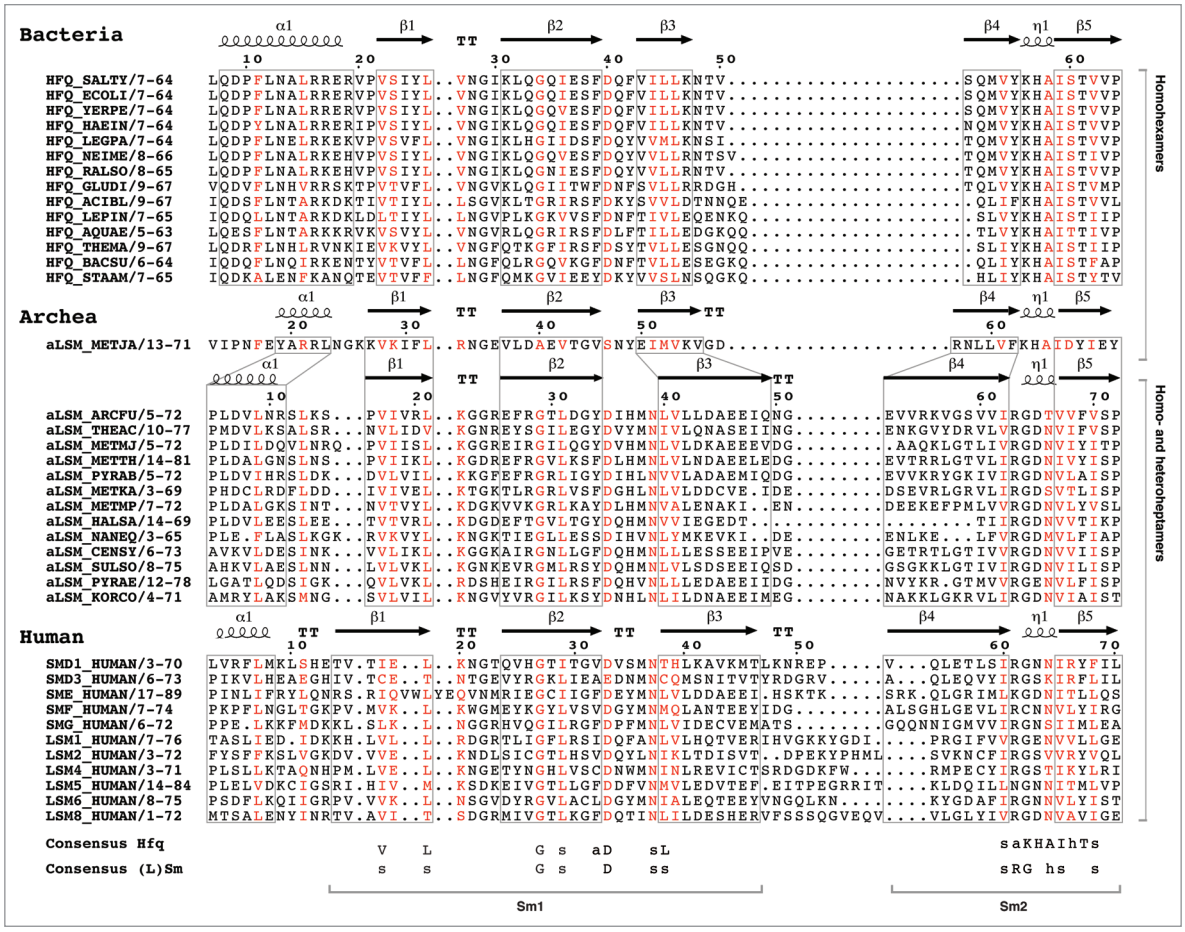

Figure 2. The LSm fold is conserved in all domains of life. Multiple sequence alignment of the LSm domain found in bacterial (Hfq), archeal (aLSM) and human (L)Sm proteins. Residues with > 70% sequence conservation are shown in red. The secondary structure of always the top species of each subgroup is indicated on top of the primary sequence. Although the sequence conservation of the different LSm domains is low, the LSm fold is conserved. The main differences are the length of the N-terminal α-helix, the strands β3 and β4 as well as loop L1 and L4. Interestingly, the only known archeal homohexameric Hfq protein (aLSM_METJA) comprises an Sm2 signature very similar to its homohexameric bacterial homologs, while the Sm2 motif of homoheptameric archeal homologs is more related to the human (L)Sm proteins. Species abbreviations and UniProt accession numbers for the selected bacterial sequences are given in **Figure 1**. Species abbreviations and UniProt accession numbers of archeal LSm proteins are: Methanococci: METJA, *Methanococcus jannaschii* (Q58830); Archeoglobi: ARCFU, *Archeoglobus fulgidus*, (O29386); Thermoplasmata: THEAC, *Thermoplasma acidophilum*, (P57670); Methanomicrobia: METMJ, *Methanoculleus marisnigri*, (A3CS14); Methanobacteria: METTH, *Methanobacterium thermoautotrophicum*, (O26745); Thermococci: PYRAB, *Pyrococcus abyssi*, (Q9V0Y8); Methanopyri: METKA, *Methanopyrus kandleri*, (Q8TYS2); Methanococci: METMP, *Methanococcus maripaludis,* (Q6LY45); Halobacteria: HALSA, *Halobacterium salinarum*, (Q9HPS2); Nanoarcheoata NANEQ, *Nanoarcheum equitans,* (Q74N54); Thaumarcheoata CENSY, *Cenarcheum symbiosum,* (A0RZA4); Crenarcheoata SULSO, *Sulfolubus solfataricus*, (Q97ZQ0); Crenarcheoata PYRAE, *Pyrobacculum aerophilum*, (Q8ZYG5); Koracheoata CORCO, *Korarcheum cryptofilum*, (B1L734). The accession numbers for the HUMAN (*Homo sapiens*) Sm proteins are: SmD1, (P62314); SmD3, (P62318); SmE, (P62304); SmF, (P62306); SmG, (P62308). LSm proteins: LSm1, (O15116); LSm2, (Q9Y333); LSm4, (Q9Y4Z0); LSm5, (Q9Y4Y9); LSm6, (P62312); LSm8, (O95777).

two conserved sequence signatures Sm1 and Sm2, where residues from Sm1 reside in the first three β-strands and residues from Sm2 reside in strands β4 and β5 (**Fig. 1C**). The Sm1 signature can be identified in all (L)Sm proteins, while Sm2 is divergent in the bacterial Hfq proteins (**Fig. 2**). Although, the sequence conservation between prokaryotic,[6,34] archeal[35,36] and eukaryotic[37-40] ring-forming (L)Sm proteins is low, the characteristic LSm fold is preserved.

All classical eukaryotic Sm (SmB/B', SmD1, SmD2, SmD3, SmE, SmF, SmG) and LSm (LSm1-8 and LSm10-11) proteins assemble into ring-shaped heteroheptamers, which represent the functional biological entity (for review, see ref. 32). The interface between two subunits is made up from residues in the β4 and β5* strands (* indicates a neighboring subunit) and adjacent subunits interact in an oriented way, resulting in an oligomerization where all N-terminal α-helices are located on the same face of the ring (**Fig. 1B**).

In bacteria, there is usually only one (L)Sm homolog, the Hfq protein, which assembles into homohexamers.[3,46] Interestingly, enterobacterial Hfq proteins comprise unusually long C-terminal extensions (**Fig. 1C**). The sequence of this unstructured region is not conserved and its biological function is controversial.[41-45] It has been shown that Hfq proteins, which lack the C-terminal extension, can functionally replace the full-length protein[27,46]

and, recently, it has been suggested that the C-terminal tails may be involved in additional RNA interactions.[47]

Although several crystal structures of ring-forming (L)Sm proteins have been determined, their oligomerization state is still a matter of debate. One reason complicating the interpretation of experimental data is the intrinsic propensity of the wedge-shaped LSm domain to assemble into oligomers and consequently pentameric,[48,49] hexameric,[6,34] heptameric[36,40,50] and octameric[51] rings have been described for (L)Sm proteins of bacterial, archeal and eukaryotic origin. For bacterial Hfq, a homopentameric form has once been suggested,[48] but no further experimental evidence supporting this hypothesis could be provided in the following years. Furthermore, the published crystal structures of bacterial Hfq proteins[6,9,34,42,52-54] as well as electron microscopy[55] and small-angle X-ray scattering[43] data have established the hexamer as the functional state of Hfq. In contrast to eukaryotic Sm protein heteroheptamers,[37-39,56,57] the assembly of archeal LSm and bacterial Hfq oligomers is less well understood. Recent in vitro evidence however, suggests that *E. coli* Hfq exists in a monomer-hexamer equilibrium in solution with the hexamer being most active in RNA binding and annealing.[58]

The RNA Binding Properties of Hfq and the Initial sRNA Binding Model

Hfq achieves its different cellular tasks by the differential use of distinct RNA binding sites and already very early experiments indicated a preference of Hfq for single-stranded adenosine- and uridine-rich sequences.[59,60] Subsequently, two independent RNA binding sites—distal and proximal—with respective specificities, could be characterized on opposite surfaces of the Hfq hexamer.[8]

The distal RNA binding site. The so-called distal RNA binding site shows specificity for purine-rich sequences and is thought to be relevant for the interaction of Hfq with internal adenosine-rich sequences of mRNAs[47,61,62] and with poly-$(A)_n$ tracts found at the 3' end of RNA degradation intermediates.[21,22] In 2008, Soper et al. originally showed that an adenosine-rich motif (5'-AAYAA-3'; A, adenosine; Y, pyrimidine) in the leader sequence of the *rpoS* mRNA is important for the stimulation of *rpoS* translation by the Hfq-dependent sRNA DsrA, ultimately resulting in the expression of the *rpoS* encoded transcription factor σ^S.[61] Similar Hfq-binding sequence motifs could also be identified in the leader sequences of *flhA*[62] and *glmS*[47] mRNAs and by genomic SELEX (5'-AAYAAYAA-3'),[63] indicating that adenosine-rich sequences represent important Hfq binding sites in target mRNAs. The structural basis for RNA binding to the distal surface was provided by several crystal structures of Hfq hexamers bound to short, purine-rich oligonucleotides.[7,64,65] In 2009, the complex structure of *Escherichia coli* Hfq bound to poly-$(A)_{15}$ RNA revealed a tripartite RNA binding motif on each Hfq monomer (A-R-E motif: A site, adenosine specificity site; R site, purine selectivity site; E site, non-discriminatory entrance/exit site) resulting in specific binding of up to six poly-$(ARN)_n$ repeats (A, adenosine; R, purine; N, any nucleotide) per hexamer.[7] In contrast, the recent complex structures of Hfq homologs from the gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* in complex with poly-$(A)_4$[64] and $(AG)_3A$[65] RNA respectively, revealed a bipartite RNA-binding motif composed of a purine nucleotide-specificity site and a sequence-independent linker site. These data indicate that RNA binding to the distal surface can differ between bacterial species: Hfq proteins from gram-negative bacteria comprise a tripartite binding motif for poly-$(ARN)_n$ repeats, while Hfq homologs from gram-positive species are likely to interact with poly-$(AN)_n$ sequences via bipartite binding sites.[64]

The distal RNA binding site is thought to be important for the function of Hfq as an RNA interaction platform and it has been suggested that Hfq promotes sRNA/mRNA base pairing by simultaneous interactions with poly-$(ARN)_n$ sequences of mRNAs via its distal and with sRNA via its other RNA binding surface (for review, see ref. 5). Consistently, it has been demonstrated by Mikulecky et al. that the distal site is indeed independent and available for simultaneous RNA binding.[8]

The proximal RNA binding site. The second, so-called proximal RNA binding site of Hfq was shown to preferably bind uridine-rich sequences and has been implicated in sRNA recognition. In analogy to the eukaryotic Sm heteroheptamer, where the RNA threads through the central pore of the ring, the proximal site of Hfq has been assumed to bind internal A/U-rich sequences and *Staphylococcus aureus* Hfq was hence co-crystallized with a $A(U)_5G$ RNA substrate.[6] In the complex, the first six nucleotides were bound in specific binding pockets around the central pore and the 3' terminal guanosine was exposed. Additionally, probing experiments of Hfq/sRNA complexes indicated a preference of Hfq for single-stranded A/U-rich sequences in vicinity to secondary structure elements.[66,67] Combined, these observations led to a widely accepted binding model where Hfq interacts with internal A/U-rich sequences of sRNAs via its proximal surface.[6,7,23,66,67] This binding model however could not explain (1) the general protection of sRNAs by Hfq beyond few nucleotides and (2) the apparent specificity of Hfq for the structurally very diverse sRNAs.

Hfq and its Interaction with (s)RNAs —A Changing Perspective

The prevailing sRNA binding model has been challenged by the discovery that Hfq specifically binds 3' terminal uridine-rich sequences and that this binding specificity is utilized to recognize a common feature in bacterial sRNA transcripts, namely the ρ-independent transcription terminator. The molecular details of this new binding mode were examined using a U_6 RNA substrate and in vitro binding experiments demonstrated that a free 3' hydroxyl group is crucial for the high-affinity interaction.[9] The crystal structure of *Salmonella typhimurium* Hfq in complex with the U_6 oligonucleotide revealed that the specificity and affinity of Hfq for RNA 3' ends relies on a special mode of direct recognition of the 3' hydroxyl group in the context of a constricted RNA backbone conformation.

In parallel, the importance of sRNA 3' end binding by Hfq was demonstrated in vivo. Using the model sRNA SgrS, Otaka and colleagues found that shortening of the 3' uridine-rich sequence eliminates the interaction of the sRNA with Hfq and abolishes

target mRNA regulation.[10] Given that ρ-independent terminators are found at the 3' end of most Hfq binding sRNAs,[27] it is, therefore, very likely that 3' end binding contributes significantly to the selective recognition of sRNAs by Hfq.

A new RNA binding surface. Importantly, the interaction of the sRNA 3' end with the proximal RNA binding site of Hfq was found to be not the only determinant for sRNA binding. A subsequent analysis of Hfq RNA binding mutants identified an additional binding surface for the sRNA body on the lateral surface of the hexamer.[12] This so-called lateral RNA binding surface consists of six patches (one per monomer) of conserved polar residues (R16, R17, R19 and K47), and a mutational analysis of the model sRNA RybB demonstrated that especially single-stranded, internal uridine-rich sequences interact with these sites.

The new sRNA binding model. Combined, the results of the recent studies have changed the view of how sRNAs are thought to interact with Hfq.[9-12] Ultimately, the data suggest a new model for sRNAs recognition where, the specific interaction of Hfq with the sRNA 3' end anchors the sRNA on the proximal face of the hexamer, whereas internal uridine-rich sRNA sequences contribute additively to complex stability by interacting with several of the six lateral RNA binding sites of Hfq (**Fig. 3**). Although binding experiments with RybB mutants indicated that also the 3' terminator structure contributes to sRNA binding, its detailed binding mode remains unclear. Given the binding specificities of the proximal, distal and lateral RNA-binding sites of Hfq and its low affinity for double-stranded sequences,[68] it seems unlikely that the terminator stem-loop interacts with one these surfaces.

Therefore, recognition of the sRNA 3' end on the proximal surface of Hfq should rather position the terminator stem-loop above the proximal side of the ring. Possible interaction sites for the terminator stem-loop could be the C-terminal extensions of Hfq, which are very flexible[43,47] and contain several polar and aromatic residues that could interact with the grooves of the RNA helix.

Hfq—The RNA interaction platform. The partial sequence complementarity between sRNAs and their mRNA targets determines the specificity of sRNA-mediated regulation.[3,69] Consequently, the putative complex formation between the Hfq/sRNA complex and an mRNA target has been addressed by several studies.[61,70-74] The application of high-resolution chromatography techniques combined with optimized RNA and protein constructs recently provided a more detailed understanding of multipart Hfq/(s)RNA complexes and confirmed that the three RNA binding sites of Hfq are independent and can be used simultaneously in any combination.[12] The experimental data showed that in a binary complex composed of the model sRNA RybB and Hfq, the sRNA is presented in a hybridization competent state on Hfq and readily forms a duplex with its mRNA target. In the resulting sRNA-mediated ternary complex, the sRNA 3' end remained anchored to the proximal site of Hfq, while the sRNA body was released from the lateral surface to base pair with the mRNA target sequence. In the case of a natural, full-length mRNA, however, it has to be considered that remote $(ARN)_n$-repeats in the mRNA sequence could simultaneously interact with the distal surface of Hfq and influence complex formation and stability.[62,70] The physiological relevance of these in vitro observations is strongly supported by recent evidence, which indicates that the ternary complex of Hfq, sRNA and mRNA guides the endoribonuclease RNase E to initiate degradation of the target mRNA.[75]

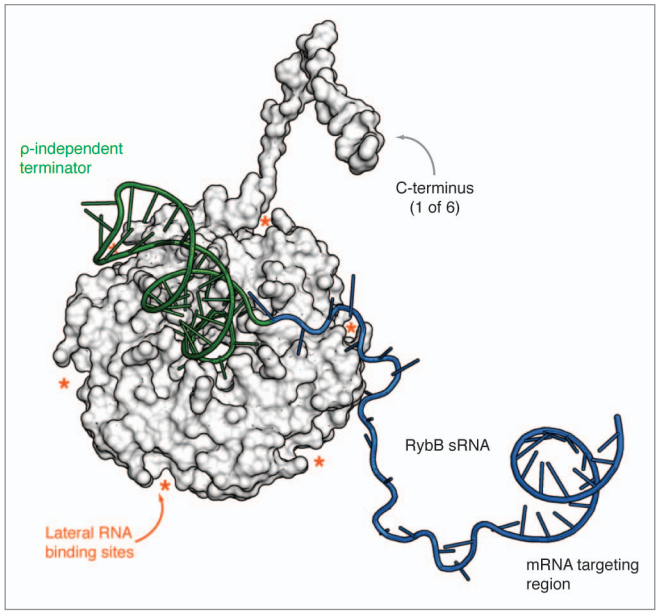

Figure 3. Model of an Hfq/sRNA complex. Proximal side view of a model of the Hfq/RybB complex. The Hfq hexamer (PDB-ID: 2YLC[9]) is shown as a surface representation with a superimposed model of only one of the six Hfq C-termini for clarity. RybB sRNA is depicted in cartoon representation with the ρ-independent terminator colored in green, the single-stranded sequence is blue and the location of the seed region is indicated. The asterisks mark the location of the six lateral RNA binding sites of Hfq. The model was assembled using COOT[98] considering the biochemical and structural evidence summarized in this review. The depicted structure of the C terminus was modeled using HHpred.[99] The model shows how Hfq might interact with RybB sRNA and also gives an impression of the proportions of the sRNA body with respect to the size of the terminator stem-loop and the Hfq protein.

sRNA Recognition by Hfq—Implications and Future Directions

sRNA 3' end binding by Hfq—The key to selectivity? The apparent selectivity of Hfq for bacterial sRNAs is still an unresolved question in the field of sRNA research. The discovery that Hfq directly recognizes sRNA 3' ends therefore suggests an elegant explanation for the selectivity of Hfq for many sRNAs despite their structural diversity. The high sequence conservation of the proximal RNA binding site (KHAI motif, **Fig. 1C**) in bacteria implies that the recognition of uridine-rich sRNA 3' ends is a general property of Hfq and likely represents the predominant function of the proximal surface. Clearly, this hypothesis has to be verified experimentally in the future, as to date, direct 3' end recognition by Hfq has only been demonstrated for a few sRNAs (RybB,[9] SgrS and RyhB[10,11]).

Although a uridine-rich 3' end is the only recurrent similarity of Hfq-binding sRNAs, the ρ-independent transcription

terminator is not a unique feature. Actually, the transcription of a majority of bacterial mRNAs is terminated using this mechanism resulting in uridine-rich 3' ends prone to bind Hfq.[27,76-78] An immediate question raised by this fact is how Hfq discriminates between these different RNA substrates in the cell. An important factor for efficient proximal site binding seems to be the accessibility of the single-stranded uridine-rich 3' sequence and a first quantitative picture of Hfq-bound RNAs was provided by deep sequencing experiments.[27] The data showed that sRNAs that display uridine-rich tails are highly enriched on Hfq. In contrast, Hfq-independent RNAs either had processed 3' ends or base-paired terminal uridines. In the future, a comprehensive analysis of RNA sequences that are directly bound by Hfq in the cell could reveal the in vivo relevance of 3' end recognition and, furthermore, show which additional sRNA sequences are involved in Hfq binding.

The observation that the sRNA/mRNA duplex remains associated with Hfq via the sRNA 3' end[12] further emphasizes the importance of this binding mode for sRNA regulation. Apparently, this high affinity interaction is sufficient to tether the sRNA to Hfq and may also explain why no additional general Hfq binding sites have been conserved among sRNAs. It is tempting to hypothesize that only those Hfq/sRNA complexes are functional in sRNA-mediated regulation, whose sRNA 3' end is bound to the proximal site and allow for ternary complex formation with the mRNA followed by RNaseE recruitment.[75,79] An interesting (in vivo) experiment to prove such a model would be to test the regulatory potential of an sRNA, which on the one hand is stably bound to Hfq via the lateral site, but where 3' binding is prevented for example by a 2'-3' cyclic phosphate.

The Lateral RNA Binding Surface of Hfq—The Missing Link?

In the past, probing experiments of several Hfq/sRNA complexes have shown that internal A/U-rich sequences of sRNAs are protected by Hfq.[61,66,72,80] However, the protection of multiple sites was difficult to reconcile with sRNA binding to the single proximal surface of Hfq. The identification of the six lateral sites as a third RNA binding surface furthermore provided a rational explanation for the protection of a much larger portion of the sRNA body.

In principle, every sRNA could occupy a different number of lateral sites depending on the length and spatial distance of its A/U-rich sequences. In an extreme scenario, it is even possible that every individual sRNA takes a different path on the lateral surface of Hfq resulting in heterogeneity of Hfq/sRNA complexes, which, in turn, could have complicated the interpretation of probing data.

Several single point mutants of lateral site residues have been described previously, however, only a partial reduction in RNA binding affinity was observed indicating that each residue additively contributes to (s)RNA binding.[8,71,81] The sequence alignment of bacterial Hfq homologs shows that the lateral surface is conserved and, therefore, likely a general property of Hfq proteins (**Fig. 1C**). Arginine 16 shows the highest sequence conservation, while in position 17 and 19, positively charged (Arginine or Lysine) and aromatic (Histidine) residues are found. The side chains of these amino acids are often involved in RNA binding interactions, as they can interact with RNA by stacking or hydrogen bonding.[82,83] Moreover, single-stranded RNA sequences are more accessible for this type of intermolecular interactions and, therefore, the lateral site may represent an ideal binding surface. Also in *Staphylococcus aureus*, where Hfq function is currently under debate (for review, see ref. 84), the sequence conservation suggests that the key biochemical properties of Hfq should be conserved (**Fig. 1C**): the proximal RNA binding site (KHAI motif) is identical with enterobacterial Hfq proteins and several residues on the lateral surface (K10, K41) could be engaged in RNA interactions.

Implications for sRNA competition. In 2010, Fender and colleagues demonstrated that sRNAs can displace each other on Hfq and similar observations were later made in vivo.[85,86] The suggested model for sRNA competition consequently implied (1) a transient association of the competitor sRNA with existing sRNA/Hfq complexes, followed by (2) an exchange of RNA binding sites and (3) the eventual dissociation of one of the sRNAs. Because the A/U content and the localization of single-stranded sequences in the sRNA body differ between individual sRNAs, binding of the sRNA body to lateral surface could be an important factor in the context of sRNA competition for Hfq. In contrast to the proximal site, which is likely to be occupied by only one sRNA 3' end, the six lateral sites could serve as individual entry points for a competitor sRNA and allow for a gradual displacement of a bound sRNA. Clearly, this hypothesis has to be tested in the future e.g., by competition experiments using selective Hfq lateral site mutants.[12] Moreover, the weak and transient interaction of the sRNA body with Hfq suggests that the lateral sites might represent the "chaperoning" surface of Hfq[87-90] (for review, see ref. 91). Evidently, structural information is required to understand the detailed interaction of RNA with the lateral surface and the associated binding parameters have to be determined. Nevertheless, the interaction of the sRNA body with the lateral surface of Hfq emerges as an additional element in the sRNA/Hfq interaction and has to be further investigated.

(s)RNA Binding to the Distal Surface of Hfq

Although the lateral and proximal RNA binding sites of Hfq seem to be sufficient for sRNA binding, this was only shown on the example of the model sRNA RybB, which does not contain poly-$(ARN)_n$ repeats.[12] In principle, other sRNAs that comprise poly-$(ARN)_n$ sequences could (also) bind to the distal site of Hfq or additional Hfq rings could associate with an Hfq/sRNA complex via the distal surface. The underlying biochemical property of the Hfq distal RNA binding site is the cooperative, length-dependent assembly of Hfq rings on poly-$(A)_n$ RNA, suggesting that for a stable interaction with the distal surface, at least four Hfq monomers have to be occupied by (four) consecutive (ARN)-repeats that are rarely found in sRNAs associated with Hfq.[7,12] Importantly, the cooperative effect could be due to the homo-polymeric sequence and, hence, the high local concentration of binding sites.[12] It is

therefore normal to expect that cooperative binding is rather limited to the interaction of Hfq with poly-$(A)_n$ sequences. Thus, in sRNA-mediated regulation, RNA binding to the distal surface of Hfq may rather be relevant for the simultaneous interaction of the Hfq/sRNA complex with an mRNA target and may contribute to the stabilization of the sRNA/mRNA duplex.[47,61,62] Again, this hypothesis could be tested in vivo by analyzing the functionality of selective Hfq distal site mutants[12] in sRNA-mediated regulation.

Hfq/sRNA/mRNA Ternary Complex Formation—Implications for sRNA Function

RNaseE was shown to directly interact with Hfq and to initiate the degradation of the mRNA target as well as the base paired sRNA by endonucleolytic cleavage.[92-94] A very recent study furthermore suggested an active role for the sRNA/mRNA/Hfq complex in stimulation of RNase E cleavage.[75] Consequently, it might be interesting to investigate whether the observed structural rearrangement of Hfq/sRNA complexes upon target mRNA binding[12,68,70,95] is recognized by downstream factors like RNaseE. Because RNaseE cleavage preferably occurs at single stranded A/U-rich sites,[66,94,96] the release of the sRNA body from the lateral surface of Hfq would expose putative RNaseE sites both in the sRNA and the mRNA target, which could initiate their degradation. Furthermore, it is possible that the freed surfaces of Hfq serve as an interaction site for the direct recruitment of RNaseE to the Hfq/sRNA/mRNA particle.

Conclusions

The systematic discovery of bacterial sRNAs and the growing understanding of their mechanism of action opened an exciting field of RNA research. Furthermore, the observation that the biological function of sRNAs depends on the Hfq protein, did finally explain the pleiotropic effects of Hfq on bacterial gene expression. The specific interaction of Hfq with sRNAs turned out to be rather complex and is still not fully understood. However, the accumulated knowledge of the RNA binding properties of Hfq and the characterization of its (s)RNA complexes, has now set the starting point for the design of new experiments toward an ever more detailed molecular analysis of Hfq function in the cell and the mechanisms of sRNA-mediated regulation.

Cleary, atomic structures of Hfq/sRNA complexes represent one of the most important but also challenging aims in the field and a first glimpse was provided by a study using small-angle X-ray scattering to analyze the overall shape of *Vibrio cholerae* Hfq in complex with Qrr sRNA.[97] The determination of high-resolution structures is complicated by the inherent flexibility and heterogeneity of Hfq/sRNA complexes, which interferes with their crystallization. The design and utilization of minimal sRNAs and Hfq mutants in combination with elaborated purification protocols (e.g., high-resolution chromatography) and stabilization (e.g., by cross-linking) techniques should identify the most promising candidate complexes for structure determination in near future.

Most importantly, the biochemical properties of Hfq and its (s)RNA complexes have to be reconciled with the in vivo situation. Therefore, a global analysis of sRNA sequences that are directly bound by wild-type Hfq (and/or its RNA binding mutants) could allow for a critical evaluation of the proposed sRNA binding model. Furthermore, the inclusion of selective Hfq RNA binding mutants in in vivo experiments should facilitate a further differentiation between the various cellular functions of Hfq and, thereby, contribute to an advanced understanding of sRNA-mediated regulation in bacteria.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here:
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