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POINT OF VIEW

RNA silencing in plant symbiotic bacteria: Insights from a protein-centric view

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ABSTRACT

Extensive work in model enterobacteria has evidenced that the RNA chaperone Hfq and several endoribonucleases, such as RNase E or RNase III, serve pivotal roles in small RNA-mediated post-transcriptional silencing of gene expression. Characterization of these protein hubs commonly provide global functional and mechanistic insights into complex sRNA regulatory networks. The legume endosymbiont *Sinorhizobium meliloti* is a non-classical model bacterium with a very complex lifestyle in which riboregulation is expected to play important adaptive functions. Here, we discuss current knowledge about RNA silencing in *S. meliloti* from the perspective of the activity of Hfq and a recently discovered endoribonuclease (YbeY) exhibiting unprecedented catalytic versatility for the cleavage of single- and double-stranded RNA molecules.

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Small non-coding RNAs (sRNAs) are recognized as ubiquitous components of complex post-transcriptional regulatory networks controlling virtually any adaptive response of bacteria to environmental changes.[1,2] The canonical activity mechanism of most RNA regulators involves base pairing to mRNAs, which may affect translation and/or turnover rates of the targeted transcripts in an either negative or positive manner.[3,4] Complementarity between sRNAs and *trans*-encoded target mRNAs is typically short and imperfect, and therefore, a protein serving a chaperone function often assists these type of interactions. The widely conserved bacterial Sm-like protein Hfq has long been perceived as the major RNA binder fulfilling a dual role as sRNA stabilizer and matchmaker in *trans*-mRNA regulation.[5-7] Conversely, *cis*-acting antisense sRNAs (asRNAs) rely on extensive perfect complementarity to regulate mRNAs transcribed from the opposite strand and are believed to be largely protein-independent.[8] Although sRNA-mediated upregulation of gene expression in bacteria is not unusual, target repression is the typical outcome of sRNA activity.[4,8] Most *trans*-acting sRNAs interact with their targets at the translation initiation region, thereby outcompeting ribosome binding and impairing protein synthesis. Primary translational repression is often coupled to irreversible target mRNA degradation, which is catalyzed by RNases that, in some cases, can be recruited to the sRNA-mRNA interplay by Hfq.[7,9,10] As component of the degradosome complex, the single-strand specific endoribonuclease RNase E is one Hfq-interacting partner in some bacterial species, initiating mRNA decay upon base pairing with *trans*-sRNAs.[11,12] Alternatively, the prototypical double-strand endoribonuclease RNase III can initiate cleavage of some of these imperfect RNA-RNA duplexes and is a major effector of mRNA decay initiation driven by antisense transcription.[13,14] RNA-binding proteins and ribonucleases are thus pivotal elements in riboregulatory networks and their characterization commonly provide primary functional hallmarks to complex sRNA landscapes. These paradigms about the biological roles and mechanistic principles of riboregulation mostly derive from extensive pioneering research on classical model bacteria (i.e., *Escherichia coli* and related enterobacteria) and clinically relevant pathogens. However, during the last two decades the availability of distantly related genomic sequences from bacteria with widely diverse lifestyles has increased exponentially. Comprehensive analysis of this overwhelming genomic information and its derived transcriptomes is expected to unveil novel insights into protein-assisted RNA regulation underlying unique ecological specializations.

Rhizobia, the nitrogen-fixing endosymbionts of legume plants, are an example of soil-dwelling microbes with complex biology and genomic architecture. During their life cycle, these bacteria shift from a free-living state in soil to a chronic intracellular residence within root nodules elicited in the plant host.[15] To establish competitive populations in soil, rhizobia must efficiently adapt their physiology to several abiotic and biotic variables typical of this harsh environment, e.g., oligotrophy, pH and temperature oscillations, drought, hyperosmolarity, native microbial populations or specific root exuded plant compounds like flavonoids. Upon root hair infection, nodule development and differentiation of nitrogen-fixing bacteroids are events governed by a coordinated exchange of signaling molecules between both symbiotic partners. Plant-derived signals in response to rhizobial infection include defensive reactive oxygen species, cysteine-rich peptides that promote bacteroid differentiation or microaerobiosis required by nitrogenase functioning within nodules.[15] These cues reprogram gene expression in rhizobia, which has been assumed to rely almost entirely on transcriptional control by protein factors, whereas post-transcriptional RNA-dependent regulation has been systematically overlooked.

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The group of root-nodule bacteria includes species mostly belonging to the Rhizobiales order within the larger α–subgroup of proteobacteria. Among them, *Sinorhizobium meliloti*, the partner of the forage legume alfalfa (*Medicago sativa*) and the model plant *M. truncatula*, has served as main genetically tractable model species to study riboregulation in rhizobia.[16-18] The closely related *S. meliloti* reference strains Rm1021 and Rm2011 have highly syntenic composite genomes consisting of 3 megareplicons; the chromosome (3.54 Mb) and the so-called symbiotic plasmids pSymA (1.35 Mb) and pSymB (1.68 Mb), the latter exhibiting chromosome-like features.[19,20] Computational comparative genomics and RNAseq-based screens have uncovered a large and heterogeneous inventory of non-coding transcripts expressed by this bacterium.[21-25] The challenge now is deciphering the function and activity mechanisms of this plethora of newly discovered *S. meliloti* sRNAs in the regulation of the adaptive responses imposed by both the saprophytic life in soil and the symbiotic transitions. Here, we discuss insights into RNA-based regulation in *S. meliloti* derived from RNA networks of two proteins functionally linked to sRNA activity; the RNA chaperone Hfq (henceforth *Sm*Hfq) and the novel recently discovered endoribonuclease YbeY (*Sm*YbeY).[26,27]

Discrete contribution of SmHfq to riboregulation

Hfq has been positioned at the core of post-transcriptional regulatory networks in bacteria. Accordingly, the identity of Hfq regulated genes and binding transcripts typically draft major pathways relying on sRNA-based regulation.[28-30] In *S. meliloti*, lack of Hfq compromises adaptation of free-living bacteria to abiotic stress and the overall symbiotic performance on legume roots.[31-34] Profiling of the *Sm*Hfq regulon and RNA ligands by a diversity of genome-wide screens has traced the pathways specifying this phenotypic outcome.[27,31,33-35] These independent studies converged in the identification of nutrient uptake, energy metabolism, stress response, biosynthesis of surface macromolecules, quorum sensing (QS) and symbiotic nitrogen-fixation as major processes influenced by Hfq in *S. meliloti*. Most *Sm*Hfq-associated RNA species derive from or are full-length mRNAs, which collectively represent ~18% of the *S. meliloti* protein coding genes. More than third of these genes have been reported to be differentially expressed in *hfq* knockout mutants. In contrast, *Sm*Hfq binds and stabilize only small fractions of the *trans*-acting and asRNAs expressed by this bacterium (14% and 2%, respectively).[27,36]

Almost half of the mRNAs concurrently bound and regulated by *Sm*Hfq encode components of ABC transport systems and cytoplasmic metabolic enzymes.[27] Remarkably, *Sm*Hfq negatively affects the steady-state levels of a large array of mRNA partners that encode periplasmic substrate binding proteins with affinity for a diversity of nitrogen-containing compounds (e.g., amino acids, peptides or polyamines), a feature common to many bacterial Hfq homologs characterized to date. In enterobacteria, the Hfq-dependent sRNA GcvB post-transcriptionally silences many of these mRNAs, which integrate one of the largest sRNA regulons described in bacteria.[37] Similarly, computer predictions in *S. meliloti* delivered a plethora of putative targets of the *Sm*Hfq-dependent homologous AbcR1 and AbcR2 sRNAs within the set of *Sm*Hfq mRNA ligands encoding proteins for the uptake of nitrogen sources.[27,31,38] Further genetic reporter assays *in vivo* confirmed AbcR1/2-mediated downregulation of some of these mRNAs, most likely by a canonical silencing mechanism involving primary occlusion of the respective ribosome binding site.[27,38] Similar regulatory roles and activity mechanisms have been described for AbcR homologs in the related α–proteobacteria *Agrobacterium tumefaciens* and *Brucella abortus*.[39,40] Rhizobial genomes encode exceptionally large inventories of ABC transport systems (e.g., 200 in *S. meliloti* compared with 67 in *E. coli*) for the uptake of widely diverse growth limiting nutrients in soil and specific plant-derived compounds in the rhizosphere and endosymbiotic compartments.[19,41] Selective silencing of unnecessary simultaneous transcription of transporter gene sets, which is commonly driven by analogous compounds, would contribute to optimize rhizobial metabolism throughout the symbiotic interaction.

From a methodological point of view, these findings provide another proof of principle validating the atlas of Hfq-binding transcripts as a reliable source of functional sRNA-mRNA regulatory pairs in bacteria. However, since most *S. meliloti* sRNAs are Hfq independent, mining of this data set would render a rather partial view of RNA regulation in this bacterium. Indeed, it has been recently reported that the Hfq-independent *trans*-RNAs RcsR1 and EcpR1 regulate other symbiotically relevant processes such as QS or cell cycle.[42,43] In line with this notion, post-genomic research on phylogenetically distant bacterial species increasingly suggests that the contribution of Hfq to riboregulation is more limited than expected from the early studies in classical model bacteria. Even in *Salmonella*, Hfq-binding sRNAs represent only a third of the non-coding transcripts expressed by this bacterium.[44] On the other hand, almost half of bacterial species lack an *hfq* gene and in others, this is fully dispensable for riboregulation.[8] These evidences have prompted searches for alternative bacterial RNA-binding proteins fulfilling Hfq-like roles as novel nodes of post-transcriptional regulatory networks.

SmYbeY is a novel endoribonuclease with unprecedented metal-dependent catalytic versatility

A few years ago, it was proposed that the YbeY protein might functionally replace Hfq for RNA regulation in *S. meliloti*.[45] YbeY belongs to the UPF0054 family of metallo-hydrolases and is almost ubiquitous in bacteria. Indeed, its coding gene is part of the minimal prokaryotic genome set, thus anticipating that it serves a fundamental function probably affecting a wide range of cellular processes.[46] Consequently, YbeY has been shown to be essential in some bacterial species, whereas in others its loss-of-function leads to severe pleiotropic phenotypes that in *S. meliloti* resemble those of the *hfq* mutants.[45,47-50] Structural modeling of *Sm*YbeY unveiled a positively charged cavity reminiscent of the MID domain of the Argonaute (AGO) proteins that assist RNA silencing in eukaryotes.[45] Further supporting a likely similar competence of bacterial YbeY homologs for RNA binding and/or regulation, recent studies in *S. meliloti*, *E. coli* and *Vibrio cholerae* have shown that lack of YbeY misregulates subsets of sRNAs and their predicted mRNA partners.[45,50,51] In particular, *Sm*YbeY and *Sm*Hfq co-regulate a handful of sRNAs in a positive manner, thus suggesting that both proteins might share a chaperone function in sRNA pathways.[45] To test this hypothesis, the *Sm*YbeY-dependent RNA network has been recently probed by genome-wide approaches (i.e., CoIP and

transcriptomics) and experimental setups similar to those used previously for *Sm*Hfq.[26] *Sm*YbeY CoIP-RNA was sparingly enriched with RNA species, contrary to what was observed for *Sm*Hfq, indicating either reduced competence of *Sm*YbeY for RNA binding or transient contacts with its partner transcripts. Further, transcriptomics revealed scarce influence of *Sm*YbeY on the expression of the ~500 *trans*-sRNAs probed with the microarrays. Together, these findings argued against a major Hfq-like role of *Sm*YbeY as RNA stabilizer and matchmaker in riboregulation.

The biochemical properties of YbeY were first reported for the *E. coli* homolog (*Eco*YbeY).[52] This protein behaved as a metallo endoribonuclease that specifically degraded *in vitro* synthetized single-stranded RNA substrates as well as several endogenous RNA molecules such as mRNAs and rRNA. This catalytic activity, which is largely conferred by the histidine triad $H(X)_3H(X)_4DH$ characteristic of metallo-hydrolases, is also required for late-stage 70S ribosome quality control and 16S rRNA processing. Similarly, purified *Sm*YbeY has metal-dependent endoribonuclease activity on synthetic and endogenous RNAs.[26] However, it is not required for 16S rRNA maturation and exhibits striking differences in substrate specificity with respect to what was proposed for its *E. coli* counterpart. *Sm*YbeY is competent for cleaving both single-stranded (ssRNA) and double-stranded RNA (dsRNA) substrates, which is an unprecedented ability among bacterial endoribonucleases characterized to date. Nevertheless, *in vitro* assays evidenced certain preference of *Sm*YbeY for degrading dsRNA. Specifically, reactivity patterns and catalytic efficiency of *Sm*YbeY on the generic highly structured R1.1 RNA, which is typically used to assay RNase III activity, resembled cleavage of RNase III on the same substrate. Interestingly, *Sm*YbeY-mediated catalysis showed strong metal co-factor dependence, with Mn^{2+} favoring overall cleavage efficiency on all substrates and Ca^{2+} specifically blocking reactivity on dsRNA and R1.1 RNA. This feature may have important implications for substrate selectivity and modulation of *Sm*YbeY activity *in vivo*. In this regard, it is known that protection against the oxidative burst elicited in the host cells upon infection requires maintenance of Mn^{2+} and Ca^{2+} homeostasis in intracellular mammal pathogens and plant endosymbionts.[53,54]

This recent discovery extends the repertoire of RNases predicted to be encoded by the *S. meliloti* genome, which includes homologs of the known silencing endoribonucleases RNase E and RNase III, as well as the 3'-5' exoribonuclease polynucleotide phosphorylase (PNPase), all of which have been shown to degrade the free pool of sRNAs in enterobacteria.[19,55] It has been shown that *S. meliloti* RNase E promotes degradation of *sinI* and *dnaA* mRNAs upon base-pairing with EcpR1 and RcsR1 sRNAs in an Hfq-independent manner.[42,43] RNase J is a ribonuclease functionally related to RNase E in the control of quorum sensing and S-adenosyl methionine (SAM) homeostasis in *S. meliloti*.[56] However, the contribution of these enzymes to RNA turnover and regulation in the group of plant symbiotic bacteria remains mostly unexplored.

Sm*YbeY influences decay of bulk and sRNA-regulated mRNAs*

Transcriptomics uncovered a large *Sm*YbeY-dependent RNA network integrated by more than 650 mRNAs (~11% of the *S. meliloti* protein-coding genes) and hardly 35 *trans*-sRNAs (less than 6% of those identified in this bacterium).[26] Two-thirds of these transcripts, including most of *trans*-sRNAs, most likely are secondary molecular targets of *Sm*YbeY, i.e., their steady-state levels are positively influenced by the endoribonuclease activity of *Sm*YbeY. This set of mRNAs encodes fundamental cellular functions related to energy metabolism, RNA turnover and translation, whose downregulation would explain the pleiotropic phenotype associated to a *Sm*YbeY loss-of-function. Comparison of the *Sm*YbeY- and *Sm*Hfq-dependent gene sets revealed a rather discrete overlap, suggesting that despite of their commonalities, the free-living and symbiotic phenotypes of *S. meliloti hfq* and *ybeY* mutants rely on the alteration of largely different pathways.

Notwithstanding this major divergence in their RNA networks, several putative *Sm*YbeY substrates can be foreseen among the known *Sm*Hfq-binding mRNAs whose intracellular concentration increases in the absence of *Sm*YbeY. The *fix* cluster (*fixN1O1Q1P1GHI1S1*) and the *nifA* mRNA, encoding cytochromes associated with the nitrogenase complex and a master regulator of nitrogen fixation, respectively, are all downregulated in *S. meliloti hfq* mutants and recovered in their entire lengths by CoIP with *Sm*Hfq (Fig. 1; upper and middle panels). These findings suggest that *Sm*Hfq interacts with these mRNAs

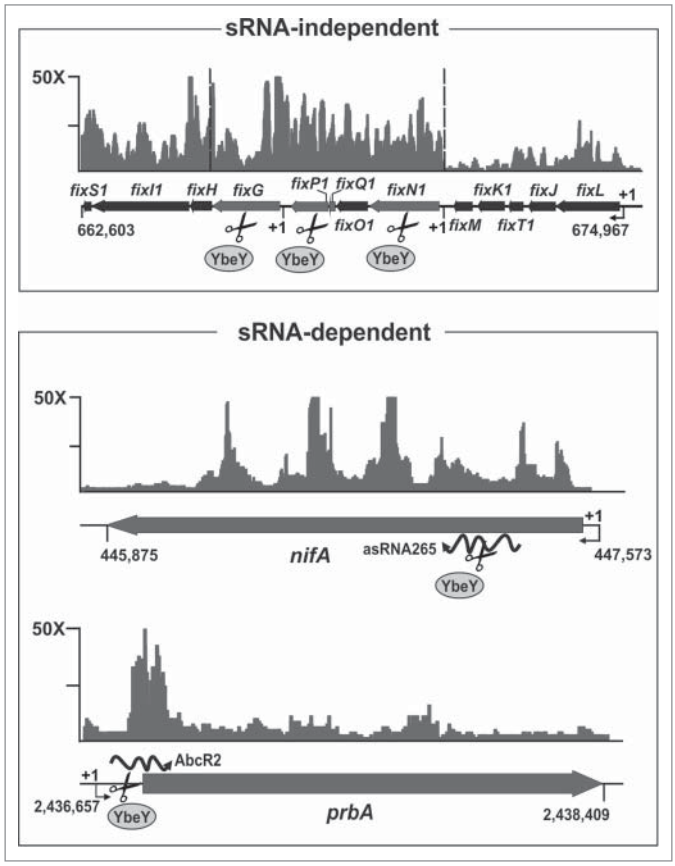

Figure 1. *Sm*YbeY influences turnover of bulk and sRNA-regulated mRNAs. Shown are enrichment patterns of putative *Sm*YbeY mRNA substrates in *Sm*Hfq CoIP-RNA.[27] The vertical axis indicate fold enrichment with respect to a control CoIP-RNA. Relevant genomic information for each mRNA is provided in the schematics below the plots. Numbering denotes coordinates in the *S. meliloti* Rm1021 genome. *Sm*YbeY-mediated silencing of *nifA* and *prbA* mRNAs is likely triggered by base-pairing with the *trans*-sRNA AbcR2 and the asRNA265, respectively. See text for further details.

in a protective mode, thus impairing their degradation by *Sm*YbeY. Therefore, *Sm*YbeY would fulfil a role in bulk mRNA turnover in rhizobia that in enterobacteria has been largely attributed to RNase E.[12] Nonetheless, silencing of the *nifA* mRNA could be driven by the yet uncharacterized asRNA265 (Fig. 1; middle panel). In this particular case, *Sm*YbeY would serve an RNase III-like role promoting degradation of the *nifA* mRNA by a primary cleavage at the RNA duplex. Recent RNA-seq surveys have shown pervasive antisense transcription in *S. meliloti*, which is strikingly biased toward nodulation and nitrogen fixation genes within the pSymA megaplasmid.[25,57] Despite of *S. meliloti* chromosome almost triples pSymA in size and gene content, transcriptomics revealed that *Sm*YbeY has similar relative impact on gene expression in both replicons.[26] It is therefore tempting to speculate on a major role of *Sm*YbeY in the antisense control of mRNA levels from symbiotic genes clustered in pSymA.

The set of *Sm*YbeY and *Sm*Hfq co-regulated mRNAs includes several from amino acid transporter genes, whose expression is negatively influenced by both proteins.[26,33] Interestingly, known targets of the *Sm*Hfq-dependent AbcR1/2 sRNAs were found among these mRNAs (e.g., *livK* or *prbA*).[27,38] The enrichment patterns of these transcripts in *Sm*Hfq CoIP-RNA are compatible with their ribonucleolytic degradation upon *Sm*Hfq-assisted base pairing with AbcR1/2 at their 5'-UTR regions (Fig. 1; bottom panel). *In vivo* reporter and *in vitro* assays using the AbcR2-*prbA* interaction as a proof of principle further supported *Sm*YbeY-mediated cleavage of this imperfect RNA duplex.[26] Existing *in silico* and experimental evidences suggest that the homologous AbcR1 and AbcR2 mRNAs govern a large regulon of transporter genes in *S. meliloti*, similar to that of GcvB in enterobacteria.[27,38,58] More recently, another *S. meliloti trans*-sRNA called NfeR1 has been shown to contribute to the Hfq-independent post-transcriptional silencing of ABC transporter mRNAs in response to external hyperosmolarity and during symbiosis.[59] Therefore, it is likely these 3 sRNAs and their suites of mRNA targets shape a dense overlapping regulon controlling nutrient uptake in response to different external cues, i.e., they show rather different expression profiles.[38,59] Competition for shared regulatory sRNAs can promote mRNA cross-regulation via target mRNA-derived sponges that base-pair with and destabilize the sRNA, thereby relieving repression of the entire regulon.[60] In the GcvB network, RNase E catalyzes both biogenesis of the RNA sponge (i.e., SroC) and GcvB degradation.[37] It is therefore plausible that *Sm*YbeY may be an active player in this layer of regulation of the particularly large network of *S. meliloti* ABC transporters.

Concluding remarks and future perspectives

As hubs in RNA networks, RNA-binding proteins and ribonucleases are global markers of sRNA function and activity mechanisms. In the α-rhizobia *S. meliloti*, the RNA chaperone *Sm*Hfq has a major role in riboregulation of nutrient uptake, but interacts with scarcely 14% of *trans*-sRNAs uncovered by RNAseq. This finding suggests that other yet unknown proteins may probably serve an Hfq-like function in *S. meliloti*. A recent screen of ribonucleoprotein complexes fractionated on a glycerol gradient (Grad-seq) identified ProQ as a new RNA-binding protein in *S. enteria*.[44] However, ProQ is not as ubiquitous as Hfq and, among α-rhizobia, only *Rhizobium leguminosarum* bv. *viceae* encodes a recognizable homolog of this protein.[61] Therefore, the search for new RNA-binding proteins assisting riboregulation in plant symbiotic bacteria remains opened.

Recent biochemical and genetic characterization of *Sm*YbeY revealed that it behaves as a metal-dependent endoribonuclease rather than as an Hfq-like chaperone, as suggested by previous structural predictions and phenotypic evidences. *Sm*YbeY indistinctly cleaves ssRNA and dsRNA, which is a unique feature among known bacterial endoribonucleases. The crystal structure, mutagenesis analysis and genome-wide mapping of *Sm*YbeY cleavage sites will shed light about the molecular bases of this catalytic versatility and its regulation by different metal co-factors. *Sm*YbeY influences turnover of bulk and sRNA-regulated mRNAs. The discrete contribution of *Sm*YbeY to the different levels of RNA regulation, i.e., sRNA turnover and target mRNA decay, as well as the identity of its protein partners must be further explored in the near future. In particular, the role of *Sm*YbeY in mRNA silencing driven by antisense transcription will be key to understand post-transcriptional regulation of genuine symbiotic genes.

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