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CURRENT REVIEW

Insights into the Noncoding RNome of Nitrogen-Fixing Endosymbiotic α-Proteobacteria

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Symbiotic chronic infection of legumes by rhizobia involves transition of invading bacteria from a free-living environment in soil to an intracellular state as differentiated nitrogen-fixing bacteroids within the nodules elicited in the host plant. The adaptive flexibility demanded by this complex lifestyle is likely facilitated by the large set of regulatory proteins encoded by rhizobial genomes. However, proteins are not the only relevant players in the regulation of gene expression in bacteria. Large-scale high-throughput analysis of prokaryotic genomes is evidencing the expression of an unexpected plethora of small untranslated transcripts (sRNAs) with housekeeping or regulatory roles. sRNAs mostly act in response to environmental cues as post-transcriptional regulators of gene expression through protein-assisted base-pairing interactions with target mRNAs. Riboregulation contributes to fine-tune a wide range of bacterial processes which, in intracellular animal pathogens, largely compromise virulence traits. Here, we summarize the incipient knowledge about the noncoding RNome structure of nitrogen-fixing endosymbiotic bacteria as inferred from genome-wide searches for sRNA genes in the alfalfa partner *Sinorhizobium meliloti* and further comparative genomics analysis. The biology of relevant *S. meliloti* RNA chaperones (e.g., Hfq) is also reviewed as a first global indicator of the impact of riboregulation in the establishment of the symbiotic interaction.

In addition to its agronomical and ecological relevance, the mutualistic symbiosis established between certain nitrogen-fixing α- and β-proteobacteria (i.e., rhizobia) and leguminous plants has long been recognized as a model experimental system to investigate chronic infection processes of eukaryotic hosts by microbes. Development of the rhizobia–legume interaction involves a complex series of events, triggered by the coordinated exchange of signal molecules between both partners, that lead to the organogenesis of root or, less frequently, stem nodule structures (Gibson et al. 2008; Jones et al. 2007; Kereszt et al. 2011; Oldroyd and Downie 2008; Soto et al. 2009). Mature nodules accommodate the differentiated nitrogen-fixing bacteroids intracellularly, thus providing the microaerobic environment required by rhizobial nitrogenases to reduce the atmospheric dinitrogen to ammonia.

Three genomic features are likely major contributors to the adaptive flexibility demanded by the transition of rhizobia from a competitive free-living state in soil to its final intracellular residence within nodules. First, with a few exceptions (i.e., *Mesorhizobium* and *Bradyrhizobium* spp.), rhizobial species exhibit a multireplicon pangenomic structure consisting of a single chromosome and a variable number of nonessential accessory plasmids of mosaic origin (Galardini et al. 2011; González et al. 2010; Schneiker-Bekel et al. 2011; Young et al. 2006). The latter host genes required for symbiosis and other ecological specializations (e.g., degradative capabilities or specific stress responses), thus being a major source of intraspecific phenotypic variability. Second, rhizobial genomes are usually large, evidencing high levels of gene duplication (i.e., paralogy) that have contributed to the evolution of new adaptive traits. Finally, the content of genes specifying transport proteins and regulators of transcription is particularly high in rhizobial genomes, which is predicted to optimize the coordination of the large-scale transcriptional, proteomic, and metabolic rearrangements underlying the symbiotic interaction.

Comparative genomics and high-throughput analysis of prokaryotic transcriptomes have revealed that bacteria express an unexpected diversity of 50- to 514-nucleotide (nt)-long untranslated transcripts, commonly referred to as s small untranslated transcripts (sRNAs), which have relevant housekeeping or regulatory roles. A handful of sRNAs carry out specific housekeeping functions, namely the transfer-messenger RNA (tmRNA), which acts as both tRNA and mRNA to tag incompletely translated proteins for degradation, thereby releasing stalled ribosomes (Moore and Sauer 2007), the 4.5S RNA component of the signal recognition particle (SRP) involved in protein secretion (Egea et al. 2005), and the ribozyme RNAse P that catalyzes the trimming of the 5′ end of tRNA precursors (Altman 2007).

Most of the known sRNAs function as regulators of gene expression through structure-based, target mimicry or antisense mechanisms. Some 5′ untranslated regions (UTR) influence transcription attenuation and translation initiation of the downstream mRNA coding sequence through secondary structure

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shifts triggered by alterations in the intracellular concentrations of specific small metabolites (i.e., riboswitches) or temperature changes (i.e., RNA thermometers) (Narberhaus et al. 2006; Serganov 2009). Two well-characterized riboregulators bind to and counteract the activity of specific proteins by mimicking features of other nucleic acids; 6S RNA resembles an open promoter that sequesters the σ^{70} RNA polymerase holoenzyme, thus contributing to transcription reprogramming during bacterial growth (Wassarman 2007), and the CsrB family of sRNAs carry the preferred binding sites (repeated GGA motifs) of the CsrA (carbon storage regulator) protein in the translation initiation regions of mRNAs (Babitzke et al. 2009; Valverde et al. 2004).

The majority of sRNAs thus far characterized use antisense mechanisms for the post-transcriptional regulation of their target mRNAs. *Cis*-sRNAs transcribed opposite annotated protein-coding genes interact with their antisense mRNA transcripts via regions of extensive perfect complementarity, most likely influencing negatively on mRNA stability. Representatives of these typical antisense RNAs were early identified in bacterial extrachromosomal genetic elements and have long been studied as regulators of plasmid, phage, or transposon functions (Brantl 2007). Unlike the *cis*-antisense sRNAs, *trans*-acting sRNAs are expressed from integenic regions (IGR) and use discontinuous, short sequence stretches of imperfect complementarity to target the 5′ UTR of one or multiple mRNAs, thereby influencing on the translation or stability of the message (Fröhlich and Vogel 2009; Storz et al. 2011).

Trans-sRNAs are likely to constitute the largest group of riboregulators expressed by bacteria. The functions of a representative subset have been deciphered in model enterobacteria (i.e., *Escherichia coli* and *Salmonella typhimurium*) and other clinically relevant pathogens (Papenfort and Vogel 2010; Sharma and Heidrich 2012; Storz et al. 2011; Waters and Storz 2009). They accumulate in the cell under specific biological conditions and their activity largely impacts bacterial physiology, contributing to the control of a wide range of cellular processes: sugar and amino acid transport and metabolism, iron and envelope homeostasis, general responses to abiotic stress, biosynthesis of virulence factors, or quorum sensing (Papenfort and Vogel 2010; Storz et al. 2011; Waters and Storz 2009). Riboregulated processes are relevant to the bacterial colonization of biotic surfaces and the establishment of chronic intracellular residences within eukaryotic hosts. Therefore, it is reasonable that a diversity of sRNAs could also have key roles in the coordination of the intricate gene networks activated in nitrogen-fixing endosymbiotic bacteria during their interaction with legume plants.

Genome-wide identification of sRNAs in rhizobia has been pioneered by work on the alfalfa symbiont *Sinorhizobium meliloti* (del Val et al. 2007; Schlüter et al. 2010; Ulvé et al. 2007b; Valverde et al. 2008). More recently, microarray- and deep sequencing-based surveys of sRNAs in other rhizobial species such as *Rhizobium etli* or *Bradyrhizobium japonicum* have been reported (Madhugiri et al. 2012; Vercruysse et al. 2010). The repertoire of sRNAs expressed by *S. meliloti* as inferred from in silico and experimental approaches that are described in detail below are summarized in Figure 1A.

Early approaches to identify sRNA genes in rhizobial genomes.

The vast majority of known bacterial sRNAs have evaded genetic, biochemical, and molecular detection until now. Unlike protein-coding genes (i.e., open reading frames [ORF]), the nucleotide sequences of sRNA genes have no obvious distinctive statistical biases such as codon usage or ribosome binding signals. Therefore, their annotation is overlooked by current single genome analysis and it must rely largely on comparative genomics (Eddy 2002).

In rhizobia, the tmRNA has been the only sRNA identified in a Tn*5*-based genetic screen performed in *B. japonicum* to search for genes involved in symbiotic nodule development on soybean roots (Ebeling et al. 1991). The corresponding chromosomal *locus* was named *sra* (for symbiotic ribonucleic acid) and its disruption by Tn*5* severely altered the ability of *B. japonicum* to colonize root nodules and fix nitrogen. The tmRNA is widely conserved in bacteria and, consequently, homology searches using the primary nucleotide sequence of the *B. japonicum* tmRNA as query allowed the identification and annotation of its homologous coding genes in other rhizobial genomes (Williams 2002). In *S. meliloti*, the tmRNA is expressed as an unstable pre-RNA molecule which is processed into two stable RNA species of 214 and 82 nt, likely corresponding to the mRNA and tRNA domains, respectively. *S. meliloti* tmRNA exhibits growth- and stress-dependent accumulation profiles (Ulvé et al. 2007a) but it still remains to be elucidated whether it has a general common symbiotic function in all rhizobial species.

Similar to tmRNA, the housekeeping RNAse P and 4.5S RNAs, and the regulatory 6S RNA are chromosomally encoded and ubiquitous in bacteria. All the sequenced rhizobial genomes have recognizable chromosomal homologs of these sRNAs, which are identifiable on the basis of both nucleotide sequence and structure conservation. Although the functions of these sRNAs, their cellular partners, and mechanisms of activity have been extensively studied in model bacteria (Altman 2007; Egea et al. 2005; Wassarman 2007), their roles in rhizobia have not been investigated yet. Interestingly, *Legionella pneumophila* requires 6S sRNA for optimal replication in human macrophages or amoeba (Faucher et al. 2010). Therefore, the contribution of the regulatory activity of 6S sRNA to the optimization of the intracellular survival within the host should be also explored in plant endosymbionts.

Another sRNA of known function and conserved in related α-rhizobia is encoded by the plasmid-borne *incA locus*. In *S. meliloti*, IncA has been characterized as a 56-nt long antisense sRNA that mediates incompatibility within the large *repABC* family of α-proteobacterial plasmids (MacLellan et al. 2005).

Both housekeeping and regulatory sRNAs that antagonize the activity of proteins are far outnumbered by base-pairing *trans*-acting riboregulators, a rapidly evolving sRNA species for which conservation is limited to phylogenetically related bacteria (Gottesman and Storz 2011). Seminal bioinformatics searches for *trans*-sRNA genes in rhizobia relied on comparisons of the IGR of *S. meliloti* with available related α-proteobacterial genomes (e.g., *Mesorhizobium loti*, *R. leguminosarum* biovars, or *Agrobacterium* and *Brucella* spp.) (del Val et al. 2007; Ulvé et al. 2007b; Valverde et al. 2008). However, primary nucleotide sequence conservation alone is usually not enough to accurately predict *trans*-sRNA encoding genes in bacterial genomes (Argaman et al. 2001; Rivas et al. 2001; Wassarman et al. 2001). Consequently, in the referred genome-wide searches, comparative sequence data were further scored with computational algorithms that implement additional genetic features common to bona-fide bacterial *trans*-encoded sRNAs: i) association of the conserved genomic regions with orphan promoter or Rho-independent transcriptional terminator signatures and ii) thermodynamic stability and conservation of the predicted RNA secondary structure. The latter was assessed by probabilistic models of expected mutational patterns in the pairwise sequence alignments that distinguish structural RNAs from a background of other conserved sequences such as ORF (del Val et al. 2007; Rivas et al. 2001; Valverde et al. 2008). As first indicator of the reliability of these

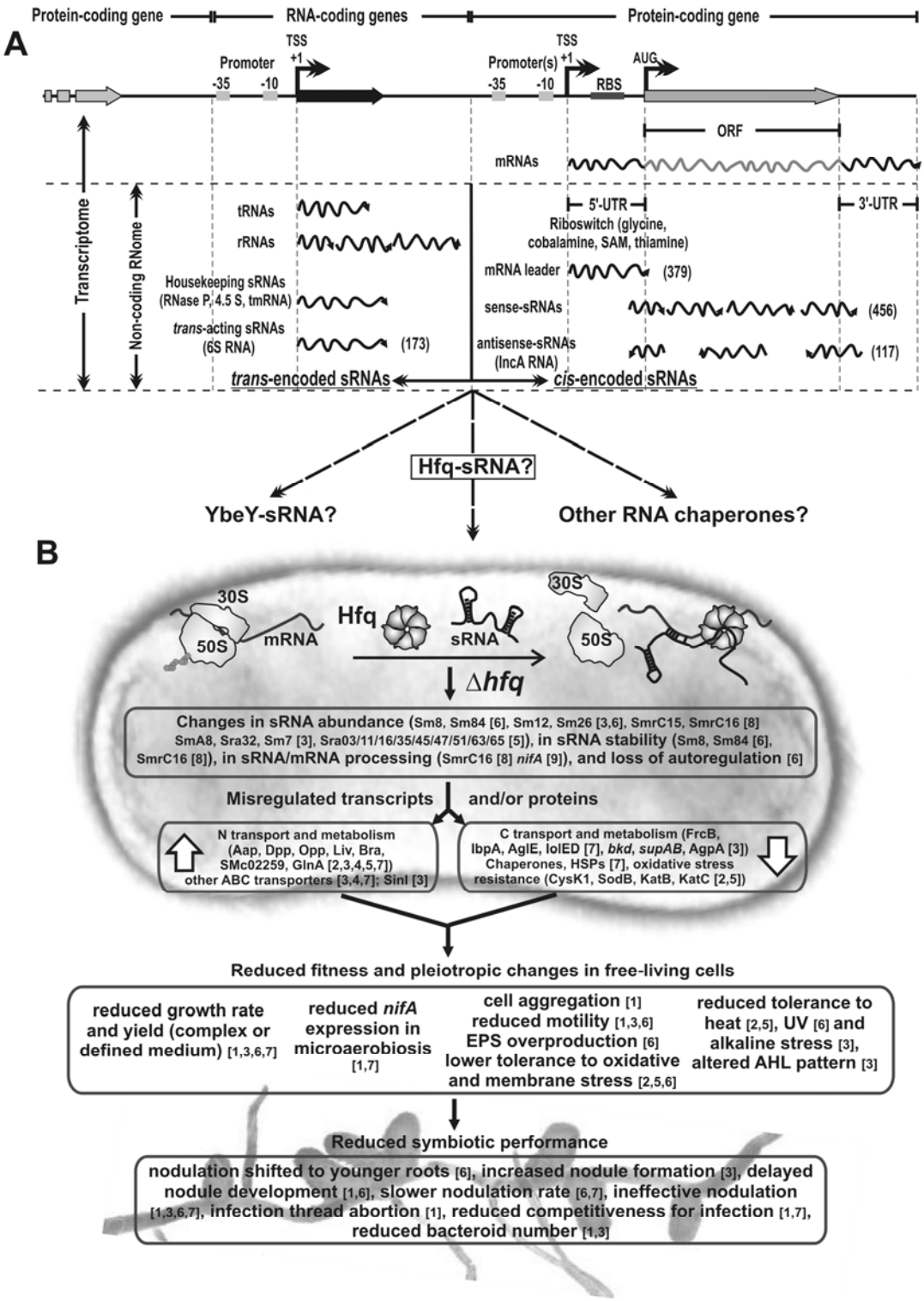

Fig. 1. Noncoding RNome structure and function of the RNA chaperone Hfq in nitrogen-fixing endosymbiotic rhizobia. **A,** Small untranslated transcripts (sRNA) species expressed by *Sinorhizobium meliloti*. *Trans*-acting sRNAs are encoded in independent transcription units with recognizable promoter and termination signatures between annotated open reading frames (ORFs). *Cis*-encoded sRNAs are transcribed sense or antisense to mRNAs. *Cis*-sense sRNAs can either form chimaeras with the coding regions of mRNAs (5′ or 3′ untranslated regions [UTR]) or be synthetized as short, discrete transcripts resulting from transcription attenuation (mRNA leaders), mRNA processing, or co-transcription with the full-length mRNA from alternative promoters. sRNAs of known function and the number of transcripts of each category identified by RNA-Seq (Schlüter et al. 2010) are quoted in brackets. TSS, transcription start site; RBS, ribosome binding site. Novel rhizobial sRNAs are predicted to regulate cell processes dependent on the activity of Hfq or alternative RNA-binding proteins such as YbeY. **B,** Summary of the documented impact of knocking out the gene encoding the RNA chaperone Hfq in symbiotic rhizobial species. By facilitating the pairing of sRNAs with target mRNAs, Hfq interferes with ribosome assembly or translation and influences turnover rates of specific transcripts, thereby contributing to complex post-transcriptional networks in enterobacteria (Vogel and Luisi 2011). In rhizobial species, the absence of Hfq results in profound changes at the transcriptional and proteomic levels, most likely due to changes in RNA metabolism, which, together, compromise fitness of free-living cells and their interaction with host legume roots at different stages of the symbiotic association. Numbers indicate sources: 1, Barra-Bily et al. 2010a; 2, Barra-Bily et al. 2010b; 3, Gao et al. 2010; 4, Mulley et al. 2011; 5, Pandey et al. 2011; 6, Sobrero and Valverde 2011; 7, Torres-Quesada et al. 2010; 8, Voss et al. 2009; and 9, Zhang and Hong 2009.

approaches, the sequences of all sRNAs of known function (i.e., RNase P, 4.5S, tmRNA, 6S, and IncA RNAs), which mostly escaped the primary annotation of the *S. meliloti* genome, were identifiable in the joint list of sRNA candidates. Northern hybridization experiments confirmed that 22 additional loci, out of more than a hundred candidates collectively predicted by the screens as sRNA genes, did express small discrete transcripts of the expected size and no polypeptide coding potential. Furthermore, the intracellular levels of these transcripts varied with the growth phase, upon application of a variety of abiotic stresses and, in some cases, in endosymbiotic bacteria (del Val et al. 2007; Ulvé et al. 2007b; Valverde et al. 2008). These newly identified sRNA loci have recognizable σ^{70}-dependent promoter and Rho-independent terminator signals, an observation that was further supported by experimental mapping of the transcription start sites of some of the RNA species detected on gels (del Val et al. 2007). The identified sRNA genes were named *smr*, *sra*, or simply *sm*, for *S. meliloti* RNA. A primary BLASTN comparison of the nucleotide sequences of these genes against all available bacterial genomes anticipated that conservation of the *S. meliloti* sRNAs was restricted to phylogenetically related α-proteobacterial species (del Val et al. 2007; Ulvé et al. 2007b; Valverde et al. 2008). All together, this evidence (i.e., differential expression from independent chromosomal transcription units and conservation patterns) catalogued these 22 transcripts as novel putative bacterial *trans*-acting riboregulators, all of which await functional characterization.

Microarray-based transcriptome profiling of *S. meliloti* and *R. etli* in different free-living growth conditions and even during the interaction with the host plant significantly expanded the list of experimentally validated candidate sRNA genes predicted by the computational screens (Valverde et al. 2008; Vercruysse et al. 2010). Among these, several loci adjacent to the 5′ boundary of annotated operons or ORF, which are recognized α-proteobacterial *cis*-regulatory elements such as glycine, cobalamine, δ-adenosyl methionine, or thiamine riboswitches, were identified. Nonetheless, interpretation of hybridization signals on oligonucleotide probes targeting the IGR is not enough to accurately catalog a newly discovered noncoding sRNA transcript. Therefore, proper characterization of bacterial sRNAs identified on the basis of microarray hybridization requires further molecular genetics analysis (e.g., Northern hybridization and experimental 5′- and 3′-end mapping).

Deep RNA sequencing uncovered the complexity of rhizobial RNomes.

Advances in sequencing technologies have pushed the characterization of the transcriptome to new levels. Second-generation sequencing, such as the 454 (Roche, Mannheim, Germany), SOLEXA (Illumina, Cambridge, U.K.), and SOLiD (Life Technologies, Gaithersburg, MD, U.S.A.) technologies, caused a boom in genome sequencing. Shortly afterward, this was followed by the development of RNA-Seq approaches promoting the characterization of the transcriptome landscape of eukaryotic and prokaryotic organisms. Different experimental set-ups allow for identification of transcription start sites, operon structures, and novel transcribed regions, including protein-coding and noncoding RNA genes that have been overlooked in annotation (Croucher and Thomson 2010; Febrer et al. 2011; Sharma and Vogel 2009; van Vliet 2010).

To date, three studies applied deep-sequencing strategies to characterize the transcriptome with the aim of identifying sRNAs in the plant symbiotic rhizobial species *S. meliloti* (Schlüter et al. 2010) and *B. japonicum* (Madhugiri et al. 2012), and the related plant pathogen *Agrobacterium tumefaciens* (Wilms et al. 2012).

The first comprehensive experimental screen for sRNAs in a symbiotic nitrogen-fixing α-proteobacterium was performed in *S. meliloti* 2011 (Schlüter et al. 2010). This dRNA-Seq and oligonucleotide microarray-based study focused on short RNAs of a size range from 50 to 350 nt obtained by size fractionation of total RNA prior to the profiling experiments. The results derived from 454 deep sequencing clearly outperformed the microarray-based approach. It suggested a total of 1,125 sRNA candidates, of which 173 were assigned to *trans*-encoded sRNAs, 117 to *cis*-encoded antisense sRNAs, 379 to mRNA leader sequences, and 456 to sense sRNAs overlapping coding regions (Fig. 1A). The putative mRNA leader sequences may include metabolite-controlled riboswitches and RNA thermometers. The reliability of the RNA-Seq data was partly demonstrated by Northern blot-based validation and the rediscovery of 82 previously reported sRNA candidates. A considerable advantage of RNA-Seq over microarray hybridizations is the ability to identify 5′ and 3′ ends. As observed in other studies, prediction of distinct 5′ ends from RNA-Seq data was quite reliable, whereas 3′ ends were more difficult to derive from this data. Nevertheless, transcriptional start sites and 3′ ends could be predicted for 466 sRNA candidates, prospectively supporting structural and functional investigations.

Transcripts of *trans*-encoded sRNA candidates ranged from 53 to 259 nt, with an average length of 114 nt. In contrast to the tremendous antisense transcriptional activity observed in other bacteria (Georg and Hess 2011), this study delivered only a low number of antisense transcripts. This may be traced to the size fractionation of RNA that aimed at the discovery of native short RNAs. Strikingly, a high number of short sense transcripts overlapping coding regions have been found corresponding to observations from other transcriptome studies in prokaryotes. The majority of these transcripts are most likely degradation derivatives of mRNAs, although primary 5′ ends and upstream promoter motifs have been predicted for a small proportion of these transcripts. Whether these transcripts have a functional role in the bacterial cell remains in question. *Trans*-encoded sRNAs mapped to all three replicons of the multipartite genome of *S. meliloti* but showed a slight prevalence on the chromosome.

Little is known about the sRNA repertoire of other members of order Rhizobiales. Recently, a dRNA-Seq study applying the 454 technology was reported for *A. tumefaciens* C58. It delivered 221 *trans*-encoded sRNAs mapping to the four replicons: the circular and linear chromosomes as well as the Ti and At plasmids (Wilms et al. 2012). Northern blot analysis validated a subset of these sRNAs and showed induction of a Ti plasmid-borne sRNA under virulence conditions. This data set provides a valuable resource for comparative studies of sRNAs in order Rhizobiales. Deep sequencing was also used to validate seven sRNA candidates predicted by comparative computational methods in *B. japonicum* (Madhugiri et al. 2012).

RNA-Seq approaches in *S. meliloti* and *A. tumefaciens* have identified numbers of *trans*-encoded sRNA candidates in a similar magnitude as reported for a broad range of other gram-positive and gram-negative bacteria. However, comparison of exact numbers is complicated by difficulties in defining appropriate consistent criteria for annotation of transcripts, including the threshold for sequence coverage. Furthermore, different RNA preparation and cDNA library construction protocols and different sequencing technologies hamper a comparison of results across different studies.

The newly identified *S. meliloti trans*-sRNAs define novel α-proteobacterial sRNA families.

Experimental screens in *S. meliloti* and *A. tumefaciens* delivered a wealth of putative sRNAs, raising the question of their

conservation and distribution in order Rhizobiales. Two comparative in silico studies combining sequence homologies with structural conservation analyses addressed this question (del Val et al. 2012; Reinkensmeier et al. 2011) (Supplementary Fig. 1S). Reinkensmeier and associates (2011) elucidated the distribution of 52 *trans*-encoded sRNAs derived from the dRNA-Seq study in *S. meliloti* 2011 (Schlüter et al. 2010) by construction of RNA family models (RFM). This approach included two strategies: the preferential construction of covariance models (CM) as stochastic models capturing sequence and structure conservation in alignments and, if CM construction was not successful, thermodynamic matcher focusing on structural features and folding energy. The latter can ignore sequence conservation in some parts and emphasize conserved sequence motifs elsewhere in the structure. The 52 sRNAs were collected in 39 RFM which showed various taxonomic distribution patterns. Further independent pieces of evidence supporting the new RFM were provided by microsynteny observed in 27 RFM and independent experimental validation of members of five RFM predicted in *A. tumefaciens* (Wilms et al. 2012).

del Val and associates (2012) similarly investigated the distribution of sRNA candidates (Smr7C/SmelC023, Smr9C/SmelC289, Smr45C/SmelC706, Smr15C/SmelC411, Smr14C/SmelC397, and Smr35B/SmelB053) identified in a previous study in *S. meliloti* 1021 (del Val et al. 2007). Two of these were also part of the aforementioned study and delivered consistent phylogenetic distributions. Combining both studies, the majority of RFM were restricted to *Sinorhizobium* spp. or members of the family Rhizobiaceae. Only eight RFM included members from families beyond the Rhizobiaceae; namely, Phylobacteriaceae and Brucellaceae. Just one RFM founded by Smr45C/SmelC706 also included members from the families Bartonellaceae, Xanthobacteriaceae, and Beijerinckaceae. In general, RFM founded by *S. meliloti* sRNA candidates mapping to pSymB and pSymA were restricted to *Sinorhizobium* spp. and *S. meliloti*, respectively, with only punctual exceptions. This is in agreement with the assumption that pSymA represents the most recently acquired replicon in genus *Sinorhizobium* (Galibert et al. 2001).

Comparative analyses were also performed for seven sRNA candidates predicted by genome comparison of *Bradyrhizobium* and *Rhodopseudomonas* spp. (Madhugiri et al. 2012). The analyzed sRNAs displayed a different degree of conservation in order Rhizobiales, and three sRNAs were validated in the free-living purple bacterium *Rhodopseudomonas palustris* 5D.

Identification of sRNA homologs is complicated by our poor knowledge of relationships between sequence, structure, and function of these molecules. In all studies, examples of extended sequence similarities and structural conservation were found for sRNA sequences deduced from closely related bacteria. However, comparative analysis is impeded if only short motifs and few structural features show similarities, which may also result in ambiguous assignments of RNAs to different RFM. This raises the question about how far beyond the phylogenetic order is a comparative analysis of sRNAs justifiable. Nevertheless, the distribution patterns in order Rhizobiales strongly suggest vertical inheritance and the occurrence of extensive ancestral duplication events in the course of evolution of this group of bacteria.

Functional characterization of recognizable RNA-binding proteins anticipates a broad impact of riboregulation in symbiosis.

RNA-binding proteins are functionally linked to sRNA activity in many bacterial species. For instance, Csr/Rsm mimic sRNAs titrate away the RNA-binding proteins of the CsrA/RsmA family from target mRNAs, thus relieving the translational control exerted by these small dimeric and basic proteins (Babitzke and Romeo 2007). Intriguingly, α-proteobacterial genomes lack sequences encoding members of the CsrA/RsmA protein family, and only one allele of this type has been detected in the replication region of a *S. meliloti* cryptic plasmid, most probably as a consequence of a lateral gene transfer event (Watson and Heys 2006). On the other hand, most of the characterized *trans*-acting sRNAs that hybridize with target mRNAs require the assistance of the RNA chaperone Hfq (Vogel and Luisi 2011). Unlike *csrA/rmsA* homologs, *hfq* genes are present in all sequenced α-proteobacteria, invariably upstream of *hflX* encoding a putative GTP-binding protein of uncharacterized biological function (Sobrero and Valverde 2012). For a comprehensive description of structural, biochemical, and genetic aspects of Hfq, we refer the reader to recent review articles (Vogel and Luisi 2011; Sobrero and Valverde, 2012). For the purpose of this review, it is worth mentioning that the sequence of α-proteobacterial Hfq proteins strongly resembles that of enterobacterial Hfq in terms of sequence motifs and predicted secondary and tertiary structure, which suggests a quaternary organization into a hexameric toroid with at least two different RNA-binding surfaces (Sobrero and Valverde 2012). In fact, cross-complementation experiments of *S. meliloti* and *E. coli* mutants support that *S. meliloti hfq* encodes an RNA chaperone that can be functionally exchanged by its homolog from *E. coli* (Kaminski et al., 1994; Sobrero and Valverde 2011). The *hfq* genes seem to be strongly expressed in α-proteobacteria (Barra-Bily et al. 2010b; Kaminski et al. 1994; Sobrero and Valverde 2011) and subjected to autoregulation at the translational level (Sobrero and Valverde 2011). These expression features suggest that Hfq is well-represented and tightly controlled in the proteome repertoire, in line with its function as a facilitator of RNA transactions.

A role for Hfq in the control of gene expression was discovered well before the realization of the magnitude of the α-proteobacterial RNomes. As part of a generalized chemical mutagenesis screening of *Azorhizobium caulinodans*, *hfq* was identified as a positive regulator of *nifA* expression at the translational level (Kaminski et al. 1994). As a consequence, *A. caulinodans hfq* mutants were defective in symbiotic nitrogen fixation (Nod^+ Fix^-) due to the lack of activation of *nif* and *fix* operons by NifA (Kaminski and Elmerich 1998; Kaminski et al. 1994). More recently, such positive translational control of *nifA* expression has been also reported in *R. leguminosarum* bv. *viciae* (Zhang and Hong 2009). However, in the past few years, a series of parallel works have provided strong evidence that Hfq has a much broader participation in the control of gene expression in rhizobia. The studies were carried out in *S. meliloti* by means of a reverse genetic approach, to assess the impact of knocking out *hfq* on free-living and symbiotic phenotypes (Barra-Bily et al. 2010a and b; Gao et al. 2010; Sobrero and Valverde 2011; Torres-Quesada et al. 2010). In spite of utilizing different *S. meliloti* strains, different growth conditions, different mutagenic strategies, and different *Medicago sativa* cultivars and growth systems, an overall common pattern emerged from those studies: the lack of Hfq compromised growth, motility, and tolerance to environmental stresses (particularly oxidative stress), and resulted in profound changes in the transcriptomic and proteomic pattern (Fig. 1B). In particular, Hfq is mainly involved in controlling expression of genes and operons that mediate transport of small molecules relevant to carbon and nitrogen metabolism, and in tolerance to a variety of stresses (Barra-Bily et al. 2010a and b; Gao et al. 2010; Torres-Quesada et al. 2010). As expected, such global changes associated with the lack of Hfq were reflected in the symbiotic

interaction with *M. sativa* roots which, depending on the strain and plant cultivar, revealed a spectrum of symbiotic defects, including reduced competitiveness for infection, a delay in nodule appearance and development, nodule developmental arrest, high proportion of ineffective nodules, and reduced nitrogen fixation and plant growth (Barra-Bily et al. 2010a and b; Gao et al. 2010; Sobrero and Valverde 2011; Torres-Quesada et al. 2010). That Hfq-dependent regulatory mechanisms are central to effective rhizobial symbioses is reflected in the observation that Nod^+ Fix^- *R. leguminosarum* GOGAT mutants were rescued by second site suppressor mutations in *hfq* (Mulley et al. 2011). In fact, *R. leguminosarum* Hfq negatively controls key amino acid transport systems (Aap and Bra) which are fundamental for N exchange between the bacteroid and the host cell, and a number of other ABC transport systems, resembling the scope of Hfq target proteins identified in *S. meliloti*.

The major impact of Hfq on the *S. meliloti* transcriptome and proteome strongly suggests that, at least partly, a number of regulatory processes are under the direct influence of Hfq and cognate partner sRNAs. Co-immunoprecipitation experiments have shown that Hfq binds to a number of *S. meliloti* sRNAs (Torres-Quesada et al. 2010), and this interaction may indirectly stabilize sRNAs (Gao et al. 2010; Sobrero and Valverde 2011; Voss et al. 2009). However, on top of this experimental evidence, RNomics of Hfq-associated RNAs from free-living and symbiotic cells would provide a straightforward and comprehensive identification of direct Hfq mRNA and sRNA substrates (Sittka et al. 2009), which will ultimately lead to the identification of Hfq-dependent mRNA-sRNA regulatory pairs.

Finally, Hfq may not be the sole rhizobial RNA-binding protein involved in riboregulation. Deletion of the widely conserved YbeY *S. meliloti* protein (SMc01113), which bears an RNA-binding region reminiscent of the MID domain of eukaryotic Argonaute proteins involved in RNA silencing, produces a number of free-living and symbiotic changes that almost completely overlap those reported for Hfq mutants (Pandey et al. 2011). Whether these effects are direct on mRNAs and sRNAs or indirect via control of *hfq* expression remains to be determined. It should not be disregarded that other proteins with RNA-binding domains, such as the cold-shock protein A family (Phadtare and Severinov 2010) and the ribosomal protein S1 (Rieder et al. 2012), do also contribute to riboregulation in rhizobia.

Concluding remarks and perspectives.

Recent comprehensive computational comparative genomics and experimental genome-wide screens (i.e., hybridization of tiling arrays and RNA-Seq) have uncovered the complex noncoding RNA output from the genomes of nitrogen-fixing α-rhizobia. Functions for the vast majority of the newly identified sRNAs remain to be assigned. Therefore, this sRNA catalog constitutes a reference data set for the forthcoming systems-level investigation of the impact of riboregulation in the adjustment of rhizobial physiology during the transition from a free-living to an endosymbiotic state.

Although RNA-Seq is revealing the identity of 5′ and 3′ UTR and high rates of antisense transcription, for which biological meaning should be addressed, *trans*-acting sRNAs likely constitute the largest class of riboregulators expressed by rhizobia. The reported differential expression profiles of a subset of *trans*-sRNAs identified in *S. meliloti* are the first clues to unravel their functions. However, the identification of their mRNA targets constitutes the major challenge in the functional characterization of *trans*-acting sRNAs. Approaches combining computational predictions and transcript or protein profiling of sRNA deletion or overexpression derivative strains as well as in vivo (e.g., based on *lacZ* or GFP reporters) and in vitro binding assays are being developed to identify sRNA-mRNA pairs on a high-throughput basis.

On the other hand, the activity of the *trans*-sRNAs has been assumed to be mainly assisted in gram-negative bacteria by the ubiquitous RNA chaperone Hfq. In line with this evidence, deletion of the conserved *hfq* gene has a broad impact on the physiology of free-living and endosymbiotic *S. meliloti* bacteria. Nonetheless, available experimental data anticipate that Hfq could not be the only rhizobial RNA chaperone involved in riboregulation. Purification of functional RNA-protein complexes (e.g., using aptamer-tagged sRNAs) will allow the characterization of the repertoire of RNA-binding proteins relevant to sRNA activity and turnover. Conversely, deep-sequencing analysis of direct sRNA and mRNA targets of the identified RNA chaperones will reveal the identity of riboregulators controlling symbiotic processes, helping in the elucidation of their mechanisms of activity. Aptamer-tagged variants of sRNAs also would be an invaluable tool to investigate the intracellular location and trafficking of *trans*-sRNAs and their protein partners in both free-living bacteria and nodule tissues. Secretion of bacterial *trans*-acting sRNAs into host cells to regulate eukaryotic functions, as suggested in some recent studies, is also an exciting possibility to be explored as a novel level of mutual recognition and signaling between the partners in the rhizobia–legume symbiosis.

The occurrence and conservation patterns of the identified sRNA genes in rhizobial genomes will shed light about their evolutionary history but also have functional implications. Reported homology searches based on co-variance models of a number of *S. meliloti trans*-sRNAs have shown that many occur with high levels of paralogy in individual genomes, thus providing an excellent model to investigate the physiological and ecological advantages of these reiterations, which has been scarcely addressed in other bacterial groups. sRNAs are expressed from the chromosome and the extra-chromosomal replicons of rhizobia. Therefore, functional RNomics will contribute to understand the sRNA-mediated cross-talk between the core and accessory portions of rhizobial genomes as well as to unravel common and unique strategies used by α-proteobacteria to establish intracellular chronic residences in their eukaryotic hosts.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

RhizoGATE–The gateway to rhizobial genomes website: www.cebitec.uni-bielefeld.de/CeBiTec/rhizogate
Wellcome Trust Sanger Institute Rfam database: rfam.sanger.ac.uk
Small non-coding RNAs in the endosymbiotic diazotroph alphaproteobacterium *Sinorhizobium meliloti*: en.wikipedia.org/wiki/User:Rcrzarg/Small_non-coding_RNAs_in_the_endosymbiotic_diazotroph_%CE%B1-proteobacterium_Sinorhizobium_meliloti