

# Regulation by Small RNAs in Bacteria: Expanding Frontiers

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DOI 10.1016/j.molcel.2011.08.022

Research on the discovery and characterization of small, regulatory RNAs in bacteria has exploded in recent years. These sRNAs act by base pairing with target mRNAs with which they share limited or extended complementarity, or by modulating protein activity, in some cases by mimicking other nucleic acids. Mechanistic insights into how sRNAs bind mRNAs and proteins, how they compete with each other, and how they interface with ribonucleases are active areas of discovery. Current work also has begun to illuminate how sRNAs modulate expression of distinct regulons and key transcription factors, thus integrating sRNA activity into extensive regulatory networks. In addition, the application of RNA deep sequencing has led to reports of hundreds of additional sRNA candidates in a wide swath of bacterial species. Most importantly, recent studies have served to clarify the abundance of remaining questions about how, when, and why sRNA-mediated regulation is of such importance to bacterial lifestyles.

## Introduction

It is firmly established that RNA transcripts are important regulators whose roles cannot be ignored in any organism. In bacteria, a large number of these RNA regulators exist as relatively short transcripts (~50–300 nucleotides) that act on independently expressed targets. These regulators, which are most commonly referred to as small RNAs (sRNAs), are the focus of this review. Regulatory RNA elements that are transcribed as part of their target messenger RNAs (mRNAs) are discussed elsewhere (Breaker, 2011).

The most extensively studied sRNAs, often called *trans*-encoded sRNAs, are those that regulate mRNAs by short, imperfect base-pairing interactions (reviewed in Waters and Storz, 2009). Many of these sRNAs base pair at or near the ribosome binding site (RBS) of their targets and block translation by occluding ribosomes. However, other family members base pair at more distant locations and thus interfere with ribosome binding by other mechanisms, increase ribosome binding by preventing the formation of inhibitory secondary structures, or decrease or increase mRNA stability. In Gram-negative bacteria, the RNA binding protein Hfq is usually required for the function and/or stability of this family of sRNAs.

Increasing numbers of sRNAs that are encoded on the opposite strand of established coding sequences, here denoted antisense RNAs (asRNAs), also have been found to impact translation and/or mRNA stability of the fully complementary sense gene (reviewed in Georg and Hess, 2011; Thomason and Storz, 2010). However, for some asRNAs, regulation may be exerted by the act of transcribing the asRNA, rather than be a function of the resulting RNA.

Other prominent sRNA regulators act by modifying protein activity, as exemplified by the very highly conserved *E. coli* CsrB and 6S RNAs. These sRNAs bind specific proteins rather

than base pair to target RNAs (reviewed in Babitzke and Romeo, 2007; Wassarman, 2007; Willkomm and Hartmann, 2005).

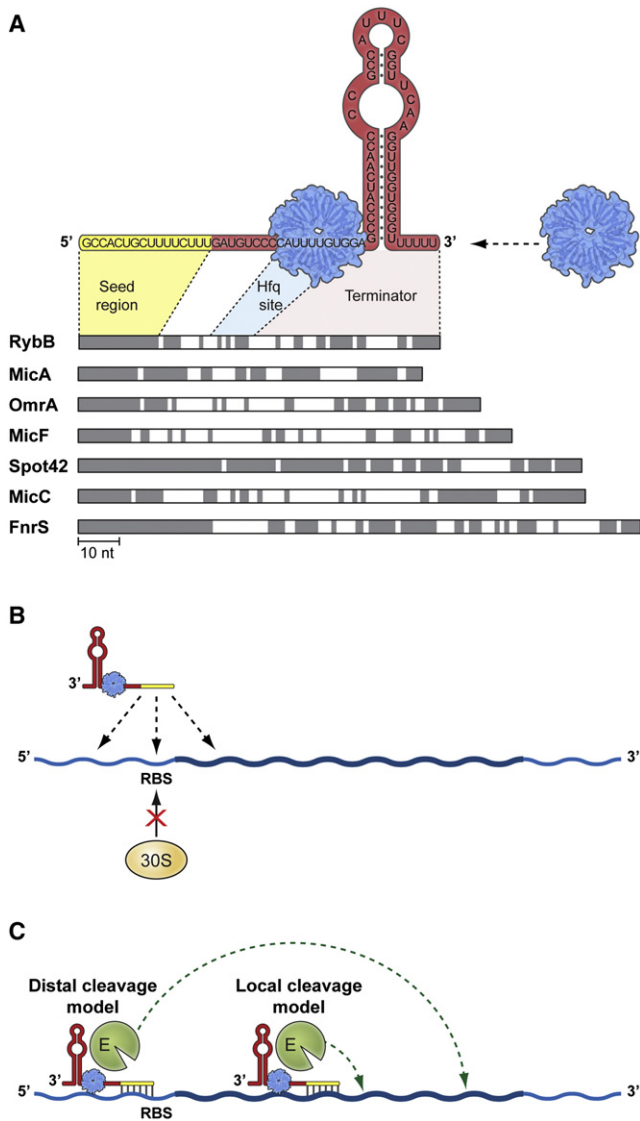
The outstanding progress in identifying and characterizing bacterial sRNAs has made it abundantly clear that RNA regulators are ubiquitous, are often well conserved, and may even exceed protein regulators in number and diversity. Here, we emphasize new concepts revealed in the last 2 to 3 years by citing a limited number of examples, but the general ideas hold true for most sRNAs. Many exciting questions still remain, even in well-studied *Escherichia coli* and *Salmonella*, and certainly in other more divergent bacteria.

## Intricate Mechanisms of sRNA Base Pairing

How base-pairing sRNAs find their target mRNAs among thousands of other cellular transcripts is key for understanding how these sRNAs function, and extensive research has focused on characterizing the RNA elements and protein chaperones required.

### RNA Elements Involved in Base Pairing

Early studies of asRNAs encoded on plasmids, phages, and transposons led to a prevalent view that structural elements and multi-step interactions were crucial in base-pairing mechanisms of bacterial RNAs (reviewed in Brantl, 2007; Wagner et al., 2002). Despite potential complementarity of hundreds of base pairs or more, these asRNAs recognize their target mRNAs with initial fast, high-affinity contacts via a few nucleotides exposed in stem-loop regions of the regulator, the target, or both. After this first “kissing” interaction, additional base pairs can form, often involving rearrangements in RNA secondary structure. Whether or not chromosomally encoded asRNAs act by similar mechanisms is only starting to be investigated, but a recent study of the *ibsC*-*SibC* sense-antisense pair in *E. coli* does indicate that some structural elements are



**Figure 1. General Properties of Trans-Encoded Base-Pairing sRNAs** (A) Diagram showing the modular structure of Hfq-binding sRNAs. The most highly conserved regions in seven enteric sRNAs are shaded gray. This conservation generally corresponds to the region(s) of the sRNA involved in base pairing, frequently occurring at the 5' end, but corresponding to two and three regions of base pairing for FnrS and Spot42, respectively. (B) Diagram showing the different positions at which sRNAs can block ribosome binding. (C) Diagram showing ways by which base paired sRNAs can direct RNase E-mediated target mRNA processing.

required and that base pairing proceeds via multiple steps (Han et al., 2010).

Structure-driven pairing with target mRNAs also has been demonstrated for a subset of sRNAs with *trans*-encoded targets. Base-pairing sRNAs in *Staphylococcus aureus* (RNAlII, RsaE, and SprD) usually recognize target RNAs using C-rich stretches that are within accessible loop regions (Bohn et al., 2010; Chabelskaya et al., 2010; Geissmann et al., 2009). Several other sRNAs in this organism carry conserved C-rich motifs, suggest-

ing that they may act similarly. A conserved C-rich apical loop in the enterobacterial CyaR sRNA also is utilized to recognize a single-stranded region in several regulated mRNAs (De Lay and Gottesman, 2009; Johansen et al., 2008; Papenfort et al., 2008).

For many other *trans*-encoded base-pairing sRNAs, however, the region required for pairing is encompassed primarily within single-stranded stretches, suggesting a complex structure is not as important (Peer and Margalit, 2011). In fact, these sRNAs appear to be modular in enteric bacteria (Figure 1A). A structured 3' end followed by poly(U) promotes Rho-independent transcription termination and protects the sRNA against 3' exonucleases. A second domain is the binding site for Hfq, the chaperone protein required for function and stabilization of many of these sRNAs. A third region is utilized for base pairing to target RNAs. This region often is highly conserved and appears to interact with target mRNAs by a “seed-pairing” mechanism similar in concept to target selection by eukaryotic microRNAs (miRNAs) (reviewed in Bartel, 2009). Experiments in which the seed regions of *Salmonella* RybB or MicC were fused to unrelated scaffold RNAs demonstrated that the seed is sufficient to guide target recognition (Papenfort et al., 2010; Pfeiffer et al., 2009).

More in depth knowledge is required to fully understand how these sRNAs regulate their targets. The optimal length and nucleotide composition of the bacterial seed have not been fully defined. Seed lengths of 6 or 7 nucleotides have been proposed for the SgrS and RybB sRNAs (Baltontin et al., 2010; Kawamoto et al., 2006; Papenfort et al., 2010), but this may vary among sRNA-mRNA pairs. Most studies examining seed requirements used overexpressed sRNAs, raising the possibility that sRNAs at endogenous levels could have somewhat different preferences. Curiously, the seed region of many sRNAs is located at their very 5' ends (Guillier and Gottesman, 2008; Papenfort et al., 2010), suggesting that position may impact function.

Less information is available regarding what features of target mRNAs influence base pairing and regulation. While initial studies suggested that pairing occurred primarily in a region overlapping or adjacent to the RBS, more recent studies have shown that sRNA base pairing as far as 70 nucleotides upstream or 15 nucleotides downstream of the start codon can block translation in *E. coli* and *Salmonella* (Bouvier et al., 2008; Holmqvist et al., 2010) (Figure 1B). The sites of base pairing for sRNAs that activate translation by preventing the formation of an inhibitory secondary structure via an anti-antisense mechanism can be more distant (reviewed in Fröhlich and Vogel, 2009). sRNAs that primarily regulate mRNA stability can do so at even more variable locations, including deep within the coding sequence of the target mRNA (Pfeiffer et al., 2009).

The impact of RNA structures, noncanonical base pairs and sequences outside the seed also remain to be further evaluated. A recent survey identified a propensity for a 3' adenosine adjacent to the region of pairing, which is somewhat reminiscent of miRNA target sites (Papenfort et al., 2010). Systematic analyses, such as recently carried out for *E. coli* RyhB (Hao et al., 2011), will be necessary to better define parameters that allow highly specific selection of bona fide mRNA targets from the thousands of other nontarget transcripts. This information will help elucidate

the advantages of more extensive, structure-driven pairing versus limited seed-driven pairing, as well as facilitate the prediction of target mRNAs and the design of synthetic regulators.

### **Roles of RNA Chaperones**

The functions of *trans*-encoded base-pairing RNAs are generally dependent on the highly studied RNA chaperone Hfq in Gram-negative bacteria (reviewed in Vogel and Luisi, 2011). In vitro experiments suggest that Hfq generally binds an A/U-rich single-stranded region often located adjacent to a stem-loop structure, reminiscent of binding sites for the related Sm and Lsm proteins in eukaryotes. However, predictions of Hfq binding sites in sRNAs are unreliable, given the weak conservation and the presence of multiple A/U-rich sites. In addition, accumulating evidence indicates that polyU at the 3' end of sRNAs also can be recognized by Hfq, possibly serving as a loading site (Otaka et al., 2011; Sauer and Weichenrieder, 2011).

Hfq binding sites on mRNAs are even less well documented, and there are very few mRNA sites with proven physiological relevance. The mRNA binding sites have been mainly predicted based on crystallographic studies of Hfq (Link et al., 2009) and genomic SELEX approaches (Lorenz et al., 2010). In light of the nonspecific binding activity of Hfq in vitro, it will be necessary to globally map Hfq contacts with cellular RNAs in vivo with approaches such as the covalent crosslinking techniques that have given unprecedented insights into binding determinants for eukaryotic RNA-binding proteins (reviewed in Licatalosi and Darnell, 2010).

Despite the widely accepted role of Hfq as an “RNA chaperone,” the detailed mechanism(s) by which it promotes productive encounters of cognate RNA partners in cells remains ambiguous. High-resolution structures of Hfq with RNA oligomers, together with in vitro binding assays with Hfq mutants, have revealed several RNA binding sites on both the proximal and distal faces of the hexameric Hfq ring (Link et al., 2009; Sauer and Weichenrieder, 2011), consistent with Hfq binding to more than one RNA simultaneously. There is evidence suggesting Hfq facilitates base pairing by increasing annealing rates (Fender et al., 2010; Hopkins et al., 2011; Hwang et al., 2011) by stabilizing cognate sRNA-mRNA duplexes (Soper et al., 2010) or by promoting structural remodeling of one of the RNA partners (Maki et al., 2010). What is not as clear is whether particular mechanisms are specific to sRNA-mRNA pairs. It is also not known which RNA binds first, how Hfq affects the first and subsequent RNA(s) and whether Hfq helps to position the sRNA seed region to interrogate potential mRNA partners. In addition, there is the question whether Hfq directly affects ribosome binding or the action of nucleases subsequent to base pairing. Structures of Hfq bound to “natural” RNA targets, mutants that separate the different activities of Hfq, and in vitro assays that recapitulate regulation in the presence of multiple specific and nonspecific RNAs as well as other accessory proteins are much needed to fully understand the mechanism of Hfq action.

While the focus has long been on *E. coli* Hfq, a role in sRNA-mediated regulation extends to distant bacteria such as the Gram-negative Spirochaetales *Borrelia burgdorferi* (Lybecker et al., 2010) and the Gram-positive firmicute *Listeria monocytogenes* (Nielsen et al., 2010), both of which have significantly

divergent Hfq proteins. Intriguingly, a growing number of bacteria such as *Burkholderia cenocepacia* (Ramos et al., 2011) are now known to contain multiple Hfq proteins indicating potential functional diversification. Studies of Hfq in a range of organisms will help uncover its full role in posttranscriptional regulation.

Obviously, other bacterial proteins also may contribute to base-pairing sRNA function. Such proteins could act as RNA chaperones in addition to Hfq or in lieu of Hfq in species where there is no obvious Hfq homolog. A chaperone role has been suggested for the *E. coli* ProQ protein, which has a C-terminal domain with predicted structural similarities to Hfq (Chaulk et al., 2011). The YbeY protein, which is ubiquitous in bacteria and shares structural similarities with a domain of eukaryotic Argonaute proteins, impacts gene expression analogous to Hfq in *Sinorhizobium meliloti* (Pandey et al., 2011). Further characterization of the contributions of these proteins, other putative chaperones, and other RNA-binding proteins such as helicases and ribosomal proteins to sRNA-mediated regulation certainly are important directions of study.

### **Contributions of Dynamic Interactions and Competition**

Other critical parameters need to be established before full understanding of base-pairing mechanisms is realized. Cellular copy numbers are unknown for most sRNAs and target mRNAs, and even the level of Hfq remains controversial in *E. coli* (400 versus 10,000 copies) and has not been determined in other organisms (reviewed in Vogel and Luisi, 2011). Binding constants have only been measured for individual RNAs in vitro (Fender et al., 2010; Olejniczak, 2011; Soper et al., 2010; Updegrove et al., 2008). Understanding the intracellular concentrations of sRNAs, mRNAs, and interacting proteins, as well as the binding constants and kinetic parameters for their interactions, is important for a number of considerations. First, since regulatory effects are observed within minutes, sRNAs and mRNAs presumably must cycle on and off Hfq rapidly to allow sufficient encounters of cognate RNA molecules in an appropriate time frame. The cycling is proposed to involve sequential binding and release of RNAs on individual subunits around the hexameric ring of Hfq (Fender et al., 2010). Second, recent studies suggest cellular RNAs compete for Hfq and that one abundant sRNA can indirectly impact the targets of others by disrupting Hfq-mediated effects (Hussein and Lim, 2011; Papenfort et al., 2009). Concentrations and rates of association and dissociation are likely to be particularly critical for systems with multiple homologous or even heterologous sRNAs acting redundantly or additively. Finally, these parameters will aid in the modeling of sRNA networks in bacteria, an area receiving increased attention (reviewed in Levine and Hwa, 2008).

The availability of sRNAs and their target mRNAs additionally must be considered. For instance, the seed and target regions might be sequestered in RNA secondary structures or by ribosomes and other proteins at various times during growth. An intriguing possibility is that subcellular localization may serve to regulate sRNA action. The *ptsG* mRNA is repressed efficiently by SgrS RNA only when the mRNA encodes transmembrane domains that mediate association with membranes via the translocation machinery (Kawamoto et al., 2005). It is proposed that ribosome access to the *ptsG* mRNA is reduced once the transcript is membrane associated, which leads to increased SgrS

access. Whether there are specific proteins to sequester sRNAs or compete with their binding to mRNAs, analogous to proteins that prevent miRNAs from acting (reviewed in Meisner and Filipowicz, 2011; Suzuki and Miyazono, 2011) remains to be seen.

### Intimate Connections between sRNAs and RNases

Modulation of bacterial RNA stability is a well-established mechanism to control gene expression, but it has only recently received attention in conjunction with sRNA-mediated regulation. Changes in mRNA stability brought about by base-pairing sRNAs initially were attributed to the connection between ribosome loading and mRNA stability. However, sRNAs modulate mRNA levels faster than can be explained by altered ribosome binding (Papenfort et al., 2006), and recent studies show direct sRNA regulation of mRNA stability by RNase E or RNase III, even for sRNAs that affect translation (reviewed in Caron et al., 2010). *Salmonella* MicC and RyhB exclusively increase degradation of some targets through base pairing within the coding region of mRNAs (Papenfort et al., 2010; Pfeiffer et al., 2009), and *E. coli* RyhB destabilizes one of its targets via interactions with an intergenic region (Desnoyers et al., 2009). sRNAs also affect mRNA stability to increase expression. In *Clostridium perfringens*, an inhibitory 5' terminal stem loop normally blocks expression of a collagenase mRNA. Binding of the VR-RNA induces an endonucleolytic cleavage that generates an mRNA with an accessible RBS and a different 5' structure predicted to increase stability (Obana et al., 2010). Protection of the 5' end of mRNAs from nucleases also is exploited by group A *Streptococcus*, where FasX RNA binding to the 5' end of the streptokinase mRNA provides a barrier to nucleases, thereby promoting mRNA stability (Ramirez-Peña et al., 2010).

RNases also contribute to sRNA activities and levels in various other ways. Most base-pairing sRNAs are transcribed as independent transcripts, but many are processed in some manner. For *E. coli* ArcZ, nuclease digestion serves to reveal the conserved seed region at the 5' end (Papenfort et al., 2009), while processing of the *Vibrio cholerae* MicX leads to increased stability (Davis and Waldor, 2007). Generation of the mature *E. coli* 6S RNA involves a multi-step pathway of 5' endonucleolytic digestion by RNase E or G and exonucleolytic trimming at both the 5' and 3' ends (Chae et al., 2011).

The stabilities of base-pairing sRNAs can be influenced by the presence of target mRNAs. *E. coli* RyhB and other sRNAs undergo coupled degradation with mRNA targets by RNase E (Massé et al., 2003). Interestingly, this mode of degradation can be discriminatory. The ChiX sRNA found in enteric bacteria is a target for RNase E cleavage when base paired with an mRNA decoy, but not when base paired to the mRNA it represses (Figuroa-Bossi et al., 2009; Overgaard et al., 2009), indicating that there must be specificity in RNase recognition or activity. Furthermore, *E. coli* MicA appears to be degraded by RNase E when not base-paired and by RNase III when paired with target mRNAs (Viegas et al., 2011).

A key for understanding the role of nucleases in sRNA-mediated regulation will be deciphering their specificity for sRNAs and mRNAs, alone or paired to each other. Cleavage of sRNA-mRNA duplexes by RNase III, such as directed by *S. aureus* RNAIII, reflects the known propensity of this nuclease to cleave

uninterrupted RNA duplex and can be recapitulated with the purified enzyme in vitro (Huntzinger et al., 2005). In contrast, specific signals directing RNase E cleavage remain ambiguous, although it is known that RNase E preferentially cleaves within single-stranded regions (Caron et al., 2010). The site of cleavage can be adjacent to the sRNA-target mRNA duplex, as proposed for MicC-directed cleavage of the *ompD* mRNA (Pfeiffer et al., 2009), or a significant distance from the region of duplex formation as found for RyhB and *sodB* (Prévost et al., 2011) (Figure 1C; local or distal cleavage model, respectively). However, even for the well studied RyhB-*sodB* pair, molecular determinants for cleavage by RNase E remain controversial (Afonyushkin et al., 2005; Prévost et al., 2011).

Other protein interactions may influence RNase cleavage of sRNAs and/or mRNAs. The RNase E-mediated inactivation of GlmZ, which removes the region required for base pairing to the target mRNA, requires the adaptor protein YhbJ (Reichenbach et al., 2008; Urban and Vogel, 2008). YhbJ is further regulated by a second sRNA (GlmY), highlighting the potential for complex regulation of sRNA activity. Studies of other proteins that modulate sRNA stability, such as polynucleotide phosphorylase, which was identified as impacting sRNA turnover in a genetic screen (De Lay and Gottesman, 2011), are still needed.

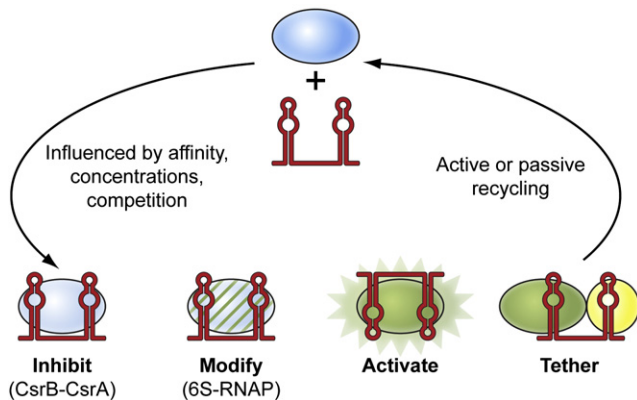
A molecular understanding of how RNases and other proteins interact and influence sRNA function and stability is only rudimentary. Hfq was reported to bind to the large C terminus of RNase E (Ikeda et al., 2011) suggesting that RNase E may be recruited to sRNA pairs via Hfq. There also have been indications that sRNAs can direct target cleavage by RNases other than RNase III and E (Opdyke et al., 2011). Further elucidation of the interactions between sRNAs and RNases await studies focused on uncovering the key players, identifying the determinants of specificity, and establishing in vitro assays.

### Modulating Protein Activity with sRNAs

Most sRNAs work in conjunction with proteins in vivo, and sRNA-protein interactions can be grouped into two general categories. In the first category, as exemplified by the base-pairing sRNAs discussed above, the sRNA provides the specificity and the primary activity to the RNA-protein partnership. The second category, which is the focus of this section, is comprised of sRNAs that interact with proteins for the purpose of regulating their activity. Some of these sRNAs act by sequestering proteins, particularly RNA binding proteins, from normal targets. However, sRNA binding to proteins also can produce more complex outcomes, such as the modification of an enzymatic activity (Figure 2).

#### Regulating RNA Binding Proteins

sRNAs that regulate RNA binding proteins typically work by mimicry, whereby the sRNA contains the protein recognition sequence, often in multiple copies. The classic example is the *E. coli* CsrB RNA, which has 18 binding sites for CsrA, an RNA binding protein known to regulate mRNA translation and stability (reviewed in Babitzke and Romeo, 2007). CsrB RNA thus acts as a direct competitor for CsrA target mRNAs. Much is known about CsrA and how its dimer recognizes a GGA motif in the loop of short stem structures in CsrB and mRNA targets. However, as diverse CsrA- and CsrB-like molecules continue to be identified, more flexibility in target site recognition may be revealed. The



**Figure 2. General Properties of sRNAs that Modulate Protein Activity**

Bacterial sRNA binding to proteins has been demonstrated to inhibit and/or modify protein activities. It is proposed that sRNA binding to proteins also can activate or to bring two or more proteins into together. The association of sRNA and proteins is likely to be influenced by many different factors, and the disassociation can be actively or passively controlled.

recent discovery of the CrcZ RNA, which has five repeats of a CA-rich motif bound by the translation repressor protein Crc in *Pseudomonas* (Moreno et al., 2009; Sonnleitner et al., 2009), raises the possibility that other classes of protein-sequestering sRNAs remain to be found.

CsrB-like sRNAs are widely distributed among bacterial species, and it is common to find multiple CsrB-like RNAs per organism, and in some cases multiple CsrA-like proteins (reviewed in Sonnleitner and Haas, 2011). The possibilities for complex regulation utilizing different RNA-protein partners thus are numerous, dispelling the idea that these sRNAs function to “simply” sequester a protein. For example, *Pseudomonas fluorescens* has three CsrB-like RNAs (RsmX/Y/Z) and two CsrA-like proteins (RsmA/E), leading to at least six possible combinations of sRNA-protein interactions.

An important open question regarding protein binding sRNAs is when and how the targeted proteins are released from sRNA sequestration. One such factor impacting release in *E. coli* is CsrD, an RNase E adaptor protein that modulates CsrB RNA stability (Suzuki et al., 2006).

### Regulating Enzymes

Other sRNAs bind proteins with enzymatic activity, and have the potential to inhibit, activate or modify protein activity. The best-studied example is the *E. coli* 6S RNA, which binds to the house-keeping form of RNA polymerase ( $\sigma^{70}$ -RNAP) (reviewed in Wassarman, 2007; Willkomm and Hartmann, 2005). The secondary structure of 6S RNA largely mimics the conformation of DNA during transcription initiation, which suggests this RNA also could act as a direct competitor. However, the binding sites of DNA and RNA on RNAP are not fully congruent (Klocko and Wassarman, 2009), and the effects on transcription are surprisingly complex (Cavanagh et al., 2008; Neusser et al., 2010). Direct downregulation is observed for only a subset of  $\sigma^{70}$ -dependent promoters, indicating that  $\sigma^{70}$ -RNAP activity is modified rather than simply inhibited.

As with all sRNAs, expanding studies into diverse species have given interesting new perspectives on potential complexities for regulation by 6S RNA. For instance, several species including *B. subtilis* and *Legionella pneumophila* express two 6S RNAs. Initial work has demonstrated that the two RNAs are functionally distinct based on different phenotypes and/or changes in gene expression in cells lacking one or the other sRNA (K.M.W., unpublished data) (Weissenmayer et al., 2011; Faucher et al., 2010). In addition, it is possible that 6S RNAs in other species act quite differently than in *E. coli*, as the regulon for one of the *L. pneumophila* 6S RNAs contains a limited number of genes, in contrast to the extensive 6S RNA regulon observed for *E. coli* (Cavanagh et al., 2008; Faucher et al., 2010; Neusser et al., 2010).

Further understanding of how sRNAs modulate enzymes and how this regulation impacts cell physiology again will require detailed analyses of the dynamics of association and disassociation in vivo and identification of other cellular components that influence these processes. A key finding was that *E. coli* 6S RNA serves as a template for RNA synthesis by RNAP to generate short product RNAs (pRNA), and that this process leads to release of 6S RNA from RNAP during outgrowth from stationary phase (reviewed in Wassarman, 2007). These types of studies provide new insights into the enzymes regulated, such as the finding that the cellular RNA polymerase can function as an RNA-dependent RNA polymerase.

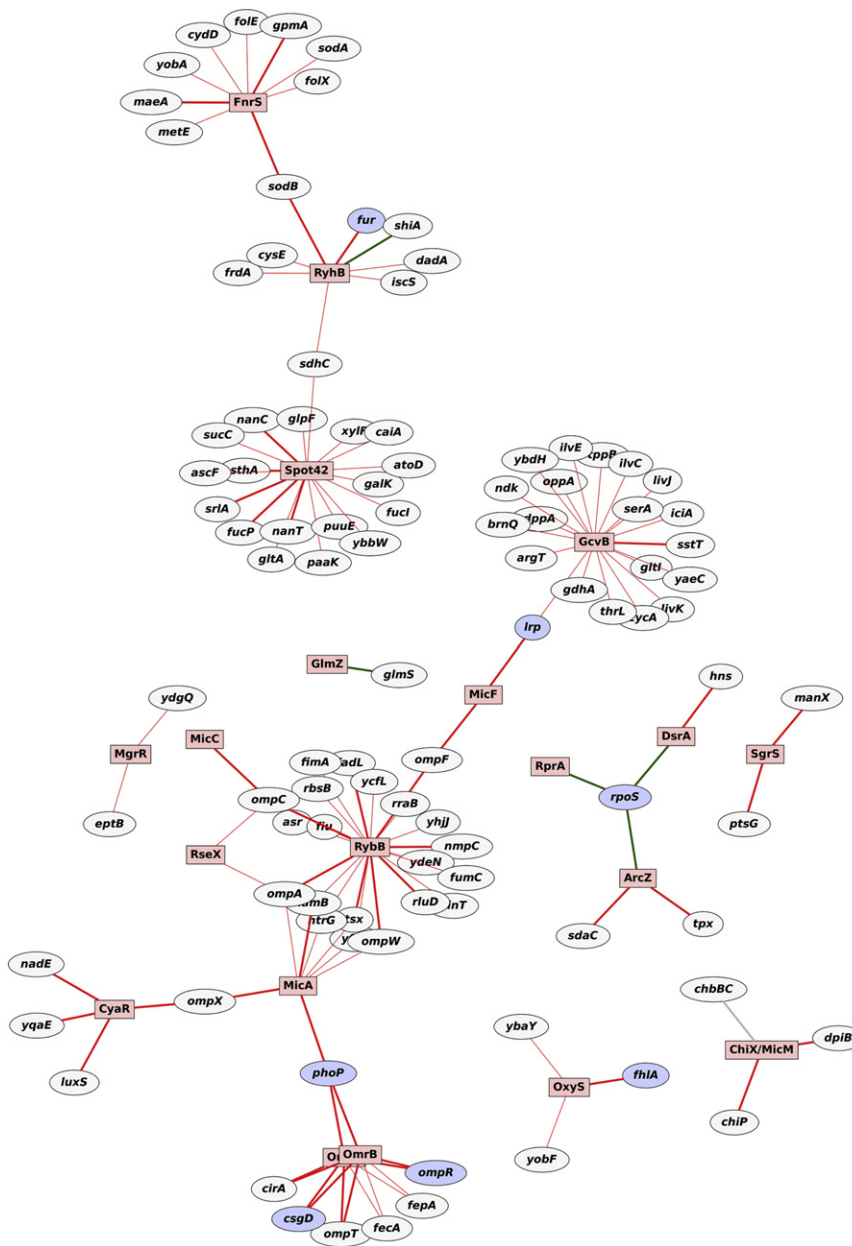
For sRNAs that regulate protein activity, a number of important mechanistic questions remain unanswered. Thus, structure determination of this class of RNAs, with and without binding partners, may be especially enlightening. It is also not clear how many of these sRNAs exist. Traditionally, sRNAs that bind to Hfq were classified as most likely to act by base pairing. In light of the increasing numbers of asRNAs that do not require Hfq for function, however, it is more difficult to assess how well the lack of Hfq binding predicts sRNAs that modulate protein activity (reviewed in Thomason and Storz, 2010). It seems probable that additional sRNAs function by non-base-pairing mechanisms, and it is prudent to continue to search for them among growing numbers of identified sRNAs. As a consequence, the diversity in sRNAs functions is expected to increase. For example, sRNAs could modulate proteins and enzymes via allosteric regulation or could tether proteins to each other, a mechanism likely to be used by a small RNA encoded by the ColE1 plasmid (Chant and Summers, 2007) (Figure 2).

### Vast and Diverse sRNA Regulons

In most of the initial studies of chromosomally encoded sRNAs, only one, or at most a few, target mRNAs were uncovered (reviewed in Wassarman et al., 1999). It is now evident that many sRNAs regulate a multitude of genes and have roles as broad as some general transcription factors in bacteria and some miRNAs in eukaryotes (Figure 3).

### Defining sRNA Regulons

For base-pairing sRNAs, targets are recognized through complementary sequences within each mRNA, while for protein binding sRNAs, targets are recognized via their interactions with the sRNA-regulated proteins. Experimental and computational approaches have been used to define the currently known



**Figure 3. Networks Regulated by Trans-Encoded Base-Pairing sRNAs in *E. coli***

The network is based on a compilation of published interactions. Boxes represent sRNAs and ovals represent mRNA targets. Ovals corresponding to mRNAs encoding transcription regulators are colored blue. Red lines indicate negative regulation, green lines indicate positive regulation, and gray lines indicate base pairing but no effect on the target. The thickness of the lines indicates the level of proof for base pairing; compensatory mutation analysis supports those interactions represented with the thickest lines. A limited number of lines emanating from an sRNA may reflect incomplete characterization or the fact that the sRNA has only few targets.

contribute to mechanistic understanding of how each sRNA functions.

Once identified, sRNA regulons often elucidate a clear physiological role for a particular sRNA within well-established regulatory networks (Figure 3). In *Caulobacter crescentus*, overexpression of the carbon starvation-induced CrfA RNA led to induction of TonB-dependent receptors, consistent with a CrfA role in facilitating nutrient uptake (Landt et al., 2010). In addition to enhancing our understanding of a particular sRNA role, the characterization of some regulons has given new insights into some physiological responses. For instance, studies of the Fur-regulated RyhB, whose levels are highest when iron is limiting, showed that RyhB contributes to metabolic remodeling under low-iron conditions by repressing expression of a serine acetyltransferase, which increases the flux of serine into siderophore production (Salvail et al., 2010). Nonetheless, the physiological roles of a subset of sRNAs have been more difficult to decipher, in part because the currently identified regulons are particularly small or large, or do not fit into known responses.

regulons (reviewed in Sharma and Vogel, 2009). The experimental approaches have included studies of sRNA-dependent changes in gene expression (by expression microarrays, deep sequencing, or comparative protein analyses), global searches for mRNAs that bind to a particular sRNA-regulated protein, and genetic screens for changes in reporter gene expression or altered growth phenotypes. Computational approaches have been based on the potential of mRNAs to base pair with sRNAs of interest or to carry binding sites for a protein whose activity is modified by the sRNA. Both direct and indirect effects will be detected by approaches that monitor altered gene expression. These effects are likely to help uncover the physiological role of a given sRNA. However, only direct targets will

Some discussion of how true direct targets can be identified is warranted. In studies using transient overexpression of sRNAs, it is often assumed that direct targets are affected before indirect targets (Massé et al., 2005; Sharma et al., 2011). However, if targets are regulated through different mechanisms, such as translation versus mRNA decay, or with different efficiencies, this assumption may not be valid. For base-pairing sRNAs, compensatory mutant analysis continues to be the gold standard for validation of direct targets, but such studies must be done on a gene-by-gene basis. In addition, mutagenesis may not always give conclusive results (Desnoyers et al., 2009), such as in cases where base-pairing regions of the sRNA or target mRNA have multiple functions. It is less clear how to

differentiate direct from indirect regulation of non-base-pairing sRNAs, especially when *in vitro* systems do not fully recapitulate *in vivo* observations such as for 6S RNA (reviewed in Wassarman, 2007).

Other considerations include the relative importance of multiple targets, whether the most strongly regulated targets are most biologically significant, and what targets are being missed with current approaches. Looking at changes in mRNA levels is the most common approach for characterizing sRNA regulons, but low abundance targets may be overlooked. In addition, some mechanisms of regulation do not result in altered mRNA levels, and changes in mRNA levels may not always be reflected as altered protein activity. Another consideration is that methods examining changes in gene expression or altered phenotypes typically utilize high overexpression of the sRNA of interest. sRNAs do not function in isolation, and therefore these high levels of expression, often at times or conditions when the endogenous sRNA is not normally expressed, may have unintended consequences that complicate interpretation of the results. In addition, mRNA species whose levels change upon sRNA overexpression might actually be decoys that regulate the sRNA under native conditions rather than being altered themselves, as has been argued in the case of eukaryotic miRNAs (reviewed in Seitz, 2009).

Computational predictions for mRNA targets of sRNA regulation have improved with additional information regarding what is required for sRNA-mRNA interactions (reviewed in Backofen and Hess, 2010). However, bioinformatic approaches alone are still fraught with high false positive rates because not all parameters are known. Conversely, since the searches are based on known, possibly biased information, false negative rates also may be a problem. For example, many computational approaches have focused on the region surrounding the start site of translation, which have missed more distant base-pairing regions of interest. Predictions of targets of protein-modifying sRNAs also can be challenging depending on the nature and understanding of the target features. Nevertheless, the combination of experimental and computational approaches for identifying sRNAs targets has contributed significantly to our understanding of the physiological relevance of sRNAs and hopefully will soon allow the definition of complete sRNA regulons.

### **sRNA Regulons as Part of Regulatory Networks**

In addition to revealing regulated genes, regulon identification has begun to elucidate the contributions of sRNAs within the context of larger regulatory networks (reviewed in Beisel and Storz, 2010). For example, *E. coli* Spot 42 RNA is part of a multi-output feedforward loop, in which Crp both directly activates target genes by binding to their promoters and indirectly activates the same genes by repressing the synthesis of the Spot 42 RNA, which acts as a repressor of the genes (Beisel and Storz, 2011). This configuration impacts the dynamics of target gene expression in cells switching between the presence and absence of glucose. Another regulatory scheme has been defined by the action of sRNAs such as the  $\sigma^E$ -regulated MicA and RybB, which endow an activator protein ( $\sigma^E$ ) with apparent repressive activity on particular targets (Gogol et al., 2011), thus leading to the opposite outcome on target genes than the

transcription regulator was designed to carry out directly. Additional levels of complexity arise from crossregulation. RyhB represses expression of its own regulator Fur (Vecerek et al., 2007), and MicA base pairs with the mRNA encoding the PhoPQ two-component system important in magnesium sensing and thus links one regulatory network with another (Coornaert et al., 2010). 6S RNA modulates expression of a number of key regulators, including *relA*, *crp*, and the translation machinery, amplifying the global response as nutrient quality decreases (Cavanagh et al., 2010; Neusser et al., 2010).

A growing number of mRNAs encoding transcription regulators appear to be targets of multiple sRNAs. The classic example, *rpoS*, is regulated directly by several Hfq-binding sRNAs (DsrA, RprA, ArcZ) and indirectly by other sRNAs by mechanisms that are not yet fully understood but may involve competition between sRNAs (OxyS) or RNA polymerase availability (6S RNA) (reviewed in Battesti et al., 2010). Other examples of mRNAs that encode key transcription factors and are targeted by multiple sRNAs include *E. coli* CsgD, which regulates curli genes (Holmqvist et al., 2010), Lrp, which regulates many genes involved in amino acid biosynthesis (Sharma et al., 2011) as well as *Vibrio* LuxR and AphA, two key regulators in quorum sensing (Rutherford et al., 2011). In fact, the balance between Qrr RNA repression of LuxR and activation of AphA is critical in ensuring appropriate gene expression at both low and high densities as well as during the transition between them. These recent results strongly suggest that additional regulatory proteins will be found to be modulated by multiple sRNAs, and that crosstalk between regulatory networks is even more extensive than currently appreciated. Methods for monitoring expression of reporter genes containing putative sRNA-targeted sequences in response to libraries of sRNA-overexpression plasmids allow efficient screening of sRNA effects on putative target genes (Mandin and Gottesman, 2010; Urban and Vogel, 2007), again with the caveat of possible artifacts due to overexpression. These systems also can be used to test the effects of mutations as well as sRNA-sRNA competition or hierarchy on specific targets.

### **Advantages of sRNA Regulators**

As sRNA regulon identification proceeds, it is worthwhile to consider possible advantages of RNA-based regulation over protein-based regulation. Previously suggested benefits include reduced metabolic cost, additional levels of regulation, faster regulation and unique regulatory properties (reviewed in Beisel and Storz, 2010). The coupled degradation of many sRNA-mRNA pairs permits sRNAs to achieve responses that are different than those of transcription factors (Levine et al., 2007). In addition, the ability to regulate a target at two levels, with both a transcription regulator and base-pairing sRNA, has been shown to reduce leakiness as well as alter the dynamics of target gene expression (Beisel and Storz, 2011). With increasingly larger and hopefully less biased data sets, it should become clear whether there are particular networks or genes that are predominant targets for sRNA regulation and whether sRNAs have reappearing positions in regulatory circuits. These data also should provide insights into why particular targets are regulated by different sRNAs and why some systems contain multiple sRNAs with apparently similar function.

### The sRNA Biome

The initial systematic searches for sRNAs based on screens for sequence conservation or orphan promoter and terminator sequences, or characterization of size-selected RNA, led to the identification of dozens of sRNAs (reviewed in Sharma and Vogel, 2009). The advent of deep sequencing has turned the discovery of regulatory RNAs on its head, revealing literally hundreds of previously undetected transcripts, as illustrated by studies in *Helicobacter pylori* (Sharma et al., 2010) and *Synechocystis* (Mitschke et al., 2011). The sequencing reads map to both intergenic and genic regions, in both sense and antisense directions relative to annotated genes and correspond to transcripts of a wide range of sizes. Multiple short transcripts most likely derived from the 5' end, 3' end, or internal regions of mRNAs also have been detected and may function as independent sRNAs as has been suggested for the SreA and SreB 5' fragments in *L. monocytogenes* (Loh et al., 2009). To date, very few of these newly reported candidate sRNAs have been functionally characterized, but their discovery raises important questions about how to define sRNAs and whether all detectable RNA transcripts have function.

Although the results of deep sequencing approaches are a potential gold mine for finding sRNA regulators, they highlight a number of challenges. At the simplest level, processing and comparing these extremely large data sets are difficult. In addition, there is a surprising lack of overlap between transcripts reported, even for studies in the same organism (reviewed in Croucher and Thomson, 2010). Use of different cDNA preparation protocols, different sequencing platforms, and different thresholds and stringencies for annotating transcripts are likely to contribute to this lack of clarity. Before results from deep sequencing analyses can be used to full advantage, the following questions will need to be addressed. What is an appropriate threshold for the number of reads, relative to what standard(s)? How should reproducibility be reported? What transcripts should be annotated? More universal platforms for sharing, analyzing, and storing data and uniformity in transcriptome annotation will greatly facilitate the utilization of the data and the application of bioinformatic screens. Ironically, the vast volume of data itself ultimately may result in barriers to its use.

Beyond the difficulties of reporting and evaluating deep sequencing data, even greater challenges lie in further validation of the identified transcripts and determination of their functions. For instance, what experiments, if any, need to be done to further validate and determine the ends of transcripts detected by deep sequencing? Northern analysis typically has been the standard for validating candidate sRNAs identified by other approaches. In addition to validating expression, northern analysis provides information about size and potential processing. Identification of 5' ends of RNAs by deep sequencing is often robust, but identification of the 3' ends has been less reliable. Finally, the possibility that the newly identified transcripts may encode small proteins that are frequently missed during genome annotation needs to be considered.

Thus far, the elucidation of sRNA function for both base-pairing and protein-binding sRNAs has proceeded with the detailed characterization of individual sRNAs. While this approach has been fruitful, without higher throughput approaches, it will take

years to characterize the hundreds of sRNAs now being reported. Collections of tagged sRNA deletion strains and libraries of sRNA overexpression plasmids are available in *E. coli* and *Salmonella* (Hobbs et al., 2010; Mandin and Gottesman, 2010; Papenfort et al., 2008). However, some of the phenotypes associated with decreased or increased expression of known sRNAs are subtle and may only be observed under specific conditions or in sensitized backgrounds. High-throughput competition screens with mixed cultures have been successful in identifying phenotypes but require uniquely tagged strains. Alternatively, individual mutants can be arrayed such that a particular sRNA deletion can be compared to all other deletion strains under a wide range of conditions or easily moved into other mutant backgrounds (Nichols et al., 2011).

There are some caveats to the current global approaches for finding phenotypes for sRNA regulators as a method to uncover function, particularly for the asRNAs prevalent in the deep sequencing approaches. Foremost, it is difficult to delete asRNA genes without affecting the oppositely encoded gene. An alternative approach may be to construct libraries overexpressing RNAs that are complementary to the asRNA with the intent of blocking the activity of the asRNA (an anti-antisense strategy). For RNAs with dual functions, such as the *E. coli* SgrS, *S. aureus* RNase III, *B. subtilis* SR1, and *Pseudomonas aeruginosa* PhrS sRNAs that encode proteins and function as regulatory RNAs (reviewed in Vanderpool et al., 2011), it may be challenging to discern the effects of the regulatory RNA activity from the effects of the encoded protein, especially since translation of the peptide could impact the riboregulatory activity. It is conceivable that some mRNAs act as RNA regulators as well, raising the possibility that the number of *trans*-acting regulatory RNAs is even higher than the hundreds of sRNAs and asRNAs already being reported.

### The Awesome Power of Studying sRNAs in Bacteria

As we move beyond our understanding of individual sRNA functions and how they integrate into known networks, sRNAs undoubtedly will serve as fodder for broader questions about bacterial physiology and evolution. It already is clear that sRNAs serve as diverse regulators that impact almost every aspect of bacterial physiology. Therefore, understanding the ways that bacteria respond to and influence communities and how they survive such diverse environments will benefit from further studies of sRNAs. The breadth of bacterial species with fully sequenced genomes, coupled with the available genome sequences of multiple isolates of the same species, enables comparisons that are likely to help elucidate the evolution of sRNA regulators and the advantages provided by sRNA-based regulation. In addition, the ability to conduct evolution experiments involving thousands of generations is unique to the bacterial world. An ongoing, long-term evolution experiment with *E. coli* has not yet revealed critical changes in sRNA genes (Barrick et al., 2009). However, in *Myxococcus xanthus*, a spontaneous mutation that abolished the regulatory function of the Pxr RNA, thereby restoring developmental proficiency in a population lacking this capacity, was identified in a short-term evolution experiment (Yu et al., 2010). It will be interesting to perform these types of experiments using otherwise isogenic strains



manipulated to examine the effects of having a particular node regulated by a protein or an sRNA.

The use of sRNAs as diagnostic tools and platforms for the development of antimicrobial therapies has long been suggested as an important outcome of sRNA studies. The promoters controlling expression of sRNAs are among the most sensitive for any particular stress and thus might serve as good reporters of conditions encountered by a cell (Mutalik et al., 2009). More and more sRNAs have been found that are induced by contact with a host cell, some of which impact survival in the host (reviewed in Papenfort and Vogel, 2010). For example, the lack of 6S RNA significantly affected *L. pneumophila* growth in a human cell line (Faucher et al., 2010). In addition, many sRNAs remodel metabolic flux. Therapies that take advantage of these observations are attractive but remain in early stages of development (reviewed in Isaacs et al., 2006). Another interesting but unresolved question is whether RNAs are ever exchanged between a bacterium and a host, perhaps acting as regulators or signaling molecules in a heterologous bacterium or eukaryotic cell.

Finally, there are many universal regulatory RNA-based questions for which bacteria may provide opportunities for study not afforded by higher organisms. For instance, the ease in growing and manipulating large quantities of many bacterial species, combined with a smaller number of genes per genome, will facilitate the measurement of important parameters including RNA concentrations and affinities as well as the dynamics of RNA-RNA and RNA-protein interactions. Directed in vivo selection experiments also may further elucidate critical sequences and structures. Such information will be necessary for the development and testing of models for the roles of RNAs in regulatory networks. The facility of using bacteria and well-studied sRNAs that can be utilized as proofs of principle also should allow for the enhanced development of techniques for studying RNAs in all organisms at both single cell- and population-wide levels.

Clearly there are many exciting frontiers and unanswered questions in research on bacterial sRNAs. It is likely that important insights will come from breakthroughs in methodology. We anticipate that these will be in the form of high-resolution structures of sRNAs, their targets and the proteins involved, further exploitation of global approaches for identifying sRNAs and examining the binding of critical proteins, and visualization of the subcellular localization of RNAs in bacteria. However, continued characterization of individual sRNAs undoubtedly also will uncover new insights, new regulatory mechanisms and novel connections within bacterial physiology.

#### ACKNOWLEDGMENTS

We thank K. Förstner for help with Figure 3, many colleagues for their comments, and the participants of the 2011 ASM Conference on Regulating with RNA in Bacteria for discussion, which stimulated many of the ideas in this review.

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