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## Prevalence of small base-pairing RNAs derived from diverse genomic loci

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### Abstract

Small RNAs (sRNAs) that act by base-pairing have been shown to play important roles in fine-tuning the levels and translation of their target transcripts across a variety of model and pathogenic organisms. Work from many different groups in a wide range of bacterial species has provided evidence for the importance and complexity of sRNA regulatory networks, which allow bacteria to quickly respond to changes in their environment. However, despite the expansive literature, much remains to be learned about all aspects of sRNA-mediated regulation, particularly in bacteria beyond the well-characterized *Escherichia coli* and *Salmonella enterica* species. Here we discuss what is known, and what remains to be learned, about the identification of regulatory base-pairing RNAs produced from diverse genomic loci including how their expression is regulated.

### Keywords

small RNAs; sRNA biogenesis; UTR; RNA-seq

## 1. Introduction

Regulatory small RNAs (sRNAs) are typically transcripts of less than 300 nt that are diverse and complex in their means of action, which involve interactions with target mRNAs, other sRNAs, RNases, and RNA chaperones. While the best characterized organisms remain *Escherichia coli* and *Salmonella enterica*, there is evidence that regulatory RNAs play important roles in modulating cellular responses to environmental stress in nearly all

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bacteria taxa. This review provides a general overview of their mechanisms of action before a detailed discussion of sRNA prevalence, biogenesis, and regulation.

### 1.1. Types of sRNA regulators

In general, there are regulatory sRNAs that sequester proteins and those that act by base-pairing with other RNAs. Only a few sRNAs that function by protein sequestration have been described mechanistically. The 6S RNA binds directly to  $\sigma^{70}$ -RNA polymerase, which inhibits its association with promoters [1], thus preventing their transcription. The regulatory sRNAs CsrB and CsrC function as molecular sinks to sequester the global regulators CsrA/RsmA away from target mRNAs (reviewed in [2]). As another example, the GlmY sRNA impacts the stability, and consequently the regulatory effect, of the GlmZ base-pairing sRNA. GlmY sequesters the RNA binding protein, RapZ, that otherwise would stimulate GlmZ turnover by recruiting the major endoribonuclease RNase E [3]. Additionally, some sRNAs that bind the RNA chaperone Hfq, which typically facilitates sRNA-target regulation (discussed in depth below), do not themselves base pair with other RNAs but instead sequester Hfq from sRNA base-pairing regulators (reviewed in [4]).

The most well-characterized sRNAs act by base-pairing. Some sRNAs are encoded antisense to their target RNA, and thus have perfect complementarity with this RNA. However, most sRNAs are transcribed at other locations on the genome (*trans*-encoded) and act by base-pairing with RNA targets with only limited complementarity. For both types of base-pairing sRNAs, the initial region of base-pairing on the sRNA is generally denoted as the “seed sequence.” The outcome of this base-pairing can vary, but ultimately leads to some regulatory effect on the target RNA.

This review is focused on base-pairing sRNAs, in particular on how they have been discovered, their various origins, and how they are regulated, but will first provide an overview of their mechanisms of action.

### 1.2. Regulation by antisense RNAs

Antisense RNAs (asRNAs) (reviewed in [5, 6]) were first discovered as regulators of *E. coli* ColE1 replication [7]. Subsequently, asRNAs were shown to control transposase expression. The first example was RNA-OUT of the *E. coli* *Tn10/IS10* transposase system, which is transcribed antisense of the transposase mRNA to inhibit translation and thus downregulate transposition [8]. Other examples of asRNAs transcribed opposite transposase genes include two in *S. enterica* [9, 10], two in *Caulobacter crescentus* [11], and three in *Listeria monocytogenes* [12]. asRNAs also serve as the antitoxin of type I toxin-antitoxin (TA) systems (reviewed in [13]). In these systems, asRNAs base-pair with and prevent expression from the mRNA encoding the toxin by competing with ribosome binding [14], blocking translation of leader peptides [15], and/or providing RNA-double stranded sequences which can be degraded by RNase III [16].

Given that the asRNA and its corresponding target RNA are linked on the genome, transcription of the asRNA itself could serve as a regulatory mechanism. Possible transcriptional interference mechanisms include the simultaneous transcription and collision of RNA polymerases from two convergently elongating complexes, occlusion of one

promoter due to elongating transcription from the other, and interference where an elongating polymerase collides with and displaces a “sitting duck” preinitiation polymerase (reviewed in [17, 18]). Some tests of these models have been carried out with asRNAs of coliphage 186 [19] and *L. monocytogenes* [12], and artificially tested in *E. coli* [20]. Other times both transcriptional interference and base-pairing could contribute to asRNA regulation, as has been suggested for the AmgR asRNA in *S. enterica* [21]. While deep sequencing indicates that there is an abundance of antisense RNAs, very few have been shown to have a physiological function. Repressing the synthesis of a potentially toxic protein is the overwhelming role in the limited documented examples of antisense sRNA function.

### 1.3. Regulation by trans-encoded base-pairing RNAs

*trans*-encoded base-pairing sRNAs can regulate mRNA targets at multiple levels. There are a few examples of sRNAs that affect transcription by base-pairing with the 5′ untranslated region (UTR) of an mRNA and modulating Rho-mediated premature termination (reviewed in [22]). However, the most prevalent mechanisms of sRNA-mediated regulation impact the stability and/or translation of an mRNA. This was the original mechanism described for the first characterized *trans*-encoded sRNAs like *E. coli* MicF [23] and *Staphylococcus aureus* RNAIII [24]. The base-pairing can block ribosomes from accessing the ribosome binding site (RBS), impede translation, or recruit RNases to process mRNAs or degrade them. In a more limited number of cases, base-pairing alters mRNA secondary structure and exposes a previously concealed RBS leading to increased translation as is the case for *E. coli* DsrA and RprA activation of *rpoS* translation [25, 26].

There are also more complex regulatory mechanisms. RNA-RNA interactions have now been observed to occur not only at 5′ UTR regions but also within coding sequences [27]. In some cases, base pairing leads to mRNA structural changes that conceal the upstream RBS and inhibit translation initiation as for *Bacillus subtilis* SR1 sRNA which binds 80 nt into the coding sequence of the *ahrC* mRNA [28]. It is also possible that coding sequence interactions could slow or inhibit elongating ribosomes. The internal base pairing can result in changes in the turnover of mRNAs. As an example, in *S. enterica*, the MicC sRNA binds 67 nt into the coding sequence of the *ompD* mRNA leading to RNase E-dependent *ompD* decay [29]. sRNAs additionally can interact with other sRNAs, serving as sponges to prevent the affected sRNAs from finding their mRNA targets, often by promoting their degradation [30]. While usually assumed to be non-coding, more and more examples of “dual-function” RNAs, encoding both a regulatory base-pairing region and a small regulatory protein are emerging (reviewed in [31]).

The region of base-pairing between the sRNA and the target is generally limited. Thus, in many bacteria, a protein chaperone has been found to mediate these RNA-RNA interactions. The best characterized bacterial RNA binding protein, is the homo-hexameric RNA matchmaker Hfq, which uses distinct surfaces to bind specific sequence motifs in sRNAs and their cognate targets (reviewed in [32]). In *E. coli* and *S. enterica*, deletion of Hfq results in the destabilization of sRNAs and decreased regulatory effects (reviewed in [33]). A relatively understudied RNA chaperone, ProQ, also has been shown to have RNA-RNA

matchmaking capabilities in *E. coli* [30], *S. enterica* [34, 35], and *Legionella pneumophila* [36] (reviewed in [37]). However, ProQ also appears to have additional roles in RNA regulation. In both *E. coli* and *S. enterica*, ProQ was shown to block RNase-mediated degradation of both mRNAs and sRNAs [30, 38].

While much work has focused on Hfq and ProQ, which are found in a range of bacterial species (reviewed in [37, 39]), in some bacteria, such as many Gram-positive organisms, Hfq can bind sRNAs but is not required for sRNA stability or regulatory function. As an example, SR1 sRNA regulates *ahrC* mRNA independent of Hfq in *B. subtilis* [40]. sRNAs also have been detected in organisms such as *Streptomyces* [41], *Chlamydia* [42], *Helicobacter* [43], and *Campylobacter* [44], which do not have known Hfq or ProQ homologs. Either other RNA binding proteins serve RNA matchmaking roles or base-pairing RNA-mediated regulation occurs independently of a protein chaperone.

The identification of *trans*-encoded base-pairing sRNA targets has exploded in recent years with the development of several techniques to detect the targets on a genome-wide level (reviewed in [45]). Some of these methodologies, like RIL-seq (RNA Interaction by Ligation and Sequencing) [46] and CLASH (UV Cross-linking, Ligation, and Sequencing of Hybrids) [47] rely on the crosslinking of RNAs to proteins followed by protein immunoprecipitation and the ligation of RNA ends. The sequencing of these products reveals RNAs that are in proximity on the immunoprecipitated protein. Other approaches, such as GRIL-seq (Global sRNA Target Identification by Ligation and Sequencing) which relies on *in vivo* expression of an RNA ligase coupled with RNA-seq [48, 49], are independent of RNA binding proteins.

## 2. Methods to identify sRNAs

The annotation of regulatory RNAs in bacterial transcriptomes followed significantly after the annotation of mRNAs and housekeeping RNAs. This can, in part, be attributed to the lack of awareness about potential regulatory RNA loci in genetic screens and because the consequences of defects in sRNA-mediated regulation generally are more subtle than phenotypes associated with defects in protein factors. However, the methods to detect sRNAs have greatly expanded in recent years.

### 2.1. Direct detection

Initially, small transcripts in *E. coli* were detected by two-dimensional polyacrylamide gel electrophoresis and RNase T1 fingerprinting [50, 51]. Later, high-density microarrays revealed signals for intergenic sRNA candidates in *E. coli* [52] and other organisms such as *Streptococcus pyogenes* [53], *Neisseria gonorrhoeae* [54], *Burkholderia thailandensis* [55], to only name a few. The development of RNA-seq brought higher resolution for sRNA identification. However, the compactness of bacterial genomes with overlapping transcripts creates challenges in mapping sRNAs using standard RNA-seq methods.

Improvements to transcriptome annotation have come with strand-specific RNA-seq and the development of methodologies to define RNA boundaries. 5' RNA-seq approaches distinguish transcriptional start sites (TSSs) from processed 5' ends. For instance, differential RNA-seq (dRNA-seq) [56] utilizes a terminator 5'-phosphate-dependent

exonuclease (TEX) to degrade RNA carrying a 5' monophosphate (as a result of processing), leaving 5' triphosphate-containing RNAs (indicative of transcripts with a native TSS) to be sequenced (reviewed in [57]). Similarly, treatment of RNA with tobacco acid pyrophosphatase (TAP) or RNA 5' pyrophosphohydrolase (RppH), which both remove the pyrophosphate from the 5' end of triphosphorylated RNA, can be used in RNA-seq approaches to distinguish TSSs from processing sites. In the Cappable-seq method, TSSs are labeled with a desthiobiotin cap for reversible binding to streptavidin and subsequent 5' RNA-seq [58]. On the other end of the transcript, 3' ends of RNAs can be identified by Term-seq through the ligation of 3' sequencing adapters in the first step of RNA-seq library preparations [59]. This can be combined with bicyclomycin (BCM), an inhibitor of the termination factor Rho, to subclassify 3' ends as Rho-termination-dependent [60]. SMRT (Single Molecule, Real-Time)-Cappable-seq utilizes single-molecule long read sequencing to map bacterial operons, concurrently identifying 5' and 3' ends [61]. Additionally, SEnd-seq (Simultaneous 5' and 3' End Sequencing) circularizes cDNA for simultaneous 5' and 3' end identification [62].

The identification of sRNAs in RNA-seq data sets is improved by approaches that enrich for these molecules. This can be via size selection for small transcripts [63, 64] or association with RNA binding proteins. A number of different methods have been developed for the sequencing of transcripts that bind proteins like Hfq and ribonucleases. Many of these such as CLIP-seq (Crosslinking Immunoprecipitation and Sequencing) [65], RIL-seq [27, 30] and CLASH [47, 66] rely on the crosslinking of RNAs to the proteins of interest followed by immunoprecipitation of either the native protein or tagged derivatives. In another approach termed Grad-seq (Gradient profiling by Sequencing), RNA binding proteins and their RNAs are fractionated on glycerol gradients and co-association is determined by sequencing all of the RNAs and deep mass-spectrometric analysis of the proteins in each fraction [34].

Collectively, these transcriptome datasets have revolutionized bacterial genetics in a variety of model and pathogenic bacteria. The datasets have challenged dogma by revealing an abundance of internal, antisense, and intergenic transcription comprising much more complex bacterial transcriptomes than first anticipated.

## 2.2. Computational detection

Initially there were certain criteria by which sRNAs were defined and computational predictions were developed to scan bacterial genomes to identify the best candidates (reviewed in [67, 68]). Early search programs focused on intergenic regions where sRNAs were first discovered. Many algorithms are based on comparative genomics, examining closely-related bacteria. In general, these programs search intergenic regions for sequence conservation, GC content, and other features such as promoter motifs, transcription factor binding sites, and intrinsic terminators. sRNA secondary structures should be of a thermodynamically favorable minimum free energy, with lower free energy than a random sequence. Therefore, RNA folding can also be helpful in the computational prediction of sRNAs. Programs that use such parameters include QRNA [69], RNAz [70], sRNAPredict [71], sRNAscanner [72], and RNASurface [73]. Machine learning based software also has been developed [74, 75]. The downside of these approaches is that they sometimes rely on

conservation, though not all sRNAs are broadly conserved. Additionally, intergenic regions also harbor mRNA transcription elements such as promoters and transcription factor binding sites that can confound the data. Limiting search parameters to intergenic regions also misses sRNAs encoded elsewhere in the genome.

One way to avoid some of the issues mentioned above is to combine direct detection and computational predictions, which is the premise of programs such as ANNOgesic [76] and APERO [77]. These approaches integrate data from RNA-seq experiments to detect small transcript boundaries and subsequently predict sRNA features from these datasets. The identification of sRNAs by multiple approaches has led to overwhelming datasets, even for just one bacterial species. For example, in *S. aureus*, sRNAs have been detected by computational predictions [78], microarrays [79], and RNA-seq methods [80–83] to generate a list of over 500 candidate sRNAs. However, combining RNA-seq methods with the sRNA computational software DETR'PROK narrowed this number to 50 putative sRNAs [84]. While some sRNAs could be missed, this type of analysis could reveal promising sRNAs for further studies, especially for organisms where few sRNAs have functionally been characterized.

### 3. Prevalence of sRNAs across bacteria species

While the true number of sRNAs is not known for any organism, it is useful to consider the numbers of sRNAs reported in different bacterial species. With the surge in research on bacterial regulatory RNAs and the availability of RNA-seq technologies, there seems to be an abundance of small transcripts. However, careful mechanistic studies which document base-pairing and regulatory actions of these RNAs is limited for most bacteria.

We have assembled a table presenting the estimated numbers of detected sRNAs across species excluding asRNAs, excepting those asRNAs that have been functionally characterized. We also note key methods in identifying sRNAs, the approximate number of characterized sRNAs, and a representative base-pairing sRNA (Table 1). The number of reported sRNAs varies between species. For instance, ~20 have been reported in *Chlamydia trachomatis*, compared to ~100 in *E. coli*, and ~600 in *Borrelia burgdorferi*. Follow-up experiments which confirm the expression of these sRNAs are important before making sweeping conclusions about the existence of these regulators. Northern blot analysis is the gold standard for validating the presence of sRNAs. This approach provides information about both the abundance and size of a transcript, which cannot be accomplished with other methods, like Reverse-Transcriptase PCR, especially for sRNAs that overlap other transcripts. Of the ~500 putative sRNAs in *Synechocystis* sp., only 14 have been tested by northern blot analysis to document their presence and discrete sizes [85]. In comparison, nearly all ~100 *E. coli* sRNAs reported by various global approaches, including size-selection RNA-seq [86], have been confirmed by northern blot analysis and many have been shown to be functional. Most bacteria in Table 1 have hundreds of sRNAs detected but fewer than 5 examples of sRNAs documented to act by base-pairing ideally by mutating the sRNA seed region to disrupt regulation and then restoring the regulation with a compensatory mutation in the target RNA. Most of the representative sRNAs listed in Table 1 have been tested for direct base-pairing. In some phyla (Acidobacteria, Fusobacteria, Planctomycetes),



we did not find any studies which specifically detected sRNAs, providing opportunities for future discoveries.

## 4. Sources of sRNAs

Abundant numbers of sRNAs have been reported across bacterial species for many years. However, the fact that the sRNAs themselves are encoded by nearly all parts of the bacterial genome (Figure 1) has only been appreciated more recently. The following sections will describe the unique genomic loci (independently transcribed, excised from mRNA 5' and 3' ends, processed from operons, and components of tRNAs) that produce small base-pairing RNA regulators.

### 4.1. Antisense sRNAs

Antisense sRNAs are encoded on the DNA strand opposite another gene, though base-pairing occurs in *trans* after both RNAs are transcribed (Figure 1A). Usually the target is the mRNA encoded on the opposite strand, though some asRNAs also have targets encoded elsewhere on the genome. RNA-seq data indicate extensive antisense transcription, with over 5,000 asRNAs detected in *E. coli* by RNA-seq [87], and up to 75% of all genes having antisense transcripts in *Prochlorococcus* sp. and *S. aureus* [88, 89]. asRNAs range in size from less than 100 nt to several thousand nt, even spanning several genes. One asRNA of ~7,000 nt spans 14 genes, comprising two adjacent operons in *Prochlorococcus* sp. [90], and a ~1,000 nt long asRNA is transcribed in opposition to an S-box riboswitch and the *mccA* gene in *Clostridium acetobutylicum* [91]. Others transcribed just opposite to the 5' or 3' ends of mRNAs such as SymR in *E. coli* [92] and SprA1 in *S. aureus* [93] are much smaller, just 77 nt and 60 nt in length, respectively. In *Synechocystis* sp., IsrR, a 177 nt asRNA completely internal to the gene encoded opposite, has also been shown to have a regulatory effect [94]. One would assume asRNAs can easily base-pair with the mRNA encoded on the opposite strand because of the perfect complementarity. However, as already mentioned, despite their abundance, few instances of *bona fide* regulatory roles have been documented.

### 4.2. Intergenic sRNAs

The largest number of functionally characterized base-pairing sRNAs are encoded as distinct transcripts in intergenic regions with their own promoters (Figure 1B). Most of these sRNAs contain an intrinsic terminator with a stable stem-loop and stretch of U residues. In *E. coli* ~50 intergenic sRNAs have been documented, ranging in size from ~50 to ~300 nt. A plethora of intergenic sRNAs also have been described in other bacteria. One study compared more than 23,000 intergenic regions and found ~900 intergenic sRNAs across 13 different bacterial species [95]. The authors also concluded that sRNA genes were significantly enriched in longer intergenic regions, which further implies that bacteria efficiently use genomic space.

One challenge is documenting the function for the numerous intergenic sRNAs in bacterial genomes. The genomic context and conservation of adjacent neighboring genes can be helpful in identifying gene functions, as these can be related. For instance, there are a number of examples, such as OxyR-OxyS and GcvA-GcvB in *E. coli* [96, 97], where the

transcription factor that controls the expression of an sRNA is encoded adjacent to the sRNA gene.

Global evolutionary analysis of *E. coli* has indicated intergenic sRNAs in this organism are mostly conserved within the *Enterobacteriales* order [98]. However, gene rearrangements can result in the gain or loss of an intergenic sRNA, even among closely related species. For instance, the intergenic region of *E. coli* *uspF* and *ompN* contains the biofilm-inhibitor sRNA, EcsR1, which in *S. enterica* was fragmented due to a translocation event leading to the loss of the sRNA gene [99]. In general, the evolution of sRNAs continues to be poorly understood because sRNAs with the same function and targets have been described even when there is little sequence similarity between the two sRNAs. FsrA, one of the first intergenic sRNA characterized in *B. subtilis*, regulates mRNA targets involved in iron metabolism and storage [100], similar to *Enterobacteriaceae* RyhB [101] but has a completely different sequence and structure.

Since the characterization of the first *E. coli* sRNA [23], 30 years of research has documented regulatory mechanisms for ~30 *E. coli* intergenic sRNAs. As regulatory RNA-networks are being delineated in a range of bacteria, important physiological functions for intergenic sRNAs continue to be discovered. For example, VqmR in *Vibrio cholerae* regulates biofilm formation [102], *B. subtilis* RoxS regulates the NAD<sup>+</sup>/NADH equilibrium [103], RmaA regulates flagellar motility in *Erwinia amylovora* [104], *S. aureus* SprC regulates virulence and contributes to phagocytosis and murine infectivity [105]. While most functional characterization has been focused on intergenic-derived sRNAs, there has been increased discovery of base-pairing sRNAs in other genomic regions, as will be discussed in the following sections.

#### 4.3. sRNAs processed from within operons

Some intergenic sRNAs have been found to be co-transcribed with other genes in operons and subsequently processed into functional small transcripts (Figure 1C). Generation of these regulators relies on transcription from the upstream genes. The 5' end of the sRNA is always generated by cleavage events, but the 3' end can result from transcription termination or cleavage. For instance, the sRNA sponge SroC, characterized in *S. enterica*, is derived from the *gltIJKL* mRNA encoding the glutamate/aspartate ABC transporter. In this case, the 5' end is generated by RNase E cleavage and the 3' end is generated by transcription termination at an internal intrinsic terminator directly proceeding *gltJ*, which must allow some read-through for the polycistronic mRNA to be transcribed [96]. The mature SroC sRNA binds another sRNA, GcvB, as a sponge to prevent GcvB-mediated regulation. The uncharacterized sRNAs 3' to *fimA* and *glnA* in *E. coli* [106] are also encoded within operons but less is known about their generation and activities.

#### 4.4. 5' derived sRNAs

The synthesis of sRNAs from 5' mRNA regions is understudied, but these regions also have the potential for encoding *trans*-acting RNA regulators (Figure 1D). Classically, 5' UTRs are hotspots for *cis*-acting RNA-regulatory elements, particularly riboswitches, RNA thermometers, and attenuators (reviewed in [107–109]). Riboswitches alter expression of



their downstream genes through the binding of specific ligands, often metabolic byproducts from the gene's enzymatic or signaling pathway. The secondary structures of RNA thermometers are changed with temperature to hide or expose ribosome binding sites. Finally, attenuators are structured leader sequences that encode a small upstream open reading frame (uORF) preceding the coding region of the mRNA. Ribosomes either translate or stall on the uORF, affecting the formation of a downstream terminator hairpin or Rho binding, which results in transcription termination and the release of an RNA corresponding to the attenuator.

In many of these regulatory scenarios, small, stable transcripts are generated. Indeed, small 5' transcripts for the transcriptional riboswitches preceding *mgtA* [110] and *lysC* [111] were detected in an *E. coli* cloning-based screen, which captured RNAs of 30–65 nt [106]. Northern blot analysis verified that distinct fragments are detected at reasonable levels. Similarly, two uncharacterized *E. coli* sRNAs, named SroA and SroG [112], are produced from the thiamine riboswitch upstream of *thiCOGE* [113] and riboflavin riboswitch upstream of *ribB* [114], respectively.

While the functional properties of most 5' UTR fragments have not been explored, there are some suggestions that 5' sRNAs can have independent functions. For instance, two S-adenosylmethionine (SAM) riboswitches in *L. monocytogenes* leave behind stable, small 5' RNAs denoted SreA and SreB, which have been shown to base-pair with and downregulate in *trans* the mRNA encoding the virulence regulator PrfA [115]. As another example, adenosyl cobalamine (B<sub>12</sub>) responsive riboswitches in *Enterococcus faecalis* and *L. monocytogenes* produce *trans*-acting sRNAs, EutX and Rli55 respectively [116, 117]. Both of these sRNAs bind the two-component response regulator EutV and thus prevent the protein from acting on its downstream *eut* target mRNAs. In *Sinorhizobium meliloti*, there are three tryptophan biosynthesis operons, but only one, *trpE(G)*, encodes a known uORF. Interestingly, the attenuator RNA byproduct from this operon has been reported to function as a base-pairing sRNA. This sRNA, named mTrpL, appears to base-pair with the *trpDC* mRNA from another tryptophan operon leading to its decay when tryptophan is abundant [118].

Outside of known *cis*-regulatory elements, other examples of small transcripts derived from 5' UTR regions have been noted. In the same *E. coli* cloning based screen mentioned above, 15 others were detected [106]. Another study using RNA decay coupled with RNA-seq identified 12 fragments in 5' UTRs [119]. One study examining the global consequences of deleting the 3'-to-5' phosphorolytic exoribonuclease PNPase (*pnp*) revealed that several 5' UTR fragments increased in a *pnp* strain, suggesting a subset of these transcripts could be regulated via this RNase [120]. Indeed stable 5' derived small transcripts can arise both from *cis*-regulatory events and perhaps early termination or cleavage events at 5' UTRs.

There are two characterized 5' derived sRNAs for which there is no known *cis*-regulatory element. The *E. coli* SgrS RNA corresponds to the 5' end of the *setA* mRNA and is generated by termination upstream of *setA* [121]. RhIS described in *Pseudomonas aeruginosa* provides another example [122]. This sRNA was discovered by mapping premature termination using Term-seq. A 3' end was noted 34 nt upstream the start codon of

*rhII*, which encodes a protein involved in regulating N-acylhomoserine lactone quorum sensing. This region did not appear to encode a riboswitch when tested with N-butanoyl-homoserine lactone, and no other regulatory elements were predicted. RhlS contains a stem-loop followed by a polyuridine sequence, a hallmark of sRNAs. For both SgrS and RhlS, considering that they are part of the *setA* and *rhII* 5' UTR, respectively, there must be at least occasional transcriptional readthrough to fully transcribe the full-length mRNAs. As more studies demonstrate the abundance of 5' mRNA fragments, the importance and biological relevance of these transcripts should be explored.

#### 4.5. Intragenic sRNAs

Various RNA-seq studies have documented RNA regions within coding sequences that are more abundant than the rest of the mRNA (Figure 1E). Some bacteria seem to contain more intragenic transcription than others, although a subset could be due to misannotation. Nevertheless, 5' RNA-seq mapped 63% of TSSs to internal coding regions in *B. burgdorferi* [123], 62% in *Leptospira interrogans* [124] and 61% in the haloarchaeon *Haloferax volcanii* [125]. Within *E. coli*, 37% of sequenced TSSs mapped to intragenic coding regions [87]. It is possible some of the transcripts initiating from internal promoters encode small proteins in the same or different frame as annotated gene [126]. However, an RNA decay experiment in *E. coli* using RNA-seq revealed 21 short, stabilized intragenic RNAs that the authors postulated could be base-pairing regulatory RNAs [119]. Furthermore, RIL-seq analysis indicated several are bound by Hfq and provided evidence that putative intragenic sRNAs base-pair with 5' regions of mRNAs [27, 30]. Since no mechanistic studies to demonstrate base-pairing have been carried out, this class also deserves further study.

#### 4.6. 3' derived sRNAs

A quickly-expanding number of *trans*-acting sRNAs transcribed and/or processed from the 3' end of mRNAs (Figure 1F) have been shown to have regulatory effects. Some 3' UTR regions were first noticed as discrete small transcripts in early *E. coli* sRNA screens [106, 112]. However, their role as base-pairing sRNAs was first recognized in a *S. enterica* RNA-seq experiment [127] analyzing RNAs that co-immunoprecipitated with the RNA-RNA matchmaker, Hfq. Eight of these sRNAs were detected by northern blot analysis and one, DapZ, was shown to have its own promoter sequence internal to its overlapping mRNA, *dapB*. Other 3' sRNAs can be processed from the larger mRNA, like SorX in *Rhodobacter sphaeroides* [128]. Even 3' sRNAs transcribed as separate entities can be processed into a shorter form, like the 83 nt MicL-S derivative of the 300 nt *E. coli* MicL sRNA [129]. 3' derived sRNAs can be identified by searches for 5' ends in mRNA 3' UTRs in RNA-seq data. For instance, 17 were identified and classified as transcribed or processed in *Neisseria meningitidis* using TEX treatment and dRNA-seq [130]. These sRNAs tend to be named based on their overlapping mRNA, keeping the first three letters of the mRNA and the fourth with a late letter of the alphabet, like CpxQ derived from the 3' region of *cpxP* [131] or RbsZ which overlaps *rbsB* [30].

Given the known features of base-pairing sRNAs, 3' UTR regions are ideal for evolution of these sRNAs, which require a stable stem-loop and stretch of poly-U's to bind with Hfq. Thus, mRNA intrinsic terminators, which contain these features can be exploited. Co-

evolution of 3' sRNAs with their overlapping mRNAs eliminates evolving this structure *de novo*. In line with this suggestion, it has been noticed that mRNAs encoding 3' derived sRNAs tend to have a longer stretch of poly-U's than mRNAs without an sRNA [27]. Natural selection may favor rapid evolution in 3' UTRs where mutations are less likely to be deleterious. The linked genetic location could imply similar physiological roles for the protein and sRNA encoded by the overlapping sequences. For example, the sRNA s-SodF is cleaved from the 3' region of *sodF* (iron superoxide dismutase) and targets the nickel superoxide dismutase, *sodN* in *Streptomyces coelicolor* [132]. Therefore, understanding the genomic context of 3' sRNAs may help to elucidate their functions.

Mechanistic studies have now documented that a number of 3' derived sRNAs function as base-pairing regulators, and the association with Hfq strongly implies base-pairing potential for others. Consistent with this implication, ~300 *E. coli* RNA pairs were mapped to 3' UTRs and other coding sequences in stationary phase using the RIL-seq approach, which detects Hfq-bound sRNAs and their target RNAs [27]. We expect more and more functional 3' derived sRNAs to be discovered and characterized from numerous bacteria in the future.

#### 4.7. tRNA precursors and fragments

tRNAs are either transcribed independently or in polycistronic tRNA transcripts. These tRNA precursors are then processed by a series of 5' and 3' end cleavages to complete their maturation (reviewed in [133]). Mature tRNAs also are subject to endonuclease cleavage. The byproducts of these cleavage products, tRNA-derived fragments (tRFs), range in size from 13 to 20 nt and have been identified in all domains of life (Figure 1G) (reviewed in [134]). tRFs are implicated in various biological functions in eukaryotes, but direct evidence for functional tRFs in bacteria is more limited. Here we present some indication that prokaryotic tRNA precursors and tRFs can function as base-pairing regulators.

Association of tRNA precursors with Hfq has suggested a regulatory function for these transcripts. Microarray analysis of Hfq co-immunoprecipitated RNA showed enrichment of the *proM* internal transcribed spacer (ITS) region from the *argX-hisR-leuT-proM* polycistronic tRNA precursor [135]. Subsequent northern blot analysis demonstrated *proM* tRNA precursors, but not mature tRNA<sup>Pro</sup>, were enriched in Hfq-bound RNA samples. Similarly, Hfq co-immunoprecipitation coupled with RNA-seq and northern blot analysis showed the *metZ<sub>WV</sub>* transcript, but not mature tRNA<sup>Met</sup>, binds Hfq [136]. Some mature tRNAs have been shown to bind Hfq *in vitro* [137]. Additionally, the sRNAs RybB and MicF were found to co-purify with the ITS regions on the *metZ<sub>WV</sub>* tRNA precursor [138]. This interaction was only observed with full length *metZ<sub>WV</sub>* and not the ITS fragments. However, the physiological relevance of these interactions is still unknown.

There is one example of a characterized tRNA precursor-derived fragment with a function. 3' ETS<sup>*leuZ*</sup> is processed from the 3'-external transcribed spacer (ETS) of the *glyW-cysT-leuZ* polycistronic tRNA precursor. 3' ETS<sup>*leuZ*</sup> functions as a base-pairing regulator by sponging and repressing the activity of the RybB and RybB sRNAs [138]. Targets of these sRNAs include genes related to the TCA cycle and antibiotic sensitivity. Experiments assaying these regulons demonstrated 3' ETS<sup>*leuZ*</sup> sponges the sRNAs from their targets affecting cellular fitness. Given that sRNAs are powerful regulators, it may be beneficial to

repress their activity via sponging from a constitutive tRNA transcript, such that specific induction of the sRNA must overcome the sponge to elicit effects. The RIL-seq approach found tRNA sequences in RNA duplexes with coding sequences, UTRs and sRNAs on the RNA chaperones Hfq and ProQ [27, 30], again implying base-pairing and perhaps new regulatory networks.

Some tRFs are generated by cleaving tRNAs into halves or quarters. In the archaeon *H. volcanii*, a stress-induced 26 nt tRF cleaved from tRNA<sup>Val</sup> reduces protein synthesis by directly binding to the small ribosomal subunit [139]. One study describes three tRFs cleaved from tRNA<sup>Val</sup>, tRNA<sup>Gly</sup>, and tRNA<sup>Gln</sup> in the Rhizobium *Bradyrhizobium japonicum* [140]. Intriguingly, the targets of these tRFs are not in the bacterium itself, but in its symbiotic partner the soybean, *Glycine max*. Overexpression and silencing of the Rhizobium tRFs affected root hair curling and nodule formation in the soybean via base-pairing with root hair and plant developmental gene targets. This emerging concept of RNA-mediated regulation across species and kingdoms is discussed in more detail below.

There are likely underexplored types of tRNA-linked regulatory elements. The cellular pool of tRNAs has been shown to be dynamic; for instance tRNAs are subject to degradation after amino acid starvation [141]. Conceivably, such byproducts could be recycled as regulators during such stress conditions. Other forms of tRNA precursors also could serve as regulators, like the tRNA-linked repeats, which are located immediately downstream tRNAs and share the last 18–19 nt of the 3' end of a tRNA sequence [142]. Given the abundance of these transcripts, tRNA precursors and fragments could be a significant source of other regulatory RNAs.

## 5. Other potential sRNAs sources

There is now a greater appreciation of the diverse origins of base-pairing RNAs. Undoubtedly, sRNA regulators will be found to be encoded by still other genomic locations, and we highlight a few conceivable sources below.

### 5.1. sRNAs from ribosomal RNAs

The three rRNAs (5S, 16S, and 23S) are co-transcribed in most bacteria and require processing into mature species through a series of cleavage and nucleotide/sugar modification events (reviewed in [143]). One can imagine that precursor or mature rRNA fragments (rRFs) could be functional, similar to tRFs. Fragments of mature *E. coli* rRNAs have been studied and shown to interact with translation factors [144], antibiotics [145, 146], mRNA/tRNA analogs [146], and to alter translation termination [147]. In eukaryotes small ribosome-derived RNAs have been functionally characterized (reviewed in [148]). For instance, the qiRNAs of the filamentous fungus *Neurospora crassa* are processed from 28S rRNA during DNA damage and mediate gene silencing in the DNA damage response pathway [149]. Such rRNA-derived regulators could also be active in bacteria.

### 5.2. sRNAs from other species

The concept of RNA-mediated crosstalk between bacteria, phage, and eukaryotic hosts is quite attractive. This is especially true for bacterial biofilms, microbiomes, bacteria-

eukaryote symbionts, and host-pathogen interactions. Given that bacteria are excellent adaptors and manipulators of their environment, it is conceivable that molecules like regulatory RNAs participate in bacterial systems for hijacking host cells or for inter-organism communication.

Phage infections are likely instances of RNA-mediated crosstalk, in part because the virus can hijack the host genome for its replication. An obvious example of base-pairing crosstalk involving RNA is the CRISPR-Cas13 immunity system [150, 151]. Evidence is accumulating that phages also harbor other base-pairing sRNAs that modulate phage or host derived transcripts. Several putative phage sRNAs were detected during  $\phi$ R1–37 infection of *Yersinia enterocolitica* [152], and two sRNAs transcribed from the PAK\_P3 phage during *P. aeruginosa* infection have been described [153]. Genomic regions containing bacterial prophage also have been shown to encode sRNAs, such as the AgvB sRNA found in pathogenic Enterohemorrhagic *E. coli* (EHEC) [154].

Bacterial-eukaryotic relationships are another likely environment for RNA-mediated crosstalk. The study described previously documenting regulatory RNAs from *B. japonicum* modulating its symbiotic host the soybean [140] provides one such example. *L. monocytogenes*, a Gram-positive intracellular bacterium, has been reported to secrete bacterial RNA/DNA which is detected by host cytosolic nucleic-acid sensing receptors [155]. It has also been reported that this bacterium secretes an RNA binding protein, Zea, which binds *L. monocytogenes* RNA extracellularly in the host cell cytoplasm and interacts with host proteins [156]. As detection methods improve to distinguish and categorize sRNAs from different species, we expect more examples of RNA-mediated crosstalk.

One question that arises is how sRNAs might be delivered from one species to another in their environment. The formation of extracellular vesicles to secrete molecules has been observed for both eukaryotes and prokaryotes. The most extensive characterization of bacterial vesicles is in Gram-negative bacteria, where they are termed outer membrane vesicles (OMVs), however, their biological significance is largely unknown. Several groups have shown OMVs play roles in pathogenesis and elicit host immune responses (reviewed in [157]). There has been much interest in the composition of OMVs, especially since it has been postulated that they harbor inter- and intra-kingdom “communication” molecules, like regulatory RNAs (reviewed in [158]). Some groups have sequenced extracellular RNA (exRNA) from OMVs and found them to be shorter than 250 nts and frequently derived from intergenic regions or fragments of tRNAs or rRNAs [159–161]. In one example, a tRNA-derived sRNA in *P. aeruginosa* has been reported to be transferred into human airway cells via OMVs to reduce the inflammatory response during infection [162]. The exRNA Rli32 of *L. monocytogenes* induces interferon- $\beta$  production and promotes intracellular growth during macrophage infection [163]. Some caution is warranted given differences in vesicle isolation between groups and potential contamination of lysed cells or other secreted/extracellular factors. Given that the RNAs found in OMVs are so short, it is also conceivable that they just correspond to random, degraded RNA fragments. Nevertheless, there is considerable interest in the topic [164].

## 6. Regulation of sRNA synthesis

Like any other regulatory molecule, sRNAs themselves need to be expressed in a controlled manner to elicit targeted effects. Several mechanisms control sRNAs at the levels of transcription as well as processing/degradation.

### 6.1. Transcription initiation

The levels for sRNAs can be regulated by transcription from specific promoter sequences by an interplay of transcription activators and repressors. This is especially true for antisense and intergenic sRNAs.

Sigma factors ( $\sigma$ ) compete with one another for binding to the RNA polymerase core subunits and direct transcription by interacting with promoter sequences. There are seven known sigma factors in *E. coli*, but the number in other bacteria varies from three in *B. burgdorferi* to 19 characterized/predicted in *B. subtilis* and more than 100 in the soil dwelling myxobacterium *Sorangium cellulosum* (reviewed in [165]). Some sigma factors like the stress response factor RpoS ( $\sigma^S$ ) and envelope stress factor RpoE ( $\sigma^E$ ), are relatively broadly conserved and affect sRNA expression in many species. Under membrane stress for instance,  $\sigma^E$  upregulates periplasmic proteases, protein chaperones, and specific sRNAs to counteract the misfolding of outer membrane proteins. In *E. coli*, the  $\sigma^E$ -dependent sRNAs MicA and RybB base-pair with and negatively regulate the mRNAs encoding membrane proteins, including *ompA* [166–169]. This is similar to *V. cholerae*, where  $\sigma^E$  directs increased transcription of the MicV and VrrA sRNAs, which share conservation with the *E. coli* RybB seed sequence and function to downregulate *ompA* and other outer membrane protein transcripts [170]. Sigma factors also can be species specific. In *B. subtilis*  $\sigma^K$  and  $\sigma^G$  direct transcription during late stages of sporulation and induce several sRNAs that have been postulated to regulate genes during sporulation [171]. Similarly, the alternative sigma factor SigB, regulates a subset of intergenic sRNAs in *L. monocytogenes* and *S. aureus*, such as Rli47 [172] and RsaA [78], respectively, in addition to some uncharacterized asRNAs [88, 173]. Feedback loops also exist, where an sRNA negatively regulates the synthesis of its own sigma factor, as for *E. coli* RybB and  $\sigma^E$  [168]. Through sigma factors, sRNAs can be targeted for expression under specific conditions where they are most needed.

Transcription factors can be used in conjunction with sigma factors to regulate the expression of sRNAs, either positively or negatively. They too can be broadly conserved, with the same transcription factor controlling sRNA transcription in many organisms. A classic example is the ferric uptake regulator (Fur). Under conditions of high iron, *E. coli* Fur represses synthesis of the sRNA RyhB, which targets mRNAs encoding iron-containing proteins during iron-limiting growth [174, 175]. Functional analogs of RyhB, such as PrrF in *P. aeruginosa* [176], NrrF in *N. meningitidis* [177], HrrF in *Haemophilus influenzae* [178] and FsrA in *B. subtilis* [100], are all transcriptionally regulated by Fur in these species. There are also species-specific transcription factors that regulate sRNA genes like ToxT, which activates expression of the TarA sRNA in *V. cholerae* [179]. As described for sigma factors, there are feedback loops involving transcription factors and sRNAs. For example, there are a number of two-component systems, where a membrane sensor kinase phosphorylates a transcription factor response regulator in response to environmental cues



(reviewed in [180]) and thereby activates sRNA transcription. In a few examples, such as for the OmrA/B sRNAs and OmpR-EnvZ sensor kinase-response regulator in *E. coli* [181], the induced sRNAs base pair with and repress the mRNA encoding the two-component system, forming a negative feedback loop.

Often, sRNAs are the most strongly induced transcript in a specific stress response. For instance, the sRNAs OxyS and MicA are among the most highly-induced RNAs upon H<sub>2</sub>O<sub>2</sub> and cell envelope stress, respectively [129, 182, 183]. Thus, sRNAs can serve as reporters of a particular cellular stress, and further understanding of the transcriptional regulation of sRNA genes can give insights into the response.

## 6.2. Transcription termination

Termination is a regulated process that can either result from formation of a stem-loop (hairpin) structure causing RNA polymerase pausing, and subsequent dissociation, or the binding of the termination factor Rho which recognizes specific sequences and dissociates the RNA polymerase elongation complex (reviewed in [184]). Given that sRNAs can be derived from 5' UTRs, operons, and perhaps intragenic regions of mRNAs, there likely are mechanisms to favor production of the functional sRNA under some conditions and the functional mRNA under other conditions. Some evidence suggests that efficiency of sRNA termination is enhanced with the same stress conditions that induce transcription initiation of the sRNA [185]. It is also possible that the level of termination is constant in some cases with the basal level of termination sufficient to still elicit sRNA regulatory effects. The mechanisms by which transcription termination of sRNAs is regulated and also the role of the Rho protein are aspects that require further exploration.

## 6.3. Modification

One possible mechanism for controlling sRNAs could be RNA modifications. Post-transcriptional base modifications are widespread across the kingdoms of life and also have been detected for bacterial sRNAs (reviewed in [186–188]). Several sequencing studies have identified nicotinamide adenine dinucleotide (NAD) capped transcripts [189] and internal N<sup>6</sup>-methyladenosine (m<sup>6</sup>A) modifications in bacterial RNA [190]. Such modifications have been found in higher frequency for sRNAs than mRNAs, though only low percentages of the sRNAs are modified. This raises questions about the physiological roles of the modifications. It is possible that modifications contribute to processing, turnover and/or regulatory effects, but the biological consequences need to be further investigated.

## 6.4. Processing

Northern blot analysis of sRNAs frequently show multiple bands, indicative of precursors and/or processed forms of the sRNAs. Some sRNAs are cleaved from operons, 3' UTRs, or tRNAs to produce functional regulators. However, even sRNAs that are transcribed as separate entities may be cleaved. 3' UTR-derived sRNAs can have their own TSS intragenic to the protein coding sequence and also be processed from the mRNA, which contains the 3' UTR. The *E. coli* sRNA RbsZ is likely made from its own promoter at the 3' end of the ribose transporter *rbsB*, but is also processed from the mRNA itself and further cleaved into a shorter sRNA (RbsZ-S) [30]. The mechanisms for sRNA maturation can be complex and

require the activity of multiple RNases. For example, the DicF sRNA in *E. coli* is believed to be cleaved from the *dicBF* operon by RNase III and then further processed by RNase E [191].

Sometimes different forms of an sRNA can have different regulatory consequences or be present at different levels throughout growth. Different forms of the *E. coli* SdsN sRNA have distinct mRNA targets, likely attributed to differences in secondary structure [192]. In *Myxococcus xanthus*, both the longer (Pxr-L) and shorter (Pxr-S) forms of the Prx sRNA are present in high amounts in vegetative cells, while Pxr-S levels decrease during starvation [193]. Conceivably a sRNA precursor could exist in the cell with some low level of activity, to then be processed under a specific stress condition to elicit a stronger effect. For example, compared to full-length *E. coli* ArcZ, the processed form of ArcZ binds much stronger to the *rpoS* target in the presence of Hfq *in vitro* [194]. The same may be true for other sRNAs. Processing results in a monophosphate at the 5' end of the sRNA, which also can be advantageous. Indeed, RNase E-dependent cleavage has been shown to be stimulated by a 5' monophosphate provided by an sRNA to activate degradation of the target RNA [195]. Thus, processing may provide an extra layer of regulation of sRNA activity beyond transcriptional synthesis.

The precise cues and players which contribute to such complex regulation of sRNA regulators continue to be discovered. RNA structures and specific sequences can dictate the cleavage of 3' derived sRNAs [196]. Some of the cleavage sites for RNase E and III, which have been globally predicted by activating or deleting the RNase and performing RNA-seq in *E. coli* or *S. enterica* [197, 198], map to sRNA genes.

Obviously, another function of RNases is to turnover sRNAs. Across bacteria some of the key exo- and endoribonucleases in these processes are PNPase, RNase J, RNase E, RNase Y, and RNase III (reviewed [199, 200]). Control of degradation is crucial for regulating sRNA activity and can vary with conditions and the type of RNA. Some sRNAs are co-degraded with their base-pairing targets via RNase E or RNase III [201]. In this mechanism, one sRNA can only regulate one target transcript before it is eliminated. PNPase has also been shown to degrade some sRNAs, particularly those not bound by Hfq [202]. However, for other sRNAs this RNase can have a stabilizing effect, particularly for Hfq-bound sRNAs [120].

Other RNA binding proteins can affect sRNA turnover either generally or individually. In Gram-negative bacteria, Hfq is associated with the RNA degradosome promoting cleavage of bound targets and sRNAs. Paradoxically, however, deletion of Hfq results in the destabilization of many sRNAs. Thus, Hfq is required for both stabilizing the cellular pool of sRNAs as well as facilitating their turnover. The *E. coli* RapZ protein is an example of an RNA binding protein that specifically promotes turnover of just one sRNA, GlmZ, by acting as an adapter for RNase E [3].

Several transcripts that base-pair with and thus sponge sRNAs have been characterized. In some instances, an mRNA target sequesters and induces turnover of the sRNA, like the *E. coli* and *S. enterica chb* operon and ChiX [203, 204]. Other cases involve sRNA-sRNA

pairing, where the sponging can result in RNase-mediated degradation. These types of sRNA sponges, like SroC [96], PspH [27], and RbsZ [30], have mainly been found in *E. coli* and *S. enterica*, but likely constitute an understudied mechanism to control sRNA-mRNA regulation in other bacteria.

## 7. Perspectives

Research in RNA biology is booming with more regulatory transcripts being discovered than ever before. The era of RNA-seq has facilitated the global identification of sRNAs from previously underappreciated genomic loci. However, finding functions for these sRNAs can be challenging. Moving forward it will be exciting to tease out new types of sRNA regulatory mechanisms and networks and to characterize sRNAs in bacteria which have not been extensively studied. Although they are small, sRNAs have big effects in the RNA world.

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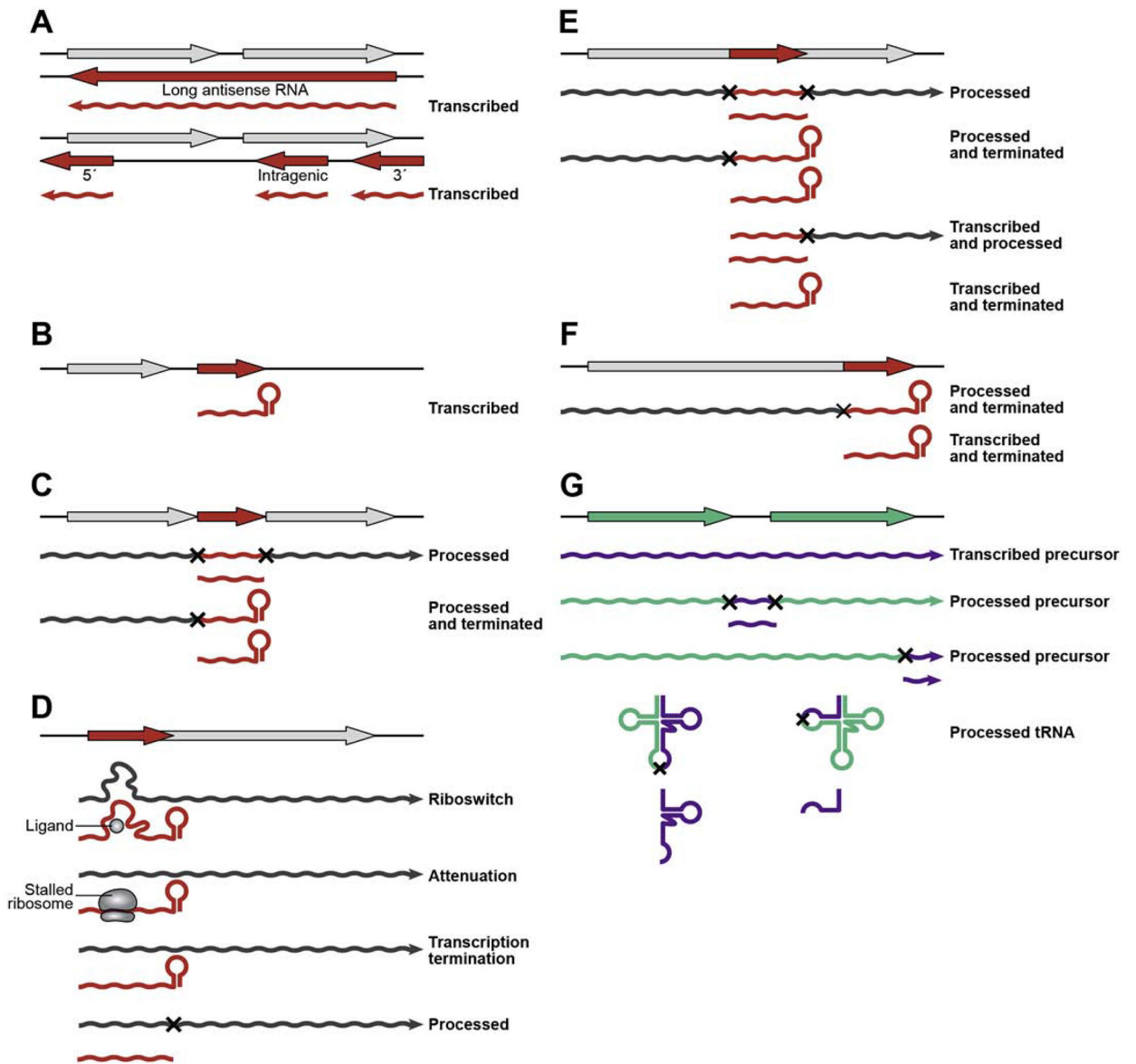
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**HIGHLIGHTS**

- Varied numbers of regulatory sRNAs have been reported for many different bacteria
- Most characterized sRNAs act via base-pairing with target RNAs
- sRNAs are produced from diverse genomic loci including portions of mRNAs and tRNAs
- sRNA levels are controlled by transcriptional and post-transcriptional mechanisms



**Figure 1.**

Origin of sRNAs from diverse genomic loci. **(A)** antisense **(B)** intergenic **(C)** operon-derived **(D)** 5' derived **(E)** intragenic **(F)** 3' derived **(G)** tRNA-derived. Coding sequences are denoted in gray, sRNA sequences in red, tRNA sequences in green, and regulatory tRNA sequences in purple. Processing cleavage sites are indicated with an "x". Instances of known transcription termination are indicated by a hairpin. 3' ends corresponding to known processed RNAs are blunt. An arrow is used if the mechanism of 3' end generation is unknown.

**Table 1.**

sRNAs detected across bacteria species.

Phylum	Example Species	Estimated sRNAs <sup>1</sup>	Example Methods <sup>2</sup>	Example sRNA <sup>3</sup>	Functionally Characterized <sup>4</sup>
Acidobacteria		none			
Actinobacteria	<i>Streptomyces coelicolor</i>	~100	size selection RNA-seq [205]	scr5239 [206]	<5
	<i>Mycobacterium tuberculosis</i>	~200	size selection RNA-seq [207]	MrsI [207]	<5
Bacteroidetes	<i>Porphyromonas gingivalis</i>	~30	size selection microarray and RNA-seq [208]	none	<5
Chlamydiae	<i>Chlamydia trachomatis</i>	~20	5' RNA-seq [42]	IhtA [209]	<5
Cyanobacteria	<i>Synechocystis</i> sp.	~500	5' RNA-seq [210], size selection RNA-seq [211]	RbIR [85]	<5
Firmicutes	<i>Bacillus subtilis</i>	~150	5' RNA-seq [212]	FsrA [100]	<10
	<i>Staphylococcus aureus</i>	~50	total RNA-seq and computational pipeline [84]	RsaD [213]	<15
	<i>Listeria monocytogenes</i>	~150	size selection RNA-seq [214]	LhrA [215]	<10
Alpha-proteobacteria	<i>Caulobacter crescentus</i>	~150	total RNA-seq [216]	CrfA [217]	<5
	<i>Rickettsia prowazekii</i>	~40	Total RNA-seq [218]	none	<5
Beta-proteobacteria	<i>Neisseria meningitidis</i>	~70	5' RNA-seq [130]	AniS [219]	<10
Gamma-proteobacteria	<i>Escherichia coli</i>	~100	cDNA library [112], size selection RNA-seq [86], Hfq and ProQ RIL-seq [30]	OxyS [97]	<70
	<i>Legionella pneumophila</i>	~90	5' RNA-seq [220]	RocR [36]	<5
	<i>Pseudomonas aeruginosa</i>	~170	5' RNA-seq [221]	RhIS [122]	<20
	<i>Vibrio cholerae</i>	~100	5' RNA-seq [102]	Qrr1-4 [222]	<20
	<i>Yersinia pestis</i>	~220	size selection RNA-seq [223]	Ysr141 [223]	<5
Delta-proteobacteria	<i>Myxococcus xanthus</i>	unknown	n/a <sup>5</sup>	Pxr [193]	<5
Epsilon-proteobacteria	<i>Helicobacter pylori</i>	~60	5' RNA-seq [56]	RepG [224]	<5
Spirochaetes	<i>Borrelia burgdorferi</i>	~600	size selection RNA-seq [225]	DsrA [226]	<5
Fusobacteria		none			
Planctomycetes		none			

<sup>1</sup>Numbers of sRNAs (excluding uncharacterized asRNAs) for each example species were estimated using global approaches which directly reported sRNAs.

<sup>2</sup>Example methods used for sRNA estimations.

<sup>3</sup>One example of a characterized base-pairing sRNA.

<sup>4</sup>Estimation of functional sRNAs where base-pairing has been experimentally documented.

<sup>5</sup>Not applicable.