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A broadening world of bacterial small RNAs

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Summary

The ubiquity of small RNAs (sRNAs) in bacteria is now well established. These transcripts are members of regulatory circuits involved in diverse processes ranging from stress adaptation to virulence to metabolism. Recent large-scale searches suggest that there exist many times more sRNAs than previously predicted even in the best studied of bacterial transcriptomes. Based on these and other recent findings of regulatory sRNAs that do not function in a "classical" manner, we propose that the working definition of sRNAs be broadened.

Introduction

It has become abundantly evident that small RNAs (sRNAs) can act as regulators of gene expression in all organisms in which they have been investigated. In the past decade, there has been an explosion in the number of sRNAs identified in bacteria [1-5]. Although still a young field, the study of bacterial sRNAs has already greatly extended our knowledge of genetic regulatory circuits in bacteria [6-13]. Historically, the bacterial sRNA field has focused on *trans*-encoded sRNAs, which differ from the *cis*-encoded antisense RNAs of plasmids, bacteriophages and chromosomes in that they have only imperfect complementarity with their RNA targets [14]. Small RNAs are often defined as short non-coding transcripts that, together with the RNA chaperone, Hfq, act in *trans* to control the translation or stability of target mRNAs. Indeed, many of the sRNAs originally identified, primarily in the model organism *Escherichia coli*, function in this manner (Figure 1A,B) [15-20].

Recent reports have revealed that there are more types of sRNA in bacteria than were predicted [21-24]. Additionally, it is becoming increasingly apparent that the established sRNA paradigm does not describe all or even most sRNAs in bacteria. Many *cis*-acting, chromosomally-encoded antisense sRNAs have now been identified, and Hfq-independent sRNAs have also been described. In addition, the known functions of sRNAs are broader than just the control of translation or mRNA stability. For example, the 6S and CsrB/C/D sRNAs directly bind protein transcription factors to affect downstream gene expression (Figure 1C) [25,26]. There are even sRNAs that serve both as regulatory RNAs and translated messenger RNAs. These observations are compelling reasons for broadening the working definition of sRNAs to accommodate all of these examples, as well as to leave room for the inevitable additional types of sRNAs yet to be reported.

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sRNAs that do not fit the mold

Classically, sRNAs that regulate mRNA expression were known as non-coding transcripts that regulate gene expression by binding Hfq and an mRNA expressed in *trans*. Below, we provide examples from the recent literature that suggest that there are many aspects of this definition that must be relaxed or redefined.

sRNAs that code

There are now several examples of sRNAs that have regulatory functions as RNA but are also translated into small proteins. One of the first sRNAs to be identified, RNAIII, a *cis*-acting regulatory RNA in *Staphylococcus aureus* involved in quorum-sensing, also encodes a 26-amino acid peptide that may be involved in biofilm integrity [27]. In *E. coli*, the 227-nucleotide (nt) sRNA SgrS, which is expressed when phosphosugar intermediates reach high, toxic levels, is a translational repressor of *ptsG* encoding the glucose transporter component of the phosphoenolpyruvate phosphotransferase system (PTS) [28]. SgrS also codes for the 43-amino acid SgrT that inhibits glucose transport through PtsG [29]. Finally, transcriptional profiling of *Listeria monocytogenes* recently revealed, among many putative sRNAs, five that are also predicted to encode small proteins ranging from 28–64 amino acids [24]. These findings collectively suggest that sRNAs are not necessarily non-coding, but can have dual functions as both messenger RNA and regulatory RNA.

Hfq is not always required

Originally identified as a host factor for replication of the RNA phage Qβ in *E. coli* [30], Hfq is an RNA binding protein that has been studied extensively for its role in sRNA-mediated gene regulation. Many of the best-characterized sRNAs require Hfq for stability and/or activity [15,19,31]. In several cases, Hfq has been shown to aid activity by enhancing the rate of duplex formation between sRNA and target RNA [32,33], which is often followed by hydrolysis of target, sRNA, or both, by ribonucleases such as RNase E [34,35] (Figure 1A).

The presumed central role of Hfq in sRNA biology has led to the use of this protein in the identification of new sRNA species [5,23,36]. Most recently, this approach was used towards the identification of 31 putative new sRNAs in *Salmonella enterica* serovar Typhimurium [23]. RNA sequences that co-immunoprecipitated with Hfq were cloned and sequenced *en masse* [23]. In addition to identifying candidate sRNAs, an advantage of this method is that 727 mRNAs that are Hfq-bound *in vivo* were also identified [23]. Thus, this method provides potential targets for each of the newly identified sRNAs. Through this analysis, the total number of known and putative *S*. Typhimurium sRNAs was increased to 64, and the authors were able to identify post-transcriptional regulons involving Hfq and sRNAs.

Obviously, sRNAs that do not bind Hfq will be missed in any Hfq-based sRNA identification strategy. There are several examples of Hfq-independent sRNAs, and we anticipate that there are additional such sRNAs remaining to be discovered and characterized. For example, a recently characterized sRNA in *V. cholerae*, VrrA, modulates colonization of the host small intestine and is involved in regulating outer membrane vesicle formation, but is not dependent upon Hfq for these activities [37]. In *E. coli*, the *cis*-acting sRNA, SymR, represses the translation of *symE*, encoding a toxic protein, in an Hfq-independent manner [38]. Additionally, using purified components, it was recently demonstrated that SgrS does not require Hfq for translational silencing of *ptsG*, though it does enhance the rate of duplex formation, which, in this case, still supports the role of Hfq as a chaperone [39]. Finally, multiple searches in both *Streptomyces* [22,40,41] and *Mycobacterium tuberculosis* [42] have identified numerous candidate sRNAs. However, an Hfq homolog has yet to be identified in these bacteria [40]. While it remains possible that these bacteria employ a novel RNA chaperone that is functionally

equivalent to Hfq, it is also possible that these newly identified sRNAs do not require a protein chaperone for activity.

A multitude of cis-encoded sRNAs

The emphasis on sRNAs being expressed in *trans* to their targets needs to be reevaluated, for there is growing evidence that bacterial transcriptomes contain numerous chromosomally-encoded *cis*-acting sRNAs [2,42-44]. Though this class of sRNA is not yet fully characterized, it appears that most *cis*-encoded sRNAs are transcribed antisense to their target RNA. As a result, the sRNA shares perfect complementarity over at least a portion of its length with its target, resulting in highly specific, high affinity binding (Figure 1 D,E). Following are several recently described examples of chromosomally *cis*-encoded sRNAs.

Several *cis*-acting sRNAs in *E. coli* (SibA-E, SymR) have been characterized as antitoxins belonging to type I toxin-antitoxin pairs [38,45]. These sRNAs are transcribed antisense to, and repress the expression of, ORFs encoding toxic proteins. This mode of regulation is similar to the well-characterized plasmid sRNAs that are involved in maintenance and replication [46,47]. For example, SymR is transcribed in *cis* to the 5′ end of *symE*, which encodes an RNA-associating protein with toxin-like properties (Figure 1D) [38].

There exist additional classes of *cis*-encoded sRNAs besides antitoxins. In *E. coli*, the GadY sRNA base-pairs with the 3′ UTR of *gadX* mRNA, which encodes a transcriptional activator of the acid response system. The pairing of the two RNAs significantly increases the stability of the message and ultimately results in increased expression of downstream acid resistance genes (Figure 1E) [48]. Working with *V. cholerae*, our lab recently identified MtlS, an sRNA transcribed in *cis* to, and in antisense orientation relative to the 5′ UTR of *mtlA*, encoding the mannitol-specific transporter of the PTS [21]. MtlS and *mtlA* mRNA share 70 nt of perfect complementarity, and their expression, which is dependent upon carbon source, is inversely correlated. MtlS is also able to repress *mtlA* expression when expressed in *trans*. This suggests that MtlS represses the translation and/or the stability of *mtlA* mRNA by direct base-pairing (Figure 1D), as opposed to *cis* affects on *mtlA* transcription, such as changes in DNA supercoiling caused by transcription of *mtlS*.

A recent computational screen for sRNAs encoded within the pathogenicity islands of *S.* Typhimurium identified 19 candidate sRNA genes, 11 of which are complementary to a flanking gene [49]. Subsequent work examining the expression patterns of these sRNAs suggested that they play a role during invasion and virulence. Interestingly, three of the sRNAs (IsrC, IsrH-1 IsrN) that overlap with the 3′ end of a flanking gene each appear to affect the *cis*-encoded gene in a distinct manner (Figure 1D,E). The expression pattern of IsrC sRNA and *msgA* mRNA is the same, and the two transcripts undergo mutual degradation when expressed in *cis* from a plasmid [49]. In contrast, IsrH-1 sRNA appears to inhibit the expression of the *cis*-encoded gene, *glpC*, and the two transcripts have inverse expression patterns [49]. Lastly, overexpression of sRNA IsrN from a plasmid results in accumulation of the *cis*-encoded STM2765 mRNA. Thus, not only are *cis*-acting sRNAs common genetic elements in *S.* Typhimurium, but they appear to function through several different mechanisms. These and other studies not highlighted here show that *cis*-acting sRNAs are encoded in the genomes of numerous bacteria. Furthermore, these regulatory RNAs should not be considered exceptions but, instead, consistent with a common property of sRNAs as regulators of gene expression.

Experimental identification of sRNAs

One of the dangers of limiting the definition of bacterial sRNAs is that experimental approaches designed to identify new sRNAs may provide incomplete catalogues. For example, the search for new sRNAs continues to rely heavily on computer programs that often search exclusively

within intergenic regions (IGRs), and that define sRNAs based on a combination of sequence conservation, specific promoters and Rho-independent terminators [22,40,43,49]. Although many sRNAs have been successfully identified in this manner, sRNAs that are transcribed within or antisense to ORFs [45,49] will be missed. Moreover, sRNAs whose 3′ ends are formed by Rho-dependent termination and/or processing by RNases or ribozymes will be missed by computational methods that rely on the presence of Rho-independent terminators. Likewise, experimental sRNA searches that use Hfq-binding as a prerequisite will miss Hfq-independent sRNAs. Thus, there is a need to supplement these approaches with additional, less biased approaches for the discovery of bacteria sRNAs. One such approach, mentioned above, used transcriptional profiling with whole genome tiling microarrays to reveal many new candidate sRNAs in *L. monocytogenes*, including ones that are antisense to ORFs [24]. Several of these candidate sRNAs exhibit the same expression patterns as virulence genes, suggesting their involvement in regulating pathogenic processes. Below, we review two additional experimental approaches for discovering sRNAs that do not depend on the classical definition of sRNAs.

Identification of candidate sRNAs by massively parallel sequencing

The use of massively parallel sequencing as a tool for sRNA discovery has the advantages of precisely mapping the 5′ and 3′ ends of the sRNA transcripts and any sRNA identified is automatically known to be transcribed under the condition used. Our lab recently developed “sRNA-Seq” to identify the total population of sRNAs in any bacterial transcriptome with no assumptions *a priori* with respect to the nature of the sRNAs (e.g., genome location, promoter, terminator, Hfq-binding) [21]. Our method differs from previously reported direct cloning protocols [2,3] in that during the cloning, tRNAs and 5S rRNA are depleted from the sRNA population by using RNase H and a mixture of oligonucleotides complementary to the tRNAs and 5S rRNA. Applying this method to *V. cholerae*, we identified many hundreds of candidate sRNAs plus all 20 previously known sRNAs [21]. This population was enriched for candidate sRNAs encoded within IGRs, but we also observed many that were antisense to, or sense to annotated ORFs. The vast majority of these candidate sRNAs are of unknown function. Thus, a great challenge for the future will be deciphering the biological roles of candidate sRNAs identified by this and other methods.

Targeted identification of sRNAs

A recently reported genetic approach for sRNA discovery begins with the mRNA target, and then searches for sRNAs that regulate it [31]. DNA encoding the target transcript including its 5′ UTR is cloned under the control of an inducible promoter as a translational fusion to *lacZ*, thereby decoupling transcription from post-transcriptional control. The researchers applied this method to the *E. coli dpiBA* operon transcript encoding a two-component system involved in the SOS response to β-lactam antibiotics. The *dpiBA-lacZ* construct was introduced into the *E. coli* chromosome and a screen with a multicopy plasmid library successfully identified one *trans*-acting sRNA, RybC, as a direct negative regulator. Although RybC requires Hfq for stability, this method does not exclude Hfq-independent regulatory factors, nor is it specific to sRNAs since protein regulators of *dpiBA* may have been identified as well. This method should be broadly applicable to the search of sRNAs in any genetically tractable system.

Conclusions

The ubiquity of sRNAs in bacteria is now widely accepted. What remains is the challenge of identifying the complete repertoire of sRNAs, and the even greater challenge of deciphering the roles of these regulators within the context of bacterial regulatory circuits and perhaps other processes. We anticipate that many new signaling systems involving sRNAs will be uncovered, and even well known circuits may need to be revisited as the number of identified sRNAs

continues to increase. The recent contributions to the field suggest that as we continue to identify and characterize sRNAs, we will benefit by maintaining a broad definition of what sRNAs are and how they function. We suggest that any bacterial RNA molecule <500 nt that functions in the regulation of gene expression or protein activity be considered an sRNA, regardless of its mechanism of action or other functions. The recently developed methods to identify new sRNAs in bacterial transcriptomes should facilitate the identification of additional "non-canonical" sRNAs and reveal how common these genetic elements actually are. It is likely that we are just beginning to uncover the vast repertoire of sRNAs, and that novel functions for sRNAs remain to be discovered.

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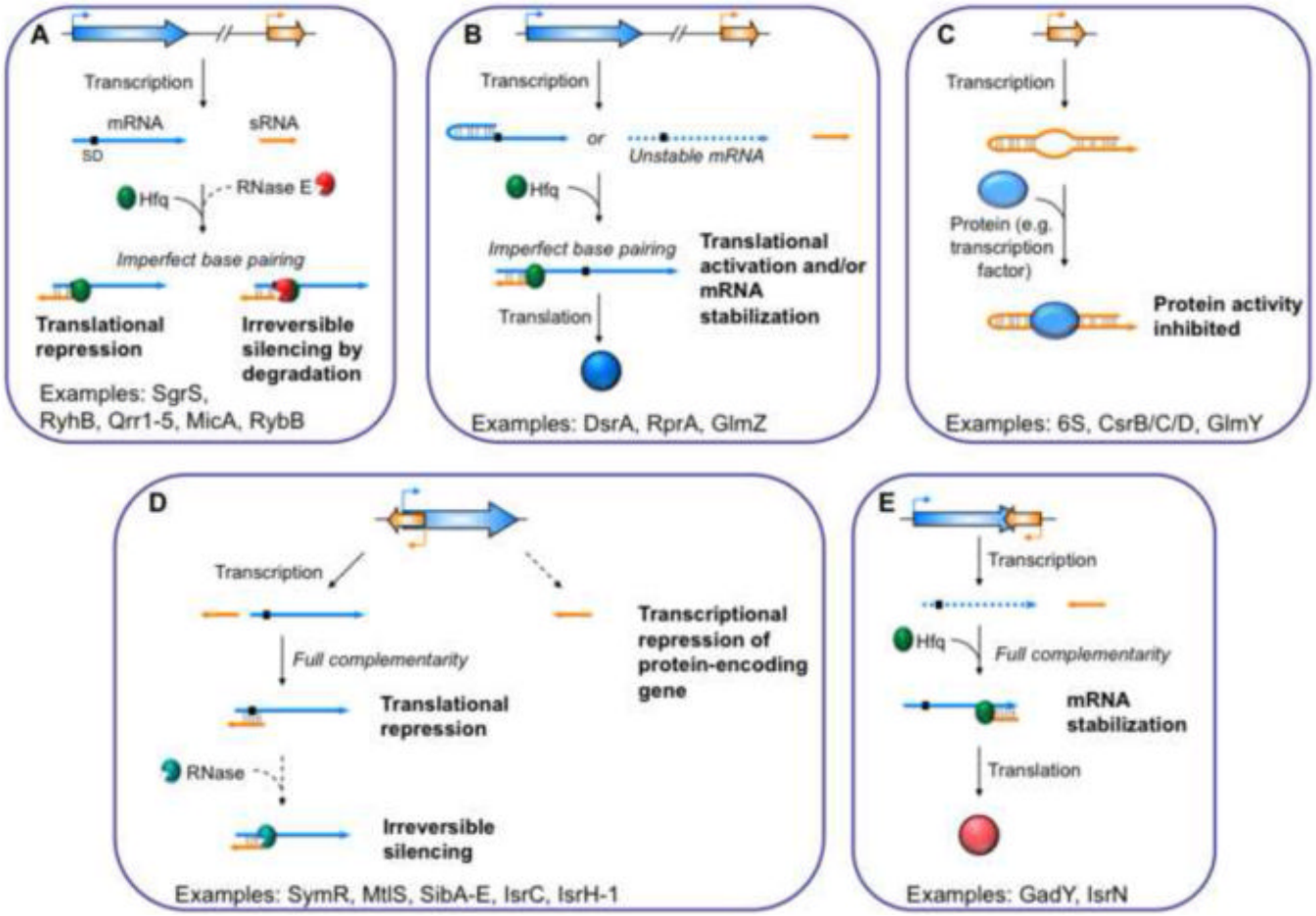

Figure 1. Different types of sRNAs based on their mechanism of action

Trans-encoded sRNAs can base-pair imperfectly with mRNA targets and either (A) repress or (B) activate translation. Alternatively, (C) some *trans*-encoded sRNAs interact with proteins, including transcription factors, and inhibit their activity. *Cis*-encoded antisense sRNAs can also either (D) activate or (E) repress protein expression. Colored arrows represent RNA transcripts; black boxes indicate Shine-Dalgarno (SD) sequences. Dashed colored arrows represent unstable transcripts. Dashed black arrows represent hypothetical mechanistic steps of sRNA-mediated regulatory pathways.