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Regulatory roles for small RNAs in bacteria

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Small RNAs can act to regulate both the synthesis of proteins, by affecting mRNA transcription, translation and stability, and the activity of specific proteins by binding to them. As a result of recent genome-wide screens, around 50 small RNAs have now been identified in *Escherichia coli*. These include many that require the RNA-binding protein Hfq for their activity; most of these RNAs act by pairing with their target mRNAs. Small RNAs can both positively and negatively regulate translation, can simultaneously regulate multiple mRNA targets, and can change the pattern of polarity within an operon.

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Abbreviations

CRP	cAMP receptor protein
Csr	carbon storage regulator
Hfq	host factor for Qβ replicase
ORF	open reading frame

Introduction

Recent work has highlighted a dizzying array of unexpected functions by regulatory small RNAs in bacteria and eukaryotes. Traditionally, such small RNAs were antisense RNAs that regulated only one mRNA target, which was encoded on the opposite strand and therefore was fully complementary. These *cis*-encoded antisense RNAs are most common in plasmid replication control systems and in bacteriophage immunity systems (reviewed in [1,2]). For instance, RNAI controls ColE1 plasmid copy number by binding to the RNA primer of replication and preventing the formation of the RNA–DNA hybrid required for replication initiation. In plasmid R1, the small antisense RNA CopA binds CopT, the mRNA encoding the replication protein, inhibiting its translation. In Gram-positive bacteria, copy number control of plasmids pT181 and pIP501 by counter-transcribed RNA (ctRNA) and RNAIII, respectively, occurs via a transcription-attenuation system; the RNA causes transcription termination, rather than interfering with the translation of the replication protein. *Cis*-encoded antisense RNAs can also act as antitoxins, as in the Sok RNA of plasmid R1 that affects the translation of the open reading frame (ORF) situated just upstream of the Hok toxin protein, ultimately interfering with the translation of Hok itself (reviewed in [3]).

The recent increased recognition of important regulatory roles for small RNAs encoded far from their targets (*trans*-acting), acting on multiple targets, or both, has expanded interest in how to find such regulatory RNAs and how they work. In this review, we focus on the recent progress that has been made in finding and understanding this class of chromosomally-encoded small regulatory RNAs.

Finding small RNAs

The first small RNAs, including 6S and Spot 42, were discovered 30 years ago. By 2000, 13 small RNAs had been detected in *E. coli*. Studies on some of these have demonstrated their involvement in cellular functions varying from regulating mRNA stability and translation to modulating RNA polymerase activity to tagging proteins for degradation (reviewed in [4–7]). These studies prompted four groups to investigate how many other small RNAs are present in the chromosome of *E. coli*. The searches involved the use of conservation, orphan promoters and secondary structure elements to identify likely candidates within intergenic regions. Many of these were confirmed with northern blots. Over 35 confirmed new small RNAs were found, bringing the total number to close to 50 [8••–10••,11•]. This suggests that bacteria such as *E. coli* might have in the range of 100 small RNAs, but not thousands. Whereas most of these newly found small RNAs (and some of the older ones) remain of unknown function, accumulating data suggests that bacteria use these small RNAs to fine-tune their physiology and adapt to rapidly changing environments. Therefore, understanding small RNA function has opened up a new area of research, because these small molecules create a new network that brings an additional level of complexity to cell physiology.

Regulation by small RNAs: an explanation for regulatory mysteries

The small RNA RyhB (also called SraI) was discovered in two of the genome-wide searches [8••,9••]. The promoter region and the 90 nt sequence of *ryhB* are very well conserved in *E. coli*, *Salmonella* and *Klebsiella pneumoniae*; a core sequence of 27 nt within RyhB is also well conserved in *Yersinia pestis* and *Vibrio cholerae* and in a second, partially homologous and similarly regulated small RNA

found in *Salmonella* and *Yersinia* [12••]. The promoter of *ryhB* is repressed by the Fur repressor and by iron (Fe). In the event of Fe limitation or in a *fur* mutant, *ryhB* is strongly expressed. RyhB causes a dramatic decrease in the level of several mRNAs. The targeted genes encode for Fe-containing proteins, or are involved in the intracellular storage of Fe. Among these are three enzymes involved in the tricarboxylic acid (TCA) cycle, aconitase A (encoded by *acnA*), fumarase A (encoded by *fumA*) and succinate dehydrogenase (*sdhCDAB*), two Fe storage proteins, bacterioferritin (*bfr*) and ferritin (*ftnA*) and the enzyme Fe-superoxide dismutase (*sodB*) [12••].

The work on RyhB provided an explanation for the previously observed inability of a *fur* mutant to grow on minimal succinate media [13]. Overexpression of RyhB results in the destruction of *sdhCDAB* mRNA, blocking growth on succinate. Also previously unexplained was the positive regulation of *acnA*, *fumA*, *bfr*, *ftnA* and *sodB* genes by the Fur repressor (reviewed in [14,15]). Because Fur negatively regulates *ryhB*, and RyhB negatively regulates these genes, the resulting Fur regulation is positive and dependent upon RyhB [12••].

Another mysterious regulatory phenomenon was recently found to be caused by another small RNA, the 109 nt Spot 42 RNA. Spot 42 was found in 1973 and identified as a stable RNA, present at about 200 copies per cell; the gene (*spf*) is negatively regulated by the cAMP–CRP (cAMP receptor protein) complex [16,17]. Møller *et al.* [18••] recently reported that Spot 42 RNA is responsible for the discoordinate gene expression in the galactose operon. It was known that the ratio of expression of the third gene of the operon, *galK*, to the first gene, *galE*, is negatively regulated by cAMP and CRP, which is believed to reflect a requirement for GalE but not GalK under specific conditions. Previously, the mechanism for this change in ratio was unclear. Spot 42 has now been shown to inhibit translation of *galK* by base-pairing near its ribosome-binding site [18••]. Since Spot 42 production is itself regulated negatively by cAMP–CRP, this leads to cAMP-regulated discoordinate regulation. The discoordinate expression is lost in an *spf* mutant. This is the first example of a small RNA acting within an operon, suggesting a level of regulated polarity not previously appreciated. At least one other operon might be regulated similarly by Spot 42. *sucC*, part of the *sucABCD* operon encoding enzymes involved in the TCA cycle, has complementarity with Spot 42. When the small RNA is overproduced, the strain cannot grow on minimal succinate media [18••].

Regulation by small RNAs: multiple RNAs regulate RpoS

The regulation of the stress/stationary phase sigma factor RpoS (σ^S) is somewhat unique in *E. coli*. Three small RNAs, OxyS, DsrA and RprA, regulate its translation, and DsrA and RprA RNAs act as positive regulators. The only other documented example of positive regulation by a small RNA translational regulator is found in *Staphylococcus aureus*, where RNAIII positively regulates translation of α toxin while negatively regulating other genes [19]. Translational initiation of *rpoS* is repressed by a secondary structure in the upstream message, occluding the Shine-Dalgarno (S-D) site in a hairpin structure; a similar inhibitory structure is found in the 5′UTR (untranslated region) of the α toxin of *S. aureus*. Both DsrA and RprA RNA have complementary sequences that allow them to pair with the upstream leader region of *rpoS* and relieve the inhibition by freeing the S-D site [20,21•]. In contrast, OxyS RNA represses *rpoS* translation, possibly by modulating the activity of host factor for Qβ replicase (Hfq), an RNA-binding protein that is required for this process (see below and [22]). So why would three RNAs be needed to regulate the expression of one target gene? Indications are that the small RNAs are synthesized in response to different environmental signals. DsrA is strongly synthesized at low temperature and its stability also varies with temperature [23,24•]. RprA is regulated by the capsule phospho-relay pathway RcsC→YojN→RcsB and responds to undetermined signals, but not to temperature [21•]. OxyS is only induced when cells are subjected to an oxidative shock [25]. In addition, DsrA is an antisense repressor of the global regulator protein H-NS [23,26], whereas OxyS acts as an antisense translational repressor of *fhlA* (a transcriptional regulator) and as a general anti-mutator [25,27,28]. Taken together, these data suggest that the small RNAs act as signal transducers of environmental conditions and coordinate gene expression in response to a particular condition.

Most interestingly, RyhB, Spot 42, DsrA, RprA and OxyS all require the abundant RNA-binding protein Hfq for activity [12••,22,29,30,31•]. Because of its critical role, this protein, isolated in the late 1960s, has enjoyed a renaissance, becoming the focus of intense research in the past couple of years.

Role of Hfq in small RNA function

Hfq, first discovered as a host factor for RNA phage Qβ replicase, has emerged as a common requirement for the large family of small regulatory RNAs that act by complementary pairing to their targets. Hfq is conserved, abundant [32,33], and binds strongly to single-stranded RNA that is rich in As and Us [31•,34•]. Hfq mutants are viable, but grow poorly and have a range of defects [35]. Both sequence and structure comparisons demonstrate the similarity between Hfq and the eukaryotic Sm proteins [31•,34•,36••] — components of the spliceosomal complex, also involved in other RNA metabolic steps in eukaryotes (reviewed in [37]). Hfq binds strongly to many small RNAs [8••] and is required for the activity of many of them. Part of this requirement might be a result of the

stabilization of at least some of these small RNAs by Hfq (Spot 42 [31•]; DsrA [29], RyhB (Massé *et al.*, unpublished data)). However, it also stimulates pairing between small RNAs and their complementary targets *in vitro* [31•,34•], suggesting that it acts as an RNA chaperone.

Besides interacting with small RNAs, Hfq has also been implicated in some other RNA interactions. It has been found to affect the degradation of several mRNAs by binding to their polyA tail, but can also stimulate polyA adenylation, which itself affects message stability [38]. Translation of the message of a major outer membrane porin, *ompA*, is inhibited by Hfq, by competing with ribosome binding and therefore preventing ribosome loading [39]. Stability of the *hfq* message itself, as well as that of the *ompA* message, is perturbed in an *hfq* mutant [40,41]. So far, no small RNA has been implicated in these processes (modulating polyA tailing or *ompA* and *hfq* message stability).

These roles for Hfq might involve the recognition of some loosely defined RNA binding sites (AU-rich and unstructured), followed by changes in RNA structure that change susceptibility to nucleases and propensity to interact with itself or other RNAs. It is not yet clear whether any other RNA-binding proteins play as ubiquitous a role in small RNA action in bacteria; certainly, none are significantly redundant with Hfq, given the profound effect of an *hfq* mutant on the activity of many small RNAs.

Other modes of action for small RNAs

Whereas the small RNAs discussed above apparently work by pairing with target mRNAs, other small RNA regulators act by binding a specific target protein, inhibiting or changing its activity. 6S RNA binds RNA polymerase, changing its promoter preference [42]. The global regulatory system Csr (carbon storage regulator) is involved in the modulation of several stationary-phase genes. CsrA is a small translational regulatory protein of 61 amino acids which is involved in positive regulation of flagella synthesis, acetate metabolism and glycolysis, in negative regulation of biofilm formation, glycogen biosynthesis and catabolism, and in gluconeogenesis in *E. coli* [43–46]. CsrA acts by binding to sequences close to the ribosome binding site of the *glgC* (glycogen metabolism gene) transcript and inhibits the initiation of translation [47•]. The 366 nt CsrB RNA carries 18 repeats of the CsrA binding site, and so acts as an antagonist for CsrA action by sequestering it. Thus, the amount of CsrB determines the availability of CsrA [43]. Interestingly, the transcription of CsrB is indirectly activated by CsrA itself, via the response regulator UvrY, suggesting a homeostatic regulation [48,49]. Further adding to the complexity of the system is the recent discovery of a second small RNA antagonist of CsrA, CsrC [50]. The CsrA/CsrB paradigm has been found in a wide variety of bacteria, and is involved in regulation of invasion and virulence in plants and animals [51–53].

Yet another role for small RNAs that is not primarily regulatory is exemplified by tmRNA, so named for its shared tRNA and mRNA functions (reviewed in [54]). While tmRNA acts primarily as part of the quality-control apparatus for protein translation, releasing stalled ribosomes and causing degradation of incompletely translated proteins, on occasion, it may also play a regulatory role [55].

Conclusions and future considerations

In this review, we have emphasized some of the recent findings in the rapidly expanding world of small regulatory RNAs. These RNAs are poised to sense the environment via their synthesis or stability, and transduce that signal to regulate gene expression. More importantly, they allow the coordination of a response by having more than one target, or targeting global regulators of gene expression.

Although small RNAs were frequently overlooked by traditional approaches, probably in part because they are small targets for mutagenesis, and because methods are often focused on protein regulators, the recent genome-wide searches have made it clear that they are present in significant numbers and carry out critical regulatory steps. These searches have focused mostly on conserved, stand-alone genes in inter-ORF regions and will therefore have missed un-conserved small RNAs, ones encoded within ORFs, or processed from the mRNA of an ORF. Nonetheless, the approaches developed for *E. coli*, and the demonstration that many of these small RNAs bind Hfq, should all be applicable to finding small RNAs in other microbes.

Even with those already found, mechanisms and targets of action are not well defined for most of these. Understanding when they are made will provide the strongest clue to when they might be important. Targets cannot yet be defined with confidence simply by looking for complementarity, so methods to improve this will be important. Even when regulation and target are defined, how similar will the outcome be for different regulatory RNAs? Do they act positively or negatively, or both? Are they Hfq-dependent, implying action by RNA–RNA pairing? If not, is another RNA chaperone necessary for their action, or do they act by modulating the activity of a protein? Do other factors regulate the maturation, activity and stability of the regulatory RNA? For instance, the efficiency of regulation of ColE1-type plasmid replication by the antisense RNAI is decreased by polyadenylation of RNAI [56]. The polyA tail both interferes with the RNA–RNA interaction and affects RNA decay. Any modifications or interactions affecting either the activity or the half-life of the regulatory RNA will be important to understanding its regulatory function.

The recent increase in activity in this field promises to continue to accelerate in the coming years, uncovering a level of regulation by small RNAs that might eventually rival the classic protein regulators, adding greatly to our understanding of the multiple levels of control cells use and how they interact.

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