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Current Opinion in Microbiology

Activation of gene expression by small RNA

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Small regulatory RNAs (sRNAs) commonly act to downregulate gene expression. In bacteria, however, sRNAs have also been shown to activate genes by a variety of direct or indirect mechanisms. Several sRNAs (DsrA, GlmZ, RNAIII, RprA, RyhB, and Qrr) act as direct translational activators by an 'anti-antisense mechanism' in the 5′ mRNA region to liberate a sequestered ribosome binding site, while pairing of GadY sRNA to the 3′-end alters processing and increases stability of its target mRNA. Indirect activation includes cases of RNA mimicry in which degradation of the activating GlmZ sRNA is suppressed by the highly similar GlmY sRNA, or where a pseudo-target mRNA traps MicM sRNA to derepress porin synthesis.

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Current Opinion in Microbiology 2009, **12**:674–682

This review comes from a themed issue on
Growth and development: prokaryotes
Edited by Patrick Viollier

Available online 31st October 2009

1369-5274/$ – see front matter

DOI 10.1016/j.mib.2009.09.009

Introduction

The two most prominent classes of regulatory small RNAs to date, the ~22 nt microRNAs of eukaryotes [1] and the 50–400 nt sRNAs of bacteria [2], both act to modulate gene expression at the post-transcriptional level. The microRNAs in general, and most of the bacterial sRNAs, exert their regulatory function by short base-pairing to target mRNAs. The interactions of microRNAs with the 3′-untranslated region (UTR) or coding sequence (CDS) of targets almost exclusively results in the repression of the encoded gene, by a variety of mechanisms including translational control, induced mRNA cleavage and deadenylation [3]. Few cases of microRNA-mediated activation have been reported [1].

Likewise, the common base-pairing of bacterial sRNAs to the 5′ mRNA region most often represses the target [2]. Typically, sRNA pairing masks the ribosome binding site (RBS) of the target, thus inhibiting 30S ribosomal subunit association and translational initiation. As a consequence, the untranslated target mRNA is destabilized, usually by the action of RNase E [4,5] or RNase III [6,7], although cases of translational repression without mRNA stability changes were also reported (e.g. [8]). Downregulation by sRNAs is not limited to RBS targeting: several sRNAs inhibit translation in the upstream 5′-UTR [9–11], or promote target decay without translational repression in the CDS [12] or intergenic space of polycistronic mRNA [13].

At least in Gram-negative bacteria, a considerable number of base-pairing sRNAs require the RNA chaperone Hfq [2] for both intracellular stability and productive target pairing [14]. In fact, most of the sRNAs described below are Hfq-dependent. Deletion of the *hfq* gene causes diverse phenotypes impairing both general physiology and virulence of a wide range of bacteria, and deregulates up to one-fifth of all genes in enteric bacteria such as *Escherichia coli* and *Salmonella* [15,16]. Not only upregulation of distinct genes (indicating loss of repression) but also downregulation is observed in *hfq* deletion mutants; as far as post-transcriptional regulation is concerned, such downregulation may in part reflect a loss-of-function of activating sRNAs.

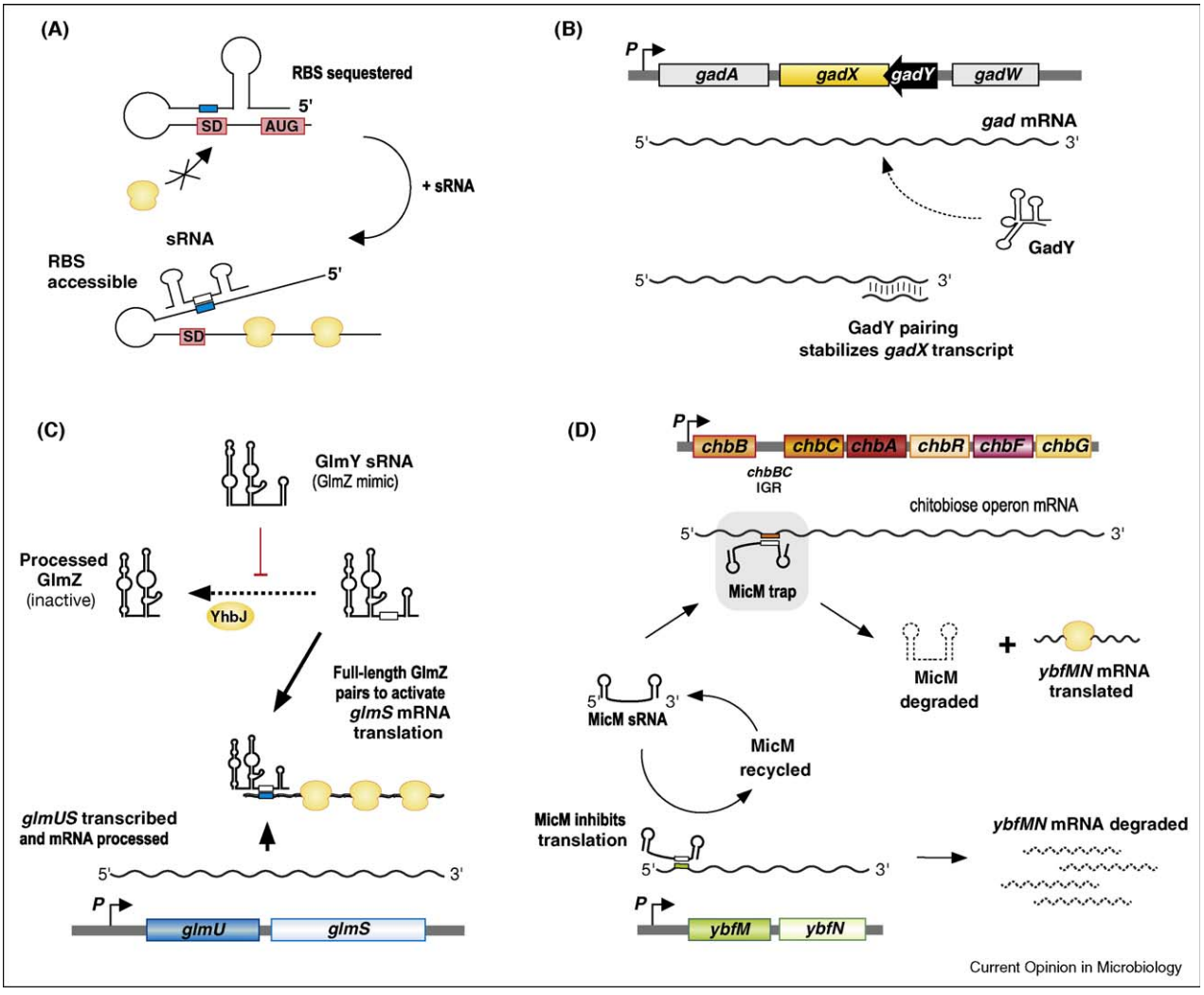
The present review covers the two mechanisms known to date by which direct base-pairing of sRNAs activates a target mRNA, that is the 'anti-antisense mechanism' and the 3′-processing-mediated transcript stabilization (Figure 1A,B). In addition to direct activation, we will describe the cases of the GlmYZ and MicM sRNAs in which RNA mimicry leads to target activation through the backdoor (Figure 1C,D).

Bacterial messengers are not the only targets, and a number of sRNAs are known to bind cellular proteins to modulate their activity. There is currently no well-established molecular mechanism for direct stimulation of protein activity by sRNA. However, both the antagonistic binding of the CsrB-like sRNAs to CsrA/RsmA proteins, and the association of 6S RNA with RNA polymerase (RNAP), can eventually lead to increased gene expression. These regulations and their physiological consequences have been recently reviewed [17–19].

Direct translational activation of mRNA: the anti-antisense mechanism

Translation is initiated by sequence-specific anchoring of 30S ribosomes at the RBS as defined by the Shine–Dalgarno (SD) sequence and the AUG start codon. If any part of the extended RBS region from approximately −35 relative to the translational start down to the fifth codon is sequestered by a stable RNA structure, the rate-limiting

Figure 1

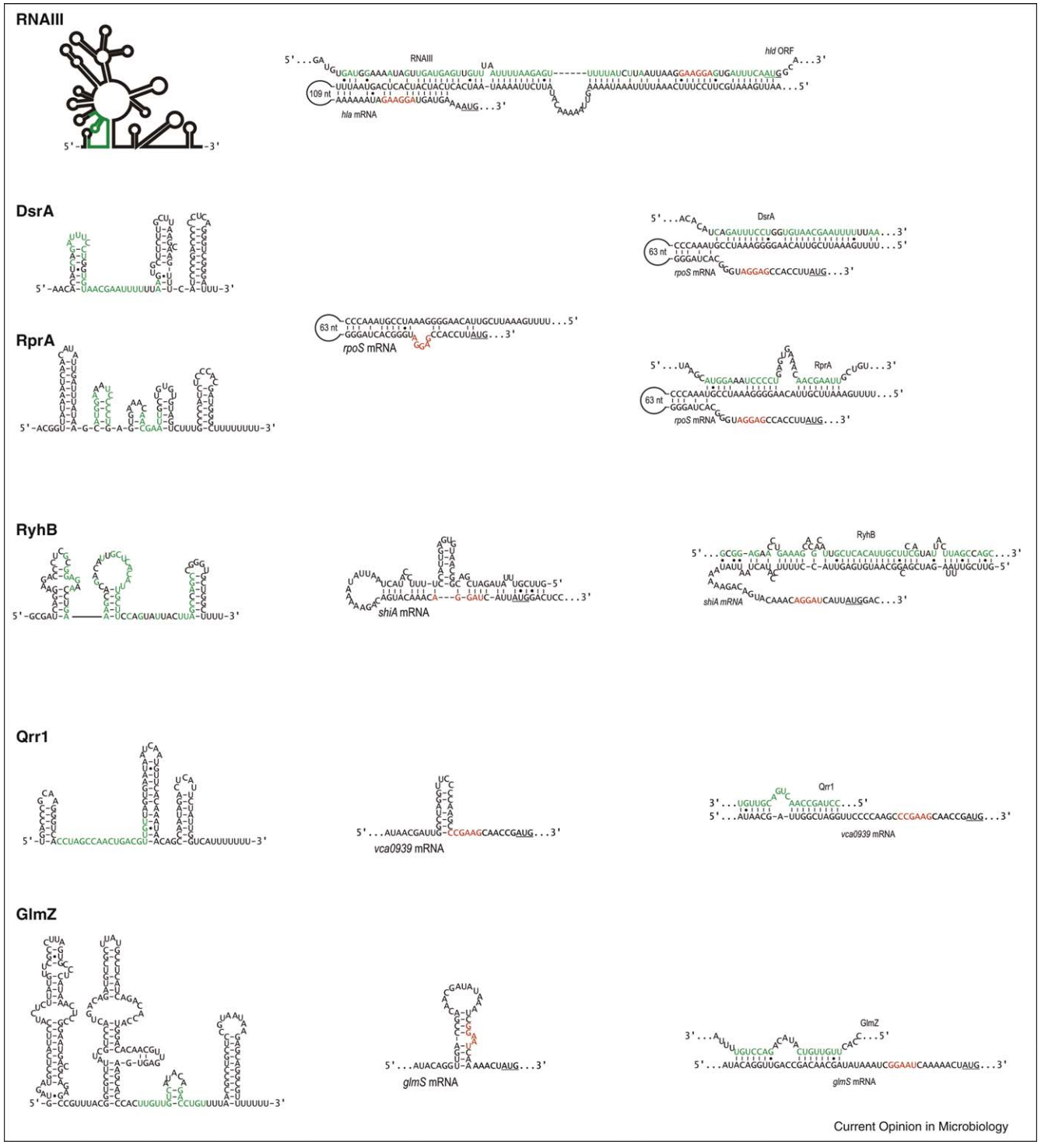


Mechanisms of target gene activation by sRNAs. **(A)** Translation efficiency of a transcript can be markedly reduced due to the formation of a secondary structure within the 5′-UTR covering the RBS. Competitive binding of a *trans*-encoded sRNA can induce structural rearrangement to unmask the RBS and thus increase target translation. **(B)** GadY sRNA encoded on the opposite strand in the *gadX–gadW* IGR interacts with the 3′-UTR of *gadX* to stabilize this transcript. **(C)** While being highly similar regarding both sequence and structure, GlmZ but not GlmY contains a sequence stretch to facilitate *glmS* translation by direct base-pairing via the anti-antisense mechanism. However, by competing for factors promoting GlmZ inactivation, GlmY sRNA contributes to the maintenance of the activating effect of GlmZ RNA on *glmS* mRNA translation. **(D)** In the absence of chitosugars, the constitutively expressed *ybfMN* mRNA is bound by the highly abundant MicM sRNA. While the interaction results in transcript decay, MicM is recycled and remains functional. The inhibitory effect of MicM on *ybfMN* is abrogated by the expression of the chitobiose operon in the presence of inducing chitosugars. An intergenic region of the *chbBCAFRG* messenger shares substantial complementarity with MicM and functions as a molecular trap for the sRNA, leading to the degradation of MicM and consequent expression of *ybfMN*.

initiation step is inefficient, resulting in low protein synthesis [20]. Hairpins that sequester the RBS are not uncommon in bacterial mRNAs, and often underlie *cis*-encoded regulation, for example in RNA thermometers and riboswitches in which an occluded RBS is conditionally liberated by temperature-dependent melting or ligand-induced structural rearrangement, respectively [21].

The first sRNA discovered to activate gene expression, the ~514 nt RNAIII of the Gram-positive pathogen *Staphylococcus aureus* [22], disrupts an inhibitory RBS hairpin of the *trans*-encoded *hla* mRNA producing α-toxin [23]. Computational searches and *in vitro* structure probing suggested that an anti-SD sequence located ~140 nt upstream in the 5′-UTR folds back to pair with the SD of *hla* mRNA (Figure 2). By binding to the anti-SD and competing with the formation of the hairpin structure, the 5′-end of RNAIII releases the SD and promotes *hla* translation [23]. Absence of RNAIII causes a dramatic ~70-fold drop in α-toxin synthesis,

Figure 2



Secondary structures of sRNAs and their respective target mRNAs activated by the anti-antisense mechanism. Discussed sRNA/mRNA pairs comprise RNAIII/*hla* of *Staphylococcus aureus*; DsrA/*rpoS* and RprA/*rpoS* of *E. coli*; $DsrA_{Bb}$/*rpoS* of *B. burgdorferi* (not shown); Qrr/*vca0939* from *V. cholerae* (Qrr1 sRNA is shown; the interaction site for *vca0939* is conserved among Qrr1–4); RyhB/*shiA* of *E. coli*; GlmZ/*glmS* from *E. coli*. See text for references. SD sequences are marked in red. Interaction sites in the sRNA are marked in green.

although part of this reduction might be accounted for by the simultaneous loss of the independent stimulatory effect of RNAIII on *hla* transcription [22,23].

RNAIII has many other targets, and its structured 3′-region acts as a translational repressor by base-pairing with the RBS of many virulence-associated mRNAs [6,22,24]. The inverse regulation of α-toxin and the other virulence proteins might help *S. aureus* precisely coordinate its virulence program. In addition, the 5′-region of RNAIII encodes δ-hemolysin [22]. Intriguingly, the ORF for δ-hemolysin (*hld*) starts only ∼50 nt downstream of the *hla* activating region of RNAIII, and there is base-pairing potential of the *hla* 5′-UTR with the RBS of *hld* [23]. In other words, as the RNAIII–*hla* pairing activates α-toxin production, δ-hemolysin synthesis might become repressed [23].

The discovery of the conserved ∼87 nt DsrA as a positive regulator of *rpoS* mRNA in *E. coli* at low growth temperature [25] prompted deeper molecular and physiological studies of the above-described anti-antisense mechanism. The stationary phase sigma factor, σS (encoded by *rpoS*), is a major stress regulator with a large regulon in many Gram-negative bacteria; its expression is regulated at various levels [26]. The 5′-UTR of *rpoS* folds into a ∼100 nt hairpin including the RBS, resulting in poor basal translation [27].

Direct pairing of DsrA to the self-inhibitory *rpoS* hairpin (Figure 2), freeing the RBS and increasing σS synthesis, has been supported by a wealth of data [28–32]. The molecular dissection of the DsrA–*rpoS* regulation gave unprecedented insight into how Hfq, which by itself often represses mRNA translation [33,34], is reprogrammed by sRNAs into an activating factor [32]. The hexameric Hfq protein, which is essential for *rpoS* translation [26,35], binds DsrA and *rpoS* mRNA at two distinct interaction surfaces [36], but by itself does not alter the structure of either RNA partner [37,38]. This suggests that Hfq promotes the melting of the *rpoS* structure as an RNA chaperone, increasing the concentration of DsrA at the site of action, and also facilitating the transition from the inhibitory *rpoS* structure to the activated form in which DsrA is paired to the anti-SD of *rpoS* [31,39]. The major double-strand-specific nuclease, RNase III, was proposed to have a dual role in this regulation [28]: RNase III processes *rpoS* mRNA in the absence of DsrA, and also cleaves the DsrA–*rpoS* duplex, presumably to prevent the reuse of DsrA. As such, the RNase III-mediated removal of the activator sRNA resembles the coupled degradation of sRNA and target by RNase E or III in cases of mRNA repression [5,7,40], which is generally thought to render translational repression robust and irreversible.

The general importance of sRNA/Hfq-mediated *rpoS* control was echoed by several findings. First, work in the spirochete *Borrelia burgdorferi* identified $DsrA_{Bb}$ sRNA as an activator of *rpoS* translation following an increase in temperature [41], an important virulence signal in this causative agent of Lyme disease. $DsrA_{Bb}$ is much longer (∼300 nt) than, and shares no sequence homology with, *E. coli* DsrA, but uses the same anti-antisense mechanism to act as a molecular thermometer of σS expression in *Borrelia* [41]. Intriguingly, $DsrA_{Bb}$ activity on *rpoS* mRNA requires a *Borrelia* protein of otherwise unknown function, which shows some similarity to *E. coli* Hfq and can substitute Hfq function when expressed in an *hfq* mutant of *E. coli* (MC Lybecker, DS Samuels, personal communication). Second, a multi-copy plasmid screen for additional *rpoS* regulators in *E. coli* uncovered the conserved 105 nt RprA RNA as a suppressor of a *dsrA* mutant [42]. RprA interacts with roughly the same region in the *rpoS* message as DsrA to activate translation (Figure 2); unlike DsrA, which is induced upon cold-shock, RprA is strongly upregulated upon cell surface stress by the RcsC/YojN/RcsB phosphorelay system [43]. Third, several more Hfq-dependent sRNAs are known to upregulate an *rpoS–lacZ* reporter fusion in *E. coli*, indicating the existence of a larger network of sRNAs that reinforce σS synthesis under a variety of stress conditions [44].

Additional sRNAs translationally activate unrelated mRNAs by anti-antisense pairing. The iron-starvation-induced RyhB sRNA of *E. coli* (Figure 2), which is foremost known as a negative regulator of genes encoding iron-containing proteins, promotes translation of the *shiA* mRNA [45,46]. Regarding its physiological relevance, the increased production of ShiA (shikimate permease) might be important for siderophore synthesis under iron-starvation conditions [45]. The Qrr sRNAs of *Vibrio* species, which are best known as mRNA repressors that time the synthesis of the quorum-sensing master regulators, LuxO/HapR, activate the *vca0939* mRNA in the classical *Vibrio cholerae* El Tor strain (Figure 2) [47]. Intriguingly, the translational activation of VCA0939, a GGDEF protein involved in cyclic-di-GMP synthesis, induces virulence factors and biofilm formation by a HapR-independent pathway [47].

Aside from acting on 5′-UTRs of monocistronic transcripts, sRNAs can bind within polycistronic messengers to selectively inhibit protein synthesis from downstream cistrons as Spot42 does in the *gal* operon mRNA [8]. Reciprocally, the conserved GlmZ sRNA acts on the downstream cistron of the *glmUS* operon mRNA to increase GlmS synthesis, presumably at the level of a processed monocistronic *glmS* mRNA whose SD is normally sequestered within a hairpin (Figure 1C) [34,48]. Hfq-dependent base-pairing of GlmZ by a ∼15 nt single-stranded stretch releases the sequestered *glmS* SD (Figure 2) [34,48] to increase translation, which also strongly stabilizes the *glmS* mRNA [34]. Note that the synthesis of GlmU and GlmS, two key enzymes in amino sugar metabolism, is strictly coupled at the transcriptional

Table 1

Characteristics of sRNA/mRNA interactions activating translation

sRNA	Target mRNA	Organism	UTR length in nucleotides	sRNA binding site relative to AUG	Length of inhibitory structure	Length of sRNA/mRNA interaction
RNAIII	*hla*	*S. aureus*	328	−132	15	54
DsrA	*rpoS*	*E. coli*	567	−94	14	24
$DsrA^{Bb}$	*rpoS*	*B. burgdorferi*	50–170	−51	?	31–68
RprA	*rpoS*	*E. coli*	567	−93	14	19
RyhB	*shiA*	*E. coli*	77	−27	26	37
Qrr1–4	*vca0939*	*V. cholerae*	?	−23	6	15
GlmZ	*glmS*	*E. coli*	161 (IGR between *glmU* and *glmS*)	−22	8	15

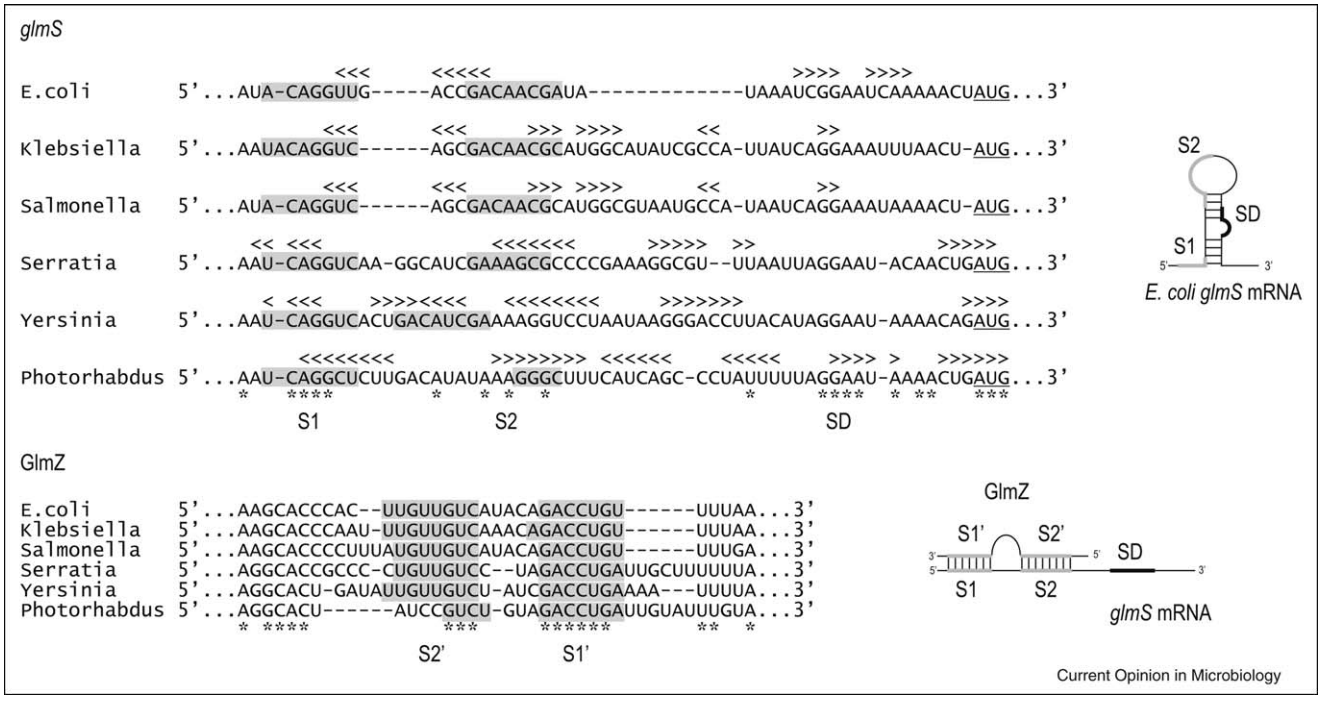
level. GlmU is always required since it turns over both externally and internally derived substrates, whereas GlmS is only essential for the alternative synthesis of glucosamine-6-phosphate (GlcN-6-P) from fructose and glutamine in the absence of external amino sugars. Thus, GlmZ (and its seeming homologue, GlmY; see below) facilitates a metabolic switch by specifically activating *glmS* at the post-transcriptional level.

Comparison of translational activation and repression

The known activating and inhibitory sRNAs act by similarly short and imperfect RNA interactions; the Qrr sRNAs even use the very same region to activate and repress mRNAs [49]. Nonetheless, the known activation sites are ≥22 nt upstream of the target AUG (Table 1), whereas repression commonly takes place proximal (<20 nt) to AUG [2,50] and also in the CDS [12,20,51]. However, since sRNAs can repress translation 100 nt upstream of AUG [9], the sRNA binding site position by itself cannot safely predict the regulatory outcome. Thus, the intrinsic ability of an mRNA to form an inhibitory hairpin on its own, and the ability of an sRNA to outcompete such structure, are currently the only common denominators for translational activation.

Enterobacterial sRNAs that act as inhibitors often carry deeply conserved target binding sites, for example GcvB

Figure 3



Sequence conservation of *glmS* mRNA and GlmZ sRNA binding sites. Sequence alignments reveal the conservation of an inhibitory stem-loop structure within *glmS* mRNA among different enterobacteria. Activation of *glmS* translation by GlmZ sRNA via the anti-antisense mechanism depends on the interaction at two sites, S1 and S2 with S1′ and S2′, respectively. The S1–S1′ interaction appears to be highly conserved, while the S2–S2′ pairing seems be more heterogeneous in both sequence and position, with least conservation in *Photorhabdus*.

targets multiple ABC transporter mRNAs by a conserved G/U-rich region [10]. Similarly, sequence alignments argue that *glmS* activation by GlmZ might occur — and involve similar interactions — in many other enterobacteria (Figure 3).

To date, the validated cases of repression by far outnumber those of activation, both in general and at the level of the individual sRNAs. GlmZ and RprA are only known as activators, though neither of them has been subjected to a global target screen. In contrast, DsrA, RNAIII, and RyhB directly interact with many mRNAs, and mostly repress these targets. For example, four repressed versus one (*hla*) activated targets are experimentally validated for RNAIII [6,23,24]. Regarding *E. coli* RyhB, the activated *shiA* mRNA is outnumbered by three repressed targets (*sodB*, *fur*, and *iscRSUA*) with experimentally confirmed interactions [13]. We note that whereas conserved sRNAs often repress more than one target [52], direct activation of multiple targets by a sRNA is as yet unknown.

3′-End-mediated mRNA stabilization

Antisense RNAs that are encoded in *cis* and in opposite orientation to their target mRNAs constitute a large class of bacterial regulators. Originally identified on bacterial mobile elements [53], and now increasingly so in bacterial chromosomes as well [54], the *cis*-encoded antisense RNAs have consistently been reported to repress mRNAs, with one exception: GadY of *E. coli* [55]. This sRNA is encoded on the opposite strand in the IGR of *gadX* and *gadW*, the two downstream ORFs in the *gadAXW* acid response cluster. GadY synthesis requires σS, GadW, and GadX, which is consistent with the accumulation of the sRNA in stationary phase and upon acid stress [55,56]. The primary GadY sRNA is 105 nt long but two shorter, 5′-processed species (90 and 59 nt) were also detected; all of the GadY species overlap the 3′-UTR of *gadX* mRNA by >30 bp [55]. Although one would intuitively expect that a perfect 3′-antisense match causes target repression, overexpression of GadY stabilizes the *gadX* mRNA, and a *gadY* promoter mutation abrogating GadY synthesis reduces *gadX* mRNA levels (Figure 1B) [55]. Perhaps the GadY–*gadX* duplex constitutes a roadblock that slows down *gadX* decay. The involved nucleases are yet to be identified: RNase E was tested and found to be essential for GadY accumulation [57], preventing conclusions as to whether it is required for *gadX* stabilization.

Intriguingly, GadY is also an Hfq-dependent RNA [55,58], and its overexpression directly or indirectly affects four other abundant proteins encoded at various loci unrelated to the *gad* genes [55]. Given the current knowledge of cellular Hfq functions, GadY might be the first example of a bacterial sRNA that acts on multiple mRNAs both by Hfq-dependent pairing in *trans* and by a novel 3′-end mechanism in *cis* [55].

Indirect activation by saving the direct activator: the GlmYZ sRNAs

We outlined above how *glmS* mRNA is activated by GlmZ through anti-antisense pairing. However, the first sRNA found to stimulate GlmS synthesis was GlmY [59], and overexpression of the two sRNAs invariably causes GlmS accumulation [34]. GlmY and GlmZ share substantial sequence and structure homology; crucially, however, GlmY lacks the complementary region to *glmS* mRNA [34,60], and unlike GlmZ fails to promote *glmS* translation *in vitro* [34]. How does then GlmY stimulate GlmS synthesis?

In wild-type *E. coli* with intact YhbJ (a protein of unknown function), the primary GlmZ transcript is rapidly 3′ processed to an abundant shorter RNA species that is deprived of the *glmS* target site [34,48]. GlmY suppresses the detrimental GlmZ processing to promote the accumulation of functional (full length) GlmZ sRNA [34,60]. The exact mechanism of GlmY action on GlmZ has remained elusive; it is likely though that GlmY acts by RNA mimicry to intercept the factor(s) promoting GlmZ cleavage (Figure 1C).

Intriguingly, GlmY itself is subject to repression at the post-transcriptional level; its intracellular stability is negatively regulated by 3′-end polyadenylation [34,60]. Consequently, in a strain lacking PAP I (polyA polymerase) GlmY accumulates, which suppresses GlmZ cleavage and upregulates GlmS synthesis [34,60]. As such, the discovery of GlmY explained an earlier observation of GlmS overproduction in polyadenylation-deficient *E. coli* [61]. In summary, the seemingly homologous GlmYZ sRNAs act in a hierarchical manner to form a regulatory cascade with several input functions to ultimately increase GlmS synthesis from the *glmUS* operon mRNA [34,60].

An mRNA trap removes an inhibitory sRNA to activate another mRNA

While the GlmYZ case has been suggestive of RNA mimicry, direct RNA competition that ultimately increased mRNA expression has recently been identified in the regulation of YbfM, a chitosugar-specific porin of *E. coli* and *Salmonella* [62,63] (Figure 1D). The *ybfM* mRNA is strongly repressed under standard growth conditions by RBS pairing of the constitutively expressed, *trans*-encoded MicM sRNA [64]. Unlike in other Hfq-dependent repressions [5], the MicM–*ybfM* pairing does not entail coupled degradation of the sRNA with its target: whereas *ybfM* mRNA is degraded by RNase E [63], MicM is recycled and remains in large excess relative to its target [62].

Genetic screens have identified factors that reverse the constitutive *ybfM* repression. Besides enzymes with established functions in RNA metabolism (PAP I, RppH), the transcription of the first IGR of the *chbBCARFG* chitobiose operon was found to antagonize the repressor, MicM, by

promoting its degradation [62,63]. The *chbBC* IGR exhibits short complementarity to almost exactly the same region in MicM that pairs with *ybfM* [62,63]. However, the MicM–*chbBC* interaction is slightly longer, and engages two nucleotides of the 3′-terminal stem-loop of MicM, whose partial opening seems to render MicM more susceptible to degradation [63]. Thus, *chbBC* triggers irreversible decay of MicM to clear the repressor. In other words, it functions as an RNA trap through target mimicry, facilitating the activation of one mRNA by another via the elimination of an inhibitory sRNA.

The novel mechanism has much biological meaning: as chitobiose, the major product of chitin catabolism, becomes the primary carbon source, *E. coli* strongly induces the *chb* operon. Transcription of the RNA trap causes about tenfold reduction of MicM levels, permitting the synthesis of the YbfM porin selective for chitosugar uptake. Interestingly, both *ybfM* and the chitobiose operon are transcriptionally activated by the same inducer, that is chitosugars [63], but only the concomitant removal of MicM eliminates the translational block, thus providing a positive feed-forward loop that unleashes the synthesis of the desired porin.

The molecular details of the 'trapping' mechanism, and consequently, of indirect *ybfM* activation remain to be fully understood. For example, which nuclease destabilizes the *chbBC*-bound MicM sRNA, and how is protein synthesis from the *chb* operon ensured when its polycistronic messenger interacts as 'pseudo-target' with MicM? The *chbBC* RNA is liberated as a ~300 nt species [63]; is this an independently acting RNA species? Otherwise, the MicM pairing might also alter the ratio of proteins produced from the *chb* operon mRNA, as seen in the regulation of *galETKM* or *glmUS* mRNAs by sRNAs [8,59]. At a more general level, the indirect activation involving a pseudo-target bears similarity to previously observed modulation in microRNA activity in plants, in which the expression of a noncoding pseudo-target titrates microRNA-399 to prevent it from downregulating its 'real' target mRNA [65].

How many activator RNAs are there?

It is now well established that conserved sRNAs often regulate multiple target mRNAs [2,52]. We expect that, similar to DsrA, RNAIII, and RyhB, sRNAs with multiple targets might often include an activated mRNA in their regulon. Biocomputational algorithms such as *TargetRNA* [66] or *RNAhybrid* [67] can predict target interactions yet do not take possible activation into account. Candidates for activation through anti-antisense pairing might be identified in the available catalogs of Hfq-associated mRNAs [15,58,68], by focusing on mRNAs that are downregulated in the absence of Hfq, and combining predictions of stable RBS structure and of complementarity with known or candidate sRNAs.

Pulse expression of sRNAs [46,66,69], currently the method of choice for the experimental target prediction, can achieve up to a ~45-fold repression [12] of a directly targeted mRNA within the typical 10 min period of sRNA expression. In contrast, translational activation usually entails a milder increase in mRNA levels, for example *shiA* mRNA levels rose 5.1-fold within 15 min of induced RyhB overexpression [46]. Thus, constitutive overexpression of an sRNA might provide a more robust approach [25,42,55], despite the risk of scoring indirect regulations such as GlmY–*glmS* [59].

New mechanisms might also be discovered as more sRNAs are analyzed. For instance, Rcd RNA was recently reported to stimulate tryptophanase activity by a mechanism that remains yet to be understood [70]. Base-pairing of an sRNA could mask a nuclease entry site in the mRNA, suppress translation at a cryptic RBS to redirect ribosomes to a target ORF, or prevent the formation of a transcriptional attenuator, all of which would increase gene expression.

Acknowledgements

We thank the members of our laboratory for comments on the manuscript, and colleagues for sharing unpublished results. Work in the Vogel lab is supported by the Deutsche Forschungsgemeinschaft (DFG) Priority Program SPP1258 *Sensory and Regulatory RNAs in Prokaryotes*. KSF is supported by the International Max Planck Research School for Infectious Diseases and Immunology Program.

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