REVIEW

Noncoding RNA control of the making and breaking of sugars

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Noncoding RNA regulators have been implicated in almost all imaginable cellular processes. Here we review how regulatory small RNAs such as Spot42, SgrS, GlmY, and GlmZ and a *cis*-encoded ribozyme in *glmS* mRNA control sugar metabolism. Besides discussing the physiological implications, we show how the study of these molecules contributed to our understanding of the mechanisms and of general principles of RNA-based regulation. These include the post-transcriptional repression or activation of gene expression within polycistronic mRNAs; novel ribonucleoprotein complexes composed of small RNA, Hfq, and/or RNase E; and the hierarchical action of regulatory RNAs.

Any life on earth depends on carbohydrates, which provide cells with energy and the building blocks for biosynthesis of all macromolecules. It is therefore not surprising that the uptake and metabolism of carbohydrates are extensively regulated at all levels. Organisms typically express the genes required for the utilization of a particular sugar substrate only if it is available in the environment. Regulation of the *Escherichia coli* lactose operon by the Lac repressor provides the classical paradigm for this kind of control (Miller-Hill 1996). However, substrate-dependent control of gene expression can also be achieved after the initiation of transcription by a variety of mechanisms, including transcriptional attenuation, translational control, and modulation of mRNA decay.

Organisms often encounter in their habitats a mixture of different carbon sources and therefore possess means to selectively take up and metabolize those substrates that permit the most rapid growth and promise the best success in competition with other species. Many heterotrophic bacteria and fungi prefer glucose as a carbon source, and usually repress the use of other sugars when glucose is available. This phenomenon of “catabolite repression” relies on sensing of the activities of carbohy-

drate transporters or of intracellular glycolytic metabolites. These cues modulate the synthesis of intracellular signaling molecules such as cAMP, or the activity of protein kinases, which in turn control the activity of global regulatory proteins; e.g., Crp (cAMP receptor protein) in enteric bacteria or cAMP-controlled protein kinase A in eukaryotic cells (Deutscher 2008; Gancedo 2008; Görke and Stülke 2008).

Once inside the cell, carbohydrates enter a central metabolic pathway i.e., glycolysis, the pentose phosphate pathway, or the Krebs (TCA) cycle. Flux through these pathways must be tightly coordinated with all other processes in the cell to avoid deleterious accumulation or depletion of metabolites. Global regulators such as the *Escherichia coli* Cra protein coordinate the transcription of glycolytic and gluconeogenic genes (see Nanchen et al. 2008 and references therein), whereas the widespread family of CsrA/RsmA RNA-binding proteins control metabolic genes at the post-transcriptional level. Notably, the CsrA/RsmA-like proteins are commonly associated with antagonistic small RNAs—e.g., *E. coli* CsrB (Liu et al. 1997)—that control the pool of active regulator (Babitzke and Romeo 2007; Lapouge et al. 2008). Moreover, there is extensive regulation at the metabolic enzyme level; e.g., by allosteric regulation or phosphorylation (Bott 2007; Lomas-Lopez et al. 2007; Macek et al. 2007).

In this review, we focus on a novel class of post-transcriptional regulators of bacterial sugar pathways; i.e., small noncoding RNAs (sRNAs) that act on *trans*-encoded target mRNAs. To date, sRNA functions have been studied most extensively in Gram-negative species including *E. coli* and *Salmonella*, in which a variety of approaches (Vogel and Sharma 2005) have identified more than a hundred chromosomally encoded sRNAs. These molecules, which typically range from 50 to 250 nucleotides (nt) in length, have been known in bacteria since the early 1970s, but their prevalence and impact on various biological control processes have been appreciated only recently (Majdalani et al. 2005; Storz et al. 2005; Romby et al. 2006). Since many *E. coli* sRNAs are conserved in a wider range of species (Hershberg et al. 2003), their functional analysis is often predictive of physiological roles beyond this model organism. Recent examples include the discovery of *E. coli* RyhB sRNA

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The *gal* operon is expressed at a rather high level even in the absence of the operon-specific inducer galactose, allowing the cell to synthesize the UDP sugars from G6P. This anabolic pathway also generates galactose whose fate depends on the available carbon source. If it is glucose, the galactose is excreted, and therefore the GalK enzyme (encoded by the third gene in the operon) is idle. If there is no glucose, the cells recycle the intracellularly generated galactose in a GalK-dependent manner. To match the differential need of GalK, the *gal* operon proteins are differentially synthesized depending on the physiological condition (Wilson and Hogness 1969). That is, the presence of glucose decreases GalK synthesis by fourfold relative to the other Gal proteins, whereas normally equimolar amounts of all four Gal operon proteins are produced when glucose is absent. The underlying mechanism remained obscure until the discovery of Spot42 RNA action in this pathway.

Spot42 regulates *galK* translation within the *galETKM* operon mRNA

The *galETKM* operon and its functions

The diagram illustrates the metabolic pathways for galactose and glucose in *E. coli*, showing the flow of carbon from the exterior through the membrane into the interior. Key components include:

- Exterior:** The environment outside the cell.
- Membrane:** The boundary of the cell, containing transporters like IICB^{Glc} (PtsG), GalP, and NagE.
- Interior:** The cytoplasm where metabolic reactions occur.
- Glucose Pathway:**
 - Glucose enters via IICB^{Glc} (PtsG).
 - Glucose is converted to Glucose-6-P by Pgi.
 - Glucose-6-P can be converted to Glucose-1-P by Pgm, which then enters the LPS synthesis pathway via GalU/GalF and GalE.
 - Glucose-6-P enters Glycolysis to produce Fructose-6-P.
 - Fructose-6-P is converted to GlcN-6-P by GlmS, which then enters the LPS synthesis pathway via GlmM and GlmU.
 - Fructose-6-P can also be converted to GlcN-6-P by GlmY.
 - GlcN-6-P is converted to GlcN-1-P by GlmM, which then enters the LPS synthesis pathway via GlmU.
 - GlcN-1-P is converted to UDP-GlcNAc by GlmU, which then enters the LPS synthesis pathway via GlmU.
 - UDP-GlcNAc is used for the synthesis of Peptidoglycan and LPS.
- Galactose Pathway:**
 - Galactose enters via GalP.
 - Galactose is converted to Galactose-1-P by GalK.
 - Galactose-1-P is converted to UDP-galactose by GalT, which then enters the LPS synthesis pathway via GalE.
 - UDP-galactose is converted to UDP-glucose by GalF, which then enters the LPS synthesis pathway via GalU.
 - UDP-glucose is used for the synthesis of LPS.
- Regulation:**
 - SgrS inhibits IICB^{Glc} (PtsG) and GlmZ.
 - GlmZ inhibits GlmS.
 - GlmY inhibits GlmM.
 - Spot42 inhibits GalK.
- Other Pathways:**
 - Amino sugars enter via NagE.
 - ManXYZ is involved in the synthesis of LPS.

cosine epimerase; (GalF and GalU) subunits of the glucose-1-phosphate uridylyltransferase; (Pgm) phosphoglucosmutase; (Pgi) phosphoglucose isomerase; (GlmM) phosphoglucosamine mutase; (GlmU) N-acetylglucosamine-1-phosphate uridylyltransferase; (NagE) N-acetylglucosamine PTS transporter; (ManXYZ) mannose PTS transporter; (UDP) uridine diphosphate; (GlcN-1-P) glucosamine-1-phosphate; (GlcNAc) N-acetylglucosamine.

A **B**

Figure 2. Small RNAs controlling bacterial sugar pathways. (A) Secondary structures of Spot42, GlmY, and GlmZ sRNAs as predicted by structural probing or by Mfold software analysis (note that the secondary structure of SgrS is unknown). sRNA residues that pair with target mRNAs are highlighted in red. The sgrT ORF in SgrS is highlighted in green. Previously mapped processing sites in GlmY/Z are indicated by vertical arrows. (B) Pairing of sRNAs with their target mRNAs according to computational and experimental analysis. The relevant portions of the RNAs are shown. Shine-Dalgarno (SD) sequences are highlighted by gray boxes, and start/stop codons underlined. Base-pairing is indicated by black dots. (Bottom left) The stem-loop that sequesters the *glmS* SD in the absence of active GlmZ is shown for comparison.

and impaired the adaptation to changes in media composition (Rice and Dahlberg 1982). While the early studies fell short of defining a physiological role, they did

suggest that Spot42 was likely a regulatory RNA (Rice et al. 1987).

The mystery of Spot42 function was solved when Val-

entin-Hansen and coworkers (Møller et al. 2002b) noticed that Spot42 exhibited significant complementarity to the *galK* start codon region. The inverse correlation of *spf* and *galK* expression in response to glucose prompted them to pursue the *galETKM* mRNA as a potential antisense target (Møller et al. 2002b). That is, the *spf* gene is subject to repression by the cAMP–CRP complex (Sahagan and Dahlberg 1979; Polayes et al. 1988), which reduces Spot42 levels threefold to fivefold in the absence of glucose; concomitantly, the glucose-dependent fourfold repression of GalK synthesis is alleviated. It was demonstrated that the sRNA selectively inhibits the synthesis of GalK but not of the GalE and GalT proteins encoded upstream in the *gal* operon mRNA (Fig. 3A; Møller et al. 2002b).

Spot42 specifically binds to the 5' part of the *galK* mRNA and acts as a translational inhibitor that prevents 30S ribosome binding to the *galK* moiety of the operon mRNA. Probing of the Spot42–*galK* mRNA complex identified three predominantly single-stranded regions in Spot42 RNA that antisense-paired with the AUG region of *galK* mRNA (Fig. 2B). The target site overlaps with the 3' end of the *galT*, thus Spot42 binding must withstand the helicase activity of a ribosome elongating along the upstream *galT* gene. The selective repression of GalK synthesis by Spot42 established a novel principle in bacterial gene regulation; i.e., the intraoperonic targeting of a gene at the post-transcriptional level (Fig. 3).

A crucial role of Hfq protein in Spot42–target RNA interaction

Similar to many other sRNAs (see below), the pairing of Spot42 with its target *galK* is imperfect and discontinuous. How is efficient base-pairing accomplished? It was known at the time that the bacterial Sm-like protein Hfq interacted with several *E. coli* sRNAs, and that its presence is required for regulation in vivo (Zhang et al. 1998; Sledjeski et al. 2001; Wassarman et al. 2001). Spot42

RNA was also found to be associated with Hfq protein in vivo, and Hfq was shown to target three short single-stranded A/U-rich regions of the sRNA (Møller et al. 2002a). Interestingly, these binding motifs resembled those of eukaryotic Sm proteins, which are involved in various aspects of RNA regulation and metabolism. Moreover, the homohexameric Hfq protein was shown to form ring-like doughnut-shaped structures reminiscent of the oligomeric structures of Sm proteins. Structural analyses unequivocally confirmed that Hfq is an Sm homolog (Brennan and Link 2007). One Hfq hexamer binds a Spot42 molecule, and Hfq increases the interaction of Spot42 with its *galK* target RNA by 150-fold, resulting in a tripartite ribonucleoprotein (RNP) complex composed of the regulatory RNA, its target, and Hfq. These findings, together with parallel work on Hfq and OxyS sRNA by the Storz laboratory (Zhang et al. 1998, 2002), laid the ground for the meanwhile fully appreciated role of Hfq as a key facilitator of sRNA–mRNA interactions.

SgrS RNA combats phosphosugar stress

Many bacteria take up glucose by the phosphoenolpyruvate phosphotransferase system (PTS) (Plumbridge 2002; Deutscher et al. 2006). PTS enzyme IICB^{Glc}, encoded by *ptsG* in *E. coli*, is a key player in this process; it transports and concomitantly phosphorylates glucose to G6P. While G6P is an essential intermediate in glucose metabolism, its intracellular accumulation causes the phenomenon of sugar-phosphate toxicity, also called “phosphosugar stress.” The Aiba laboratory made the seminal observation that this stress condition destabilizes the *ptsG* mRNA, thereby limiting enzyme IICB^{Glc} synthesis and further G6P accumulation (Kimata et al. 2001). Intriguingly, this post-transcriptional repression of *ptsG* required Hfq (Morita et al. 2004), indicative of the hidden action of an sRNA.

The sRNA to combat phosphosugar stress in *E. coli*

Figure 3. Intraoperonic targeting by sRNAs as a novel principle in gene regulation that permits the selective regulation of a downstream cistron within a polycistronic messenger in response to a specific signal. (A) Spot42 sRNA selectively represses *galK* translation within the polycistronic *galETKM* mRNA. (Top) Aided by Hfq, Spot42 binds to the translation initiation region of *galK* and reduces synthesis of the GalK protein threefold to fourfold, whereas synthesis of the GalE and GalT proteins remains unaffected. (B) Following transcription, the *glmUS* cotranscript is processed by RNase E at the *glmU* stop codon. Whereas the *glmU* message is rapidly degraded, the *glmS* monocistronic transcript can be stabilized by Hfq-aided

base-pairing with the GlmZ sRNA. GlmZ binds to the 5' UTR of the *glmS* mRNA and removes a translational block allowing high GlmS synthesis rates. The homologous GlmY sRNA accumulates in response to depletion of the intracellular GlcN6P concentration and activates *glmS* indirectly by suppressing the endonucleolytic inactivation of GlmZ sRNA.

was identified as SgrS by Vanderpool and Gottesman (2004), an sRNA originally discovered by global microarray analysis of Hfq-associated RNA (Zhang et al. 2003). Induced SgrS expression from a plasmid caused a rapid loss of the *ptsG* messenger, and reciprocally, deletion of the *sgrS* gene abrogated the *ptsG* down-regulation that normally follows phosphosugar stress (Vanderpool and Gottesman 2004). With a length of ~220 nt, *E. coli* SgrS is at the longer end of the enterobacterial sRNA size range (Fig. 2A). This reflects the fact that SgrS also encodes a polypeptide (see below). SgrS is transcribed from the intergenic region that separates *sgrR* and *setA*, two genes that directly or indirectly relate to SgrS action.

Phosphosugar stress-induction of *sgrS* requires the adjacently encoded SgrR protein, a novel type of transcriptional regulator, and based on genetic and molecular evidence, SgrR is now regarded as a dedicated transcriptional activator of *sgrS* (Vanderpool 2007). Genomic linkage of an sRNA gene (*sgrS*) with its cognate transcription factor (*sgrR*) gene has been a recurring theme in *E. coli*; e.g., *gcvB* and *oxyS* are also next to (and divergent from) the genes of their transcriptional factors (Altuvia et al. 1997; Urbanowski et al. 2000).

SgrS targets *ptsG* mRNA by short antisense pairing

The observations that SgrS is Hfq-associated (Zhang et al. 2003) and that Hfq is mandatory for stress-induced *ptsG* repression (Morita et al. 2004) correctly predicted that SgrS regulation relies on an antisense mechanism. The SgrS–*ptsG* interaction, initially proposed by Vanderpool and Gottesman (2004), was extensively studied by Aiba and colleagues (Kawamoto et al. 2005, 2006). As depicted in Figure 2B, residues 168–187 of SgrS pair with the 5′ untranslated region (UTR) of *ptsG* mRNA around the SD sequence, forming an imperfect 20-base-pair (bp) RNA duplex that contains several C:U or A:G mismatches. The detailed genetic evidence available (Kawamoto et al. 2006) makes the SgrS–*ptsG* interaction attractive for further RNA structure analysis. Work on several sRNAs unrelated to sugar control circuits revealed that the interacting residues are often presented by distinct structural elements—e.g., apical loop regions (Argaman and Altuvia 2000; Boisset et al. 2007; Papenfort et al. 2008)—and this quite mirrors the mechanisms of target recognition by *cis*-encoded antisense RNAs (Wagner et al. 2002).

Alignment of the predicted SgrS RNAs of diverse bacteria reveals great sequence diversity, but the *ptsG* pairing site is visible as a short conserved region (Vanderpool and Gottesman 2004). Given the considerable length of bacterial sRNAs, on the one hand, and short imperfect mRNA interactions, on the other hand, reliable sRNA target predictions have remained a challenge. Yet exploiting the conservation of “antisense domains” containing the target mRNA-binding site(s), such as seen in SgrS, should improve target predictions; it has already aided the identification of binding sites of the ~200-nt GcvB sRNA on multiple ABC transporter target mRNAs (Sharma et al. 2007).

Concerted action of Hfq and RNase E

Rapid loss of *ptsG* mRNA is the most apparent outcome in the phosphosugar stress response (Kimata et al. 2001; Vanderpool and Gottesman 2004). However, SgrS pairing covers the SD sequence of *ptsG* mRNA (Fig. 2B), indicating that it could also inhibit *ptsG* translation. Since the half-life of bacterial mRNA is strongly affected by the association with ribosomes (Deana and Belasco 2005), one may infer a simple model in which *ptsG* mRNA decay stems from an SgrS-mediated translation block. Yet, there is more than one layer of SgrS action, and this includes a novel ribonucleoprotein complex (RNP) essential for *ptsG* mRNA decay.

This novel RNP is composed of RNase E, Hfq, and SgrS (Morita et al. 2005), but might as well form with other Hfq-dependent sRNAs (Massé and Gottesman 2002; Morita et al. 2005). RNase E, the major ribonuclease of *E. coli*, is typically found in the “degradosome,” a complex assembled by PNPase, RhlB, and enolase binding to the C-terminal “scaffold” region of RNase E (Carpousis 2007). Strikingly, whereas RNase E including its scaffold region was essential for stress-induced *ptsG* mRNA decay, other degradosome components did not seem to be required (Morita et al. 2005). Analysis of the proteins that copurified with epitope-tagged RNase E, Hfq, or RhlB indicated that Hfq interacted with the RNase E scaffold independently of degradosome assembly. This association recruits the Hfq-associated SgrS sRNA to RNase E, and SgrS can then direct RNase E/Hfq to the *ptsG* target (Morita et al. 2005). Thus, Hfq has dual function: It facilitates stable SgrS–*ptsG* pairing (Kawamoto et al. 2006) and hands the regulatory RNAs a nucleolytic cofactor that will irreversibly inactivate the target. If Hfq and RNase E are always in a complex, this poses the intriguing question why other sRNA targets are repressed without concomitant mRNA decay. For example, Spot42 does not seem to recruit RNase E to the *gal* operon mRNA albeit it is tightly associated with Hfq. However, recent work from the Luisi laboratory (Worrall et al. 2008) suggested that the RNase E–Hfq interaction may not be direct but may be brought about by RNA; if so, not all regulatory sRNAs may be able to bring the two proteins into a complex.

The association of SgrS with RNase E could also provide an anticipatory control mechanism that allows the cell to rapidly reverse the down-regulation of *ptsG* when phosphosugar stress is lifted. Massé et al. (2003) demonstrated by elegant *in vivo* experiments that several Hfq-dependent sRNAs decay along with their targets in an RNase E-dependent fashion. In the absence of target pairing, Hfq may protect the sRNAs from RNase E, but upon target pairing, both the mRNA and the sRNA become labile (Massé et al. 2003). Since RNase E and Hfq have coincident binding sites (single-stranded A/U-rich regions) (Moll et al. 2003), RNase E presumably displaces Hfq on its RNA targets.

Targeted mRNA destabilization turned out to be just one side of the SgrS story. In an RNase E mutant strain defective in *ptsG* mRNA decay, SgrS still reduced the

levels of the *ptsG*-encoded IICB^{Glc} protein (Morita et al. 2006). Metabolic protein labeling immediately after phosphosugar stress induction showed that SgrS inhibited IICB^{Glc} protein synthesis in vivo without concomitant target mRNA destruction (Morita et al. 2006). Moreover, following earlier assays of sRNA-induced inhibition of translational initiation using 30S ribosomes—e.g., for *OxyS/fhlA* mRNA (Altuvia et al. 1998)—it has now been demonstrated that SgrS by itself can inhibit *ptsG* mRNA translation in a reconstituted in vitro translation system (Maki et al. 2008). In more general terms, this translational repression mechanism is in striking contrast to the function of eukaryotic microRNAs, which achieve target repression by the recruitment of dedicated proteins and only function to select the correct mRNA.

mRNA localization matters

The nascent *ptsG* mRNA will first be cytoplasmic but later on near the membrane as the IICB^{Glc} protein will be cotranslationally integrated into the inner membrane. Kawamoto et al. (2005) made the crucial observation that SgrS action on *ptsG* mRNA is strictly dependent on intact protein localization. The investigators discuss several models of why membrane-localized but not cytoplasmic *ptsG* mRNA is an SgrS target, including the accumulation of RNase E near the membrane (Liou et al. 2001) and a reduced translational initiation rate during the course of cotranslational integration of the nascent peptide into the membrane. Note that since most bacterial sRNAs can be expected to act on nascent target mRNAs, competition between sRNA pairing and ribosome binding is very likely a general principle (Unoson and Wagner 2007). In line with this model, SgrS could regulate *ptsG* mRNA regardless of membrane localization when the *ptsG* translation rate was artificially reduced (by a weaker SD sequence) (Kawamoto et al. 2005). We have yet to see to what extent mRNA localization matters in sRNA circuits. Interestingly, another SgrS target—i.e., *manXYZ* mRNA—seems to be regulated independently of mRNA localization (C. Vanderpool, pers. comm.). However, given that many sRNA targets encode extracytoplasmic proteins—e.g., outer membrane, periplasmic, or secreted proteins (Guillier et al. 2006; Vogel and Papenfort 2006; Boisset et al. 2007; Sharma et al. 2007)—spatial control may not be uncommon.

SgrS action taken beyond antisense regulation

Strictly speaking, SgrS is not a “noncoding” but a bifunctional RNA that is both antisense regulator and mRNA. Wadler and Vanderpool (2007) discovered that in the long 5′ region upstream of the *ptsG* interaction site there is a conserved 43-amino-acid ORF, named *sgrT* (Fig. 2A). The SgrT peptide and *ptsG* base-pairing are equally sufficient to protect *E. coli* from phosphosugar stress, suggesting physiological redundancy. By a yet-unknown mechanism, SgrT may antagonize the preexisting IICB^{Glc} pro-

tein, while the pairing of SgrS with *ptsG* blocks ongoing IICB^{Glc} synthesis (Wadler and Vanderpool 2007).

Dual functionality has been known for the 514-nt RNA III of *Staphylococcus aureus*, whose 5′ end codes for a short virulence peptide, whereas the 3′-end-located antisense domain regulates a multitude of virulence factor mRNAs (Novick et al. 1993; Huntzinger et al. 2005; Boisset et al. 2007). It will be interesting to understand how such RNAs can control *trans*-encoded mRNAs if they are also ribosome targets.

The *sgrS* gene may hold more surprises owing to its location right upstream of the *setABC* operon (Vanderpool and Gottesman 2004). The SetABC proteins belong to a family of sugar efflux transporters, and SetA has broad substrate specificity including glucosides (Liu et al. 1999). The *sgrS* terminator lies only ~25 bp upstream of the *setA* start codon (Vanderpool and Gottesman 2004), and read-through transcripts from *sgrS* may also activate the *setABC*-encoded sugar efflux pump when elevated G6P levels become a threat. If so, this *cis* effect would be yet another novel function of this exciting sRNA gene.

Intraoperonic gene targeting the other way around: the GlmYZ sRNAs activate a downstream cistron

We discussed that Spot42 modulates the expression of the *galETKM* operon by selective repression of a downstream cistron. The opposite case—i.e., the selective activation of a downstream cistron by sRNA—leads to differential expression of *E. coli glmUS* mRNA. This operon encodes enzymes of the biosynthetic pathway for amino sugars, which are essential precursors for the peptidoglycan and lipopolysaccharide components of the cell wall of Gram-negative bacteria (Fig. 1). Whereas GlmU is constantly needed, GlmS only becomes essential for the synthesis of glucosamine-6-phosphate (GlcN-6-P) when no external amino sugars are available. Owing to strict transcriptional coupling of the *glmUS* genes (Plumbridge 1995), the relative synthesis of GlmS in response to substrate availability can only be adjusted after transcription (Fig. 3B). Although RNase E-dependent processing was shown to generate a monocistronic *glmS* from the dicistronic *glmUS* mRNA (Joanny et al. 2007; Kalamorz et al. 2007), there was no evidence that the cleavage was metabolically regulated. In contrast, independent studies from our two laboratories recently identified two *E. coli* sRNAs, GlmY and GlmZ (Fig. 2A), as suboperonic stimulators of GlmS synthesis (Kalamorz et al. 2007; Urban et al. 2007; Reichenbach et al. 2008; Urban and Vogel 2008).

The GlmY and GlmZ sRNAs were originally described in multiple *E. coli* screens (Argaman et al. 2001; Rivas et al. 2001; Wassarman et al. 2001; Vogel et al. 2003), yet their now obvious functional and structural relationship was not realized before each of them was revealed as an activator of GlmS synthesis. Overexpression of GlmY caused such drastic GlmS accumulation that this protein became readily visible on standard SDS gels; the levels of the upstream encoded GlmU protein, however, were un-

affected (Urban et al. 2007). In parallel, a transposon screen discovered the *glmZ* gene as the cause of chronic GlmS overproduction in a certain *E. coli* mutant (*yhbJ*; see below); again, only GlmS and not GlmU levels were altered (Kalamorz et al. 2007). Note that the latter study constitutes a rare case in which a transposon mutagenesis screen led to the recognition of an sRNA's function, whereas sRNA overexpression was the typical route to success (Vogel and Wagner 2007).

The two sRNAs are highly similar in length, sequence, and structure (Fig. 2A). Yet these seemingly homologous sRNAs activate GlmS synthesis by entirely different mechanisms and act hierarchically in a noncoding RNA cascade.

Mechanism of translational activation by bacterial sRNAs

Translation of the *glmS* mRNA is normally weak since an internal hairpin structure containing the *glmS* SD sequence (Fig. 2B) limits ribosome access to the downstream *glmS* RBS and therefore GlmS synthesis. This situation is reminiscent of *rpoS* mRNA control in *E. coli* (*rpoS* encodes the general stress sigma factor, σ^S): Translation of this messenger is normally low owing to an intrinsic RBS hairpin. Yet, *rpoS* translation can be activated by the *trans*-encoded DsrA and RprA sRNAs via an "anti-antisense" mechanism; base-pairing of these sRNAs with the *rpoS* mRNA leader competes with the formation of the inhibitory RBS hairpin, thus promoting translation (Lease et al. 1998; Majdalani et al. 1998, 2002).

In silico analysis (Kalamorz et al. 2007) and RNA structure probing experiments (Urban and Vogel 2008) indicated the same mechanism for *glmS* mRNA: Some 15 residues of an accessible, single-stranded region of GlmZ RNA base-pair with the 5' flank of the *glmS* RBS hairpin (Fig. 2B); this alternative pairing liberates the SD and activates *glmS* mRNA translation. Mutational analyses in vivo support this interaction; point mutations in the upper and lower helices of the GlmZ–*glmS* interaction abrogate *glmS* induction, but when combined in a compensatory fashion, activation by GlmZ is restored (Urban and Vogel 2008). In addition, the *glmS* mRNA activation by GlmZ has also been recapitulated in an in vitro translation system (Urban and Vogel 2008). This illustrates that the anti-antisense mechanism in essence requires only the translational machinery and an activator sRNA. Following its encounter in other bacterial sRNA circuits (Hammer and Bassler 2007; Prevost et al. 2007), the anti-antisense mechanism is now regarded as the paradigm of sRNA-mediated mRNA activation.

Note, however, that in vivo GlmZ not only enhances *glmS* translation but also stabilizes this mRNA (Kalamorz et al. 2007; Urban and Vogel 2008). Whether the increase in mRNA translation and stability is directly coupled remains to be determined. In vitro the addition of purified Hfq protein increases the effect of GlmZ on translation by almost 10-fold (Urban and Vogel 2008), and in vivo, Hfq is ultimately required for *glmS* activa-

tion (Kalamorz et al. 2007; Urban and Vogel 2008). In vivo Hfq not only associates with the GlmY or GlmZ sRNAs (Zhang et al. 2003; Sittka et al. 2008) but also specifically with the *glmUS* intergenic region (Sittka et al. 2008), in line with Hfq's common function as a matchmaker of sRNA–target mRNA interactions.

A regulatory RNA cascade—GlmY acts on GlmZ to activate GlmS synthesis

GlmY and GlmZ look much alike, yet GlmY lacks the residues of GlmZ that target the *glmS* hairpin (Fig. 2A) and also fails to promote GlmS synthesis in vitro (Urban and Vogel 2008). How can it then activate the *glmS* gene in vivo? It does so by counteracting the inactivation of GlmZ.

The primary 207-nt GlmZ transcript is subject to a 3'-RNA processing that involves the action of YhbJ, a conserved protein of as-yet-unknown function (Kalamorz et al. 2007). The processing generates a 153-nt GlmZ species (Argaman et al. 2001) that is deprived of the *glmS*-binding site and does not promote GlmS synthesis in vitro (Urban and Vogel 2008). By a yet-unknown mechanism, GlmY antagonizes the deleterious GlmZ processing and thereby increases the levels of the direct *glmS* activator (Reichenbach et al. 2008; Urban and Vogel 2008). This antagonism may involve RNA mimicry of GlmY to compete with the factor(s) that tag the highly similar GlmZ RNA for cleavage.

Families of homologous sRNAs have been reported from diverse bacteria other than *E. coli*; e.g., *Vibrio*, *Pseudomonas*, and cyanobacterial species (Lenz et al. 2004; Wilderman et al. 2004; Axmann et al. 2005; Lapouge et al. 2008). Where known, these sRNAs act redundantly and/or additively, and to some degree substitute for each other's function. In contrast, the hierarchical action of the GlmYZ sRNAs is novel such that GlmY strictly requires GlmZ for *glmS* activation (Reichenbach et al. 2008; Urban and Vogel 2008).

Polyadenylation controls sRNA expression

Hfq and YhbJ take decisive roles in the GlmYZ cascade by controlling GlmZ activity at the RNA level (Kalamorz et al. 2007; Urban and Vogel 2008). Yet another protein regulates GlmY levels, and again at the post-transcriptional level; it is the main enzyme responsible for 3'-end RNA polyadenylation, PAP I [poly(A) polymerase I encoded by *pcnB*] (Cao and Sarkar 1992).

Historically, PAP I was the first factor implicated in post-transcriptional *glmS* regulation when the study of an *E. coli pcnB* mutant revealed chronic overproduction of both *glmS* mRNA and GlmS protein (Joanny et al. 2007). Since poly(A) tails typically destabilize bacterial transcripts by accelerating the 3' → 5' exonucleolytic degradation of RNA (Dreyfus and Regnier 2002; Kushner 2004), it was speculated that the loss of polyadenylation directly impeded the decay of *glmS* mRNA (Joanny et al. 2007). However, the observations that *glmS* reporter fu-

sions with 3' ends different from native *glmS* mRNA, as well as that the GlmYZ sRNAs were also up-regulated in the PAP I-deficient strain (*pcnBΔ1*), hinted at a different mechanism (Kalamorz et al. 2007; Reichenbach et al. 2008; Urban and Vogel 2008). Assessment of the stability and the poly(A) status of the GlmY, GlmZ, or *glmS* RNAs revealed that GlmY was the only RNA for which differential stabilities and expression levels in *pcnBΔ1* cells correlated with the presence or absence of 3'-end poly(A) tails. Thus, if GlmY is no longer polyadenylated (normally >50% of all GlmY molecules), it is stabilized and accumulates, which in turn stabilizes GlmZ and increases *glmS* translation (Reichenbach et al. 2008; Urban and Vogel 2008).

Polyadenylation-dependent destabilization had for long been known to affect *cis*-encoded antisense RNAs of bacterial plasmids (Xu et al. 1993; Dam Mikkelsen and Gerdes 1997; Söderbom et al. 1997), yet GlmY was the first chromosomal sRNA whose polyadenylation impacts on the synthesis of a cellular protein.

Multiple input functions in sRNA-mediated amino sugar regulation

The analyses of the GlmYZ RNAs solved the long-standing puzzle of how GlmS activity, which is feedback-inhibited at the enzyme level in eukaryotic systems (Milewski 2002) and *cis*-controlled in Gram-positive bacteria (Winkler et al. 2004), was autoregulated in Gram-negative bacteria. Physiological studies using various inhibitors of amino sugar and cell wall synthesis pathways demonstrated that the GlmYZ RNAs are essential for feedback control of *E. coli* GlmS synthesis by its product, GlcN6P. Depletion of GlcN6P causes the accumulation of GlmY, which induces GlmS synthesis by stabilizing GlmZ (Kalamorz et al. 2007; Reichenbach et al. 2008).

It will now be important to understand how this RNA cascade senses the environmental signals that determine *glmS* epistasis. Moreover, the precise mechanism of how YhbJ affects GlmZ RNA processing is yet to be solved. The protein, which is conserved in many bacteria, contains a nucleotide-binding motif, and software analysis predicts a putative RNA-binding domain at the C terminus. Regardless of the precise molecular mode of action, the unexpected discovery of YhbJ as a mediator of sRNA processing hints at the existence of additional proteins that modulate the activity of regulatory sRNAs.

Regulation of *glmS* expression by a ribozyme in Gram-positive bacteria

In low GC Gram-positive bacteria, *glmS* expression is also regulated at the post-transcriptional level yet by a *cis*-encoded ribozyme (Winkler et al. 2004) rather than *trans*-encoded sRNAs. The *glmS* ribozyme was discovered during a biocomputational search for conserved sequence and structural elements in unusually large intergenic regions of bacterial genomes (Barrick et al. 2004). The ribozyme resides in the 5' UTR of the *glmS* mRNA.

Unlike classical riboswitches, it lacks a transcriptional or translational attenuator. In contrast, *in vitro* experiments showed that it undergoes self-cleavage specifically in the presence of GlcN6P (Winkler et al. 2004).

In the *in vivo* situation, this cleavage event liberates the *glmS* mRNA from a larger polycistronic transcript (Winkler et al. 2004) and down-regulates GlmS synthesis. The Winkler lab has deciphered the *in vivo* mechanism of gene regulation by the *Bacillus subtilis* ribozyme (Collins et al. 2007). By altering the intracellular GlcN6P concentration, they showed that GlcN6P depletion highly up-regulated *glmS* expression. Under this condition, the uncleaved full-length *glmS* transcript accumulated, whereas no *glmS* transcript was detectable in the presence of high GlcN6P concentrations. Mutations in the ribozyme that prevent cleavage also cause accumulation of the full-length *glmS* mRNA, regardless of the GlcN6P concentration. RNase J1, an essential nuclease with a key role in RNA processing and degradation in *B. subtilis* (Even et al. 2005; Mathy et al. 2007), was subsequently identified as the enzyme that is responsible for the rapid degradation of the 3' cleavage product of the ribozyme (Collins et al. 2007).

Cleavage of the *glmS* ribozyme was shown to occur by an internal phosphoester transfer yielding products with 2',3'-cyclic phosphate and 5' hydroxyl moieties at the termini (Winkler et al. 2004; Collins et al. 2007). RNase J1 specifically degrades RNAs with a 5' hydroxyl group, which is radically different from the paradigmatic model of global mRNA decay in *E. coli*, in which the unrelated enzyme RNase E specifically recognizes 5' monophosphate mRNA, whereas 5' hydroxyl RNA is stable (Ceslesnik et al. 2007). There is accumulating evidence that Gram-positive and Gram-negative bacteria evolved completely different RNA decay mechanisms.

The unique features of the *glmS* ribozyme

Among the known metabolite-sensing RNAs in nature, the one in *glmS* is unique because it represents the only ribozyme so far that controls gene expression, and because its ligand GlcN6P is a cofactor in the underlying acid-based catalysis of RNA self-cleavage. The *glmS* ribozyme has become a model RNA for biochemical and biophysical studies, including the determination of its crystal structures, alone or in complex with GlcN6P (Klein and Ferre-D'Amare 2006; Cochrane et al. 2007). Collectively, these studies demonstrated that unlike in classical riboswitches, no conformational changes occur in the ribozyme upon GlcN6P binding and RNA cleavage. A recent study demonstrated that the ribozyme can be physically separated from its target RNA without losing activity (Tinsley et al. 2007); if an engineered *glmS* ribozyme can regulate gene expression *in trans*, small metabolite-sensing RNAs may also exist in nature.

Conclusion and outlook

Noncoding RNAs have been implicated in ever more cellular pathways and stress responses (Majdalani et al.

2005; Storz et al. 2005). Besides the control of virulence and transcription factors (Romby et al. 2006; Wassarman 2007) and outer membrane protein biogenesis (Guillier et al. 2006; Vogel and Papenfort 2006), the regulation of metabolic sugar pathways has emerged as a major domain of bacterial sRNA action.

We have highlighted how the SgrS, Spot42, GlmY, and GlmZ sRNAs control the flux through central carbohydrate metabolic pathways. Much remains to be learned about each of these circuits. For example, the various phenotypes upon Spot42 overproduction suggest the existence of additional targets—e.g., in succinate metabolism—and Spot42 is predicted to pair with the 5' end of *sucC* mRNA, encoding the β -subunit of succinyl-CoA synthetase (Møller et al. 2002b). Regarding SgrS, microarray analyses have predicted that in addition to *ptsG*, the mRNAs of the PTS sugar transporters specific for fructose and mannose are SgrS targets (see Vanderpool 2007). Similar analysis in *Salmonella* has shown that SgrS action extends beyond sugar regulation, and that by using the same binding region as for *ptsG*, SgrS regulates a laterally acquired *Salmonella* virulence factor (K. Papenfort and J. Vogel, in prep.). Note that there is an intimate connection between carbohydrate utilization and virulence in many pathogenic bacteria (Görke and Stülke 2008).

Global transcriptome analyses of *hfq* mutants (Ding et al. 2004; Sonnleitner et al. 2006; Guisbert et al. 2007; Sittka et al. 2008) have revealed an extensive role of Hfq in the regulation of sugar pathways. Moreover, microarray- or deep sequencing-based identification of Hfq-associated RNA (Zhang et al. 2003; Sittka et al. 2008) suggests that Hfq targets mRNAs not only of many sugar transporters and metabolic enzymes, but also of transcription factors that coordinate the uptake and utilization of carbohydrates. It is reasonable to predict that numerous of these Hfq-associated mRNAs will turn out as being targeted by sRNAs. Hfq also binds the mRNA coding for Crp (Sittka et al. 2008), and this global transcription factor in turn controls not only metabolic genes (Gutierrez-Rios et al. 2007), but also the synthesis of two sRNAs, Spot42, and CyaR (Polayes et al. 1988; Papenfort et al. 2008). All these observations suggest that we are just beginning to understand the manifold and widespread role for sRNAs in control of sugar metabolism.

This might also be true for metabolite-sensing ribozymes and riboswitches. We note that a potential riboswitch was identified upstream of the *sucA* gene in β -proteobacteria (Weinberg et al. 2007); SucA catalyzes a reaction in the Krebs cycle. In *E. coli*, the same function is encoded by *sucC* mRNA, which is a putative target of Spot42 sRNA (see above). Since riboswitches are abundant in low G + C Gram-positive bacteria such as *B. subtilis* but rare in *E. coli* (Barrick and Breaker 2007), it is tempting to speculate that regulation by *trans*-encoded sRNAs generally compensates for the much lower number of riboswitches in enteric bacteria. The documented differences in *glmS* regulation in *B. subtilis* (ribozyme) and *E. coli* (GlmYZ sRNAs) are in favor of this speculation.

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