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MicroCommentary

RNA mimicry, a decoy for regulatory proteins

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Summary

Small non-coding RNA molecules (sRNA) are key regulators participating in complex networks, which adapt metabolism in response to environmental changes. In this issue of *Molecular Microbiology*, and in a related paper in *Proc. Natl. Acad. Sci. USA*, Moreno *et al.* (2011) and Sonnleitner *et al.* (2009) report on novel sRNAs, which act as decoys to inhibit the activity of the master post-transcriptional regulatory protein Crc. Crc is a key protein involved in carbon catabolite repression that optimizes metabolism improving the adaptation of the bacteria to their diverse habitats. Crc is a novel RNA-binding protein that regulates translation of multiple target mRNAs. Two regulatory sRNAs in *Pseudomonas putida* mimic the natural mRNA targets of Crc and counteract the action of Crc by sequestrating the protein when catabolite repression is absent. Crc trapping by a sRNA is a mechanism reminiscent to the regulation of the repressor of secondary metabolites (RsmA) in *Pseudomonas*, and highlights the suitability of RNA-dependent regulation to rapidly adjust cell growth in response to environmental changes.

Introduction

Bacteria have evolved complex mechanisms that co-ordinate gene expression in response to their ever-changing lifestyles. Current works show how RNA-dependent regulation is well appropriate to rapidly adjust cell growth in response to environmental changes. All bacteria produce a significant number of *trans*-acting sRNAs (small RNA) that directly or indirectly regulate a multitude of genes and hence, generate broad effects in a way very similar to the transcriptional regulatory proteins (Beisel and Storz, 2010). Mechanistic studies reveal that the majority of these sRNAs share limited or extended complementarities to mRNAs thus promoting the formation of base pairings. Pioneering works performed in *Escherichia coli* and *Salmonella typhymurium* showed that these sRNAs primarily target mRNAs, which encode membrane proteins, enzymes involved in metabolic pathways, virulence factors and transcriptional regulators (Storz *et al.*, 2011). In addition, transcriptional regulatory proteins can induce the synthesis of sRNAs to regulate gene expression in an opposite manner. Such mixed regulatory networks mediated both by sRNAs and regulatory proteins extend the number of regulated genes (Beisel and Storz, 2010). Although the most popular mode of action of sRNA is regulation of expression via base-pairing with mRNAs, few sRNAs exert their function on proteins (Fig. 1). Some of these sRNAs, which are the focus of this micro-commentary, interact with post-transcriptional regulatory proteins to inhibit their regulatory activities (Sonnleitner *et al.*, 2009; Moreno *et al.*, this issue).

Global post-transcriptional regulatory proteins as main targets of sRNAs

Several features differentiate sRNAs targeting proteins from sRNAs regulating mRNAs through base-pairings. In general, these sRNAs work by mimicking the mRNA substrate of the protein, the binding site being often present in multiple copies in the sRNA. A well-characterized example involves members of the CsrB/CsrC family of sRNAs of *E. coli*, which counteract the activity of the major carbon storage regulator protein CsrA (Babitzke and Romeo, 2007). *E. coli* CsrA and its orthologues RsmA/RsmE (repressor of secondary metabolites) in *Pseudomonas* regulate the expression of proteins involved in carbon metabolism, peptide transport, biofilm formation, quorum sensing and virulence (reviewed in Babitzke and Romeo, 2007; Bejerano-Sagie and Xavier, 2007; Brencic and Lory, 2009). These proteins are homodimeric and bind to the ribosome binding site (RBS) of multiple mRNAs to regulate translation. The NMR structure of *Pseudomonas fluorescens* RsmE (homologue of CsrA/RsmA) bound to a stem-loop structure containing the RBS of a target mRNA (Schubert *et al.*, 2007) shows that each monomer of RsmE

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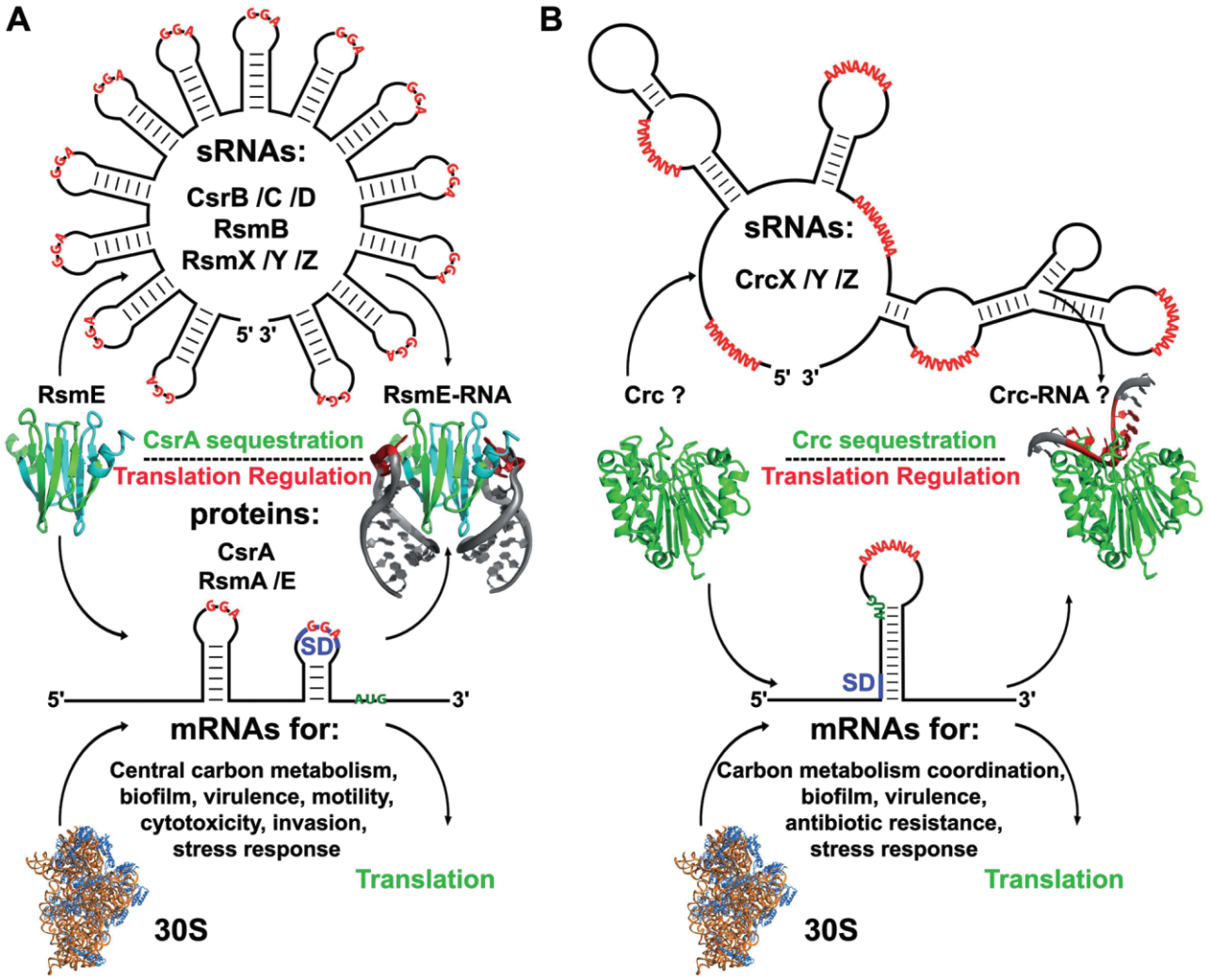

Fig. 1. sRNAs trapping global post-transcriptional regulatory proteins.
A. CsrA/RsmA/RsmE circuits. In *Pseudomonas*, the two-component system GacS–GacA activates transcription of CsrB-like sRNAs (RsmY/RsmZ), which sequester CsrA-like RsmA/RsmE protein. The CsrB-like sRNAs found in various bacteria are: CsrB (22 AGG) and CsrC (13 AGG) in *Escherichia coli* and *Salmonella enterica*; CsrB (28 AGG), CsrC (23 AGG), CsrD (21 AGG) in *Vibrio cholerae*; RsmB (20 AGG) in *Erwinia carotovora*; RsmX (5 AGG), RsmY (7 AGG), RsmZ (8 AGG) in *P. fluorescens*; RsmY (7 AGG) and RsmZ (7 AGG) in *P. aeruginosa* (Babitzke and Romeo, 2007; Lapouge *et al*., 2008). RsmA/RsmE are post-transcriptional proteins, which bind to mRNAs at the ribosome binding site when they are free in solution. The structures of the dimeric RsmE free or bound to an RNA hairpin were from Schubert *et al*. (2007). Schematic secondary structures of a typical mRNA target and of CsrB-like sRNA are shown. The number of AGG repeats is given for each sRNA. The AGG motif recognized by RsmA/E is in red, the Shine and Dalgarno sequence (SD) is in blue.
B. *Pseudomonas putida* Crc circuits. Schematic secondary structures of CrcZ/CrcY sRNAs and of *alkS* mRNA (a Crc target) are shown (Moreno *et al*., 2009b; Moreno *et al*., this issue). The CrcY-like sRNAs found in various *Pseudomonas* are: CrsZ (5 repeats) in *P. aeruginosa*; CsrZ and CsrY (6 repeats) in *P. putida* and *P. fluorescens*; CrcY, CrcZ and CrcX in *P. syringae*. In both RNAs, the conserved sequence motif AANAANAA recognized by Crc is shown in red. When bacteria are grown with a non-preferred carbon source, the levels of CrcZ/CrcY strongly increase, sequestering Crc protein. In the presence of a preferred carbon source, CrcZ/CrcY levels decrease and Crc preferentially binds to its target mRNAs to repress translation. Crc shows 37% identity with an AP endonuclease (Carpenter *et al*., 2007). A model based on the crystal structure of the bacterial AP endonuclease was built using I-TASSER server (Roy *et al*., 2010) and an unpaired AAUAAUAA sequence was positioned on the surface of the protein based on the structure of the orthologue human APE1 endonuclease bound to DNA (Mol *et al*., 2000). The Crc regulatory model is adapted from Moreno *et al*. (this issue). The co-ordinate used for the structure of the 30S ribosomal subunit is from Jenner *et al*. (2010).

makes base-specific contacts with the GGA sequence of the Shine and Dalgarno (SD) sequence located in the apical loop, and interacts with the phosphate backbone of the small helix (Fig. 1A). This structure of the complex explains how, by sequestering the SD sequence, RsmE protein represses translation. This is reminiscent of the mechanism by which many sRNAs, which bind to the SD sequence of target mRNAs, repress translation. Hence, sequestration of the SD sequence can be directed through specific protein–mRNA or sRNA–mRNA interactions. The

E. coli CsrB and CsrC sRNAs contain 22 and 13 GGA repeats respectively, which are predicted to bind several CsrA dimers. The formation of stable ribonucleoparticles (RNP) titrates out CsrA from its mRNA targets and inactivates it. CsrA has been shown to upregulate the expression of CsrB/CsrC sRNAs through a two-component system BarA–UvrY, thereby producing an autoregulatory circuit (Babitzke and Romeo, 2007; Timmermans and Van Melderen, 2010).

Two recent studies discovered a very similar system of protein-sequestering sRNAs (Sonnleitner *et al*., 2009; Moreno *et al*., this issue). This system operates in *Pseudomonas* species to regulate Crc, the master regulator of catabolite repression (MacGregor *et al*., 1991; Morales *et al*., 2004; Moreno *et al*., 2007). Crc is an RNA-binding protein, which modifies the expression of more than 135 different genes (Moreno *et al*., 2009a). At the post-transcriptional level, Crc inhibits the transport and/or assimilation of substrates that are not optimal for growth in *Pseudomonas*, such as glucose (a non-preferred compound in *Pseudomonas*), some amino acids or many hydrocarbons. Crc acts by repressing the translation of mRNAs (e.g. *alkS*, *benR*, *xylR*) encoding transcriptional regulatory proteins, and/or inhibiting the synthesis of proteins involved in the transport and/or catabolism of the non-preferred compounds (Collier *et al*., 1996; Hester *et al*., 2000; Moreno *et al*., 2009a). Furthermore, adding further evidence for links between bacterial metabolism and virulence, Crc has also been reported to affect biofilm formation, stress response, antibiotic resistance and virulence of *Pseudomonas aeruginosa* (Linares *et al*., 2010). Thus, like the CsrA/RsmA, Crc protein appears to be a truly global regulatory protein (reviewed in Rojo, 2010; Sonnleitner and Haas, 2011). Interestingly, although Crc does not contain a known RNA binding motif, the protein binds with high affinity an unpaired AANAANAA sequence (where N is C = U > A) located upstream or downstream the AUG initiation codon of target mRNAs (Fig. 1B; Sonnleitner *et al*., 2009; Moreno *et al*., 2009b). This interaction prevents the formation of the initiation ribosomal complex to inhibit translation initiation (Moreno *et al*., 2009b). Another key component of the catabolite regulation in *Pseudomonas* is the two-component system CbrA–CbrB, which is required for growth utilizing a large variety of carbon sources and which is inactive in the presence of preferred carbon sources (Li and Lu, 2007). Thus, CbrA–CbrB counteracts the action of Crc. The discovery of the sRNA CrcZ in both *P. aeruginosa* (408 nt) and *P. putida* (368 nt) suggests a link between Crc and the two-component system CbrA–CbrB (Sonnleitner *et al*., 2009; Moreno *et al*., this issue). Indeed, the expression of this conserved sRNA gene, which is always located downstream *cbrB* in *Pseudomonas* species, appears to be under the positive control of CbrA–CbrB. However, the nature of the signals, which act on the sensor kinase CbrA, is not known. CrcZ contains several Crc recognition motif (AANAANAAN), 5 in *P. aeruginosa* and 6 in *P. putida*. *In vitro* assays show that Crc binds strongly to CrcZ with a similar binding affinity as for the target mRNAs, and forms high molecular weight complexes (Sonnleitner *et al*., 2009; Moreno *et al*., this issue). Furthermore, the six repeats of CrcZ individually bind to Crc albeit with different affinities (Moreno *et al*., this issue). How many Crc molecules bind to CrcZ? Does Crc bind in a cooperative manner to CrcZ? How does Crc recognize its mRNA targets and sRNAs? Does Crc act as a dimer? These questions need further experimental evidence. The 3D structure of Crc is not known. However, the protein shares 30% identity with an AP endonuclease involved in BER reparation pathway (Carpenter *et al*., 2007), which has allowed generating a model that might be of help for future studies (Fig. 1B).

Due to the specific AANAANAAN signature, CsrZ acts as an antagonist and sequesters Crc in a similar manner to what was described for RsmA/CsrA to relieve the regulations exerted by Crc (Sonnleitner *et al*., 2009; Moreno *et al*., this issue). Therefore, the following model has been proposed. When the bacteria are grown with a non-preferred carbon source, the steady state level of CrcZ strongly increases, sequestering Crc protein and allowing translation of the target mRNAs. Conversely, in the presence of a preferred carbon source, the activity of the two-component system CbrA–CbrB decreases, and hence, the expression of CrcZ is stopped allowing Crc to preferentially bind to its target mRNAs (Fig. 1B).

One, two, three antagonist sRNAs for one global regulator

CrcZ-like sRNAs are widely distributed in *Pseudomonas* species. In this issue, Moreno *et al*. have demonstrated that, in addition to CrcZ, *P. putida* contains a second sRNA, CrcY, which contains six Crc-specific binding motifs and, like CrcZ, forms a stable RNP with Crc to counteract its activity. In contrast to CrcZ, CrcY is not found in every *Pseudomonas* species. Furthermore, three CrcZ-like sRNAs exist in *P. syringae* (Moreno *et al*., this issue). The expression of highly similar sRNAs has several biological implications. Such sRNAs might act either in an additive manner or might be fully redundant to translate the signal. In *P. putida*, elimination of one of the two sRNAs led to a compensatory increase in the levels of the remaining sRNA. Only the deletion of the two sRNA genes led to deregulated high levels of Crc, which impaired growth when a non-preferred substrate was used as the sole carbon source. These observations indicate that CrcZ and CrcY sRNAs have redundant functions. Despite this functional redundancy, the inactivation of the response regulator CbrB had a stronger effect on CrcZ expression than on

CrcY synthesis, suggesting that the two sRNAs might be differentially expressed in various conditions in *P. putida* (Moreno *et al.*, this issue). This would allow the bacteria to integrate multiple signals and to enhance the dynamics of the signalling circuits that converge to Crc. Differences in the number of sRNAs antagonist to RsmA/RsmE in *Pseudomonas* species have also been described (Lapouge *et al.*, 2008). In *P. aeruginosa*, for instance, the expression of the two similar sRNAs RsmY and RsmZ is differentially regulated through multiple signalling pathways that converge to the two-component system GacS–GacA (Ventre *et al.*, 2006; Bordi *et al.*, 2010). Intriguingly, the two RNAs exert a redundant action on the repression of T3SS genes, while they have an additive effect for the activation of the *pel* genes (Bordi *et al.*, 2010).

These examples illustrate the complexity of these sRNA-dependent circuits that fine-tune the action of master regulatory proteins and in turn the expression of competing metabolic pathways. Crc belongs to the group of master regulators that do not simply switch on and off gene expression but are essential to optimize the metabolic versatility and interactions with the environment (Rojo, 2010). Hence, the use of RNA antagonists for which the levels can be rapidly adjusted, is well appropriate to control the activity of such a protein. Another advantage of protein sequestration is the reversibility of the regulation. However, how Crc protein can be released from the stable RNP is not known. One obvious factor that can contribute to the release is the modulation of the levels of the sRNAs, which are determined by their relative synthesis and turnover. Interestingly, in *E. coli*, CsrD protein serves as an RNase E adaptor to specifically induce degradation of CsrB, thus impacting on the release of CsrA (Suzuki *et al.*, 2006). Whether such a specific turnover mechanism accounts for CrcY and CrcZ has to be studied.

Molecular mimicry in gene regulation

The work of Moreno *et al.* illustrates how mimicry between two different nucleic acids (sRNA and mRNA) has important functional implications, the most obvious being that similar structures or sequence signatures allow for competitive inhibition of binding to a common partner. In that case, the sRNA serves as a decoy to antagonize the regulatory activity of Crc. Molecular mimicry is often used in post-transcriptional regulation of gene expression (Romby and Springer, 2003). A typical example is the *E. coli* 6S RNA, which binds to the housekeeping form of RNA polymerase (σ70-RNAP) (reviewed in Wassarman, 2007). The secondary structure of 6S RNA mimics the open conformation of a promoter region during transcription initiation that excludes promoter DNA binding, and 6S RNA can serve as a template to produce a 14- to 20-nucleotide pRNA, which allows the release of 6SRNA from RNAP (Wassarman and Saecker, 2006). However, the binding sites of DNA and 6S RNA on RNAP do not totally overlap (Klocko and Wassarman, 2009), and this partial mimicry explains why downregulation is only observed for a subset of promoters. It also indicates that 6S RNA does not totally inhibit RNAP but restricts its function. Molecular mimicry is also often used in feedback regulatory mechanisms. In *Enterobacteriaceae*, the synthesis of many RNA-binding proteins is regulated by a feedback mechanism at the translational level that involves a competition between the natural substrate and the binding site of the protein on its mRNA, which often resemble each other. This mimicry between the primary function and regulation was demonstrated for primary ribosomal proteins (reviewed in Springer *et al.*, 1998), *E. coli* threonyl-tRNA synthetase (reviewed in Romby and Springer, 2003), and ribonucleases (Matsunaga *et al.*, 1996; Schuck *et al.*, 2009). This dual property of these essential RNA-binding proteins is required to adjust their cellular concentration to that of their natural substrates according to cellular needs. Interestingly, decoys not only regulate the activity or the synthesis of proteins, but can also modulate the interaction between sRNAs and mRNA targets. Regulation of chitin metabolism is controlled by several antagonist RNA-RNA complexes (Figueroa-Bossi *et al.*, 2009; Overgaard *et al.*, 2009). The *chiP* gene which encodes a chitoporin is repressed by a constitutive sRNA, ChiX, which interacts with the 5′ leader of *chiP* mRNA. Conversely, the presence of chitooligosaccharides alleviates the repression due to the accumulation of an RNA that pairs with ChiX to promote its degradation, and in turn activates *chiP* mRNA translation. These works gave the new concept of 'target mRNA mimic' in which a target mRNA could also be a competitive inhibitor of sRNA function. Another RNA decoy was described to activate the translation of *E. coli glmS* mRNA, which codes for an essential enzyme involved in sugar metabolism (Reichenbach *et al.*, 2008; Urban and Vogel, 2008). This activation is co-ordinated by two very similar sRNAs, which act in a hierarchical manner. GlmZ sRNA contains a sequence complementary to the 5′ leader region of *glmS* mRNA and in concert with the RNA chaperone protein Hfq, prevents the formation of an inhibitory structure which sequesters the SD sequence. In contrast, GlmY sRNA indirectly positively regulates *glmS* by preventing the 3′ end degradation of GlmZ. In addition, YbhJ protein was shown to counteract the action of the two sRNAs by promoting GlmZ processing (Urban and Vogel, 2008). Although the mechanism is not fully understood, it is tempting to propose that GlmY acts as a decoy to prevent the action of YbhJ on GlmZ processing. Whether YbhJ is an RNA-binding protein remains to be studied. All these examples show that RNA mimicry regulates many processes that can directly alter the function of regulatory sRNAs or of nucleic acid-binding proteins.

Perspectives

With the development of high throughput sequencing and proteomic approaches, many new regulatory pathways involving specific sRNA–protein interactions in bacteria are expected to be discovered. Since many RNA–protein interactions involve mutual conformational changes, we cannot exclude that sRNA might regulate/modify the activity of an enzyme by inducing allosteric changes, recruit other protein partners to form large complexes, or induce specific modifications of proteins. Structural details and deep mechanistic studies of sRNA–protein interactions also remain crucial to their understanding. How many of these interactions will involve the kind of mimicry described for Crc is an open question.

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