Regulatory RNA molecules

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Numerous examples of antisense RNA-mediated gene regulation have been found in bacteria. Such regulatory systems were first identified on accessory genetic elements such as plasmids, transposons and phages, and it is from these that most of our current knowledge of regulatory RNAs is drawn.

Antisense RNAs and their RNA targets typically exhibit considerable secondary structure. Initial interactions between the molecules usually involve complementary unpaired sequences within stem loops, giving rise to 'kissing' complexes. A U-turn motif is often present in one of these loops, which increases antisense RNA pairing kinetics [1]. Intermolecular base pairing can then extend from the hybridised loops, although full duplex formation may not be required for regulatory effect. In the majority of cases, antisense RNAs act to repress translation of their mRNA targets; however, in other cases, antisense RNAs induce transcript attenuation, translational activation and/or accelerated target RNA degradation.

The intricacies of the molecular interactions and the alterative RNA structures induced upon antisense RNA binding show remarkable diversity. More recently, it has become apparent that many small, untranslated RNA molecules (sRNAs) are encoded by bacterial chromosomes. Several of these sRNAs have been shown to be involved in the regulation of virulence genes and of proteins that take part in adaptive stress responses, demonstrating that antisense RNA-mediated gene regulation is more widespread in bacteria than previously anticipated.

Here we illustrate the mechanistic diversity of antisense RNA-mediated gene regulation using plasmid replication control systems as examples, and describe cases where regulatory RNAs have been implicated in the regulation of virulence and stress responses in *Escherichia coli* and *Staphylococcus aureus* (Table 1).

Regulatory RNA control of plasmid copy number

Naturally occurring antisense RNAs were first discovered 25 years ago by independent research groups studying the replication of plasmids ColE1 and R1 from *E. coli* [2, 3]. Both plasmids were found to produce small, untranslated RNA molecules that negatively regulated plasmid replication frequency. Since the abundance of these antisense regulators is proportional to the copy number of the plasmid, they form part of a feedback loop that maintains a steady-state copy number.

Table 1. Examples of bacterial systems controlled by regulatory RNA molecules.

Biological system	Antisense RNA	Target(s)	Primary mode(s) of action
Plasmid replication			
ColE1	RNAI (108 nt)	RNAII preprimer	Prevention of primer maturation
R1	CopA (90 nt)	*repA* mRNA	Inhibition of translation by preventing leader peptide translation
pMU720	RNAI (~70 nt)	*repA* mRNA	Inhibition of translation by preventing pseudoknot activation
pT181	RNAI (87 nt)	*repC* mRNA	Transcription attenuation
pSK41	RNAI (~83 nt)	*rep* mRNA	Direct inhibition of distal translation
Bacterial stress response and virulence			
E. coli	MicF (93 nt)	*ompF* mRNA	Inhibition of translation and degradation
	OxyS (109 nt)	*rpoS*	Sequestration of Hfq activator protein
		fhlA	Inhibition of translation
S. aureus	RNAIII (514 nt)	*hla*	Activation of translation
		spa	Inhibition of transcription, inhibition of translation and RNase III degradation

Plasmid ColE1 is the basis of many commonly used cloning vectors and is the prototype for a large number of closely related high copy number plasmids that are unusual in that they do not encode their own replication initiator protein. Host RNA polymerase synthesises a preprimer, RNAII, approximately 550 nt in length, that hybridises to complementary DNA sequences in the replication origin via single-stranded regions exposed in the loops of RNAII (Figure 1).

RNAII in the hybrid is specifically cleaved by RNase H and the 3'-end of the cleaved RNA primes DNA synthesis by DNA polymerase I. This essential replication step is controlled by a 108 nt antisense RNA, RNAI, that is transcribed from the same region as the primer, but in the opposite direction.

During specific stages of RNAII transcription, RNAI has the opportunity to bind nascent RNAII, resulting in an alteration of its secondary structure that prevents the preprimer transcript forming a persistent hybrid with the origin of replication [4]. RNAI interaction must occur when RNAII is 100-150 nt in length for inhibition to occur. The interaction between RNAI and RNAII is stabilised by the plasmid-encoded Rom protein but this protein is not essential for inhibition and its absence results in only modest increases in plasmid copy number.

Plasmid R1 is typical of many low copy number plasmids, encoding a replication initiator protein, RepA, that is rate limiting for replication. Thus, by regulating RepA synthesis, the frequency of plasmid replication is controlled. RepA synthesis is inhibited at the post-transcriptional level by an antisense RNA, CopA (90 nt), and also at the level of transcription by a repressor protein CopB (not discussed here). The *repA* mRNA contains a short open reading frame in its leader region, *tap,* translation of which causes the unfolding of a stable stem-loop structure that otherwise sequesters the RepA ribosome binding site (RBS), thereby allowing *repA* translation [5]. The CopA countertranscript binds to a region in the *repA* message (CopT), which sterically blocks *tap* translation, and hence indirectly prevents RepA translation. CopA binding also induces RNase III-dependent cleavage of the target RNA although the effect on control is minor.

The replication regions of another group of low copy number plasmids, represented by pMU720 (IncB), are structurally similar to R1-like plasmids but display important differences in replication control. The pMU720 RepA transcript contains a long-range tertiary structure, termed a pseudoknot, that forms between complementary sequences located approximately 100 nt apart in the leader region [6]. Formation of the pseudoknot is required to activate RepA translation.

The RepA transcript also contains an open reading frame encoding a leader peptide to which *repA* is translationally coupled, since translation of the leader peptide induces formation of the pseudoknot. Interaction of the antisense RNA, RNAI, with complementary sequences in the

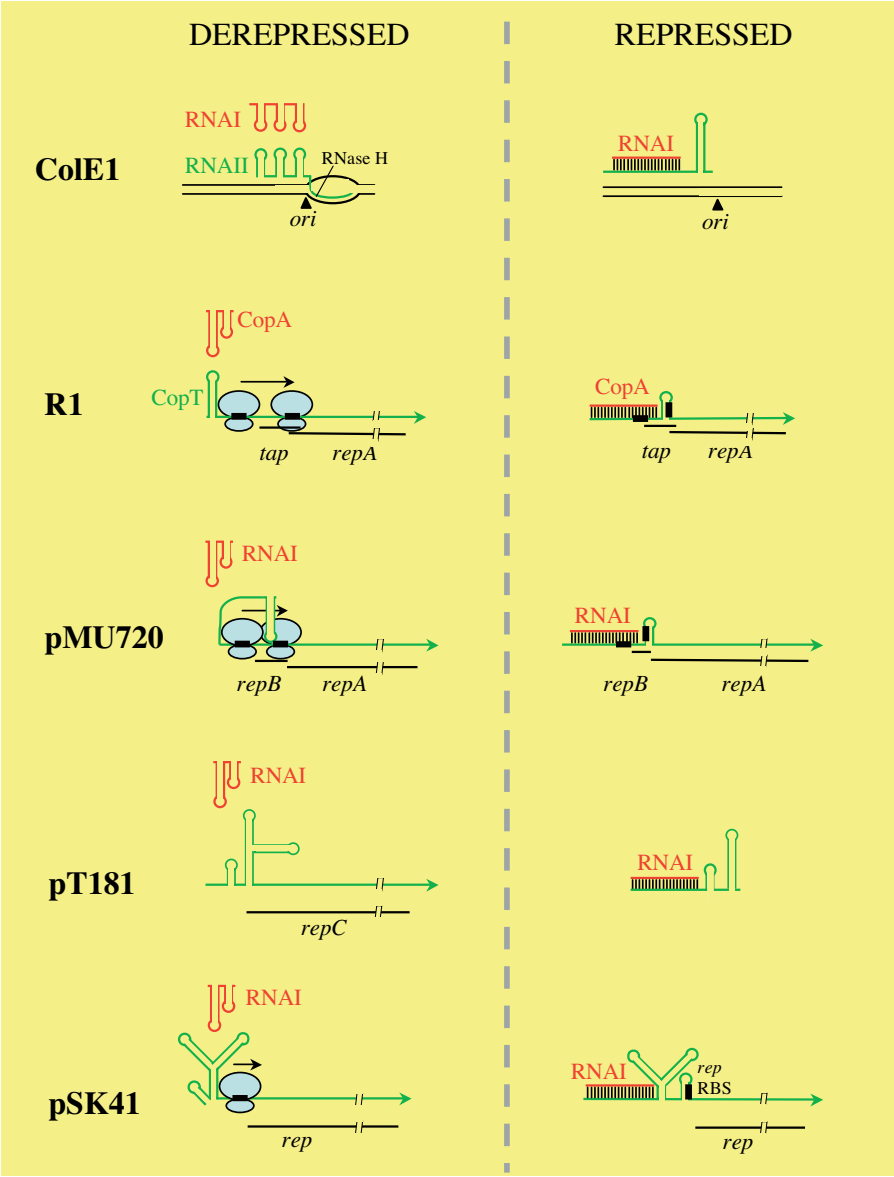

Figure 1. Schematic representation of mechanisms of antisense RNA regulated plasmid copy number control; derepressed (unpaired) and repressed (paired) states are presented. Antisense countertranscripts are shown in red, whereas target transcripts are shown in green. Solid boxes denote ribosome binding sites and blue ribosomes indicate translational proficiency (see text for details).

MICRO-FACT

The E. coli genome encodes 4322 proteins and 122 rRNAs and tRNAs; 91 proteins have signal transducer activity.

repA message blocks translation of the leader peptide, preventing the formation of the pseudoknot activator and therefore repressing *repA* translation.

Multi-drug resistant strains of staphylococci frequently contain one or more plasmids that can vary in size from several kilobases up to around 60 kb. Plasmid pT181 is a small staphylococcal plasmid that utilises a rolling-circle replication mechanism and its copy number is controlled by an antisense RNA-mediated attenuation mechanism [7]. Binding of the antisense RNA induces a stem-loop structure in the *repC* mRNA leader that functions as a rho-independent transcriptional terminator. In the absence of antisense binding, mutually exclusive secondary structures form in the leader that pre-empt formation of the terminator, resulting in read through transcription.

Larger staphylococcal plasmids (>15 kb) frequently carry multiple drug resistance determinants and many have been shown to utilise an evolutionarily related theta-type replication system [8]. A prototype of this family, pSK41, utilises an approximately 83 nt antisense RNA (RNAI) to control replication initiation [9].

The major effect of RNAI binding is translational repression of the *rep* mRNA transcript. RNAI binding is thought to trigger a distal transition in *rep* mRNA leader structure, such that the *rep* RBS becomes sequestered within a step-loop structure. RNAI binding also reduces the amount of *rep* mRNA transcript approximately 3-fold by an unknown mechanism, possibly a novel type of attenuation or through accelerated RNA degradation.

Chromosomally-encoded regulatory RNAs

Small non-coding RNAs (sRNAs) that are encoded by bacterial chromosomes are generally distinguished from classical plasmid-encoded antisense RNA regulators in that they are usually transcribed from a different DNA region to that of their target RNA molecules. Because of this, sRNAs frequently display only limited complementarity to their target RNAs, a feature that can allow them to have multiple regulatory targets. Additionally, sRNAs can act as transcriptional activators by forming structures capable of binding repressor proteins, thereby indirectly activating transcription.

Genome wide searches have uncovered more than 70 sRNA genes encoded by the chromosome of *E. coli* [10]. While many of the regulatory functions have yet to be elucidated, the majority of the sRNAs are present in pathogenic strains, suggesting they may be important for virulence.

MicF was the first sRNA identified and its synthesis is induced by environmental stresses, such as changes in temperature, low osmolarity and redox stress. MicF is a 93 nt antisense RNA that binds its target *ompF* mRNA and regulates *ompF* expression by inhibiting translation and inducing degradation of the message, resulting in alterated membrane properties [11].

OxyS effects the expression of more than 40 genes in *E. coli* and is strongly induced by oxidative stress. OxyS-mediated regulation has been characterised for two genes, *rpoS* (stationary phase sigma factor) and *fhlA* (transcriptional activator of formate metabolism), in which different regions of the 109-nt sRNA are involved.

In the case of *rpoS,* OxyS acts indirectly by sequestration of the Hfq protein, which is required to unfold and activate *rpoS* mRNA, thereby indirectly affecting *rpoS* expression [12]. In the case of *fhlA,* OxyS directly interacts with the *fhlA* mRNA, pairing with the RBS and inhibiting translation [13].

In *S. aureus,* virulence is controlled by the *agr* (accessory gene regulator) locus consisting of two operons that function as a density-sensing cassette and a two-component sensory transduction system. The key effector within *agr* is the multifunctional RNAIII transcript (514 nt), which serves as a messenger RNA, an antisense RNA activator and an antisense RNA repressor [14]. The 5'-region of RNAIII contains the *hld* gene that gives rise to the 26 amino acid δ-hemolysin. RNAIII also stimulates expression of extracellular toxins and enzymes and, in the case of *hla* (α-hemolysin), activates translation by direct interaction with the leader region of *hla* mRNA [15].

In addition, RNAIII inhibits expression of the cell surface protein, protein A (encoded by *spa),* by repressing *spa* mRNA transcription by an unknown mechanism and also by blocking *spa* mRNA translation by directly binding to the RBS. A further 12 sRNA transcripts have been identified in *S. aureus,* five from the core genome and seven from pathogenicity islands that confer virulence and antibiotic resistance [16]. This suggests that sRNAs play a prominent role in complex regulatory networks that coordinate virulence gene expression.

The examples described above demonstrate considerable mechanistic and phenotypic diversity. However, it seems likely that the significance of RNA-mediated regulation in bacteria is yet to be fully realised.

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