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Fresh layers of RNA-mediated regulation in Gram-positive bacteria

Philippe Bouloc¹ and Francis Repoila²

Bacterial regulatory RNAs have been defined as diverse classes of *cis* and *trans* elements that may intervene at each step of gene expression, from RNA and protein synthesis to degradation. Here, we report on a few examples from Gram-positive bacteria that extend the definition of regulatory RNAs to include 5' and 3' UTRs that also act as *cis* and *trans* regulators. New examples unveil the existence of *cis* and *trans* acting regulatory RNAs on a single molecule. Also, we highlight data showing that a key RNA chaperone in *Enterobacteriaceae*, Hfq, does not fulfill the same role in Gram-positive *Firmicutes*.

Addresses

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Introduction

Bacterial survival results from the capacity of the cell to coordinate the expression of genes in response to changing environmental conditions, thus allowing it to adapt and gain novel niches. Besides many proteins, RNA-mediated controls are crucial actors modulating gene expression in bacteria [1]. RNA-mediated regulation involves (i) a target element, either RNA or a protein, and (ii) an RNA-based element made up of a sequence intrinsic to the RNA target (*cis*-regulatory RNA) or a transcript encoded elsewhere in the genome (*trans*-regulatory RNA). *Cis*-regulatory RNAs are usually located at 5' ends of messenger RNAs (mRNA). They fold in alternative structures that respond to physicochemical cues (e.g. temperature, pH) [2] or ligands (e.g., vitamins, nucleotides, ions, tRNA, or amino acids). These latter regulatory RNAs are usually called 'riboswitches' [3]. *Trans*-regulatory RNAs are encoded at loci that are distinct from their

targets. They can bind to proteins, thereby affecting their activity, or pair to mRNAs, changing their translation efficiency, stability or termination.

Seminal publications in the last 20 years have defined these diverse classes of regulatory RNAs and unveiled their modes of action. However, recent studies in *Firmicutes* add 'fresh layers' to the diversity of RNA-mediated processes. This review highlights recent discoveries of diverse mechanisms whereby RNA mediates gene regulation in Gram positive bacteria.

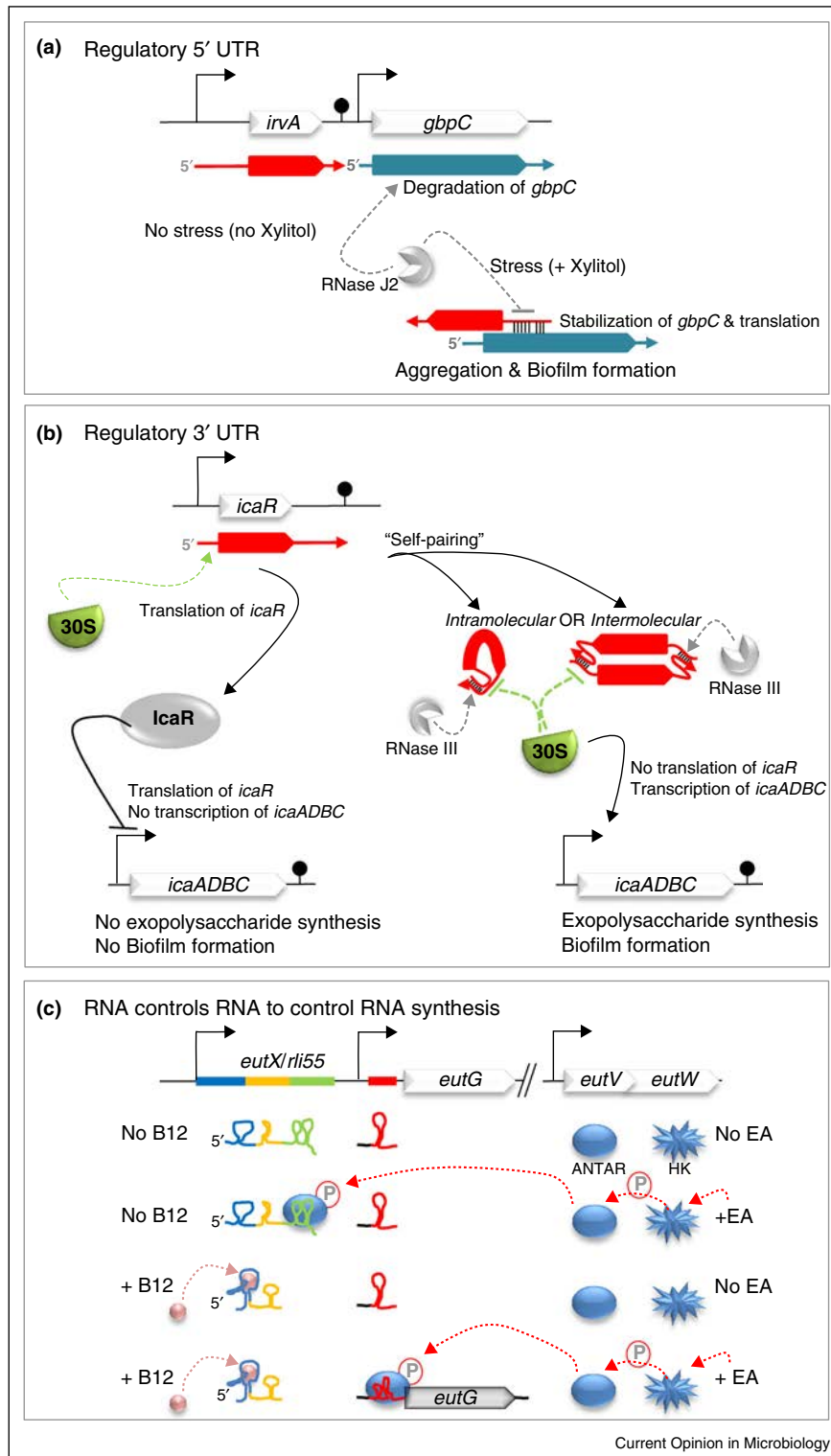
5'UTR, not only the beginning; 3'UTR, not only the end; CDS, not only the coding sequence

A few regulatory RNAs containing an open reading frame, such as SgrS in enteric bacteria [4], SR1 in *Bacillus subtilis* [5], or RNAlII in *Staphylococcus aureus* [6], have been described. In these cases, the expressed proteins are small and tend to be seen as peculiarities of regulatory RNAs. More recently, additional mRNAs encoding transcriptional regulators were demonstrated to act as regulatory RNAs via their 5' or 3' untranslated regions (UTRs), suggesting that the duality 'protein-encoding and regulation' in RNA is more frequent than anticipated.

Bacterial 5' untranslated regions (5'UTR) of mRNAs differ in length from a few to hundreds of nucleotides, and are central to numerous *cis*-regulatory functions. These regions may also sequester regulatory RNAs [7–10] or act as a *trans*-acting factor [11].

Streptococcus mutans is a Gram-positive oral commensal causative agent of dental cavities in humans, producing glucan-binding proteins (Gbp). One of them, GbpC, contributes to biofilm formation, cariogenesis, bacteremia, and infective endocarditis. Its transcription is stimulated by an unusual mechanism involving the *irvA* transcriptional repressor gene [12*]. Surprisingly, the IrvA protein is not the regulatory element required for *gbpC* expression; the process depends solely on an *irvA* mRNA/*gbpC* mRNA interaction, and only the *irvA* mRNA 5'UTR is required. Interestingly, the *gbpC* mRNA target region is not its 5'UTR, but the coding sequence (Figure 1a). The *gbpC* mRNA is susceptible to RNase J2-mediated degradation but its pairing with *irvA* mRNA promotes its stabilization and consequently its translation (Figure 1a). Expression of *irvA* is controlled by IrvR, a self-cleaving LexA-like regulator [13], and environmental stresses, including the sweetener xylitol, trigger *gbpC* expression by affecting its activation regulatory cascade.

Figure 1



(a) Regulatory 5' UTR. In *S. mutans*, expression of glucan-binding protein C is enhanced by *irvA* mRNA. *irvA* mRNA pairs to the coding sequence of *gbpC* mRNA and stabilizes it by preventing RNase J2 degradation [12*]. **(b) Regulatory 3' UTR.** *icaR* mRNA translation in *S. aureus* is auto-controlled. *icaR* encodes a transcriptional repressor of exopolysaccharide synthesis. Depending on environmental conditions, *icaR* mRNA is translated (left part of panel) or not (right part). Repression of *icaR* translation is due to pairing of the 3'UTR to the 5'UTR of the mRNA. The reaction can be intra- or inter-molecular. The formed RNA duplex is recognized and cleaved by RNase III [15]. **(c) RNA controls RNA to control RNA synthesis.** Utilization of ethanolamine (EA) as a source of carbon and nitrogen involves the *eut* operon (symbolized by the first ORF, *eutG*),

Demonstration of the dual function of the *irvA* gene highlights the possibility that many 5'UTR may contribute to regulation independently of their associated coding sequence.

The Hfq protein is a key RNA chaperone in *Enterobacteriaceae* involved in the vast majority of RNA duplexes formed between a *trans*-regulatory RNA and its mRNA target. The recent discovery of mRNA 3' regions enriched by co-immunoprecipitation with Hfq in *Salmonella* and *Escherichia coli* suggested that these sequences were functionally active in an Hfq-dependent manner (reviewed in [14]). These 3' RNA regions could originate (i) from internal promoters to mRNA genes (Type I) or (ii) from mRNA processing (Type II). In both cases, they are a source of regulatory RNAs that end at mRNA terminators [15]. The interactions between 3' RNA ends and Hfq are documented in *Enterobacteriaceae* and suggest a regulatory role for these RNA sequences. However, it is not the case in Gram-positive bacteria where the function of Hfq remains elusive (see below). Nevertheless, tiling-arrays and RNA-seq data revealed an unexpected high density of transcription, with 3'UTRs that are long and sometimes conserved [16–18,19^{*}]. 3'UTRs may also participate in RNA-mediated control: about one-third of characterized staphylococcal mRNAs have long 3'UTR (>100 nt), underscoring their potential importance. Recently, the function of one of these extensions was tackled [20^{**}]. The *icaR* mRNA is about 1000 nt long and encodes a translational regulator from the TetR family (IcaR), which down-regulates synthesis of a main biofilm exopolysaccharide. The *icaR* 390 nt long 3'UTR pairs with the *icaR* 5' end over about 40 nt. This 3'UTR includes a C-rich region that interacts with the *icaR* Shine–Dalgarno (SD) sequence. Deletion of the 3'UTR leads to increased levels of IcaR due to stabilization and improved translation of *icaR* mRNA. Interestingly, authors favor the hypothesis that the UTR acts in *cis*, bridging the 5' and 3' ends and leading to a circular mRNA structure with a stem–loop (Figure 1b). These results are reminiscent of the intermolecular interactions of the *hok/sok* mRNA of plasmid R1 modulating toxin translation [21]. While 3'UTR sequences controlling RNA stability and translation in eukaryotic mRNAs are widely acknowledged, *icaR* expression control by a 3'–5' intramolecular mRNA pairing is thus far an unconventional mode of regulation in bacteria.

Long and overlapping RNAs

Transcription of two opposite DNA strands generates RNAs that can associate by base pairing and contribute

to antisense RNA (asRNA) regulation. In *Listeria monocytogenes*, the presence of unusually long 3' or 5' UTRs, extending over the opposite strands of adjacent genes, led to the concept of 'excludon', which defines the class of genomic loci encoding long asRNAs spanning divergent neighboring genes with related or mutually exclusive functions [22,23]. The mechanistics behind the excludon are not yet characterized. However, one actor in its regulation is likely the double-stranded RNA (dsRNA)-specific nuclease RNaseIII: a recent high-throughput study in *E. coli* using dsRNA-specific antibody (at least 40-mers) led to the identification of asRNAs associated with their cognate sense RNAs in an RNase III-dependent manner, supporting the existence of numerous functional complexes [24]. Observations in *S. aureus* also identify widespread antisense transcription and the role of RNase III in mediating degradation of overlapping transcripts [18,25].

Transcription termination is an obvious means of controlling antisense organization. Interestingly, it can be modulated by its 5' sequence either being a riboswitch or pairing to a *trans*-acting regulatory RNA. In both cases, they affect the activity of the transcription termination factor Rho [7,8,26]. Genome-wide alteration of termination may have a drastic impact; for instance, Rho is an essential protein in *E. coli* [27]. However, *rho* mutants in *S. aureus* and *B. subtilis* are viable. In *B. subtilis*, mRNA extensions can reach up to 12 kb, and Rho appears as a general inhibitor of antisense transcription [19^{*}]. Yet in its absence, *Firmicutes* survive even with extensive inappropriate antisense transcripts, therefore questioning why this aberrant transcription is not deleterious and consequently what is the impact of pervasive transcription in this phylum.

RNA controls RNA to control RNA synthesis

A key element of RNA-based regulation is their transcription, which is often conditionally controlled [19^{*}]. Recently, a new level of complexity was observed in Gram-positive bacteria, with a riboswitch-controlled *trans*-acting RNA. Ethanolamine (EA) is an abundant molecule of the gastrointestinal environment produced by hydrolysis of phosphatidylethanolamine, a crucial component of cell membranes. EA is used as a nitrogen source by many bacteria, but is also utilized as a carbon source by enteropathogens to outcompete the resident microbiota [28]. Among them, *Firmicutes Enterococcus faecalis* and *L. monocytogenes* employ a sophisticated RNA-mediated regulation strategy, combining riboswitch-termination, sRNA-termination and antitermination dependent processes to coordinate EA catabolism

(Figure 1 Legend Continued) the two-component system *eutVW* and the sRNA-riboswitch *eutX* or *rli55* (described in *E. faecalis* or *L. monocytogenes*, respectively; [29^{**},30^{**}]), and requires vitamin B12 (B12). In the presence of EA, the anti-terminator 'ANTAR' protein (i.e., containing an RNA binding domain; [32]), EutV, is activated by phosphorylation, and binds preferentially to an RNA-sequestering sequence present at the 3' end of EutX/Rli55. Transcription of the *eut* operon is stopped by a transcriptional terminator upstream of *eutG*. When B12 is present, it binds to the riboswitch structure at the 5' end of EutX/Rli55, and transcription stops upstream of the RNA binding sequence recognized by the activated EutV. The latter binds to the transcription terminator upstream of *eutG*, and transcription of the *eut* operon may then proceed.

with nutrient availability [29**,30**]. In these species, EA utilization requires expression of the *eut* operon, comprising over a dozen open reading frames (ORFs), and a sRNA encoding gene, *eutX* in *E. faecalis* or *rli55* in *L. monocytogenes*, which lies upstream of *eut*. Transcription of *eutX/rli55* is controlled by a B12-riboswitch aptamer [31]. Full expression of *eut* is achieved in the presence of EA and vitamin B12, an essential cofactor for enzymes encoded by the operon. In *E. faecalis*, EA in the environment is sensed by a two component system comprising a histidine kinase, EutW, and its cognate response regulator EutV that belongs to the ANTAR RNA-binding protein family. ANTAR regulatory proteins act as transcription antiterminators [32]. When EA is absent, *eut* transcription stops at a terminator preceding the first ORF of the operon (*eutG*), and EutV is not phosphorylated and inactive; in this condition, *eutX/rli55* produces a full length sRNA. In the presence of EA, EutV is phosphorylated and binds to EutX/Rli55. EutV is thereby inactive on *eut* expression as it is sequestered by the sRNA. When EA and B12 are both present, B12 binds to the aptamer of EutX/Rli55 and provokes formation of a transcription terminator upstream of the EutV binding sequence, generating a truncated sRNA that no longer sequesters the active response regulator. EutV then binds to the nascent *eut* operonic RNA and allows transcription of downstream ORFs (Figure 1c).

The Hfq mystery in Gram-positive bacteria

Hfq is a well-characterized RNA binding protein involved in post-transcriptional regulation (reviewed in [33]). In *Enterobacteriaceae*, Hfq stimulates base-pairing of *trans*-encoded RNAs with their RNA-partner molecules and consequently, its absence produces highly pleiotropic phenotypes.

However, within the *Firmicutes* phylum, Hfq is either not found (e.g. *Lactobacillales*) or when present, it seems to marginally affect adaptation and gene expression. The absence of Hfq in *B. subtilis* and *S. aureus* does not impact growth in nearly two thousand tested growth conditions [34,35], nor *L. monocytogenes* in standard laboratory growth conditions [36]. Nevertheless, in *L. monocytogenes*, Hfq does contribute to osmotic and ethanol stress adaptation [36]. Comparative transcriptome studies in these organisms with their respective *hfq* derivatives reveal moderate or no transcriptional variations as compared to *Enterobacteriaceae* [17,34,37,38]. Although Hfqs of these Gram-positive species bind to RNAs [37,39,40], their contribution in sRNA-mediated regulation remains enigmatic. So far, no sRNA-dependent regulation in *S. aureus* and *B. subtilis* was shown to require Hfq (e.g. in *S. aureus* [41–44] and in *B. subtilis* [45–49]). In *B. subtilis* Hfq is required for *ahrC* mRNA translation, a target of SR1 sRNA; oddly, however, the stimulatory effect of Hfq is SR1-independent [46,47]. Within the class of Bacilli, the only documented sRNA/Hfq-dependent regulation concerns the *L.*

monocytogenes sRNA LhrA, which directly down-regulates expression of at least three genes and is protected from degradation by Hfq [50,51]. However, a transcriptome study indicated that in the tested conditions, the absence of Hfq did not impact the abundance of over 60 sRNAs (including LhrA) [17,52]. Consequently, as was seen in *S. aureus* and *B. subtilis*, sRNA stabilization by Hfq in *Listeria* is likely not a general trait. Complementation of either *Salmonella* or *E. coli* Δhfq mutants has often been obtained by *hfq* genes from other species (e.g. [53]); however Hfq from *B. subtilis* or *S. aureus* does not compensate the absence of Hfq in *Salmonella* [34,54]. These results indicate that the ability of those Hfq proteins to stimulate sRNA/mRNA regulation is either lost or restricted to specific pairs.

As genomes of *Firmicutes* have a low GC content, it was suggested that RNA pairing properties would be different in this phylum and consequently Hfq would not be required for sRNA-mediated regulation [55]. However, recent results show that Hfq plays an essential role in *Clostridium difficile* [56*] which is 29% GC. Depletion of Hfq drastically affects its growth and gene expression, and notably, alters the levels of sRNAs shown to bind Hfq [56*]. Hfq from *C. difficile* restores phenotypes associated to the Δhfq mutation in *E. coli* [53], further supporting its functional role in sRNA regulation.

Hfq forms a homo-hexameric donut-like structure. A conserved arginine rich sequence (RRER) on the outer rim of Hfq is required for *E. coli* Hfq chaperone function [57]. This motif is not conserved in *S. aureus* Hfq, and the presence of just one arginine in *B. subtilis* and *Listeria* [57] may explain why Hfq is not associated with general sRNA regulations. In contrast, two arginines present in the *Clostridium* Hfq motif (RKER) may contribute to its activity.

The *hfq* gene is present in most, if not all, natural isolates of *B. subtilis*, *S. aureus* and *Listeria*. It was recently shown that Hfq is involved in *B. subtilis* stationary phase survival and therefore provides a selective advantage for this soil bacterium, which encounters starvation periods [34,38]. Similarly, Hfq contributes to the long-term survival of *L. monocytogenes* under amino-acid-limiting conditions, which could explain its contribution to pathogenicity [36]. Possibly, the observed *hfq* virulence-associated phenotype in *S. aureus* [37] could be related to adaptation to stationary phase. In contrast to the generalized activity of Hfq proteins in *Enterobacteriaceae*, the orthologs present in the *Firmicutes* appear to act on a restricted set of substrates, indicating a more specialized role.

Concluding remarks

Past discoveries on posttranscriptional regulation initially emerged from studies in *E. coli* and *Salmonella*. The new results from Gram-positive bacteria, some of which are highlighted here, bring to light a wide diversity of

mechanisms that regulate bacterial physiology. Different mechanisms may have been selected to cope with specific sets of enzymes acting on RNA metabolism (e.g. RNases [58,59]), alternate composition (e.g. to accommodate low G + C genome composition) or other evolutionary constraints. Analysis of the distribution of short and long RNA fractions in several *Firmicutes* revealed the existence of a generalized antisense transcription with processing patterns that differ from those in Gram-negative bacteria [18]. Different phyla were shown to have distinct regulatory features, for example, with respect to sRNA Hfq-dependency, Rho essentiality, and RNase components. We expect that novel alternative regulatory solutions established in Gram-positive bacteria remain to be found.

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