

An overview of RNAs with regulatory functions in gram-positive bacteria

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Abstract During the last decade, RNA molecules with regulatory functions on gene expression have benefited from a renewed interest. In bacteria, recent high throughput computational and experimental approaches have led to the discovery that 10–20% of all genes code for RNAs with critical regulatory roles in metabolic, physiological and pathogenic processes. The *trans*-acting RNAs comprise the noncoding RNAs, RNAs with a short open reading frame and antisense RNAs. Many of these RNAs act through binding to their target mRNAs while others modulate protein activity or target DNA. The *cis*-acting RNAs include regulatory regions of mRNAs that can respond to various signals. These RNAs often provide the missing link between sensing changing conditions in the environment and fine-tuning the subsequent biological responses. Information on their various functions and modes of action has been well documented for gram-negative bacteria. Here, we summarize the current knowledge of regulatory RNAs in gram-positive bacteria.

Keywords Regulatory RNAs · Small RNAs · Noncoding RNAs · Riboswitches · CRISPR · Gene expression regulation · Virulence · Gram-positive bacteria

Introduction

Until recently, research on the control of gene expression in bacteria had focused mostly on the regulatory functions of proteins. Besides proteins, RNA molecules have emerged lately as key players in gene regulation in adaptive responses [1–3]. Natural antisense RNAs were first discovered as plasmid-encoded elements that are major regulators of plasmid copy number control (i.e., ColE1 and R1 plasmids) [4, 5]. A couple of years later, the first chromosomally encoded regulatory RNA was revealed to act as an antisense RNA and inhibit the translation of an outer membrane porin in *Escherichia coli* (i.e., *micF* RNA/OmpF porin) [6, 7]. With the development of genome-wide searches, more than >80 small RNAs were discovered encoded on the chromosome of *Escherichia coli* [1]. To date, impressive technical advances such as high throughput computational searches [8, 9], deep-sequencing [10] and tiled microarrays [11, 12] have led to the prediction and/or characterization of numerous novel RNAs in various bacteria. Overall 10–20% of genes in bacteria code for RNAs that are likely to have regulatory functions on gene expression. Due to their rather small sizes (from 80 to 500 nucleotides), these RNAs are usually called small RNAs (sRNAs).

Substantial efforts have been made to determine sRNA functions in various physiological responses, identify their primary targets, decipher the mechanisms by which they affect the targets and analyze their participation in more complex regulatory networks in the cell [1–3, 13]. The

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diverse biological roles of RNA-based elements encompass the regulation of metabolism (e.g., carbohydrate metabolism, metabolite transport, synthesis and degradation), growth processes (e.g., toxicity, biofilm formation), adaptation to stress and varying culture conditions (e.g., temperature, iron limitation, cell density). Moreover, recent evidence shows that regulatory RNAs play key roles in microbial pathogenesis [1, 14–17]. During infection, bacterial pathogens have to quickly react to changing environmental conditions in the host. Subsequent adaptive responses include survival strategies in specific body niches, avoidance of the immune system and systemic toxicity. In the multiple infectious strategies that bacterial pathogens have developed, regulatory RNAs are considered as signal transducers of environmental cues by participating in the precise coordination of gene expression. Several sRNAs have been shown to regulate the production of virulence factors, while others regulate pathogenic traits by adapting the metabolism of the bacteria in response to the host. Recent studies have focused on species-specific sRNAs and their direct implication in virulence. However, a number of regulatory RNAs, such as riboswitches, are conserved across gram-negative and gram-positive bacteria. These elements play critical functions in metabolism and contribute to the maintenance of cell survival and cell integrity, especially during exposure to stress. Bacteria also use regulatory RNAs to interfere with foreign DNA invasion processes, such as lytic bacteriophage infection or plasmid conjugation [18, 19].

Regulatory RNAs employ diverse mechanisms to modulate expression of their targets, functioning primarily at the post-transcriptional level. By using mRNAs as targets, they can affect transcription, translation, RNA stability, maturation and processing [1]. Protein targets can be sequestered away from their biological functions by regulatory RNAs, thus inducing numerous downstream effects [20–22]. When binding to metabolites, tRNAs, proteins and *cis*-acting regulatory regions of mRNAs can change conformation and affect transcription or translation of the downstream genes [23, 24]. More recently, DNA has been postulated to function as a novel target for regulatory RNAs (i.e., CRISPR family), broadening further the range of RNA-mediated strategies [18, 19].

RNA-based elements in gram-negative bacteria have been extensively reviewed in a number of recently published manuscripts [1–3, 13–17, 20–22]. Except for the class of riboswitches, information on RNA-mediated gene regulation in gram-positive species is rather limited. Here, we provide a comprehensive summary of the information available in the literature on RNAs with regulatory functions in gram-positive bacteria.

A diversity of regulatory RNAs in gram-positive bacteria

As shown for gram-negative bacteria [1, 3, 17, 23, 24], gram-positive bacteria have developed numerous RNA-dependent regulatory mechanisms to respond to internal and external signals. These regulatory RNAs can be divided into four main classes: (1) The *trans*-acting sRNAs act as antisense RNAs by basepairing with target mRNAs (Fig. 1). Two different mechanisms have been elucidated for this class of RNAs. In the first case, the sRNA and the target mRNA are encoded on the same DNA locus, but are transcribed in opposite directions. As a result, both RNAs form extended basepairings. In the second case, the sRNA and target mRNAs are encoded on different DNA loci and share only partial basepairing complementarities. These sRNAs can target multiple mRNAs. Some of these sRNAs are bona fide non-coding RNAs (ncRNAs), while others may carry small open reading frames (ORFs). (2) The second class involves RNAs, which can sequester regulatory proteins, thus perturbing their activity. RNAs that are part of ribonucleoprotein complexes and exert cellular housekeeping functions are included in this class. (3) The third class consists of a unique family of small processed RNAs, the CRISPR RNAs, recently described to regulate DNA maintenance. (4) The fourth class comprises the *cis*-acting regulatory regions of mRNAs, which respond to *trans*-acting agents [e.g., intracellular concentration of metabolites (riboswitches), uncharged tRNAs (T-boxes) or proteins] or to environmental cues [e.g., temperature (thermosensors)] (Fig. 2).

sRNAs acting at the mRNA level in gram-positive bacteria

Generalities

In gram-positive bacterial pathogens, all reported biological functions of the class of sRNAs are linked to adaptation or virulence processes. Of interest, several sRNA-encoded genes are embedded within pathogenicity islands (e.g., *Staphylococcus aureus*, [25]). Among the sRNAs encoded on the genome, some are associated with loci expressing two-component regulatory systems (e.g., *S. aureus* RNAIII, *Streptococcus pyogenes* *fasX*) or transcriptional regulators (e.g., *S. pyogenes* *rivX*) (Table 1) [26]. In non-pathogenic species such as *Bacillus subtilis*, sRNAs are suspected to regulate metabolism and sporulation. Most sRNAs acting at the mRNA level are described as ncRNAs. However, two sRNAs are larger in size compared to conventional sRNAs and encode proteins with virulence functions (i.e.,

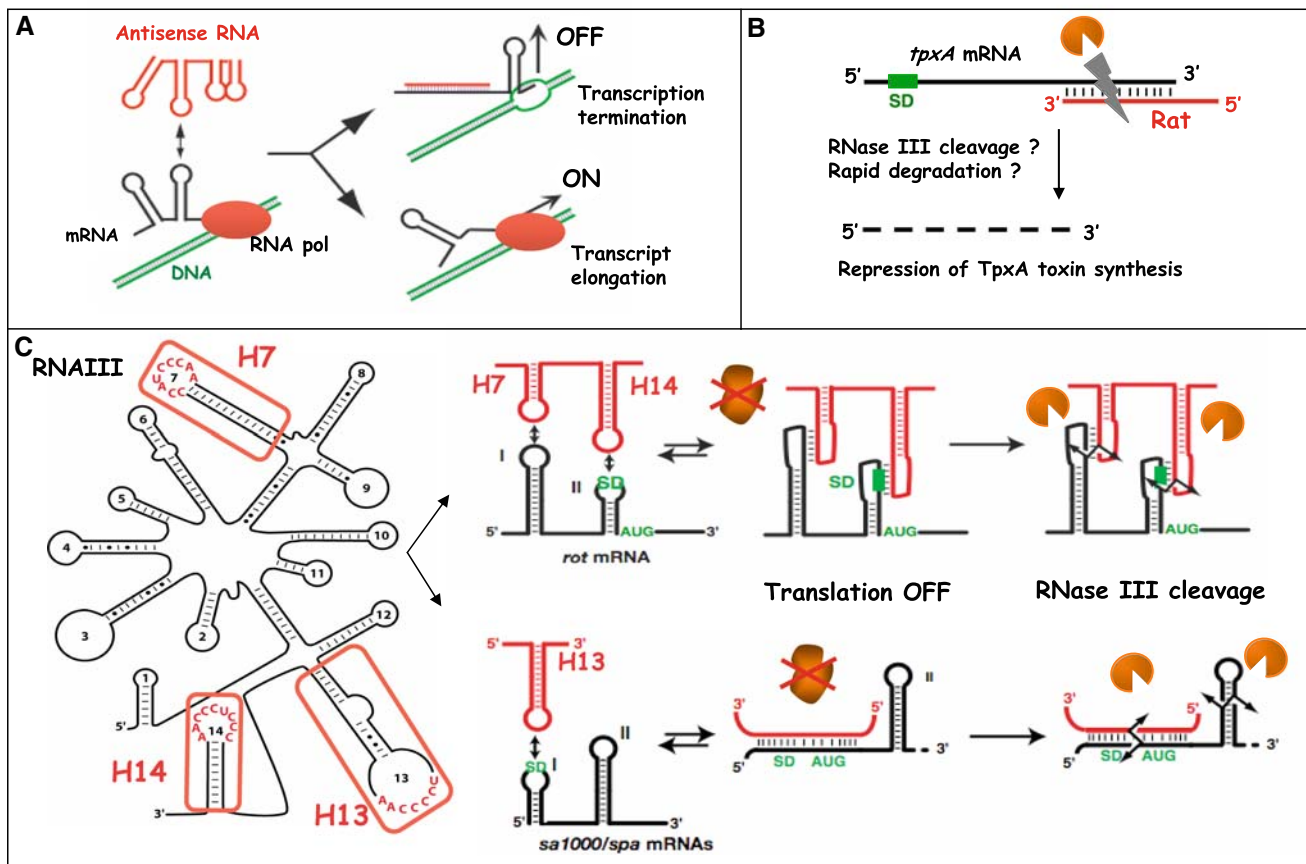


Fig. 1 Mechanisms of action of *trans*-acting antisense sRNAs in gram-positive bacteria. **a** Schematic drawing of antisense RNA-mediated transcription attenuation in plasmid pT181 (according to [155]). The antisense RNA (red) initiates binding with the mRNA (black) by a loop-loop interaction, which rapidly propagates to form a long duplex. The formation of the duplex stabilizes the formation of a transcription terminator and arrests the elongation of transcription. In the absence of the antisense RNA, the mRNA structure forms an anti-terminator structure allowing the synthesis of RepA protein. **b** Schematic drawing of the antisense RNA Rat-mediated degradation of *tpxA* mRNA from *B. subtilis* (according to [43]). The antisense

RNA is fully complementary to the 3' end of the mRNA and induces degradation of the target mRNA. **c** Structure and mechanism of action of the quorum-sensing RNAIII from *S. aureus*. The secondary structure of RNAIII is from [52] with the three hairpins 7, 13 and 14. RNAIII uses different hairpin motifs to bind various mRNAs encoding virulence factors (protein A, fibrinogen-binding protein SA1000) and the transcriptional regulatory protein Rot. Formation of RNAIII-mRNA complexes blocks the access of the 30S ribosomal subunit and concomitantly recruits the endoribonuclease III to cleave the repressed mRNAs [27]. SD is for Shine-Dalgarno sequence

S. aureus RNAIII, *S. pyogenes pel*) (Table 1). The sRNAs that have been investigated to date are expressed mainly in a growth phase-dependent manner, some being under the control of quorum-sensing systems (e.g., *S. aureus* RNAIII) or sporulation-specific transcriptional factors (e.g., *B. subtilis* SurA, SurC) (Table 1). Although it is assumed that the sRNAs are part of complex regulatory processes involved in adaptation and pathogenicity, the knowledge of factors, stimuli or environmental conditions being responsible for modulation of sRNA expression is lacking overall. Only a few transcriptional regulators and two-component systems have been reported to control their expression (Table 1).

Investigation into the mode of action of *trans*-acting sRNAs in gram-positive bacteria has revealed antisense

mechanisms of the sRNAs with their target mRNAs, which leads to either inhibition or enhancement of mRNA translation initiation (e.g., *B. subtilis* SR1, *S. aureus* RNAIII) and/or mRNA degradation (e.g., *S. aureus* RNAIII) [27–33]. The knowledge of protein contribution in these mechanisms is also limited. The role of the Sm-like Hfq protein, which functions as a RNA chaperone in gram-negative bacteria, is dispensable in many gram-positive bacteria, although the protein was found to be important for virulence in some species.

In the following section we will discuss separately the sRNAs encoded on the same locus as the target mRNA (fully complementary to the target mRNA) and the sRNAs encoded in an autonomous locus on the chromosome (partial complementarity with the target mRNAs). Selected

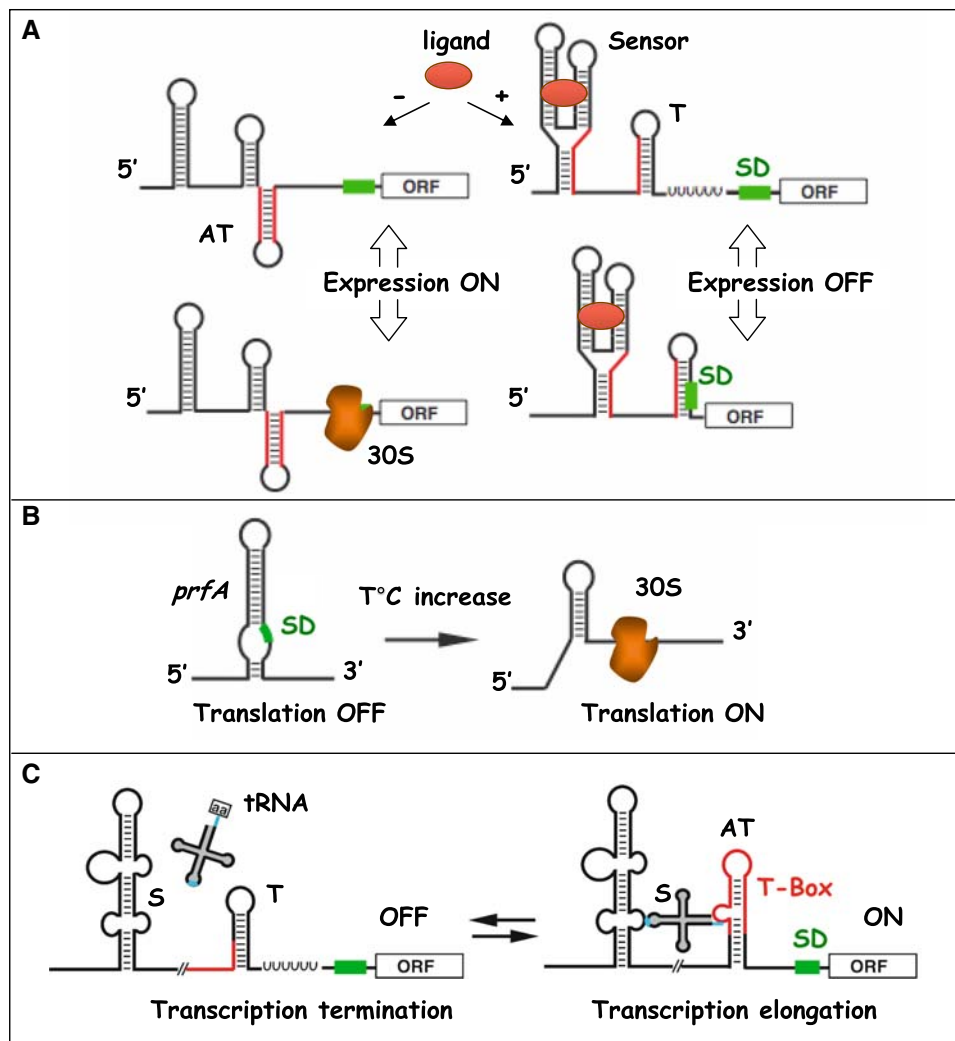


Fig. 2 Mechanisms of action of *cis*-acting regulatory regions of mRNAs in gram-positive bacteria. **a** Transcriptional attenuation (*top*) and translational (*below*) control mediated by the binding of a ligand. In the absence of ligand, the mRNA forms an anti-terminator hairpin and transcription proceeds through the open reading frame (ORF), or alternatively, the ribosome binding site (RBS) is available for translation initiation. In the presence of ligand, the sensor domain is stabilized by the direct recognition of ligand, causing the formation of a transcription terminator or alternatively a structure that sequesters the RBS. SD is for Shine-Dalgarno sequence. **b** *L. monocytogenes prfA* mRNA is regulated by a temperature-dependent mechanism [115]. At low temperature, the mRNA adopts a secondary structure, which blocks access of the 30S ribosomal subunit. At 37°C, the structure melts, allowing access of the 30S subunit and translation of PrfA, a major transcriptional regulator of virulence gene expression.

c The T-box family of tRNA-mediated riboswitches. The uncharged tRNA acts as the signal molecule directly sensed by the 5' untranslated region of the mRNA. When no uncharged tRNA is sensed, a transcription terminator in the T-box of the controlled mRNA is formed, leading to its transcription termination. Upon sensing of the uncharged tRNA, two interactions (*blue*) with the leader region including the T-box (*red*) take place. The first one is the pairing of the uncharged tRNA anticodon with the so-called specifier sequence (S) acting as a codon mimic. The second interaction involves pairing of the 3'CCA end of the tRNA with a complementary sequence in the leader region. As a result, an anti-terminator sequence is stabilized, and continued transcription of the downstream controlled ORF is allowed. T is for terminator and AT for anti-terminator of transcription

examples are described to illustrate the diversity of mechanisms and functions of these regulatory RNAs. Many novel sRNAs have been recently predicted or analyzed in various gram-positive bacteria and are waiting further functional and mechanistic studies (Table 1). It is very likely that many of them might directly target mRNAs through basepairing.

Fully complementary antisense sRNAs control plasmid replication or act as antitoxins

Natural antisense RNA-regulated systems were first described in accessory genetic elements such as plasmids, phages and transposons [34–36]. These antisense RNAs are rather small (around 55–150 nt long), diffusible and

Table 1 *Trans-acting sRNAs in gram-positive bacteria*

Bacterial species/sRNA	Target ^a	Target function	Mechanism of action	Other biological function	References
<i>B. subtilis</i>					
BS190	ND	–	ND	ND	[140, 141]
BS203	ND	–	ND	ND	[140, 141]
BsrE, BsrF, BsrG, BsrH, BsrI	ND	–	ND	ND	[140, 141]
SR1 ^a	<i>ahrC</i>	Transcriptional activator of arginine catabolic operons	Antisense sRNA	Regulated by sugars	[38–40]
SurA, SurC, <i>polC-yksI</i> IGR	ND	–	–	Activated under sporulation-specific conditions	[36, 41]
<i>C. perfringens</i>					
VR RNA	<i>colA</i>	Kappa-toxin	ND	Regulated by the Vir/VirS system	[123, 124]
	<i>cpd</i>	2', 3'-cyclic nucleotide phosphodiesterase	ND		
	<i>plc</i>	Protein tyrosine phosphatase	ND		
	<i>ptp</i>	Cystathionine gamma synthase (<i>metB</i>)	ND		
	<i>ycgJ-metB-cysK-ygaG</i>	cysteine synthase (<i>cysK</i>)	ND		
<i>L. monocytogenes</i> ^b					
LhrA	ND		ND	Binds Hfq	[12, 62, 63]
LhrB	ND		L20 regulatory region	Binds Hfq	[12, 62, 63]
LhrC1-5	ND		ND	Binds Hfq	[12, 62, 63]
<i>rilA</i>	ND		ND		[37]
<i>rilB</i>	<i>lmo2104</i>	Ferrous iron transport protein FeoA	Antisense sRNA	Carries 5 long repeats	[37]
	<i>lmo2105</i>	Ferrous iron transport protein FeoB	Antisense sRNA		[37]
<i>rilC</i>	ND		ND		[37]
<i>rilD</i>	ND		ND		[37]
<i>rilE</i>	<i>comEA</i>	Competence factors	Antisense sRNA		[37]
	<i>comFA</i>	Competence factors	Antisense sRNA		
	<i>lmo0945</i>	Competence factors	Antisense sRNA		
<i>rilF</i>	ND		ND		[37]
<i>rilG</i>	ND		ND		[37]
<i>rilH</i>	ND		ND		[37]
<i>rilI</i>	<i>lmo1035</i>	Phosphotransferase system	Antisense sRNA		[37]
<i>sbrA</i>	ND		ND	Activated by Sigma B	[12, 64]
<i>rlt22</i>	ND		ND	ND	[12]

Table 1 continued

Bacterial species/sRNA	Target ^a	Target function	Mechanism of action	Other biological function	References
<i>rli23, rli25, rli35</i>	<i>Imo0172</i> , 0330, 0828	Transposase	Antisense homologous sRNAs	ND	[12]
<i>rli24, rli26-27</i>	ND		ND	ND	[12]
<i>rli28, rli50</i>	ND		Homologous sRNAs	ND	[12]
<i>rli29</i>	<i>Imo0471</i>		Antisense sRNA complementary to the 5'UTR	ND	[12]
<i>rli30</i>	<i>Imo0506</i>		Antisense sRNA	ND	[12]
<i>rli31, rli32</i>	ND		ND	ND	[12]
<i>rli33</i>			ND	Activated by Sigma B	[12]
<i>rli34, rli36</i>	ND		ND	ND	[12]
<i>rli37</i>	ND		Carries 1 ORF 58 aa	ND	[12]
<i>rli39</i>	ND		Cobalamine riboswitch? (RFAM)	ND	[12]
<i>rli40</i>	ND		Carries 1 ORF 64 aa	ND	[12]
<i>rli41</i>	ND		Carries 2 ORF 45 and 35 aa	ND	[12]
<i>rli42</i>	ND		ND	ND	[12]
<i>rli43</i>	ND		Carries 1 ORF 35 aa	ND	[12]
<i>rli44</i>	ND		Carries 1 ORF 28 aa	Repressed by SigmaB	[12]
<i>rli45, rli46</i>	ND		rli45 antisense to rli46	sRNA regulatory networks	[12]
<i>rli47</i>	ND		ND	Activated by Sigma B	[12]
<i>rli48-49</i>	ND		ND	ND	[12]
<i>S. aureus</i>					
RNAIII ^a	<i>hla</i>	Alpha-hemolysin	Antisense sRNA (translation initiation)	Regulated by the <i>agr</i> system, global effect on virulence factor expression and phenotypes	[51–53]
	<i>rot</i>	Repressor of toxin	Antisense sRNA (translation initiation, RNase III dependent hydrolysis)		
	<i>spa</i>	Protein A			
	<i>sal000</i>	Fibrinogen-binding protein			
	<i>lyrM</i>	Peptidoglycan hydrolase			
	Coa	Coagulase			
<i>sprA</i>	<i>sa2216</i>	ABC transporter	Possible antisense sRNA	ND	[25]
<i>sprA2, sprA3, sprB, sprC, sprD, sprE, speF, sprG, sprFG2, sprFG3</i>	ND		ND	ND	[25]
<i>rsaOA, rsaOB</i>	ND		ND	ND	[9]

Table 1 continued

Bacterial species/sRNA	Target ^a	Target function	Mechanism of action	Other biological function	References
<i>S. pneumoniae</i>					
csRNA1	ND		ND	Controlled by CiaR of the CiaRH system	[131]
csRNA2	ND		ND		
csRNA3	ND		ND	csRNA4 and 5 involved in stationary phase autolysis	
csRNA4	ND		ND		
csRNA5	ND		ND		
<i>S. pyogenes</i>					
<i>faxX</i>	<i>fbp54</i>	Fibronectin-binding protein	ND	Regulated by amino-acid starvation, the FasBAC and <i>luxS</i> systems	[125–128]
	<i>mrp</i>	Fibrinogen-binding protein	ND		
	<i>Ska</i>	Streptokinase	ND		
	<i>SLS</i>	Streptolysin S	ND		
<i>pel</i>	<i>emm</i>	M protein	ND	Regulated by cell-density mechanism	[129]
	<i>sic</i>	Streptococcal inhibitor of complement	ND		
	<i>SpeB</i>	Cysteine protease	ND		
<i>rivX</i>	<i>emm</i>	M protein	Dependent on RivR, ND	Controlled by the regulator CovR	[130]
	<i>fbpA</i>	Fibronectin-binding protein	Dependent on RivR, ND		
	<i>grm</i>	Gene-regulated by Mga	Dependent on RivR, ND		
	<i>Mga</i>	Response regulator	Dependent on RivR, ND		
	<i>scpA</i>	C5a peptidase	Dependent on RivR, ND		
	<i>scrA</i>	Phosphotransferase system	Dependent on RivR, ND		
	<i>speB</i>	Cysteine protease	Dependent on RivR, ND		

^a *hla*, *rot*, *spa*, *sal000*, *lytM*, *coa* mRNAs are direct targets of RNAlIII; *ahvC* mRNA is a direct target of SRI

^b The expression of all *L. monocytogenes* sRNAs changed under various experimental conditions and after growth in the intestinal lumen and blood: the sRNAs (lhrC, rliB, rli24, rli26, rli33, rli37–38, and rli42) were induced in blood; sRNAs (rli28, rli36, rli39, rli45–48) were found enhanced in the intestinal lumen; other sRNAs (rli22, rli27, rli29, rli40) were found modified in blood and intestinal lumen. The expression of all sRNAs is not modified in the mutant Δhfq strain. Data according to [12], and see supplementary materials

^c Chevalier, Geissmann, Romby, unpublished data

This table does not include the housekeeping ncRNAs (RNase P, rRNA, tRNA, SRP) and 6S RNA (see text)

untranslated. They form long duplexes with their target mRNA. Several plasmid-encoded antisense RNAs hosted by gram-positive bacteria regulate replication, conjugation or post-segregational killing using various mechanisms, i.e., transcriptional attenuation and translational repression [35]. For example, replication control of the *S. aureus* rolling-circle-replicating plasmid pT181 [37] and the *B. subtilis* theta-replicating plasmid pIP501 [38] involves transcription attenuation. In both cases, the plasmid-encoded antisense RNAs bind to the 5' untranslated region (5'UTR) of *repA* mRNA encoding the initiator replication protein RepA. This results in the stabilization of an alternative conformation of the mRNA allowing the formation of a terminator structure in the leader region of *repA* mRNA [39]. In consequence, elongation is arrested, and the expression of the target mRNA is inhibited (Fig. 1a). In contrast, for the *S. aureus* pSK41 replicon, an antisense RNA was shown to repress indirectly the translation of *repA* mRNA. This antisense RNA binds the target mRNA far upstream of the ribosome binding site (RBS) and thereby induces the stabilization of a hairpin structure that sequesters the RBS [40]. In *Enterococcus faecalis*, the *par* locus of plasmid pAD1, which encodes the toxic protein Fst and an antisense RNA as the antidote, is the first plasmid-killer system identified in gram-positive bacteria [35, 41, 42]. The two genes are transcribed convergently and overlap at the level of the bi-directional terminator. The two RNAs contain two additional short regions of complementarity, which correspond to two distant repeats [35]. These two repeat motifs in the mRNA are located close to the *fst* RBS and downstream of the *fst* gene. Interaction between antisense and target RNAs within these two distant complementary regions suffices to block ribosome binding and thus *fst* translation [41, 42]. It was shown that plasmid-encoded antisense RNAs associate rapidly with the target mRNA, and it is the rate of formation rather than the affinity of duplexes that determines efficient inhibition [34–36].

Several fully complementary antisense RNAs encoded on the chromosome of several gram-positive bacteria have been also described [12, 43, 44]. Some of these chromosomally encoded antisense RNAs repress the synthesis of potentially toxic proteins homologous to plasmid-encoded or mobile element-encoded toxins [13]. In *B. subtilis*, RatA is described as an antidote sRNA, which is encoded on a cryptic prophage. This RNA affects the accumulation of the overlapping convergently transcribed mRNA, which encodes the toxic peptide TxpA (for toxic nucleotides). RatA basepairs with the 3' end of the *txpA* mRNA (Fig. 1b), and experimental data suggest that the duplex triggers target mRNA degradation [43]. Several short and long antisense RNAs have also been recently discovered in *L. monocytogenes* ([12], see below).

sRNAs regulate metabolism, and sporulation in *Bacilli*

SR1 was identified as a ncRNA involved in the fine-tuning of arginine catabolism. Expression of SR1 is derepressed under conditions of gluconeogenesis but repressed under glycolytic conditions [31]. The primary target of SR1 is *ahrC* mRNA, which encodes the transcriptional activator of arginine catabolic operons [28, 29, 31]. SR1 acts as an antisense RNA that interacts with the coding region of *ahrC* mRNA and prevents translation initiation by changing the structure of the target mRNA in the vicinity of the RBS [29].

Transcriptional profiling using microarrays combined with the use of comparative genomic algorithms revealed a number of additional sRNAs, the expression of which is activated under sporulation-specific conditions [45]. SurA and SurC expression is under the control of the master sporulation regulator Spo0A and the sigma factor Sigma K, respectively. Expression of three other intergenic transcripts from the operon *polC-ylxS* is induced by the alternative Sigma G and Sigma K factors [45]. Other highly expressed sRNAs were identified in *B. subtilis*, but their functions remain unknown [46].

A multifunctional sRNA regulates virulence in *S. aureus*

The best-studied case of sRNAs controlling bacterial infectious processes is RNAIII from *S. aureus*, the intracellular effector of the *agr* (accessory gene regulator) operon. *S. aureus* has evolved a plethora of sensory systems that turn on/off the expression of virulence genes in response to both environmental and host signals. Complex regulatory networks exist that involve several two-component systems, transcriptional regulatory proteins and regulatory RNAs [47, 48]. Among these systems, the *agr* operon, which functions as a sensor of the population density, was shown to be required for virulence in animal models [49–51]. The *agr* operon encodes the two-component system (sensor kinase AgrC and response regulator AgrA) and the quorum-sensing cassette (membrane protease AgrB and secreted auto-inducing peptide AIP). At high cell density, AgrA activates the transcription of its own operon from the P2 promoter and of the regulatory RNAIII from the P3 promoter. RNAIII acts as the intracellular effector of the *agr* regulon and controls the switch between the early expression of surface proteins and the late expression of exotoxins [33]. Detailed mechanistic studies show that RNAIII acts primarily at the post-transcriptional level and that it targets multiple mRNAs (Table 1). The RNA uses independent structural domains to perform its different tasks [52]. RNAIII is a mRNA that encodes the hemolysin δ along with noncoding regions that carry

specific regulatory functions (Fig. 1c). The 5' domain of RNAIII activates translation of *hla* mRNA encoding α -hemolysin by competing directly with an intramolecular mRNA structure that sequesters the *hla* RBS [32, 33]. The 3' end and the central domain of RNAIII repress the synthesis of major cell surface virulence factors (protein A, coagulase, fibrinogen-binding protein) as well as the transcriptional regulator Rot [27, 30, 53]. Repression of the synthesis of multiple proteins by RNAIII involves a shared mechanism whereby three C-rich hairpin loops of RNAIII bind to the Shine and Dalgarno (SD) sequence of the target mRNAs (Fig. 1c). The RNAIII-mRNA interactions block the access of the ribosome at its loading site and concomitantly recruit the endoribonuclease III, which cleaves the formed complexes [27, 30]. By repressing the synthesis of the regulator Rot, RNAIII also induces many downstream effects and indirectly activates the synthesis of many exotoxins.

Besides the major regulatory RNAIII, several stable RNAs were identified in infectious *S. aureus* strains. Seven of those were found encoded on pathogenicity islands, which carry important virulence factors [25]. Remarkably, expression of the sRNAs showed large variation among clinical isolates. Based on these observations, it was suggested that these RNAs contribute to *S. aureus* niche adaptation and pathogenicity [25]. One of the prophage sRNAs, *sprA*, was shown to form a stable complex with an ABC transporter encoding mRNA [25].

Other numerous stable RNAs that are differentially expressed under stringent, cold and heat shock responses were identified by microarrays in *S. aureus* [54, 55]. Many of them might originate from protein coding transcripts, while others might be antisense RNAs. Another study based on a high-scale computational approach predicted numerous ncRNA candidates in 932 bacterial strains including 47 novel ncRNA candidates in *S. aureus* [8, 56]. A more recent phylogenetic profiling study analyzing information on intergenic and coding regions of all available bacterial genomes has shown that five regulatory regions of mRNAs and one novel sRNA are expressed in *S. aureus* N315 strain [9]. Another group's evaluation of the intergenic regions of the *S. aureus* genome using bioinformatics and expression analysis has revealed multiple regulatory regions in the genome and the existence of ten additional sRNAs [57]. The transcription of three sRNAs is activated by the alternative SigmaB factor. Most of these novel sRNAs carry conserved and unpaired C-rich sequences as found in RNAIII. One of these RNAs has been shown to bind to the ribosome binding site of mRNAs encoding metabolic enzymes through its C-rich motif [57]. Thus, the unpaired C-rich motif might be considered as a signature for ncRNAs, which interact with mRNAs in low GC gram-positive bacteria.

Is the RNA chaperone protein Hfq associated with sRNA-mediated regulation in gram-positive bacteria?

In gram-negative bacteria, Hfq is an RNA chaperone that plays a crucial role in sRNA-dependent regulation by either stabilizing the sRNAs or optimizing sRNA/mRNA duplex formation [58, 59]. However, the function of Hfq as a critical co-factor in sRNA-mediated regulatory mechanisms in gram-positive bacteria is unclear. For example, the streptococcal and lactobacillal genomes do not contain any obvious Hfq homologue-encoding gene [60]. In *S. aureus*, *hfq* mRNA appears to be weakly transcribed [53], and the function of Hfq in stress response, virulence factor production or metabolism has not yet been determined [61]. Although *S. aureus* Hfq binds to RNA in vitro, the protein does not stimulate RNAIII-dependent repression of target mRNAs in vivo and is dispensable for RNA-RNA interaction such as RNAIII-*spa* mRNA [27, 30, 61] and *sprA* sRNA-mRNA [25]. Furthermore, *S. aureus* Hfq was reported to not fully complement a *hfq*-deficient mutant of *E. coli* [62]. This study also shows that the C-terminal extension of the *E. coli* Hfq constitutes an RNA interaction surface with specificity for mRNAs. This C-terminal extension is lacking in *S. aureus*, thus suggesting that the protein has lost some RNA binding properties. In *B. subtilis*, Hfq is dispensable for the stability of SR1 RNA and the interaction of SR1 RNA with its *ahrC* mRNA target, but is required for *ahrC* mRNA translation [28]. Hfq is also not required for the action of the *cis*-acting antitoxin RatA sRNA on its *tpxA* mRNA target [43]. In all these cases, the sRNA is able to rapidly form sRNA-mRNA basepairings. In *L. monocytogenes*, Hfq contributes to tolerance to osmotic and ethanol stress, long-term survival under amino acid-limiting growth conditions and pathogenesis in mice [63, 64]. Transcription of *hfq* mRNA is weak under rich medium culture conditions, but is induced under stress exposure in a Sigma B-dependent fashion [63, 64]. Hfq was used as bait to fish the sRNAs LhrA, LhrB and LhrC [64, 65]. However, recent work shows that LhrB and LhrA are not bona fide ncRNAs, but originate from mRNA leaders, therefore questioning the role of Hfq for these putative *cis*-acting RNA elements [12, 65]. Finally, the stability of many newly discovered *L. monocytogenes* ncRNAs was not affected in a *hfq*-deficient mutant [12, 44, 65].

Taken together, the lack of *hfq* homologues in some gram-positive species, the weak expression of *hfq* in others and the poor evidence for a requirement of Hfq in sRNA stability or sRNA-mRNA interaction cast a direct involvement of Hfq in regard to sRNA-mediated regulation in gram-positive bacteria. Whether other RNA chaperones, helper or helicase proteins substituting for Hfq are required in the basepairing process of sRNAs with their target mRNAs remains to be determined.

RNAs modulating protein activity in gram-positive bacteria

RNAs with protein sequestration activity

Regulatory RNAs also have the property to bind proteins and thereby regulate their activities. This protein sequestration ability was demonstrated in *E. coli* for CsrB sRNA (targeting the global carbon storage regulatory CsrA protein), GlmY sRNA (targeting YhbJ protein) and 6S RNA (targeting Sigma 70-RNA polymerase) [20–22]. These sRNAs have the common feature to be able to mimic structures of the natural substrates for target proteins and thereby counteract their activity. For example in *E. coli*, 6S RNA mimics open promoters that bind the Sigma 70-RNA polymerase and thus can titrate the transcriptional factor away from the promoters. Control of gene expression by 6S RNA was shown to occur in response to the shift from exponential to stationary phase of growth, suggesting that 6S RNA is a critical factor in bacterial adaptation processes [22].

In gram-positive bacteria, no protein sequestration mechanism such as CsrB/CsrA and GlmY/YhbJ has been identified yet. However, 6S RNA, which is conserved among bacterial species, is likely to control gene expression in gram-positive bacteria by sequestering the alternative sigma factor of RNA polymerase as suggested for *B. subtilis* [66]. In this species, two abundant 6S RNAs are discoordinately expressed during growth, emphasizing the critical role of 6S RNA in bacterial adaptation [66, 67].

RNAs of ribonucleoprotein complexes

The class of protein-binding RNAs includes RNAs that are part of ribonucleoprotein complexes (RNPs). The RNPs have important housekeeping functions. They are involved in protein biogenesis and metabolism and as such are crucial for the maintenance of cell integrity when bacteria are challenged by stresses encountered in their environments.

The bacterial RNase P consists of a single catalytic RNA component associated with a protein subunit. In *E. coli*, this holoenzyme catalyzes the removal of the 5' leader sequence of pre-tRNAs to produce the mature 5' terminus of tRNAs [68–70]. RNase P has been studied in *B. subtilis*, *Clostridium sporogenes* and *S. aureus* [71–73]. Interestingly, an additional function for RNase P was reported in *B. subtilis*. RNase P cleaves the coenzyme B12 and adenine riboswitches in the absence of metabolite binding. It was proposed that this cleavage might contribute to the stabilization of mRNA under normal conditions [74, 75].

In bacteria, the signal recognition particle (SRP) consists of the 4.5S RNA (also called scRNA for small

cytoplasmic RNA) and the Ffh protein [76]. SRP is a universally conserved pathway for targeting polypeptides to the cell membrane via the cotranslational machinery. SRP binds protein signal sequences as they exit the ribosome and targets them to the bacterial membrane. Here the SRP complex binds the membrane-associated receptor FtsY, which releases the signal peptide from the Sec translocon. In gram-positive bacteria, SRP is thought to be the main mechanism for targeting polypeptides. Although SRP is considered an essential cellular component, this pathway is dispensable in *Streptococcus* where it plays important roles in the secretion of various virulence factors and in biofilm formation [77–81].

Another RNA modulating protein activity is tmRNA, also called *ssrA* RNA. In bacteria, tmRNA is a specialized RNA with properties of both a tRNA and an mRNA [82–84]. tmRNA bound to the small protein SmpB interacts with the translational ribosomal complexes stalled at the 3' end of a truncated mRNA and accepts the nascent polypeptide in the same fashion as does the elongator tRNA. Translation is continued on the short mRNA encoded within tmRNA by adding a peptide tag to the truncated protein at its C-terminus. The peptide tag, which contains recognition determinants for intracellular proteases, then targets the tagged protein for rapid degradation. This mechanism, called *trans*-translation, plays a crucial role in protein quality control pathways. It is involved in cell viability, development, response to stress and virulence when bacteria need to execute large changes in their genetic programs [82]. In this regard in *B. subtilis*, tmRNA was shown to be required for sporulation events [85, 86].

CRISPR RNAs in gram-positive bacteria

CRISPR RNAs emerged recently as a unique family of regulatory RNAs [87]. They are encoded within the CRISPR loci that are widely present in bacteria and archaea. To date CRISPR has been associated with two biological functions: resistance to lytic phages in *Streptococcus thermophilus* [88] and interference with plasmid conjugation in *S. epidermidis* [18]. Besides these functions, CRISPR has been considered for a long time as a valuable tool for strain typing and classification, as well as for the evaluation of the historical background of bacterial clones. The CRISPR system has retained recent attention based on certain similarities with the eukaryotic RNAi-driven gene silencing system [1, 87]. Functional CRISPR loci consist of highly variable DNA sequences including a leader sequence followed by a series of repeat-spacer units and of several CRISPR-associated (Cas, Csn) genes. The DNA arrays can contain from 2 to 249 copies of the same repeat. The CRISPR repeats can vary from 24 to 47 base pairs and

show significant divergence in sequence among bacteria. However, significant sequence similarity among the repeats is observed within species. The repeats are regularly interspaced with unique spacers of 26–72 base pairs. The spacer sequences are not conserved and often vary among strains of the same species. All sequence spacers studied so far are homologous to DNA from phages or plasmids. The CRISPR-associated genes were predicted to code proteins containing putative RNA- and DNA-binding domains, helicase motifs and endo- and exo-ribonuclease activities.

CRISPR DNA loci are transcribed as full-length RNAs, which are rapidly processed into shorter RNAs (crRNAs, psiRNAs). In *E. coli*, the CasE protein from the Cascade complex CasA-E was shown to be responsible for the processing event [89]. Of note, in the thermophilic Archaea *Pyrococcus furiosus*, Cas6 functions as a novel endoribonuclease that cleaves specifically CRISPR RNAs within the repeat sequences to release the individual invader targeting RNAs [90]. Based on these data and on the sequence analysis of CRISPR loci, a model for the mode of action of CRISPR RNAs has been proposed. The full-length CRISPR locus is transcribed as a RNA that is further processed by the Cascade complex of CAS proteins into unique single repeat-spacer units called crRNAs [18, 89]. Similar to the RNAi machinery of eukaryotes, CAS proteins would act as essential cofactors to direct basepairings of the crRNA spacer sequences with phage or plasmid target sequences. Although it has been postulated in the past that CRISPR RNAs targeted mRNAs [91], it recently became evident that they target phages (Charpentier, personal communication) and plasmids [18] at the DNA level. Future studies are necessary to decipher the exact molecular mechanism whereby the CRISPR RNAs recognize the extrachromosomal DNA target, the subsequent fate of the target and the exact contribution of the CAS proteins in this process. Efforts are also needed to understand why foreign DNA is preferentially used for integration in the CRISPR loci as well as the mechanisms of spacer recognition, selection and insertion in between the repeats within the DNA region. It is worth noting that so far knowledge available on CRISPR biological function was produced mainly in *Streptococcus* and *Staphylococcus* species [18].

Cis-acting RNA elements in gram-positive bacteria

A plethora of *cis*-acting mRNA elements in gram-positive bacteria

RNA molecules have the extraordinary ability to adopt various structures that can efficiently regulate the expression of genes they encode. This characteristic is largely exploited by the *cis*-acting RNAs, which are usually

located in the 5' untranslated mRNA leader regions of the regulated genes. They can be recognized by a variety of *trans*-acting regulators (metabolites, uncharged tRNAs, proteins) but can also sense environmental signals (temperature, divalent ions) without the contribution of any additional regulatory factor [23, 24, 92–98]. As a result of binding to their ligands, the leader regions of mRNAs undergo significant structural changes modifying expression of the transcripts (Fig. 2). The *cis*-acting mediated regulatory effects include attenuation of transcription, translation initiation and mRNA stability [23, 99, 100]. Globally, this class of RNA elements is involved in cellular metabolism and is expected to be critical in adaptive strategies of bacteria. Some of these elements can also directly affect virulence as exemplified by the *pfrA* mRNA thermosensor in *L. monocytogenes*. Overall, more than 70 *cis*-acting regulatory RNAs were identified in *B. subtilis* [92, 101, 102]. More recently, 40 functional riboswitches (metabolites, T-boxes, leaders of r-protein operons and PyrR protein-sensing riboswitch) were described in *L. monocytogenes* [12]. This study also reveals 13 novel regulatory regions of mRNAs. Of interest, two sense the temperature, while expression of most of these RNAs is regulated during infection in the intestinal lumen of mice or in blood [12]. The study also demonstrates that the metabolism of bacteria is submitted to large variations during the infectious process. Such a panel of regulatory RNAs thus offers the advantage for the bacteria to be able to integrate multiple signals to express particular gene products. In the complexity of pathogenic processes, the bacteria need to adapt to numerous changing environments within the host and to coordinate accordingly gene expression at appropriate times and spaces during infection, and these sophisticated RNA-based sensors become critical. From the knowledge available in the literature, gram-positive bacteria seem to rely more on *cis*-acting RNAs to regulate gene expression than gram-negative bacteria. Furthermore, riboswitches in gram-positive bacteria more frequently utilize transcription attenuation, whereas those in gram-negative bacteria favor translational inhibition [92, 103, 104]. In contrast to *trans*-acting sRNAs, the *cis*-acting regulatory RNAs are quite well conserved among firmicutes. Selected typical examples of riboswitches in several gram-positive bacteria are described in the following section.

Riboswitch RNAs sensing metabolites

Riboswitches consist of two functional domains [23, 24, 92–98]. The aptamer or the sensor domain is a conserved and structured receptor that is specifically recognized by a defined ligand. The second domain is the expression platform that is responsible for the expression switch of the

downstream ORFs as a result of ligand binding. Depending on the positioning of the riboswitch on the mRNA, regulation can occur at the transcriptional or post-transcriptional levels. These regulations lead either to repression or to activation of gene expression. At the transcriptional level, ligand binding can alter the ability of RNA polymerase in the process of transcription elongation through the formation of intrinsic transcription terminator/anti-terminator structures. At the translational level, ligand binding can lead to the formation of structures that either sequester the RBS or render the RBS accessible, thereby affecting the ability to translate the mRNA (Fig. 2a). Riboswitches can also induce site-specific cleavage in the mRNA, inducing its rapid degradation [100]. So far, riboswitches were shown to interact with cofactors [e.g., thiamin pyrophosphate (TPP), flavin mononucleotide (FMN), adenosyl cobalamin, *S*-adenosyl methionine (SAM)], nucleobases (e.g., adenine, guanine), amino acids (e.g., glycine, lysine), sugar derivatives (e.g., glucosamine-6 phosphate) or divalent ions (Mg^{2+}). The controlled genes are often involved in the biosynthesis and/or transport of the ligands. As a result of this control, the expression of metabolic enzymes or transporters becomes adapted to the intracellular concentration of the metabolite. This metabolite sensing system can be considered as a feedback regulation mechanism allowing the maintenance of physiological levels of the metabolites.

Most of the riboswitches sense one single metabolite. Remarkably, riboswitches arranged in tandem and able to respond to two distinct metabolites exist as well. This is the case of the *metE* mRNA from *Bacillus clausii*, which contains in its 5'UTR one riboswitch responding to *S*-adenosylmethionine and a second one responding to coenzyme B12 [105]. The two ligands can repress independently the tandem riboswitch, thereby exerting a higher degree of control on gene expression.

Another interesting example is the *glmS* regulatory region that is present in certain gram-positive species, which affects mRNA stability in response to ligand binding [106–109]. This “riboswitch” presents unique characteristics. The structure of the regulatory domain was solved in the absence and in the presence of glucosamine 6-phosphate (GlcN6P), revealing that the ribozyme adopts a pre-formed compact structure in the absence of the metabolite. Therefore, this region does not behave as a classical “riboswitch” [107]. Binding to GlcN6P stimulates a self-cleaving ribozyme activity whereby the ligand itself acts as a coenzyme in the self-cleavage reaction by binding to a pre-folded active site pocket in the RNA. In this reaction, Mg^{2+} works as a cofactor in ribozyme folding and might be rate-limiting for catalytic activity [110].

RNA elements in 5'UTRs can also function as metal-sensing regulatory RNAs, i.e., “M-box” in *B. subtilis*

[111]. The M-box functions as a divalent metal sensor for the Mg^{2+} -specific regulation of the *mgtE* mRNA encoding a Mg^{2+} transport protein. The box appears to utilize a transcription attenuation-like genetic control mechanism through predicted Mg^{2+} -regulated switching between mutually exclusive helices in the RNA. Thus, the M-box constitutes an example whereby control of gene expression by an RNA sensor can be achieved by simply tuning the tertiary structure formation to a particular metal binding affinity.

Recent X-ray structures of aptamer domains of several metabolite-sensing riboswitches have unraveled novel recognition modes [99, 100] and provided a molecular explanation for the selective specificity of the metabolite to bind the RNA sensor. These structures contain various junctions and tertiary contacts between distant domains that generate compact folds and specific clefts for ligand recognition. Some of the metabolites bind to regulatory sequences, while others participate in tertiary interactions. In general, the common feature of these structures is to sequester part of the sequence of the expression platform, leading to a different genetic response (Fig. 2a). Recent single-molecule and NMR analyses of a purine riboswitch show that the mRNA structures fold sequentially and that metabolite binding stabilizes the pre-formed folding of the sensor domain [94, 112]. Furthermore, the rate of RNA transcriptional elongation and the extent of the RNA polymerase pausing were shown to influence the folding of the sensor and the genetic response [113].

Riboswitch RNAs sensing environmental cues

Besides sensing metabolites, RNAs can also respond to environmental cues such as temperature. The RNA thermosensors, also called thermometers, constitute a unique class of structural RNA elements. They do not require any ligand recognition domain, but instead the regulatory function of the RNA element is adjusted in response to a change in temperature. These RNA elements affect expression of the downstream-located genes usually by sequestration of the RBS. In this system, the RBS is sequestered within an inhibitory helix (Fig. 2b). An increase of temperature affects the stability of the helix by melting, which results in an enhanced accessibility of the RBS to the ribosomal initiation complex. These regulatory elements are often used to control the expression of genes involved in cell response to sudden changes in temperature, such as heat-shock genes [114]. Interestingly, this type of regulatory element is also directly involved in virulence. This is the case for the *pfrA* mRNA thermosensor in *L. monocytogenes*, which is responsible for expression of the global regulator of virulence factor expression PfrA at 37°C [115] (Fig. 2b).

tRNA-mediated riboswitches

In gram-positive bacteria, the T-box family of riboswitches commonly controls the expression of genes involved in tRNA aminoacylation, amino acid transport and biosynthesis [103, 104]. T-box RNAs are also found in leader regions of mRNAs encoding transcriptional regulators and enzymes involved in quorum sensing [103]. The uncharged tRNA is the signal molecule directly sensed by the T-box mRNA element (Fig. 2c). The 5'UTRs of the controlled mRNAs, including the T-box element, exhibit a conserved set of sequence and structural features, which can fold into two alternative hairpin structures, an intrinsic transcription terminator or a competing transcription anti-terminator. Specificity of regulation is based on pairings between the anticodon of the uncharged tRNA and a codon-mimick, the so-called specifier sequence, located in the sensor domain of the T-box mRNA. A second interaction common to all tRNAs involves the 3'CCA end of the tRNA and a complementary sequence in the T-box leader region. As a result of sensing uncharged tRNA, the anti-terminator structure is stabilized, and continued transcription of the downstream genes is allowed (Fig. 2c). T-box RNA leaders can also control translation initiation. In this case, binding of the uncharged tRNA induces structural rearrangements in the leader region of mRNAs, thus promoting accessibility of the RBS to the ribosome [103, 104]. T-box RNAs are widespread among firmicutes but underrepresented in gram-negative bacteria. Furthermore, T-box RNAs that regulate transcription termination are observed primarily in low GC gram-positive bacteria, while T-box RNAs that control translation initiation predominate in high GC gram-positive and in some gram-negative bacteria [104]. It is intriguing that tandem T-box sequences were also found upstream of biosynthetic genes, thus adding a level of complexity in the fine-tuning of regulation. An example of amino acid metabolism regulated by T-boxes is illustrated below with tryptophan biosynthesis.

Cis-acting RNAs sensing proteins

A number of RNA-binding proteins mediate structural switches within 5'UTRs of mRNAs that can affect either transcriptional termination or translational initiation of the genes located downstream. They are key regulatory players in auto-regulation processes of ribosomal protein synthesis, amino acid metabolism and pyrimidine metabolism. Many of these proteins are often expressed constitutively, and their activity is modulated in response to the regulated effector (metabolite, RNA).

In gram-negative bacteria, the expression of ribosomal proteins is feedback regulated at the level of translation. The regulatory r-proteins have the properties to bind tightly

to the rRNAs and to play a role in the assembly process of the ribosome. When the intracellular concentration of the r-protein exceeds that of the rRNA, the free regulatory r-protein (not bound to the ribosome) acts at the mRNA level to repress the synthesis of its whole operon. In many cases, the regulatory r-proteins recognize structurally similar rRNA and mRNA binding sites [116]. The mechanism is based on a competition between the rRNA and the mRNA for binding to the r-protein. The general principle of this autoregulation mechanism was also demonstrated for the *B. subtilis* *rpsD* mRNA [117] and *infC* operon [118], and for the *B. stearothermophilus* *rpsO* mRNA [119]. For the *B. subtilis* *infC* operon, L20 protein binds to the conserved leader region of the operon mRNA that mimics the 23S rRNA binding site, and thereby stabilizes the formation of a transcriptional terminator that induces premature transcription arrest [118].

In *Bacillus* species, expression of the *pyr* genes for the de novo pyrimidine nucleotide biosynthesis is regulated by a transcriptional attenuation mechanism involving the PyrR protein [120]. An RNA segment with conserved sequence and secondary structure located in the 5'UTR of *pyr* genes can alternatively form transcription anti-terminator/terminator stem-loop structures. Interestingly, PyrR senses the intracellular concentrations of the nucleotide UMP and binds to UMP only when present at high concentrations in the cell. Under these conditions, PyrR binds to a *pyr* regulatory RNA element in the leader region of the mRNA and induces formation of a transcription terminator. With this mechanism, the expression of downstream genes responsible for biosynthesis of pyrimidines is regulated by both exogenous metabolite (UMP) together with protein PyrR in a coordinated manner. Of interest, recent work shows that transcription of the *pyr* operon in *L. monocytogenes* is enhanced during blood infection [12].

Another regulatory protein in *Bacillus*, which senses the intracellular concentration of a metabolite, is the *trp* RNA-binding attenuation protein (TRAP) [121]. This protein plays a central role in the control of tryptophan biosynthesis and transport. At high concentrations of tryptophan, the amino acid binds to TRAP and induces allosteric activation of the protein. As a result, TRAP-Trp binds to multiple tryptophan codon repeats in the leader regions of target transcripts. For expression of the *trpEDCFBA* operon (encoding the tryptophan biosynthetic enzymes), TRAP-trp induces transcriptional termination and blocks translation of the mRNAs that have escaped termination by promoting the formation of a structure that sequesters the *trpE* RBS. Recent works in *B. subtilis* show that the transcription elongator factor NusA, a protein that binds to RNA polymerase, stimulates the intrinsic termination and pausing of the polymerase in the *trpEDCFBA* operon leader [122]. RNA polymerase pauses contribute to the

transcription attenuation and translational control mechanisms, presumably by providing time for TRAP-Trp to bind to the nascent *trp* leader transcript during transcription of the mRNA. One may expect that NusA protein might have a more general role in the regulation mediated by *cis*-acting mRNA structures. Another degree of complexity is the involvement of a protein called anti-TRAP (AT), which antagonizes the activation of TRAP by tryptophan when the charged tRNA^{Trp} levels in the cell decrease [123]. Recent X-ray structures of the AT-TRAP complex reveal that AT binds to TRAP as a trimer and sterically blocks the binding to RNA [124]. In addition, expression of AT is regulated at the transcriptional level by a T-box located in the leader sequence of its own mRNA and by a leader peptide-dependent translational control mechanism [125]. The accumulation of uncharged tRNA^{Trp} promotes transcription anti-termination of the *at* operon and induces translation of the *rtpA* (encoding AT) coding region. The consequence of AT synthesis is inactivation of TRAP and elevated expression of all genes controlled by TRAP. To insure an adequate supply of charged tRNA for protein synthesis during this process, the uncharged tRNA coordinately increases transcription of the *trpS* operon encoding the tryptophanyl-tRNA synthetase. Taken together, control of tryptophan metabolism involves interplay of several auto-regulatory systems that sense the free tryptophan levels and the uncharged tRNA^{Trp} [121].

Another example of protein-dependent riboswitch is the control of genes for the metabolism of carbohydrates that are taken up by the phosphotransferase system (PTS) in *B. subtilis* [126]. Four homologous proteins (SacY, SacT, LicT, GlcT) of the BglG/SacY family act as antiterminators of transcription of genes and operons involved in sucrose metabolism (SacY, SacT), oligo- β -glucoside and aryl- β -glucoside metabolism (LicT) and glucose assimilation (GlcT) [127–133]. In the absence of the cognate PTS-sugar, a transcriptional terminator present in the untranslated mRNA leader of the target genes prevents transcriptional elongation. In the presence of the cognate PTS-sugars, the proteins become activated, bind to and stabilize a conserved motif called the ribonucleic antiterminator (RAT) present in the mRNA leader. As a consequence, the formation of an overlapping transcription terminator is prevented and gene expression can take place. Remarkably, the antiterminator proteins can be negatively or positively controlled through phosphorylation [126].

Cis-acting RNAs sensing antibiotics

Translational attenuation by site-specific ribosome stalling is a mechanism used for the control of expression of antibiotic resistance genes such as *cat* (chloramphenicol acetyl transferase) and *erm* (methyltransferase) [134–136].

In the case of erythromycin resistance gene control, the inducible *ermC* gene in *B. subtilis* is preceded by a short ORF encoding a regulatory leader peptide, ErmCL. In the absence of an inducing antibiotic, the RBS of *ermC* is sequestered in an mRNA secondary structure and *ermC* expression is repressed. Sub-inhibitory concentrations of an inducer (erythromycin or similar macrolides) cause the ribosome to stall at the *ermCL* ORF, triggering a conformational change in the mRNA that releases the *ermC* RBS sequestration and activates expression of the methylase gene. A similar mechanism was proposed to occur for *S. aureus* *ermA* whose product confers resistance to the macrolide-lincosamide-streptogramin B family of antibiotics [137].

Other RNAs with regulatory functions in gram-positive bacteria

Many novel chromosomally encoded RNAs have been recently identified in the gram-positive *Bacillus*, *Clostridium*, *Listeria*, *Staphylococcus* and *Streptococcus* (Table 1). For most of these RNAs described to date, their direct target(s) and mechanism(s) of action remain to be experimentally validated. However, many have been indirectly or directly associated with adaptation of bacteria to the host and to pathogenesis. Because these RNAs are expected to act in *trans*, the nomenclature of sRNAs is applied here. A brief overview is given for several bacterial species.

VR-RNA regulates virulence in *Clostridia*

VR-RNA is a sRNA, the expression of which is positively regulated by the two-component VirR/VirS system [138]. VR-RNA is responsible for the transcriptional regulation of the toxin-encoding genes *colA* and *plc*, and thus functions as a mediator of the regulatory information originating from the VirR/VirS system. The 3'-region of the VR-RNA molecule was shown to act as the positive effector in regulation, but the exact mechanism of action remains to be addressed [138, 139].

sRNAs regulate virulence gene expression in *Streptococcus*

In *S. pyogenes*, three sRNAs have been described: ncRNA (*fasX* RNA), a protein coding RNA (*pel* RNA) and a putatively processed RNA (*rivX* RNA). All participate in the control of virulence factor expression; however, the regulatory mechanisms used by these RNAs have not been determined yet. *fasX* RNA is part of an operon encoding two histidine kinases (FasBC) and one response regulator (FasA) [140]. In a M49 serotype, the *fasBCA* regulon, via

the effector *fasX* RNA, negatively controls the expression of two matrix protein-binding adhesins (fibronectin-binding protein FBP54 and fibrinogen-binding protein MRP) at the transcriptional level and has a positive influence on the activities of two secreted virulence factors (streptokinase and streptolysin S) [140]. Whether *fasX* RNA regulates synthesis of a transcriptional regulator or binds to a regulatory protein, thereby inducing indirect effects at the transcriptional level remains to be addressed. Interestingly, *fasX* positively controls the interaction of *S. pyogenes* with epithelial laryngeal cells and bacterial aggressiveness in terms of host cell response [141]. Its expression is under the influence of amino acid starvation in a M49 serotype and regulated by the *luxS/AI2* signalling system in a M19 serotype [142, 143]. The second sRNA, *pel* RNA of the pleiotropic effect locus functions as a regulator of virulence factor expression at both transcriptional (*emm* encoding the surface-exposed virulence M protein and *sic* encoding a streptococcal inhibitor of the complement system) and post-transcriptional levels (SpeB encoding a cysteine protease) in a M1 strain [144]. These regulations however are indirect, and the primary targets are not known. The *pel* locus comprises *sagA* encoding Streptolysin S, one of the two hemolysins of *S. pyogenes*. Similar to *S. aureus* RNAIII, *pel* RNA displays bi-functionality, being an effector of virulence factor expression and encoding at the same time a hemolysin. More recently, a study revealed *rivX* RNA is co-expressed with the downstream gene encoding the response regulator RivR (RALP4) [145]. *rivX* RNA together with RivR was shown to positively affect expression of virulence genes that are activated by the global transcriptional regulon Mga [145]. Data suggest that *rivX* RNA is a processed form of the *rivRX* cotranscript and may act as a *trans*-acting sRNA [145]. Lately, a genome-wide computational and experimental search for novel sRNAs in *S. pyogenes* led to the prediction and validation of a number of candidates encoded within the intergenic regions of the genome (Charpentier, personal communication). In *S. pneumoniae*, five sRNAs sharing a high degree of similarity among each other were recently identified. The so-called csRNAs are expressed under control of the response regulator CiaR of the two-component system, CiaRH. Two of these sRNAs, csRNA4 and csRNA5, are involved in stationary-phase autolysis [146].

Multiple regulatory RNAs in *L. monocytogenes*

As mentioned above, in *L. monocytogenes*, the protein Hfq contributes to stress tolerance and virulence. This suggested that RNA-dependent regulation might be associated with these phenotypes [64]. By using coimmunoprecipitation, three sRNAs conserved among *Listeria* species were identified (LhrA, LhrB, LhrC). The stability of these

sRNAs is Hfq-dependent. LhrB appears to be produced by a transcriptional attenuation mechanism and was recently annotated as the regulatory region of the L20 r-protein operon. LhrC is present in five copies within the *L. monocytogenes* genome. The expression of the three sRNAs is dependent on growth phase, and all of them are expressed in *L. monocytogenes* multiplying within mammalian cells [63]. Using *in silico* approaches, nine additional sRNAs were identified [44]. In contrast to the previous study, the stability of these sRNAs seems to be Hfq-independent. Among these sRNAs, five were found only in the pathogenic strains. Three of these sRNAs act as antisense RNAs: (1) RliD is complementary to *pnp* mRNA encoding the 3'-5' exoribonuclease polynucleotide phosphorylase; (2) RliH is complementary to a mRNA encoding a putative regulator of transcription; (3) RliE acts as antisense RNA of *comC* mRNA and forms stable complexes with several mRNAs encoding proteins that are part of a complex similar to the competence machinery of *B. subtilis*. All of these ncRNAs overlap the RBS of the mRNAs, suggesting that they would regulate translation. Of interest, RliB carries five repeats of 29 nucleotides reminiscent of a CRISPR RNA except that none of the genes in the vicinity of *rliB* shows significant homology with the *cas* genes. More recently, transcription of the entire genome of *L. monocytogenes* was probed using tiling arrays from wild-type and mutant strains grown in rich medium or purified from infected cells and mice [12]. In addition to 21 previously known sRNAs, 29 additional ones were characterized. All of them have a size between 77 and 534 nucleotides, and three contain a small ORF. Although the expression of none of the sRNAs seems to be dependent on Hfq or the major transcriptional regulator of virulence PrfA, the synthesis of three sRNAs was significantly induced by the stress-response alternative Sigma B factor. One of these RNAs (SbrA) was already characterized [65]. Remarkably, major changes in the expression pattern of many sRNAs were observed in the intestinal lumen or in blood after infection of mice [12]. For instance, expression of the five LhrC and of RliB is strongly enhanced after blood infection. Prediction of basepairings of the Rli sRNAs with mRNAs provided some ideas on their functions and suggest that many of the sRNAs would target mRNAs involved in metabolism or mRNAs encoding virulence factors such as the internalins. Several sRNAs could potentially interact with other sRNAs [12]. Two of the sRNAs (*rli38* and *rliB*) affect virulence in different ways. The expression of *Rli38* is strongly induced in blood and in response to oxidative stress, and the *rli38* deletion mutant is attenuated when bacteria are orally inoculated in mice. In contrast, the *rliB* deletion mutant strain colonizes the liver faster than the wild-type strain. Therefore, this study highlights the crucial role of sRNAs in adaptation of the

bacteria to the host and during the infection process [12]. Besides sRNAs, tiling arrays experimentally validated 40 *cis*-acting regulatory regions of mRNAs, which respond to metabolites, uncharged tRNAs or regulatory proteins. Of relevance, long RNAs corresponding to 5'-UTRs of mRNAs were revealed for known virulence genes and genes that encode putatively virulence-associated surface proteins [12, 147]. Three long antisense RNAs complementary to more than one ORF were identified, and several RNAs corresponding to long 5' or 3'UTRs of mRNAs and partially overlapping with mRNAs produced from convergent genes were also shown to be expressed. Such a long RNA, the expression of which is induced by Sigma B, was proposed to regulate flagellum biosynthesis [12]. In addition to the coordinated global transcriptional changes observed during the infection process, this study further emphasizes the importance of RNA-dependent regulation in *L. monocytogenes* virulence.

Concluding remarks and perspectives

In recent years, major findings have been made in the field of regulatory RNAs in gram-negative bacteria. In gram-positive bacteria, while many riboswitches have been discovered and studied, the information on the modes of action of *trans*-acting sRNAs is often missing. This picture may change considerably given the fact that in silico and experimental genome-wide approaches have allowed the identification of a battery of regulatory RNA candidates in several gram-positive species. Recent data show that some of the identified RNAs play critical functions in adaptive responses of the bacteria and in pathogenesis events and thus constitute key players in the signaling networks of gene expression control. It is very likely that other newly revealed RNAs interfere as well with these biological processes.

Interestingly several regulatory signals on mRNAs (e.g., metabolite-sensing riboswitches, leader regions of r-protein operon mRNAs) are conserved in the two distant bacteria, *E. coli* and *B. subtilis*, which have been proposed to have diverged about 10^9 years ago. These conserved regulatory signals reflect primitive regulatory mechanisms. On the other hand, these bacteria have retained/evolved different strategies for regulation. For instance, RNA-dependent regulation in firmicutes provides a genetic response primarily focused at the transcriptional termination/anti-termination level, while in gram-negative bacteria gene expression is regulated at the translational level (e.g., metabolite-sensing riboswitches, uncharged tRNAs, proteins). Firmicutes also use different RNA-based strategies to regulate the expression of similar genes. For example, in low GC gram-positive bacteria, *glmS* expression is

regulated by a *cis*-acting regulatory element, whereas in enterobacteriae, a regulatory cascade, involving two *trans*-acting sRNAs, leads to the activation of *glmS* mRNA translation [21]. Gram-positive and gram-negative bacteria utilize distinct machineries for mRNA degradation and different strategies to initiate translation initiation that might contribute to explaining why the bacteria use different RNA-based regulatory mechanisms. In gram-positive bacteria, many mRNAs carry strong SD sequences, and the ribosomal 30S subunit lacks the ribosomal protein S1. Furthermore, stalled ribosomes can have a long-distance effect on mRNA stability in gram-positive bacteria. This protection can be the consequence of the mRNA decay by RNase J1, a 5'-3' exoribonuclease, which does not exist in *E. coli* [148–150]. In addition regulation by sRNAs in gram-positive bacteria can regulate translation, but degradation is not always a consequence as in gram-negative bacteria. The RNA chaperone Hfq does not seem to play a major role in the action of sRNAs on their target mRNAs in gram-positive bacteria, and a system analogous to the RNase E–Hfq–sRNA complex has not been identified yet. Whether the sRNAs associate with other *trans*-acting proteins to exert their function remains to be determined. Another point of interest is how do sRNAs recognize their targets? Are there some specific recognition signatures on sRNAs that target mRNAs, like the C-rich motif of *S. aureus* RNAIII, and in *Listeria* and *B. subtilis* sRNAs? Do the sRNAs target the coding sequence and the 3'UTR of target mRNAs? The current data accumulating from high-throughput genome analyses (e.g., deep-sequencing, tiling arrays, biocomputational tools) should expand not only the number, but also the diversity of regulatory RNAs. What is the proportion of *trans*-acting sRNAs versus *cis*-acting regulatory elements? What are the roles of the class of long RNAs? Would they be involved in RNA quality control (processing and maturation events, elimination of non-translated mRNAs)? Recently, in *E. coli* and *Salmonella*, a novel RNA-based regulatory mechanism has been identified whereby a mRNA mimics a mRNA target and serves as a sRNA trap to induce the degradation of a constitutively expressed sRNA [149, 150]. Therefore, a mRNA can be potentially regarded as a target of sRNA, but also as a *trans*-acting regulator of sRNA [151, 152]. In *L. monocytogenes* and *S. aureus*, several sRNAs could also potentially interact with other sRNAs [12]. In addition to classical transcriptional control, it might be possible that RNA-mediated degradation of sRNA is also playing an important role in gram-positive bacteria. How widespread are multifunctional RNAs like *S. aureus* RNAIII, which is a mRNA and acts as an antisense RNA? What are the mechanisms used by the CRISPR RNAs to target extra-chromosomal DNA? Are there some RNAs that would be involved in control of genome maintenance? Like viruses,

phytopathogenic bacteria have evolved strategies to suppress or counteract cellular RNA silencing to cause disease in the host plant [153]. There is increasing evidence that endogenous plant small RNAs have broad regulatory functions in response to various bacteria. Whether pathogenic bacteria derived sRNAs would be produced/secreted within the host cell awaits further demonstration [154].

The diversity of RNA-mediated regulatory systems described so far in gram-positive bacteria might predict that additional regulatory mechanisms are still to be expected. Future research is therefore necessary to identify the exact mechanisms by which regulatory RNAs, in particular the *trans*-acting sRNAs, act on their targets. This information will help our understanding of the regulatory circuits involved in bacterial adaptation and virulence. One of the next challenges in gram-positive regulatory RNA biology will be to understand how regulation by RNAs occurs at a single cell level within a population of cells to coordinately regulate gene expression. In their environments, bacteria are forming heterogeneous populations of cells that communicate with each other and respond distinctly to specific external signals. In this context, it is predicted that regulatory RNAs are expressed at specific times and in defined spaces. Future development of mechanistic and cell biology studies will be necessary to obtain knowledge of the spatial and temporal dynamics of RNA-mediated regulation in the cell.

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