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ADVANCED REVIEW

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Riboregulation in bacteria: From general principles to novel mechanisms of the *trp* attenuator and its sRNA and peptide products

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Abstract

Gene expression strategies ensuring bacterial survival and competitiveness rely on *cis*- and *trans*-acting RNA-regulators (riboregulators). Among the *cis*-acting riboregulators are transcriptional and translational attenuators, and antisense RNAs (asRNAs). The *trans*-acting riboregulators are small RNAs (sRNAs) that bind proteins or base pairs with other RNAs. This classification is artificial since some regulatory RNAs act both in *cis* and in *trans*, or function in addition as small mRNAs. A prominent example is the archetypical, ribosome-dependent attenuator of tryptophan (Trp) biosynthesis genes. It responds by transcription attenuation to two signals, Trp availability and inhibition of translation, and gives rise to two *trans*-acting products, the attenuator sRNA rnTrpL and the leader peptide peTrpL. In *Escherichia coli*, rnTrpL links Trp availability to initiation of chromosome replication and in *Sinorhizobium meliloti*, it coordinates regulation of split tryptophan biosynthesis operons. Furthermore, in *S. meliloti*, peTrpL is involved in mRNA destabilization in response to antibiotic exposure. It forms two types of asRNA-containing, antibiotic-dependent ribonucleoprotein complexes (ARNPs), one of them changing the target specificity of rnTrpL. The posttranscriptional role of peTrpL indicates two emerging paradigms: (1) sRNA reprograming by small molecules and (2) direct involvement of antibiotics in regulatory RNPs. They broaden our view on RNA-based mechanisms and may inspire new approaches for studying, detecting, and using antibacterial compounds.

This article is categorized under:

RNA Interactions with Proteins and Other Molecules > Small Molecule-RNA Interactions

RNA Interactions with Proteins and Other Molecules > RNA-Protein Complexes

Regulatory RNAs/RNAi/Riboswitches > Regulatory RNAs

KEYWORDS
antisense RNA, ARNP, leader peptide, sRNA, transcription attenuator

1 | INTRODUCTION

The importance of gene regulation by RNA for bacterial competitiveness and survival is well established, but new facets and even new mechanisms are still emerging. In transcriptomic studies, usually steady-state RNA levels are determined, which result from the rates of transcription and decay. Bacterial mRNAs are highly unstable, with typical half-lives within the range of few minutes. Furthermore, the mRNA half-lives change with changing environmental conditions (Bernstein et al., 2002; Klug, 1991; Nilsson et al., 1984; Vargas-Blanco et al., 2019). Such stability changes are often directly or indirectly caused by stimuli-induced regulatory RNAs (Adnan et al., 2015; Durand et al., 2015; Lenz et al., 2004; Pfeiffer et al., 2007) or by sensory RNA elements (Nou & Kadner, 1998; Richards & Belasco, 2021b). In addition, regulatory RNAs influence the initiation or the progress of mRNA transcription (Bossi et al., 2012; Sedlyarova et al., 2016; Silva et al., 2019; Wassarman & Storz, 2000).

Regulation with RNA (riboregulation) is widespread, mechanistically diverse, and affects virtually all aspects of the bacterial life. Among others, it helps bacteria to adapt to stress conditions (Adnan et al., 2015; Altuvia et al., 1997; Augagneur et al., 2020; Lalaouna, Baude, et al., 2019; Morita et al., 1999; Nocker et al., 2001), maintain metabolic homeostasis (Hoyos et al., 2020; Khan & Görke, 2020; Papenfort et al., 2013; Wilderman et al., 2004), regulate photosynthesis genes (Dühring et al., 2006; Georg et al., 2014; Mank et al., 2012), remodel gene expression during growth (Majdalani et al., 1998; Sedlyarova et al., 2016; Wassarman & Storz, 2000), produce biofilms (Holmqvist et al., 2010; Liu et al., 2016; Papenfort et al., 2015; Schoenfelder et al., 2019), coordinate expression of virulence factors (Boisset et al., 2007; Brewer, Twittenhoff, et al., 2021; Eisenbart et al., 2020; Jia et al., 2021; Pappesch et al., 2017; Twittenhoff, Heroven, et al., 2020; Westermann et al., 2016), and to survive antibiotic treatment (Borgmann et al., 2018; Peschek et al., 2020; Zhang et al., 2017). Numerous excellent and comprehensive reviews on prevalence, mechanisms, and evolution of regulatory RNAs are available (e.g., Adams & Storz, 2020; Babitzke et al., 2019; Carrier et al., 2018; Chen, Morita, & Gottesman, 2019; Dersch et al., 2017; Felden & Cattoir, 2018; Hör, Matera, et al., 2020; Ignatov & Johansson, 2017; Santiago-Frangos & Woodson, 2018; Turnbough, 2019; Wagner & Romby, 2015; Waters & Storz, 2009). Here, after a general overview of the principles of RNA regulation in bacteria, I will focus on new, unexpected functions of the conserved *trp* attenuator in Gram-negative bacteria, which indicate emerging mechanisms in response to antibiotics exposure.

2 | PROTEIN-BINDING, *TRANS*-ACTING SRNAS

Regulatory RNA, which acts independently of its genomic locus, is *trans*-acting RNA. In bacteria, many small RNAs (sRNAs), typically ranging from 50 to 300 nucleotides, act in *trans* (Mizuno et al., 1984; Zhang et al., 2003). They have two principle modes of action: protein binding or base pairing.

The prime example for a protein-binding sRNA is 6S RNA. In *Escherichia coli*, it accumulates throughout growth, binds the sigma^{70}-RNA polymerase (RNAP) holoenzyme, and contributes to the changes in gene expression in the stationary phase (Cavanagh et al., 2008; Wassarman & Storz, 2000). 6S RNA is highly conserved and some bacteria have two or more 6S RNA genes (Wehner et al., 2014). The two *Bacillus subtilis* 6S RNA paralogs 6S-1 and 6S-2 have different expression profiles during growth and 6S-1 seems to be functionally similar to *E. coli* 6S RNA. Using an undomesticated *B. subtilis* strain, it was shown that they both are important for the bacterial fitness under harsh environmental conditions (Thüring et al., 2021). In *Bacillus*, 6S-1 RNA contributes to sporulation (Cavanagh & Wassarman, 2013; Li, Zhu, et al., 2020). In anoxygenic photosynthetic bacteria, 6S RNA is important for salt stress tolerance (Elkina et al., 2017) and in cyanobacteria, for recovery from nitrogen depletion (Heilmann et al., 2017). Interestingly, in *Mycobacterium smegmatis*, the sRNA Ms1, which is not related to 6S RNA, interacts with the core RNAP and influences its amount (Šiková et al., 2019). Since also natural RNA aptamers were found to regulate transcription by interacting with RNAP in *E. coli* (Sedlyarova et al., 2017), it is possible that RNAP-binding riboregulators still remain to be discovered in other bacteria.

Other functions of protein-binding sRNAs are to sequester transcription factors or posttranscriptional regulators. Such protein-sponging functions are important for bacterial virulence. For example, B12-riboswitch-regulated sRNAs sequester the two-component response regulator EutV in *Listeria* and *Enterococcus* (DebRoy et al., 2014; Mellin et al., 2014). In *E. coli*, *Erwinia*, and *Yersinia*, the CsrB/RsmB sRNA and its paralogs sequester the global regulator and RNA-binding protein (RBP) CsrA/RsmA, which otherwise represses translation of genes for glycogen biosynthesis, extracellular enzymes, or transcription factors (Heroven et al., 2012; Liu et al., 1997). Furthermore, in *E. coli*, the sRNA GlmY titrates the RNA-processing factor RapZ away from its regular target, the sRNA GlmZ. The GlmZ sRNA is responsible for posttranscriptional activation of *glmS*, which is needed for cell envelope synthesis (Göpel et al., 2013; Khan et al., 2020). Protein-binding sRNAs have been much less investigated than base-pairing sRNAs, probably because not much is known about RBPs in bacteria. New high-throughput approaches such as Grad-seq (Hör, Di Giorgio, et al., 2020; Smirnov et al., 2016) and organic phase separation (Smith et al., 2020; Urdaneta et al., 2019) will certainly unravel novel RBPs and protein-binding riboregulators.

3 | BASE-PAIRING, *TRANS*-ACTING SRNAS

3.1 | Basic principles

Base-pairing, *trans*-acting sRNAs affect translation and/or stability of mRNA in a positive or, more often, in a negative way. Inhibition of translation is often due to binding of an sRNA to the ribosome binding site (RBS) as described for the OxyS sRNA and *fhlA* mRNA (Altuvia et al., 1998), but translation inhibition was also observed upon sRNA binding more upstream in the 5′-UTR (Andreassen et al., 2018; Sharma et al., 2007), or even in the coding sequence (Bouvier et al., 2008). In addition to blocking the ribosome access by the sRNA (Maki et al., 2008) several other translation inhibition mechanisms were deciphered in *E. coli*: The RyhB sRNA downregulates *fur* expression by blocking a short, upstream open reading frame (uORF), whose translation is coupled to that of the *fur* gene (Vecerek et al., 2007). The OmrA and OmrB sRNAs inhibit the formation of translation-activating stem-loops in the coding regions of *fepA* and *bamA* mRNAs (Jagodnik et al., 2017). Translation of *manX* mRNA is repressed by the RNA chaperone Hfq, and this interaction is supported by the SgrS and DicF sRNAs (Azam & Vanderpool, 2018). Finally, the SgrS sRNA interferes with the interaction between a translation enhancer and ribosomal protein S1 of *manY* mRNA (Azam & Vanderpool, 2020). Translation inhibition by an sRNA is often accompanied by degradation of the ribosome-free RNA (Deana & Belasco, 2005).

In bacteria, *trans*-acting sRNAs mostly bind their mRNA targets via imperfect complementarity, with only limited sRNA–mRNA contacts (Argaman & Altuvia, 2000; Poddar et al., 2021). Probably therefore, efficient sRNA–mRNA binding is often mediated by a proteinaceous RNA-chaperone such as Hfq (homolog of the eukaryotic Sm proteins) or ProQ (Attaiech et al., 2016; Chaulk et al., 2011; Møller et al., 2002; Smirnov et al., 2016). The mechanism of the Hfq hexamer was intensely studied, mainly in *E. coli*. It binds RNA via three distinct areas and according to the binding pattern, sRNAs can be divided into two classes. The majority of the studied sRNAs belong to Class I. They bind with their Rho-independent terminator to the proximal face of Hfq and with 5′ or internal regions to its arginine-rich rim, while their mRNA targets bind to the distal surface of the chaperone. Upon RNA rearrangement, annealing of the sRNA and the mRNA takes place at the Hfq rim. In contrast, Class II sRNAs bind to the proximal pore and the distal surface, and their targets bind to the rim of Hfq (Schu et al., 2015). Unstructured Hfq regions at the N- and C-terminus contribute to its RNA chaperone activity (Santiago-Frangos & Woodson, 2018).

Hfq interacts with the degradosome-organizing endoribonuclease RNase E via sRNA (Bruce et al., 2018). In this way, Hfq-dependent sRNAs recruit the RNA-degrading machinery and destroy their targets (Lalaouna, Morissette, et al., 2015; Morita et al., 2005). Thereby, the sRNA and the mRNA are co-degraded (Massé et al., 2003), or only the mRNA is degraded and thus the sRNA acts catalytically (Feng et al., 2015; Overgaard et al., 2009). Since the single-strand-specific RNase E has a monophosphate-binding pocket, it was proposed that a processed sRNA having a 5′ monophosphate more efficiently recruits RNase E (Bandyra et al., 2012), and that this works even without the help of an RNA chaperone (Baek et al., 2019). However, in vivo evidence for this mechanism remains to be provided.

The binding of an sRNA to an mRNA often results in an endonucleolytic cleavage of the mRNA. Besides RNase E, the double-strand specific RNase III can cleave the sRNA–mRNA duplex in a mechanism resembling RNAi in eukaryotic cells. The latter is exemplified by the downregulation of the porin-encoding *ompA* by the sRNA MicA (Viegas et al., 2011). However, since the double-strand binding domain of RNase III optimally binds to duplexes in the length

of approximately 20 bp (Nicholson, 2014), RNase III-dependent cleavage was rarely described in conjunction with *trans*-acting sRNAs and seems to be more often used in regulation by *cis*-transcribed antisense RNAs (asRNAs) (Oliva et al., 2017; Vogel et al., 2004; Wen et al., 2014).

The fewer examples of positive mRNA regulation include sRNA base paring which alters an mRNA secondary structure to expose previously hidden RBS (Majdalani et al., 1998; Urban & Vogel, 2008). Additionally, sRNA binding can mask an RNase E cleavage site and increase the mRNA stability (Chen, Previero, & Deutscher, 2019; Fröhlich et al., 2013). Moreover, an sRNA can stabilize an intermediate of RNase E-dependent mRNA decay and thus selectively activate gene expression (Papenfort et al., 2013; Papenfort & Vanderpool, 2015).

Trans-acting sRNAs can base-pair not only with mRNAs, but also with other regulatory sRNAs. In such cases, one of the sRNAs acts as a "sponge" and sequesters its sRNA target and/or downregulates its level (Acuña et al., 2016; Lalaouna, Carrier, et al., 2015). Recently, in several studies, identification of sRNAs and their mRNA or sRNA base pairing partners was boosted by using high-throughput methods: Affinity purification of MS2-tagged sRNA together with interacting molecules followed by RNA sequencing (MAPS) (Carrier et al., 2016); in vivo UV crosslinking, coimmunoprecipitation, and sequencing of RNA interacting with 3 × FLAG-ProQ (CLIP-seq; Holmqvist et al., 2018); RIL-seq (RNA interaction by ligation and sequencing) of interacting RNA–RNA pairs associated with Hfq and ProQ (Melamed et al., 2016, 2020); UV cross-linking, ligation and sequencing of hybrids (CLASH) co-purified with RNase E or Hfq (Iosub et al., 2020; Waters et al., 2017); and RNA-Seq of sRNAs that are derived from 5′-UTRs or are internal to ORFs (Adams et al., 2021).

Regarding the genomic sRNA loci, the first systematic searches were focused on non-annotated regions between ORFs, and therefore in early studies, mostly sRNAs having own promoter and transcription terminator were detected (Argaman et al., 2001). Later, many 3′-UTR-derived sRNAs were identified (Chao et al., 2012), while sRNAs originating from 5′-UTRs or internal to ORFs were rarely described (Guo et al., 2014; Loh et al., 2009; Thomason et al., 2019). This currently changes with the application of Term-seq detecting 3′ ends of transcripts and of multilayered RNA-Seq approaches (Adams et al., 2021; Dar et al., 2016; Dar & Sorek, 2018).

Most of the base-pairing sRNAs are non-coding, but several dual-function sRNAs harboring small, functional ORFs were described (Gimpel & Brantl, 2017). A well-known example is the *E. coli* SgrS sRNA, which encodes the small protein SgrT. The base pairing of SgrS to at least nine mRNAs and the inhibition of the glucose transporter PtsG by SgrT are mechanistically independent, but both counteract the glucose-phosphate stress, which induces SgrS production (Bobrovskyy et al., 2019; Lloyd et al., 2017; Vanderpool & Gottesman, 2004). In *Staphylococcus aureus*, the multifaceted sRNA RNAIII, which has a length 514 nt and is one of the largest *trans*-acting riboregulators in bacteria, represses or activates several mRNA targets by base-pairing, and encodes the hemolytic toxin Hld (Bronesky et al., 2016; Novick et al., 1993).

3.2 | Regulatory networks of sRNAs

The imperfect complementarity between sRNAs and their targets also accounts for the capability of certain sRNAs to regulate multiple genes, as shown for the Spot42, GcvB, and RybB sRNAs in *E. coli* (Lalaouna, Eyraud, et al., 2019; Storz et al., 2011). To base-pair with different mRNAs, an sRNA can use the same seed region as described for the 5′ target recognition domain of RybB in *Salmonella* (Papenfort et al., 2010). However, sRNAs also use separate modules for binding to different mRNAs, as shown for RNAIII in *S. aureus* (Bronesky et al., 2016), AbcR1 sRNA in *Agrobacterium tumefaciens* (Overlöper et al., 2014), and GcvB in *E. coli* (Lalaouna, Eyraud, et al., 2019). Furthermore, an sRNA can affect its multiple targets by different mechanisms. For example, the *Vibrio harveyi* Qrr3 sRNA deploys four distinct mechanisms to regulate quorum sensing: catalytic degradation, coupled degradation, repression through sequestration, and activation by revealing a ribosome binding site (Feng et al., 2015).

On the other hand, specific, key mRNAs can be targeted by several differently regulated sRNAs. For example, the enterobacterial curli activator mRNA *csgD*, which is important for biofilm formation, is regulated by no less than seven sRNAs, which relay signals such as carbon source availability, osmotic or envelope stress, and amino acid metabolism (Andreassen et al., 2018; Boehm & Vogel, 2012). Another prominent example is *rpoS*, which encodes the general stress sigma factor in Gammaproteobacteria. In *E. coli*, it is upregulated by the low-temperature activated sRNA DsrA, the cell-envelope stress-related sRNA RprA, and a processed form of the energy limitation-activated sRNA ArcZ. These sRNAs act by base-pairing within the 5′-UTR of the *rpoS* mRNA (McCullen et al., 2010; Mika & Hengge, 2014). Recently, it was reported that in *E. coli*, the CyaR sRNA downregulates *rpo*S expression by base pairing next to the ArcZ

binding site. Additionally, the short, active form, or ArcZ binds CyaR and promotes its degradation by RNase E. Thus, ArcZ upregulates *rpoS* expression by two different mechanisms, mRNA activation, and sRNA sponging (Kim & Lee, 2020). Furthermore, the *rpoS* expression in *E. coli* is downregulated by the oxidative-stress induced sRNA OxyS by a still unknown mechanism (Zhang et al., 1998). Moreover, in enterohemorrhagic *E. coli*, *rpoS* is regulated by StsX, a processed sRNA, which is derived from a constitutively initiated and constitutively terminated Shiga toxin transcript (Sy et al., 2020). In *Legionella*, *Pseudomonas*, and *Vibrio*, *rpoS* is also regulated by several base-pairing sRNAs (Han & Lory, 2021; Lu et al., 2018; Saoud et al., 2021; Thi Bach Nguyen et al., 2018).

The capability of a single sRNA to bind multiple mRNAs and vice versa, and the sRNA regulation of genes encoding sigma factors and other transcription regulators result in complex sRNA networks (Bossi & Figueroa-Bossi, 2016; Nitzan et al., 2017; Storz et al., 2011). In these networks, an mRNA target can compete with other mRNAs for a shared sRNA regulator (Miyakoshi et al., 2015) and a sponge-sRNA with mRNAs for binding to sRNAs or RBPs (Lalaouna, Carrier, et al., 2015; Liu et al., 1997; Sonnleitner & Bläsi, 2014). Furthermore, many sRNAs compete for binding to the global RNA chaperones Hfq and ProQ. A recent study analyzing RNA–RNA pairs associated with Hfq and/or ProQ in *E. coli* revealed that they generally bind to different sRNAs, but share a set of overlapping interactors, for example, a RybB–RbsZ sRNA–sRNA pair. It was suggested that when this pair is bound to Hfq, the RbsZ sponge downregulates RybB, while binding to ProQ protects RybB from degradation (Melamed et al., 2020).

In enterobacteria, many sRNAs are functionally dependent on Hfq, the disordered C-terminal domain of which regulates the RNA competition and ensures the release of double-stranded RNA complexes (Santiago-Frangos et al., 2016). The abundance and the Hfq-binding kinetics of the particular sRNAs determine the hierarchy in their Hfq occupancy, which is important for the regulatory outcome (Faigenbaum-Romm et al., 2020; Kwiatkowska et al., 2018). By acting as a decoy for Hfq, certain sRNAs could prevent the action of other sRNAs by limiting their access to Hfq (Moon & Gottesman, 2011) or by preventing an Hfq-dependent mRNA remodeling needed for base pairing with the sRNA (Hoekzema et al., 2019). Alternatively, an sRNA can displace Hfq from its mRNA target (Park et al., 2021). Furthermore, sRNA binding to Hfq probably affects its interactions with asRNA (Bilusic et al., 2014), rRNA (Andrade et al., 2018), tRNA (Lee & Feig, 2008), and DNA (Malabirade et al., 2017), and thus its multiple functions in the cell (Dos Santos et al., 2019). Altogether, dynamic interactions, competition, displacement, and sponging determine the cross-talk between subregions of the sRNA networks and finally the posttranscriptional regulation of a specific gene in the cell.

3.3 | Small RNAs in diverse bacteria

The majority of the above sRNA examples originate from Gammaproteobacteria. In the last years, it became clear that similar mechanisms also operate in other bacteria, but certain differences were also observed. This is a fast-growing research field (Adams & Storz, 2020) and only few examples can be mentioned here.

Most Alphaproteobacteria have Hfq but not ProQ (Olejniczak & Storz, 2017). In vitro studies and heterologous expression in *E. coli* suggested that Hfq of the cell-cycle model organism *Caulobacter crescentus* is functionally similar to its *E. coli* homolog. However, the involvement of *C. crescentus* Hfq in regulation by base pairing sRNAs was not shown experimentally (Fröhlich & Velasco Gomariz, 2021; Santiago-Frangos et al., 2019). In the soil-dwelling plant symbiont *Sinorhizobium meliloti*, the sRNAs AbcR1 and AbcR2 are Hfq-dependent (Torres-Quesada et al., 2013). They are conserved in related pathogens belonging to *Agrobacterium* and *Brucella*, where they target multiple mRNAs (Overlöper et al., 2014; Sheehan & Caswell, 2017; Wilms et al., 2011). However, other *S. meliloti* sRNAs such as EcpR1 and rnTrpL (alias RcsR1), which bind to the RBS of their mRNA targets and destabilize them in an RNase E-dependent manner, do not need Hfq (Robledo et al., 2015; Robledo, García-Tomsig, & Jiménez-Zurdo, 2020). In *S. meliloti*, Hfq binds only a minor sRNA fraction and interacts predominantly with mRNAs (Torres-Quesada et al., 2014). To find novel RBPs in *S. meliloti*, recently pull-down with MS2-tagged sRNAs was conducted. Surprisingly, the S-adenosylmethionine synthetase MetK was identified as an RBP associated with functionally diverse sRNAs (Robledo, García-Tomsig, Matia-González, et al., 2021). Furthermore, in the facultative phototrophic species *Rhodobacter sphaeroides*, recently, the DUF1127 protein CcaF1 was described as a globally acting RBP, which influences the stability of sRNAs (Grützner, Billenkamp, et al., 2021). Currently, *R. sphaeroides* is the alphaproteobacterial species with the highest number of functionally characterized sRNAs. It harbors orphan, 3′-UTR and 5′-UTR derived, Hfq-dependent and Hfq-independent sRNAs, which mostly act in conjunction with RNase E (Adnan et al., 2015; Billenkamp

et al., 2015; Eisenhardt et al., 2018; Grützner, Remes, et al., 2021; Hess et al., 2014; Mank et al., 2012; Müller et al., 2016; Peng et al., 2016).

In the pathogenic *Helicobacter pylori* (Epsilonproteobacteria), which lacks Hfq and ProQ, highly variable simple sequence repeats (SSRs) contribute to the regulation of virulence genes by phase-variation. A SSR in the 5′-UTR of the target mRNA encoding the chemotaxis receptor TlpB was found to regulate the mode of action of the RepG sRNA. This sRNA harbors a C/U rich terminator loop region, which base pairs with a homopolymeric G-repeat in the *tlpB* leader. Depending on the length of the G-repeat in different *H. pylori* strains, *tlpB* translation is affected differently by RepG (Pernitzsch et al., 2014). Furthermore, a variable thymine stretch in the promoter of the *H. pylori* sRNA NikS determines its phase-variable transcription and thus the posttranscriptional regulation of major virulence factors (Eisenbart et al., 2020).

In cyanobacteria, the sRNA AcpZ, which is derived from the 3′-end of a phycobilisome operon, downregulates the expression of the orange carotenoid protein by binding to the first codons of its ORF, thus mediating a reverse regulation of light harvesting and photoprotection (Zhan et al., 2021). Also in cyanobacteria, the iron-responsive sRNA IsaR1 has at least 15 direct target mRNAs and deploys a RBS-binding mechanism (Georg et al., 2017). However, the role of the cyanobacterial Hfq homolog is related rather to type IV pilus assembly than to sRNA metabolism (Bøggild et al., 2009; Schuergers et al., 2014; Yegorov et al., 2021), and other RNA chaperones were not described yet (Riediger et al., 2021).

The power of RNA-Seq enabled the transcriptome-wide detection and the subsequent functional analysis of sRNAs not only in well-established Gram-negative model bacteria, but also in emerging species with potentially important roles. For example, this approach led to the discovery of the sRNA *donS*, which influences 15 polysaccharide utilization loci in *Bacteroides*, a major component of the human gut microbiota. Thus, this sRNA controls 30% of the polysaccharide utilization loci, which are important for the predominance of this genus (Cao et al., 2016). Furthermore, very recently, the RNA landscape of five cancer-associated *Fusobacterium* strains, which lack CsrA, Hfq, and ProQ, was analyzed at different growth stages, and an oxygen-induced sRNA regulating a major porin gene was discovered (Ponath et al., 2021).

In the Gram-positive pathogen *Streptococcus pyogenes*, many sRNAs were detected by transcriptomics and more recently by Grad-seq (Hör, Garriss, et al., 2020; Le Rhun et al., 2016; Patenge et al., 2012). Several sRNAs were found to regulate competence and virulence genes, among them FasX, which targets multiple mRNAs (Danger et al., 2015; Hör, Garriss, et al., 2020; Pappesch et al., 2017). The virulence of the related pathogen *Streptococcus pneumoniae* is also controlled by sRNAs (Mann et al., 2012). In *Staphylococcus*, which also belongs to Bacilli, the large sRNA RNAIII uses several regulatory domains to target multiple mRNAs encoding transcriptional regulators, major virulence factors, and metabolic enzymes. Upon base-pairing with an mRNA, RNAIII mediates translation inhibition and/or RNase III cleavage, or activates gene expression by RBS liberation and/or mRNA stabilization (Bronesky et al., 2016). In *S. aureus*, the RsaA sRNA blocks translation of the global transcriptional regulator MgrA by imperfect base pairing with the Shine–Dalgarno sequence and by an additional loop–loop interaction within the coding region of its mRNA. It was proposed that this sRNA supports commensalism (Romilly et al., 2014). Furthermore, in the same species, the glucose limitation-activated sRNA RsaI uses two distinct base-pairing domains: a CU-rich unpaired region of RsaI binds to the RBS or the 3′-UTR of mRNAs, thereby repressing the glucose uptake system and inducing exopolysaccharide production, while a G-track sequences located in the first hairpin of RsaI bind to C-rich sequences of several sRNAs (Bronesky et al., 2019). In the related species *Staphylococcus epidermidis*, the sRNA RsaE is spatiotemporally expressed in biofilms. It exists in a full-length and a processed (20 nt shorter) form, and the two forms bind to the 5′-UTRs of different mRNAs: the full-length form facilitates extracellular DNA release, while the processed form regulates the central carbon metabolism (Schoenfelder et al., 2019).

In *B. subtilis*, the RsaE homolog RoxS is regulated by nitric oxide. It affects the *ppnKB* mRNA (encoding an NAD +/NADH kinase) by translation inhibition and destabilization. Interestingly, the latter is mediated by two independent mechanisms involving RNase III or RNase Y. Upon processing, RoxS also shows a change in the target specificity toward *sucCD* mRNA encoding succinyl-CoA synthase (Durand et al., 2015). The RoxS sRNA is regulated by an RNA sponge (Durand et al., 2021). Only few *B. subtilis* sRNAs were characterized functionally and available data were reviewed recently (Ul Haq et al., 2020). Similar to *Staphylococcus*, *Bacillus* also does not have ProQ but harbors Hfq, for which, however, no RNA chaperone properties were described yet (Zheng et al., 2016). Instead, CsrA was shown to promote the annealing of the sRNA SR1 with its *ahrC* mRNA target encoding an arginine repressor/activator protein (Müller et al., 2019). Furthermore, in *Listeria monocytogenes*, which together with *B. subtilis* and *S. aureus* belongs to *Bacillales*, the LhrC sRNAs destabilize the *tcsA* mRNA encoding a T-cell-stimulating antigen (Ross et al., 2019). Also in

Listeria, the Rli27 sRNA is upregulated during the intracellular life cycle, when it targets a long 5′-variant to regulate a cell wall protein (Quereda et al., 2014). In contrast to the *Bacillales* members which have Hfq but not ProQ, *Mycobacterium* belongs to Actinobacteria and lacks both RNA chaperones (Olejniczak & Storz, 2017). In *M. smegmatis*, RNA chaperone-independent interactions were described between a C-rich loop of 6C sRNA and its multiple targets including *dnaB* mRNA, which is essential for replication (Mai et al., 2019).

3.4 | Current and emerging sRNA topics

Despite the huge progress in knowledge on sRNAs in bacteria, certain topics still remain understudied and new aspects are emerging. As mentioned above, one of the current challenges is to identify the protein interaction partners of the sRNAs. Besides Hfq, the identification of ProQ as a global RNA chaperone (Smirnov et al., 2016) and of RapZ as an sRNA-specific adaptor protein that activates RNase E to degrade the GlmZ sRNA (Durica-Mitic et al., 2020; Göpel et al., 2013) demonstrated the importance of RBPs for RNA regulation. It is reasonable to propose that RBP binding to sRNA could be regulated by covalent modification of the RBP and/or the sRNA, or by their allosteric interactions with small molecule effectors, but such mechanisms were not shown experimentally yet. However, it was shown that interaction of the RBP RapZ with glucosamine-6-phosphate (GlcN6P) accounts for its second function in the GlmZ-*glmS* regulation paradigm: At low GlcN6P level, the GlcN6P-free form of RapZ stimulates the phosphorylation of a two-component system inducing the GlmY sRNA, which, in turn, sequesters RapZ to prevent GlmZ degradation. This leads to the accumulation of the base-pairing sRNA GlmZ, which activates the translation of *glmS*, the gene responsible for GlcN6P synthesis (Khan et al., 2020; Khan & Görke, 2020).

Another interesting topic is processed (truncated) sRNA forms. Shorter sRNA forms were often described, but for most of them, function is unknown. As mentioned above, sRNA processing could maturate/activate the sRNA or change its target specificity (Papenfort et al., 2009; Schoenfelder et al., 2019). Moreover, the processed sRNA can have a role in feedback regulation: Upon binding of the RBP RapZ to the GlmZ sRNA, the latter is cleaved by RNase E. Thereby, a processed sRNA form, GlmZ*, arises, which binds RapZ but does not allow for RNase E activation. The cleavage product GlmZ* competes with full-length GlmZ for RapZ binding, thus preventing complete GlmZ turnover and contributing to basal *glmS* expression (Durica-Mitic & Görke, 2019).

A continuous challenge is the identifications of sRNA targets and factors determining target specificity/prioritization. As mentioned above, high throughput methods such as MAPS, CLIP-seq, RIL-seq, and CLASH greatly contributed to the sRNA target detection in enterobacteria under specific conditions. In addition, bioinformatic prediction remains an important tool for target identification (Li, Edelmann, et al., 2021; Wright & Georg, 2018). Since most base-pairing sRNAs bind their targets by imperfect complementarity, theoretically many more mRNAs could be bound by an sRNA than experimentally found. Two studies addressed features distinguishing mRNA targets from nontargets of Hfq-dependent sRNAs in *E. coli*, showing the importance of the base-pairing extent, accessibility of the RNA seed regions necessary for the initial interaction, and no overlap between interaction site and Hfq binding site (Beisel et al., 2012; Bobrovskyy et al., 2019). Integration of such aspects in the bioinformatic predictions and combination of experimental and computational methods maximize target identification (Georg et al., 2020; Raden et al., 2020). It was proposed that subcellular RNA localization also influences target prioritization (Teimouri et al., 2017).

Subcellular sRNA localization in ribonucleoprotein (RNP) bodies organized by liquid–liquid phase separation (LLPS) is an emerging research topic (Irastortza-Olaziregi & Amster-Choder, 2021). The prokaryotic bacterial cell is spatiotemporally organized, having uneven distribution of macromolecules. It is known that in *C. crescentus*, tmRNA (responsible for *trans*-translation) is localized in a cell-cycle-dependent manner (Russell & Keiler, 2009). Furthermore, in *E. coli* and *B. subtilis*, the key endoribonucleases RNase E and RNase Y, which organize the RNA degradosome complexes in these bacteria, are localized at the cytoplasmic membrane, where they form short-lived foci (Hamouche et al., 2020; Strahl et al., 2015). Similarly, an RNase J-based degradosome forms foci at the cytoplasmic membrane in *H. pylori* (Tejada-Arranz et al., 2020), and RNase E assembles in RNP bodies via LLPS in *C. crescentus* (Al-Husini et al., 2018). These bacterial RNP-bodies (also called BR-bodies) were suggested to represent localized sites for mRNA degradation and can be considered phase-separated, membrane-less organelles. In *E. coli*, RNA-dependent DEAD-box helicases form LLPS foci (Hondele et al., 2019), and even Hfq was detected in distinct and reversible foci upon long-term nitrogen starvation (McQuail et al., 2020). The intracellular localization of Hfq, components of the RNA degrading machinery and even certain mRNAs (Fei & Sharma, 2018) suggests that base pairing sRNAs could also be localized. Indeed, using a *C. crescentus* RNase E variant with a mutation in the active site, which allows for isolation and analysis

of otherwise labile BR-bodies, it was shown that they are enriched in poorly translated mRNAs, asRNAs, and sRNAs (Al-Husini et al., 2020). Future analyses of the role of BR-bodies will certainly lead to deeper understanding of the sRNA mechanisms in bacteria.

Specific challenges are encountered in analyses of sRNA functions in host–microbe interactions. The dual RNA-seq approach enables the analysis of sRNA-dependent changes in the transcriptomes of bacterial pathogens and their hosts without their physical separation (Westermann et al., 2016). Recently, a triple RNA-seq analysis of the response to co-infection with a bacterial pathogen and a virus was described (Seelbinder et al., 2020). An important aspect is also the development of suitable systems to study the mechanisms of bacterial infections. For example, when an intestinal three-dimensional (3D) tissue model was compared to a two-dimensional (2D) model, different phenotypes of mutant *Campylobacter jejuni* strains lacking the small regulatory RNA pair CJnc180/190 were observed during infection (Alzheimer et al., 2020). Furthermore, communication between bacteria and their hosts with extracellular vesicles harboring functional sRNA cargo has drawn attention in the last years (Frantz et al., 2019; Koeppen et al., 2016; Moriano-Gutierrez et al., 2020; Ñahui Palomino et al., 2021). Extracellular vesicles are also used for communication between bacteria (Knoke et al., 2020). In *Vibrio cholerae*, the release of outer membrane vesicles is affected by regulation with the sRNA VrrA sRNA (Song et al., 2008).

Particularly difficult are also studies of sRNAs in bacterial communities and in environmentally or medically important species, which cannot be cultured and/or are not accessible by genetic methods (Lambrecht et al., 2019; Stazic et al., 2016). Metatranscriptomic approaches enable sRNA identification and differential analysis of the abundance of sRNAs and possibly of their predicted targets in microbiome communities. For functional validation and mechanistic analysis, related laboratory strains are used (Lott et al., 2020).

In Eukarya, internal posttranscriptional RNA modifications such as m^6A methylation are important for gene regulation (Yue et al., 2015). This and other modifications could change the sRNA structure, stability, and base-pairing properties, but no functional examples for sRNA modification in bacteria are known yet (Felden & Gilot, 2018). However, a fraction of certain bacterial sRNAs carry a 5′ NAD modification introduced by RNAP, which can initiate transcription using the NAD dinucleotide (Cahová et al., 2015). In *E. coli*, NAD-RNAs are preferentially produced from genes differentially expressed in the exponential and stationary growth phases, suggesting that the NAD-modification plays a regulatory role (Zhang et al., 2021). In *S. aureus*, the NAD-cap of RNAIII was suggested to modulate the production of the two hemolytic toxins Hla (translation of which is activated by base-pairing with RNAIII) and Hld (encoded by RNA III; Morales-Filloy et al., 2020). It was suggested that other nucleotide-containing cofactors could also be used for transcription initiation, leading to variety of 5′ RNA caps with putative regulatory functions (Barvík et al., 2017). Altogether, the analysis of sRNA modifications in bacterial gene regulation is just beginning.

4 | *CIS*-ACTING RIBOREGULATORS

The *cis*-acting riboregulators comprise antisense RNAs (asRNAs) and RNA elements with alternative structures that regulate transcription, translation, and mRNA decay.

4.1 | Antisense RNAs

Antisense RNAs are transcribed from the opposite DNA strand of the same genomic locus and are thus, at least in part, perfectly complementary to their targets. Initially, bacterial asRNAs were shown to regulate phage propagation and plasmid replication (Lacatena & Cesareni, 1981; Spiegelman et al., 1972). With the advance of RNA-seq and genome-wide transcription start site (TSS) mapping, widespread antisense transcription of chromosomes and plasmids were detected in various bacteria (Mitschke et al., 2011; Schlüter et al., 2013; Sharma et al., 2010). Most asRNAs have low reads number in RNA-seq analyses and probably therefore, they are often considered the result of transcriptional noise (Lloréns-Rico et al., 2016; Wade & Grainger, 2014). However, their low abundance could be explained by fast decay (Lybecker et al., 2014) and meanwhile physiological functions are assigned to growing number of asRNAs (Georg & Hess, 2018).

Probably not surprising, the base-pairing effects of *trans*-acting sRNAs and *cis*-acting asRNAs are similar: They regulate transcription termination (Stork et al., 2007), translation (Blomberg et al., 1992) and stability of mRNA targets (Stazic et al., 2011). Furthermore, when the promoters of the asRNA and the gene target are in close proximity,

initiation or elongation of asRNA transcription can interfere with those of the target (Sesto et al., 2013). Chromosome-borne asRNAs influence diverse processes from ribosome synthesis (Mars et al., 2015) to iron transport (Waldbeser et al., 1995) or photosynthesis (Dühring et al., 2006; Reuscher & Klug, 2021).

Because asRNAs show extended full complementarity to their targets, it could be assumed that they easily form perfect duplexes and do not need support by protein partners. However, after transcription, the asRNAs and their targets do not necessarily directly base pair with each other. As shown for the asRNA CopA, the copy number regulator of plasmid R1, each transcript first adopts its own structure, and then initial, intramolecular interactions between complementary nucleotides in hairpin loops take place, forming "kissing complexes" (Hjalt & Wagner, 1995). The loop–loop interaction is followed by transcripts rearrangement and extended duplex formation (Kolb et al., 2000; Malmgren et al., 1997). In some cases, an RBP or even an RNA chaperone might be needed for binding of an asRNA to its target. In the copy number regulatory system of plasmid ColE1, interaction between RNAI and RNAII starts with the kissing complex formation, which is stabilized by the Rop protein (Di Primo, 2008; Helmer-Citterich et al., 1988). Furthermore, in *E. coli* Hfq facilitates the asRNA annealing in the Tn10/IS10 system (Ross et al., 2013) and acts together with the asRNA GadY, which activates *gadX* encoding a transcriptional regulator of the acid response (Fröhlich & Vogel, 2009; Opdyke et al., 2004) In *R. sphaeroides*, Hfq directly interacts with the asRNA asPufL and influences the stability of the asRNA and of its mRNA target, which are co-degraded by RNase III (Reuscher & Klug, 2021). Moreover, asRNAs were found to be associated with Hfq in *B. subtilis* (Dambach et al., 2013). Finally, FinO and FopA, two members of the emerging FinO/ProQ-like RBPs, act on plasmid-encoded asRNAs (El Mouali et al., 2021; Gerovac et al., 2020; van Biesen & Frost, 1994).

As for sRNAs, the definition borders for asRNAs are blurry. In *B. subtilis*, the asRNA regulating translation of the S4 ribosomal protein (and thus the level of 30 S subunits) has dual function, since it also encodes a small protein regulating enolase and RNase Y in the RNA degradosome (Mars et al., 2015; Ul Haq et al., 2021). Furthermore, in *S. aureus*, the asRNA $SprA1_{AS}$ is acting in *trans*. It is transcribed in *cis* to the sRNA SprA1 and their 3′-ends overlap. However, $SprA1_{AS}$ represses production of a SprA1-encoded cytolytic peptide by base-pairing in *trans*, using a domain outside its target overlapping (complementary) sequence (Sayed et al., 2011). Moreover, recently a direct antisense interaction between the noncoding regions of two mRNAs was found to regulate the production of virulence factors in *L. monocytogenes* (Ignatov et al., 2020).

4.2 | Transcription and translation attenuators without ORFs

Many mRNAs comprise regulatory elements that change their structure in response to a signal. Usually, they are located in the 5′-UTR and deploy a variety of intricate mechanisms to regulate conditional transcription termination, translation, and accessibility of RNase cleavage sites. Here only few examples of transcription and translation attenuators are mentioned. For more comprehensive information and deeper understanding, see corresponding reviews (Bastet et al., 2018; Battaglia & Ke, 2018; Ignatov & Johansson, 2017; Kortmann & Narberhaus, 2012; Richards & Belasco, 2021a; Sherlock & Breaker, 2020; Sherwood & Henkin, 2016; Turnbough, 2019; Zhang, 2020; Zhang et al., 2017).

RNA thermosensors or RNA thermometers are RNA elements, which regulate translation by rearrangement of their structures in response to temperature changes (Altuvia et al., 1989; Kortmann & Narberhaus, 2012). Zipper-type thermosensors sequester the RBS in a helix structure and upon temperature increase, the helix melts rendering the RBS accessible for ribosomes. This mechanism is used for translation activation of the heat shock sigma factor in *E. coli* (Morita et al., 1999), small heat shock proteins in rhizobia (Nocker et al., 2001), the virulence master regulator PrfA in *L. monocytogenes* (Johansson et al., 2002), a secreted toxin in *Yersinia pseudotuberculosis* (Twittenhoff, Heroven, et al., 2020), and a zinc resistance determinant in *C. jejuni* (Barnawi et al., 2020). In contrast, switch-type thermosensors adopt mutually exclusive structures at different temperatures. Such a mechanism activates translation of the *E. coli* cold shock protein CspA at low temperature (Giuliodori et al., 2010). New RNA temperature-responsive elements are discovered by bioinformatic prediction (Brewer, Twittenhoff, et al., 2021; Churkin et al., 2014; Hussein et al., 2019) or by in vitro or in vivo analysis of the RNA structurome at different temperatures, as shown for *Y. pseudotuberculosis* (Righetti et al., 2016; Twittenhoff, Brandenburg, et al., 2020).

Protein-binding attenuators are based on RBP-mediated regulation of secondary structures in the nascent transcript (Stülke, 2002; Turnbough, 2019). A prime example is the posttranscriptional regulation of the *bgl* operon, which encodes genes for utilization of certain aromatic β-glucosides by *E. coli*. In the presence of β-glucosides,

unphosphorylated BglG forms a dimer and binds to ribonucleic antiterminator (RAT) sites partially overlapping transcription terminators flanking the first gene of the operon (*bglG*). This prevents formation of terminator hairpins and activates expression of the *bgl* genes (Raveh et al., 2009; Schnetz & Rak, 1988). Another basic example is the posttranscriptional regulation of the L-tryptophan (Trp) biosynthesis operon in *B. subtilis* by the *trp* RNA-binding attenuation protein TRAP. Under conditions of excess Trp, activated TRAP binds to the mRNA leader of the polycistronic *trp* mRNA, prevents formation of an antiterminator structure, and enables formation of a transcription terminator (Gollnick et al., 1990, 2005; Yang et al., 1997).

Riboswitches use a sensor domain (aptamer) to bind a small molecule (Mironov et al., 2002; Winkler et al., 2002), an ion (Dann et al., 2007), or tRNA (Grundy & Henkin, 1993). This binding usually induces a conformational change in the expression platform of the riboswitch, which influences in a positive or negative way transcription or translation of downstream genes (Serganov & Nudler, 2013). Binding of GlcN6P to the riboswitch of the *glmS* gene in Gram-positive bacteria, however, induces a ribozyme cleavage (Winkler et al., 2004). A 5′-UTR can also harbor two ligand-binding riboswitch aptamers: In *Bacillus clausii*, *metE* mRNA is regulated by a tandem of S-adenosylmethionine and coenzyme B12 riboswitches (Sudarsan et al., 2006). Control of gene expression at multiple levels by riboswitches was also described (Bastet et al., 2018; Richards & Belasco, 2021a). For example, in *E. coli*, binding of thiamin pyrophosphate to the *thiM* riboswitch triggers Rho-dependent transcription termination early in the *thiM* ORF as an indirect consequence of translation repression (Bastet et al., 2017). Similarly, binding of lysine to the *E. coli lysC* riboswitch aptamer leads to rearrangement of the expression platform which inhibits translation and exposes an RNase E cleavage site for initiation of mRNA decay (Caron et al., 2012). Another mechanism for regulation of mRNA stability by a riboswitch was described in *Legionella pneumophila*: In its ligand-bound state, the aptamer domain of a guanidine III riboswitch folds into a pseudoknot, which blocks the path of RNase E scanning the transcript from the 5′-end. This mRNA stabilizing function of the aptamer does not depend on the separate expression platform of the riboswitch (Richards & Belasco, 2021b). In *Bacillus* and *Clostridium*, a minimal-size riboswitch was described. Its HMP-PP (thiamin precursor) binding aptamer is embedded in an intrinsic transcription terminator, and ligand binding relieves transcription termination (Atilho et al., 2019). Novel riboswitches are discovered by comparative sequence analyses (Brewer, Greenlee, et al., 2021).

In addition to regulating protein-coding genes, riboswitches can control regulatory RNAs. An example is the protein binding sRNAs EutX and Rli55 in *Enterococcus faecalis* and *L. monocytogenes*, respectively, which harbor adenosylcobalamin (vitamin B12) responding riboswitches. Using their 3′-part, these sRNAs sequester EutV, activator of ethanolamine-utilization (*eut*) genes. Vitamin B12 binding to the riboswitch aptamer causes premature transcription termination, thus leading to the production of a shorter sRNA form, which cannot sequester EutV. As a consequence, the *eut* genes, whose products require B12 as a cofactor, are activated (DebRoy et al., 2014; Mellin et al., 2014).

Transcription attenuation leads to the release of a small RNA, which may have own function in *trans*. In *L. monocytogenes*, two *S*-adenosylmethionine (SAM) riboswitches correspond to the SreA and SreB sRNAs, which base pair with and downregulate the mRNA encoding the virulence regulator PrfA. Although this sRNA function does not depend on SAM binding (Loh et al., 2009), it is reasonable to propose that functions of sRNAs, which harbor elements capable to adopt mutually exclusive structures, can be regulated by those elements in response to signals.

4.3 | ORF-containing transcription and translation attenuators

Ribosome-dependent attenuators harbor a small uORF encoding a leader peptide. In the case of transcription attenuators, uORF translation by the pioneering ribosome determines whether the downstream genes will be transcribed. This prokaryotic mechanism is often used for regulation of amino acid biosynthesis operons in Gram-negative bacteria. The prototype is the transcription attenuator of the *trp* operon *trpEDCBA* in *E. coli* (Bertrand et al., 1975; Lee & Yanofsky, 1977; Yanofsky, 1981; Zurawski et al., 1978). The 162-bp leader region preceding *trpE* contains the uORF *trpL*, which harbors two consecutive Trp codons. When there is enough Trp (and thus enough charged $tRNA^{Trp}$), their fast translation prevents the formation of an antiterminator structure in the nascent transcript (Figure 1). Instead, transcription terminator is formed and transcription is aborted before RNAP can reach the structural genes for Trp biosynthesis. In contrast, under conditions of Trp insufficiency, the low level of charged $tRNA^{Trp}$ leads to ribosome pausing at the Trp codons. This allows for antiterminator formation and the structural genes are transcribed. (Yanofsky, 1981). It is remarkable that two consecutive Trp codons ensure this posttranscriptional regulation. In similar attenuators,

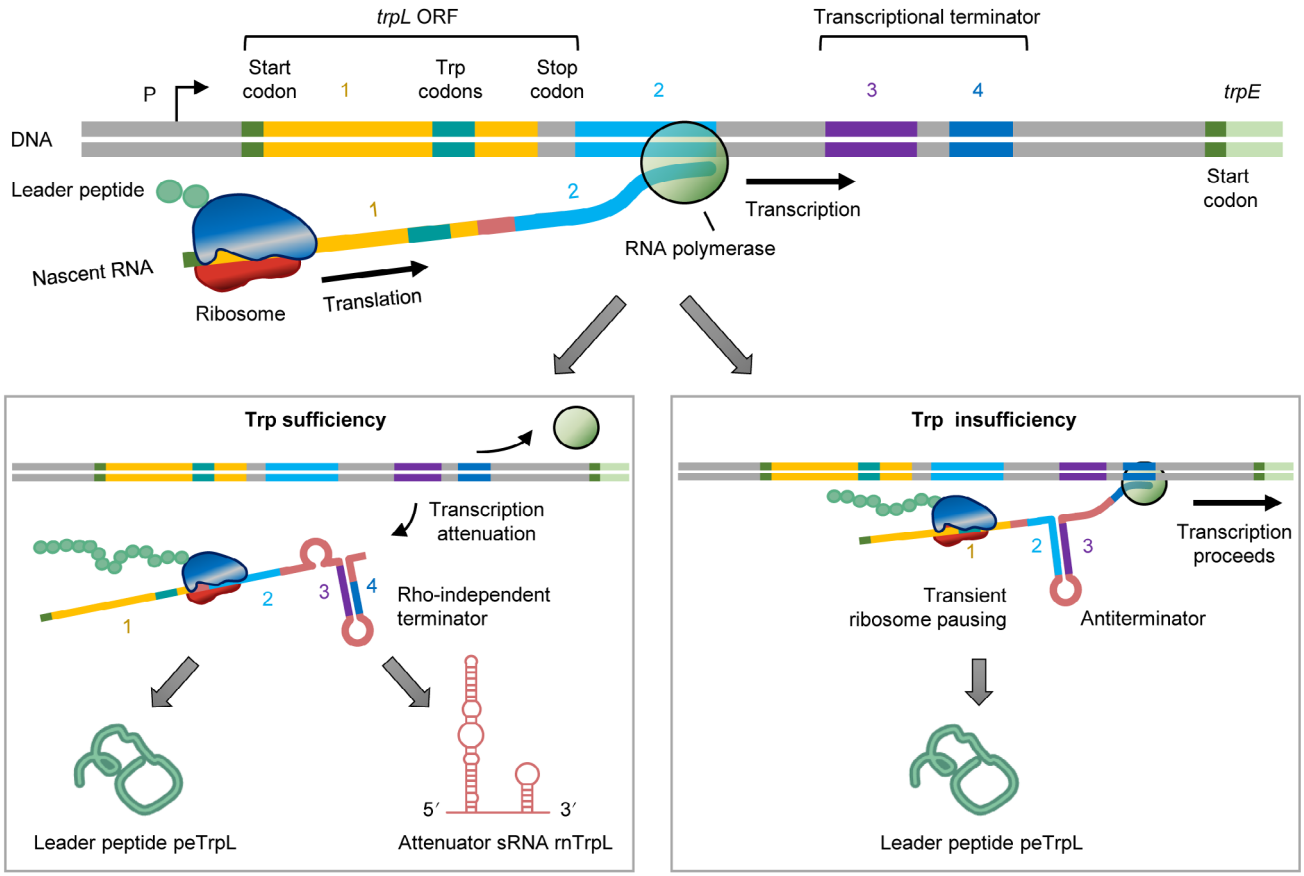

FIGURE 1 Transcription attenuation at the ribosome-dependent *trp* attenuator and arising small RNA and leader peptide with (potential) functions in *trans*. The *trp* attenuator is located upstream of *trpE* (in *E. coli*, upstream of the *trpEDCBA* operon). Regions 1–4 of the nascent transcript can undergo alternative base-pairing interactions, which are determined by the speed of translation of consecutive Trp codons in the uORF *trpL*. Under conditions of Trp sufficiency, translation of the regulatory Trp codons by the pioneering ribosome is fast and base pairing between Regions 2 and 3 is prevented. Therefore, Regions 3 and 4 can base pair and form a Rho-independent terminator. This leads to transcription attenuation. As by-products, the leader peptide peTrpL and the attenuator sRNA rnTrpL are generated. In contrast, under conditions of Trp insufficiency, transient stalling of the pioneering ribosome at the Trp codons leads to base pairing of Regions 2 and 3 in the nascent transcript. This prevents the formation of the transcriptional terminator and *trpL* is cotranscribed with the downstream genes. In this case, only peTrpL is generated as a by-product. P, promoter; the transcription start site is indicated by a flexed arrow (adapted from Melior & Evguenieva-Hackenberg, 2021)

usually, more than two regulatory codons are present in the uORF (Keller & Calvo, 1979; Vitreschak et al., 2004). For example, the enterobacterial histidine and leucine attenuators harbor seven consecutive histidine and four consecutive leucine codons, respectively (Di Nocera et al., 1978; Gemmill et al., 1979). For alternative mechanisms of ribosome-dependent transcription attenuation of metabolic genes, see the recent review of Turnbough (Turnbough, 2019). Upstream ORFs can also be used for regulation of mRNA stability (Ben-Zvi et al., 2019) or for translation attenuation (Dar et al., 2016).

Antibiotic resistance genes are often regulated by attenuation (Dersch et al., 2017). For example, in *B. subtilis* and *S. aureus*, the macrolide resistance gene *ermC* is translationally attenuated: In the absence of antibiotic, its uORF is efficiently translated, rendering the RBS of *ermC* hidden in a secondary structure. Upon erythromycin exposure, the ribosome stalls at the uORF. This results in a leader structure with exposed RBS and translation of *ermC* is activated (Bechhofer, 1990; Catchpole et al., 1988; Dubnau, 1985). This mechanism is not restricted to Gram-positive bacteria: Chloramphenicol resistance (*cat*) genes in *Agrobacterium* and *Pseudomonas* are also activated by ribosome-dependent relief of translation attenuation (Rogers et al., 2002). In *Bacillus licheniformis*, antibiotic-induced ribosome stalling at an uORF in the leader of a macrolide resistance gene prevents the formation of a transcription terminator (Kwak et al., 1991). In *B. subtilis*, the tylosin resistance gene *tlrB* is repressed by transcription attenuation and translation attenuation, which are relieved by tylosin-dependent ribosome stalling in the uORF (Yakhnin et al., 2019). The widespread

existence of antibiotic-responsive transcription attenuators was shown by Term-seq, a method for quantitative, transcriptome-wide detection of 3′-ends (Dar et al., 2016).

Ribosome stalling in uORFs in response to macrolides involves interactions of the ribosomal peptide exit tunnel with the antibiotic and with the nascent leader peptide at specific arrest motif (Davis et al., 2014; Yakhnin et al., 2019). Thus, the leader peptide can participate in *cis* in the posttranscriptional regulation of downstream genes (Cruz-Vera et al., 2011; Ramu et al., 2009). Leader peptides are widespread by-products of uORF-mediated regulation events and some of them could evolve into functional *trans*-acting small proteins. Currently, only the leader peptide peTrpL of the *trp* attenuator in *S. meliloti* was shown to have its own functions (Melior et al., 2020). By-products of ribosome-dependent transcription attenuation are also attenuator sRNAs, which could also adopt functions in *trans* or even act as dual-function sRNAs playing a role as a riboregulator and a small mRNA (Figure 1). The first example of a *trans*-acting attenuator RNA is the rnTrpL sRNA derived from the *trp* attenuator in *S. meliloti* and *E. coli* (Li, Edelmann, et al., 2021). Interestingly, some of the rnTrpL functions in *S. meliloti* are not related to the Trp metabolism but rather to antibiotic sensing (Li, Edelmann, et al., 2021; Melior et al., 2019, 2021).

5 | THE VERSATILE *TRP* ATTENUATOR AND ITS *TRANS*-ACTING PRODUCTS

Since Trp is the most costly amino acid to synthesize, strict regulation of the Trp biosynthesis genes is inevitable. This is reflected by the development of several posttranscriptional mechanisms for *trp* gene regulation in bacteria during evolution. One of these mechanisms, the ribosome-dependent transcription attenuation, is conserved in many Proteobacteria, some Actinobacteria, Chlamydiales, and Deinococci (Merino et al., 2008; Pokorzynski et al., 2020). Although the *trp* attenuation is very similar in *E. coli* (Gammaproteobacteria) and *S. meliloti* (Alphaproteobacteria), the organization of the *trp* genes and their transcriptional control differ (Merino et al., 2008; Panina et al., 2001). These differences account for the functional diversification of the *trans*-acting attenuator sRNA rnTrpL in these species.

5.1 | The rnTrpL sRNA links tryptophan availability to initiation of chromosome replication in *E. coli*

In *E. coli*, an intrinsic function of the leader peptide peTrpL has not yet been documented, but recently, the attenuator sRNA rnTrpL was shown to downregulate *dnaA*, the master regulator of initiation of chromosome replication (Li, Edelmann, et al., 2021). Activated DnaA (ATP-DnaA) binds to the origin of replication *oriC* and helps to unwind the DNA helix to start replication. Expression of *dnaA* is regulated at the transcriptional and protein level (Hansen & Atlung, 2018; Leonard et al., 2019; Skarstad & Katayama, 2013). An additional layer of regulation is the destabilization of *dnaA* mRNA by base pairing with rnTrpL. Several lines of evidence were presented to verify this sRNA–mRNA interaction, which was originally predicted by CopraRNA (Wright et al., 2013): (i) the *dnaA* mRNA level was decreased 3 min after induction of rnTrpL sRNA overproduction, suggesting that this is a primary effect; (ii) using suitable reporter constructs, base pairing in vivo was validated by introducing mutations in rnTrpL and compensatory mutations in *dnaA*; (iii) comparison of the wild type to mutant strains harboring chromosomal rnTrpL deletion or point mutations in the predicted interaction region also supported direct interaction between *dnaA* mRNA and rnTrpL (Li, Edelmann, et al., 2021). The importance of this riboregulation is underlined by the estimation that each *dnaA* mRNA transcript gives on average one molecule of DnaA polypeptide (Hansen & Atlung, 2018).

In *E. coli* under conditions of Trp excess, transcription of the *trp* operon is repressed by the transcription factor TrpR (Figure 2a), which allosterically binds Trp (Hurlburt & Yanofsky, 1992; Zubay et al., 1972). When Trp becomes scarce, it dissociates from the TrpR repressor, which falls off DNA and transcription is initiated. Then, transcription progress to the structural genes is regulated by the ribosome-dependent transcription attenuation in the mRNA leader (Figure 1; Merino et al., 2008), which from time to time leads to rnTrpL liberation (Figure 2b). TrpR-mediated regulation affects the *trp* operon expression 100-fold, while regulation at the attenuator allows for a 8-fold difference in expression (Yanofsky & Horn, 1994). In rich and in minimal medium, there are conditions, under which transcription is initiated and then attenuated, leading to the production of the attenuator sRNA rnTrpL, which can act in *trans*.

In LB-medium in the exponential phase, there is enough Trp for transcription repression of the *trp* operon. However, during transition to the stationary phase, the Trp level obviously drops to a level allowing for transcription

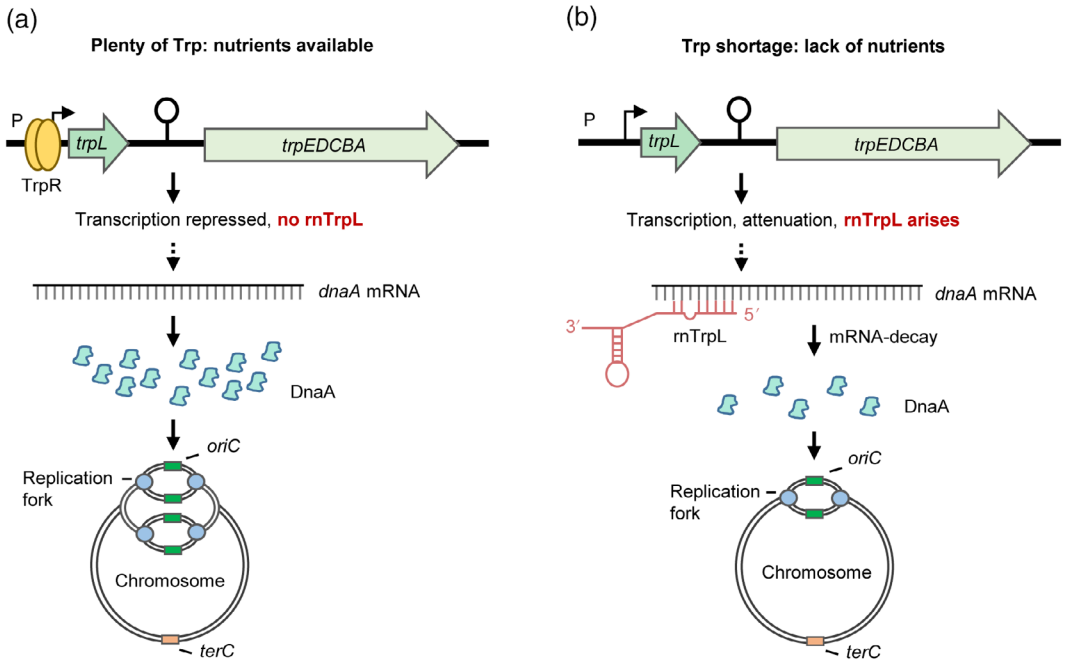

FIGURE 2 Tryptophan as a signal for nutrients availability, which indirectly regulates the initiation of chromosome replication in *E. coli*. (a) During exponential growth in rich LB medium, the *trp* operon is repressed by TrpR in its Trp-bound form. This prevents the production of rnTrpL and thus the *dnaA* mRNA destabilization by this sRNA. Under these conditions, more DnaA is synthesized in the fast-growing cells and replication of the chromosome is initiated more frequently. (b) During exponential growth in minimal medium, the *trp* operon is derepressed. Expression of the *trp* genes leads to Trp biosynthesis and eventually, intracellular Trp concentration is reached, which allows for transcription attenuation and rnTrpL production. Destabilization of *dnaA* mRNA by the rnTrpL sRNA contributes to lower DnaA production in the slow-growing cells and replication of the chromosome is initiated less frequently. P, promoter, *oriC*, origin of replication; *terC*, terminus of replication. The transcription start site is indicated by a flexed arrow and the transcription terminator of rnTrpL by a hairpin (adapted from Melior & Evguenieva-Hackenberg, 2021)

initiation: Using reporter *egfp* fusions with the *trp* promoter or the promoter and the attenuator, it was shown that at ODs between 1.5 and 4, the promoter is active but transcription is attenuated, leading to rnTrpL production. Despite this, using Northern hybridization, rnTrpL was detected only at OD of 4, but not at ODs of 1.5 and 2.5 (Li, Edelmann, et al., 2021). Most probably, at ODs of 1.5 and 2.5, the sRNA was rapidly degraded together with its target(s) and was therefore below the detection limit in the Northern blot analysis. Indeed, its interaction with the *hdeD* mRNA was detected by Hfq CLASH analysis in cells grown in LB medium to an OD of 1.2–1.8 (Iosub et al., 2020). The expression pattern of rnTrpL during the growth of *E. coli* in LB medium fits well with its function in destabilizing *dnaA* mRNA, since in the transition to the stationary phase, cells stop initiating chromosome replication. However, role of rnTrpL for regulation of *dnaA* and chromosome replication in rich medium was not shown yet.

In minimal medium, the intracellular concentration of free Trp (which can bind to the TrpR repressor and activate it) and charged $tRNA^{Trp}$ (which determines transcription attenuation) roughly corresponds to the amount of Trp made by biosynthesis minus that incorporated into proteins. During growth, the *trp* operon is transcribed (attenuation is relieved) and the structural genes are translated to biosynthesize Trp. Eventually, conditions of Trp sufficiency are reached, transcription attenuation takes place and the attenuator sRNA rnTrpL is generated. Indeed, rnTrpL was easily detected by Northern blot analysis in RNA purified form *E. coli* MG1655 exponentially growing in M9 medium (Li, Edelmann, et al., 2021). Expression of rnTrpL during exponential growth in minimal but not in rich medium is in line with the less frequent initiation of chromosome replication in the minimal medium (Figure 2b; Hansen et al., 1991; Løbner-Olesen et al., 1989). Comparison of wild-type and *ΔtrpL* strains in minimal medium revealed increased *oriC* levels in the mutant, supporting the view that *dnaA* downregulation by rnTrpL influences replication initiation. Thus, the posttranscriptional regulation of *dnaA* by the attenuator sRNA rnTrpL is a factor contributing to the nearly constant concentration of DnaA in *E. coli* cells growing with different doubling times due to different nutrients supply: To maintain DnaA homeostasis, higher production is needed in faster-growing LB cultures when compared to slower-growing cultures in minimal medium (Figure 2; Hansen & Atlung, 2018; Li, Edelmann, et al., 2021).

With its Rho-independent terminator, rnTrpL has features of a typical Hfq-binding sRNA. In line with this, it regulates *dnaA* in an Hfq- and RNase E-dependent manner (Li, Edelmann, et al., 2021). As mentioned above, another Hfq-dependent target of rnTrpL is most probably *hdeD* mRNA, which is regulated by the sRNAs CyaR and RprA (Iosub et al., 2020; Lalaouna et al., 2018). The *hdeD* gene encodes an acid-resistance membrane protein important for virulence and is needed at high cell densities (Gao et al., 2020; Mates et al., 2007). It was not investigated whether *hdeD* is negatively or positively regulated by rnTrpL. Furthermore, rnTrpL seems to regulate *mhpC* (part of an operon for degradation of aromatic compounds) in a positive way, and *sanA* (plays a role in role in the RpoS-dependent SDS resistance in carbon-limited stationary phase) in a negative way (Li, Edelmann, et al., 2021; Manso et al., 2009; Mitchell et al., 2017). Thus, the attenuator sRNA rnTrpL has its own regulon and participates in regulatory networks in *E. coli*.

Why did the attenuator RNA of the *E. coli trp* operon evolve into a sRNA, which indirectly regulates such a central event as the initiation of chromosome replication? Most probably, because it is useful for the cells to link the monitoring of their Trp status (and thus rnTrpL expression) to the regulation of chromosome replication. Due to the high metabolic costs of its biosynthesis, Trp is well suited as a signal for nutrient availability, and nutrient availability determines cell growth and must be coordinated with DnaA homeostasis (Figure 2).

5.2 | Tryptophan-related functions of the sRNA rnTrpL in soil alphaproteobacteria

In the soil alphaproteobacterium *S. meliloti*, the Trp biosynthesis genes are organized in three operons: *trpE(G)*, *trpDC*, and *trpFBA* (Johnston et al., 1978), of which only the *trpE(G)* operon is preceded by a *trpL* ORF and regulated by transcription attenuation (Bae & Crawford, 1990). Another difference to *E. coli* is the lack of TrpR and of transcriptional control of *trpE(G)* and *trpDC* expression during growth (the *trpFBA* regulation is not clear). In rich and in minimal medium, transcription of the *trpE(G)* operon is constitutively initiated and, under conditions of Trp availability, prematurely terminated by attenuation. The liberated attenuator sRNA rnTrpL base pairs with a *trpD* region in the polycistronic *trpDC* mRNA and leads to its destabilization (Figure 3). Thus, in *S. meliloti*, the same genetic locus corresponds to two riboregulators, which use fundamentally different mechanisms: the *cis*-acting ribosome-dependent *trp* attenuator and the *trans*-acting sRNA arising by the attenuation. In this way, the expression of the *trpE(G)* and *trpDC* operons is coordinated at the RNA-level according to the Trp availability. A similar mechanism for coordinated expression of the *trpE(G)* and *trpDC* operons seems to operate in the related Alphaproteobacteria *A. tumefaciens* (plant pathogen and biotechnology tool) and *Bradyrhizobium japonicum* (soybean symbiont). In contrast, in *E. coli* where a single *trp* operon

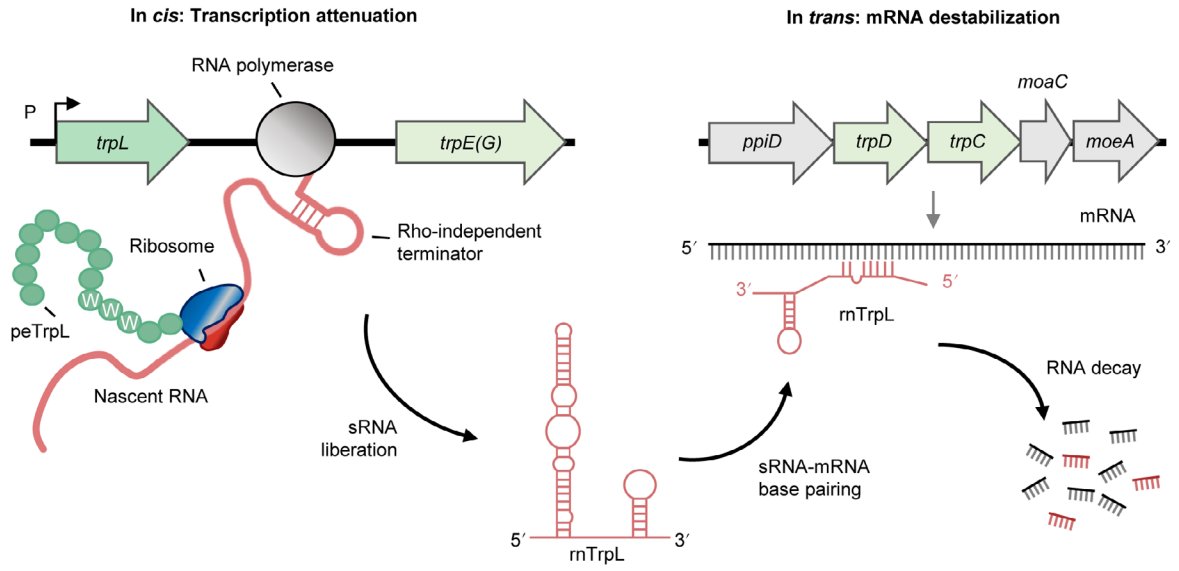

FIGURE 3 Coordination of the expression of split *trp* operons at the posttranscriptional level by the attenuator sRNA rnTrpL. The same RNA sequence acts in *cis* as transcriptional attenuator of *trpE(G)* and, after attenuation in response to high level of charged $tRNA^{Trp}$, in *trans* as base-pairing sRNA to downregulate *trpDC* together with cotranscribed genes (adapted from Melior & Evguenieva-Hackenberg, 2021)

contains all genes for Trp biosynthesis, induction of rnTrpL in *trans* does not influence the level of the polycistronic *trpEDCBA* mRNA (Melior et al., 2019).

The *trpDC* genes are part of a larger operon (*ppiD-trpDC-moaC-moeA*) in *Sinorhizobium*, *Agrobacterium* and *Bradyrhizobium*. The operon genes *ppiD*, which encodes peptidyl–prolyl isomerase required for folding of outer membrane proteins/protein translocation (Dartigalongue & Raina, 1998; Fürst et al., 2018), and *moaC* and *moeA*, which encode enzymes for biosynthesis of the molybdenum cofactor MoCo (Iobbi-Nivol & Leimkühler, 2013), are also post-transcriptionally regulated by rnTrpL and thus in response to Trp availability (Melior et al., 2019). Several other genes, among them genes for transcription factors, were predicted to base pair with rnTrpL (alias RcsR1), suggesting that it has an extended regulon. Experimentally validated was the negative regulation by base-pairing of rnTrpL to *sinI* mRNA, which encodes an autoinducer synthase (Baumgardt et al., 2016). This suggests that *S. meliloti* uses Trp as a signal for the availability of nutrients to regulate bacterial communication. It is known that in addition to the cell density, the nutrient availability determines the quorum sensing onset (Xu et al., 2018).

5.3 | Response of the *trp* attenuator to translation inhibition

The *trp* attenuators of *E. coli* and *S. meliloti* bear even more surprises. Recently, we proposed that these attenuators respond to translation inhibition as an additional signal. This proposal is based on their molecular mechanism relying on mutually exclusive secondary structures in the nascent transcript. These alternative structures are formed with participation of four RNA regions (Regions 1–4 in Figure 1). In the above description of the ribosome-dependent attenuation in response to availability of charged $tRNA^{Trp}$, the antiterminator and terminator hairpins were mentioned. To understand why the *trp* attenuator could respond to additional signals, it is important to consider the anti-antiterminator structure, which is formed by base pairing of Regions 1 and 2 of the nascent RNA (Figures 1 and 4). When Trp is scarce, ribosome pausing at the Trp codons of *trpL* prevents this pairing and thus the anti-antiterminator stem-loop. This enables the base pairing between Regions 2 and 3, leading to the formation of the antiterminator helix, which in turn, precludes the formation of the transcription terminator containing Regions 3 and 4.

However, the tandem of anti-antiterminator and terminator is shaped also in the absence of translation. Indeed, in *E. coli*, mutation of the *trpL* start codon AUG to AUA increased the efficiency of attenuation 3- to 5-fold (Zurawski et al., 1978). Also for the *leu* operon in *Salmonella*, transcription attenuation in the absence of translation was described (during in vitro transcription), which was explained by the anti-antiterminator formation directly after transcription of the Regions 1 and 2, which precludes the antiterminator and supports the terminator formation (Gemmill et al., 1979). Similarly, mutations in the RBS of *trpL* increased transcription termination at the attenuator of *Serratia marcescens*. Since transcription attenuation in the absence of translation is stronger than under conditions of Trp excess, it was named superattenuation (Stroynowski et al., 1982). Superattenuation at the *trp* attenuator was also described in *S. meliloti* (Bae & Stauffer, 1991).

The superattenuation phenomenon suggests that the availability of charged $tRNA^{Trp}$ is not the only factor promoting transcription attenuation and thus rnTrpL expression. It is known that in *E. coli*, ribosome stalling before the 10th (Trp) codon does not relieve attenuation. More precisely, attenuation is relieved by ribosome pausing at the *trpL* codons 10–12 (Trp Trp Arg) which correspond to the *trpL* codons 10–12 (Trp Trp Trp) in *S. meliloti* (Zhu & Meyer, 2015). Thus, disturbance in the initiation of *trpL* translation or in translation of the codons 1–9 would lead to transcription attenuation even under conditions of Trp starvation (Figure 4). This could be due to antibiotics affecting the ribosome function or due to stringent response (Durfee et al., 2008). Since rnTrpL expression is a consequence of transcription attenuation, the sRNA could adopt functions in adaptation to attenuation-promoting conditions. This scenario is compatible with the newly described downregulation of *dnaA* by rnTrpL in *E. coli* (Li, Edelmann, et al., 2021) since the stringent response results in downregulation of replication initiation (Irving et al., 2021). So far, experimental evidence for response of the *trp* attenuator to translation disturbance was provided only in *S. meliloti* exposed to subinhibitory tetracycline (Tc) amounts. After the addition of Tc to cultures growing under Trp-limiting conditions, the attenuator sRNA rnTrpL accumulates and has an antibiotic-related function in regulating specific ribosomal genes (see below; Melior et al., 2021).

Transcription attenuation and release of corresponding attenuator sRNA in response to (partial) translation inhibition or amino acid starvation is expected at attenuators, which include an uORF in an anti-antiterminator structure. Such attenuators are often located upstream of amino acid biosynthesis genes (Keller & Calvo, 1979).

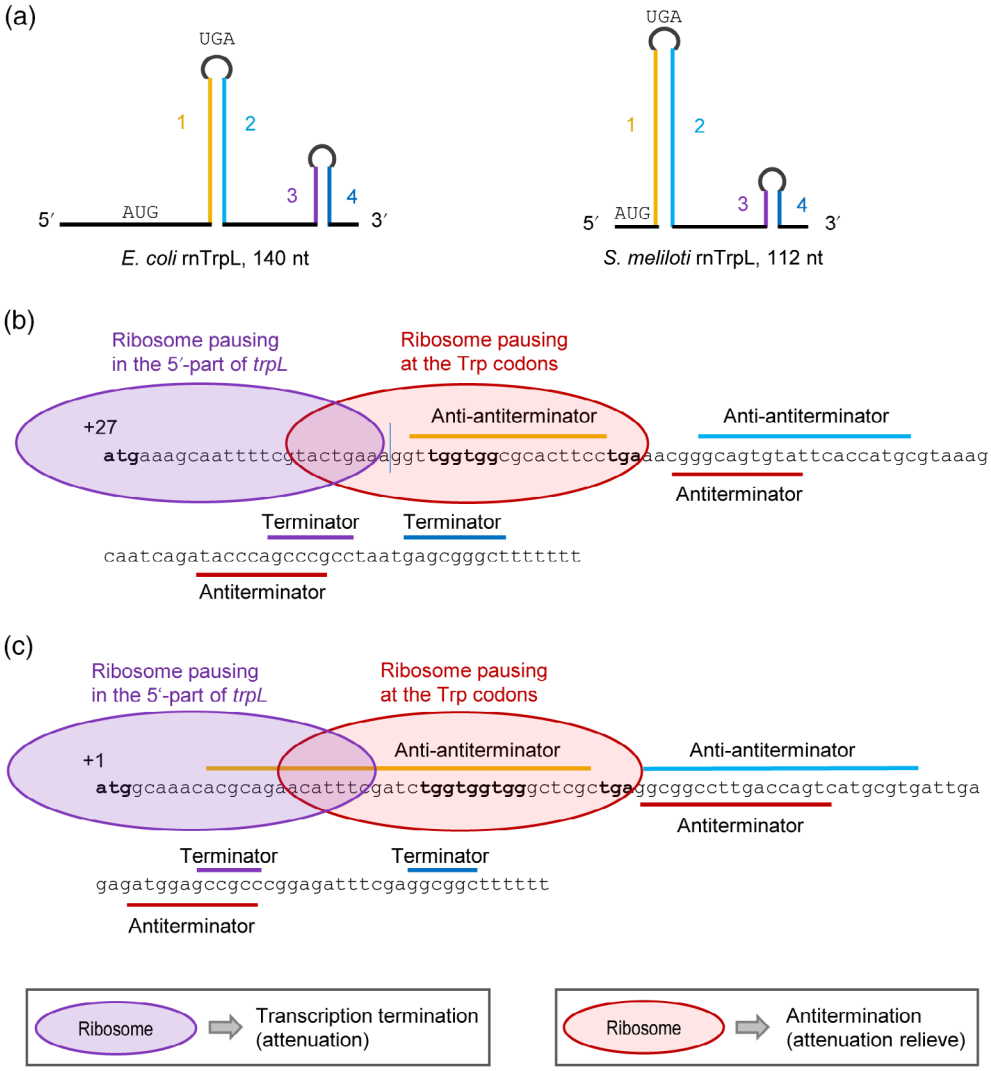

FIGURE 4 Model for transcription termination at the *trp* attenuators of *E. coli* and *S. meliloti* under different conditions. (a) Schematic of the anti-antiterminator and terminator stem-loops leading to transcription attenuation and liberation of the rnTrpL sRNAs in both species. Base pairing of Regions 1 and 2 in the nascent RNA lead to the formation of the anti-antiterminator hairpin, which causes formation of the terminator hairpin by base pairing of Regions 3 and 4, since base pairing between Regions 2 and 3 and thus antiterminator formation is prevented. Start and stop codons of *trpL* are indicated. In *E. coli*, *trpL* is preceded by a 5′-UTR, while *S. meliloti* rnTrpL is leaderless and directly starts with AUG. (b,c) Sequences of the rnTrpL genes in *E. coli* (b) and *S. meliloti* (c) starting with *trpL* (position of the *trpL* start in respect to the transcription start site is indicated). Start, Trp, and stop codons of *trpL* are in bold. Regions involved in the formation of mutually exclusive secondary structures in the nascent RNA are indicated. Red ellipses indicate ribosome coverage during transient pausing at the consecutive *trp* codons, which prevents anti-antiterminator formation, thus leading to the formation of the antiterminator and relieving attenuation (cotranscription of *trpL* with the structural genes). Purple ellipses indicate transient ribosome pausing at the begin or at the first codons of *trpL*, for example, due to subinhibitory amounts of translation inhibiting antibiotics. This still allows for (partial) anti-antitermination formation, thus leading to transcription attenuation and rnTrpL liberation

5.4 | Reprograming of the rnTrpL sRNA by the peTrpL leader peptide and antibiotics

While analyzing the interaction of rnTrpL with its predicted targets *trpDC* and *rplUrpmA* in *S. meliloti*, we made the puzzling observations that *rplUrpmA* is downregulated only in the presence of translation inhibitors such as Tc, and that for this the sRNA works in conjunction with the leader peptide (Figure 5). We found that base pairing between an *rplU* region and the sRNA takes place in an antibiotic-dependent ribonucleoprotein complex (ARNP), which obviously serves to destabilize the *rplUrpmA* transcript. The complex was isolated from *S. meliloti* by coimmunoprecipitation using 3 × FLAG-peTrpL produced additionally to the chromosomally encoded, wild type peTrpL, or by affinity chromatography using MS2-tagged rnTrpL.

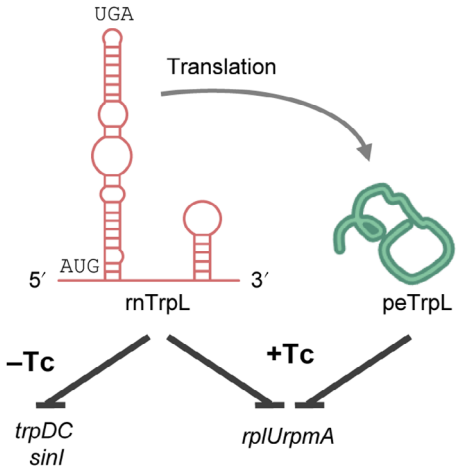

FIGURE 5 Reprograming of the base-pairing sRNA rnTrpL in response to antibiotic exposure. The attenuator RNA rnTrpL is a *trans*-acting sRNA, which also encodes the small protein peTrpL. In the absence of antibiotics, rnTrpL downregulates *ppiD-trpDC-moaC-moeA* (short *trpDC*) and *sinI* in a peTrpL-independent manner. Upon exposure to antibiotics such as Tc, Em, Cl, ot Km, the specificity of the rnTrpL sRNA changes in favor of *rplUrpmA*. The interaction between *rplUrpmA* and rnTrpL takes place in a peTrpL-containing ARNP

During the isolation, it was essential to use washing buffer containing the ARNP-triggering antibiotic, to avoid complex dissociation. The purified complex comprised peTrpL, rnTrpL, *rplUrpmA* mRNA, and corresponding asRNAs (Melior et al., 2021).

The antibiotic dependence of the *rplUrpmA*-ARNP was observed by its disassembly in buffer without an antibiotic and reassembly by adding Tc to the sample. For in vitro ARNP reconstitution, four components were necessary: the sRNA, a mini-*rplU* transcript representing the target mRNA, the peptide, and the antibiotic. Furthermore, reconstitution was dependent on the imperfect base pairing between *rplU* and rnTrpL. Alanine scanning mutagenesis revealed that T4, S8 and W12 are crucial to the peTrpL activity in vivo and for ARNP reconstitution in vitro (Melior et al., 2021). Thus, the peptide probably adopts alpha-helical conformation in the complex, while the pure peptide is insoluble (Kubatova et al., 2020). Physiological studies and mass spectrometry revealed that 3 × FLAG-peTrpL interacts with the chromosomally encoded peptide, indicating that peTrpL oligomerizes in the ARNP. Mass spectrometry also detected a strong increase in the level of the chromosomally encoded peTrpL 10 min after the addition of subinhibitory Tc amount to the culture, and reporter fusion constructs suggested that this is due to peptide stabilization in the ARNPs (Melior et al., 2020).

According to in vivo data, the *trpDC* and *sinI* mRNAs are a Tc- and peTrpL-independent targets of *S. meliloti* rnTrpL, while *rplUrpmA* is a Tc- and peTrpL-dependent target (Figure 5). In other words, the specificity of the constitutively transcribed rnTrpL sRNA in the cell is reprogramed with the help of peTrpL in response to antibiotic exposure. Evidence for this change in target specificity was also presented in vitro: When short *rplU* and *trpD* transcripts comprising the base-pairing regions compete for interaction with rnTrpL in a sample containing peTrpL, the *trpD* transcript is preferentially bound if the Tc concentration is below a threshold. However, an increase in the Tc concentration leads to exclusive binding of *rplU*. Besides Tc, several other, structurally different translation inhibitors are able to support the *rplUrpmA*-ARNP in vivo and in vitro: erythromycin (Em), chloramphenicol (Cl), and kanamycin (Km), while the transcriptional inhibitor rifampicin (Rf), or the plant flavonoids genistein (Gs) and luteolin (Lt) have not this capability.

Transcription of the asRNAs, which were found in the ARNP, was induced only by the ARNP-supporting translation inhibitors. The asRNAs were not necessary for in vitro complex reconstitution, but increased the reconstitution efficiency, suggesting that their role in vivo is to support the *rplUrpmA*-ARNP formation (Melior et al., 2021).

The sRNA rnTrpL is the first sRNA shown to regulate ribosomal genes in *trans*. It also exemplifies the first example of sRNA reprograming, suggesting that small molecules can increase the riboregulation flexibility in response to metabolic or environmental changes.

5.5 | Regulation of multiresistance by a second, peTrpL-containing ARNP type

In addition to participating in the *rplUrpmA*-ARNP together with the rnTrpL sRNA, the leader peptide peTrpL has its own, rnTrpL-independent function in regulating the *smeABR* operon (Figure 6; Melior et al., 2020). This operon

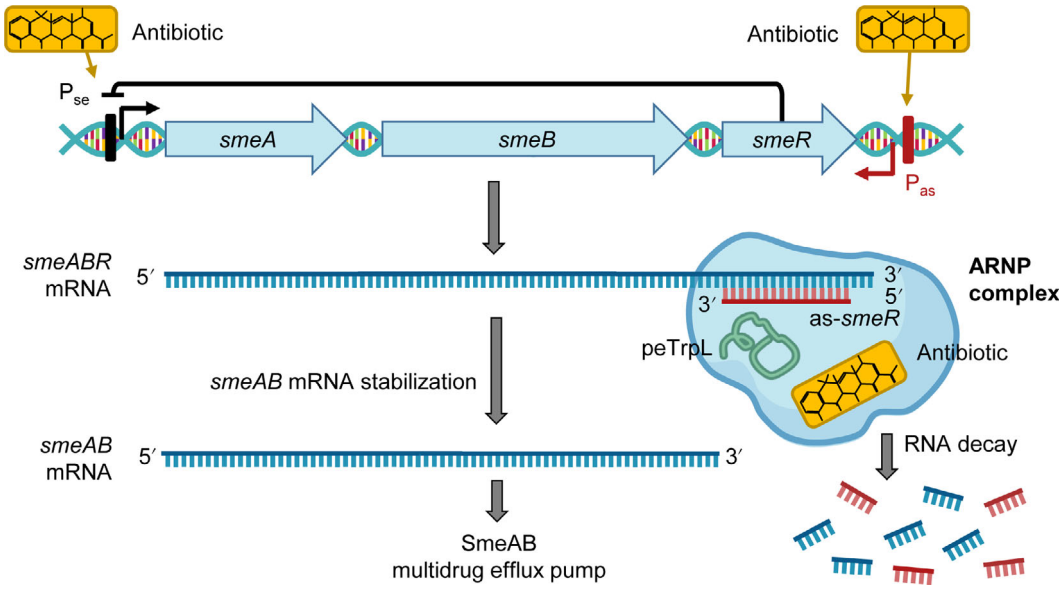

FIGURE 6 Model of the differential regulation of the multidrug resistance operon *smeABR* in response to antibiotic exposure. Antimicrobial compounds such as Tc, Em, Cl, Rf, and Gs, which are extruded by the MDR efflux pump SmeAB, induce transcription of *smeABR* from the sense promoter (P_{se}) and as-*smeR* RNA from the antisense promoter (P_{as}). The as-*smeR* RNA, the antibiotic (shown is Tc), and peTrpL form together with the *smeR*-part of the *smeABR* transcript an ARNP, in which the RNAs are degraded, while *smeAB* mRNA is stabilized. This prevents the production of the SmeR repressor and supports the production of the SmeAB efflux pump (adapted from Melior & Evguenieva-Hackenberg, 2021)

encodes the major multidrug resistance (MDR) efflux pump SmeAB, which is important for the symbiotic competitiveness of *S. meliloti* (Eda et al., 2011). Additionally, it encodes SmeR, the multidrug-binding repressor of the operon. Since in the initial phase of adaptation to antibiotic exposure *smeAB* but not *smeR* should be expressed, the operon is differentially regulated at the level of RNA. This posttranscriptional regulation is accomplished with the help of the peTrpL peptide and an as-*smeR* RNA, which is complementary to the *smeR* part of the *smeABR* transcript. Transcription of as-*smeR* is induced by compounds, which are extruded by the SmeAB pump: Tc, Em, Cl, Rf, and Gs (but not Km or Lt, which are not extruded by SmeAB). The asRNA uses a specific region to bind peTrpL in ARNP complexes depending on Tc, Em, Cl, Rf, and Gs (but not Km or Lt), and to destabilize the *smeR* part of the *smeABR* transcript, while the *smeAB* part is stabilized. The peTrpL mechanism in the *smeR*-ARNP is probably similar to that in the *rplUrpmA*-ARNP, because the peptide amino acid residues Thr4, Ser8, and Trp12 are crucial to the formation of both ARNP types.

The two ARNP types, *rplUrpmA*-ARNP and *smeR*-ARNP, depend on distinct, but overlapping sets of antimicrobial compounds (Figure 7). Complexes of the *rplUrpmA*-ARNP type are supported by translation inhibiting antibiotics, while those of the *smeR*-ARNP type are held together in the presence of substrates of the MDR efflux pump SmeAB (Melior & Evguenieva-Hackenberg, 2021). Thus, a separate analysis of the two ARNP types is possible by separate induction of their assembly. For example, Km can be used to induce *rplUrpmA*-ARNP formation, and Rf or Gs for the formation of *smeR*-ARNP. Upon exposure to Tc, Em, or Cl, both ARNPs are assembled in parallel in the cell, but their separation is possible by using MS2-tagged variants of their specific RNA components. The sRNA rnTrpL is the regulatory RNA component of the *rplUrpmA*-ARNP, while in the *smeR*-ARNP this function is occupied by the as-*smeR* RNA (Melior et al., 2021).

The roles of the asRNAs in the *rplUrpmA*-ARNP seem not to be essential, but they share important features with the *as-smeR* RNA. As shown by *egfp* promoter fusions, transcription of the asRNAs is induced by the specific antimicrobial compounds supporting the respective ARNPs. However, the as-*rplUrpmA* RNA and the as-*smeR* RNA were detected only after enrichment in the ARNPs, but not by RNA-seq of total RNA. This could be explained by the rapid co-degradation of the asRNAs with their targets, as suggested by qRT-PCR analysis of changes in their levels upon Tc exposure. Thus, the ARNP purification revealed functional asRNAs that escaped detection by other methods (Melior et al., 2020, 2021).

The above shows that the *trans*-acting products of the *trp* attenuator play roles in adaptation to antibiotics. However, are peTrpL and rnTrpL synthesized upon antibiotic exposure? In nature, antibiotics are usually present at low, subinhibitory concentrations. In the presence of subinhibitory amounts of a translation inhibitor such as Tc, rnTrpL is still transcribed, and *trpL* translation is occasionally initiated. Disturbance in initiation and elongation of *trpL*

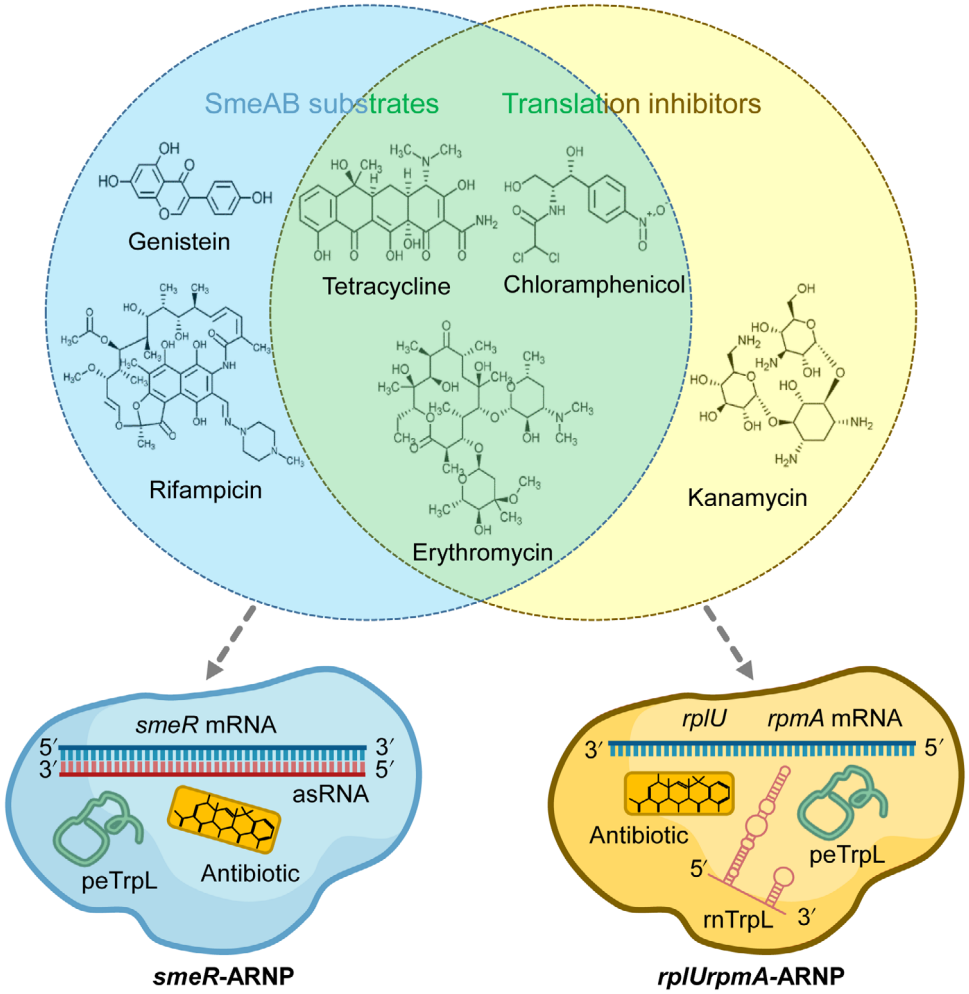

FIGURE 7 Structurally different antimicrobial compounds support the formation of two different, peTrpL-comprising ARNP types. The *smeR*-ARNP type is supported by SmeAB-substrates, while the *rplUrpmA*-ARNP type is supported by translation inhibitors. The SmeAB-substrates induce the as-*smeR* RNA, which plays a central role in the *smeR*-ARNP. The central riboregulatory role in the *rplUrpmA*-ARNP is exerted by rnTrpL. The antibiotics supporting this ARNP type are also inducing asRNAs, but since these asRNAs were not required for ARNP reconstitution in vitro, they are not shown here (adapted from Melior & Evguenieva-Hackenberg, 2021)

translation leads to superattenuation, because the pioneering ribosome transiently stalls in *trpL* before the Trp codons. Due to the subinhibitory antibiotic amount, peTrpL is eventually translated. Thus, both rnTrpL and peTrpL should be available for gene regulation under such conditions.

The ARNP mechanism is well suited for fast adaptation to antibiotic exposure, because the antibiotics directly participate in complex assembly and thus their intracellular concentration governs the stability of specific mRNAs. For example, at the begin of Tc exposure, when the antibiotic concentration in the cell is relatively high, *smeR*- as well as *rplUrpmA*-ARNPs are formed, and corresponding mRNAs are destabilized. The *smeR*-ARNP supports *smeAB* expression and the MDR efflux pump SmeAB is produced. The pump extrudes Tc, until intracellular concentration is reached, at which the ARNPs are disassembled. This leads to an increase in SmeR production and thus repression of the *smeABR* operon, and to normalization of the *rplUrpmA* expression. Antibiotics such as Km, which are not extruded by SmeAB, are probably removed by another efflux pump (Eda et al., 2011).

5.6 | Conservation of antibiotic-related functions of the *trp* attenuator

The role of rnTrpL in downregulation of *trpDC* and *rplUrpmA*, as well as the role of peTrpL in regulation of *smeABR* (*acrABR*) homologs seems to be conserved in other soil-dwelling and plant-interacting Alphaproteobacteria such as

Bradyrhizobium and *Agrobacterium* (Melior et al., 2020, 2021). The *Rhizobiales* members *Sinorhizobium*, *Bradyrhizobium*, and *Agrobacterium* have similar habitats, a similar arrangement of the *trp* genes, and most probably similar regulation of the three *trp* operons with constitutive *trpE(G)* transcription during growth, which leads to almost constitutive *trpL* expression. The latter probably enabled the development of peTrpL-dependent ARNP mechanisms in these bacteria, which coexist with antibiotic producers in the soil and are also exposed to plant-derived antimicrobial compounds. However, despite the conservation of the *trp* attenuator, the amino acid sequence of peTrpL does not show highly conserved motif(s) besides the consecutive Trp residues. In line with this, overexpression of the respective *trpL* ORF increased resistance to Tc in *Sinorhizobium*, *Agrobacterium*, or *Bradyrhizobium*, but heterologous overexpression had no effect (Melior et al., 2020).

Antibiotic-related functions of rnTrpL could also exist in Gammaproteobacteria, for which superattenuation was described. It will be interesting to see whether medically relevant strains or species belonging to *Escherichia*, *Salmonella*, *Vibrio*, and *Pseudomonas*, also use rnTrpL and/or peTrpL for antibiotic-induced or even ARNP-mediated regulation. *Trans*-acting rnTrpL and/or peTrpL can also be expected in *Bordetella*, *Brucella*, *Corynebacterium*, *Streptomyces*, *Chlamydia*, and *Deinococcus* (Merino et al., 2008).

5.7 | The novel mechanisms of the *trp* attenuator and its products: Outlook

The recent findings on the *trp* attenuator and its *trans*-acting products rise many questions that still need to be answered in future studies to complete the rnTrpL and peTrpL regulatory networks, to understand the mechanistic details of the posttranscriptional regulation by ARNPs, and to determine the ARNP prevalence in other organisms. For example, the proposed transcription attenuation in soil Alphaproteobacteria in response to translation inhibition by antibiotics other than Tc or under conditions of stringent response should be proved. Its validity in other bacteria including *E. coli* or for other anti-antiterminator containing attenuators also needs to be tested. Superattenuation at the *trp* operon is possible in enteric bacteria (Stroynowski et al., 1982), but is it used in response to antibiotics?

The existence of ARNPs and their importance for destabilization of specific mRNAs in response to antibiotics exposure was clearly shown, but they await thorough characterization. The structural rearrangements needed for a productive interaction between rnTrpL and *rplU* in the ARNP and the exact mechanisms of mRNA destabilization upon ARNP assembly should be uncovered. The most striking feature of the ARNPs is the capability of structurally diverse compounds to form complexes with the peTrpL peptide and with specific RNAs. This raises two important questions: (1) How is the specificity in RNA binding achieved and (2) do such compounds play a role in riboregulation beyond resistance, for example, in their producers and in microbial communities (Sengupta et al., 2013). To answer the first question, structural probing of RNA in the complexes, (further) peptide, and RNA mutagenesis followed by complex reconstitution and analysis, crystal structures determination, and/or NMR analyses of ARNPs could be helpful. To address the second question, the existence of ARNPs in antibiotic producers and natural communities should be studied. The diversity of the ARNP-supporting compounds also suggests that similar RNP complexes could be assembled in the cell with the help of metabolites, for example, Trp or its precursors, and play a role in processes different from an antibiotic response.

In *S. meliloti*, the peTrpL level is strongly increased under conditions of Tc exposure and it was proposed that this is mainly due to peptide stabilization in the ARNPs. To understand the regulation of peTrpL accumulation, the *trpL* transcription and translation should be analyzed in detail, peptide stabilization in vivo in the presence of Tc should be tested experimentally, and it should also be tested whether peTrpL specifically accumulates upon exposure to other ARNP-supporting antimicrobial compounds.

The ARNP mechanism seems to be conserved in the presence of Tc at least in *Agrobacterium* and *Bradyrhizobium*. However, ARNPs were not purified from these bacteria, corresponding complexes were not reconstituted and the involvement of other antimicrobial compounds was not tested yet. The molecular analyses of ARNPs from bacteria other than *S. meliloti* may help to understand how this presumably conserved mechanism relies on small peptides lacking strong sequence conservation. Furthermore, it will be interesting to see whether peTrpL is functional in more distantly related bacteria.

Another open question is the mechanism of induction of the ARNP-associated asRNAs in response to structurally diverse antimicrobial compounds. Transcription of the *smeABR* operon and the as-*smeR* RNA seems to be induced by the same set of antimicrobial compounds (Tc, Em, Cl, Gs, and Rf) (Melior et al., 2020) and thus it is tempting to speculate that the SmeR repressor regulates the transcription at the *smeABR* locus in both directions. However, the sequence

proposed to be bound by SmeR upstream of *smeA* (Eda et al., 2011) was not found in the region of the as-*smeR* promoter. Furthermore, transcription of the asRNAs complementary to rnTrpL and *rplUrpmA* was induced by an overlapping set of antibiotics (Tc, Em, Cl, and Km; Melior et al., 2021). Altogether, this suggests that *S. meliloti* harbors at least two multidrug binding transcriptional regulators with partially overlapping specificities and/or transcription of the ARNP-associated asRNAs is controlled by several transcription factors. This and the question about the in vivo impact of the asRNAs, which in vitro only increase the ARNP reconstitution, should be addressed in future studies.

The physiological role of *rplUrpmA* downregulation by rnTrpL reprogramming in response to antibiotic exposure also needs to be clarified. As mentioned previously (Melior et al., 2021), this response may cause downregulation of ribosome biogenesis in order to save resources for SmeAB production and antibiotic extrusion. Alternatively or in addition, the posttranscriptional *rplUrpmA* downregulation may result in the production of a ribosome subpopulation lacking L21 and L27, which may play a role in adaptation to antibiotic exposure. Indeed, it was shown that a 2-fold reduction of *rplUrpmA* expression in *Pseudomonas aeruginosa* results in the assembly of specialized ribosomes, which cause increased expression of multidrug efflux pump genes (Lau et al., 2012).

Another non-solved question is why genes with no clear relevance for Trp metabolism are cotranscribed and thus directly co-regulated with *trp* genes in many bacteria including *S. meliloti*. Finally, it is not known whether rnTrpL and peTrpL play a role in the symbiosis of *S. meliloti* with plants.

In summary, it is fascinating that the *trp* attenuator, the prototype of transcription attenuators described in textbooks, responds to more than one signal and gives rise to multifaceted, *trans*-acting products. In *S. meliloti*, its locus comprises overwhelmingly dense genetic information: The same RNA sequence performs transcription attenuation when charged $tRNA^{Trp}$ is available or upon translation inhibition, acts as a reprogrammable base-pairing sRNA having multiple targets, encodes a small protein (leader peptide) which works alone and in conjunction with this sRNA in ARNPs, and, last but not least, corresponds to an antisense RNA which supports the ARNPs formation.

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CONFLICT OF INTEREST

The author has declared no conflicts of interest for this article.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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