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Regulation of antibiotic-resistance by non-coding RNAs in bacteria

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Antibiotic resistance genes are commonly regulated by sophisticated mechanisms that activate gene expression in response to antibiotic exposure. Growing evidence suggest that *cis*-acting non-coding RNAs play a major role in regulating the expression of many resistance genes, specifically those which counteract the effects of translation-inhibiting antibiotics. These ncRNAs reside in the 5′UTR of the regulated gene, and sense the presence of the antibiotics by recruiting translating ribosomes onto short upstream open reading frames (uORFs) embedded in the ncRNA. In the presence of translation-inhibiting antibiotics ribosomes arrest over the uORF, altering the RNA structure of the regulator and switching the expression of the resistance gene to 'ON'. The specificity of these riboregulators is tuned to sense-specific classes of antibiotics based on the length and composition of the respective uORF. Here we review recent work describing new types of antibiotic-sensing RNA-based regulators and elucidating the molecular mechanisms by which they function to control antibiotic resistance in bacteria.

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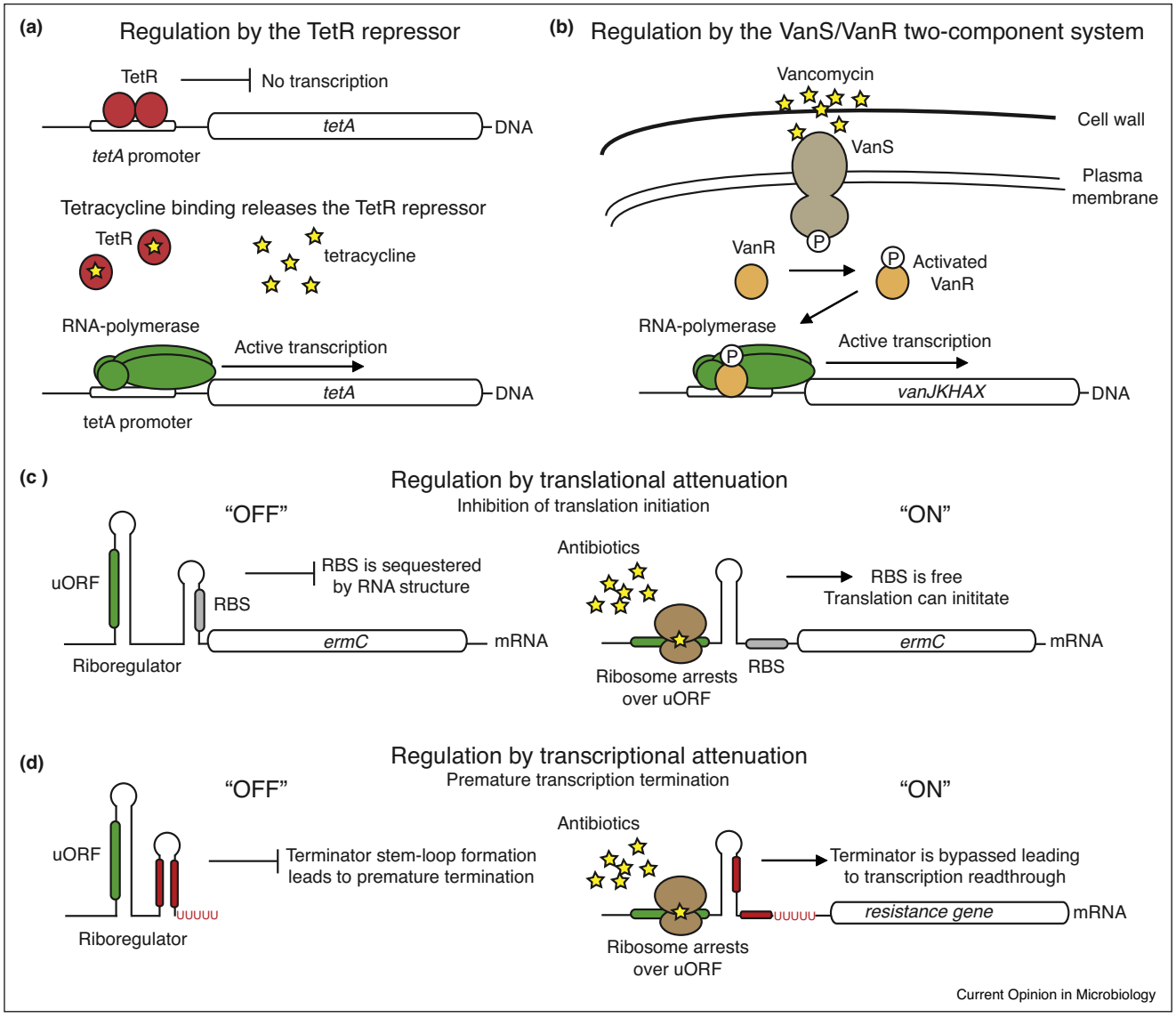
Introduction

The discovery of antibiotic compounds had made a profound impact on modern medicine, extending both life span and life quality. Presently, antibiotics are pervasively administered in clinical and in veterinary care, as well as in agricultural applications, resulting in a tremendous flow of antibiotics into the environment [1]. The immense selective pressure caused by exposure to antibiotic has driven the rapid spread and evolution of antibiotic-resistance genes among pathogenic and commensal bacteria [2,3]. These resistance genes allow bacteria to overcome antibiotics through various mechanisms, such as the ejection of antibiotics from the cell via efflux pumps, enzymatic deactivation of the antibiotic molecules, and protection of antibiotic cellular targets via chemical modifications (*e.g.* modifying the ribosome or the cell wall) [4]. Together, such resistance mechanisms threaten the continued efficacy of antibiotics in medicine [3].

Although resistance genes provide bacteria with a distinct advantage during exposure to antibiotic, they are generally thought to carry a fitness burden at times when antibiotics are not applied [2,5,6]. For example methylation of specific residues in the 23S ribosomal RNA protects bacteria from macrolide antibiotics, but also causes cell-wide disruption in protein synthesis, which lead to major fitness defects [7•]. Therefore, to mitigate the negative effects of antibiotic resistance, bacteria employ regulatory mechanisms that can sense the antibiotic molecule, and then selectively activate the expression of the relevant antibiotic resistance genes only during exposure to the antibiotic, effectively circumventing the fitness burden and facilitating the long term maintenance of such genes in the genome [2,5,6,7•].

One of the common mechanisms underlying antibiotic-dependent activation of resistance genes is via specific transcription factors that can sense the presence of the antibiotic [4]. In Gram-negative bacteria such regulation is manifested, for example in the context of the tetracycline efflux pump, *tetA*. In the absence of tetracycline, the transcriptional repressor TetR constitutively binds the *tetA* promoter and inhibits the expression of the *tetA* resistance gene [8]. When tetracycline antibiotic is present in the cell, direct binding of tetracycline to the *tetR* repressor leads to its dissociation from the DNA and drives *tetA* expression, leading to antibiotic resistance [8] (Figure 1a). Transcription regulation has also been detected in the vancomycin resistance operon, v*anHAX*, commonly found in *Enterococci* [9]. In this case, however, the presence of vancomycin is detected by a membrane sensory kinase (VanS) which, in turn, phosphorylates and activates VanR, a transcription regulator that drives the expression of the *vanHAX* resistance operon [9] (Figure 1b). Analogous forms of transcription regulation occur in many bacteria, where they play important roles in controlling the expression of antibiotic resistance genes [2,5].

Figure 1



Control of antibiotic resistance by protein or RNA-based regulators. **(a)** The TetR DNA-binding repressor targets the promoter of *tetA* and blocks transcription. Upon binding to tetracycline, the repressor dissociates, promoting *tetA* transcription initiation. **(b)** The membrane protein VanS senses vancomycin and then phosphorylates the response regulator VanR, which activates the transcription of the vancomycin resistance gene operon. **(c)** Schematic representation of translational attenuation. In the absence of antibiotics the riboregulator folds into an RBS sequestering structure, such that translation of the mRNA of the resistance gene cannot be initiated. Subsequent exposure to translation inhibiting antibiotics causes the ribosome to stall over a specific position in the uORF, resulting in structural reshaping of the riboregulator such that the RBS is released, thus enabling translation to initiate. **(d)** Transcriptional attenuation regulates expression by controlling the formation of a premature transcription terminator—a stem-loop structure immediately followed by a poly uridine tract. In the absence of antibiotics, transcription begins, yet terminates prematurely. Ribosome stalling over the uORF inhibits terminator stem-loop formation and promotes transcription of the resistance gene.

RNA-mediated regulation of antibiotic resistance

Growing evidence show that, in addition to classic transcription-factor mediated gene regulation, bacteria frequently employ *cis*-regulatory non-coding RNAs (ncRNAs) that sense the presence of antibiotics and regulate resistance genes accordingly [10–12,13••]. These ncRNAs, also known as riboregulators, are structured RNA elements that reside within 5′ untranslated regions (5′UTRs) of antibiotic resistance genes, in particular those that provide resistance to ribosome-inhibiting antibiotics. In the absence of antibiotics these RNA regulators inhibit the expression of the resistance gene in *cis* by either masking the ribosome binding site (RBS) or

generating a premature transcriptional terminator within the 5′UTR [14,15] (Figure 1c,d). Conversely, when the antibiotic is present in the cell, the RNA structure of these regulators is altered, resulting in alternative base-pairing patterns that do not attenuate transcription or translation, and so activate the expression of the regulated gene (Figure 1c,d).

The majority of antibiotic responsive riboregulators have been found to sense the presence of translation-inhibiting antibiotics by directly measuring the activity the ribosome, that is the target of the antibiotic [11]. This regulatory mechanism, also known as attenuation, is enabled by the utilization of short upstream open reading frames (uORF) that recruit translating ribosomes to the regulatory 5′UTR element. In the presence of certain antibiotics, translating ribosomes enter a prolonged arrested state over a specific part of the uORF, which physically disrupts the formation of the inhibitory RNA structure thus releasing the repression over the gene [11,16] (Figure 1c,d). In addition to these common ribosome-dependent regulators, only one riboregulator, the aminoglycoside riboswitch, was so far suggested to sense and respond to antibiotics via direct binding of the antibiotic molecule itself [17•], although the validity of this finding is still under debate [18,19].

The best studied case of ribosome-dependent riboregulation is the one controlling the *Staphylococcus aureus ermC* gene, which provides resistance against Macrolide–Lincosamide–Streptogramin B (MLS) antibiotics by methylating the 23S ribosomal RNA residue required for their binding [7•,20,21•]. In the absence of antibiotics, the *ermC* riboregulator tightly represses the expression of *ermC* by base-pairing with its RBS sequence, preventing its translation [10,22,23]. When the antibiotic is bound to the ribosome, ribosome pausing over the *ermC* sensory uORF triggers a conformational change and leads to alternative base-pairing that releases the RBS and induces ErmC protein synthesis [10,22,23] (Figure 1c). This form of regulation by translational attenuation was found to control other resistance genes, including many additional 23S rRNA methylases [11,24,25] and chloramphenicol inactivating proteins [26,27].

In addition to regulation of translation initiation, drug-dependent riboregulators were also shown to control the expression of antibiotic-resistance genes at the transcriptional level [15]. For example in the absence of lincosamide antibiotics, the *Bacillus subtilis bmrB* riboregulator inhibits the expression of the *bmrCD* antibiotic efflux genes by preventing RNA polymerase from synthesizing the *bmrCD* mRNA. This pattern of transcriptional attenuation is dependent on the formation of a premature transcription terminator signal composed of a stable stem-loop structure immediately followed by a stretch of uridine residues [13••,28,29]. In this case, antibiotic-dependent ribosome stalling over the short uORF disrupts the terminator stem-loop base-pairing, enabling transcriptional readthrough and synthesis of the *bmrCD* mRNA [28] (Figure 1d). Transcriptional attenuation has been implicated in the regulation of several antibiotic resistance genes including ribosome methylating enzymes [15], antibiotic efflux pumps [28,30], and ribosome rescue factors [31,32].

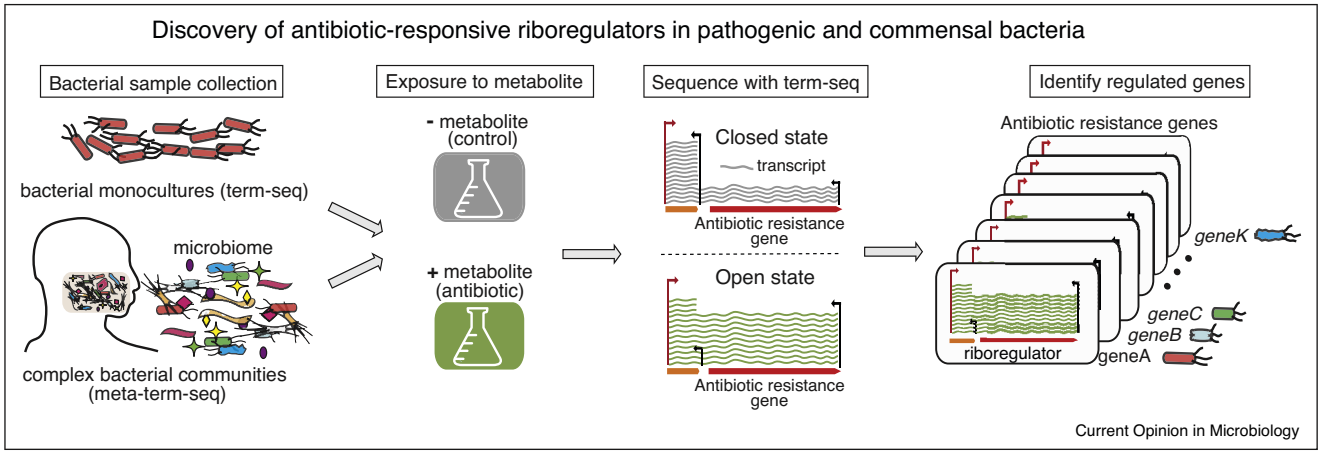
Until recently, most antibiotic-responsive riboregulation was thought to function via RBS-mediated translational attenuation, with transcriptional attenuation (regulation via the generation of a premature transcriptional terminator in the 5′UTR) considered relatively rare [11,15]. A recent study, which employed term-seq, a high-throughput RNA-sequencing method for detecting premature transcription termination events, discovered numerous riboregulators that control antibiotic resistance genes via transcription attenuation [13••]. In this study, term-seq was applied to bacteria exposed to sublethal doses of various antibiotics, and the resulting transcriptomes were compared to identify riboregulators that specifically activated gene expression in response to antibiotic exposure (Figure 2). This genome-wide approach was then extended to the human oral microbiome, a complex community comprising hundreds of bacterial species, where term-seq revealed that antibiotic-dependent riboregulation is common in human commensal bacteria and in pathogens, suggesting important roles for riboregulation in shaping the microbiome response to antibiotic treatment [13••] (Figure 2).

Furthermore, the above-described multi-bacteria study found that for most classes of resistance genes against ribosome-inhibiting antibiotics there were instances showing their regulation by such antibiotic-responsive riboregulators. These included several predicted classes of multidrug antibiotics efflux pumps and exporters [33], rRNA methylases that confer antibiotic resistance via modification of the ribosomal RNA [7•], acetyltransferases that acetylate the antibiotic molecule thus directly deactivate it [12], genes known to rescue antibiotic-bound ribosomes [34], and additional genes that may have antibiotic resistance properties not yet described [13••]. Therefore, while resistance to ribosome-inhibiting antibiotics can be manifested by many types of enzymatic activities, regulation of this resistance commonly leans on the principle of ncRNA-mediated regulation.

Surprising antibiotic specificity of ribosome-sensing riboregulators

The ribosome is a central target for multiple types of natural antibiotics, and so devising specific sensors for each type of antibiotic may inflict significant regulatory burden on bacteria. Sensing the inhibition of ribosome activity via short uORFs in riboregulators provides bacteria with an elegant solution to this problem, as this

Figure 2



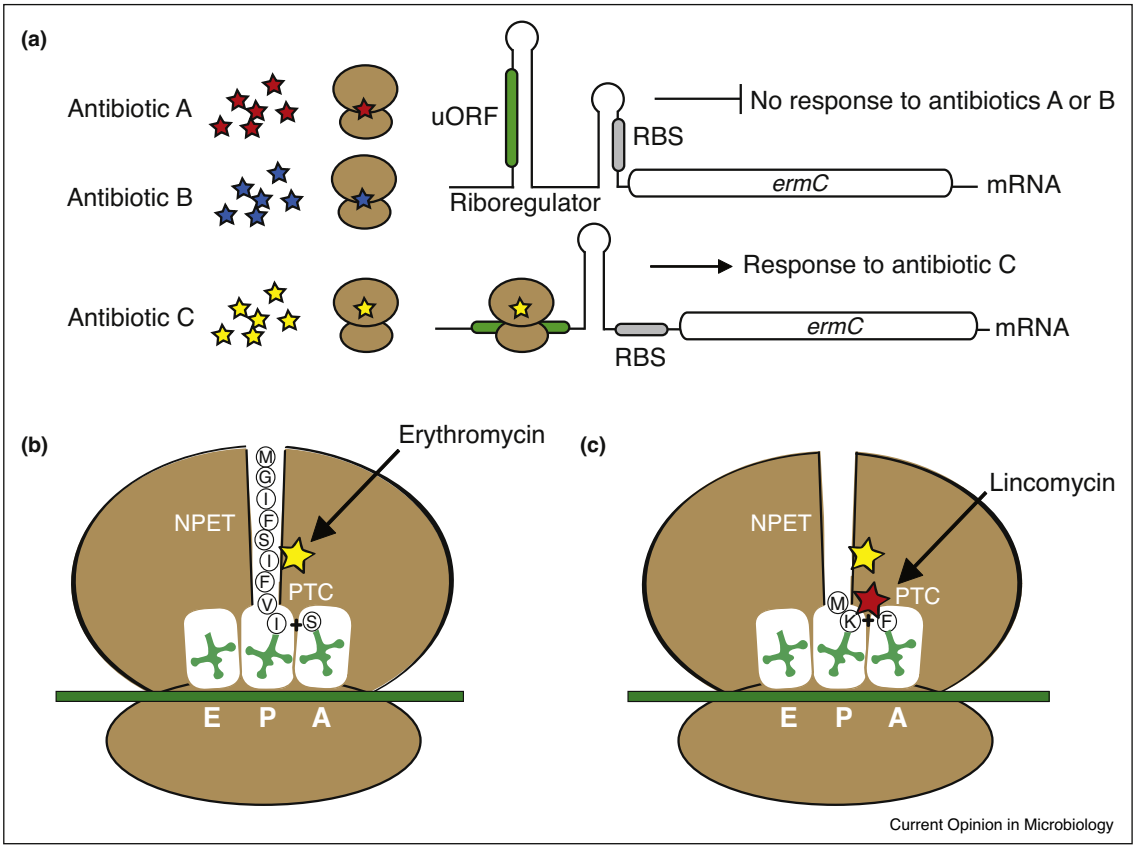
Discovery of antibiotic-responsive riboregulators in bacteria using RNA sequencing. Bacterial cultures or complex communities collected from the human microbiome are briefly exposed to a sublethal dose of antibiotics, followed by term-seq and RNA-seq. Transcriptome-wide analyses reveals the genomic loci of riboregulators that specifically activate gene expression in response to antibiotic exposure by bypassing a premature transcriptional terminator [13••].

allows sensing the initial exposure to any ribosome-inhibiting antibiotic. However, in principle, this form of regulation should be relatively indiscriminate, as various antibiotics and environmental stresses, including amino-acid starvation, may lead to ribosome stalling over the riboregulator uORF [35,36]. Nonetheless, while some riboregulators respond to multiple classes of antibiotic [13••], surprising specificity has been reported for others. For example the *lmo0919* gene in *Listeria monocytogenes*, which confers resistance to lincomycin, is regulated by a riboregulator that specifically responds to lincomycin but not to erythromycin or chloramphenicol, although all three antibiotics inhibit translation elongation [13••]. Similar observations in additional regulators have previously led to the suggestion that there is a 'code' which may dictate specificity to particular antibiotics [10,13••,37••,38]. Indeed, different classes of ribosome targeting antibiotics bind and inhibit different functional regions in the ribosome, which may provide a clue to how riboregulators can specifically sense one antibiotic over another [39,40].

Interestingly, mechanistic studies of the *ermC* regulator have suggested that the particular amino-acid sequence of the uORF plays a major role in directing the specificity of this regulator to particular macrolide antibiotics [21•,37••,41,42]. Macrolide antibiotics were previously shown to bind within the ribosome nascent peptide exit tunnel (NPET), through which the synthesized polypeptide emerges [35,43]. This positioning in the tunnel facilitates interactions between the ribosome components, the antibiotic molecule and the amino-acids generated during synthesis [16,21•]. It was suggested that when the macrolide erythromycin is bound to the ribosome, specific amino-acid combinations in the uORF promote translational arrest while others are thought to allow progress of the nascent polypeptide chain through the tunnel [11,42]. This provides a potential discriminatory system, based on the amino acid sequence of the uORF, for differentiating between various macrolide antibiotics (Figure 3a). Recent ribosome profiling experiments in bacteria exposed to different macrolide antibiotics revealed that drug-bound ribosomes preferentially pause at specific short sequence motifs, providing further support to the amino-acid code for guiding programmed ribosome stalling [44••,45••]. Furthermore, a recent study has demonstrated that the antibiotic specificity of two different riboregulators can be changed via single amino-acid mutations in the uORF sequence [37••], suggesting that riboregulators can rapidly evolve to discriminate between different types of macrolides.

While much progress has been made towards understanding the 'code' guiding the specificity of *ermC* and closely related riboregulators to different macrolides, not much is known as to the principles dictating specificity to other, non-macrolide antibiotics. For example the recently discovered *Listeria monocytogenes lmo0919* riboregulator has been shown to specifically respond to lincomycin (a lincosamide antibiotic) but not to erythromycin, and the mechanism of specificity in this case is still unclear [13••]. Interestingly, the uORF in the *lmo0919* riboregulator is only three amino-acid long, the shortest uORF documented to participate in transcriptional attenuation-based regulation. The length of this uORF, which is conserved across lincomycin-responsive riboregulators, stands in contrast to uORFs of macrolide-sensitive regulators that are usually longer than 10 amino-acids [11,42].

Figure 3



Specificity in ribosome-dependent riboregulation of antibiotic resistance. **(a)** While many different antibiotics inhibit ribosome progression, riboregulators often display specificity to a single class of antibiotics. **(b)** Ribosome (brown) translating the *ermC* uORF mRNA (green line) with the nascent peptide, synthesized in the peptidyl-transferase-center (PTC) and emerging from the nascent polypeptide exit tunnel (NPET). tRNAs, shown in green occupy the A, P and E sites. Erythromycin, shown as a yellow star, is bound within the NPET, where its interaction with the emerging polypeptide determines the position of ribosome stalling. **(c)** Translation of the *lmo0919* 3-amino-acid long uORF. Lincomycin and erythromycin are shown as red and yellow starts, respectively. In this riboregulator, the uORF is too short to interact with erythromycin. In contrast, lincomycin, which binds the PTC region, can induce ribosome arrest, providing a potential explanation for why this riboregulator responds to lincomycin but not to erythromycin [13••].

We propose that the uORF length may act as an important determinant of the riboregulator specificity to lincomycin: while macrolides bind the NPET, lincomycin binds near the ribosome peptidyl-transferase-center (PTC), where the nascent peptide and newly arrived amino-acid donors are physically linked. In accordance, it was shown that lincosamide antibiotics pause the ribosome after the addition of 1–2 amino acids [46], while erythromycin generally require the synthesis of 6–8 amino-acids before the nascent chain interacts with the antibiotic at the NPET [46] (Figure 3b). Therefore, uORFs sized 3aa could lead to preferential stalling of lincomycin-bound ribosomes, but not of erythromycin-bound ones, suggesting a plausible mechanism for the specificity (Figure 3c). Nevertheless, it is likely that additional factors play a role in determining this specificity, as chloramphenicol, which also binds the PTC, does not activate the *lmo0919* riboregulator [13••].

Outlook

While numerous ribosome-sensing riboregulators are estimated to reside in bacterial genomes [13••,47], there is still little evidence for *cis*-encoded RNA regulators that respond to other antibiotic classes such as beta-lactams, fluoroquinolones, glycopeptides and other non-ribosome targeting drugs. Nevertheless, the natural diversity and abundance of ncRNA-based regulation in various biological processes, as well as the benefits of controlled antibiotic-resistance, suggests that such riboregulator classes may exist and remain to be discovered [13••,48]. The development and application of genome-wide discovery techniques is expected to play an essential role in comprehensive and more detailed understanding of antibiotic regulation principles [13••,47,49].

In addition to *cis*-acting regulators, bacteria commonly utilize other regulatory types of ncRNAs such as small

trans-acting RNAs (sRNAs) or antisense transcripts, which generally target specific mRNA molecules and modulate their translation rate or stability [50]. Significant advancements over the last decade have demonstrated broad roles for sRNAs in fine-tuning numerous biological processes, including many which can potentially influence antibiotic susceptibility [50,51]. A recent transcriptome-wide study in *Pseudomonas putida* has found that many sRNAs are differentially expressed in response to antibiotic exposure, suggesting a possible role for these ncRNAs in antibiotic resistance [52]. Thus, future studies may uncover additional forms of RNA-based regulation of antibiotic resistance [51].

Antibiotic-responsive *cis*-acting ncRNAs have emerged as frequent regulators of resistance to translation-inhibiting drugs. While major progress has been made towards understanding the details of ribosome sensing by these riboregulators, significant challenges remain on the road for clinical application. In principle, compounds that target such riboregulators could mitigate antibiotic resistance, marking these ncRNA elements as plausible drug targets. It remains to be seen whether such drugs will be developed.

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