Chapter 24
Influence of Regulatory RNAs on Antimicrobial Resistance and Efflux Mechanisms

Xu Jia, Bao-Dong Ling, and Xian-Zhi Li

Abstract Regulatory RNA molecules in bacteria have increasingly been shown to play an important role in influencing gene expression, particularly during the response to intracellular and environmental signals or stress conditions (including exposure to antimicrobial agents). These RNAs include the noncoding small RNA (sRNA) molecules and structured noncoding domains termed riboswitches. sRNA molecules can often have pleiotropic effect by targeting multiple mRNAs, and their activities are frequently dependent on the RNA chaperone Hfq protein. While sRNA molecules play their regulatory role through two major mechanisms, base pairing with RNAs and binding to effector proteins, riboswitches control transcription or translation by selectively binding to metabolites including antibiotics. This chapter provides an overview of regulatory RNA characteristics with a focus on their role in influencing antimicrobial resistance including the expression of drug efflux pumps. Effects of other RNA structural change-related mechanisms, such as ribosome stalling on antimicrobial resistance, are also described.

Keywords Antimicrobial resistance • Efflux pump • Regulatory RNA • Small RNA • sRNA • Riboswitch • Ribosome stalling • Hfq

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24.1 Introduction

Bacteria possess remarkable abilities to adapt to various environments including the development of antimicrobial resistance [1]. The latter can be adaptive or mutational [2, 3] and is caused by one or several of the major biochemical mechanisms such as the prevention of the access of antimicrobials to their cellular targets by reduced influx and increased efflux, drug inactivation, and target alterations [3–6]. Mutations or acquisition of genetic materials related to the action of antimicrobials provides the molecular basis of antimicrobial resistance. Moreover, various regulatory pathways also play an important role in influencing antimicrobial resistance [7]. In this regard, numerous proteins are well known to exert their regulatory functions within a biological system and thus participate in the regulation of gene expression. For instance, regulatory changes can lead to upregulation of antimicrobial-inactivating enzymes (e.g., β-lactamases) [8] and multidrug efflux pumps [4]. However, even in bacteria, gene expression regulatory networks/cascades are far more complex than we previously expected. The increasing studies on regulatory RNAs, including noncoding small RNA (sRNA) molecules and riboswitches, have provided such an example in showing the intricate regulation of the gene expression at multiple levels of transcription, RNA processing, and translation [9–11]. Consequently, regulatory RNAs affect a wide range of cell functions, which include bacterial stress response, virulence, and drug resistance [12–15]. Additionally, structural changes of mRNAs also significantly influence transcriptional and translational gene expression [16, 17]. This chapter provides an overview of regulatory RNAs and structural mRNA changes as well as our current understanding of their influences on gene expression and cellular functions that affect antimicrobial resistance, in particular drug efflux pumps in bacteria.

24.2 Regulatory RNA Molecules

There are a plethora of regulatory RNAs; two major groups include sRNA and the riboswitches, which are described below. Interestingly, noncoding sRNA molecules and riboswitches can also function together in controlling gene expression [18] as evident by the discovery of a riboswitch-containing sRNA in *Enterococcus faecalis* [11] and a riboswitch-regulated sRNA in *Listeria monocytogenes* [19]. However, in this chapter we exclude the discussion on other regulatory RNAs including clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) systems which are also involved in gene editing and regulation and serve as a defense mechanism in bacteria (the CRISPR-Cas systems also provide a revolutionary technical approach to alter any organism's DNA in a relatively easy manner) [20–26]. It should be noted that the role of CRISPR-Cas in antimicrobial resistance has been uncovered, such as in enhancing the stability of cell envelope and promoting resistance to polymyxins [27]. Meanwhile, multidrug-resistant enterococci were found to lack CRISPR-Cas, possibly due to inadvertent selection, by antimicrobial use, of resistant strains with compromised genome defense [28].

24.2.1 sRNA Molecules

sRNA molecules are referred to as regulatory, noncoding RNA transcripts, usually ~50–550 nucleotides in length, including *cis*- or *trans*-acting antisense RNAs [29, 30]. These sRNAs are encoded by both chromosomes and plasmids and can be produced as primary transcripts or via processing. While sRNAs are mostly derived from the 5′ regions to act via base pairing [31–33], there is an increasing recognition of sRNAs from the 3′ regions of mRNA [34, 35]. The sRNA molecules possess multiple functions, especially as ubiquitous regulators of gene expression, and are known to affect numerous physiological responses, in many cases, stress responses [32, 33, 36]. There is an advantage for the sRNA-based regulation mechanism since it provides a fast response to environmental signals by the fine-tuning of gene expression [37]. sRNAs function via two major mechanisms, i.e., base pairing with RNAs (including mRNAs) and binding to proteins to impact their activity (Figs. 24.1 and 24.2) [9, 29, 30, 32, 33, 35, 36]. Of particular note, the RNA chaperone protein Hfq is an RNA-binding protein that is often essential in promoting pairing between the sRNA molecules and their target mRNAs and subsequently influences translation and turnover rates of specific RNA transcripts [32, 35, 38].

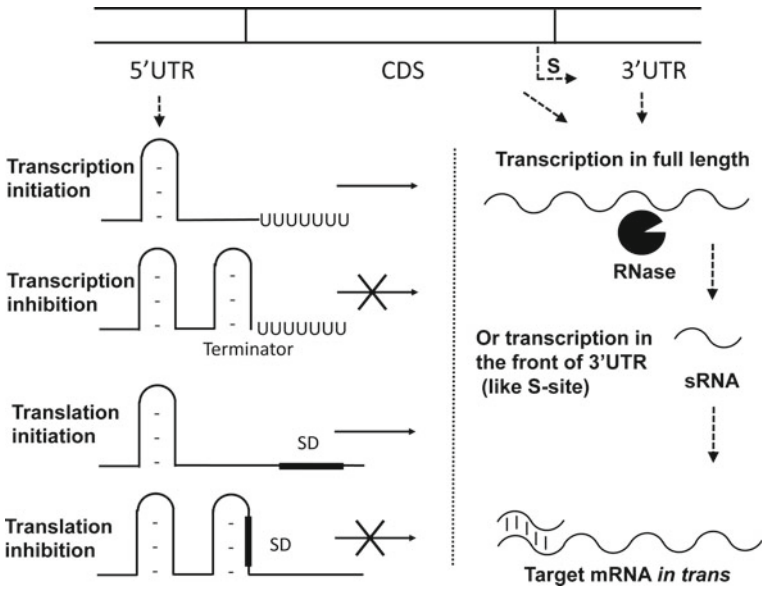

Fig. 24.1 Roles of sRNAs from the 5′ and 3′ regions of bacterial mRNAs in the regulation of gene expression. Bacterial sRNAs repress or activate their gene expression based on the configuration of the corresponding 5′ untranslated regions (5′UTR) (shown on the *left* side). They control transcription termination or translation initiation of the coding DNA sequence (CDS) in response to the change of the microenvironment, through formation of the stem-loop structure of the terminator or as a sequester of the ribosome-binding site (SD). In contrast, sRNAs from the 3′ region (3′ UTR) can be either transcribed from an mRNA-internal promoter (S site) or processed from its parental mRNA with full length (shown on the *right* side). These sRNAs regulate multiple *trans*-encoded mRNAs through short base pairing

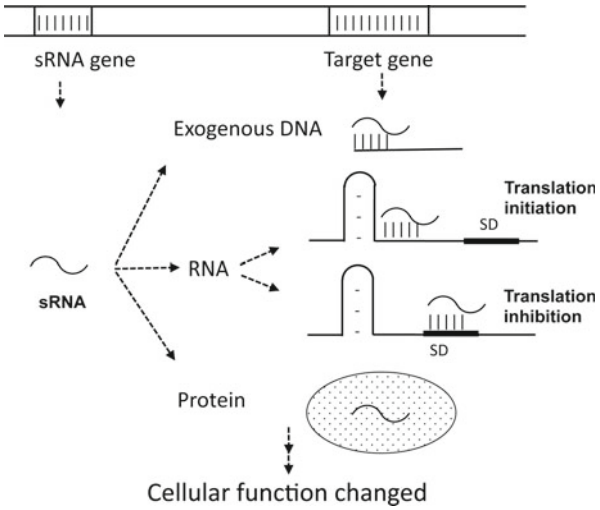

Fig. 24.2 Model for common mechanisms of sRNA-mediated gene regulation. sRNAs control exogenous gene stability, influence protein activity or regulate target mRNA fate, and consequently modulate various aspects of bacterial physiology, virulence, and behavior, including antimicrobial resistance

Functional sRNA transcripts were first discovered in bacteria in the 1980s [9]. A plasmid-specific 108-nucleotide sRNA was reported in 1981 to be untranslatable and to function as an inhibitor to block ColE1 plasmid replication [39, 40]. In 1984, the expressional downregulation of the major outer membrane (OM) porin OmpF of *Escherichia coli* by sRNA (termed as mRNA-interfering complementary RNA [micRNA]) was described [41]. This sRNA is generated from a gene (termed *micF*) that is located upstream of another gene encoding the major OM porin OmpC and is complementary to the 5′ end region of the *ompF* RNA [41]. Initially identified as a non-translated 174-nucleotide RNA [41], the primary sRNA transcript of the *micF* gene was instead found to be smaller as a 93-nucleotide MicF sRNA [42]. The MicF sRNA post-transcriptionally affects the efficient expression of OmpF. *micF* gene expression is now known to be controlled by numerous environmental and internal stress factors including oxidative stress and antibiotics such as cationic antimicrobial peptides [43, 44]. The discovery of the MicF sRNA represents the first example of a chromosomally encoded RNA regulator. Given the effect of porin production on the access of antimicrobials to drug targets in Gram-negative bacteria, the MicF sRNA is also the earliest example of sRNA effects on antimicrobial resistance.

Currently, there is a growing list for the identification and characterization of sRNAs from bacteria; these sRNAs play critical roles in many biological functions [9, 20, 36, 45, 46]. An early study, reported in 2003, summarized 55 sRNA genes in *E. coli* [47] that, as expected, include MicF and SdsR (RyeB) sRNAs currently known to implicate in antimicrobial resistance as described later. Previous studies had also showed the involvement of various sRNAs as regulators in primary and secondary metabolism in *Pseudomonas aeruginosa* [48]. A more recent report has described a genome-wide identification of sRNAs that include 44 known and >500 novel intergenic sRNAs [49]. A study targeting *Acinetobacter baumannii* showed the identification of 31 putative sRNAs, some of which were involved in stress response [50]. Sixty putative sRNAs (including three riboswitches) were also identified in *Stenotrophomonas maltophilia* [51].

As for Gram-positive bacteria, sRNAs of *Staphylococcus aureus* were demonstrated to participate in biological processes related to metabolism, stress response, and virulence [52, 53]. sRNAs related to *S. aureus* genomic and pathogenicity islands were found to be involved in the virulence regulation [54]. A database of 575 staphylococcal sRNAs has recently been made available (http://srd.genouest.org; accessed as of March 25, 2016) [55]. A recent review has discussed sRNAs of low-GC Gram-positive bacteria (such as *Bacillus subtilis*, *S. aureus*, and *Streptococcus pyogenes*); some of the known sRNAs are described to target RNAs that are related to transporters or virulence factors [56]. Additionally, more than 200 sRNAs were found in mycobacteria with certain sRNAs involved in gene expression under environmental stresses [45, 57, 58].

24.2.2 Riboswitches

Riboswitches, also known as RNA switches, are a class of RNA sensors that were first described in 2002 in bacteria in sensing small intracellular vitamin derivatives [59–61]. Over the last decade, remarkable advances have been made toward the in-depth understanding of structural, genetic, and biochemical aspects of riboswitches, which are known to be present in bacteria, archaea, and eukaryotes [62]. A total of 17 riboswitches had been determined as of 2013 [63]. We expect only a continuous dissemination of knowledge regarding the mechanisms behind the riboswitches [64, 65].

Riboswitches include two parts, an aptamer region and an expression platform. For bacteria, these regulatory elements are mainly present in the 5′ untranslated region of mRNA. Despite being composed of only four chemically similar nucleotides, RNAs can base pair with themselves and also interact with other molecules to form complex secondary and tertiary structures [66, 67]. A riboswitch requires its aptamer region to have a local structural flexibility or the ability to transition from one conformation to another in response to environmental small ligand molecules, which leads to the regulation of the downstream gene expression [62, 63]. Riboswitches control gene expression by binding small molecules without the need for protein factors [63]. This mechanism can quickly and correctly allow bacteria in response to the environmental metabolites. Antibiotics are common secondary metabolites of microorganisms for their defense against competitors [68]. It is thus reasonable to predict that antibiotics could serve as a group of potential ligands of the riboswitches and subsequently influence gene expression [69, 70].

24.3 Effect of Antimicrobial Exposures on Expression of sRNAs

The remarkable advances in molecular biology over last two decades have facilitated studies on gene expressions, such as genome-wide transcriptional profiles in bacteria following their exposure to antimicrobial agents [71–75]. Antimicrobial

exposure can affect the expression of a wide range of genes including resistance genes. In recent years, there have been an increasing number of studies that have described the sRNA production in bacteria treated by antimicrobial agents with possible impact on antimicrobial resistance [75–77]. For instance, challenging *Salmonella enterica* serovar Typhimurium with a subinhibitory level of tigecycline or tetracycline resulted in elevated expression of four sRNAs known to be conserved in several bacterial species. One of the sRNAs, sYJ20 (also known as SroA), acts *in-trans* to influence antimicrobial susceptibility [76]. The upregulation of sYJ20 was also seen in cells treated by ampicillin [76], suggesting that this sRNA may be involved in response to a broad range of stresses. More than 400 potential sRNAs were identified in two multidrug-resistant strains of *S. aureus* (with different levels of vancomycin resistance) following their exposure to one of the four antimicrobials tested (ceftobiprole, linezolid, tigecycline, and vancomycin at the half level of the minimal inhibitory concentrations), revealing that a subset of sRNAs contribute to the transcriptional response to specific drug exposures [77]. Recently, a study showed unique transcriptional response profiles (including >150 sRNAs) in multidrug-resistant *Pseudomonas putida* following exposure to a wide range of antimicrobials including ampicillin, chloramphenicol, ciprofloxacin, gentamicin, rifampicin, kanamycin, spectinomycin, and tetracycline, which have different modes of action, again supporting the role of sRNAs in fine-tuning resistance gene expression [75].

24.4 Influence of Regulatory RNAs on Antimicrobial Resistance Including Drug Efflux Pump-Mediated Resistance

Although regulatory proteins such as local or global regulators and two-component regulatory proteins have demonstrated influence on resistance gene expression [4], regulatory RNAs also participate in affecting gene expression including those involved in bacterial stress responses and drug resistance [78]. Indeed, regulatory RNAs can regulate the stability or maintenance of DNA, RNA, and proteins and consequently influence gene expression [32]. Below we describe several pathways by which expression of antimicrobial resistance genes is affected by regulatory RNAs (Table 24.1).

24.4.1 sRNAs

One major mechanistic characteristic for sRNA function is the ability of the sRNA to base pair with the targeted mRNA molecules, which can either increase or decrease the stability and translation of the targeted mRNAs (depending on the circumstances) [96, 97]. This base pairing event often occurs through imperfect

Table 24.1 Influence of sRNA molecules on antimicrobial resistance

Species	sRNA	Target mRNA	Susceptibility or resistance phenotype	Reference
E. coli	DsrA	MdtEF	Multidrug resistance	[79]
	MicA and GcvB	PhoP	Unknown	[80, 81]
	MicC	OmpC	Multidrug resistance	[82]
	MicF	OmpF	Multidrug resistance	[41, 83–85]
	MgrR	EptB	Polymyxin susceptibility	[86]
	RalA	RalR	Fosfomycin resistance	[87]
	SdsR (RyeB)	TolC	Multidrug resistance	[88, 89]
	SdsR (RyeB)	MutS	Unknown	[90]
N. gonorrhoeae	NrrF	MtrF	Multidrug resistance	[91]
S. enterica	SdsR (RyeB)	OmpD	β-Lactam resistance	[92, 93]
	sYJ20	?	Multidrug/tigecycline resistance	[76]
S. aureus	RsaA	MgrA	Unknown	[94]
	SprX	SpoVG	Vancomycin and oxacillin resistance	[95]
	sRNA10	MecA	β-Lactam resistance	[77]

pairing with the ribosome-binding site (the Shine-Dalgarno [SD] sequence) of the targeted mRNAs and consequently leads to the inhibition of effective translation and the degradation of mRNAs [97]. There are numerous examples which demonstrate the role of base pairing RNA in influencing antimicrobial resistance genes.

Membrane permeability The above mentioned sRNA MicF in *E. coli* acts as a *trans*-encoded antisense RNA that negatively regulates the production of OmpF through its binding to OmpF mRNA [41, 43]. The ribosomal binding sites and the start codon of *ompF* transcript base pair with MicF sRNA in an RNA-RNA duplex [98]. Furthermore, MicF can target a diverse number of mRNAs including that of the lipid A-modifying enzyme, LpxR [83]. (LpxR is involved in lipid A deacylation and can thus affect the integrity of lipopolysaccharide [99].) Since OmpF is the major diffusion channel for many small hydrophilic antimicrobial agents such as β-lactams [100], the diminished level or lack of OmpF is well known to contribute to antimicrobial resistance in both laboratory-generated and clinical isolates of *E. coli* [84, 85, 101]. In fact, the MicF-based mechanism constitutes a part of the overall multidrug resistance mechanisms attributable to the decreased influx and increased efflux of drugs. Several global regulators (e.g., MarA, Rob, and SoxS) positively control the expression of the *micF* gene and the predominant drug efflux pump *acrAB* genes (reviewed in [4]). Additionally, the expression of another porin, OmpC, is also affected by an sRNA, the MicC sRNA, which is Hfq associated and inhibits ribosomal binding to the *ompC* mRNA leader [82].

To date, numerous sRNAs are known to be involved in the regulation of the OM composition in response to environmental changes [102–104]. OmpA is a major OM protein which has a structural role and also functions as a slow porin [105,

106]. The sRNA, MicA (initially known as SraD), base pairs with the ribosomal binding region of the *ompA* transcript to inhibit translational initiation and enhance *ompA* mRNA degradation [107–109]. OmrA (also known as RygA) and OmrB (RygB) sRNAs of *E. coli* negatively control production of several OM proteins [110]. MicC sRNA can silence the OmpD translation by endonucleolytic mRNA destabilization [111]. The SdsR sRNA downregulates OmpD production in *Salmonella* via Hfq-dependent base pairing [92]. The reduction of OmpD expression is observed in isolates resistant to ceftriaxone [93] and multiple drugs [112]. OmpD is also one of the genes necessary for the efficient efflux of methyl viologen [113]. The major *E. coli* lipoprotein Lpp resides in the OM and is the most abundant protein in the cell [114, 115]. MicL sRNA specifically targets Lpp mRNA, preventing its translation [115]. Moreover, MicA, RybB, and MicL allow the transcriptional factor δ^E to downregulate the synthesis of all abundant OM proteins in response to stresses [107, 108, 115–120]. RybB also plays a role in the inhibitory effect of the green tea polyphenol epigallocatechin gallate on the biofilm matrix curli fibers via δ^E-dependent cell envelop stress response to reduce biofilm antimicrobial resistance [121].

In addition to porins of the OM, lipopolysaccharide serves as a major barrier for antimicrobials to cross the outer membrane of Gram-negative bacteria [105]. The PhoPQ two-component regulatory system is pleiotropic and often responds to cell envelope stress, for example, its involvement in lipopolysaccharide modifications that affect antimicrobial susceptibility [122]. The expression of *phoP* is also subjected to the negative regulation by multiple sRNAs, including MicA and GcvB, independently via base pairing between the sRNAs and *phoP* mRNA [80, 123]. In fact, GcvB sRNA is pleiotropic and controls expression of multiple target mRNAs [81]. Interestingly, the Hfq-dependent sRNA MgrR of *E. coli* is regulated by PhoPQ system, and this sRNA negatively influences the translation of two mRNAs, which include *eptB* for a lipopolysaccharide-modifying enzyme and *ygdQ* for a hypothetical protein [86]. Deletion of *mgrR* renders the mutant more resistant to polymyxin B [86], which targets lipopolysaccharide. In *Salmonella*, a PhoP-activated sRNA, PinT, affects the expression of invasion-associated effectors and virulence genes required for intracellular survival of the microbe [124]. Overall, these data link sRNA to virulence and/or antimicrobial resistance.

Drug efflux pumps sRNA involvement in the regulation of drug efflux pump expression has also been demonstrated in literature. Nishino et al. [79] showed that the expression of the MdtEF drug efflux pump of the resistance-nodulation-cell division (RND) superfamily is positively influenced by DsrA sRNA, which is 85-nucleotide in length and represses the translation of the global regulator H-NS through its base pairing with H-NS mRNA [125, 126]. The H-NS regulator is one of the complex components involved in the regulation of multiple drug efflux operons including *acrEF*, *emrKY*, and *mdtEF* [127]. Another sRNA, RyeB, produced during stationary phase, represses the expression of TolC, an OM channel component of many tripartite drug efflux pump systems including AcrAB-TolC in *E. coli* [88]. RybB overexpression was shown to reduce resistance to novobiocin and crystal violet [89].

MtrCDE is the major RND-type drug efflux system in *Neisseria gonorrhoeae* (reviewed in [4]), and its regulation also involves a *trans*-acting sRNA, NrrF, which responds to iron availability and acts as a pleiotropic regulator including inhibition of *mtrF* expression [91]. In *A. baumannii*, an sRNA named AbsR25 was recently suggested to negatively influence the expression of the A1S_1331 transporter gene [50]. Putative base pairing between AbsR25 and AIS_1331 mRNA was identified [50].

The RNA chaperone Hfq interacts with sRNAs and mRNA [38]. Deletion of Hfq in *S. maltophilia* resulted in altered production of sRNAs including the accumulation of several RNAs [51]. Hfq-inactivated mutants showed an overall higher resistance to multiple antimicrobials (≥4-fold MIC increase for chloramphenicol, ciprofloxacin, tetracycline, tigecycline, and trimethoprim-sulfamethoxazole) with slightly increased susceptibility to amikacin, colistin, tobramycin, and vancomycin (two- to threefold MIC reduction) [51]. This susceptibility phenotype may possibly suggest the effect of Hfq on gene expression related to cell membranes and drug efflux pumps.

S. aureus expresses a plethora of sRNAs, most of which have unknown biological functions [52, 53, 55]. The RsaA sRNA exerts translational inhibition on the MgrA global regulator [128] via an imperfect base pairing of RsaA with the ribosome-binding site of *mgrA* transcript and a loop-loop interaction within the coding region of the *mgrA* mRNA; this interaction subsequently promotes bacterial persistency but reduces virulence [94]. Since MgrA is implicated in the posttranslational modification of several drug efflux pumps such as NorA and NorB [129, 130], it remains to be seen whether RsaA sRNA can impact these efflux pumps.

Resistance to various antimicrobials Recently, the sRNA SprX was shown to function as a base pairing sRNA in influencing resistance to glycopeptides (such as vancomycin) and β-lactams (e.g., oxacillin) [95]. The *yabJ-spoVG* operon of *S. aureus* encodes YabJ with unknown function and the site-specific DNA-binding protein SpoVG (stage V sporulation protein G) [131]. SprX negatively regulates SpoVG expression through direct antisense pairings at the *spoVG* ribosomal binding site of *yabJ-spoVG* mRNA [95], which is also the target of the abovementioned pleiotropic RsaA sRNA regulator [94]. In another study investigating antimicrobial exposures and sRNA production, the expression of several sRNAs was inhibited by two cell wall-targeting antibiotics, ceftobiprole and vancomycin [77]. One sRNA dubbed sRNA1 is antisense to the *gyrA* gene that encodes the target of quinolone antimicrobials, and another sRNA dubbed sRNA10 is antisense to the penicillin-binding protein 2a-encoding gene *mecA*, suggesting that these sRNAs may facilitate the adaption of *S. aureus* to the presence of antimicrobials [77].

RalR-RalA, encoded by a cryptic prophage in *E. coli*, constitutes a toxin/antitoxin system. RalR functions as a nonspecific DNase, and RalA is an Hfq-dependent antitoxin sRNA with 16 nucleotides that can base pair with the RalR mRNA [87]. Genetic inactivation of *ralR* and *ralRA* renders mutants more susceptible to the peptidoglycan synthesis inhibitor fosfomycin (which inhibits phosphoenolpyruvate transferase), suggesting that RalR-RalA plays a role in fosfomycin resistance [87].

As mentioned earlier, production of several RNAs was elevated in *Salmonella* following exposure to antimicrobials [76]. Deletion of the gene encoding sYJ20 sRNA reduced the survival of the cells in the presence of tigecycline, indicating the role of this sRNA in intrinsic antimicrobial resistance [76].

MutS plays an important role in DNA mismatch repair [132]. An RpoS-dependent sRNA SdsR targets *mutS* mRNA to repress the mismatch repair activity of MutS, and this mechanism contributes the increased mutagenesis frequencies in the presence of subinhibitory concentrations of β-lactam antibiotics (which induce SdsR expression), suggesting a possible role for sRNAs in the emergence of mutational resistance [90]. sRNAs produced by prophage in *E. coli* were reported to contribute to bacterial response to osmotic, oxidative, and acid response including resistance to ampicillin and nalidixic acid [133], and one of the sRNAs named DicF was found to control metabolism and cell division in *E. coli* [134].

24.4.2 Influence of Riboswitches on Antimicrobial Resistance

Aminoglycoside resistance A decade after riboswitch discovery, Jia et al. [135–137] reported an aminoglycoside-sensing RNA in the leader RNA of mRNAs encoding aminoglycoside acetyl transferase (AAC) and aminoglycoside adenyl transferase (AAD), two drug-modifying enzymes conferring high-level aminoglycoside resistance (Fig. 24.3). The 5′ leader RNA shows a typical structure which masks the ribosome-binding site (SD2) of the mRNAs for these enzymes in the absence of aminoglycosides [135, 136, 138, 139]. In the presence of aminoglycosides, these antibiotics bind to the leader RNA and induce a change in its structure such that exposing of the ribosome-binding site becomes beneficial for ribosomal binding and translation of the resistance genes [135, 136, 138, 139]. This instance represents the first description of a riboswitch in antimicrobial resistance. In fact, a sequence in the 5′ leader RNA for the genes encoding acetyl or adenyl transferases is highly conserved in a wide range of microorganisms [135]. The aminoglycoside-binding riboswitch is speculated to help save energy and thus benefit the bacteria in surviving during antimicrobial selection. This example suggests that antibiotic-specific sRNA interference of the 5′ untranslated regions of resistance genes could play an important role in controlling resistance gene expression.

Fluoride resistance Fluorine is one of the abundant elements in the earth's crust and can serve as the ligand of riboswitches [140]. The fluoride-responsive riboswitches, present in bacterial and archaeal species (including oral disease-associated *Streptococcus mutans*), are selectively triggered by fluorine anions (but not by chlorine anions) to activate gene expression of fluoride transporters and fluoride-inhibiting enzymes [140]. These fluoride riboswitches contain a conservative domain termed the *crcB* motif, which is located upstream of genes encoding of diverse functions (including CrcB, enolase, *E. coli*-derived chloride ion channel protein EriC, major facilitator superfamily transporters, MutS, and Na^+/H^+ antiporters). (Overproduction of plasmid-borne *crcB* in *E. coli* was found to confer resistance to camphor and chromosome condensation [141].) An *E. coli* mutant

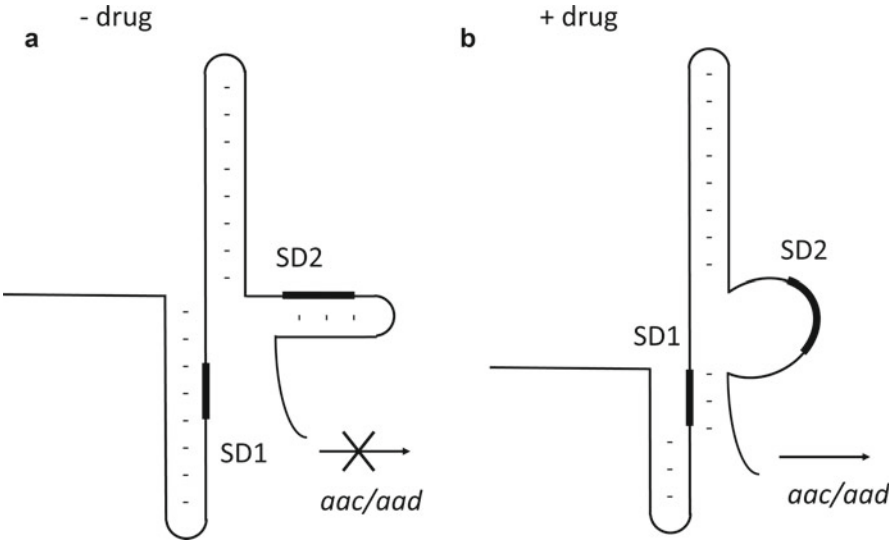

Fig. 24.3 Drug induction of *aad*/*aad* via a mechanism of regulatory riboswitch. Schematic representation of the model for the induction of aminoglycoside resistance. Aminoglycoside binding to the 5′ leader RNA induces a change in the leader RNA structure such that the anti-SD2 sequence base pairs with SD1 consequently unmasking SD2 for ribosomal binding and translation of the resistance gene. In the absence of drugs, the ribosome-binding site SD2 of *aac*/*aad* is sequestered in the mRNA secondary structure (**a**). Therefore, it is inaccessible to initiating ribosomes and *aac*/*aad* is not expressed. When cells are exposed to low concentrations of inducing aminoglycoside antibiotics, the drug bound to leader RNAs engaged in the translation of *aac*/*aad* (**b**). The drugs destabilize the ground-level mRNA secondary structure and shift the equilibrium to the induced conformation. SD2 becomes accessible, and *aac*/*aad* can then be translated by the ribosomes, which is the translation attenuation riboswitch that regulate protein synthesis

carrying *crcB* inactivation showed increased susceptibility to fluoride with a fluoride MIC of ca. 1 mM in comparison with the MIC value of 200 mM for the wild-type strain [140]. Subsequently, fluoride riboswitch-controlled antiporters were shown to be a subclass of bacterial chloride channel anion-transporting proteins which function as F^-/H^+ antiporters and protect bacteria from fluoride toxicity [142]. Moreover, in eukaryotes, resistance to fluoride toxicity is also attributable to fluoride export proteins [143].

24.5 Influence of Other RNA Structural Changes on Antimicrobial Resistance and Efflux Gene Expression

Ribosome stalling causes one of the most dramatic leader RNA structure changes, which results in translational or transcriptional attenuation of downstream gene expression in both bacteria and eukaryotes [144–146]. With this mechanism, the ribosome checks the structure of the polypeptide it is assembling, in response to

certain nascent peptide "stalling" sequences and, often, to specific cellular cues (e.g., antibiotics), which together forms the stable stalled ribosome complex [144, 146–148]. The first description of ribosome stalling dates back to the early 1980s when it was found that inducible macrolide resistance gene expression can be activated by stalling of the ribosome at the leader peptide encoded [149, 150]. In regard to the involvement of antibiotics, ribosome stalling can be grouped into either antibiotic-independent or antibiotic-dependent ribosome stalling [148]. For example, both SecM-mediated ribosome stalling and expression of the tryptophanase *tnaCAB* operon by ribosome stalling in *E. coli* are antibiotic independent. SecM controls the expression of the SecA ATPase that is involved in the protein translocation in *E. coli* via a ribosome stalling mechanism (SecM-encoding gene is located upstream of *secA*) [151–153]. The *tna* operon includes a leader peptide gene, whose product acts in *cis* via ribosome stalling to regulate the *tna* operon [145, 154, 155]. These two examples have emphatically revealed an amazing ability of RNA structures to monitor microenvironmental changes.

The macrolide-induced case [149, 150] provides an example for antibiotic-dependent ribosome stalling-based translational attenuation such as expression of the macrolide-inducible resistance genes, e.g., *ermC*. The *ermC* gene expression is activated by ribosome stalling at the leader peptide encoded by *ermCL* (Fig. 24.4). The stalling occurs in the presence of an inducing antibiotic (e.g., erythromycin) that binds in the nascent peptide exit tunnel [11]. The induction of

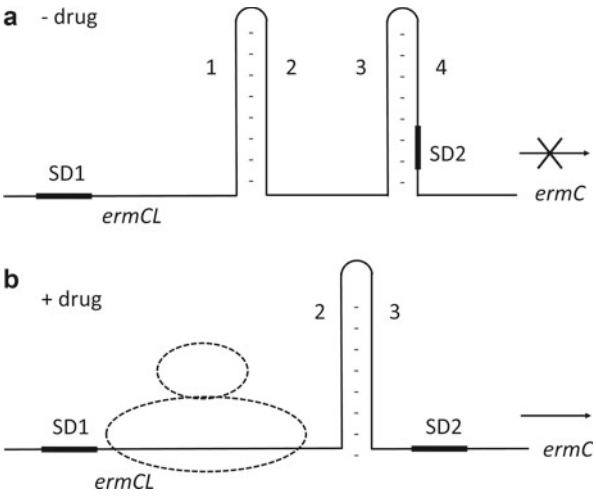

Fig. 24.4 Drug induction of methyltransferase gene *ermC* via a mechanism of translational attenuation. A segment of mRNA spanning the regulatory *ermC* leader peptide (*ermCL*), the intergenic region, and the SD2 of *ermC* are shown in an uninduced (**a**) and induced (**b**) conformation. In the absence of drug, *ermCL* is translated, while *ermC* is not because its ribosome-binding site SD2 (shown in *bold*) is sequestered in mRNA secondary structure. The mRNA segments involved in the conformational switch are marked by (*1–2*) and (*3–4*). During induction, an erythromycin-bound ribosome stalls at *ermCL* leading to a change in the mRNA conformation allowing translation of *ermC*. The mRNA segments involved in the conformational switch are marked by (*2–3*)

ermC expression by ribosome stalling is critically dependent on the ErmCL peptide sequences [11]. In the absence of erythromycin, *ermCL* is translated, while *ermC* is not because its ribosome-binding site is sequestered in the mRNA secondary structure [156]. When erythromycin is available, an erythromycin-bound ribosome stalls at *ermCL* leading to a change in the mRNA conformation that allowings the translation of *ermC* [156]. Expression of another macrolide resistance gene, *ermB*, is also similarly regulated via the macrolide-dependent ribosome stalling. The structure of the erythromycin-dependent ErmBL leader peptide-stalled ribosome complex has become available, providing structural understating of ribosome stalling regulatory process [157].

In *P. aeruginosa*, the RND-type MexXY multidrug/aminoglycoside efflux system undergoes regulation by the MexZ repressor and is inducible by ribosome-targeting antimicrobials including aminoglycosides and macrolides [158, 159]. Dimerized MexZ binds to a 20-bp palindromic sequence of the promoter of *mexXY* to only allow very low-level MexXY expression [4, 160–162]. However, MexZ expression is dependent on the antirepressor ArmZ encoded by *PA5471* (*armZ*) [163], whose own expression is controlled by a transcriptional attenuation mechanism. Drug inducibility of ArmZ requires the participation of the 367-bp *PA5472-PA5471* intergenic region which can be translated to a short 13-amino acid leader peptide, PA5471.1 [164]. In the absence of a drug, the transcribed *PA5471.1* sequence is predicted to form a stem-loop structure with adjacent regions of the leader mRNA ahead of PA5471; this structural form causes transcription termination prior to the PA5471 coding region (Fig. 24.5) [164]. When a ribosome-perturbing antibiotic is present, the PA5471.1 sequence would preclude the formation of these secondary mRNA structures and thus prevent the formation of a transcriptional terminator, permitting the transcription into the PA5471 coding region [164]. However, this structural model does not provide explanation for certain observations such as that elimination of PA5471.1 translation via an M1T (AUG→CUG) mutation also increases PA5471 expression [164] and that PA5471 is substantially upregulated in cells after exposure to oxidative stress caused by hydrogen peroxide [165] or peracetic acid [166, 167], but not by antibiotics. Recently, a novel ribosome-associated protein named SuhB was shown to modulate ribosome stalling activity toward MexXY expression [168]. Deletion of *suhB* resulted in the elevated expression of MexXY and ArmZ and reduced susceptibility to aminoglycosides [168]. SuhB was shown earlier to be a regulator of virulence genes including downregulation of several sRNAs [169].

Lastly, various other examples have also suggested the possible involvement of ribosome stalling in the regulation of antimicrobial resistance gene expression. For example, leader peptide sequences encoded by gene upstream of relevant resistance genes have been identified such as the *armA* gene for 16S rRNA methylase (aminoglycoside resistance) [170]; *cat* for chloramphenicol acetyltransferase [171]; *cfr* and *cml* for chloramphenicol efflux pumps [172]; *ermA*, *ermC*, and *ermD* for macrolide methylases [150, 173, 174]; *lasB*/*mefE*/*msrA* for multidrug or macrolide efflux pumps [175–177]; *tet*(*L*) for tetracycline efflux pump [178]; and *tet*(*M*) for ribosomal protection-based tetracycline resistance [179].

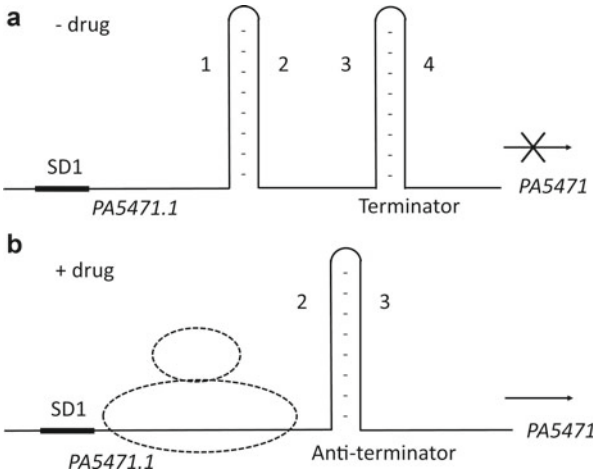

Fig. 24.5 Drug induction of efflux pump antirepressor ArmZ via a mechanism of transcriptional attenuation. Transcription of *armZ* (*PA5471*) of *P. aeruginosa* from an upstream promoter also results in the transcription of an open reading frame of *PA5471.1*, which encodes a 13-residue leader peptide. (**a**) In the absence of a drug, ribosomes bind to the SD1 site of *PA5471.1* and translation proceeds. This event permits the *PA5471.1* mRNA to form a stem-loop structure with a downstream sequence (*1–2*). In the presence of (*1–2*) stem-loop formation, an additional stem-loop is also created (*3–4*) downstream, acting as a transcriptional attenuator located just before the PA5471-coding sequences. Under drug-free growth conditions, transcription is terminated prior to the PA5471-coding region. (**b**) When a ribosome-perturbing antibiotic is present, ribosome stalling within the *PA5471.1* sequence during translation makes 1 unavailable for stem-loop formation with 2, leading to alternate mRNA folding and a stem-loop (*2–3*). The latter loop constitutes an anti-terminator structure to prevent the formation of the transcriptional terminator (*3–4*), and the downstream *PA5471* is transcribed

24.6 Concluding Remarks

This chapter provides examples regarding the contribution of regulatory RNAs and mRNA structural changes to antimicrobial resistance. It should be noted that investigation of the relationship between RNA-mediated regulation and antimicrobial resistance is a relatively new area of research in comparison with the available large amount of studies on regulatory RNAs. Therefore, more studies are warranted for better understanding of the involvement of regulatory RNAs on the development of antimicrobial resistance. Moreover, as a naturally evolved mechanism, RNA-mediated regulation of gene expression provides an efficient means toward the complex gene expression process. In this regard, targeting regulatory RNAs is already regarded as a possible important strategy for new antimicrobial research and development [24, 180]. For example, artificial antisense sRNAs, ligand analogs of riboswitches, and CRISPR system cleaving nucleotides have been utilized for potential candidates of novel antimicrobial agents [24, 181].

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