



Bacterial adaptation to antibiotics through regulatory RNAs

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1 **Bacterial adaptation to antibiotics through regulatory RNAs**

2

3 **Running title:** Antibiotics and regulatory RNAs

4

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17 regulatory RNA; sRNA; gene regulation; drug target.

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20

21 **Abstract**

22 The extensive use of antibiotics has resulted in a situation where multidrug-resistant
23 pathogens have become a severe menace to human health worldwide. A deeper
24 understanding of the principles used by pathogens to adapt, respond and resist against
25 antibiotics will pave the road to drugs with novel mechanisms. For bacteria, antibiotics are
26 clinically-relevant stresses that induce protective responses. The recent implication of
27 regulatory RNAs (sRNAs) into antibiotic response and resistance in many bacterial pathogens
28 suggests that they should be considered as innovative drug targets. This review discusses
29 sRNA-mediated mechanisms exploited by bacterial pathogens to fight against antibiotics. A
30 critical discussion of the newest findings in the field is provided, with emphasis on the
31 implication of sRNAs in major mechanisms leading to antibiotic resistance: drug uptake,
32 active drug efflux, drug target modifications, biofilms, cell wall and LPS biosynthesis. Of
33 interest is the lack of knowledge about sRNAs implicated in Gram-positive resistance,
34 compared to Gram-negative bacteria.

35

36

37 **Worldwide burden of antimicrobial resistance**

38 Bacterial resistance to antibiotics has become a main challenge for public health worldwide.
39 The World Health Organization has claimed 'antibiotic resistance' as one of the three most
40 important public health threats of the 21st century (1). Most pathogens are becoming
41 multidrug resistant (MDR), with an increased risk of failure of conventional therapies with
42 higher morbidity, mortality, hospitalization lengths and treatment costs (1). Resistant Gram-
43 positive pathogens responsible for health-care associated infections include methicillin-
44 resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and MDR
45 *Streptococcus pneumoniae*. For infections caused by Gram-negative bacteria (such as
46 *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter*), treatment choices are
47 also becoming limited. In the USA, more than 2 million people annually develop infections
48 due to MDR organisms, resulting in more than 23,000 deaths. Deaths attributable to MDR
49 bacteria every year are now 700,000 worldwide and the projected mortality rates by 2050
50 are 10 million, more than deaths caused by all cancers (2). In the near future, there are
51 serious odds that no treatment options will be available for the "ESKAPE" pathogens, which
52 comprise *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*,
53 *P. aeruginosa* and *Enterobacter* spp. (3). Therefore, development of novel antibacterial
54 agents is essential to keep up with the constantly evolving resistance in bacteria. However,
55 very few novel classes of antibacterial drugs have been discovered in the last three decades
56 (4).

57 Antibiotics kill bacteria or inhibit their growth by blocking key cellular pathways. They also
58 allow our natural defenses, including the host immune system, to eliminate the invading
59 microorganisms. Resistance against any antibiotic drug, regardless of its mechanism of
60 action, was reported soon after its clinical use. The unreasonable use of antibiotics in

61 animals and humans has promoted evolution of resistance. Using natural selection, bacterial
62 pathogens evolve to survive and evade drugs designed to eliminate them, as they have done
63 for millions of years against natural antibiotics produced by competing organisms in their
64 environment (5).

65

66 **Bacterial adaptation to antibiotics**

67 Bacterial genome plasticity is mandatory to adapt and respond to environmental threats,
68 including antibiotic stress. Antibacterial resistance is ancient, resulting from the interaction
69 among organisms and their environment. Most antibiotics were produced naturally by
70 bacteria and fungi for millions of years (6), and bacteria evolved mechanisms to overcome
71 their action, survive, and spread. As a consequence, many bacteria are naturally resistant to
72 one, several, or even most of antibiotics. Acquired resistance develops with gene mutations
73 or *via* external genetic acquisition from nearby resistant organisms, through horizontal gene
74 transfer (HGT). Non- or slow-growing bacteria can survive most bactericidal antibiotics that
75 require active growth for action, a property called “tolerance”, leading to persistence.
76 Neutral mutations during bacterial genomes evolution can pave the way for the subsequent
77 evolution of resistance (7). Mutations triggering resistance alter antibiotic action by either
78 drug target modifications, reducing drug uptake, stimulating drug efflux or by modifying
79 regulatory networks implicated in general metabolism. Another parameter that influences
80 the emergence and evolution of antibiotic resistance is the existence of antibiotic
81 concentration gradients in the environment, livestock and humans. It implies that pathogens
82 are frequently exposed to non-lethal, subinhibitory drug concentrations (SICs) shaping the
83 evolution of antibiotic resistance (8). The rationale behind dosing for antibiotic treatments is
84 to maintain a concentration higher than the MIC (i.e., the lowest concentration of a chemical

85 which prevents visible growth of a bacterium) in the relevant body compartments for long
86 enough, to clear the infection. Under MIC antibiotic concentrations, bacterial growth is
87 inhibited but cells are not killed and the infection can resume later. Low antibiotic
88 concentrations in body fluids and tissues can therefore favor resistance development.

89 Bacteria come across many stresses in their natural habitat. Pathogens fight or adapt to their
90 host's innate or adaptive defenses. Various stresses (including oxidative, acidic, osmotic,
91 temperature, nutrient starvation, and antibiotic) trigger adaptive responses from the
92 pathogen (9). Antibiotic exposure, when not lethal, induces stress responses in bacteria.
93 Since antibiotics are stresses, they often elicit protective responses in bacteria that will
94 reduce antibiotic activity. Conversely, stress can impact antimicrobial susceptibility. As
95 specific example, stress-induced growth arrest impact antibiotic susceptibility since
96 antimicrobials usually act on growing cells. Stresses raise tightly regulated adaptive and
97 protective responses, including gene expression reprogramming by signaling pathways
98 including transcriptional factors (10) and regulatory RNAs (sRNAs) (11).

99

100 **sRNAs as stress response regulators including antibiotics**

101 sRNAs participate in many regulatory events, from plasmid copy number control in bacteria
102 to X-chromosome inactivation in mammals. Sensing the environment also requires
103 appropriate sRNA-mediated responses to adapt gene expression fast and efficiently. Once an
104 external or internal signal is detected, sRNAs, alone or in cooperation with additional
105 regulators, tune target gene product levels and control expression timing, for optimal
106 adaptation. That regulation is reversible once the signal vanishes, and sRNAs as well as
107 antisense RNAs (asRNAs, transcribed from the opposite DNA strand of their target mRNA)
108 are usually consumed upon their action since they often are co-degraded with targets (12).

109 As in eukaryotes (13), many sRNAs are expressed and implicated in complex gene regulatory
110 networks in *eubacteria* and *archaea* (14). sRNAs are ~50 to 600 nucleotide-long, usually
111 stable and non-coding (there are exceptions, 15). They act on their own or require
112 associated RNA-binding proteins, such as Hfq (16). They are implicated in physiological
113 responses influenced by signals from their surroundings. sRNAs modulate DNA maintenance
114 and silencing, transcription and/or translation of target genes, protein quality control and
115 secretion mechanisms. They enhance bacterial fitness to many stresses, inducing adaptive
116 metabolic changes. They optimize utilization of available nutrients and improve survival,
117 virulence (17) and persistence (18).

118 Very few sRNAs are constitutively expressed. The majority are transcriptionally induced
119 under specific conditions (19) such as cold or heat, pH or nutrient changes, iron homeostasis,
120 membrane remodeling, virulence gene expression, motility, biofilm production, virus or
121 plasmid invasions (clustered regularly interspaced short palindromic repeats or CRISPRs, 20),
122 and antibiotic exposure (21). sRNAs, transcription factors (TFs) and small signaling
123 molecules frequently interact with regulatory networks, for gene reprogramming during
124 stress (22). TFs influence gene expression at the transcriptional level, whereas sRNAs
125 intervene essentially post-transcriptionally. We will not cover 5' untranslated regions (UTRs)
126 of antibiotic resistance genes. These sensory RNAs respond to environmental signals by
127 inducing or preventing expression of downstream genes (for recent review (23)).

128

129 **sRNAs as modulators of antibiotic response and resistance**

130 Antibiotics differ based on the cellular component(s) they affect, in addition to whether they
131 induce cell death (bactericidal) or merely inhibit cell growth (bacteriostatic). Most antibiotics
132 act by perturbing bacterial cell wall synthesis, DNA replication, RNA transcription or protein

133 synthesis (24). Recent evidence indicates that bacterial sRNAs are important actors during
134 stress responses and for the development of resistance to various antibiotics (21). Several
135 sRNAs are involved in regulatory circuits controlling antibiotic resistance (Table 1), and we
136 anticipate that this only represents the tip of the iceberg. Resistance to antimicrobial agents
137 commonly results from the following mechanisms: 1) enzymatic antibiotic inactivation, 2)
138 decreased affinity of the antibiotic for its target, by target modification or protection, and 3)
139 decreased of intracellular antibiotic concentration due to decreased permeability and/or
140 overexpression of efflux pumps (25). Also, biofilm formation is clinically relevant since
141 bacteria associated with biofilms are resistant/tolerant to many antibiotics (26). As specific
142 examples, sRNAs influence antibiotic resistance by pairing with target mRNAs expressing
143 drug efflux pumps, antibiotic transporters or enzymes involved in drug catabolism.

144

145 **sRNAs and drug uptake**

146 In Gram-negative bacteria, antibiotics must cross over the outer membrane to reach their
147 intracellular targets through a lipid-mediated pathway (for hydrophobic antibiotics), or *via*
148 water-filled porins (for hydrophilic antibiotics) (27). To become resistant, bacteria can alter
149 permeation of antibiotics through the outer membrane by modifying these uptake
150 pathways. Interestingly, the expression of some of these macromolecules can be regulated
151 by sRNAs, and therefore impacts resistance.

152 In *Escherichia coli*, GcvB sRNA regulates *sstT*, *oppA* and *dppA* involved in amino acid,
153 dipeptide and oligopeptide transports. GcvB also negatively regulates *cycA* mRNA, which
154 encodes a permease for glycine, D-alanine, D-serine and D-cycloserine transport into the
155 bacteria (28). Note that D-cycloserine is an analogue of D-alanine that interferes with
156 bacterial cell wall synthesis, used in the treatment of multi- and extensively-drug-resistant

157 tuberculosis (29). Interestingly, a $\Delta gcvB$ mutant is more susceptible to D-cycloserine than the
158 parental strain, due to increased CycA levels and increased transport of the antibiotic (28).
159 GcvB also negatively regulates the PhoPQ two-component system by translational
160 repression of PhoP and could be involved, through *eptB*, in LPS modifications and resistance
161 to antimicrobial peptides (see below).

162 Colicin Ia is a pore-forming *E. coli*-specific bacteriocin, which targets outer membrane
163 protein (OMP) CirA. The latter is a TonB-dependent transporter involved in ferric iron
164 uptake. RyhB is a Hfq-dependent sRNA that regulates iron homeostasis (Fur represses *cirA*
165 and *ryhB*, while RyhB activates *cirA*). RyhB is essential for CirA synthesis during iron
166 starvation by pairing to *cirA* mRNA, leading to its translational activation and prevention of
167 degradation by RNase E. Consequently, increased CirA levels render cells more susceptible to
168 colicin Ia bactericidal action (30). An interesting class of sRNAs is those acting as RNA
169 ‘sponges’ that interact and repress the functions of other base-pairing sRNAs. An example is
170 3’ETS^{leuZ} RNA that is a 3’ external transcribed spacer of the *glyW-cysT-leuZ* polycistronic tRNA
171 produced *via* RNase E-mediated processing (31). This RNA ‘sponge’ pairs with RyhB and RybB
172 (a sRNA that downregulates CsgD – see below), suppressing transcriptional noise from those
173 sRNAs. Accordingly, a 3’ETS^{leuZ} deletion mutant is killed by colicin Ia, compared to the
174 parental strain.

175 In *E. coli*, MicF sRNA regulates *ompF* expression by pairing with *ompF* mRNA, inducing
176 translation inhibition and mRNA degradation, in turn reducing permeability to several
177 antibiotics (32). When overexpressed in *E. coli*, MicF increases cephalosporin, norfloxacin,
178 and minocycline MICs while depletion of this sRNA reverses those phenotypes, except for
179 minocycline (33).

180 Two novel sRNAs, Sr0161 and ErsA, have recently been identified in *P. aeruginosa* using a
181 new method called High-throughput Global sRNA target Identification by Ligation and
182 sequencing (Hi-GRIL-seq) (34). They interact with the *oprD* 5' UTR, which expresses a major
183 porin required for carbapenem uptake. Both sRNAs negatively regulate *oprD* expression
184 leading, when induced, to reducing OprD protein expression and, in turn, increasing
185 carbapenem resistance. Mutant strains lacking Sr0161 or ErsA are therefore more
186 susceptible to carbapenems.

187

188 **sRNAs and active drug efflux**

189 Categorized into five families, multidrug efflux pumps in bacteria are widely distributed in
190 both Gram-positive and negative bacteria. By expelling a broad range of structurally varied
191 molecules, they lower the intracellular antibiotic concentration, and are involved in intrinsic
192 and acquired bacterial resistance (35). Mostly encoded on the chromosome, efflux pumps
193 are implicated into stress adaptation, detoxification, pathogenesis and bacterial virulence
194 (36). Their expression is subjected to tight regulations in response to environmental and
195 physiological stimuli.

196 The *yejABEF* operon encoding an ATP-binding cassette (ABC) transporter in several Gram-
197 negative bacterial species confers antimicrobial peptide (AMP) resistance to *Salmonella* (37)
198 and *Brucella melitensis* (38) by stimulating active AMP efflux. AMPs induce *yej* operon
199 expression, allowing bacteria to counteract antibiotic activity by decreasing AMP
200 intracellular concentrations. RydC sRNA pseudoknot, with the aid of the Hfq chaperone,
201 regulates curli synthesis and biofilm formation in enteric bacteria (39). In *Salmonella*, RydC
202 also remodels phospholipid composition of the membrane by controlling the cyclopropane
203 fatty acid (CFA) synthase (40). The *yej* mRNA is degraded when RydC expression is stimulated

(41). Since RydC negatively regulates the expression the *yejABEF* mRNA, this sRNA may be associated with an increase in susceptibility to AMPs.

DsrA sRNA is a key regulator of essential pathways in *E. coli*, including general stress response (σ^S), genome compaction (H-NS), cell wall biosynthesis (MreB), and ribose metabolism (RbsD) (42). DsrA is also involved in antimicrobial resistance by regulating the expression of the MdtEF efflux pump (43). Indeed, when overexpressed in efflux-defective Δ *acrB* mutants, DsrA significantly increases oxacillin ($\times 8$), erythromycin ($\times 4$), and novobiocin MICs ($\times 4$) *via* an RpoS-dependent pathway.

In *E. coli*, while overexpression of RyeB increases susceptibility to quinolones, depletion of this sRNA reverses that phenotype (33). By overexpressing RyeB, there is a decrease in the expression level of *tolC* mRNA, whereas *tolC* mRNA expression is upregulated in a Δ *ryeB* mutant. TolC is an OMP of the 'AcrAB-TolC' efflux system, which has a broad spectrum of substrates including most of lipophilic antibiotics, and is also a component of other efflux transport systems (44). Named SdsR in *Salmonella* spp., RyeB is an abundant and stationary-phase Hfq-dependent sRNA, of whose transcription depends on σ^S (45). SdsR represses *tolC* mRNA levels by pairing with its 5' UTR, 33 nucleotides upstream of target mRNA ribosome binding site (RBS) (46). SdsR overexpression also increases susceptibility to other antibiotics, such as novobiocin and, to a lesser extent, erythromycin and rifampin. SdsR represses biofilm formation independently of pairing with *tolC* mRNA, suggesting additional targets. SdsR is a conserved sRNA from enterobacteria and its role in *tolC* mRNA repression was also found in *Salmonella* (47).

MtrF is an inner membrane protein belonging to the AbgT family described in *Neisseria gonorrhoeae* (48, 49). This membrane protein is required for gonococcal high-level resistance to hydrophobic antimicrobials (e.g. penicillins, erythromycin, rifampin) mediated

228 by the MtrCDE efflux system (48, 49). MtrF is also by itself a proton-motive-force (PMF)-
229 dependent antibiotic efflux pump that expels sulfonamides from the bacteria (50).
230 Interestingly, *trans*-acting, iron-regulated sRNA NrrF directly controls the MtrF expression by
231 reducing *mtrF* mRNA stability by increasing its turnover (51, 52). Thus, NrrF attenuates MtrF
232 action in antibiotic resistance. MtrF transcripts are also repressed by Fur, MtrR (repressor of
233 *mtrCDE*), and MpeR (repressor of *mtrR*) (52), implying that expression of that inner
234 membrane protein is tightly controlled by several additional regulators.

235

236 **sRNAs and drug target modifications**

237 Spontaneous or acquired variations in antibiotics target sites preventing drug binding is a
238 widespread resistance mechanism (25). Noteworthy, modest alterations of the targets can
239 induce substantial variations on antibiotic binding affinity. A common mechanism
240 of resistance to AMP in Gram-negative bacteria is LPS modifications (53). While several
241 sRNAs are known to regulate the expression of different proteins involved in LPS metabolism
242 MgrR, a Hfq-dependent sRNA expressed in *E. coli* and other *Enterobacteriaceae*, is part of
243 the PhoPQ regulon, a two-component system (TCS) activated under low Mg^{2+} conditions or
244 by AMPs. PhoPQ has been extensively studied; it consists in the sensor kinase PhoQ and the
245 cognate response regulator PhoP (54). MgrR actually downregulates *eptB* mRNA, which
246 encodes a phosphoethanolamine transferase involved in LPS modifications (55). EptB
247 modifies the keto-deoxyoctulosonate (KDO) residue (part of the core oligosaccharide of the
248 LPS), which reduces the net anion charges and electrostatic repulsion between LPS
249 molecules, leading to polymyxin resistance. An *mgrR*-deleted mutant is 10 times more
250 resistant to polymyxin B than the parental strain whereas complementation of the *mgrR*

251 mutation restores polymyxin susceptibility. Noteworthy, the reduction of *eptB* mRNA levels
252 by MgrR was also demonstrated in *Salmonella* (56).

253 SroC is an RNA sponge that originates from the GcvB-mediated decay of the polycistronic
254 *gltIJKL* mRNA (57). SroC negatively controls GcvB action and activates the GcvB-repressed
255 genes involved in amino acid metabolism. In *Salmonella*, SroC also pairs with the MgrR sRNA
256 to interfere with its action, thus indirectly activating *etpB* expression (56). SroC
257 overexpression increases EtpB expression and a Δ *sroC* mutant is more susceptible to
258 polymyxin B than the parental strain. Finally, Δ *mgrR* and Δ *sroC* Δ *mgrR* mutants exhibit a
259 similar resistance phenotype, suggesting that *mgrR* mutation may be epistatic to the *sroC*
260 mutation.

261 As previously mentioned, the PhoPQ TCS is induced in response to low Mg^{2+} and Ca^{2+}
262 concentrations and in the presence of AMPs. The PhoPQ regulon includes genes involved in
263 Mg^{2+} transport, LPS modifications, acid resistance, virulence, and resistance to AMPs (54). In
264 *E. coli*, the expression of *phoPQ* is directly repressed by MicA sRNA (also named SraD). MicA
265 transcription is activated by σ^E , which is induced under envelope stresses (58). More
266 precisely, MicA pairs with *phoPQ* mRNA around the *phoP* initiation codon, probably to
267 modulate translation initiation. MicA may influence AMP resistance since it downregulates
268 MgrR, *via* its action on PhoP, that itself represses *eptB* mRNA expression (Figure 1).

269 Another sRNA involved in *P. aeruginosa* antibiotic resistance is Sr006, which is a positive
270 post-transcriptional regulator of *pagL* mRNA expression that encodes an enzyme responsible
271 for lipid deacylation (34). When overexpressed, Sr006 confers increased polymyxin
272 resistance through PagL-mediated LPS modifications. The upregulation of *pagL* by Sr006
273 appears Hfq-independent.

274

sRNAs regulating cell wall biosynthesis

Cell wall synthesis and recycling are critical cellular processes essential for cell growth, elongation and division, and peptidoglycan is the main component of this complex entity (59). Peptidoglycan synthesis involves an array of enzymes across all cellular compartments (cytoplasm, inner membrane, and periplasm) and the expression of some of these enzymes can be regulated by sRNAs.

GlmS catalyzes synthesis of glucosamine-6-phosphate (GlcN6P) from fructose-6-phosphate and glutamine, a key metabolite in cell wall biosynthesis. GlcN6P is further converted by GlmM and GlmU enzymes to UDP-N-acetyl-glucosamine (UDP-GlcNAc), a common precursor for peptidoglycan and LPS synthesis. Bacilysin (tetaïne) and Nva-FMDP are dipeptide antibiotics that impair cell envelope synthesis by GlmS inhibition through covalent modification (60). In *E. coli* and presumably in most *Enterobacteriaceae*, *glmS* expression is controlled by GlmY and GlmZ sRNAs (61). GlmZ pairs with and activates *glmS* mRNA translation. Although GlmY is similar to GlmZ sequence and predicted structure, GlmY lacks a complementary region to *glmS* mRNA and does not directly activate *glmS* translation. Instead, GlmY expression inhibits a GlmZ processing event, disallowing *glmS* translation activation. Thus, GlmY functions by titrating an RNA processing factor away from homologous GlmZ sRNA. The GlmY/GlmZ pair provides resistance to bacilysin. Both *E. coli* and *Salmonella* respond to these antibiotics by increasing *glmS* expression to compensate for GlmS activity inhibition (62). GlmS inhibition by antibiotics leads to GlcN6P deprivation, sensed by GlmY sRNA, triggering its accumulation. Cells adjust GlmS expression levels to overcome growth inhibition by the GlmS inhibitor.

sRNAs modulating biofilm formation and antibiotic activity

299 Bacterial biofilms are multicellular populations, with cells surrounded by self-produced
300 extracellular matrix that can include exopolysaccharides, proteins, amyloid fibers, and DNA.
301 They are typically less susceptible to antimicrobial agents than non-adherent, planktonic
302 cells, because of the poor drug diffusion inside the biofilm structure, and also since they
303 contain metabolically inactive cells. Biofilm formation is tuned by complex regulatory hubs
304 that integrate various environmental signals *via* alternative sigma factors, two-component
305 systems, second messengers, and sRNAs. Many chronic infections are associated to bacterial
306 biofilms, which increase tolerance to antibiotics and biocides as well as resist host cell
307 phagocytosis (63). Conventional antibiotic resistance mechanisms in bacteria also include
308 survival as biofilm communities. Those mechanisms include nutrient gradient (less nutrient
309 availability in the biofilm core), compact exopolysaccharides matrices, extracellular DNAs,
310 stress responses, genetic determinants specifically expressed in biofilms, multidrug efflux
311 pumps, intercellular interactions and persister cells (64). In *Enterobacteriaceae*, RpoS and
312 CsgD transcription factors control regulons implicated in biofilm formation, and their mRNA
313 levels are controlled by numerous sRNAs (65). At least seven sRNAs (namely GcvB, McaS,
314 OmrA, OmrB, RprA, RybB, and RydC) downregulate CsgD expression by direct binding with
315 the *csgD* mRNA and, in turn, reduce biofilm formation (Figure 1). Thus, these sRNAs, when
316 expressed, are expected to increase antibiotic susceptibility for biofilm-associated bacteria.

317

318 **sRNA-control of transcription factors involved in antibiotic resistance**

319 Regulatory systems in bacteria (including two-component, transcription, and sigma factors)
320 respond to extracellular signals to modulate gene expression, contributing antimicrobial
321 resistance genes.

322 SpoVG is a transcription factor (66) contributing to *S. aureus* methicillin and glycopeptide
323 resistances, acting as a DNA-binding protein in *eubacteria* (67). In *S. aureus*, SprX sRNA (*alias*
324 RsaOR), modulates resistance to glycopeptides (68), antibiotics that inhibit cell wall
325 peptidoglycan synthesis and are treatment of choice of MRSA infections (69). Modifying SprX
326 levels influences both vancomycin and teicoplanin susceptibility profiles. SprX negatively
327 regulates SpoVG expression by direct pairings at the SpoVG translation initiation signals.
328 SpoVG is not the unique target of SprX (70, 71), and those other targets could also impact
329 glycopeptide resistance and pathogenicity.

330 *Trans*-encoded sRNAs often rely on sRNA-binding proteins for function (72). RNA chaperone
331 Hfq promotes pairings between sRNAs and their mRNA targets to induce post-transcriptional
332 regulations affecting mRNA stability and/or translation (73). Consequently, all the Hfq-
333 binding sRNAs involved in antibiotic response (several in Gram-negative bacteria) will be
334 impacted by the presence/absence of the protein. In *E. coli*, Hfq regulates a multidrug efflux
335 pump at post-transcriptional level, and therefore impacts multidrug resistance (74).
336 Compared to Gram-negative bacteria, the role of Hfq in sRNA functions seems less important
337 in Gram-positive bacteria (75).

338

339 **tmRNA and antibiotic resistance**

340 *Trans*-translation, monitored by an hybrid transfer-messenger RNA (the so-called tmRNA
341 [76]) with the SmpB protein, is a conserved quality control in *eubacteria* activated in
342 response to ribosome stalling on truncated or non-stop mRNAs that can arise in cells due to
343 premature transcription termination or mRNA damage (77). Accumulation of stalled
344 ribosomes on those problematic mRNAs is toxic and needs to be rescued, otherwise protein
345 synthesis would come to halt. As a consequence, impairment of *trans*-translation leads to

346 increased sensitivity to antibiotics targeting protein synthesis in several bacterial pathogens
347 (78, 79). Mutations that inactivate tmRNA or SmpB are lethal in some bacteria, including
348 *Neisseria gonorrhoeae* and *Shigella flexneri* (80). In other species, deletion phenotypes
349 include deficiencies in virulence, sporulation, cell cycle progression and antibiotic resistance
350 (81). Apart from ribosome rescue, these phenotypes could be due to the misregulation of
351 specific regulatory proteins in the absence of tmRNA. Also, *trans*-translation may be
352 coordinated with other essential co-translational processes such as protein folding and
353 secretion. In *S. pneumoniae*, the lack of tmRNA protects bacteria against fluoroquinolones
354 (82). In *S. pneumoniae*, deletion of tmRNA prevented chromosome fragmentation associated
355 to levofloxacin treatment. Such protective effect mainly depends on protein synthesis
356 inhibition. The increased susceptibility to translation inhibitors in different bacteria defective
357 in *trans*-translation implies that tmRNA is an attractive target for the development of novel
358 antibacterial agents (83). Indeed, the components of *trans*-translation were detected in
359 every sequenced bacterial genome, and mutations in these components affect viability or
360 virulence in many bacteria, suggesting that *trans*-translation inhibitors could be effective
361 'broad-spectrum' antibiotics.

362

363 **Concluding remarks and perspectives**

364 Antibiotic stress responses usually include sophisticated regulatory networks that were
365 recently investigated by extensive whole genome RNA-seq studies, with and without
366 antibiotics at SIC, in various bacteria (*S. aureus* [84, 85]; *E. faecium* [86]; *P. putida* [87]; and *S.*
367 *enterica* [88]). These global transcriptomic studies revealed that the expression of several
368 sRNAs is induced or repressed as a result of antibiotic SIC exposure, but the roles and

369 mechanisms connecting those sRNAs with the bacterial antibiotic responses await to be
370 uncovered.

371 A major challenge with antibiotic use in human and veterinary medicine is bacterial
372 resistance. Pioneering investigations in *E. coli*, *Salmonella*, *P. aeruginosa* and *S. aureus*,
373 indicate that some sRNAs contribute to antibiotic response, susceptibility and resistance.
374 Accumulating evidence indicates that specific sRNAs are essential players in adaptive
375 networks to control key processes (such as drug efflux or uptake, LPS and cell wall syntheses,
376 and biofilm formation) involved in resistance to the major classes of antibiotic drugs. Also,
377 growing evidence suggests that *cis*-acting RNAs also regulate the expression of many
378 resistance genes, sensing the presence of antibiotics and regulating resistance genes
379 accordingly (89). Because there is an international spread of MDR opportunistic organisms
380 including several Gram-positive bacteria, the implication of sRNAs in antibiotic resistance in
381 these pathogens should be investigated thoroughly in the coming years. The development
382 and application of genome-wide transcriptomic approaches will facilitate the identification
383 of the set of riboregulators implicated in antibiotic response and resistance in many bacterial
384 pathogens, a starting point to uncover and analyze the underlying regulation principles. A
385 better understanding of the implication of sRNAs in antibiotic resistance networks will allow
386 the design of new compounds preventing their actions in the future. They could be used
387 with existing drugs to enhance their activities and lower the development of resistance.
388 However, the development of resistances against new drugs targeting sRNA-regulated
389 processes cannot be ruled out. However, since each sRNA usually impacts the expression of
390 several targets (sRNA-associated regulon), it may be more complicated for bacteria to
391 produce resistances against each regulated target. Many challenges remain to be solved,
392 prior to clinical application.

393

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398

399

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616 **Legend of the figure**

617 **FIG 1 sRNAs regulating antibiotic resistance in bacteria.** Mechanisms subjected to sRNA-
618 mediated antibiotic response and resistance were divided into five main sections (dotted
619 color boxes): drug uptake (green), active drug efflux (blue), lipopolysaccharide (LPS) and cell
620 wall impairments (orange), biofilm formation (red), and transcription factors (TF) regulations
621 (purple). The antibiotics subjected to sRNA-induced controls are indicated. sRNA targets
622 involved are presented. Arrows correspond to sRNA-induced target gene expression
623 upregulations; broken line are sRNA-induced target gene downregulations. Riboswitches
624 were excluded. Of interest is the lack of knowledge for Gram-positives relative to Gram-
625 negatives bacteria. AMP, antimicrobial peptides.

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TABLE 1 The *trans*-acting regulatory RNAs associated with antibiotic resistance in Gram-negative and positive bacteria.

sRNA	Bacterial species	Mechanism(s) of resistance	Antibiotics ^a	Targets	Regulation	References
Gram-negative bacteria						
DsrA	<i>E. coli</i>	Active drug efflux	Oxacillin, erythromycin, novobiocin	<i>mdtEF</i>	+	43
ErsA	<i>P. aeruginosa</i>	Drug uptake	Meropenem	<i>oprD</i>	-	34
GvcB	<i>E. coli</i>		D-cycloserine	<i>cycA</i>	-	28
GlmY, GlmZ	<i>E. coli, Salmonella</i>	Cell wall changes	Bacilysin (tetaïne), Nva-FMDP	<i>glmS</i>	+	62
MicA	<i>E. coli</i>	LPS changes	AMP	<i>phoPQ</i>	-	58
MicF	<i>E. coli, Salmonella</i>	Drug uptake	Cephalosporins, norfloxacin	<i>ompF</i>	-	33
MgrR	<i>E. coli</i>	LPS changes	Polymyxin B	<i>eptB</i>	-	55
Nrrf	<i>N. gonorrhoeae</i>	Active drug efflux	Sulphonamides	<i>mtrF</i>	-	50, 52
RydC	<i>E. coli, Salmonella</i>	Active drug efflux	AMP, Microcin C	<i>yejABEF</i>	-	41
RybB	<i>E. coli</i>	Biofilm formation	EGCG	<i>csgD</i>	-	90
RyhB	<i>E. coli</i>	Drug uptake	Colicin Ia	<i>cirA</i>	+	30
SdsR	<i>E. coli, Salmonella</i>	Active drug efflux	Quinolones	<i>tolC</i>	-	33, 46, 47
(RyeB)						
Sr006	<i>P. aeruginosa</i>	LPS changes	Polymyxin	<i>pagL</i>	+	34
Sr0161	<i>P. aeruginosa</i>	Drug uptake	Meropenem	<i>oprD</i>	-	34
SroC	<i>Salmonella</i>	LPS changes	Polymyxin B	MgrR	-	56
3'ETS ^{euZ}	<i>E. coli</i>	Biofilm formation, drug uptake	Colicin Ia	RybB, RyhB	-	31
Gram-positive bacteria						
SprX	<i>S. aureus</i>	Global effect	Glycopeptides	<i>spoVG</i>		68
(RsaOR)						

^aAMP, Antimicrobial peptide; EGCG, Epigallocatechin gallate (catechin from green tea); LPS, Lipopolysaccharide.

