Small RNAs in Bacterial Virulence and Communication

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ABSTRACT Bacterial pathogens must endure or adapt to different environments and stresses during transmission and infection. Posttranscriptional gene expression control by regulatory RNAs, such as small RNAs and riboswitches, is now considered central to adaptation in many bacteria, including pathogens. The study of RNA-based regulation (riboregulation) in pathogenic species has provided novel insight into how these bacteria regulate virulence gene expression. It has also uncovered diverse mechanisms by which bacterial small RNAs, in general, globally control gene expression. Riboregulators as well as their targets may also prove to be alternative targets or provide new strategies for antimicrobials. In this article, we present an overview of the general mechanisms that bacteria use to regulate with RNA, focusing on examples from pathogens. In addition, we also briefly review how deep sequencing approaches have aided in opening new perspectives in small RNA identification and the study of their functions. Finally, we discuss examples of riboregulators in two model pathogens that control virulence factor expression or survival-associated phenotypes, such as stress tolerance, biofilm formation, or cell-cell communication, to illustrate how riboregulation factors into regulatory networks in bacterial pathogens.

INTRODUCTION

Many pathogenic bacteria transit between free-living lifestyles and the markedly different environments presented by their hosts. This requires detection, integration, and response to different external and intracellular conditions and subsequent realignment of physiology and metabolism, as well as virulence factor expression via coordinated gene expression changes. Signals detected by pathogens include not only changes in temperature, pH, or nutrient availability, but also cues from the host and neighboring bacteria. How virulence genes are regulated at the transcriptional level has been studied extensively, and the regulons of several master regulators of virulence and survival gene transcription have been described in detail (1, 2). This has provided insight into both general regulatory mechanisms used by bacteria, and the lifestyles of pathogenic species.

Besides transcriptional control, gene expression can also be regulated at the posttranscriptional level. The importance of diverse posttranscriptional mechanisms (i.e., regulation of mRNA translation, stability, and processing) in bacterial virulence gene expression is becoming increasingly apparent (3–5). The central players in posttranscriptional control in bacteria are small non-coding (sRNAs), which mainly use their base-pairing capacity to directly interact with mRNAs, ultimately leading to repression or activation of protein expression (3). Recently, there has been an explosion in identification of candidate sRNAs in bacterial pathogens by global transcriptome studies using deep sequencing, revealing a previously unappreciated capacity for RNA-based regulation (6–9). In addition, an ever-increasing diversity of mechanisms of action, beyond the canonical translational repression via base-pairing interactions,
has been described for riboregulators. These include activation of target gene expression, direct modulation of protein activity, and effects on transcription (3). Relatively limited conservation of sRNA repertoires between even closely related bacteria (10) suggests that this class of regulators is diverse. Many of the known mechanisms and functions of sRNAs are based on work in Gammaproteobacteria spp. and especially model organisms such as Escherichia coli and Salmonella. Insights from ongoing functional characterization of sRNA candidates outside the enterobacteria, including in a variety of important pathogens, is identifying additional mechanisms of gene regulation by sRNAs. This work is deepening the understanding of how virulence programs are regulated in bacterial pathogens. Also, in addition to sRNAs, endogenous highly conserved and structured regions within mRNAs themselves, such as riboswitches, are now known to factor centrally in posttranscriptional regulation. An emerging class of dual-function mRNAs that act both as templates for protein synthesis and as trans-acting regulators themselves, or act by sequestering proteins or sRNAs, has been reported (11, 12). These mechanisms also utilize various protein accomplices, such as RNA-binding proteins or ribonucleases (RNases). For example, the RNA chaperone Hfq is required for sRNA stability and function in enterobacteria and has also been linked to virulence regulation in pathogens (13, 14).

While the first sRNAs characterized had relatively restricted functions in bacterial regulatory networks, it is now also clear that sRNAs can regulate multiple genes in the same pathway or phenotypic response (15). Similar to the protein master-regulators of transcription, they can be global regulators of metabolic processes or pathways that are central to pathogenesis, including outer membrane integrity, quorum sensing, iron homeostasis, biofilm formation, or host–pathogen interactions (4). Several sRNAs are also either induced in response to antibiotic treatment or are required for antibiotic tolerance (16, 17). Many transcription factor master-regulators of virulence and survival are themselves regulated by sRNAs or include sRNAs as part of their regulons. sRNAs encoded on pathogenicity islands (PAIs) or virulence plasmids are prime candidates for regulators with a central role in virulence (18–20). Nevertheless, PAI-encoded sRNAs can also regulate genes of the core genome (21, 22).

While once thought to be rare novelties of bacterial gene regulation, sRNAs are now taking center stage in bacterial virulence control. Many recent reviews discuss the emerging roles and mechanisms of sRNAs in human pathogens such as Salmonella enterica (23, 24), one of the main model organisms for riboregulation, but also, for example, in diverse pathogens such as Listeria (25, 26), Vibrio spp. (27, 28), Yersinia spp. (29, 30), Staphylococcus aureus (31, 32), Mycobacterium tuberculosis (33), Helicobacter pylori (34), Pseudomonas aeruginosa (35), and streptococci (36).

In this article, we provide an overview of the diversity of regulatory mechanisms used by sRNAs, especially in pathogens, as well as their various protein “accomplices.” We apologize for any studies or sRNA examples that cannot be mentioned here due to space restrictions. We provide a general overview of how deep sequencing methods have revolutionized prokaryotic transcriptome analysis and sRNA identification and how they have provided novel means to study their functions and regulons on a genome-wide scale (37). We refer the reader to more in-depth reviews for more detailed descriptions of methods to identify and verify sRNAs (38–40) and to study their functions (41, 42). Finally, pathways central to virulence and bacterial communication, such as quorum sensing, that employ sRNAs in the pathogens S. aureus and Vibrio cholerae, will be discussed in more detail to illustrate how riboregulation is integrated into gene regulatory circuits.

IDENTIFICATION OF A WEALTH OF sRNAs IN BACTERIAL GENOMES

The first RNAs known were serendipitously discovered as curious noncoding transcripts in biochemical or functional genetic screens, aided by their relative abundance in the bacterial cell (38). Many of the first sRNAs to be functionally characterized were identified on mobile genetic elements such as plasmids, phages, and transposons, where they were shown to control replication, maintenance, or transposition (43). The abundant OmpR-dependent sRNA MicF was identified as the first chromosomally encoded sRNA that represses translation of an mRNA (encoding the OmpF porin) (44), making it a prototype for environmental posttranscriptional regulation by sRNAs. The first sRNA shown to directly affect virulence, RNAIII of S. aureus (see section on RNAIII, below), was then revealed to regulate multiple virulence factor-encoding transcripts (45).

Genome-Wide Approaches to Identify sRNAs

Approximately 15 years ago, numerous global studies were initiated to gauge the potential for sRNA-mediated regulation in bacteria, mainly in E. coli but also in
pathogens (reviewed in 38, 40). These genome-wide searches used comparative genomics, secondary structure predictions, and/or computational searches for promoters and terminators in “intergenic” regions to identify potential sRNA candidates (46–49). Confirmation of transcriptional activity with high-density or tiling microarrays increased the confidence of predictions (50–52) and also revealed many sRNAs that are expressed under pathogenesis-related conditions or involved in virulence of bacterial pathogens (53). RNomics approaches (either shotgun cloning or microarray analysis of cDNA) in multiple species, including E. coli and the pathogens P. aeruginosa and S. aureus, added to the pool of putative sRNAs (18, 54, 55). Identification of RNAs that copurify with the RNA chaperone Hfq by either microarrays (56) or direct enzymatic sequencing (37) was also utilized.

Going Deeper: Global Sequencing Approaches Identify Numerous sRNA Candidates and Place Riboregulators Centrally into Posttranscriptional Regulatory Networks

The development of a variety of next-generation sequencing technologies (so-called deep sequencing) to sequence cDNA (“RNA-sequencing” or “RNA-seq”) has revolutionized transcriptome annotation and identification of novel noncoding transcripts in many pro- and eukaryotes and vastly increased the number of potential riboregulators (37). Furthermore, an increasing number of novel deep sequencing applications have been developed, such as global mapping of transposon insertion sites (transposon-sequencing) (58), surveying and measuring translatomes using ribosome profiling (59), and analysis of RNA-protein complexes (60). These approaches greatly facilitate the identification, verification, and functional characterization of sRNAs and are summarized below.

RNA-seq-based transcriptome analysis

Global transcriptome analyses at single-nucleotide resolution using RNA-seq technology have identified a wealth of sRNA candidates in prokaryotic genomes in recent years (6–8, 37, 39). While initial approaches in V. cholerae and Listeria employed deep sequencing of size-selected RNA that had been depleted of tRNA and 5S rRNA (“sRNA-seq”) (2, 61, 62), a variety of RNA-seq methodologies to cover full transcriptomes have also been developed (8). These include approaches that specifically analyze primary transcriptomes and annotate transcriptional start sites, such as differential RNA-seq (dRNA-seq; reviewed in reference 9). RNA-seq studies have revealed a wealth of novel sRNAs and regulatory elements in diverse prokaryotes including numerous human pathogens such as Burkholderia cenocepa (63), H. pylori (64), Salmonella (65–67), Campylobacter jejuni (68–71), Neisseria gonorrhoeae (72), V. cholerae (73, 74), Chlamydia spp. (75, 76), Listeria spp. (77, 78), P. aeruginosa (79), M. tuberculosis (80), Streptococcus pneumoniae (81), staphylococci (82, 83), and Legionella (84). Comparative transcriptome analysis in different pathogenic species or strains has revealed both conserved sRNAs and strain-specific sRNAs that might contribute to differences in pathovar gene regulation and colonization of different niches or hosts (68, 77). Strikingly, a large reservoir of potential noncoding regulatory RNAs have also been identified transcribed antisense to open reading frames (ORFs) (85–87). It remains unclear how many of these “pervasive” transcripts are due to spurious transcription and how many have real functions.

Besides genome-wide transcriptome analysis, global RNA-seq analysis of RNA copurifying with RNA-binding proteins (so-called RIP-seq: RNA-immunoprecipitation-sequencing) has also played an important part in the recent large-scale identification of sRNA candidates. RIP-seq of the RNA chaperone Hfq has revealed a surprising number of sRNA candidates arising not only from intergenic regions, but also in untranslated regions (UTRs) of mRNAs, such as 3’UTRs (88–90). As a result, mRNAs/coding regions are now accepted as an important source of sRNAs. Sequencing of cross-linked Hfq-binding RNAs in enterohemorrhagic E. coli has identified numerous candidate sRNAs, including some that are encoded by PAIs (22). Likewise, RIP-seq of S. aureus RNase III revealed global regulatory functions for this enzyme, as well as novel mechanisms of gene coregulation (91).

These approaches have together greatly expanded the number of candidate sRNAs in bacteria. In Salmonella, for example, there are 280 strong sRNA candidates (66). However, functional characterization of sRNAs and identification of their mRNA targets can be laborious. For most of the candidate sRNAs their functions and targets still need to be identified. The limited number of currently characterized sRNAs available for comparative analyses, together with their short overall length (50 to 400 nucleotides [nt]) and short region of complementarity to target mRNAs (sometimes as few as 7 nt) has made in silico predictions of regulons challenging (42). A variety of methods have been developed to identify and study sRNA regulons...
using global approaches (39). Quantitative RNA-seq now serves as the gold standard for studying and comparing bacterial transcriptomes (92), replacing hybridization-based technologies such as microarrays and providing both single-nucleotide resolution and a higher dynamic range. Comparative transcriptomics of sRNA deletion and overexpression strains, as well as following pulse-expression of sRNAs, has both identified direct sRNA targets and revealed the global physiological functions of sRNAs and bacterial RNases (93).

Transposon-sequencing
The power of deep sequencing has also been harnessed for unbiased, global genetic screens for sRNAs. The location of transposon insertions in high-density mutant libraries can be identified by “transposon-sequencing” (Tn-seq) based on deep sequencing of transposon-chromosome junctions (58, 94). Due to the use of deep sequencing, a high insertion density (so far, as high as one insertion for every 8 bp) can be screened. This provides the resolution necessary to identify short genomic regions that might encode sRNAs or regulatory regions, which might have been missed in traditional transposon screens. Furthermore, combination of Tn-seq with whole-transcriptome RNA-seq data allows for the identification of transcriptionally active regions. As proof-of-principle, Tn-seq of two serovars of pathogenic *Salmonella* (Typhimurium and Typhi) with different host ranges and tissue tropisms identified an essential core genome conserved with nonpathogenic *E. coli* K12, as well as differences in sRNA repertoires that may underlie variations in pathogenicity (95). Tn-seq has been used to define the essential gene content and/or candidate virulence-associated sRNAs of numerous other pathogens, including *N. gonorrhoeae* (72), *S. pneumoniae* (81, 96), *M. tuberculosis* (97), *Salmonella* (95, 98, 99), enterohemorrhagic *E. coli* (100), *C. jejuni* (101), and *Haemophilus influenzae* (102).

**HOW REGULATORY RNAs POSTTRANSCRIPTIONALLY REGULATE GENE EXPRESSION IN BACTERIA**

While many sRNAs directly interact with mRNAs via base-pairing, other mechanisms of riboregulation have also been described. These include sRNAs that directly modulate protein activity, *cis*-acting elements of mRNAs themselves, and even dual-function sRNAs, which act as both riboregulators and code for small proteins (11). In this section, we provide an overview of the steadily expanding diversity of mechanisms employed by regulatory RNA (3, 103). We focus, where possible, on examples identified in pathogenic species that control virulence- and survival-associated phenotypes or regulate virulence genes (Table 1).

**Regulation by cis-Encoded Antisense RNAs**
Most of the functionally characterized sRNAs regulate their target mRNAs by an antisense mechanism using direct base-pairing interactions. Base-pairing sRNAs are generally divided into two categories based on their genomic relationship with their mRNA targets: (i) *Cis*-encoded sRNAs are transcribed from the opposite DNA strand and therefore show perfect, often extensive (>75 nt), complementarity with their target mRNAs (Fig. 1A), and (ii) *Trans*-encoded sRNAs are transcribed from different genomic locations than their targets and interact, often with multiple mRNAs, via imperfect base-pairing (Fig. 1B).

The first identified *cis*-encoded antisense RNAs (asRNAs) were found opposite to replication genes of plasmids or to transposase genes. They control maintenance and stability of mobile genetic elements through, for example, inhibition of primer maturation, transcriptional attenuation, translational repression, or induction of RNA degradation or cleavage (43). Many *cis*-encoded asRNAs are components of so-called type I toxin–antitoxin (TA) systems (104). These are two-gene elements consisting of a stable protein toxin and an unstable *cis*-encoded RNA antitoxin that base-pairs with the toxin mRNA, thereby inhibiting its translation and leading to degradation (105). While type I TA systems were initially found to be involved in plasmid stability and addiction, an increasing number of TA loci have since been identified in the chromosomes of many bacteria, including pathogens (106). The function of these chromosomally encoded TA systems remains enigmatic, but they may mediate adaptation of metabolism under stress, persister cell formation, or antibiotic resistance (107, 108). For example, TisB is an SOS-induced membrane-interacting toxic peptide that has been implicated in persister cell formation, which may increase intrinsic antibiotic resistance of bacterial populations (reviewed in 109). Its asRNA antitoxin, IstR, acts by sequestering a ribosome standby site on the *tisB* mRNA, thereby preventing its translation (110). TisB-dependent persister cells are tolerant to multiple antibiotics, and deletion of *Salmonella istR* also reduces fitness in pooled deletion mutant infections in mice, supporting a role in pathogenesis (111).

Besides the asRNAs of ORFs encoding small toxic proteins, an increasing number of transcripts have been
reported that are expressed antisense to other mRNAs, including examples that are antisense to UTRs or coding regions (Fig. 1A) or even to sRNAs (85, 86). Some asRNAs have, in fact, been shown to regulate virulence-associated genes. The Salmonella PhoPQ two-component regulatory system (TCRS) responds to the acidified environment of the phagolysosome, among other signals, to activate expression of factors that allow for intracellular replication. These include MgtC, an inner membrane protein required for survival in macrophages and virulence in mice (112, 113), as well as the MgtB Mg²⁺ transporter. PhoP also activates expression of the 1.2-kb AmgR transcript, which is encoded antisense to mgtCBR. AmgR promotes decay of mgtC mRNA via an RNase E–dependent mechanism (114). Absence of the AmgR asRNA both increases MgtC and MgtB levels and enhances bacterial virulence. A second asRNA, lesR-1, has also been identified on the pSLT virulence plasmid of S. Typhimurium (115). Expression of lesR-1 is 300-fold higher during infection of fibroblasts compared to its expression in broth culture, and it is required for replication in this cell type. In the gastric pathogen H. pylori, the urease enzyme, encoded in part by the ureAB operon, is required for adaptation to the acidic environment of the stomach. An sRNA (named 5′ureB-sRNA) on the opposite strand to ureAB is expressed dependent on the ArsRS TCRS and binds to the 5′-end of the ureB message (116, 117). This promotes premature transcription termination of the ureAB transcript and thereby represses urease levels at neutral pH, where the enzyme is presumably dispensable.

Recent work has identified asRNAs in Neisseria that may act at the DNA level, rather than by base-pairing with mRNA. Adherence to the epithelium of the urogenital tract by N. gonorrhoeae is mediated by type IV pili. Pilin genes are a prime target of antigenic variation, which provides population-level fitness and mediates immune evasion and persistent infection. A G-quadruplex (G4) structure upstream of the promoter of pilE has been shown to be required for recombination between the “expressed” pilE locus and one of the 19 remaining “silent” pilS pilin loci (118). Transcription of a short, C-rich RNA antisense to this G4 structure is also required for switching (119). It has been suggested that transcription of the asRNA may aid in formation of the G4 structure via a DNA:RNA hybrid between the asRNA and the complementary C-rich strand of the pilE G4. However, it is not yet clear whether it is solely the act of transcription (rather than the sRNA itself) that mediates G4 formation. Recombination also contributes to pilin antigenic variation in N. meningitidis. However, in meningococci, an asRNA conditionally expressed from a region downstream of pilE, complementary to the entire pilE coding sequence and 5′ UTR, may instead influence the rate of variation (120).

A large reservoir of potential cis-encoded sRNAs is the pervasive antisense transcription that has been noted in recent global bacterial transcriptome studies (121). For example, dRNA-seq analysis of H. pylori found that approximately half of all ORFs had at least one antisense TSS (64). Likewise, it has been proposed that 75% of S. aureus coding genes could be subject to regulation by asRNAs resulting from widespread transcription antisense to coding mRNAs, which provides double-stranded substrates for processing and/or degradation by RNase III (87). Extensive asRNA expression has also been noted from the Ti plasmid of the plant pathogen Agrobacterium, many of which are antisense to known virulence genes (122). Presumably, these transcripts are well suited to interfere with transcription or translation of mRNAs on the opposite strand or to stimulate their degradation by RNase III. However, whether these asRNAs are simply transcriptional noise or play a general regulatory role is still unclear, as many are neither significantly conserved nor very abundant.

Transcriptome maps of Listeria spp. identified a new class of asRNA species, the long antisense RNAs (lasRNAs), and a new concept in riboregulation, the “excludon” (53, 77) (reviewed in 123) (Fig. 1C). Often, lasRNAs arise in divergently transcribed ORFs/operons with mutually exclusive or related functions and can overlap multiple ORFs. Expression from a conditional promoter upstream of one operon provides an asRNA, likely part of an extended leader, which can base-pair with the mRNA of the operon on the opposite strand (Fig. 1C, top panel). One such “excludon” in the Listeria flagellum biosynthesis locus has been identified and characterized (53). The mogR mRNA, encoding the MogR repressor of flagellum biosynthesis, can be expressed from a promoter immediately upstream of the start codon. However, expression of mogR from a second σ⁵²-dependent promoter further upstream leads to a transcript with a longer 5′ UTR. This extra leader (lasRNA Anti0677) extends into the adjacent divergently transcribed operon, which encodes three genes required for flagellum export. Overexpression of the longer mogR transcript leads to decreased motility and flagellin transcript levels, possibly due to antisense interactions with this adjacent motility-associated operon. Likewise, transcription of extended 3′ UTRs in divergently transcribed operons could potentially interfere
**TABLE 1** Examples of PAI-encoded sRNAs and of riboregulators of bacterial virulence/colonization factors

<table>
<thead>
<tr>
<th>Type</th>
<th>RNA</th>
<th>Pathogen</th>
<th>Target(s)</th>
<th>Related phenotypes/comment</th>
<th>Reference(s)</th>
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<tr>
<td><strong>cis-acting sRNAs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>S1ureB</td>
<td></td>
<td><em>Helicobacter pylori</em></td>
<td>ureB</td>
<td>Urease expression/acid adaptation</td>
<td>116</td>
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<tr>
<td>AmgR</td>
<td></td>
<td><em>Salmonella enterica</em></td>
<td>mgtC</td>
<td>Survival in macrophages</td>
<td>114</td>
</tr>
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<td>lesR-1</td>
<td></td>
<td><em>S. enterica serovar</em></td>
<td>Unknown</td>
<td>Expressed from pSLT virulence plasmid, required for replication in fibroblasts</td>
<td>115</td>
</tr>
<tr>
<td><strong>trans-acting sRNAs</strong></td>
<td></td>
<td><em>Brucella abortus</em></td>
<td>Unknown</td>
<td>Infection of macrophages and chronic infection of mice</td>
<td>152</td>
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<tr>
<td>AbcR-1, AbcR-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>DapZ</td>
<td></td>
<td><em>S. Typhimurium</em></td>
<td>oppA, dppA</td>
<td>3′ UTR-encoded sRNA that regulates amino acid/oligopeptide metabolism</td>
<td>88</td>
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<tr>
<td>DsrA</td>
<td></td>
<td><em>Borrelia burgdorferi</em></td>
<td>rpoS</td>
<td>Expression of virulence-associated surface proteins</td>
<td>163</td>
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<td>FasX</td>
<td></td>
<td>Group A streptococci</td>
<td>ska, cpa</td>
<td>Streptokinase and pilus expression</td>
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<td>IhmT</td>
<td></td>
<td><em>Chlamydia trachomatis</em></td>
<td>hctA</td>
<td>Differentiation between the RB and EB developmental stages</td>
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<td>InvR</td>
<td></td>
<td><em>S. Typhimurium</em></td>
<td>ompD</td>
<td>PAI-encoded, activated by SPI-1 regulator, HilD, represses a porin of the core genome</td>
<td>21</td>
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<td>IsrJ</td>
<td></td>
<td><em>S. Typhimurium</em></td>
<td>Unknown</td>
<td>Affects translocation of SptP and invasion into nonphagocytic cells</td>
<td>19</td>
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<tr>
<td>IsrM</td>
<td></td>
<td><em>S. Typhimurium</em></td>
<td>sopA, hilE</td>
<td>Invasion, intracellular replication, virulence, and colonization of mice</td>
<td>155</td>
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<tr>
<td>IstR</td>
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<td><em>Escherichia coli</em></td>
<td>tisB</td>
<td>Persister cell formation</td>
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<tr>
<td>IstR</td>
<td></td>
<td><em>Salmonella</em></td>
<td>tisB</td>
<td>Type I TA system, colonization</td>
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<tr>
<td>LhrC</td>
<td></td>
<td><em>Listeria monocytogenes</em></td>
<td>lapB</td>
<td>Represses a virulence-associated adhesin</td>
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<td>PapR</td>
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<td>Uropathogenic <em>E. coli</em></td>
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<td>Population-level type I fimbriae expression</td>
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<td><em>H. pylori</em></td>
<td>tipB</td>
<td>Chemotaxis receptor levels</td>
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<td><em>L. monocytogenes</em></td>
<td>Lmo0514</td>
<td>Activation of expression of a cell wall protein inside cells</td>
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<td>RyhB</td>
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<td><em>Shigella dysenteriae</em></td>
<td>virB</td>
<td>Affects T3SS/effector and virulence</td>
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<td>RprA</td>
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<td><em>S. enterica</em></td>
<td>rpoS, ricl</td>
<td>Control of conjugation of the pSLT virulence plasmid in response to membrane conditions</td>
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<td>SgrS</td>
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<td><em>S. Typhimurium</em></td>
<td>sopD</td>
<td>Core genome sRNA that regulates a SPI-1 effector</td>
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<td>SpdD</td>
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<td><em>Staphylococcus aureus</em></td>
<td>sbi</td>
<td>Immune evasion</td>
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<td><em>S. aureus</em></td>
<td>spoVG</td>
<td>Glycopeptide resistance</td>
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<td>SroA</td>
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<td>Riboswitch-derived sRNA required for infection of mice</td>
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<td>sX13</td>
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<td><em>Xanthomonas campestris</em></td>
<td>hrpX</td>
<td>Affects levels of T3SS regulator</td>
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<td>TarB</td>
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<td><em>Vibrio cholerae</em></td>
<td>tcpF</td>
<td>Regulates a virulence factor and colonization</td>
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<td>VqrR</td>
<td></td>
<td><em>V. cholerae</em></td>
<td>vpsT, rtx, coIA</td>
<td>Biofilm formation and toxin expression</td>
<td>73</td>
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<td>VR-RNA</td>
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<td><em>Clostridium perfringens</em></td>
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<td>Toxin expression</td>
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<td>fmr 5′ UTR</td>
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<td><em>S. Typhimurium</em></td>
<td>CsrA</td>
<td>Hierarchical fimbriae expression</td>
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<td>RNAIII</td>
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<td><em>S. aureus</em></td>
<td>spa, hla, coa,</td>
<td>δ-haemolysin-encoding; quorum sensing control of toxin and surface protein expression</td>
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<td>rot, sbi</td>
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<td><em>S. aureus</em></td>
<td>SprA1-encoded</td>
<td>Cytolysin-encoding, cis-encoded trans-sRNA</td>
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<td>PhrS</td>
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<td><em>Pseudomonas aeruginosa</em></td>
<td>pqS</td>
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<td>Riboswitches</td>
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<td><em>Salmonella</em></td>
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<td>Intracellular magnesium uptake</td>
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<td>(ligand/signal)</td>
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<td>mgtA 5′ UTR (Mg⁡⁺²)</td>
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<td><em>Salmonella</em></td>
<td>mgtA</td>
<td>Expression of type IV pili and aggregation</td>
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<tr>
<td>pilA1 5′ UTR (c-di-GMP)</td>
<td></td>
<td><em>Clostridium difficile</em></td>
<td>pilA1</td>
<td>Present in the mRNA encoding a TfoX competence regulator homologue</td>
<td>248</td>
</tr>
<tr>
<td>Vc2 (c-di-GMP)</td>
<td></td>
<td><em>V. cholerae</em></td>
<td>VC1722</td>
<td>Controls expression of the AIX transporter by forming a translationally active structure at low pH</td>
<td>246</td>
</tr>
<tr>
<td>alx 5′ UTR (pH)</td>
<td></td>
<td><em>Salmonella</em></td>
<td>alx</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(continued)
TABLE 1  Examples of PAI-encoded sRNAs and of riboregulators of bacterial virulence/colonization factors (continued)

<table>
<thead>
<tr>
<th>Type</th>
<th>RNA</th>
<th>Pathogen</th>
<th>Target(s)</th>
<th>Related phenotypes/comment</th>
<th>Reference(s)</th>
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</thead>
<tbody>
<tr>
<td>Thermosensors</td>
<td>css 5′UTR</td>
<td>Neisseria meningitidis</td>
<td>css</td>
<td>Controls capsule biosynthesis and immune evasion</td>
<td>264</td>
</tr>
<tr>
<td></td>
<td>lasl 5′UTR</td>
<td>P. aeruginosa</td>
<td>lasl</td>
<td>Quorum sensing</td>
<td>266</td>
</tr>
<tr>
<td></td>
<td>prfA 5′UTR</td>
<td>L. monocytogenes</td>
<td>prfA</td>
<td>Virulence factor expression</td>
<td>263</td>
</tr>
<tr>
<td></td>
<td>rhoAB 5′UTR</td>
<td>P. aeruginosa</td>
<td>rhoR</td>
<td>Quorum sensing</td>
<td>266</td>
</tr>
<tr>
<td></td>
<td>toxT 5′UTR</td>
<td>V. cholerae</td>
<td>toxT</td>
<td>Virulence factor expression</td>
<td>289</td>
</tr>
<tr>
<td>Protein-modulating</td>
<td>6S</td>
<td>L. pneumophila</td>
<td>RNAP</td>
<td>Required for optimal replication in amoeba and mammalian host cells</td>
<td>231</td>
</tr>
<tr>
<td></td>
<td>CsrB/C</td>
<td>S. Typhimurium</td>
<td>CsrA</td>
<td>Quorum sensing</td>
<td>306</td>
</tr>
<tr>
<td></td>
<td>CsrB/C</td>
<td>S. Typhimurium</td>
<td>CsrA</td>
<td>Motility and pathogenesis</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>Ms1</td>
<td>Mycobacterium</td>
<td>RNAP</td>
<td>Implicated in stationary phase adaptation</td>
<td>232</td>
</tr>
<tr>
<td></td>
<td>Rli55</td>
<td>L. monocytogenes</td>
<td>RsmA</td>
<td>Ethanolamine utilization</td>
<td>259</td>
</tr>
<tr>
<td></td>
<td>RsmX/Y/Z</td>
<td>P. aeruginosa</td>
<td>RsmA</td>
<td>T3SS, T6SS, virulence, exopolysaccharide</td>
<td>222, 223</td>
</tr>
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<td></td>
<td>RsmX/Y/Z</td>
<td>L. pneumophila</td>
<td>CsrA</td>
<td>Motility and replication in macrophages</td>
<td>84, 225, 226</td>
</tr>
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<td></td>
<td>CsrB/C</td>
<td>Yersinia pseudotuberculosis</td>
<td>CsrA</td>
<td>Virulence regulator RovA</td>
<td>216</td>
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<tr>
<td>lastRNA/excludon</td>
<td>Anti0677</td>
<td>L. monocytogenes</td>
<td>Flagellar operon</td>
<td>Motility/flagellum biosynthesis</td>
<td>53, 289</td>
</tr>
<tr>
<td>CRISPR/Cas</td>
<td>Cas2</td>
<td>L. pneumophila</td>
<td>Unknown</td>
<td>Cas2 is required for infection of amoeba DNA uptake and capsule genotype</td>
<td>331</td>
</tr>
<tr>
<td></td>
<td>CRISPR/Cas</td>
<td>Streptococcus</td>
<td>Unknown</td>
<td>Foreign DNA</td>
<td>327</td>
</tr>
<tr>
<td></td>
<td>CsrB/C</td>
<td>Enterococcus spp.</td>
<td>Foreign DNA</td>
<td>Mobile DNA/antibiotic resistance uptake</td>
<td>326</td>
</tr>
<tr>
<td></td>
<td>RibB</td>
<td>L. monocytogenes</td>
<td>Unknown</td>
<td>Deletion of ribB affects virulence in mice</td>
<td>53, 342</td>
</tr>
<tr>
<td></td>
<td>scla/tracr/Cas9</td>
<td>Francissella novicida</td>
<td>FTN_1103</td>
<td>Regulates a lipoprotein and affects immune evasion, antibiotic resistance</td>
<td>328, 329</td>
</tr>
<tr>
<td>Other</td>
<td>pilE upstream</td>
<td>Neisseria gonorrhoeae</td>
<td>pilE upstream G-quadruplex</td>
<td>Recombination of expressed pilin locus/antigenic variation</td>
<td>343</td>
</tr>
</tbody>
</table>

*Abbreviations: ORF, open reading frame; RB, reticulate body; EB, elementary body; PAI, pathogenicity island; TA, toxin-antitoxin; T3/4/6SS, type III/IV/VI secretion system RNAP, RNA polymerase; UTR, untranslated region*

with transcripts on the opposite strand, also leading to coregulation (Fig. 1C, bottom panel). Numerous examples of such situations were identified in tiling-array analysis of the Listeria monocytogenes transcriptome (53) but still await functional characterization.

### Regulation by trans-Encoded sRNAs

Trans-encoded sRNAs (Fig. 1B) are a diverse family of short (50 to 300 nt), usually untranslated, transcripts. While trans-sRNAs (also referred to as simply “sRNAs”) are often encoded intergenically, they can also be derived from UTRs (5’ or 3′UTRs) or even from coding regions themselves (89) (Fig. 1B, bottom). Many sRNAs are global regulators of gene expression and are often induced in response to stress or inside host cells (3, 4, 13, 124). They can control extensive regulons that include numerous target mRNAs in related pathways, and second, one mRNA may be targeted by multiple sRNAs and can thus be regulated posttranscriptionally in response to numerous signals. Yet, still, the seed-pairing strategy maintains regulatory specificity. Only a single base pair difference in the targeting region of the mRNAs encoding two paralog effector proteins of S. Typhimurium, sopD and sopD2, is sufficient for the sRNA SgrS to discriminate between them (132). While expression of sopD is repressed by SgrS, sopD2 is not regulated by this sRNA.

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A. *cis-encoded sRNAs*
- Complementary to 5'/3'UTR
- Complementary to ORF

B. *trans-encoded sRNAs*
- Stand-alone (intergenic)
- mRNA (3'UTR)-derived

C. *excludon / overlapping UTRs*
- lasRNA / excludon
- Extended 3'UTR

D. *cis-acting mRNA elements*
- Riboswitch
- Thermosensor
Functional characterization of sRNAs outside the enterobacteria and in those lacking Hfq has uncovered other common themes of sRNA structure and function. Many sRNAs use unpaired sequences in apical hairpin loops to interact with target mRNAs. An increasing number of sRNAs with C- or C/U-rich loops have been reported that are well suited for base-pairing with G-rich sequences of mRNAs, such as the consensus ribosome binding site (RBS). Examples of these C-rich sRNAs are widespread in bacterial pathogens, such as L. monocytogenes (133), S. aureus (134, 135), V. cholerae (73), Salmonella (136), H. pylori (137), and Xanthomonas campestris (138). Thus, like the seed-pairing sRNAs of enterobacteria, such C-rich sRNAs may represent another common family of riboregulators.

While some sRNAs share structural themes, the diversity of mechanisms and regulatory consequences of trans-acting sRNAs is ever-increasing. Base-pairing by sRNAs can lead to repression or activation of target gene expression (3, 139) (Fig. 2A). Here, we will discuss a selection of trans-acting sRNAs that control genes associated with virulence or survival in bacterial pathogens (see also Table 1).

**trans-encoded sRNAs that repress virulence gene expression**

The prototypical sRNA represses protein levels by interfering with translation initiation and/or elongation through base-pairing with the translation initiation region (TIR), often at or near the RBS and start codon (Fig. 2A, left). However, RNAs can also interact with other mRNA regions (Fig. 2B), such as far upstream of the TIR in the 5′UTR. For example, ribosome standoff sites or translational enhancers can be targeted (110, 140). Targeting can also occur within coding sequences (141). As the ribosome protects mRNAs from the RNA turnover machinery of the cell (142), translation inhibition often results in degradation of a message since it can be more directly targeted for degradation by RNases. In addition, interaction of some sRNAs with their target can expose RNase cleavage sites, recruit ribonucleases such as RNase E, and/or stimulate the cleavage activity of RNase E by providing a 5′ monophosphate (141, 143, 144), thereby affecting mRNA stability. sRNAs can be catalytic (recycled to repress other mRNA molecules) or noncatalytic (codegraded with their targets) (145, 146). Single sRNAs can show amazing versatility, using different mechanisms depending on the target mRNA and nature of base-pairing interaction (145).

Several sRNAs that repress virulence genes, such as the above-mentioned Salmonella SgrS sRNA that represses expression of the SopD effector protein (132), or genes crucial for replication within the host have been reported (4) (see Table 1). The virulence plasmid of the gastrointestinal pathogen Shigella dysenteriae encodes genes required for invasion of host cells and disease, including a type III secretion system (T3SS) and its effectors. These genes are coordinately regulated in response to environmental signals by two transcriptional regulators, VirF and VirB (147). The iron-responsive sRNA RyhB represses levels of VirB, thereby indirectly repressing its entire regulon (148). Overexpression of RyhB decreases VirB levels, reduces effector secretion, and causes defects compared to the wild-type strain for invasion in vitro. In uropathogenic E. coli, comparative deep-sequencing of Hfq-bound RNAs during infection identified PapR, an sRNA that represses a protein involved in phase variation and appears to affect expression of P-fimbriae, an important epithelial adhesion factor at the population level (149).
FIGURE 2  Mechanisms of posttranscriptional control by regulatory RNAs. (A) Gene repression (left) and activation (right) mechanisms used by base-pairing sRNAs (depicted in red) for direct regulation of target mRNAs (shown in blue) at the level of translation or stability. Base-pairing interaction sites in mRNAs and sRNAs are shown with blue- and red-lined boxes, respectively. Potential RNase cleavage sites are indicated with an orange asterisk. Also participating are ribosomes and RNases. TIR, translation initiation region including RBS and start codon. (B) Potential sRNA interaction sites in regulated target mRNAs, starting from the TSS (transcriptional start site) to the transcriptional terminator (TERM). (C) Targeting/titration of other regulatory molecules by riboregulators acting as so-called sponges to affect gene expression. RNA sponges can be stand-alone sRNAs, regions of mRNAs themselves (either intact or processed), or those derived from housekeeping RNAs such as the 3′ external transcribed spacer (3′ ETS) of tRNAs. They can target either sRNA or protein regulators and have been shown to sequester them away from their targets, trigger their degradation, and/or modulate their regulatory activity.
The obligate intracellular pathogen *Chlamydia trachomatis* cycles between two physiological states during infection: elementary bodies and reticulate bodies. The infectious, extracellular elementary bodies are transcriptionally and translationally silent due to chromatin condensation, which is mediated in part by the histone-like protein HctA (also called Hc1). During differentiation into metabolically active reticulate bodies, HctA levels are reduced, and this correlates with expression of the IhtA sRNA (150). IhtA interacts with the hctA mRNA *in vitro* and appears to repress its translation (151).

Two apparently redundant sRNAs, AbcR-1 and AbcR-2, are conserved in *Alphaproteobacteria* such as *Brucella abortus* and *Agrobacterium tumefaciens* (152, 153). In *B. abortus*, these sRNAs regulate genes involved in amino acid transport and are required for infection of macrophages, as well as chronic infection of mice.

Several *Salmonella* sRNAs are induced under virulence-associated stress conditions or inside eukaryotic cells, including PAI-encoded sRNAs (19, 66, 154). One such sRNA of *S. Typhimurium*, IsrM, regulates virulence factors essential for invasion of host cells (155). IsrM targets the 5′UTR of *sopA* mRNA, encoding a SPI-1 effector, as well as the leader of *hilE*, a global regulator of the expression of SPI-1 proteins. Deletion of *isrM* results in defects in epithelial cell invasion and replication inside macrophages, as well as reduced virulence and extraintestinal growth in mice. A second PAI-encoded sRNA, InvR, is activated by the SPI-1 regulator HidD and represses the *ompD* porin mRNA encoded in the core genome (21). A third PAI-encoded sRNA, IsrJ, is required for invasion into host cells and secretion of the SPI-1 T3SS effector SptP (19). However, the mRNA targets and mode of regulation for IsrJ have not yet been determined. The conserved enterobacterial sRNA OxyS is induced under oxidative stress by OxyR and acts as both a repressor and activator of gene expression in *E. coli*. OxyS represses levels of the FhlA regulator and the RpoS sigma factor (156–158), and interestingly, an *oxyS* deletion mutant in *Salmonella* shows increased fitness in mice during infections with pooled deletion mutants (111).

Small RNAs that repress important virulence and colonization factors have also been identified in Gram-positive pathogens. Profiling of *L. monocytogenes* sRNA expression in macrophages has detected 71 noncoding transcripts not previously identified under routine laboratory conditions (62). *L. monocytogenes* requires the surface protein LapB for adhesion and entry into host cells. The multicopy LhrC sRNA, induced by the stress-associated LisRK TCRS, uses three UCCC sequences to interact with G-rich sequences near the start codon of the lapB message to inhibit its translation (133). ChIP-seq of the *M. tuberculosis* PhoP regulon identified the sRNA Mcr7, which was predicted to interact with *tatC* mRNA, encoding a component of the twin-arginine translocation system (159). Deletion of *pboP*, which is required for Mcr7 expression, increases twin-arginine translocation–dependent substrates in the *M. tuberculosis* secretome, some of which contribute to infection of the host.

**trans-encoded sRNAs that activate virulence gene expression**

sRNAs can activate gene expression by mechanisms that increase mRNA translation or transcript stability (139, 160) (Fig. 2A, right). Increased translation can result from base-pairing interactions that disrupt inhibitory secondary structures sequestering the RBS, as observed for activation of the alpha toxin–encoding *bla* mRNA by RNAIII in *S. aureus* (161). In the Lyme disease spirochete *Borrelia burgdorferi*, the alternative sigma factor RpoS reciprocally regulates expression of two virulence-associated surface proteins, OspC and OspA (162). *B. burgdorferi* RpoS expression is activated at higher temperatures, and this requires the sRNA DsrA, which may allow unfolding of a structured region in the 5′UTR of the *rpoS* mRNA that sequesters its RBS (163). Inside host cells, *L. monocytogenes* upregulates cell-wall-associated proteins, including Lmo0514, which is encoded by an mRNA with two different 5′UTR lengths of 28 and 234 nucleotides (164). The longer message is induced inside cells, and the extended leader of this transcript can base-pair with Rli27 sRNA, which is also upregulated inside cells. This interaction activates translation of the mRNA.

Like decreased mRNA stability due to translational repression, increased translation can also protect transcripts from the RNA turnover machinery of the cell (Fig. 2A). Pathogen sRNAs have also been reported to activate mRNA transcripts by selective stabilization of decay intermediates or longer isoforms (165, 166) or by mediating processing into more stable transcripts (167). For example, VR-RNA of *Clostridium perfringens* activates expression of collagenase by mediating processing of the leader of the *colA* transcript to increase its stability (168).

Activation by sRNAs can occur by mechanistic twists on the above themes that combine different regulatory modules. *P. aeruginosa* uses multiple quorum sensing systems to coordinately control expression of group-associated phenotypes, including virulence. The *Pseu-
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*domonas* quinolone system (PQS) generates and detects the signal 2-heptyl-3-hydroxy-4-quinolone and controls expression of virulence genes, including elastase, phospholipase, and phenazine via the activator PqsR (169). Levels of PqsR are regulated indirectly by an sRNA via translational activation of a short upstream ORF (uORF) in the *pqsR* leader (170). Translation of *pqsR* is coupled to translation of the uORF; i.e., increased translation of the uORF releases a structure that occludes the RBS of *pqsR*. The RBS of the uORF is also occluded by the inhibitory secondary structure. Upon induction under low oxygen conditions, the PhrS sRNA base-pairs with a region upstream of the uORF and releases sequestration of its RBS. The resulting translation of the uORF thereby allows expression of the translationally coupled *pqsR* gene.

sRNAs can also activate multiple targets posttranscriptionally, and this can integrate different signals into regulatory logic modules, such as those commonly seen in transcription factor networks (124). In feed-forward loops, one regulator is controlled by a second regulator, and both act upon a third target. In *Salmonella*, the RicI protein inhibits conjugation of the virulence plasmid pSLT and is the target of such a feed-forward loop, modulated by an sRNA (171). Full expression of *RicI* requires translational activation of both its transcriptional regulator (RpoS) and its own mRNA by a single sRNA, RprA. RprA has two separate targeting seed sequences (one for *rpoS* and one for *ricI*). While the full-length RprA, which has both seed sequences, is induced under conditions such as envelope stress, the sRNA is processed by RNase E to a more stable 3′ form with only the *ricI* seed. This lower stability of full-length RprA allows another level of control: while activating conditions lead to immediate upregulation of both RpoS and RicI seed pairing regions, differential decay allows for resetting of the signal or even activation by only the second (ricI-targeting) seed when input from other conditions that activate RpoS are present. This module ensures that expression of the RicI conjugation inhibitor occurs only under conditions of significant membrane damage.

**trans-acting sRNAs that both activate and repress virulence genes**

The versatility of sRNAs as gene regulators also lies in their potential to either repress or activate different targets. One of the best-characterized sRNA that can both activate and repress targets is RNAIII of *S. aureus* (see section on RNAIII, below). However, RNAIII is far from the exception. The Qrr3 sRNA involved in *Vibrio* quorum sensing uses four distinct mechanisms to either repress or activate different target mRNAs depending on a particular pairing strategy that dictates the regulatory mechanism (145). FasX, conserved in group A streptococci (i.e., *Streptococcus pyogenes*), activates expression of the secreted virulence factor streptokinase via base-pairing interactions that stabilize the *ska* transcript (172). The same sRNA can also bind to the 5′-end of a pilus operon mRNA, thereby reducing mRNA stability and repressing translation of the first gene of this mRNA, *cpa*, encoding a minor pilin component (173). The gastric pathogen *H. pylori* expresses at least 60 candidate sRNAs (64). The abundant, conserved sRNA RepG regulates expression of the TlpB chemotaxis receptor, involved in pH, autoinducer, and urea chemotaxis (174–176), at the level of *tlpB* mRNA stability and translation. Interaction between a C-rich terminator loop of RepG with a variable homopolymeric G-repeat in the *tlpB* mRNA leader differentially represses or activates expression of TlpB depending on the G-repeat length (137). Therefore, RepG has the potential to act as either an activator or repressor based on phase variation in its targeting sequence.

**The RNA chaperone Hfq and auxiliary protein factors that cooperate with trans-acting sRNAs**

The Hfq RNA chaperone is present in about 50% of bacteria with sequenced genomes (14). Hfq is a homohexameric, doughnut-shaped protein, with each subunit containing two domains similar to the Sm proteins of eukaryotes (177). It binds sRNAs on one of two binding faces, with a preference for single-stranded A-, U-, or A/U-rich sequences near structured regions (178–180). Through these interactions, Hfq mediates numerous aspects of sRNA metabolism and activity (reviewed in 13). Hfq aids the interaction between many trans-acting sRNAs and their targets and can either facilitate presentation of seed regions for base-pairing to targets, open target structures, or increase the local concentration of sRNAs, thereby lowering the effective dissociation constant between sRNA and mRNA targets. It is also required for the stability of some sRNAs by protecting A/U-rich sites from degradation by RNase E (181). Together with sRNAs, Hfq can also recruit RNase E to degrade the target (182). The chaperone itself can also bind and affect translation of some mRNAs (183, 184), even being recruited to target mRNAs by an sRNA, such as has been reported for the Spot42 sRNA and *sdbC* mRNA (185). Competition for Hfq by sRNAs may be yet another layer of global regulation, with sRNAs actively cycling on Hfq in a concentration-dependent...
manner (186–188). In fact, in S. Typhimurium, the Hfq-bound sRNA profile changes with bacterial growth phase (14), and in uropathogenic E. coli, the Hfq-bound sRNA profile differs between intracellular and extracellular environments (149).

Not surprisingly, deletion of hfq has pleiotropic effects in numerous bacteria, including virulence (14). However, not all bacteria encode Hfq, and not all sRNAs in Hfq-containing bacteria require this protein for their activity or stability (189). In fact, sRNAs are often divided into Hfq-dependent and Hfq-independent, based on whether stability and/or interaction with target mRNAs by the sRNA is dependent on the RNA chaperone. Numerous sRNAs of Gram-positive bacteria show Hfq-independence in stability and/or target interactions, despite being identified in Hfq-binding screens. LhrC of L. monocytogenes was first identified in an Hfq-RNA coIP study (57), although it regulates at least one target, lapB mRNA, in the absence of hfq (133). This raises the question as to whether unidentified RNA chaperones exist that replace or complement the functions of Hfq.

Several ribonucleases also factor centrally into multiple levels of riboregulation, including the biogenesis/maturation and activity of trans-encoded sRNAs (190). Many RNases are essential for viability in diverse species and/or have pleiotropic effects on bacterial physiology or virulence of pathogens, including RNase III, RNase E, RNase R, PNPase (polynucleotide phosphorylase), and RNase Y (191–198). Some RNases, such as RNase E, cooperate with sRNAs to degrade target mRNAs, after either exposure of specific cleavage sites or release from the protection of the translational machinery (199, 200). RNase III can cleave double-stranded regions formed by loop-loop interactions between sRNAs and mRNAs, such as those between S. aureus RNAIII and several virulence factor–encoding mRNAs (201–204) (see section on RNAIII, below). RNases themselves also have direct effects on gene expression independent of an sRNA accomplice via cleavage of specific coding transcripts implicated in virulence. This has been especially apparent for RNases III and Y in Gram-positive bacteria (reviewed in 205, 206). For example, RNase Y of S. aureus is required for processing and stabilization of the transcript encoding the SaePQRS TCRS, a global regulator of virulence, thereby contributing to activation of expression of virulence genes (192). RIP-seq of a catalytically inactive mutant has likewise identified substrates of RNase III in S. aureus (91). RNase III was revealed to process mRNAs, such as the cspA major cold shock protein mRNA, to increase their stability, as well as to cleave overlapping transcripts from divergently transcribed genes to generate leaderless mRNAs. Therefore, widely conserved RNases in bacterial pathogens, such as RNase III, RNase Y, and RNase E, have key roles in virulence-associated posttranscriptional control, despite their central housekeeping roles. The diverse repertoire of RNases in different pathogens suggests there may be yet unidentified examples of RNases that act either alone or in concert with sRNAs on virulence-associated transcripts.

**RNAs that Regulate or Antagonize Protein Activity**

While the above-described cis- and trans-acting sRNAs function through base-pairing interactions with their target RNAs, some sRNAs instead interact with proteins and modulate their activity (3, 207) (Fig. 2C). A hallmark of these RNAs is substrate mimicry, and activity modulation is thus often directed toward RNA-binding proteins. Some abundant sRNAs, such as ChiX and CrcZ, can even titrate the RNA chaperone Hfq away from target mRNAs (208, 209).

**Csr/Rsm regulatory networks**

Perhaps the most widespread and well-studied posttranscriptional regulatory network in bacteria is the Csr (carbon storage regulator)/Rsm (repressor of secondary metabolites) network (210). Csr/Rsm has been linked to virulence control in several pathogens (29, 211). The central protein regulator of the system, CsrA (RsmA in some species) is a global posttranscriptional regulator conserved in many Gram-positive and Gram-negative bacteria. Generally, CsrA binds GGA-containing stem-loops near the TIR of target mRNAs, inhibiting translation and/or affecting their stability. CsrA can also stabilize or activate transcripts, such as the flbDC mRNA of E. coli, encoding the master regulator of the flagellar biosynthesis cascade (212). PGA (poly-N-acetylglucosamine) is a major component of the E. coli and Salmonella biofilm matrix, exported by the PGA export protein (213). In Salmonella, CsrA binds the 5’UTR of pga mRNA (214). However, this binding unexpectedly releases a secondary structure that sequesters a Rho entry site, which stimulates transcription termination. A hallmark of the Csr/Rsm system of Gammaproteobacteria is the presence of multiple environmentally regulated sRNAs (CsrB/C or RsmX/Y/Z), which bind and antagonize CsrA (RsmA) function. These sRNAs contain multiple CsrA-binding sites and sequester the regulator from target mRNAs, thereby interfering with their repression or activation.
In many bacteria, effects of csrA deletion are pleiotropic, and CsrA directly controls translation of mRNAs related to carbon metabolism (reviewed in 211). Csr networks also reciprocally control motility and biofilm formation, two phenotypes central to pathogen survival and colonization. In several pathogens, the CsrA regulon also includes important virulence factors (5, 29). In Yersinia pseudotuberculosis, CsrA has global effects on the transcriptome (215). In particular, CsrA has been shown to indirectly repress RovA, a global regulator of virulence that is required to activate expression of virulence factors such as the primary invasin and pili (216). Transcription of the antagonizing sRNAs CsrB/C in Y. pseudotuberculosis is controlled by at least three regulators, including Crp (the cAMP regulatory protein, involved in catabolite repression), the Mg^{2+}-dependent PhoPQ TCRS, and the BarA/UvrY TCRS, indicating that the Yersinia Csr system links multiple environmental cues to virulence gene expression (29, 217, 218).

In Salmonella, CsrA specifically represses translation of biiD, which activates the positive regulators of the SPI-1 and SPI-2 PAIs, HiiA and SsrAB, respectively, as well as multiple proteins involved in c-di-GMP metabolism (219, 220). Gram-negative phytopathogens, such as various xanthomonads, use a T3SS to deliver effectors that modify the plant host. Biogenesis of the Xanthomonas T3SS machinery, encoded by the hrp/hrc loci, is controlled transcriptionally by the master regulator HrpG, and in Xanthomonas citri, RsmA binds and stabilizes the 5′UTR of the hrpG mRNA (221). In P. aeruginosa, RsmA represses translation of factors associated with chronic colonization involving a type VI secretion system (T6SS) and biofilm matrix exopolysaccharide (222, 223). A second RsmA homologue, RsmF, has also been identified in P. aeruginosa, which has in part overlapping but also unique regulatory roles (224). In Legionella, the RsmX and RsmY sRNAs are induced in stationary phase and relieve CsrA-mediated repression of Dot/Icm effectors (225). Deletion of both sRNAs attenuates growth in the protozoan host Acanthamoeba castellanii and macrophages (225, 226). Overall, CsrA is the most highly conserved posttranscriptional regulator of virulence (5, 211).

6S RNA
Another widespread sRNA that modulates protein activity and can also affect pathogen survival is the highly conserved and abundant housekeeping 6S RNA, which accumulates during stationary phase and controls activity of RNA polymerase (RNAP) (227). 6S RNA forms an extended hairpin with a single-stranded bulge, which mimics the DNA structure of an open transcription complex. Binding of 6S to the σ^{70}-containing holoenzyme reduces transcription from housekeeping promoters and reprograms RNAP (228). 6S RNA also acts as a template for transcription of short pRNAs (“product RNAs”) that might facilitate unwinding of 6S from RNAP and thus recycling of the polymerase during outgrowth of bacteria (229). The 6S homologue of Coxiella burnetii, an obligate intracellular pathogen, is induced in Vero cells, suggesting a role during infection (230). L. pneumophila 6S is also required for its replication inside cells (231). A novel RNAP-interacting sRNA has also been identified in Mycobacterium smegmatis (232). Like 6S RNA, Ms1 sRNA is most highly expressed in stationary phase. However, Ms1 interacts with core RNAP, rather than the housekeeping holoenzyme bound to σ^{70}. Whether homologues of Ms1, or other novel sRNA regulators of RNAP, exist in pathogens is not yet clear. Overall, this class of RNAP-interacting sRNAs has the potential to globally effect transcription during stationary phase and persistent infection.

**cis-Regulation by Endogenous mRNA Elements: Riboswitches, Thermosensors and 3′UTR Elements**

**Riboswitches**
Global TSS mapping in multiple species suggests that 5′UTRs of bacterial mRNAs are relatively short (20 to 40 nt). However, some transcripts have longer, structured leaders that can mediate their own regulation posttranscriptionally (Fig. 1D, upper panel). Metabolite-sensing “riboswitches” are structured 5′UTR elements that provide allosteric feedback control for many biosynthetic pathways (reviewed in 233, 234). Riboswitches often detect pathway intermediates and end products related to the protein encoded by the mRNA. As such, conserved riboswitch elements have been identified that detect metabolites such as thiamine pyrophosphate, Mg^{2+}, purines, amino acids, charged tRNAs, and phosphosugars. In Gram-positive bacteria, up to 2 to 4% of genes may be regulated by these structures (233, 235, 236). Riboswitches are generally composed of two independently structured domains that determine signal sensitivity (the “aptamer”) and regulatory output (the “expression platform”), respectively. Binding of a specific small-molecule ligand to the aptamer domain causes structural changes that affect the conformation of the adjacent expression platform. Conformational changes of the output domain can affect RBS accessibility,
exposure of RNase E cleavage sites, or transcription termination/antitermination of the associated mRNA. 

Salmonella senses numerous metabolic cues inside host cells. Magnesium levels, detected by the PhoPQ TCRS, represent an important signal for expression of virulence factors, including MgtC and the high-affinity Mg\textsuperscript{2+} transporter MgtA (237, 238). The promoter of mgtA is induced by PhoPQ under low Mg\textsuperscript{2+}. However, the leader of the mgtA transcript also contains a magnesium-sensing riboswitch element which maintains a Rho termination factor binding site under low Mg\textsuperscript{2+}, thereby promoting premature termination of transcription (239, 240). High magnesium also allows for translation of a short proline-rich leader peptide, MgtL (241, 242). Under proline limitation, ribosome stalling on the leader favors formation of a structure that promotes transcript elongation. Therefore, the mgtA leader integrates two disparate intracellular signals to affect elongation of the mgtA transcript. In addition to Mg\textsuperscript{2+}, riboswitches can also sense other metal ions such as Mn\textsuperscript{2+}, Co\textsuperscript{2+}, and Ni\textsuperscript{2+} (243–245). Also in Salmonella, a pH-responsive riboswitch has been reported upstream of the alc gene encoding a transporter (246). This element increases expression of the transporter in response to high pH through formation of a translationally active structure depending on transcriptional pausing.

Cyclic diguanylate (c-di-GMP) is a nucleotide second messenger that regulates biofilm formation and virulence in many bacteria (247). Protein receptors for c-di-GMP have been identified; however, bacteria have also evolved two riboswitch types to respond to this second messenger. Many genes that are part of c-di-GMP metabolism themselves contain such riboswitches (248), suggesting a role in feedback control or signal adaptation. Some are present in leaders of ORFs encoding factors known to be under control of c-di-GMP. For example, a riboswitch in the leader of a Clostridium difficile mRNA binds c-di-GMP to stimulate alternative RNA processing by an allosteric group I ribozyme (249). Alternative splicing produces an mRNA with an unmasked start codon and canonical ribosome-binding site, supporting translation of the putative virulence factor encoded by the mRNA. A second C. difficile c-di-GMP riboswitch has been identified upstream of pilA1 of the pilin locus which allows transcription elongation under high second messenger levels (250) and thus may influence cell aggregation. In V. cholerae, a c-di-GMP responsive GEMM (Genes for the Environment, Membranes and Motility) motif is found in the leader of gbpA (248). The gbpA gene encodes an N-acetylglucosamine-binding protein that mediates adhesion to GlcNAc oligosaccharides and is required for both environmental survival and colonization (251, 252). A second GEMM element is present upstream of VC1722, which is similar to the TfoX regulator of competence (248). Recently, a riboswitch specific for another emerging second messenger, cyclic diadenylate (c-di-AMP) has also been reported (253, 254).

Riboswitches can also either be a source for trans-acting sRNAs or regulate expression of sRNAs. The Salmonella sroA sRNA appears to arise from terminated transcription of the thiB riboswitch element (54). A mutant carrying a deletion in sroA shows reduced fitness during pooled mutant infections in mice (111). Two terminated SAM (S-adenosyl methionine) riboswitches of L. monocytogenes, SreA and SreB, bind the 5′ UTR of the mRNA encoding the PrfA virulence regulator and repress its expression, thereby linking metabolism and virulence (255). Riboswitches not only control mRNAs, but can also control expression of antisense RNAs in response to cellular conditions. For example, in Clostridium acetobutylicum, a sulfur metabolic operon is controlled by T-box and S-box riboswitch-controlled antisense RNAs (256). In Listeria, a vitamin B12 riboswitch is transcribed as part of a noncoding antisense RNA (257). The riboswitch controls the length of the asRNA depending on B12 levels by a transcription termination mechanism. In the absence of the cofactor, the asRNA is transcribed and can repress the cis-encoded pocR transcript, encoding an activator of the Pdu propanediol catabolism pathway, which requires B12. When ligand levels are sufficient, the riboswitch prevents expression of the asRNA and allows activation of the pathway. The ANTAR (AmiR and NasR transcriptional antiterminator regulator) domain family of response regulators is characterized by an output domain that binds target mRNAs to affect transcription antitermination. (258). A B12 riboswitch-controlled sRNA was recently found to sequester an ANTAR-family response regulator EutV in L. monocytogenes and Entero- coccius faecalis to control ethanol utilization genes (259, 260). This exemplifies the potential for high complexity through integration of multiple RNA-based regulatory mechanisms.

Thermosensors

Structured regions of 5′UTRs can also regulate gene expression in response to temperature changes (Fig. 1D, lower panel). These thermosensor modules, or “RNA thermometers,” generally fold in a temperature-dependent manner into alternative structures that occlude or expose the RBS to affect translation (261). Hallmarks of these
elements, such as the FourU or ROSE thermosensors, are structured regions that base-pair with and sequester the TIR at low temperatures (262). Elevated temperature unfolds this region and allows ribosome access. Thermosensor modules have, not surprisingly, been identified in many heat shock genes, playing a role in sensing and adaptation to heat stress. Temperature is also a key signal for bacterial pathogens, because changes in temperature are experienced upon transition between environment and host or during inflammation. RNA thermosensors have been identified in virulence genes of many bacterial pathogens, including master regulators of virulence gene expression (reviewed in 262).

L. monocytogenes relies on a master regulator, PrfA, for induction of virulence gene transcription. PrfA regulates virulence factors such as listeriolysin O, the InlA and InlB invasions, and the phospholipases PlcA and PlcB (1). Expression of these factors is almost undetectable at lower temperatures (i.e., 30°C). The 5′UTR of prfA contains a structured region that sequesters the RBS at low temperature but allows its translation at 37°C (263) and thereby activation of virulence genes once Listeria is within the host. N. meningitidis is primarily a commensal of the human nasopharynx but can cause serious diseases such as sepsis and meningitis upon entry into other body sites. Expression of the capsule, sialylated lipopolysaccharide, as well as recruitment of complement regulator factor H, contribute to complement resistance and immune evasion of N. meningitidis. In response to elevated temperature, i.e., upon inflammation in the nasopharynx, expression of immune evasion factors and complement resistance increases as a result of thermosensor-based control of three genes required for expression of these phenotypes (264). This also has implications for meningococcal success upon coinfection with inflammation-inducing pathogens such as influenza.

P. aeruginosa is ubiquitous in environmental niches such as soil and water. Many P. aeruginosa virulence phenotypes, such as rhamnolipid biosynthesis and type III secretion, are under control of RhlR, the regulator of the Rhl C4-HSL (butanoyl-homoserine lactone)—sensing quorum sensing system (265). Many RhlR-dependent phenotypes are more highly expressed at 37°C, and a thermosensitive ROSE-like element has been identified in the 5′UTR of rhlA, upstream of rhlR (266). The thermosensor is structured at lower temperatures, having a polar negative effect on transcription of downstream rhlB and rhlR. However, thermosensor melting at the permissive temperature (37°C) allows transcription of rhlR. A second thermosensor has also been identified in the 5′UTR of lasI mRNA (266). In this context, melting of the ROSE-like element at 37°C allows translation of LasI, which is responsible for production of 3O-C12-HSL (3-oxo-dodecanoyl-homoserine lactone). A global screen for RNA thermometers in P. aeruginosa revealed four additional thermosensor candidates, including one that regulates ptxS, which is involved in exotoxin A production (267).

Expression of many virulence genes on the Yersinia virulence plasmid, such as those encoding the T3SS and Yop effectors, is minimal outside the host but strongly induced at the 37°C host body temperature. Temperature-dependent expression of these genes is controlled by the transcriptional regulator LcrF. While transcription of lcrF is controlled by the thermo-labile transcription factor YmoA, a second, posttranscriptional, layer of temperature control of LcrF has been shown (268). A novel, structured element in the intergenic region of the yscW-lcrF polycistron sequesters the lcrF RBS at 25°C. Melting of the element at 37°C allows LcrF translation and induction of downstream virulence genes. Y. pseudotuberculosis strains with stabilized or destabilized versions of the thermosensor are attenuated for dissemination following oral infection in mice, suggesting that the lcrF thermosensor is critical for virulence factor expression.

3′UTR elements

While eukaryotic 3′UTRs are thought to actively participate in gene regulation, for example, by acting as microRNA sponges (269, 270), the role of bacterial 3′UTRs in posttranscriptional control is only now being revealed. In S. aureus, up to one third of mRNAs were found to have long 3′UTRs (greater than 100 nt) which could have regulatory function (271). The long 3′UTR of tcaR, encoding a repressor of the major exopolysaccharide, was revealed to use a C-rich sequence to interact with its own RBS, thereby interfering with translation initiation and providing a substrate for RNase III cleavage (271). Whether this interaction is inter- or intramolecular is not yet clear. Likewise, the Salmonella bilD mRNA, encoding an activator of pathogenicity island 1 (SPI-1), carries a 310-nt long 3′UTR which is important for turnover of its own mRNA (272). However, the underlying molecular mechanism remains unclear.

Dual-Function mRNAs with trans-Regulatory Functions and RNA Sponges

The function of mRNAs has traditionally been viewed as a template for protein synthesis. The identification of UTR elements with endogenous regulatory activity,
such as riboswitches and thermostectors, demonstrated that mRNAs themselves can have regulatory functions. An increasing number of coding transcripts have been reported in bacteria that also regulate other targets, i.e., in trans (Fig. 2C). First, coding or UTR regions can be sources of trans-acting sRNAs, such as the terminated transcripts resulting from riboswitch repression, as mentioned above (see “cis-Regulation by Endogenous mRNA Elements”). Also, bacterial 3′UTRs are a source of base-pairing sRNAs (see “Regulation by trans-Encoded sRNAs”), via processing from coding transcripts or via transcription from internal promoters (89). Salmonella SroC sRNA is derived from the 3′UTR of the gltI gene, which is part of the gltIJKL mRNA, a target of repression by the sRNA GcvB (140, 273). The mRNA-derived SroC sRNA acts as a sponge to sequester and trigger degradation of GcvB, thereby regulating the expression of its own parent mRNA (273).

Full-length messenger RNAs themselves can also regulate other genes by base-pairing or modulating activity of regulatory proteins. The 5′UTR of S. pyogenes irvA mRNA interacts with the gbpC transcript, protecting it from RNase J-mediated degradation (274). The role of mRNAs as sRNA sponges and/or decoys has also become apparent. In Salmonella, carbohydrate utilization pathways are hierarchically regulated. ChiP is a porin required for transport of chitin-derived oligosaccharides across the outer membrane, while the ChbBCA transporter mediates uptake of chitosugars across the inner membrane. Expression of ChiP is repressed by the ChiX sRNA, which base-pairs with the 5′-end of chiPQ mRNA and causes premature transcription termination (275–277). In this interaction, ChiX activity toward chiPQ is catalytic; i.e., ChiX is not codegradated with its target, which preserves the pool of the inhibitory sRNA. Induction of ChiP expression in the presence of chitosugars requires silencing of ChiX by a third player, the chbbC mRNA. The intercistronic region of chbbC acts as a so-called trap-mRNA that acts as a decoy via base-pairing with ChiX (276, 278). Because this interaction opens the ChiX terminator, which in turn increases its susceptibility to degradation, repression of chiPQ mRNA is relieved. This ensures that translation of the porin component occurs when transcription of the inner membrane transporter is induced.

Cross-talk by mRNA leaders is not limited to base-pairing interactions with other RNAs, and mRNA interactions can also affect activity of protein regulators such as CsrA (see “RNAs that Regulate or Antagonize Protein Activity,” above). For example, in Salmonella, in addition to the CsrB/C sRNAs mentioned above, a fimbrial mRNA (fimAICDHF) modulates CsrA activity to mediate hierarchical expression of chromosome-encoded (fim) or plasmid-encoded (pef) fimbriae (279). In C. jejuni, which lacks homologues of the CsrA antagonizing sRNAs, the mRNA of the major flagellin FlaA is not only translationally repressed by CsrA but also acts as an RNA antagonist of CsrA. The abundant flaA mRNA controls, together with the main CsrA antagonist the protein FliW, CsrA-mediated regulation of other flagellar genes (G. Dugar, S. Svensson, C. Sharma, unpublished). Finally, other cellular RNAs that were previously thought to be metabolic byproducts, which have sRNA sponge activity, have been identified using deep sequencing–based approaches. RNA-seq of potential RNA binding partners that copurified with aptamer-tagged sRNAs revealed that the 3′ external transcribed spacers (ETSs) of tRNA genes can also act as sRNA sponges to absorb transcriptional noise from repressed sRNAs (280) (Fig. 2C). Together, the increasing number of newly identified regulatory functions for bacterial mRNAs as well as RNAs not previously thought to have regulatory function demonstrates the complexity of bacterial posttranscriptional networks and shows that riboregulators are not restricted to non-coding transcripts.

RIBOREGULATORS IN QUORUM SENSING AND VIRULENCE REGULATORY NETWORKS OF BACTERIAL PATHOGENS

Quorum sensing allows pathogenic bacteria to sense cell density and coordinate group behaviors such as virulence factor expression or biofilm formation that would be of little benefit in solitary cells and are also important for survival and colonization (281). Quorum sensing involves the release of signaling molecules (autoinducers [AIs]) by producer cells and detection of the signal above a certain threshold by neighbors expressing the appropriate receptors. Signal detection elicits broad changes in gene expression and, subsequently, physiology. Quorum sensing networks are highly regulated at both the transcriptional and posttranscriptional levels. In this section we discuss examples of bacterial pathogens where riboregulation has been shown to factor centrally in quorum sensing and virulence.

Multiple Riboregulators Control V. cholerae Quorum Sensing and Virulence

The Gram-negative gammaproteobacterium V. cholerae causes epidemics of waterborne diarrheal illness that sweep the globe (282). Its pathogenesis and life cycle
are well studied, and it serves as a model for understanding quorum sensing and regulation of virulence gene expression during transmission or colonization. *V. cholerae* is primarily a marine bacterium and lives in brackish waters, either free-living or associated with biofilms. Following ingestion by a human host, expression of two primary virulence factors mediate colonization of the intestine and induce diarrheal disease (reviewed in [283]). The TCP (toxin-coregulated pilus), encoded by the tcpA-F genes of the phage-encoded *Vibrio* PAI (VPIΦ), mediates adhesion to the intestinal epithelium. The TCP also serves as a receptor for the CTXΦ prophage, which carries the ctxAB genes, encoding the cholera toxin (CT). CT is an AB-type toxin which stimulates cyclic-AMP production in host enterocytes, leading to a massive efflux of chloride and water into the intestinal lumen to cause diarrheal disease. In addition to these two virulence factors, competence, the T6SS, and biofilms are also central to *V. cholerae* fitness ([284–286]).

Virulence phenotypes of *V. cholerae* are regulated in response to numerous environmental signals. In the intestine, expression of TCP and CT is activated by the AraC-type transcriptional activator ToxT ([287]), considered a master regulator of virulence. ToxT activates transcription of numerous operons encoding factors required for colonization and disease (Fig. 3A). Expression of toxT itself is regulated at multiple levels, including direct induction of transcription from the toxT promoter by the cooperative action of the inner membrane-localized regulators TcpPH and ToxR ([288]) and transcriptional autoregulation. However, ToxT protein levels are also activated posttranscriptionally in response to temperature using an RNA thermometer ([289]). At environmental temperatures (20°C), a FourU element in the toxT mRNA leader, containing an anti-Shine-Dalgarno sequence, sequesters the TIR (Fig. 3A, left). At the host temperature of 37°C, destabilization of this inhibitory structure allows for translation of toxT and, in turn, expression of downstream virulence factors such as CT, Acf (accessory colonization factor), and TCP, as well as its own autoregulation (Fig. 3A). A strain carrying a more stable thermosensor variant, which remains folded at 37°C in vitro, was found to be defective for mouse colonization, providing evidence that “RNA-melting” in *vivo* is required for regulation of virulence factors.

sRNAs also contribute to *V. cholerae* virulence regulation. ToxT induces transcription of two trans-acting sRNAs expressed from the VPIΦ: TarA and TarB (ToxT activated RNAs) (Fig. 3A, right). TarA represses transcription of *ptsG*, encoding a glucose transporter, and deletion of tarA negatively affects colonization in an infant mouse model in the classical biotype of *V. cholerae* ([290]). TarA was also reidentified in a global sRNA-seq screen for virulence-regulating sRNAs induced by overexpression of ToxT, together with pull-down and sequencing of ToxT-bound promoters ([2]). The same study also identified a second ToxT-dependent sRNA, TarB. TarB interacts with tcpF of the tcpA-F transcript, encoding the secreted TcpF colonization factor. Loss of TarB has a mild effect on colonization, depending on infection conditions ([2]). Thus, TarB may be required to fine-tune or temporally control expression of TcpF. The RpoE-regulated VrrA sRNA posttranscriptionally regulates multiple genes, including those encoding the outer membrane protein OmpA and TcpA of the TCP. VrrA also represses the mRNA encoding the OmpT porin, which is important during infection ([291]), as well as the stationary phase survival factor Vrp ([292]). VrrA may negatively control virulence since a vrrA deletion mutant out-competes the wild type during infant mice colonization ([293]). Many *V. cholerae* virulence and survival factors, including those activated by ToxT, are under quorum sensing control. It is thought that *V. cholerae* enters the host intestine at low density, where it expresses virulence factors such as CT. At high cell density, virulence factors are repressed. The *Vibrio* quorum sensing network relies on a set of sRNAs that posttranscriptionally control expression of the reciprocally acting master transcription regulators of quorum sensing, AphA and HapR ([294, 295]) (Fig. 3B). AphA is the regulator of low-density gene expression (Fig. 3B, left) and activates the ToxT virulence regulon as well as genes required for biofilm formation by activation of the VpsT biofilm transcriptional activator ([296]). In contrast, high-density phenotypes are regulated by the master regulator HapR (Fig. 3B, right). HapR activates genes involved in dispersal (e.g., the *hapA* hemagglutinin/protease gene) and competence (com) and also reduces virulence gene expression and biofilm formation through repression of *aphA* and *vpsT*, respectively.

There is a reciprocal gradient of the two transcription factors, depending on bacterial cell density, which is largely mediated via riboregulation ([295]). *Vibrio* quorum sensing pathways converge on a set of four nearly homologous, Hfq-dependent base-pairing sRNAs called Qrrs (quorum-regulated RNAs) (reviewed in [27] (Fig. 3B)). *Vibrio* secretes and detects two AI signals to sense population density. The two AIs, CAI-1 (cholera autoinducer-1, produced by CqsA) and AI-2 (synthe-
sized by LuxS), are detected via the CqsS and LuxPQ TCRS proteins, respectively. Both CqsS and LuxPQ converge on the LuxU-LuxO phosphorelay system. At low density, when autoinducer concentrations are low and phosphotransfer to the LuxO response regulator is active, transcription of the Qrr sRNAs is activated by phosphorylated LuxO−P and the alternative sigma factor σ^54 (297) (Fig. 3B, left). In contrast, at high density and high extracellular AI concentrations (Fig. 3B, right), reverse phosphorelay is favored, and phosphorylated LuxO is not available to activate the qrr genes.

The “sibling” Qrr sRNAs (Vibrio species variably harbor one to five Qrrs) show apparent regulatory redundancy (297), a recurring theme in bacterial sRNA biology (298). They also have regulatory flexibility and interact with different target mRNAs, including both quorum sensing master regulator mRNAs (aphA and hapR mRNA), using diverse regulatory mechanisms with distinct outcomes (145, 299). The molecular details of Qrr-mediated posttranscriptional quorum sensing regulation have been studied in detail in V. cholerae as well as its cousin V. harveyi. The Qrrs each have four conserved stem-loops with different functions (300). The first mediates base-pairing with a subset of targets and protects the sRNAs from RNase E–mediated degradation. The second stem-loop contains conserved sequences required for base-pairing with many of the target mRNAs, while the third plays an accessory role in base-pairing and stability. The fourth and final stem-loop, like for many sRNAs, functions as a Rho-independent terminator. Each Qrr conserves a 32-nt core region that allows each to interact with many of the confirmed target mRNAs (27). However, some differences in Qrr sequence and mRNA targeting do exist. For example, Qrr1 of V. harveyi lacks nine nucleotides near its 5′-end, which precludes its interaction with some targets, such as aphA (300).

In V. cholerae, at low density, the Qrrs, aided by Hfq, interact with the aphA and hapR mRNAs with reciprocal consequences to determine phenotypic output (Fig. 3B, left). Interactions with aphA are activating, whereas base-pairing with the hapR transcript is destabilizing. At low AI concentrations, Qrr activation of aphA translation allows downstream activation of regulons controlled by AphA, such as the ToxT virulence regulon and the VpsT biofilm regulon. However, the Qrrs also activate other mRNAs. For example, they promote translation of a diguanylate cyclase domain protein (Vca0939), thereby raising c-di-GMP levels to, like VpsT activation, promote biofilm formation (301). In addition to activating low-density genes, the Qrrs also repress high-density gene expression by destabilizing the transcript of the high-density regulator HapR (Fig. 3B, left). Therefore, high-density HapR-dependent phenotypes, such as dispersal, competence, and the T6SS are not expressed. The Qrrs also directly repress the mRNA encoding the large T6SS gene cluster (VCA0107-0123) (302). In contrast, low-density genes that are repressed by HapR (but activated by AphA) such as those of the ToxT regulon and biofilm genes, are de-repressed in the absence of Qrr repression of HapR translation. This ensures that when few bacteria are present, AphA-dependent, low-density genes are expressed/de-repressed, whereas HapR-dependent high-density phenotypes are repressed.

At high cell density, accumulation of AI promotes de-phosphorylation of LuxO, and the LuxO−P-dependent Qrr sRNAs are not expressed. Absence of the Qrrs allows expression of high-density phenotypes (Fig. 3B, right panel), largely due to de-repression of hapR translation. HapR promotes expression of genes mediating dispersal (hapA) and competence (com), as well as encoding the T6SS, while also inhibiting expression of virulence factors by repressing aphA transcription. The tspT gene, activated by AphA, is also the target of HapR repression (303). HapR also controls transcription of 14 c-di-GMP-metabolizing genes, with the net effect of decreasing c-di-GMP levels, which further represses biofilm formation (303). The sRNA VqmR, which is activated by the transcriptional regulator VqmA, represses multiple mRNAs including those of the RTX toxin as well as of VpsT, thereby also inhibiting biofilm formation (73). Overall, absence of Qrr sRNA-mediated posttranscriptional repression and activation of multiple targets, including the master-regulator mRNAs, at high density suppresses virulence and biofilm formation and promotes phenotypes such as dispersal and competence.

Vibrio quorum sensing also incorporates feedback control of the sRNAs. The HapR protein, itself repressed by the Qrrs, has also been shown to activate their transcription (304). This feedback is most significant under high density, which allows efficient switching to low-density expression. The Qrr sRNAs can also repress the mRNA encoding their activator, LuxO (305), providing additional feedback. The V. cholerae CsrA system also feeds into quorum sensing upstream of LuxO (306). The Qrrs are some of the best-characterized bacterial sRNAs. Their functional characterization has been central to understanding not only V. cholerae quorum sensing and pathogenesis, but also to revealing mechanisms of Hfq-
Numerous riboregulators participate in quorum sensing and virulence regulation of *Vibrio cholerae*. (A) Riboregulation of the *V. cholerae* ToxT virulence regulon in response to temperature. The central transcriptional regulator ToxT (blue circles, center) activates virulence and colonization factor genes, such as tcp (toxin-coregulated pilus), ctxAB (cholera toxin), and acf (accessory colonization factor). ToxT also autoregulates its own transcription. Levels of ToxT are also modulated in response to temperature by a FourU RNA thermometer, with increased translation at the 37°C host temperature. ToxT also activates the sRNAs TarB, which represses translation of the tcpF ORF of tcpA-F mRNA, and TarA, which represses ptsG mRNA (glucose uptake). The VrrA sRNA also represses tcpA. (B) The Qrr sRNAs mediate the switch between *Vibrio* low and high cell-density physiologies via reciprocal posttranscriptional regulation of the master regulators AphA and HapR. *Vibrio* autoinducers (AI-2 and CAI-1) are made by LuxS and CqsA, respectively, and accumulate extracellularly. Phosphorelay systems headed by LuxPQ or CqsS (AI-2 and CAI-1, respectively) detect autoinducers. Left panel: Low bacterial density. Continued phosphorylation of LuxO at low autoinducer conditions leads to transcription of the Qrr sRNAs, which act along with the RNA chaperone Hfq to activate translation of aphA mRNA. In turn, AphA expression induces the ToxT virulence regulon (see panel A), as well as genes required for biofilm formation (*vpsT*). The Qrrs also repress the *hapR* mRNA, which encodes the high-density master regulator (see right panel). Right panel: High bacterial density. High autoinducer concentration reduces levels of phosphorylated LuxO and, thus, Qrr expression. The *hapR* mRNA is no longer destabilized, allowing translation of the HapR regulator. HapR activates genes that mediate biofilm dispersal and competence. In addition, genes activated by AphA at low density, such as *vpsT*, as well as *aphA* itself and its regulated genes, are repressed by HapR. Genes encoding the type VI secretion system are also induced. Finally, feedback regulation occurs via HapR activation of Qrr expression and Qrr repression of the *luxO* mRNA. The sRNA VqmR is activated by the transcriptional regulator VqmA and posttranscriptionally represses *vpsT* mRNA and *rtx* mRNA, encoding the RTX toxin, as well as six other mRNAs.

*Figure 3 continues on next page*
dependent sRNAs, the integration of posttranscriptional control with transcription factors, and the regulatory logic of bacterial gene regulation circuits.

**RNAIII: A Dual-Function sRNA Mediates Quorum Sensing Control of *S. aureus* Virulence**

*S. aureus* is a model for understanding sRNA-mediated posttranscriptional control in Gram-positive pathogens, including sRNA-mediated cell density control of virulence factor expression (31, 307). *S. aureus* is an opportunistic human pathogen that resides on the skin and the respiratory tract. Secretion of toxins, exoenzymes, superantigens, and capsule modulate and/or subvert the host immune system and underlie serious manifestations of *S. aureus* infection. Biofilm formation and antibiotic resistance also contribute to recalcitrant nosocomial infections.

*S. aureus* biofilm formation and virulence factor expression are controlled by products of the *agr* (accessory gene regulator) quorum sensing locus, which employs a cyclic octapeptide autoinducer to reciprocally control expression of surface-associated proteins and secreted virulence factors based on bacterial cell density (308). The *agr* locus is transcribed as two transcripts, RNAII and RNAIII (Fig. 4A). The RNAII transcript encodes the AgrB and AgrD enzymes required for synthesis of the Agr octapeptide pheromone. It accumulates upon high cell density to signal when a quorum of the pathogen—suitable for phenotypic switch—has been reached. RNAII also encodes the components of the AgrAC TCRS that detects and responds to the accumulating...
autoinducer and subsequently activates expression of high-density phenotypes. The primary effector of high-density phenotypes that is activated by the AgrA response regulator is the transcript RNAIII (Fig. 4B), the first sRNA shown to have a central role in bacterial virulence (45). Expression of RNAIII is induced by the AgrA response regulator in late exponential phase upon accumulation of autoinducers (309). RNAIII encodes δ-haemolysin (hld ORF) and is the major mediator of Agr regulation. Overall integration of RNAIII posttranscriptional activities promotes toxin expression and represses expression of secreted proteins. Center: General structure of RNAIII with the hld coding region (light blue) and C-rich loops (red). The RNAIII molecule directly activates the mRNA encoding α-haemolysin (hla). Also, together with the double-strand-specific RNase III, the sRNA directly represses numerous genes encoding surface-associated proteins (coa, spa, SA2353, SA1000). RNAIII also represses translation of Rot, a repressor of toxin gene expression.

**FIGURE 4** The dual-function sRNA RNAIII of *Staphylococcus aureus* reciprocally regulates expression of secreted virulence factors and surface proteins in response to cell density. (A) Genomic context and transcriptional regulation of the *S. aureus* agr quorum sensing locus, including the dual-function RNAIII. The RNAII mRNA (black) encodes proteins required for synthesis and detection of the peptide pheromone (agrBDCA, green and red open reading frames [ORFs]). Under high cell density and high autoinducer concentration, phosphorylated AgrA (red) activates transcription of the RNAIII sRNA (blue). RNAIII encodes δ-haemolysin (hld ORF) and is the major mediator of Agr regulation. (B) Overall integration of RNAIII posttranscriptional activities promotes toxin expression and represses expression of secreted proteins. Center: General structure of RNAIII with the hld coding region (light blue) and C-rich loops (red). The RNAIII molecule directly activates the mRNA encoding α-haemolysin (hla). Also, together with the double-strand-specific RNase III, the sRNA directly represses numerous genes encoding surface-associated proteins (coa, spa, SA2353, SA1000). RNAIII also represses translation of Rot, a repressor of toxin gene expression.

quorum sensing system (202). It directly represses surface protein A, as well as a fibrinogen-binding protein, via base-pairing with the spa mRNA and SA1000 mRNAs, respectively. In both cases, translation is inhibited and RNase III-mediated degradation of the message is promoted (202, 203). A similar mechanism also represses SA2353, encoding a secretory antigen, and coa, encoding coagulase (201). RNAIII also exerts positive regulatory effects both directly and indirectly. Indirect effects on toxin gene expression are a consequence of direct repression of the mRNA encoding the transcription factor Rot (repressor of toxins) by RNAIII. Rot represses numerous genes encoding toxins that contribute to tissue invasion, including α-haemolysin, staphylococcal protease, and lipase (311). Rot also activates spa expression. RNAIII represses translation of rot mRNA through base-pairing interactions that promote RNase III–mediated cleavage of the transcript (202, 314), resulting in downstream release of Rot repression.
of toxin expression. However, RNAIII can also directly activate toxin expression. Direct activation of bla occurs via base-pairing interactions that interfere with mRNA structures that inhibit translation (161). In summary, RNAIII is a dual-function transcript: it both acts as an mRNA, encoding the δ-haemolysin toxin and acts as an sRNA that posttranscriptionally regulates expression of surface proteins and additional secreted toxins in response to cell density cues. The S. aureus Agr system thus demonstrates not only the importance of bacterial communication in control of virulence factors, but also the importance of sRNAs as versatile posttranscriptional regulators, allowing the reciprocal and/or coordinate regulation of virulence genes.

Besides RNAIII, several other S. aureus sRNAs have been identified that affect virulence and antibiotic resistance. S. aureus Sbi is an immunoglobulin-binding protein that interferes with host complement and mediates immune evasion. Transient expression of sbi during infection is controlled by the cooperative activities of the sRNA SprD, expressed from the Staphylococcus PAI, and RNAIII (315, 316). RNAIII interacts directly with multiple sites on the sbi transcript, including its translation initiation region, to suppress translation (315). However, the SprD sRNA also interacts with the 5′-region of the sbi mRNA (316). Emergence of S. aureus strains resistant to last-line antibiotics is also central to prevalence of the species as a cause of untreatable infections. Resistance to vancomycin is affected by changes in expression of the SprX sRNA, which represses translation of SpoVG, a regulator known to affect glycopeptide resistance (135). The 3′-dependent RsaA sRNA promotes bacterial persistence through suppression of acute infections (317). RsaA represses translation of the global transcriptional regulator MgrA via two interactions with mgrA mRNA: a duplex with the RBS and a loop-loop interaction within the coding region. MgrR repression leads to increased biofilm formation and decreased capsule synthesis, which in turn increases opsonophagocytic killing of S. aureus by polymorphonuclear leukocytes. Mice infections showed that RsaA contributes to reduced severity of systemic infections and enhanced chronic infections.

The gene encoding the S. aureus SprA sRNA is present in varying copy numbers, depending on the strain, and is encoded on both the core genome and PAIs (18). One copy, SprA1, is encoded in the SaPln3-PAI of strain Newman. Messenger RNA targets of SprA1 are so far unknown. However, the sprA1 locus encodes a second sRNA, antisense to SprA1 (SprA1AS), and the locus was originally proposed to be a TA system, due to the presence of a short ORF in SprA1 (318). Intriguingly, the cis-encoded asRNA interacts with SprA1 with imperfect trans-sRNA-like interactions. These involve the 5′-end of SprA1AS and the RBS/start codon of SprA1, rather than the perfectly complementary 3′-ends. SprA1AS binding prevents translation of the SprA1 ORF, and possibly even more interesting for virulence, the protein encoded by SprA1 is cytotoxic to human cells.

OUTLOOK: RIBOREGULATORS AS CENTRAL PLAYERS IN VIRULENCE NETWORKS

sRNAs are now accepted as central global regulators of bacterial gene expression that facilitate stress adaptation or control virulence traits. Study of sRNA regulons has added to our understanding of how pathogens sense and adapt to different environments, has identified new virulence strategies, and has provided many previously missing links in the complex regulatory networks that control virulence and group-associated behaviors, such as quorum sensing. Identification of structured mRNA leaders with regulatory function, such as thermosensors, has expanded our understanding of how bacteria sense the host environment. Many master regulators of virulence gene transcription are also direct targets of posttranscriptional regulation by sRNAs or UTR elements, such as Listeria PrfA, V. cholerae ToxT, and S. aureus Rot, adding a layer of posttranscriptional control to that contributed by transcription factors. sRNAs have also provided insight into the evolution of bacterial regulatory networks, including regulatory cross-talk between horizontally acquired, virulence-associated genomic elements and the core genome (10).

Functional characterization of pathogen sRNAs has identified novel mechanisms of RNA-mediated regulation. This includes the ability of RNAs to play both regulator and target of regulation, such as mRNA sponges that regulate proteins or mRNAs, or the sRNAs that are subject to antisense regulation themselves (279, 319). RNA-seq has uncovered widespread antisense transcription that may also serve a broad regulatory role, and the functional role of these potential asRNAs remains a major area to be clarified. The “exclusion” concept also increases the potential for cross-talk between mRNAs in pathways of related and/or opposing function. One must also wonder if RNA molecules, like effector proteins from pathogens, may have roles outside the bacterial cell. Fungal pathogens have been revealed to secrete RNAs into host cells to modulate the host RNAi immunity system (320). In S. aureus, a conserved region of 23S rRNA is recognized by mouse
TLR13 and triggers an immune response (321). Moreover, L. monocytogenes was found to secrete nucleic acids, including RNAs, into host cells, and this triggers host innate immunity, including the RIG-I-dependent inflammasome (322). Whether this is an active process that benefits that pathogen is still unknown.

Study of RNA-binding proteins such as the RNA chaperone Hfq and RNases has also identified many new modes of postranscriptional gene regulation. More recently characterized RNA-protein complexes, such as components of the bacterial RNA-based CRISPR/Cas immune system, are revealing additional mechanisms of regulation. CRISPR/Cas systems provide an adaptive immunity by sequence-specific restriction of exogenous nucleic acids such as bacteriophage DNA (reviewed in 323). However, new “moonlighting” roles, outside of phage immunity, for CRISPR/Cas have been identified in bacterial pathogens. For example, they have shown also to mediate endogenous gene regulation or have even been implicated in pathogenicity (324, 325), and there is an inverse correlation between antibiotic resistance genes on mobile DNA elements with the presence of intact CRISPR loci in enterococci (326). S. pneumoniae CRISPR/Cas, when primed with specific sequences, can prevent acquisition of genes encoding virulence-associated traits, such as capsule, by horizontal gene transfer (327). Together, these observations suggest that the presence or absence of CRISPR/Cas could contribute significantly to the fitness of bacterial populations. CRISPR/Cas can also play a direct role in gene regulation. Francisella CRISPR/Cas represses an mRNA encoding a lipoprotein, which is a stimulator of innate immune receptors, and the Cas9 nuclease and RNA components are required for evasion of TLR2 signaling and inflammasome evasion, as well as polymyxin resistance (328, 329). The Cas2 nuclease of L. pneumophila, which shows significant RNase activity, is also required for replication in amoeba (330, 331). A novel CRISPR locus of L. monocytogenes, which interestingly lacks associated cas genes, expresses an sRNA RliB and has been associated with virulence (53). Certainly, additional roles in gene regulation in bacterial pathogens will be uncovered as the strong interest in CRISPR/Cas continues.

A dramatic increase in our understanding of RNA biology due to technical advances provided by deep sequencing has revealed a central role for postranscriptional regulation and sRNAs in bacterial virulence. New approaches based on deep sequencing promise to provide even more insight into postranscriptional control by RNA, as well as the regulatory events (in both pathogen and host) that underlie infection and immunity. Because posttranscriptional regulation is sometimes most apparent at the level of translation initiation or the proteome, new approaches based on RNA-seq to monitor “translatomes,” such as ribosome profiling (“Ribo-seq,” deep-sequencing of ribosome-protected mRNA fragments), hold much promise for the identification of sRNA targets, which are sometimes only regulated on the translational level (332). Ribo-seq has already demonstrated repression of the E. coli major outer lipoprotein Lpp by a σE-dependent 3′UTR-derived sRNA, MicL (333). Also, Dual RNA-seq (simultaneous sequencing of both bacterial and host transcriptomes during infection, either in batch or at the single-cell level) (334–336, 344) promises to be especially relevant for identifying riboregulators and transcriptome changes, of both pathogen and host, that are important during infection. This has the potential to identify novel RNA biomarkers, such as microRNAs or long-noncoding RNAs, that indicate infection or predict disease susceptibility/outcomes and can be readily detected with high sensitivity in biological fluids (337). Finally, as the catalogue of functionally characterized sRNA-mRNA target pairs has increased, comparative genomics studies of bacterial pathogens have shed light on the evolution of bacterial regulatory circuits and how horizontally acquired sequences can regulate genes of the core genome (21, 22). Bioinformatics tools arising from these catalogues are also aiding the process of functional characterization (338).

In addition to classical virulence factors, stress response pathways and basic physiology are central to the virulence and survival of bacterial pathogens. Many of these pathways are regulated by RNA and can also impact antibiotic resistance. RNAs are a family of bacterial molecules relatively unexploited by antimicrobial design, and those that control essential processes may serve as candidate drug targets. Many RNA modules, such as SAM and B12 riboswitches, are widely conserved in bacteria but absent in humans, interact with material molecules relatively unexploited by antimicrobial design. The central role of c-di-GMP sensors in biofilm formation, a significant contributor to antibiotic resistance in the clinic, also makes them promising targets. Recently, a phenotypic screen revealed a small molecule as a synthetic mimic of the flavin mononucleotide natural ligand of the riboflavin riboswitch (339). This structurally distinct mimic (ribocil) inhibits bacterial cell growth via repressing riboswitch-regulated biosynthesis of the essential cofactor riboflavin. Therefore, RNA regulators or RNA-controlled pathways are attractive targets for novel antimicrobials.
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RNase III is to silence foreign toxin genes.

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