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Review

Regulatory RNA in Bacterial Pathogens

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Bacteria constitute a large and diverse class of infectious agents, causing devastating diseases in humans, animals, and plants. Our understanding of gene expression control, which forms the basis for successful prevention and treatment strategies, has until recently neglected the many roles that regulatory RNAs might have in bacteria. In recent years, several such regulators have been found to facilitate host-microbe interactions and act as key switches between saprophytic and pathogenic lifestyles. This review covers the versatile regulatory RNA mechanisms employed by bacterial pathogens and highlights the dynamic interplay between riboregulation and virulence factor expression.

Introduction

Bacteria are highly adaptive organisms that inhabit a broad range of ecological niches and face a plethora of environmental conditions. In addition to saprophytic and commensal species, this domain of life harbors a wealth of pathogens that colonize eukaryotes and successfully cope with the immune defense mechanisms of their hosts. A pathogenic lifestyle requires tight control of virulence gene expression and of the general stress responses. Traditionally, these regulations have been accredited to the activity of transcription factors that switch on or off relevant sets of genes in response to environmental cues. In contrast, roles of noncoding RNA regulators in pathogenesis have only begun to be addressed.

The late appreciation of regulatory RNA might be attributed to the fact that loci encoding such regulators were rarely selected in genetic screens for virulence factors, likely owing to a usually smaller gene size, missing annotations in genome sequences, and typically subtle phenotypes, as compared to virulence-associated proteins. For example, the paradigm of a regulatory RNA linked to bacterial pathogenesis, the ~514 nt RNAIII was originally described as the δ-hemolysin mRNA of *Staphylococcus aureus*. Only subsequent molecular analysis revealed that—in addition to expressing hemolysin from its 5′ region—RNAIII acts as an antisense regulator of virulence and surface protein synthesis through its 3′ region (Chevalier et al., 2010; Novick et al., 1993).

Recent developments have heightened the interest in potential links between regulatory RNA and bacterial pathogenesis. First, biocomputational predictions in a staggering number of available microbial genomes and experimental screens using new technologies such as tiling arrays and high-throughput sequencing of RNA (RNA-seq) have discovered unexpected numbers of small noncoding RNA (sRNA) loci (Livny and Waldor, 2007; Sharma and Vogel, 2009). Subsequent functional analyses of these new sRNAs identified many of them as integral parts of the bacterial stress responses with well-established roles in bacterial survival within the host. Second, the ubiquitous RNA-binding proteins, Hfq and CsrA, have increasingly been implicated in bacterial virulence (Chao and Vogel, 2010; Lucchetti-Miganeh et al., 2008). Importantly, the activity of either protein is intimately linked to sRNAs. Third, the recent discovery of riboswitches (Roth and Breaker, 2009) and a better understanding of RNA-based thermosensors (Klinkert and Narberhaus, 2009) in bacterial 5′ untranslated regions (UTRs) has increased the appreciation of posttranscriptional control of gene expression. All of the above types of posttranscriptional mechanisms (i.e., sRNA, riboswitch, and thermosensor) are now known to operate in the control of a single virulence factor, the major transcription factor PrfA of *Listeria monocytogenes* (Loh et al., 2009).

The present review aims to highlight where and how regulatory RNAs participate in gene expression control in bacterial pathogens, with emphasis on the underlying molecular mechanisms. There are also three ubiquitous small housekeeping RNAs (RNase P RNA, 4.5S RNA, tmRNA) which have been studied in a number of pathogens—especially tmRNA (Table 1)—or used for gene targeting of virulence factors (McKinney et al., 2004). However, owing to their intrinsic global functions, specific roles of housekeeping RNAs in pathogenesis are difficult to extract and will not be covered here.

Mechanisms of RNA-Based Gene Regulation

Regulatory RNAs operate at all layers of gene regulation, ranging from transcriptional initiation to protein activity (Waters and Storz, 2009). The following section introduces general classes of bacterial riboregulators along with examples of where these act in pathogenesis.

Cis-Encoded Expression Control: Thermometers and Riboswitches

The structure of the 5′UTR of an mRNA determines its rate of protein translation. This is particularly relevant for RNA structures that involve the ribosome-binding site (RBS) containing the Shine-Dalgarno (SD) sequence and start codon (AUG), and for hairpin structures that function as transcriptional attenuators.

For many bacteria, host body temperature is a major external signal that triggers virulence or stress-related gene expression. Temperature sensing can occur by almost all mechanisms of prokaryotic gene regulation, including altered promoter recognition by changes in DNA topology, or modulation of transcription factor activity via protein conformation changes (Klinkert and Narberhaus, 2009). Importantly, temperature can also influence the folding of structured regions in mRNAs and thereby impact

on translation. These so-called "RNA thermometers" typically reside in the 5′UTR of temperature-responsive genes, and switch between two distinct structures: a "closed" conformation formed at low temperature, in which the SD and/or AUG are inaccessible to 30S ribosomes; and the "open" confirmation formed at high temperature upon melting of the inhibitory structure around the RBS. Note that a converse mechanism, i.e., translational activation by RNA structure melting in the cold, regulates a major cold shock protein of *E. coli* (Giuliodori et al., 2010).

Most RNA thermometers appear to fall into distinct structural groups, of which ROSE (repression of heat shock gene expression) and fourU elements (four consecutive uridines pair with the SD) seem most common. ROSE elements are often associated with small heat shock proteins such as *E. coli* IbpA; fourU elements are associated with both heat shock and virulence factors, including *Salmonella* Typhimurium AgsA (Klinkert and Narberhaus, 2009). Structural studies of ROSE and fourU elements revealed thermosensing by RNA as a highly dynamic process in a narrow temperature range around 37°C that should be well suited to control virulence factor synthesis in pathogens of warm-blooded animals (Chowdhury et al., 2006; Rinnenthal et al., 2010).

The first RNA thermometer to be postulated in bacterial pathogens resides in the 5′UTR of *lcrF* mRNA encoding a transcriptional activator of *Yersinia pestis* virulence genes (Hoe and Goguen, 1993); its contribution to *Yersinia* pathogenesis is still to be proven. Perhaps the most prominent example to date is *L. monocytogenes* where a 127 nt hairpin in the 5′UTR of *prfA* inhibits translation below 37°C (Figure 1A; Johansson et al., 2002). PrfA is the transcription factor instrumental for the switch of the bacterium from saprophytism to virulence and activates genes required for bacterial invasion, host cytosolic propagation, and transmission to adjacent cells (Freitag et al., 2009). Interestingly, an increase in temperature to 37°C alone is insufficient to induce PrfA-dependent genes, suggesting that thermocontrol acts in parallel to other PrfA-activating mechanisms (Scortti et al., 2007).

More "palpable" cues in addition to temperature are sensed by riboswitches in bacterial 5′UTRs, and these include a plethora of chemically diverse metabolites (Roth and Breaker, 2009), pH, and metal ions (Dambach and Winkler, 2009; Nechooshtan et al., 2009). Structurally, riboswitches are organized in two domains: the "aptamer region" binding the ligand, and the "expression platform" capable of forming two mutually exclusive RNA structures depending on whether or not a ligand is bound. In most cases, ligand-binding locks the RNA in the OFF status, either by promoting the formation of a transcriptional terminator or by RBS sequestration as in the RNA thermometers.

Riboswitches are now well-established to control many metabolic genes. In comparison, there has been little work with relevance to host-microbe interaction. However, a recently identified class of riboswitches senses the emerging second messenger, cyclic diguanosine monophosphate (c-di-GMP), which has been increasingly implicated in cell differentiation, biofilm formation, and virulence (Sudarsan et al., 2008). One member of this new class is associated with GpbA, a protein attaching *Vibrio cholerae* to human epithelial cells and zooplankton (Kirn et al., 2005), suggesting a role of RNA-based sensing of c-di-GMP in cholera pathogenesis (Sudarsan et al., 2008).

Cis-Antisense RNAs

Unlike the 5′UTR-contained environmental sensors above, the majority of riboregulators that modulate the expression of target mRNAs by base pairing mechanisms are transcribed either as *cis*-encoded antisense RNA from the opposite strand, or as *trans*-encoded sRNAs from physically unlinked loci. *Trans*-antisense RNAs, to be discussed in the next section, generally act by short and imperfect target pairing and often require RNA chaperones such as the Hfq protein, whereas the genomic origin of *cis*-antisense RNAs usually entails extensive sequence complementarity with the oppositely transcribed target, albeit not necessarily the formation of long RNA duplexes (Wagner et al., 2002).

Historically, *cis*-antisense RNAs were long restricted to copy-number regulation in mobile elements such as phages, transposons, and transmittable plasmids (Wagner et al., 2002). Plasmid-based antisense regulation affects the virulence of the fish pathogen *Vibrio anguillarum*, by targeting siderophore biosynthesis and iron uptake functions. Here the ~430 nt RNAβ antisense transcript of plasmid pJM1 differentially regulates the *fatDCBA-angRT* operon by promoting the formation of an alternative transcriptional terminator downstream of *fatA* (Stork et al., 2007). By a similar mechanism, the recently discovered RnaG transcript of *Shigella flexneri* represses the plasmid-borne *icsA* mRNA encoding an outer membrane protein (OMP) that is required for host cell invasion and intercellular spreading of *Shigella* (Giangrossi et al., 2010).

Biocomputational predictions and tiling array-based gene expression studies have now identified a wealth of candidate short and long *cis*-antisense RNAs in bacterial chromosomes as well, including repressors of toxin synthesis in the widespread type I toxin-antitoxin loci (Fozo et al., 2010; Sorek and Cossart, 2009). Likewise, differential RNA-sequencing (dRNA-seq) of the gastric pathogen, *Helicobacter pylori*, revealed massive antisense transcription opposing surface structure synthesis and acid stress genes, hinting at roles of *cis*-antisense RNAs in colonization of the stomach (Sharma et al., 2010).

There are several *cis*-antisense RNAs in *Mycobacterium tuberculosis* (Arnvig and Young, 2009) and *S.* Typhimurium (Padalon-Brauch et al., 2008) whose expression negatively correlates with convergent virulence genes, for example, the ~290 nt IsrC of *S.* Typhimurium, which is antisense to the 3′ end of the virulence-related *msgA* gene (Padalon-Brauch et al., 2008). Of longer species, the ~1200 nt AmgR antisense RNA of *S.* Typhimurium is fully complementary to *mgtC*, a gene required for Mg^{2+} homeostasis and virulence. AmgR promotes specific degradation of *mgtC* in the polycistronic *mgtCBR* mRNA. Intriguingly, both *mgtC* and *amgR* are positively controlled by the same transcription factor, PhoP, suggesting that AmgR might function as a timing device to alter MgtC and MgtB levels after the onset of PhoP-inducing conditions (Figure 1B). Importantly, AmgR is a rare example of a riboregulator whose importance was successfully validated in animal infection, demonstrating that it prevents bacterial hypervirulence in mice (Lee and Groisman, 2010).

Hfq-Dependent sRNAs

Hfq-associated sRNAs are likely to constitute the largest group of posttranscriptional regulators known to date, and model enterobacteria such as *E. coli* or *S.* Typhimurium might

Table 1. Selected Regulatory RNAs with Functions Relevant to Bacterial Pathogenesis

Species	sRNA	Size	Transcription Factor	Target Genes	Phenotype/Function	Reference
Trans-Regulatory sRNAs						
C. trachomatis	**IhtA**	120	n.d.	*hctA*	Chromatin condensation.	(Grieshaber et al., 2006)
H. pylori	**HPnc5490**	90	n.d.	*tlpB*	Antisense repressor of chemotaxis receptor mRNA.	(Sharma et al., 2010)
L. monocytogenes	**RliB**	360	SigB	*lmo2104*	*rliB* mutation increases colonization of spleen in mice.	(Toledo-Arana et al., 2009)
L. monocytogenes	**Rli38**	369	n.d.	?	*rli38* mutant is attenuated in oral mouse infection.	(Toledo-Arana et al., 2009)
S. aureus	**RNAIII**	514	ArgA	*rot*, *spa*, *hla*, *coa*, SA1000, SA2353	Global regulator of quorum-sensing and virulence gene expression.	(Chevalier et al., 2010; Morfeldt et al., 1995; Novick et al., 1993)
S. aureus	**SprD**	142	n.d.	*sbi*	Represses immune evasion factor, Sbi. Virulence phenotype in mice.	(Chabelskaya et al., 2010)
S. pneumoniae	**csRNA1-5**	87-151	CiaR	?	csRNA 4 and 5 regulate stationary phase autolysis.	(Halfmann et al., 2007)
S. pyogenes	**FasX**	250	FasA	*fpbA*, *mrp*, *ska, pel*	Increases interaction *of S. pyogenes* with epithelial cells.	(Klenk et al., 2005)
S. pyogenes	**RivX**	180/220	CovR	*mga*	Regulates expression of virulence transcription factors.	(Roberts and Scott, 2007)
S. pyogenes	**Pel**	459	n.d.	*emm*, *sic*, *speB*	Bi-functional RNA that also encodes the SagA protein.	(Mangold et al., 2004)
S. Typhimurium *(E. coli)*	**MgrR**	98	PhoP	*eptB*	Modulator of LPS modification.	(Moon and Gottesman, 2009)
S. Typhimurium	**InvR**	~80	HilD	*ompD*	Invasion gene island (SPI-1)-encoded sRNA targeting porin synthesis.	(Pfeiffer et al., 2007)
S. Typhimurium	**SgrS**	239	SgrR	*ptsG*, *sopD*	Repressor of sugar uptake that also regulates secreted virulence factor.	(Wadler and Vanderpool, 2009; K.P., D. Podkaminski, S. Lucchini, J.C.D. Hinton, and J.V., unpublished data)
S. Typhimurium	**IsrJ**	74	n.d.	?	Repressor of virulence factor translocation.	(Padalon-Brauch et al., 2008)
V. cholerae	**Qrr1-4**	96-108	LuxO, σ^{54}	*hapR*, *vca0939*	Quorum-sensing control and de-repression of virulence genes.	(Hammer and Bassler, 2007; Lenz et al., 2004)
V. cholerae	**VrrA**	140	RpoE (σ^{E})	*ompA*	Outer membrane vesicle synthesis. Colonization of mouse intestine.	(Song et al., 2008)
Cis-Acting sRNAs						
M. tuberculosis	**AsDes**	75/110	n.d.	*desA1*	Induced upon bacterial uptake.	(Arnvig and Young, 2009)
S. flexneri	**RnaG**	450	n.d.	*icsA*	Transcriptional attenuator of *icsA*.	(Giangrossi et al., 2010)
S. Typhimurium	**AmgR**	1200	PhoP	*mgtBC*	Impacts on magnesium homeostasis and virulence in mice.	(Lee and Groisman, 2010)

Table 1. *Continued*

Species	sRNA	Size	Transcription Factor	Target Genes	Phenotype/Function	Reference
S. Typhimurium	**IsrC**	288	n.d.	*msgA*	Antisense regulator of *msgA* virulence gene.	(Padalon-Brauch et al., 2008)
V. anguillarum	**RNAβ**	427	n.d.	*fatDCBA-angRT*	Control of siderophore biosynthesis.	(Stork et al., 2007)
Protein-Binding sRNAs						
E. carotovora	**RsmB**	479	GacA	RsmA	Exopolysaccharide production.	(Cui et al., 2008)
L. pneumophila	**6S**	147/ 182	n.d.	RNAP	Required for replication in macrophage and amoeba. Regulates type IV secretion.	(Faucher et al., 2010)
L. pneumophila	**RsmY,Z**	110/ 132	LetA	RsmA	RsmY and RsmZ additively affect replication in macrophages via RsmA.	(Rasis and Segal, 2009; Sahr et al., 2009)
P. aeruginosa	**CrcZ**	~400	CbrA	Crc	Regulator of catabolite repression.	(Sonnleitner et al., 2009)
P. aeruginosa	**RsmY,Z**	240/ 120	GacA	RsmA	Impact on type VI secretion.	(Brencic et al., 2009)
S. Typhimurium	**CsrB,C**	363/ 244	SirA	CsrA	Redundant regulators of CsrA and replication in macrophages.	(Fortune et al., 2006)
V. cholera	**CsrB,C,D**	~300-400	VarA	CsrA	Quorum sensing control via CsrA.	(Lenz et al., 2005)
Y. pseudotuberculosis	**CsrB,C**	~320-350	UvrY	CsrA	Control of virulence genes via RovM. Inverse expression of CsrB and CsrC.	(Heroven et al., 2008)
Housekeeping RNAs						
H. pylori	**tmRNA**	~390	n.d.	damaged mRNA	Involved in competence, response to antimicrobial compounds.	(Thibonnier et al., 2008)
S. pyogenes	**tmRNA**	~300	n.d.	damaged mRNA	Increased expression upon contact with antibiotics.	(Steiner and Malke, 2001)
S. Typhimurium	**tmRNA**	~360	n.d.	damaged mRNA	Mutant attenuated in murine and macrophage infection models.	(Ansong et al., 2009; Julio et al., 2000)
Y. pseudotuberculosis	**tmRNA**	~363	n.d.	damaged mRNA	Required for effector secretion and motility.	(Okan et al., 2006)

Only the most relevant or recent references are listed.

express >100 of them. Hfq-dependent sRNAs are typically between ~50 and 250 nt in length, structurally diverse, and transcribed from free-standing chromosomal genes. They commonly recognize the 5′ region of mRNAs via short and imperfect RNA interactions (10–25 base pairs) to negatively regulate translation or stability (Waters and Storz, 2009). In addition, sRNA-mediated translational activation by preventing inhibitory structure around the RBS of a target is well-established, and the same sRNA can act to both repress and activate targets (Fröhlich and Vogel, 2009). Regulation of multiple targets by a single sRNA is common; in fact, deep sequencing analysis of Hfq-associated mRNA in *Salmonella* predicts about 7-fold more potential targets than sRNAs (Sittka et al., 2008).

To date, *Salmonella* is the pathogen with the largest network of reported Hfq-dependent regulations, most of which involve porins, transcription factors, and stress-responsive genes (Vogel, 2009). However, mutation of the *hfq* gene impairs virulence in a variety of other bacterial pathogens (Chao and Vogel, 2010), and although regulation by Hfq-dependent sRNAs was long restricted to Gram-negative bacteria, it was recently discovered in Gram-positive *Listeria* as well (Nielsen et al., 2009). Phenotypic alterations in *hfq* mutants range from loss of effector secretion in *Salmonella* and *Yersinia pseudotuberculosis* (Schiano et al., 2010; Sittka et al., 2007) to effector overproduction in pathogenic *E. coli*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, and *Vibrio* species (Hansen and Kaper, 2009; Nakano et al., 2008;

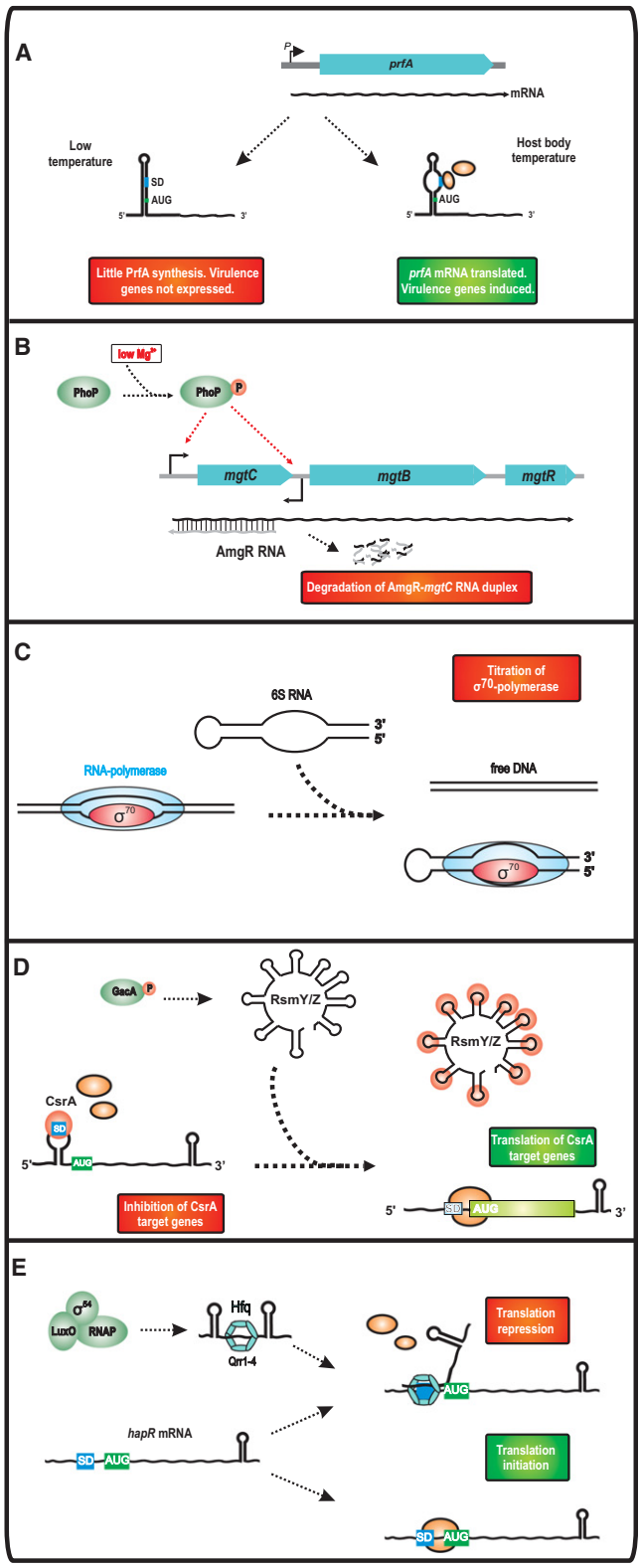

Figure 1. Overview of Mechanisms Employed by Bacterial Riboregulators

(A) The mRNA of the PrfA virulence transcription factor of *L. monocytogenes* is posttranscriptionally controlled by an RNA thermometer in the 5′UTR. This regulatory structure permits translation initiation at the high temperature of the environment of a mammalian host but inhibits ribosome binding at low temperature outside a host.

(B) AmgR is a *cis*-encoded regulatory RNA that is transcribed convergent to the *mgtC* ORF in S. Typhimurium. Expression of AmgR and *mgtC* is controlled by the PhoPQ two-component system, while interaction of both RNAs results in degradation of the RNA duplex.

(C) 6S RNA is a ubiquitous riboregulator that targets the σ^{70} version of RNAP. 6S is active in stationary phase cells to repress transcription from σ^{70}-dependent promoters favoring usage of promoters that are recognized by the alternative σ^{S} factor.

(D) The RNA-binding protein, CsrA, modulates mRNA expression by interfering with translational initiation. Activity of CsrA is counteracted by CsrB-like RNAs that carry multiple CsrA-binding sites to sequester the protein.

(E) The *trans*-encoded Qrr sRNAs of *V. cholerae* inhibit translation of the *hapR* mRNA by sequestration of the ribosome-binding site. This mechanism, as observed for most *trans*-antisense sRNAs, often requires the RNA-chaperone, Hfq.

Nakao et al., 1995; Shakhnovich et al., 2009; Sonnleitner et al., 2003). These phenotypes raise the possibility that Hfq-dependent sRNAs directly regulate bacterial virulence factors. In line with this prediction, the conserved SgrS sRNA has been shown to control the fate of a secreted *Salmonella* effector protein (K.P., D. Podkaminski, S. Lucchini, J.C.D. Hinton, and J.V., unpublished data).

Other Trans-*Antisense RNAs*

Antisense regulation of multiple targets does not necessarily require Hfq. The Gram-positive pathogens, *S. aureus* and *L. monocytogenes*, both express sRNAs with more than one *trans*-encoded target mRNA (Bohn et al., 2010; Chabelskaya et al., 2010; Geissmann et al., 2009; Mandin et al., 2007) despite limited evidence of a prominent role for Hfq in these organisms (Chao and Vogel, 2010). The *S. aureus* sRNAs tend to recognize target mRNAs at the RBS via C-rich loops, although other types of base pairing were also reported (Bohn et al., 2010; Chabelskaya et al., 2010; Geissmann et al., 2009).

H. pylori and other important ε-proteobacterial pathogens such as *Campylobacter jejuni* clearly lack Hfq (Valentin-Hansen et al., 2004). Nonetheless, these species were reported to encode dozens of sRNAs, of which HPnc5490 (~90 nt) targets the upstream 5′UTR of a *H. pylori* chemotaxis receptor mRNA through a 13 bp GC-rich RNA duplex (Sharma et al., 2010). Whether the newly discovered *H. pylori* sRNAs also act on multiple targets remains to be investigated.

RNA Regulators of Protein Activity

Small RNAs that modulate the activity of proteins are far outnumbered by antisense regulators, yet are no less global players in terms of the numbers of genes they regulate. The ubiquitous 6S RNA (encoded by *ssrS* in *E. coli*) targets the bacterium's transcription machinery; it specifically binds to RNA polymerase (RNAP) to increase association with alternative stress sigma factors such as the stationary phase factor, σ^{S}, at the expense of the vegetative σ^{70} whose control dominates transcription in fast-gowing cells (Figure 1C; Wassarman, 2007; Wassarman and Storz, 2000). Homologs of *E. coli* 6S RNA have been identified in almost all eubacteria, when analyzed by secondary structure (a long RNA duplex with a central asymmetric bulge resembling an open promoter complex of DNA) rather than primary sequence conservation (Barrick et al., 2005). Lack of 6S RNA reduces survival in long-term cultivation experiments (Wassarman, 2007) and alters the expression of ~5% of all genes in *E. coli* K12 (Neusser et al., 2010). Concerning pathogens,

Legionella pneumophilia requires 6S RNA for expression of type IV secretion effectors and replication in human macrophages or amoeba (Faucher et al., 2010). The regulatory activity of 6S RNA also seems conserved in *H. pylori* (Sharma et al., 2010), as judged by detection of the tiny 12–14 nt "product RNAs" that are a hallmark of 6S RNA interaction with RNAP (Wassarman, 2007).

The CsrB family of sRNAs indirectly modulates mRNA translation by antagonizing CsrA (a.k.a. RsmA in *Pseudomonas* species), a ubiquitous bacterial RNA-binding protein encoded by ~75% of all species, including multiple homologs in some pathogens such as *L. pneumophila* and *Coxiella burnetti* (T. Romeo, personal communication; Lucchetti-Miganeh et al., 2008). CsrA proteins target mRNAs at GGA-rich elements to inhibit ribosome binding, which often entails mRNA decay. Positive regulation by CsrA is also known but little understood (Babitzke and Romeo, 2007; Brencic and Lory, 2009). The CsrB-like sRNAs, which can be several hundred nucleotides in length, antagonize CsrA by presenting multiple high-affinity sites containing the GGA motif, thus functioning as a sink for CsrA (Figure 1D). Bacteria often employ multiple sRNAs to regulate CsrA; for example, there are three CsrB-like sRNAs in *V. cholerae* or *Pseudomonas syringae* (Kay et al., 2005; Lenz et al., 2005). The redundancy often requires the deletion of all CsrB species in a given organism to observe clear phenotypes (Fortune et al., 2006; Rasis and Segal, 2009; Sahr et al., 2009), offering an explanation for why sRNA genes are poorly captured in virulence screens.

Originally discovered as a regulator of glycogen biosynthesis in *E. coli* (Babitzke and Romeo, 2007), CsrA/B is now considered the most universal posttranscriptional control system with relevance to virulence. Deletion of *csrA*/*rsmA* usually results in strong virulence phenotypes, for example, altered invasion of human airway epithelial cells by *P. aeruginosa*, an opportunistic human pathogen in which *csrA*/*rsmA* regulates ~10% of all genes (Burrowes et al., 2006; Pessi et al., 2001). Intriguingly, there is potential crosstalk between RsmA and Hfq activity such that Hfq stabilizes one of the RsmA-antagonizing sRNA (Sonnleitner et al., 2006); the significance of this interaction for Hfq or RsmA mediated virulence is yet to be addressed.

Targets of RNA-Based Gene Regulation in Pathogens

For a better perspective of the cellular pathways that might be controlled by regulatory RNAs in bacterial pathogens, it is worthwhile to look at the potential targets of Hfq and CsrA proteins. Transcriptomics studies revealed that Hfq impacts on the expression of at least 20% of all genes in *S.* Typhimurium (Ansong et al., 2009; Sittka et al., 2008), while deep sequencing of coimmunoprecipitated RNAs suggested that Hfq directly associates with >700 cellular mRNAs (Sittka et al., 2008). Collectively, these candidate targets of Hfq and its associated sRNAs belong to 26 functional groups, and include almost all virulence loci of *S.* Typhimurium (Ansong et al., 2009; Sittka et al., 2008). Sequence analysis of RsmA-bound RNA in *P. aeruginosa* revealed many virulence factor mRNAs as potential targets, including a type VI secretion system relevant for chronic infection (Brencic and Lory, 2009). The combination of these results suggests that we have so far seen only the "tip of the iceberg" of posttranscriptional control in bacteria, and that regulatory RNAs target almost all cellular processes in these bacteria.

Control of Transcription Factors

Many Hfq-dependent sRNAs regulate transcription factors and two-component systems, either directly at the level of mRNA or indirectly through feedback loops in their regulons (Coornaert et al., 2010; Tu et al., 2010). At the top end, three conserved Hfq-dependent sRNAs (ArcZ, DsrA, RprA) activate the synthesis of σ^S, a major stress sigma factor required for virulence of *E. coli* and *Salmonella* (Papenfort et al., 2009; Soper et al., 2010). Hfq also regulates transcription factors of virulence genes, for example, InvE of *Shigella* (Mitobe et al., 2008), GrlAR and Ler of enterohaemorrhagic *E. coli* (Hansen and Kaper, 2009; Shakhnovich et al., 2009), and HilD of *S.* Typhimurium (Ansong et al., 2009; Lopez-Garrido and Casadesus, 2010; Sittka et al., 2008); as of this writing, the putative cognate sRNAs have remained elusive. In *S. pyogenes*—an organism without Hfq—a locus encoding both the noncoding RivX RNA and the RivR transcription factor regulates the major transcriptional activator of virulence gene, Mga. Reduced virulence gene expression of a *rivXR* double mutant is complemented by either RivR or RivX, suggesting redundancy of riboregulator and regulatory protein (Roberts and Scott, 2007).

There are multiple layers of riboregulation in the expression of PrfA, the factor that orchestrates the genes required for host invasion, phagosome escape, cytosolic growth, and cell-to-cell spreading of *L. monocytogenes*. As already mentioned, *prfA* expression is controlled by an RNA thermometer in the 5′UTR (Johansson et al., 2002). In addition, it has been proposed that two S-adenosylmethionine (SAM)-dependent riboswitches, *sreA* and *sreB*, produce short antisense RNAs to repress the *prfA* mRNA. Intriguingly, transcription of *sreA*/*B* in part depends on PrfA, which suggests a feedback mechanism that helps balance PrfA expression (Loh et al., 2009). Note that riboswitch-derived sRNAs are also known in Gram-negative species (Vogel et al., 2003).

The regulation of transcription factors might often promote a switch between two physiological stages. For example, RNAIII might facilitate a transcription factor switch—between Rot and AgrA—during growth of *S. aureus*. Specifically, Rot (repressor of toxins) is a transcriptional regulator of virulence genes, while AgrA counterregulates many of the ~150 Rot-dependent genes. AgrA accentuates its "anti-Rot" activity by activating the transcription of RNAIII, a translational repressor of the *rot* mRNA (Figure 2; Boisset et al., 2007).

The IhtA sRNA of the obligate intracellular pathogen, *Chlamydia trachomatis*, facilitates a switch from the replicating reticulate body (RB) to the infectious elementary body (EB). The transition to EB (the transcriptionally and translationally silent form of the bacteria) requires the expression of histone-like proteins such as Hc1 for compaction of chromatin. IhtA translationally represses the Hc1 mRNA specifically in RB, and alleviation of this repression in EB should foster the necessary accumulation of Hc1 protein (Grieshaber et al., 2006). Other switch-promoting sRNAs govern quorum sensing control (see below). Given that sRNAs are often degraded along with the mRNAs they regulate—in other words, consumed in the process of regulation—the use of RNA regulators might be advantageous over

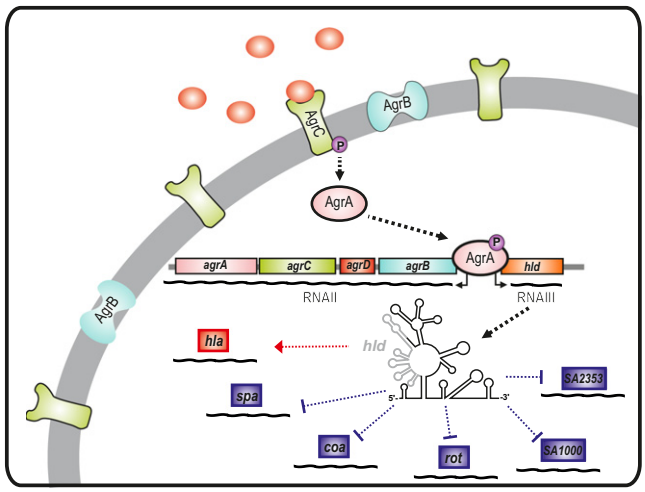

Figure 2. Quorum Sensing and RNAIII-Controlled Gene Expression
S. aureus produce an autoinducing peptide that accumulates in the medium and is sensed by a histidine kinase (AgrC). Sensing of the autoinducing peptide by AgrC leads to phosphorylation of the response regulator AgrA, which in turn is a transcriptional activator of the bifunctional RNAIII. RNAIII harbors the *hld* gene (coding for δ-hemolysin) but also acts as a posttranscriptional regulator of several target mRNAs, most of which with profound impact on virulence. While *spa*, *coa*, *rot*, SA1000, and SA2353 mRNAs are repressed, the *hla* mRNA is activated by RNAIII.

regulatory proteins in achieving a fast and irreversible transition, which is often required during pathogenesis.

Regulation of Virulence Genes

Gram-positive and Gram-negative pathogens alike produce extracellular proteins to usurp or even kill host cells. RNAIII of *S. aureus* has well-established roles in regulating such virulence factors because its antisense domain not only represses the *rot* mRNA (see above) but also those of numerous additional factors including the adhesin protein A as well as staphylocoagulase which is relevant for clotting of human plasma. Moreover, competitive binding of the same 3′ region of RNAIII prevents the formation of inhibitory secondary structure in *hla* mRNA and thereby activates the synthesis of α-hemolysin (Morfeldt et al., 1995). The pathogenicity island-encoded ~140 nt SprD sRNA is another antisense regulator of *S. aureus* virulence factors. SprD represses the *sbi* mRNA encoding an immune evasion protein, and strongly contributes to bacterial killing of infected mice (Chabelskaya et al., 2010).

VR-RNA of the Gram-positive pathogen *Clostridum perfringens* is, in principle, similar to *S. aureus* RNAIII such that it possesses a 5′ located small ORF (*hyp7*) and a 3′ located RNA domain. VR-RNA is part of the VirR/S regulon—a major system to control toxin production of *C. perfringens*—and itself acts as an inducer of α-toxin, collagenase (K-toxin), and β2-toxin synthesis by yet-to-be-understood mechanisms (Banu et al., 2000; Okumura et al., 2008).

Among Gram-negative pathogens, the global Hfq association map available for *S.* Typhimurium (Sittka et al., 2008) predicts ~60% of all secreted effector proteins to be potential targets of Hfq-dependent sRNAs. Moreover, the gifsy prophage-encoded IsrJ sRNA (74 nt) of *S.* Typhimurium contributes to host cell invasion and translocation of SPI-1 effector, SptP. Since IsrJ shows limited potential for direct base pairing with the *sptP* mRNA itself, it might regulate SPI-1 secretion at a more general level (Padalon-Brauch et al., 2008), paralleling the action of major transcription factors in the activation of invasion gene islands.

Regulation of Quorum Sensing

Quorum sensing (QS) refers to bacterial communication by the production, secretion, and detection of small autoinducer (AI) molecules. Typically, the elevated AI concentrations in the environment as cell density increases prompt bacteria to change from individual to group behavior, and this often affects bioluminescence, biofilm formation, competence, sporulation, and virulence.

A hallmark of many QS circuits is that once AI concentrations reach a given threshold, gene expression switches rather than changes gradually. In several QS systems, the switch is determined by the activity of multiple redundantly acting sRNAs, and this is best understood with the *Vibrio* Qrr sRNAs. The Qrr family of four to five highly similar, Hfq-dependent sRNAs is expressed in an AI-dependent manner at low cell density. Under this condition, they posttranscriptionally repress the master QS transcription factor, HapR, in *V. cholerae*, which in turn prevents the synthesis of HapR-controlled T3SS and other virulence genes (Figure 1E; Lenz et al., 2004). The Qrr sRNAs are instrumental in feedback regulation of QS, and their seeming redundancy permits proper dosage compensation and fine-tuning of temporal control of QS activity (Svenningsen et al., 2009; Tu et al., 2010).

Besides inhibiting *hapR* mRNA, the Qrr family activates the synthesis of VCA0939, a GGDEF protein which is involved in cyclic-di-GMP synthesis and can induce virulence factors and biofilm formation in a HapR-independent pathway. This alternative pathway seems particularly relevant in pandemic *V. cholerae* strain El Tor, in which a frameshift mutation in *hapR* prevents normal QS regulation. Thus, in the *V. cholerae* El Tor strain the same Qrr family sRNAs are recruited to regulate very different targets, with the same physiological outcome (Hammer and Bassler, 2007).

The CsrA/B system is another important player in regulating the *hapR* mRNA and QS in *Vibrio* species (Lenz et al., 2005) and generally controls QS circuits in many bacterial species. Moreover, the regulatory RNAIII of *S. aureus* is an integral part of a genetic locus (*agr*) that encodes a QS system (Figure 2). However, unlike the Qrr sRNAs, RNAIII serves an output molecule that relays QS signals for virulence factor expression (Novick and Geisinger, 2008).

Control at the Outer Layer: Outer Membrane Proteins and LPS Modification

The outer membrane of Gram-negative bacteria is a key interface in host-pathogen interactions and carries highly immunogenic OMPs. To date, more than ten *E. coli* and *Salmonella* sRNAs are known to regulate *omp* mRNAs, and most of them are conserved in other enterobacterial pathogens (Figueroa-Bossi et al., 2009; Papenfort and Vogel, 2009). Of these, the conserved RybB sRNA of *S.* Typhimurium acts globally to repress at least 11 major and minor OMPs, including the putative serum resistance protein, PagC. RybB is part of the σ^E regulon that governs envelope homeostasis under normal growth condition and in defense against antimicrobial peptides (Papenfort

et al., 2006). There are also pathogen-specific porin regulators, for example, InvR of the *S.* Typhimurium SPI-1 invasion gene island (Pfeiffer et al., 2007), or MicX and VrrA of *V. cholerea* (Davis and Waldor, 2007; Song et al., 2008). *Cis*-encoded regulators of OMP synthesis are thus far less common and exemplified by the aforementioned RnaG of *Shigella* (Giangrossi et al., 2010).

Lipopolysaccharides (LPSs) anchored to the outer membrane constitute the outmost barrier of Gram-negative bacteria and protect from cationic antimicrobials of eukaryotic cells. One path to immune evasion is the modification of LPS, in which the PhoP/Q two-component system has a primary role. Responding to low levels of divalent cations, PhoP/Q regulates genes involved in Mg^{2+} homeostasis, resistance to antimicrobial peptides and LPS synthesis; it also regulates the conserved Hfq-dependent sRNA, MgrR, which acts to repress the LPS-modification enzyme EptB and thereby fine-tunes LPS structure at intermediate levels of PhoP/Q induction in *E. coli* (Moon and Gottesman, 2009). Another Hfq-dependent sRNA to affect LPS is MicF, which regulates the synthesis of a lipid A-modifying enzyme in *S.* Typhimurium (D. Podkaminski, K.P., J.C.D. Hinton, and J.V., unpublished data).

OMP and LPS are essential for overall envelope integrity and in some cases determine host cell interactions. However, they are also a mixed blessing because of representing—with their accessibility on the outside of the bacterial cell—features recognized by the eukaryotic immune system. Thus, LPS and OMP are controlled at many levels, and this leads us to expect that more RNA regulators involved in their synthesis will emerge from work in pathogenic bacteria. Irrespective of these assumptions, any specific contributions of sRNAs to host cell interaction or evasion are yet to be proven under relevant infection conditions.

RNA Control of Horizontally Acquired Virulence Factors

Bacterial pathogens evolve by mutations, rearrangements, or horizontal gene transfer (HGT). How foreign genes are integrated into existing regulatory networks to benefit the recipient has been increasingly understood at the level of transcriptional control (Navarre et al., 2007). In contrast, if and how RNA regulators are utilized to "tame" HGT genes is less known, but again Hfq and sRNAs seem to have roles. For example, Hfq targets many mRNAs and sRNAs from HGT regions of *S.* Typhimurium (Sittka et al., 2008), and mediates the repression of the core genome-encoded *ompD* mRNA by the horizontally acquired InvR sRNA (Figure 3; Pfeiffer et al., 2007). The converse case of such posttranscriptional crosstalk between core and variable genes is the targeting of newly acquired mRNAs by Hfq-dependent sRNAs of the core genome. Examples include the glucose stress-induced SgrS sRNA (Wadler and Vanderpool, 2009), which controls the synthesis of a *Salmonella*-specific secreted effector, SopD (Figure 3; K.P., D. Podkaminski, S. Lucchini, J.C.D. Hinton, and J.V., unpublished data); and the ArcZ "core" sRNA, which represses STM3216, a horizontally acquired chemotaxis protein of *Salmonella* (Papenfort et al., 2009). Given the wide distribution of Hfq, it is reasonable to speculate that Hfq and sRNAs might significantly contribute to HGT in bacterial pathogens.

CRISPR loci (clustered interspaced short palindromic repeats) encode RNA-based immunity systems to protect bacteria from invading DNA elements such as plasmids and phages (Horvath and Barrangou, 2010), by sequence-specific degradation of the incoming nucleic acids. The sequence specificity is determined by repeat-derived, ~60 nt CRISPR RNA species that target foreign DNA (eubacteria) or RNA (archaea) through almost perfect sequence complementarity (Brouns et al., 2008; Hale et al., 2009; Marraffini and Sontheimer, 2008). CRISPR must be relevant to the evolution of bacterial virulence traits because the targeting of transmittable plasmids and phages poses a restriction to obtaining new genetic information required for host or environmental adaptation. For example, lysogenic infection of *P. aeruginosa* with bacteriophage DMS3 inhibits biofilm formation and swarming motility, and this phenotype requires an intact CRISPR system (Zegans et al., 2009). This observation suggests a role for the small CRISPR RNAs in modifying the effects of lysogeny on this pathogen, prompting speculation that some pathogens might adopt CRISPR activity to control prophage-encoded genes of virulence factors.

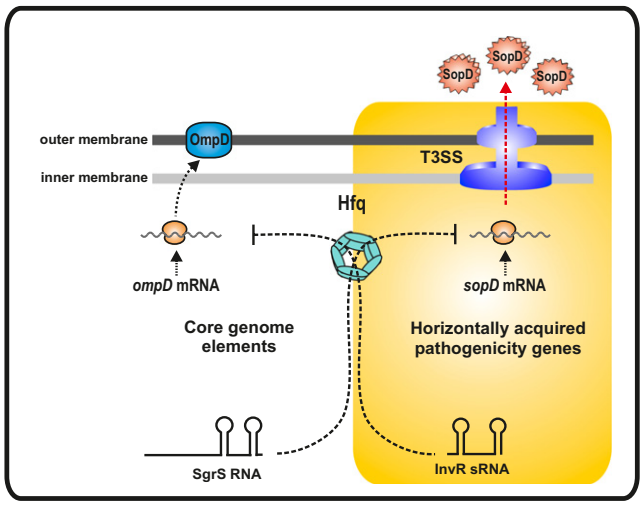

Figure 3. Posttranscriptional Crosstalk of Core and Variable Genome via Hfq and sRNAs

Hfq-dependent sRNAs originating from either the *Salmonella* core genome (SgrS) or a horizontally acquired virulence island (InvR) act in conjunction with Hfq to posttranscriptionally control mRNA targets. (Left) The conserved SgrS sRNA binds to repress the horizontally acquired *sopD* mRNA, encoding a secreted virulence protein. (Right) InvR sRNA posttranscriptionally limits the synthesis of the core genome-encoded OmpD porin.

Perspective

Genome-wide searches based on wet-lab-based methods or in silico predictions have been producing impressive lists of candidate noncoding RNAs in bacterial pathogens (Livny and Waldor, 2007; Sharma and Vogel, 2009; Sorek and Cossart, 2009). These lists are likely to grow as tiling arrays and new RNA sequencing approaches provide high-resolution pictures of transcriptomic landscapes both in vitro and in the context of host infections (Sharma et al., 2010; Toledo-Arana et al., 2009). It will now be important to systematically assay phenotypes of those sRNAs that are differentially regulated or genetically linked to virulence. For example, genome-wide expression profiling of *L. monocytogenes* revealed induction of RliB sRNA in blood and by H_2O_2; RliB was then confirmed to be essential for survival in the host, yet its actual function is yet to be elucidated

(Toledo-Arana et al., 2009). Similarly, knowledge of the genomic coordinates of sRNA genes facilitated the design of specialized knockout libraries in *S.* Typhimurium, which then discovered three sRNAs required for full virulence in mice (Santiviago et al., 2009). However, while genome-wide profiling and RNA discovery do produce promising candidates, it remains a major challenge to design genetic or biochemical screens that overcome the limitations of small genes size and redundant functions of many RNA regulators, and enable direct identification of key regulators of virulence.

Few exceptions such as *S. aureus* RNAIII notwithstanding, a mechanistic understanding of the pathogen-specific riboregulators has been limited, and assumptions have often been by inference from work in nonpathogenic model organisms such as *E. coli* K12. Arguably, the study of pathogens might not necessarily identify new mechanisms of RNA in virulence control, yet still inform new general mechanisms. Recent discoveries include the RNA-based control of catabolite repression in *P. aeruginosa* where the ~400 nt CrcZ RNA targets the RNA-binding protein Crc to allow these pathogens to adapt to various carbon sources (Sonnleitner et al., 2009). Similarly, the extensive work in *Salmonella* identified new molecular mechanisms for Hfq-dependent sRNAs, including the targeting of many ABC transporter mRNAs by a conserved "antisense domain" (Sharma et al., 2007), target recognition in the coding sequence (Bouvier et al., 2008; Pfeiffer et al., 2009), and controlled sRNA degradation by a pseudotarget (Figueroa-Bossi et al., 2009). Nonetheless, while the main interest has been on mRNA regulation, either by antisense RNA or antagonism of mRNA-regulating protein such as CsrA, it will be important to explore new avenues including the direct sequestration or activation of virulence proteins by RNA, or possible secretion of RNA into host cells akin to effector protein translocation, all of which would be logical in the context of bacterial pathogens.

Selected sRNAs could potentially be exploited as novel drug targets in order to treat and prevent disease, especially as the pipeline of conventional antibiotics is running low. Of note, riboswitches have already begun to be validated as targets for antibacterial treatment; for example, a pyrimidine compound acting as guanine riboswitch antagonist was shown to reduce *S. aureus* infection rates in mice (Mulhbacher et al., 2010). Given the progress with riboswitches, it will be desirable to exploit similar strategies of chemical biology to target individual small RNAs and/or their helper proteins by small compounds in order to combat bacterial infections in a species-specific manner.

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