Molecular Microbiology (2016) 101(5), 701–713

doi:10.1111/mmi.13428
First published online 16 June 2016

MicroReview

Interplay of regulatory RNAs and mobile genetic elements in enteric pathogens

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Summary

Horizontal transfer of genetic information is a major driving force of evolution. In bacteria, genome plasticity is intimately linked to the ability of the bacterium to integrate novel material into existing gene expression circuits. Small RNAs (sRNAs) are a versatile class of regulatory molecules, and have recently been discovered to perform important tasks in the interplay between core genomic elements and horizontally-acquired DNA. Together with auxiliary proteins such as the RNA-chaperone Hfq and cellular ribonucleases, sRNAs typically act post-transcriptionally to either promote or restrict the expression of multiple target genes. Bacterial sRNAs have been identified in core and peripheral (acquired) genome sequences, and their target suites may likewise include genes from both locations. In this review, we discuss how sRNAs influence the expression of foreign genetic material in enterobacterial pathogens, and outline the processes that foster the integration of horizontally-acquired RNAs into existing regulatory networks. We also consider potential benefits and risks of horizontal gene transfer for RNA-based gene regulation.

Introduction

Bacteria are able to thrive in nearly any ecological niche on the planet, and are characterized by their remarkable ability to rapidly detect and adapt to changes in their environment. The genetic information required for bacterial perseverance possesses extreme plasticity shaped by both differential gene loss and uptake of new material (Juhas, 2015). How is genetic information obtained from external sources? Almost one century ago, Frederick Griffith reported that a phenotypic trait – in this case virulence – could be passed from one isolate of *Streptococcus pneumoniae* to another (Griffith, 1928), and he thus obtained a first evidence for the existence of horizontal gene transfer (HGT) in bacteria. The phenomenon discovered by Griffith is termed transformation, and refers to the uptake and integration of free environmental DNA into the core genome. Transformation is one of three possible mechanisms of HGT in bacteria (Johnston *et al.*, 2014), with the other two being (i) conjugation (the transfer of chromosomal or extrachromosomal material from one cell to another), and (ii) transduction (the transfer of DNA by bacteriophages).

Conjugation is a frequent natural mode of plasmid transfer, and requires a functional Type IV secretion system (Cabezon *et al.*, 2015). Transduction propels genome reorganization and short-time evolution of prokaryotes by two mechanisms (Penades *et al.*, 2015). First, as new virus particles are assembled, bacteriophages may occasionally package host DNA fragments, which are then recombined into the genome of a naïve bacterium during the next infection cycle. Second, when infecting a susceptible host, bacteriophages may integrate into the bacterial core genome to co-replicate passively in a lysogenic state. These so-called prophages serve as integration points for additional foreign genetic material, or as initiation sites of genomic rearrangements (Brussow *et al.*, 2004).

The integration of horizontally-acquired sequences is often associated with a significant burden to the cell. While taking a chance on novel, beneficial traits and phenotypes, the bacterium faces the risk of a potential fitness loss through either disruption of core genome elements, and/or inappropriate expression of the newly acquired material. To avoid the latter scenario, physical integration of foreign DNA has to go hand in hand with the integration into established gene networks (Dorman, 2009; Will *et al.*, 2015).

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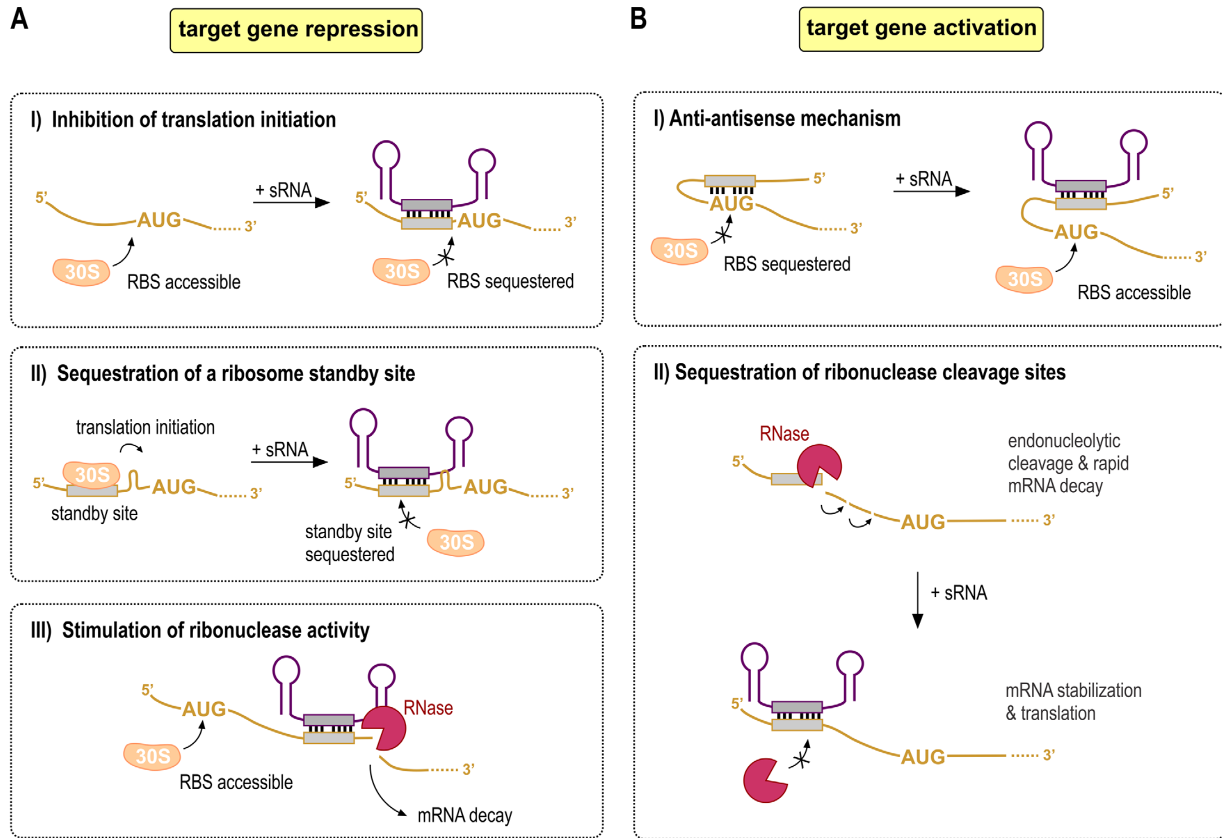

Fig. 1. Mechanisms of target gene regulation by sRNAs.
A. Post-transcriptional repression of gene expression by sRNAs. (I) Base-pairing of an sRNA with the translation initiation site of a target mRNA interferes with ribosome binding, and prevents translation initiation. (II) sRNA-binding to a ribosome standby site within the 5′ UTR of the target transcript can lead to inhibition of translation. (III) Interaction of an sRNA with the coding sequence of the target can recruit endonucleases to the base-pairing site, facilitating mRNA cleavage and decay.
B. Mechanisms of target gene activation by sRNAs. (I) Anti-antisense regulation for activation of mRNA translation. An mRNA with a long 5′ UTR forms a translation-inhibitory hairpin in the absence of any sRNA activator. Base-pairing of the sRNA prevents formation of this inhibitory structure, thus allowing ribosomes to access the RBS and initiate translation. (II) Base-pairing of an sRNA masks a ribonuclease cleavage site in the target mRNA, which stabilizes the transcript and promotes translation.

Bacterial regulation of gene expression has traditionally been linked to the activity of transcription factors. It is now accepted that RNA serves crucial regulatory functions in both prokaryotes and eukaryotes, and acts as a global modulator of gene expression. Approximately 10–15% of bacterial genomes are transcribed into non-protein-coding RNAs (Westhof, 2010), including the highly abundant classes of transfer RNAs (tRNAs), ribosomal RNAs (rRNAs) as well as several RNAs performing housekeeping functions (Wassarman *et al.*, 1999; Cavanagh and Wassarman, 2014). In addition, a highly heterogeneous group of non-coding RNAs control gene expression at the post-transcriptional level. This class of RNAs includes regulatory elements within 5′ leaders of mRNAs, such as riboswitches and RNA-thermometers (Krajewski and Narberhaus, 2014; Mellin and Cossart, 2015), antisense RNAs transcribed *in cis* to their target genes (Georg and Hess, 2011), as well as small RNAs (sRNAs). The prominent sRNAs vary dramatically in both size (50–400 nt) and secondary structure, and typically exert their regulatory function by base-pairing with the 5′ untranslated region (UTR) of a cognate target transcript encoded *in trans*, *i.e.* in a genomic locus different from the sRNA. In the cell, a large number of sRNAs associate with the RNA chaperone, Hfq, which protects them from degradation by cellular ribonucleases, thus avoiding rapid decay. Furthermore, Hfq functions as a matchmaker for RNA-duplex formation, bringing sRNA and mRNA in close proximity to allow base-pairing (Vogel and Luisi, 2011).

Most sRNAs modulate translation of their target RNAs, leading to either repression (De Lay *et al.*, 2013) or activation (Papenfort and Vanderpool, 2015) of gene expression. Negative regulation by sRNAs (Fig. 1A) is often due to direct inhibition of translation initiation (Maki *et al.*, 2008; 2010). Stable ribosome association with an

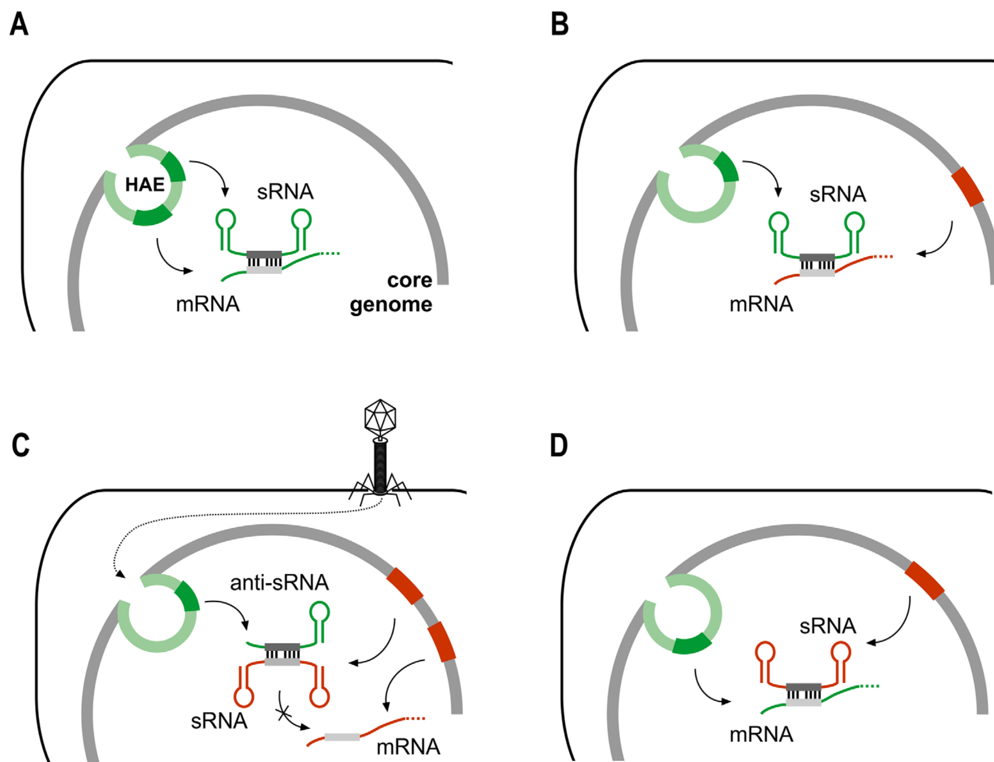

Fig. 2. Activity of bacterial sRNAs in the interplay of horizontally-acquired elements (HAEs) and the core genome.
A. Regulation of a target transcript by an sRNA both encoded on the same HAE.
B. Cross-regulation of core genes by horizontally-acquired sRNAs.
C. Phage-derived 'anti-sRNAs' indirectly promote mRNA expression by sequestration of core genome-encoded repressor sRNAs.
D. Expansion of the target repertoire of core genome-encoded sRNAs results in regulation of genes expressed from HAEs.

mRNA requires accessibility of a sequence stretch typically located between nt. −35 to +19 relative to the start codon, and base-pairing of an sRNA within this window is able to prevent assembly of the translation initiation complex (Bouvier *et al.*, 2008). Alternatively, sRNAs may interfere with translation initiation by sequestering ribosome standby-sites (Darfeuille *et al.*, 2007) or translation enhancer elements (Sharma *et al.*, 2007), and by recruiting the Hfq RNA chaperone to the respective regions (Desnoyers and Masse, 2012).

Gene activation by sRNAs involves stabilization of the target mRNA, and/or stimulation of translation (Fig. 1B). The so-called 'anti-antisense mechanism' depends on the opening of a self-inhibitory intramolecular structure in the 5′ UTR of the target mRNA through base-pairing with the sRNA. Binding of the sRNA to the transcript can result in the formation of an alternative structure favourable for mRNA translation (Morfeldt *et al.*, 1995; Majdalani *et al.*, 1998). Alternatively, sRNA-mediated sequestration of a ribonuclease cleavage site on an mRNA can prevent transcript decay, increasing its intrinsic stability and thus promoting protein production (Fröhlich *et al.*, 2013; Papenfort *et al.*, 2013).

In this review, we will focus on sRNA-mediated cross-regulation between horizontally-acquired sequences and the core genome in enterobacterial pathogens. Due to space limitations, we restrict our review to sRNAs for which target genes have been experimentally identified. For the role of regulatory RNA in plasmid maintenance, as well as the recently discovered CRISPR/Cas and BREX systems, we refer to several excellent articles (Brantl, 2014; Goldfarb *et al.*, 2015; Marraffini, 2015).

An emerging role for bacterial sRNAs in HGT

The recent development of high-throughput technologies has revolutionized the study of bacterial transcriptomes by RNA sequencing (Croucher and Thomson, 2010; Sorek and Cossart, 2010; Barquist and Vogel, 2015). Detailed analyses of total RNA pools from various prokaryotes has led to the annotation of hundreds to thousands of non-coding RNAs, many of which map to horizontally-acquired elements (HAEs) like plasmids, transposons or genomic islands (Barquist and Vogel, 2015). However, the functional characterization of

non-coding RNAs has lagged behind discovery. Consequently, we only understand the important roles played in post-transcriptional regulation and HGT for a few sRNAs. In the following sections, we will discuss the influence of core sRNAs on horizontally-acquired genes and, *vice versa*, how sRNAs that were originally imported with foreign genetic material can shape the expression of core-genome encoded elements. In certain cases, sRNAs also regulate the frequency of HGT.

sRNA and target located on the same functional unit

Occasionally, sRNAs and their target genes have been acquired as components of a regulatory circuit encoded within a single horizontally transferred module (Fig. 2A). In extra-intestinal pathogenic *Escherichia coli* (ExPEC), a computational screen for strain-specific sRNAs revealed the candidate sRNA **AfaR** (a.k.a. SQ109) located on the *afa-8* cluster of strain AL862 (Pichon *et al*., 2012). When colonizing their host, ExPEC express a variety of adhesins, including afimbrial adhesins of the Afa family, which are typically organized in clusters of six genes (Bernier *et al*., 2002). Located within pathogenicity island I (PAI-I), the *afa-8* cluster harbours three transcriptional units (*afaABCD*, *afaE* and *afaF*), as well as the gene for the AfaR sRNA. AfaR is expressed from the intergenic region (IGR) between *afaD* and *afaE*, and specifically down-regulates AfaD-VIII invasin expression while leaving *afaABC* unaffected. Base-pairing of the sRNA close to the *afaD* translational start site promotes RNase E-mediated cleavage of the mRNA, and thus reduces protein production (Pichon *et al*., 2013).

The genomes of pathogens belonging to the genus *Salmonella* have frequently been reshaped by the uptake of foreign DNA. In particular, the *Salmonella* pathogenicity islands (SPIs) contain large clusters of virulence genes that enable *Salmonella* to invade host cells, and to replicate intracellularly (Dobrindt *et al*., 2004; Sabbagh *et al*., 2010; LaRock *et al*., 2015). In *Salmonella enterica* sv. Typhimurium (hereafter referred to as *Salmonella*), the two major pathogenicity islands SPI-1 and SPI-2 both encode distinct type III secretion systems (T3SS) and effector proteins, which are transported through the apparatus into the host cell (Hensel, 2004).

Salmonella is also a well-studied model for sRNA functions in bacteria (Hebrard *et al*., 2012). A bioinformatic screen revealed the expression of 19 potential sRNAs encoded in IGRs of genetic islands of *Salmonella* (Padalon-Brauch *et al*., 2008), including the candidates IsrE, IsrM and IsrK. **IsrE** (a.k.a. RfrA or RyhB-2) is a paralogue of the core sRNA RyhB, which functions as a key regulator of iron homeostasis in many bacteria (Salvail and Masse, 2012). Expression of IsrE and RyhB is induced during the infection process (Padalon-Brauch *et al*., 2008; Ortega *et al*., 2012; Kroger *et al*., 2013; Srikumar *et al*., 2015), and both sRNAs are required for optimal replication of *Salmonella typhi* in host cells (Leclerc *et al*., 2013). Although Fur-dependent transcription of the two sRNAs is generally conserved, IsrE has been reported to receive additional regulatory input from the OxyR transcriptional regulator (Calderon *et al*., 2014a). Alike, the target profiles of the two paralogues largely overlap (Ellermeier and Slauch, 2008; Ortega *et al*., 2012; Calderon *et al*., 2014b), yet IsrE also affects motility of *Salmonella* (Kim and Kwon, 2013).

IsrK was identified as a key player in the regulation of the lysogenic Gifsy-1 prophage in *Salmonella* (Hershko-Shalev *et al*., 2016). Two different RNAs originate from the *isrK* promoter: the IsrK sRNA, as well as a longer, multicistronic read-through transcript *isrK-orf45-anrP*. The structure adopted by the longer RNA inhibits translation initiation of the two protein-coding cistrons *orf45* and *anrP*. Only when IsrK sRNA acts *in trans* and binds to the longer RNA isoform, alternative RNA folding enables protein synthesis of Orf45 and AntR, which are translationally coupled. IsrK transcription increases under various environmental conditions, including oxidative stress and iron depletion (Kroger *et al*., 2013; Hershko-Shalev *et al*., 2016), and high levels of IsrK result in inhibition of bacterial growth due to AnrP production. AnrP promotes expression of Gifsy-1 *antQ*, an anti-termination factor encoded just upstream of the *isrK* locus. Different from related proteins from other phages, AntQ not only interferes with transcription termination of distinct phage RNAs, but also supports transcription elongation throughout the bacterial genome, which results in severe damage of the chromosome and ultimately cell death. Whether the IsrK-controlled circuit serves Gifsy-1 propagation upon stress, or rather represents a host response limiting phage replication remains an open question.

The ~330 nt **IsrM** sRNA is conserved in serval *Salmonella* species, but absent from *Salmonella typhi* and the more distantly-related *Salmonella bongori* (Gong *et al*., 2011; Kroger *et al*., 2012). IsrM is expressed during infection and represses the transcript encoding the SPI-1-located effector protein SopA as well as the *hilE* mRNA. HilE is a repressor of SPI-1-mediated virulence gene expression (Baxter *et al*., 2003), and *isrM*-deficient mutants display increased HilE protein production, leading to attenuated virulence (Gong *et al*., 2011). Surprisingly, the IsrM sRNA does not associate with Hfq (Chao *et al*., 2012), indicating a regulatory mechanism distinct from canonical sRNAs. Another example of

Hfq-independent target regulation by a horizontally-acquired sRNA is **TarB** (ToxT activated RNA B). The *tarB* gene is expressed from the *Vibrio cholerae* pathogenicity island (VPI) following activation by the master virulence regulator, ToxT (Bradley *et al*., 2011). TarB inhibits the expression of the VPI-located *tcpF* mRNA, which encodes an essential colonization factor of *V. cholerae* (Kirn *et al*., 2003). Transcription of *tcpF* is tightly controlled during *V. cholerae* infection, and coordination of the regulatory circuit by TarB appears to have a positive effect on colonization (Bradley *et al*., 2011).

Horizontally-acquired sRNAs regulating core genome elements

Several HAE-encoded sRNAs have been integrated into ancestral regulatory circuits (Fig. 2B). A well-studied example of RNA-mediated cross-regulation between a pathogenicity island and the core genome is the *Salmonella*-specific sRNA, **InvR** (Pfeiffer *et al*., 2007). InvR is transcribed from the IGR between the *invH* and STM2901 genes on SPI-1, and its expression strictly depends on the SPI-1-encoded transcription factor, HilD. In contrast to many other SPI-1 genes, InvR plays no role in effector protein secretion but rather inhibits production of the abundant outer membrane protein OmpD, encoded on the core genome. Tight regulation of porin expression is vital to *Salmonella's* cell physiology: in addition to InvR (Pfeiffer *et al*., 2007), the *ompD* mRNA is repressed by at least three other sRNAs transcribed under various environmental conditions (Papenfort *et al*., 2006; Pfeiffer *et al*., 2009; Papenfort *et al*., 2010; Fröhlich *et al*., 2012). Depletion of the major surface antigen OmpD by InvR might help *Salmonella* to evade the eukaryotic host immune system during infection, and at the same time prevent crowding of the *Salmonella* outer membrane when the T3SS machinery is assembled. Indeed, *Salmonella* harbouring transposon insertions in the *invR* locus display attenuated infection of pigs, calves and chicken (Chaudhuri *et al*., 2013). However, the fundamental biology of this virulence defect requires further investigation.

The **RaoN** sRNA was identified in a transposon screen for *Salmonella* mutants showing inhibited growth under nutrient limitation in combination with low pH – two conditions relevant during infection (Lee *et al*., 2013). A transposon in the *cspH*/*envE* IGR on SPI-11 disrupted expression of a short, ~200 nt transcript termed RaoN. Although RaoN does not carry a Rho-independent terminator typical for Hfq-dependent sRNAs (Otaka *et al*., 2011), the RNA chaperone is required for full expression of RaoN. Expression of a single gene, the lactate dehydrogenase *ldhA*, is increased during oxidative stress in *raoN* mutants, but regulation via direct base-pairing between the two RNAs has not yet been validated. Interestingly, RaoN might play a role in *Salmonella* infection as *raoN*-deficient bacteria show reduced replication in mouse macrophages (Lee *et al*., 2013).

The **TarA** sRNA of *V. cholerae* is encoded on the same pathogenicity island as TarB (see previous section), and it is likewise activated by the transcription factor ToxT (Richard *et al*., 2010; Bradley *et al*., 2011). In contrast to TarB, TarA base-pairs with and inhibits the expression of *ptsG* mRNA, a transcript encoded outside of the VPI (Richard *et al*., 2010). The PtsG protein is part of a high-affinity glucose uptake system; accordingly, growth of cells that over-express TarA is severely inhibited when glucose is the sole carbon source. *V. cholerae* mutants lacking *tarA* display a mild colonization defect, which might result from *ptsG* over-production (Richard *et al*., 2010).

Bacteriophages can actively reshape prokaryotic physiology by integrating into the host chromosome and sometimes transfer additional genetic material from another bacterium. It is becoming evident that phage-derived sequences frequently influence host genetic networks, and may directly encode beneficial traits (Koskella and Brockhurst, 2014; Veses-Garcia *et al*., 2015).

A number of sRNAs are encoded by prophages, and in some cases their target repertoire is expanded to transcripts expressed from the core genome. **DicF**, for example, is a 53 nt sRNA which is processed by RNase E and RNase III-dependent cleavages from the 3′ UTR of a polycistronic transcript (Bouche and Bouche, 1989; Faubladier *et al*., 1990). The associated operon is part of a defective lambdoid prophage present in the genome of *E. coli* and several other enterobacteria (Faubladier and Bouche, 1994), and encodes two types of cell division inhibitors: the small protein DicB, and the sRNA DicF. DicB is an activator of MinC, which inhibits polymerization of the prokaryotic tubulin homologue, FtsZ (Zhou and Lutkenhaus, 2005). At the post-transcriptional level, DicF sRNA blocks translation of the *ftsZ* transcript, presumably by direct base-pairing at the translational start site (Tetart and Bouche, 1992). The expression of DicF is under the control of a C1-like repressor, and stimulated by zygotic induction (Bejar *et al*., 1988).

Studies focusing on the infection of *E. coli* by bacteriophage PA-2 revealed reduced production of the host protein OmpC when the phage-encoded porin Lc was expressed (Schnaitman *et al*., 1975). This phenotype was attributed to a region downstream of the *lc* gene harbouring a putative sRNA, which inhibits OmpC synthesis. A homologous sRNA is also present downstream of the phage-derived *nmpC* porin gene in the core genome of *E. coli*. When overexpressed, **IpeX** sRNA

likewise inhibits OmpC production, however, lack of clear sequence complementarity between IpeX and *ompC* indicated that regulation might occur indirectly (Castillo-Keller *et al.*, 2006).

Like many pathogens, enterohemorrhagic *E. coli* (EHEC) owes its virulence to foreign DNA sequences acquired through HGT (Dobrindt *et al.*, 2010). The mosaic-like genome of EHEC isolate O157:H7 str. Sakai harbours multiple islands of bacteriophage origin that together account for approximately one quarter of its genetic material (Hayashi *et al.*, 2001). Of note, there is relative enrichment of putative sRNAs on genomic islands when compared with the core genome (Tree *et al.*, 2014), arguing for an important role of sRNAs in the regulation of horizontally-acquired sequences.

A number of EHEC-specific sRNAs have been studied in more detail. For example, overexpression of **Esr41**, an sRNA from the EHEC Sakai prophage-like element (SpLE1), results in hypermotility due to increased FliC (flagellin) production (Sudo *et al.*, 2014). However, the molecular details underlying FliC activation by Esr41 remain to be determined.

Mapping of RNA sequences cross-linked to Hfq revealed 55 potential sRNAs expressed from the EHEC pathogenicity islands (Tree *et al.*, 2014). The four most abundant transcripts share a short, highly conserved sequence of 42 nt, but carry variable 5′ regions of 14–18 nt. Three of these phage-encoded RNAs, **AsxR** and two homologues of **AgvB,** function as 'anti-sRNAs' counteracting the activity of the core genome sRNAs FnrS and GcvB respectively (Fig. 2C). Expression of AsxR activates *chuS* (encoding a heme oxygenase) at the post-transcriptional level, but the lack of homology between AsxR and *chuS* mRNA excluded a mechanism based on direct base-pairing. Instead, the 5′ end of AsxR carries a recognition site for the FnrS sRNA, a repressor of *chuS* translation (Durand and Storz, 2010). By titrating the negative regulator FnrS and promoting its decay, AsxR is able to indirectly promote expression of *chuS*, and potentially additional FnrS target genes. Similarly, AgvB is able to alleviate repression of *dppA* (coding for a dipeptide transporter), a target of the GcvB sRNA. GcvB is highly conserved among the enterobacteria, and post-transcriptionally controls a large regulon of genes coding for amino acid and peptide transporters (Sharma *et al.*, 2011). The 5′ end of AgvB carries a sequence complementary to the conserved R1 seed region of GcvB. GcvB employs the R1 region to recognize the majority of its targets, and base-pairing of AgvB to the site antagonizes this function (Tree *et al.*, 2014). Interestingly, the core genome-encoded sRNA SroC uses a similar mechanism to counteract GcvB function. However, different from AgvB, SroC base-pairs with two distinct sequence elements to achieve GcvB degradation (Miyakoshi *et al.*, 2015a).

Control of foreign genetic material by core genome-encoded sRNAs

Bacterial evolution and diversity are critically influenced by HGT. However, newly acquired genetic information needs to be controlled in order to benefit the host. A general mechanism to restrict the expression of foreign DNA is transcriptional silencing through nucleoid-associated proteins such as H-NS, StpA or Fis (Dorman, 2009; Will *et al.*, 2015). Regulatory RNAs constitute an additional important layer of control over gene expression from newly acquired DNA sequences. Several recent studies have provided insight into how sRNAs encoded on the core genome are employed to regulate genes from horizontally-acquired islands (Fig. 2D), or even control the process of HGT itself.

Type VI secretion systems (T6SS) are one of the more recent additions to the list of bacterial virulence factors. The structure of the T6SS strongly resembles the tail of bacteriophage T4, pointing at a potential history as a HAE (Cascales, 2008). *V. cholerae* is one of the species that use a single T6SS to elicit both anti-eukaryotic and anti-bacterial activity, and to additionally foster uptake of foreign DNA (Metzger and Blokesch, 2015). The two master quorum sensing regulators, HapR (active under high cell density) and LuxO (active under low cell density) function as activator and repressor of the T6SS, respectively. Whereas HapR binds to the promoter sequences of T6SS genes (Zheng *et al.*, 2010), LuxO employs the four homologous sRNAs, **Qrr1-4**, to down-regulate *hapR* expression and to directly base-pair with and repress a long, polycistronic mRNA encoding key T6SS genes (Shao and Bassler, 2014).

SgrS is an integral component of the enterobacterial response to phosphosugar stress triggered by the accumulation of non-metabolizable, phosphorylated sugars in the cell (Papenfort and Vogel, 2014). SgrS is one of the rare examples of a 'dual-function sRNA' as it also encodes a small protein, SgrT, which blocks transport through the major glucose uptake machinery (Wadler and Vanderpool, 2007). When acting as a regulatory RNA, SgrS employs a conserved sequence stretch close to its 3′ end to base-pair with and repress translation of *ptsG* and *manXYZ* mRNAs, both encoding sugar transport systems (Vanderpool and Gottesman, 2004; Rice *et al.*, 2012). In addition, SgrS binds to and stabilizes a fragment of RNase E-mediated mRNA decay to promote expression of the phosphatase YigL which functions in the dephosphorylation of sugars, and thus enables their export (Papenfort *et al.*, 2013). Although the role of SgrS in the response to phosphosugar stress is conserved (Horler and Vanderpool, 2009), the sRNA is also involved in species-specific gene regulation. In

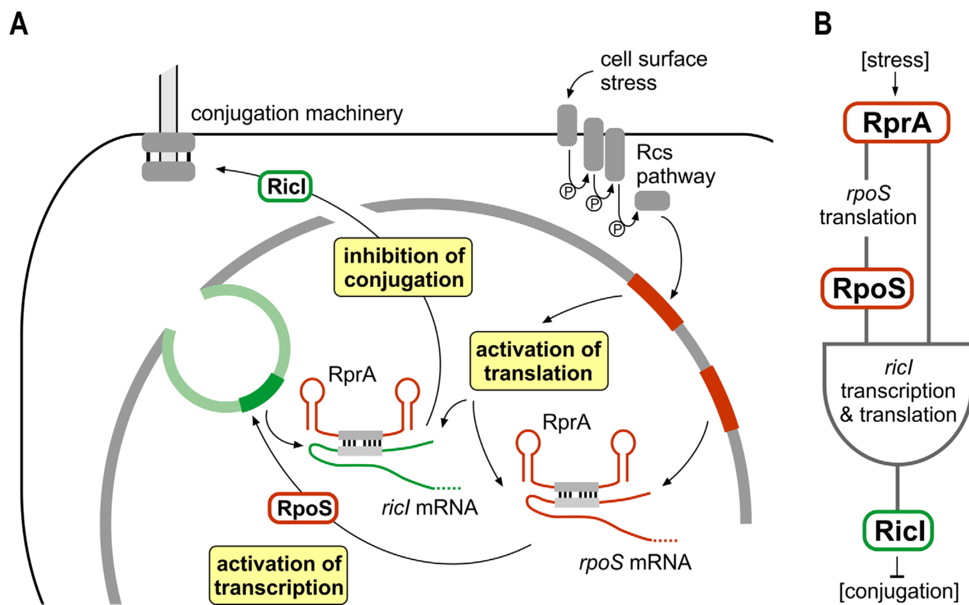

Fig. 3. The core genome-encoded sRNA RprA controls conjugation of pSLT in *Salmonella*.
A. Upon membrane perturbation, RprA is activated via the Rcs pathway. RprA promotes translation of RpoS (σ^S), which controls transcription of *ricI*. RprA is also required to facilitate *ricI* translation, and thus enables RicI-mediated inhibition of conjugation.
B. RicI production is governed by a feed-forward loop with AND-gate logic. The membrane stress-induced RprA is required to activate translation of *rpoS*, and *ricI* mRNAs. Transcription of *ricI* is dependent on the alternative sigma factor, σ^S.

Salmonella, SgrS employs its conserved seed region to repress translation and stimulate decay of *sopD* mRNA encoding an effector protein secreted into the host via both T3SSs (Papenfort *et al.*, 2012). Remarkably, a second effector gene, *sopD2*, which is most likely the result of gene duplication within *Salmonella* and shares more than 40% identity with *sopD*, is not amenable to SgrS regulation due to a single nucleotide polymorphism within the sRNA binding site. This small difference renders a stable G-C pair in the productive SgrS-*sopD* interaction into a silent G-U pair which prevents SgrS from regulating *sopD2* (Papenfort *et al.*, 2012).

SgrS is an excellent example of a core genome-encoded sRNA with a conserved target set that developed an expanded regulatory repertoire to control horizontally-acquired genes. Likewise, the **ArcZ** sRNA is conserved among the enterobacteria, and post-transcriptionally regulates a number of core-encoded mRNAs. In *Salmonella*, pulse expression of ArcZ revealed eight putative targets. Four of these transcripts locate to *Salmonella*-specific genomic regions, and base-pairing of the horizontally-acquired STM3216 mRNA (coding for a methyl-accepting chemotaxis protein) with ArcZ was confirmed experimentally (Papenfort *et al.*, 2009).

ArcZ is a processed sRNA producing two sRNA variants in the cell: a full-length transcript of ∼120 nt, and a shorter version of ∼50 nt (Argaman *et al.*, 2001; Papenfort *et al.*, 2009). Similarly, full-length **RprA** sRNA (107 nt) is cleaved by RNase E resulting in a processed variant of ∼50 nt (Madhugiri *et al.*, 2010; Papenfort *et al.*, 2015a). Interestingly, each variant of RprA controls a different set of target genes. In *Salmonella*, processed RprA inhibits the expression of two prophage-derived transcripts of *Salmonella* (SL2594 and SL2705), as well as the *traT* mRNA, which is expressed from the self-transmissible pSLT plasmid of *Salmonella*. The pSLT plasmid is specific to *Salmonella* and encodes several virulence genes required for systemic disease (Rotger and Casadesus, 1999). Conjugation of pSLT requires an intricate system of plasmid-derived and core genome-encoded control elements, which can either block or facilitate conjugation. To regulate pSLT transfer, RprA coordinately activates translation of two transcripts: first, the *rpoS* mRNA (encoding the stationary sigma-factor, σ^S) and second, the *ricI* transcript (a.k.a. *STM4242*). In both cases, RprA employs an anti-antisense mechanism to increase target gene expression (Figs 1B and 3A) (Majdalani *et al.*, 2002; Papenfort *et al.*, 2015a). Because σ^S is necessary for *ricI* transcription, RprA functions as the centerpiece of a post-transcriptional feed-forward loop with AND-gate logic for *ricI* activation (Fig. 3B). The *ricI* gene has been horizontally-acquired and integrated into the core genome of most *Salmonella* species. When expressed, RicI localizes to the cytoplasmic membrane of *Salmonella* where it binds to a conjugation apparatus protein, TraV. Interaction of RicI and TraV inhibits pSLT transfer, probably by blocking assembly of the conjugation machinery (Fig. 3A). Transcription of RprA itself is under control of the Rcs phosphorelay, which is triggered upon membrane perturbation (Majdalani and Gottesman, 2005). Indeed, exposure to membrane-damaging bile salts activates RprA production and reduces pSLT

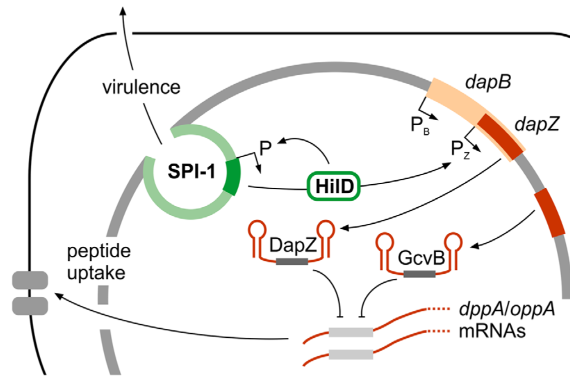

Fig. 4. The SPI-1-encoded transcription factor HilD controls DapZ sRNA expression from the core genome. DapZ is encoded within the 3′ UTR of *dapB*, and is transcribed from its own promoter in the presence of HilD (P_B: *dapB* promoter; P_Z: *dapZ* promoter). Alike, but independently from another core genome-encoded sRNA, GcvB, DapZ represses transcripts of peptide uptake systems (*oppA*, *dppA* mRNAs).

transfer in *Salmonella*. Physiologically, RicI-mediated conjugation arrest might protect the cell from the risk of conjugation apparatus assembly when membrane integrity is compromised. Many other environmental conditions activate σ^S (Battesti *et al.*, 2011), however, due to the strict requirement of RprA for RicI activation, only conditions that trigger the Rcs pathway will also affect pSLT conjugation. In summary, RprA is a multifaceted post-transcriptional regulator, which controls foreign genetic material at two levels: first, by preventing HGT itself (through inhibition of conjugation) and second, by base-pairing with horizontally-acquired genes.

Novel sRNA regulators from 3′ UTRs of mRNAs

The initial searches for novel sRNAs relied on the bioinformatic predictions of orphan promoter/terminator pairs within the IGRs of prokaryotic genomes (Altuvia, 2007). The advent of novel sequencing approaches has turned sRNA discovery on its head, revealing hundreds of regulatory RNAs residing in or overlapping with the coding sequence of bacterial transcriptomes (Barquist and Vogel, 2015). Notably, the 3′ UTRs of mRNAs have been identified as a rich source for Hfq-dependent sRNAs (Miyakoshi *et al.*, 2015b) that can also be integrated into horizontally-acquired gene networks. For example, the **DapZ** sRNA of *Salmonella* is encoded in the 3′ end of the core-genome *dapB* gene, and its transcription is activated by the SPI-1-encoded master virulence regulator, HilD (Chao *et al.*, 2012). Reminiscent of the core-encoded GcvB sRNA (Sharma *et al.*, 2011), DapZ functions as a post-transcriptional repressor of *dppA* and *oppA* mRNAs encoding amino-acid uptake systems (Fig. 4). In other words, the *Salmonella* SPI-1 locus has hijacked the 3′ UTR of the core-encoded *dapB* gene to acquire an sRNA repressor for regulation of amino-acid transport under virulence conditions (Chao *et al.*, 2012). Why 3′ ends of mRNAs are enriched for putative sRNA regulators is currently unclear. One hypothesis suggests that Hfq's high affinity for Rho-independent terminators (Otaka *et al.*, 2011; Sauer and Weichenrieder, 2011), which constitute the 3′ end of the majority of bacterial transcripts, could recruit 3′ UTR-derived transcripts into post-transcriptional networks transforming them into base-pairing regulators.

Two types of 3′ end-derived sRNAs have been described (Chao *et al.*, 2012; Miyakoshi *et al.*, 2015b; Papenfort *et al.*, 2015b). First, sRNAs can be expressed separately of their overlapping gene, *i.e.* these sRNAs possess their own promoters and independent transcriptional control (Chao *et al.*, 2012; Guo *et al.*, 2014). Second, endonucleolytic cleavage of an mRNA can give rise to sRNAs, which associate with Hfq and serve as trans-acting regulators (Miyakoshi *et al.*, 2015a; Chao and Vogel, 2016). Of note, 291 out of 770 predicted Rho-independent terminators of *Salmonella* were significantly enriched in co-immunoprecipitation experiments with Hfq (Chao *et al.*, 2012). It is interesting to speculate that mRNAs possessing a Rho-independent terminator at their 3′ end could serve as a genomic inventory for the evolution of new sRNA regulators, which can acquire regulatory roles when foreign DNA elements are integrated into preexisting regulatory circuits.

Perspective

Despite playing a major function as the coding agent for protein synthesis, an increasing number of regulatory roles have been assigned to RNA in prokaryotic organisms. RNA regulators are also gathering momentum as control devices in the interplay of core genomic sequences and HAEs. Gene expression control through sRNAs is pervasive in bacteria, and many enteric pathogens rely on sRNAs to control virulence (Papenfort and Vogel, 2010). For example, ~88% of the 280 sRNAs encoded by *Salmonella* are expressed inside macrophages, 176 of which are not conserved in other genera (Srikumar *et al.*, 2015).

The recent characterization of one of these sRNAs, PinT (a.k.a. STnc440), exemplifies how complex the entanglement of RNA-based regulation between core genome and integrated elements can be (Westermann *et al.*, 2016). Although the *pinT* gene is located on a *Salmonella*-specific HAE, its transcription is controlled by the conserved, core genome-encoded two-component system PhoPQ. PinT is induced during the infection process and down-regulates the production of the

invasion-related SopE and SopE2 effector proteins once *Salmonella* has entered the host cell. In addition, PinT inhibits two core-encoded mRNAs, *grxA* and *crp*, the gene products of which contribute to virulence gene activation in intracellular *Salmonella* (Yoon *et al*., 2009). Together, PinT facilitates *Salmonella's* transition from invasion to intracellular replication by repressing core- as well as HAE-derived transcripts.

As we discover ever more bacterial RNA regulators and classify their distinct roles in bacterial physiology, we are yet to understand the system-wide consequences of sRNA-based gene regulation. As outlined above, most sRNAs require the Hfq protein for both their stability and target regulation. The exact cellular copy number of the RNA chaperone is a matter of debate (estimates range from 400 to 10,000 hexamers per *E. coli* cell (Vogel and Luisi, 2011)), nevertheless potential RNA binding partners are certainly in molar excess over Hfq (Wagner, 2013). Consequently, expression of Hfq-binding sequences from HAEs could titrate Hfq away from core RNAs, and thus interfere with endogenous sRNA-based regulatory circuits. Of note, a number of bacterial species have acquired further copies of *hfq*, which are also located on foreign genetic sequences (Sun *et al*., 2002; Vrentas *et al*., 2015). Whether these additional Hfqs help to counterbalance the burden of gene expression from HAEs on endogenous Hfq is currently unclear.

Another potential problem associated with the integration of HAE-derived RNAs into existing post-transcriptional networks is linked to the different types of regulatory mechanisms carried out by the sRNAs. Target regulation can have three major outcomes: (i) catalytic degradation (the mRNA is degraded while the sRNA is stable), (ii) coupled degradation (sRNA and target mRNA are degraded) and (iii) sequestration (both sRNA and mRNA are stable, and regulation occurs only at the translational level). While catalytic degradation has only limited impact on the concentration of sRNA regulators, coupled degradation and sequestration both will drain the pool of available sRNAs (Feng *et al*., 2015). Thus, newly evolving interactions with mRNAs from HAEs could compromise the regulation of existing targets by limiting sRNA availability. Following this logic, catalytic degradation could be the preferred mechanism of regulation for horizontally-acquired target mRNAs. However, experimental proof for this hypothesis has yet to be obtained, and it is currently unclear if similar rules could also apply for target activation.

Clearly there are many exciting topics and unanswered questions concerning the interplay of regulatory RNAs and HAEs. We expect that the successive characterization of horizontally-acquired sRNAs will shed light on important aspects of bacterial physiology and uncover abundant cross-regulation occurring at the post-transcriptional level. Conversely, it will be interesting to study how core-encoded sRNAs can gain control over foreign genetic material, and how regulation evolves to be integrated into existing gene regulatory networks.

Acknowledgements

We apologize to all authors whose work we could not include due to space limitations. We thank Josep Casadesús, Jörg Vogel and Jay Hinton for comments on the manuscript. Work in the Papenfort laboratory is supported by the DFG (GRK2062/1 and PA2820/1). K. P. acknowledges funding by the Young Academy program of the Bavarian Academy of Sciences.

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