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Small RNAs, Large Networks: Posttranscriptional Regulons in Gram-Negative Bacteria

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Keywords

small RNA, Hfq, gene networks, regulatory motifs, sponge RNA

Abstract

Small regulatory RNA (sRNAs) are key mediators of posttranscriptional gene control in bacteria. Assisted by RNA-binding proteins, a single sRNA often modulates the expression of dozens of genes, and thus sRNAs frequently adopt central roles in regulatory networks. Posttranscriptional regulation by sRNAs comes with several unique features that cannot be achieved by transcriptional regulators. However, for optimal network performance, transcriptional and posttranscriptional control mechanisms typically go hand-in-hand. This view is reflected by the ever-growing class of mixed network motifs involving sRNAs and transcription factors, which are ubiquitous in biology and whose regulatory properties we are beginning to understand. In addition, sRNA activity can be antagonized by base-pairing with sponge RNAs, adding yet another layer of complexity to these networks. In this article, we summarize the regulatory concepts underlying sRNA-mediated gene control in bacteria and discuss how sRNAs shape the output of a network, focusing on several key examples.

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INTRODUCTION

The ability of bacteria to respond to external and internal cues is dictated by dozens of interconnected regulatory networks controlling the nature and amplitude of the necessary gene regulatory changes. In many cases, these networks are centered around major transcription factors that activate or repress the expression of genes located downstream in the regulon (59). In large regulons, one transcription factor frequently controls the production of additional transcriptional regulators, building a hierarchical relationship that determines the temporal order of the response. In addition, regulatory networks can involve the activity of small regulatory RNAs (sRNAs), which, in contrast to their transcription factor counterparts, typically function at the posttranscriptional level to control translation and transcript stability (139).

The majority of the currently known sRNAs range between 50 and 250 nucleotides and typically carry three major functional domains: at least one base-pairing sequence allowing RNA duplex formation with other transcripts, an interaction site for RNA chaperones, and a Rho-independent transcription terminator providing transcript stability (**Figure 1**). Among the RNA chaperones linked to sRNA function, Hfq, an Sm/Lsm-type RNA-binding protein, has been

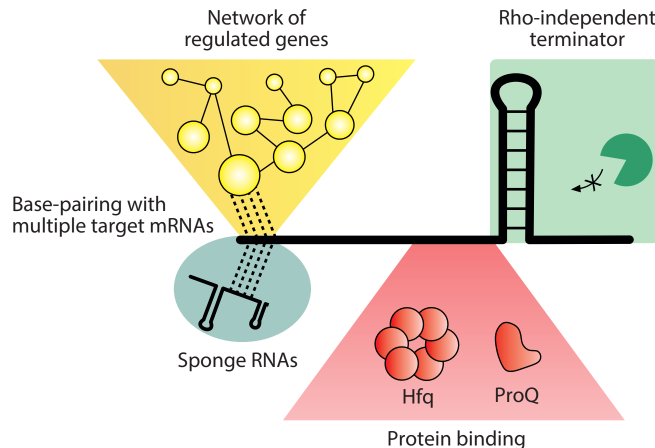

Figure 1

A canonical bacterial sRNA regulator. Many sRNAs regulate multiple target mRNAs via a conserved base-pairing sequence and thereby coordinate a network of regulated genes (*yellow*). This function can be antagonized by so-called sponge sRNAs, which also base-pair to the sRNAs and thus restrict their function (*teal*). sRNAs often work in concert with RNA chaperones, e.g., Hfq and ProQ (*red*). Interaction with an RNA chaperone typically increases sRNA stability and facilitates base-pairing with *trans*-encoded transcripts. Termination of sRNA transcription typically involves a Rho-independent termination structure (*green*), which also promotes sRNA stability and the recruitment of RNA chaperones, such as Hfq.

studied in most detail (131). Initially identified as a host factor for replication of bacteriophage Qβ in *Escherichia coli* (36), Hfq has homologs that have now been discovered in thousands of microorganisms, and mutation of their corresponding genes has frequently been associated with complex phenotypic consequences ranging from altered metabolism to lack of biofilm formation and, occasionally, loss of virulence (18). Hfq forms a hexameric ring structure with three RNA-binding surfaces (157): the distal face (binding repeats of an ARN sequence, where R is adenine/guanine and N is any nucleotide), the proximal face (binding stretches of uridine residues), and the rim (binding UA-rich sequences). Certain Hfq homologs also carry a disordered C terminus, which interacts in various ways with the different binding faces of Hfq (64) and has been implicated to reduce nonspecific RNA binding and to promote the release of sRNAs and sRNA-mRNA duplexes from Hfq (129, 130). This latter function might well accelerate cycling of RNA molecules on Hfq, which could help to globally facilitate sRNA-mediated gene regulation (160).

ProQ is another RNA-binding protein implicated in global sRNA activity and belongs to the family of FinO domain proteins (92). Mutation of *proQ* is linked to elevated resistance toward toxic proline analogs in *E. coli*, which also provided the name for the gene (84). Similar to Hfq, ProQ binds dozens of sRNAs and several hundred mRNAs in *E. coli* and *Salmonella enterica* and also promotes RNA duplex formation (50, 53, 136, 162). In contrast, other FinO-type RNA-binding proteins, such as RocC from *Legionella pneumophila* and FinO of many F-type plasmids, display only a narrow range of RNA ligands (28, 32). A comparison of the Hfq- and ProQ-associated RNAs suggested only a little overlap among their interactomes; however, recent evidence suggests that the two RNA chaperones might well have complementary and/or competing roles in the cell (80, 81).

A hallmark of many *trans*-acting sRNAs is their ability to regulate multiple transcripts. This feature is particularly well established for Hfq-binding sRNAs (52, 106); however, sRNAs associating with ProQ have also been reported to form RNA duplexes with more than just one target transcript (81). The ability to control several target genes in parallel is key for sRNAs to orchestrate gene regulation within a single gene network, but it also allows them to connect and synchronize the output of two or more simultaneously active networks. For example, the VadR sRNA from *Vibrio cholerae* adjusts cell shape by controlling the levels of the *crvA* mRNA, encoding a central regulator of cell curvature. VadR also regulates several target genes involved in biofilm formation, thus providing a link between these two regulatory pathways (109).

A single sRNA is able to inhibit and activate target gene expression, and which of the two regulatory modes is employed relies on the localization of the base-pairing site on the target transcript. Target repression most often involves sequestration of the ribosome-binding site (RBS) by the sRNA, leading to translation inhibition and increased transcript turnover (63). RNA duplex formation upstream and downstream of the RBS can also result in target inhibition (38); however, both types of regulation typically involve auxiliary factors such as ribonuclease E (RNase E) (6) or ribosomal protein S1 (3). In contrast, sRNA-mediated target activation usually occurs by base-pairing outside the RBS by one of two possible mechanisms: First, the sRNA can antagonize the formation of a stem-loop structure in the 5′ UTR (untranslated region) of the target mRNA that blocks the RBS and inhibits target translation (75, 105). Second, RNA duplex formation between an sRNA and its target can result in the sequestration of a ribonuclease cleavage site, which stabilizes the transcript and promotes translation (39, 104). Taken together, Hfq/ProQ-binding sRNAs employ a wide range of regulatory mechanisms to control gene expression, and in almost all cases, base-pairing with target mRNAs is the underlying feature that drives these processes.

In the last few years, several new methodologies have been developed to identify RNA-RNA interactions at a global scale (48, 68, 82), resulting in a surge of potential (and validated) sRNA-target mRNA interactions. However, how these interactions integrate into existing gene networks and how regulation is divided among sRNAs and transcriptional regulators is often not

clear. Combined transcriptional and posttranscriptional control creates so-called mixed network motifs that oftentimes differ in their performance from analogous network motifs involving only transcription factors (16). In fact, simple network motifs, such as feedback inhibition, have been shown to act merely at the posttranscriptional level and thus can be independent of transcriptional regulation (54). In this review article, we focus on the role of sRNA-mediated gene control in four well-studied regulatory pathways in gram-negative bacteria, i.e., carbon metabolism, envelope stress, iron homeostasis, and quorum sensing. We use these examples to illustrate how sRNAs contribute to the overall performance of the network and in which manner posttranscriptional control mechanisms differ from conventional transcriptional regulation.

CARBON METABOLISM

The proliferation and survival of many if not all microorganisms largely depend on the availability of at least one suitable carbon source that can fuel central metabolic pathways. Thus, it may not be surprising that bacteria evolved intricate regulatory pathways to ensure optimal carbon uptake and utilization (41). In enteric bacteria these pathways frequently involve regulatory RNAs (27, 107). Two prominent examples are the Hfq-dependent sRNAs Spot 42 and SgrS. Both are considered model sRNAs, as studying their physiological roles has also led to the discovery of some of the mechanistic principles underlying RNA-based gene expression control in bacteria, as well as how sRNAs modulate network performance at the posttranscriptional level.

Transcription of Spot 42 (encoded by *spf*) is repressed by the global carbon regulator CRP (113). CRP activity is increased by the second messenger cAMP (3′,5′-cyclic AMP), which is produced in the presence of carbon sources other than glucose (41). Thus, the sRNA is transcribed in the presence of glucose, whereas expression ceases when secondary carbon sources are dominant. Spot 42 was most thoroughly studied in *E. coli*, where it has been documented to base-pair with and regulate dozens of mRNAs (9, 10, 86). Functional characterization of these targets revealed a clear overarching picture: Spot 42 inhibits target mRNAs encoding proteins involved in the transport and utilization of secondary carbon sources, which enforces glucose metabolism by limiting leaky expression of nonrelevant genes. Importantly, numerous Spot 42 targets are transcriptionally activated by CRP generating a multi-output feed-forward loop with the sRNA in the middle position (**Figure 2*a***). This regulatory setup affects the steady-state levels as well as the regulatory dynamics upon sudden shifts in carbon source availability. For example, Spot 42 accelerated the inhibition of the sorbitol-specific transporter SrlA and two fucose utilization genes when *E. coli* cells were transferred to high-glucose conditions. Conversely, when shifted to nonpreferred carbohydrates, Spot 42 caused a delay in target mRNA accumulation (9).

Mechanistically, Spot 42–mediated regulation of target mRNAs has been associated with three base-pairing sites located in the first 60 nucleotides of the regulator. All three base-pairing regions are unstructured, i.e., are not involved in intramolecular base-pairing, and for several targets RNA duplex formation involved more than one site (10). Interestingly, recent evidence suggests that Spot 42 activity is further influenced by CsrA, another RNA-binding protein involved in posttranscriptional gene control. Specifically, CsrA binds to and protects Spot 42 from ribonucleolytic decay by RNase E and thereby enhances target mRNA repression (66). Given that CsrA activity is also controlled by CRP (30), one can speculate that Spot 42 and CsrA together modulate the overall output of the CRP regulon at the RNA level. In fact, in pathogenic microorganisms, such as *S. enterica*, CRP in concert with cAMP has been shown to inhibit virulence gene expression through a posttranscriptional mechanism (31). Intriguingly, the *spf* gene not only generates a regulatory RNA but also encodes the small protein SpfP (**Figure 2*a***) (2). SpfP reinforces the feed-forward loop regulated by Spot 42 as it binds to CRP and blocks the ability of CRP to

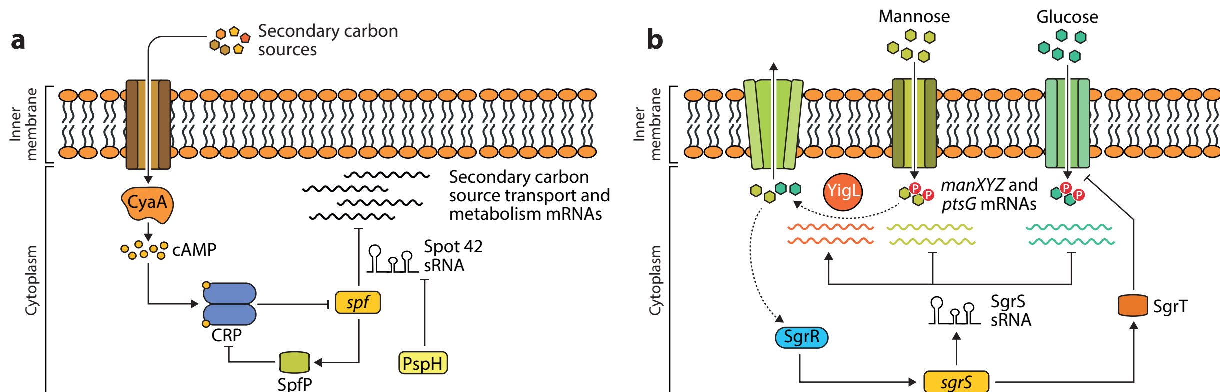

Figure 2

The role of the Spot 42 and SgrS sRNAs in carbon metabolism regulation. (*a*) In the absence of glucose, cAMP-CRP represses *spf* expression and activates secondary carbon sources' transport and metabolism genes. When glucose is present, cAMP levels are reduced and Spot 42 is expressed. Spot 42 inhibits the expression of mRNAs encoding proteins involved in secondary carbon sources' transport and metabolism and inhibits CRP activity through the small protein SpfP. Spot 42 levels are also controlled posttranscriptionally by the sRNA sponge PspH. (*b*) Glucose-phosphate stress activates the SgrR transcription factor, which drives *sgrS* transcription. SgrS inhibits PTS (phosphotransferase system) glucose/mannose transporters by binding to *ptsG* and *manXYZ* mRNAs. SgrS upregulates the phosphatase YigL, which allows the export of dephosphorylated sugars. The *sgrS* gene also encodes the SgrT small protein. SgrT binds to and inhibits the activity of the PTS glucose transporter.

activate specific genes. Spot 42 therefore belongs to the emerging group of dual-function RNAs, which are posttranscriptional regulators that also produce small proteins (120).

Virulence gene expression of *S. enterica* is also inhibited by another sRNA, SgrS (sugar-phosphate stress sRNA). Specifically, SgrS inhibits expression of the secreted effector protein SopD (102); however, this regulation might be considered an accessory function of SgrS, as its main (and conserved) physiological role is linked to the control of carbohydrate transport and utilization in various gram-negative bacteria (13).

RNA-mediated regulation by SgrS is mediated by a single conserved base-pairing region (13, 76), and regulation of six target mRNAs (*ptsG*, *manX*, *purR*, *asd*, *yigL*, and *sopD*) has been investigated in more detail (12). With respect to its role in bacterial physiology, identification of SgrS-mediated regulation of *ptsG* and *manX* indicated a function of the sRNA in carbohydrate uptake, as these genes encode PTS (phosphotransferase system) sugar transporters for glucose and mannose, respectively (123, 153). The SgrS-*ptsG* interaction has been especially instrumental for studying the molecular determinants underlying gene control by Hfq-dependent sRNAs, revealing, for example, a role for RNase E in target mRNA degradation (87) and how sRNAs are recognized by Hfq (93). Among the six targets controlled by SgrS, *yigL* is the only activated target; all others are repressed. The *yigL* gene encodes sugar phosphatase that facilitates dephosphorylation and efflux of accumulated sugars and thus aids the overall function of the sRNA, which is to control the accumulation of phosphorylated sugars in the cell (104). The role of the additional target mRNAs, e.g., *purR*, *asd*, and *sopD*, in the SgrS regulon is less obvious. However, it was speculated that regulation of *purR* and *asd* supports stress recovery (14), whereas repression of *sopD* links carbohydrate uptake with host cell invasion in *S. enterica* (102).

Transcriptional control of *sgrS*, in contrast to Spot 42, does not involve CRP but relies on the coconserved transcript factor SgrR (154). SgrR contains an N-terminal DNA-binding domain and a C-terminal solute-binding domain and is strictly required for *sgrS* transcription. The factors

controlling SgrR activity were long elusive; however, a recent screen for small-molecule binding partners of transcriptional regulators in *E. coli* indicated that interaction with glutamate could inhibit SgrR (70). SgrR also activates the transcription of *alaC* (encoding an alanine-synthesizing transaminase) and *setA* (encoding a sugar efflux pump) genes, and while their induction could potentially help to ameliorate sugar accumulation (140), regulation of *alaC* might be more relevant in the context of low glutamate levels, given that the AlaC enzyme converts glutamate and pyruvate to α-ketoglutarate and alanine (70).

In several species, the *sgrS* gene, similarly to the *spf* gene, also encodes a small protein called SgrT (159). SgrT is also involved in sugar stress, as it binds to and inhibits transport through the glucose-specific PTS transporter (72). Thus, the base-pairing and the small-protein functions encoded in *sgrS* constitute a regulatory network that counteracts stress at three interconnected levels (**Figure 2*b***): (*a*) blocking sugar import by SgrT, (*b*) reducing the synthesis of new transporters by inhibiting *ptsG* and *manX*, and (*c*) enabling sugar export by upregulation of YigL. Regulation of *purR* and *asd* by SgrS might provide an additional function to realign cellular metabolism in the presence of sugar stress (12).

SgrS and Spot 42 are not the only sRNAs involved in carbohydrate metabolism of gram-negative bacteria. In fact, quite a number of regulatory RNAs have been reported to adopt roles in sugar utilization. An interesting example is the recently identified *vcdRP* transcript from *V. cholerae*, which shows similarities to Spot 42 and SgrS (156). VcdRP is functionally related to Spot 42, as it is also repressed by CRP, and similar to SgrS, as it inhibits the translation of mRNAs encoding PTS carbohydrate transporters (by VcdR) and also encodes a small protein (VcdP). However, VcdP binds to and activates citrate synthase, a key enzyme of the citrate cycle, and thus the overall cellular function of VcdRP seems to be different from those of Spot 42 and SgrS. It might synchronize sugar uptake and central metabolism to optimize carbon utilization (156).

Other examples of sRNAs involved in carbon metabolism are GlmZ, GlmY, TfoR, MltS, DonS, CrcZ, AzuR, TarA, and ChiX (27, 119). Among those, ChiX is of overarching importance to our understanding of sRNA-mediated regulatory principles, as ChiX was the first sRNA recognized not only to act as a regulator but also to be regulated by another RNA (35, 94). This other RNA is produced from the *chb* operon mRNA and acts as a sponge (or decoy) to block ChiX activity in the presence of chitose sugars. sRNA sponges are similar to canonical sRNA regulator, but typically base-pair with other noncoding regulators, rather than mRNAs (15). In the absence of chitose sugars, ChiX efficiently inhibits the expression of genes involved in chitose sugar utilization, and the *chb*-contained sponge provides an RNA switch that inactivates ChiX. RNA sponges have now been discovered in various other pathways (34); for example, Spot 42 activity is also antagonized by a sponge RNA called PspH (82).

ENVELOPE STRESS

A hallmark of gram-negative bacteria is the presence of two membranes: The inner and outer membranes are separated by the periplasm, which includes the peptidoglycan layer. Maintaining homeostasis in both of these membranes and the periplasmic space is key for microbial growth and cell replication, and thus gram-negative cells employ several stress response systems to monitor the status of the membrane and the membrane proteins (85). These stress response systems frequently include Hfq-dependent sRNAs to alter gene regulation at the posttranscriptional level and to mitigate stress (37).

One of the most thoroughly examined regulons containing sRNAs is the σ^E-mediated envelope stress response (44, 65, 158) (**Figure 3*a***). The central transcriptional regulator of this regulon is the alternative σ^E (encoded by the *rpoE* gene). In the absence of stress, the anti-σ factor RseA

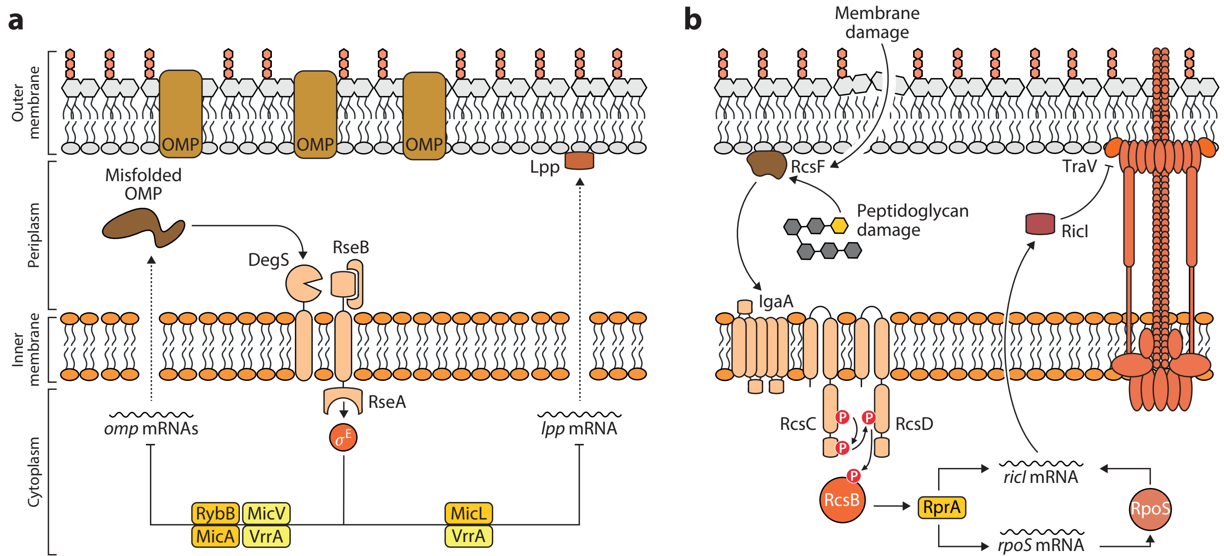

Figure 3

sRNA-mediated regulation of envelope stress response. (*a*) σ^E-Dependent sRNAs (RybB, MicA, and MicL in *Escherichia coli* and related species and MicV and VrrA in *Vibrio cholerae*) control the envelope stress response by regulating multiple target mRNAs. Accumulation of misfolded outer membrane proteins (OMPs) triggers a signal transduction cascade resulting in the release of σ^E into the cytoplasm, which activates transcription of the indicated sRNAs. These σ^E-dependent sRNAs regulate the expression of numerous mRNAs encoding outer membrane porins and the lipoprotein Lpp. (*b*) Regulation of plasmid conjugation by the RprA sRNA. Membrane damage initiates a signal transduction cascade in which the phosphorylated RcsB regulator activates the expression of the RprA sRNA. RprA induces the translation of *rpoS* and *ricI*. RpoS (σ^S) is required for transcription activation of *ricI*, encoding a membrane protein that interacts with and inhibits the anchor protein of the type IV secretion apparatus (TraV), inhibiting plasmid transfer.

sequesters σ^E at the inner membrane, which keeps the σ factor inactive. Accumulation of misfolded outer membrane proteins (OMPs) triggers a well-characterized proteolytic cascade resulting in the release of σ^E into the cytoplasm (23). σ^E then associates with the RNA polymerase core enzyme, which in well-studied model organisms such as *E. coli* and *S. enterica* has been reported to directly affect the activity of approximately 60 σ^E-dependent promoters (89). Among these are also the promoters of three sRNA genes, i.e., *rybB*, *micA*, and *micL* (47, 61, 101, 143, 149). All three sRNAs bind Hfq, yet they differ in how they are synthesized. Specifically, the promoter of *micL* is located in the *cutC* gene driving the transcription of a long MicL isoform, which is further processed by RNase E into a short variant that accumulates in the cell (47, 150). In contrast, MicA and RybB are expressed from free-standing, intergenic genes and do not require ribonucleolytic processing for maturation (61, 101, 121, 148).

Despite these differences, all σ^E-dependent sRNAs share the unifying feature that they inhibit the translation of mRNAs encoding OMPs (65, 158). Whereas MicA and RybB both control a larger set of OMP mRNAs (40, 97, 101), gene expression control by MicL seems to be specific to the *lpp* mRNA, which encodes one of the most abundant OMPs in *E. coli* and related bacteria (47). As pointed out above, misfolded OMPs also trigger the σ^E response, and thus the σ^E-dependent sRNAs empower an elegant negative feedback loop in which defects in OMP synthesis result in reduced *omp* mRNA translation through the action of the sRNAs (**Figure 3**). This regulatory logic is relevant under regular growth conditions (to optimize OMP synthesis) as well as under membrane-damaging conditions (to reduce the flow of newly synthesized OMPs into a compromised periplasmic environment) and has been reported in other organisms as well.

For example, the VrrA and MicV sRNAs of *V. cholerae* are both σ^E dependent, and both regulate several mRNAs that encode OMPs or other proteins that have to travel through the periplasm to reach their final destination (110, 127, 137, 138).

Another notable feature of σ^E-dependent sRNAs is that their target spectra frequently overlap, suggesting that they could have partially redundant functions and/or originate from a common sRNA ancestor. While our understanding of how sRNAs evolve is still limited (29, 151), and it is largely unclear why cells employ sRNAs over transcription factors for certain regulatory pathways, research on σ^E-dependent sRNAs has helped to address both of these questions. First, sequence comparison and genetic analysis revealed that the MicV, VrrA, and RybB sRNAs all share a conserved base-pairing domain that mediates *omp* mRNA binding and thus provides the regulatory foundation of the response (110). Second, *omp* mRNAs have been reported to be remarkably stable (135), and thus transcriptional control might not be sufficient to efficiently reduce the pool of existing *omp* mRNAs under stress conditions (101). Given that *omp* mRNAs have been frequently associated with sRNA-mediated control, this regulatory principle might also extend to other sRNAs that are not controlled by σ^E but also inhibit the synthesis of OMPs, such as MicF, CyaR, ChiX, MicC, OmrA/B, MicX, and InvR (22, 46, 60, 65, 100, 111, 152, 158).

Analogous to the sRNAs involved in carbon metabolism discussed above, the σ^E-controlled sRNAs are also subject to regulation by sponge RNAs. Specifically, RybB has been copurified with tRNA-associated spacer transcripts, which have been shown to reduce the activity of the sRNA in unstressed cells (67). In addition, the RbsZ sponge RNA also interacts with RybB. The *rbsZ* gene corresponds to the 3′ UTR of *rbsB*, encoding a ribose uptake protein, and interestingly RybB interacts not only with RbsZ but also with the 5′ UTR of *rbsB* (40, 81). Thus, RybB, RbsZ, and *rbsB* form an autoregulatory loop in which RbsZ can relieve the negative effect of RybB on *rbsB*. Of note, Hfq and ProQ both promote base-pairing of RybB and RbsZ; however, the two RNA chaperones have opposite effects on the fate of the interaction: Whereas Hfq facilitates fast turnover of the RNA duplex, complexes involving ProQ seem to afford a higher stability (81).

While the above sRNAs have been well documented to support outer membrane homeostasis, the integrity of the inner membrane is subject to sRNA-mediated control as well. For instance, the conserved CpxQ sRNA from *E. coli* and related organisms is part of the Cpx (conjugative pilus expression) stress pathway, which, akin to its σ^E counterpart for outer membrane stress, responds to inner membrane and periplasmic stress (44, 85). CpxQ belongs to the ever-growing class of 3′ UTR–derived sRNAs that frequently overlap with the Rho-independent terminator of an mRNA (114). In the case of CpxQ, the upstream gene is *cpxP*, encoding a periplasmic protein whose transcription is directly controlled by CpxR. CpxR is the response regulator of the Cpx two-component stress response system, with CpxA being the relevant sensor kinase (58). Accumulation of CpxQ in the cell requires RNase E–mediated cleavage of the *cpxPQ* transcript and binding of the Hfq RNA chaperone (19). The target suite of CpxQ involves several inner membrane proteins, suggesting that CpxQ could act analogously to the sRNAs of the σ^E-mediated envelope stress response. Indeed, mutants lacking *cpxQ* display increased sensitivity toward the ionophore CCCP (3-chlorophenylhydrazone) and fail to control the production of the periplasmic chaperone Skp (19, 43). Interestingly, CpxQ also regulates the expression of the *cfa* mRNA, which encodes cyclopropane fatty acid synthase (11). The Cfa enzyme allows modification of the fatty acids in the membrane under stress conditions, and thus regulation by CpxQ could establish a link between the Cpx response and the fatty acid composition of the membrane.

Another relevant scenario in which sRNAs influence the cell envelope is the regulation of microbial secretion systems. In gram-negative bacteria, secretion of proteins and nucleic acids involves transport over two membranes and demands the assembly of complex, multimeric

protein machineries involving the insertion of proteins into the inner and outer membranes (45). Depending on the environmental conditions, sRNAs can either facilitate or inhibit this process. For example, production of the SPI-1 (*Salmonella* pathogenicity island 1)-encoded type III secretion system is supported by the virulence-activated InvR sRNA, which inhibits the synthesis of the abundant OMP OmpD, thus reducing the protein load in the outer membrane (112). In contrast, RprA, an sRNA that was initially characterized in *E. coli* (74), reduces type IV secretion–mediated transfer of the *Salmonella* virulence plasmid (pSLT) (**Figure 3*b***). Specifically, RprA activates the translation of the *ricI* mRNA, which encodes a membrane protein that interacts with and inhibits the anchor protein TraV of the type IV secretion apparatus (98). Regulation of *ricI* by RprA involves two layers of regulation: First, base-pairing of RprA with *ricI* resolves an inhibitory stem-loop structure the 5′ UTR of the mRNA and thus allows translation. Second, RprA also activates expression of σ^S (74), which is necessary for *ricI* transcription. Together, RprA, σ^S, and RicI establish a coherent feed-forward loop with AND-gate logic that safeguards RicI expression. One reason for this tight control of RicI could be that σ^S is induced in various stress conditions (42), whereas plasmid transfer should only be inhibited under membrane stress. Indeed, transcription of the *rprA* gene depends on the Rcs signal transduction pathway, which is activated by membrane-damaging agents, such as bile salts and β-lactam antibiotics (161). Thus, although a member of the σ^S regulon by transcriptional control, RicI is only produced under a subset of σ^S-inducing conditions that involve activation of the Rcs system and consequently RprA expression.

IRON HOMEOSTASIS

One of the most abundant elements on earth is iron. Thanks to its redox potential, iron participates in countless reactions and related biological functions. However, iron generates a paradox for bacteria, as it is essential but also has the potential to harm bacterial cells by the generation of reactive oxygen species during aerobic metabolism. Iron homeostasis is critical for bacterial survival, and thus bacteria employ numerous regulatory systems to control iron acquisition, consumption, and storage in correlation to its availability (17).

A key regulator in iron homeostasis of gram-negative bacteria is Fur (ferric uptake repressor), which is a transcription factor controlling the expression of ∼100 genes (4, 145). Under iron-rich conditions, Fur binds Fe^{2+} and inhibits genes related to iron uptake, maintaining intracellular iron concentrations at the desired levels. In contrast, under iron-limiting conditions, Fur no longer interacts with Fe^{2+} and represses these genes, allowing the bacteria to import iron from the environment. While for many of the Fur-regulated genes the underlying regulatory mechanism could be traced back to transcription regulation, differential expression of numerous other genes affected by Fur activity remained unexplained. Studies from the Gottesman group explained this conundrum by revealing that one of the key transcripts repressed by Fur is a 95-nucleotide sRNA named RyhB (78, 79). Specifically, they discovered that RyhB is expressed under iron-limiting conditions and regulates the expression of genes involved in iron homeostasis (**Figure 4**). RyhB was further characterized to control dozens of target mRNAs and became a model sRNA for studying the mechanistic underpinnings of posttranscriptional gene regulation in bacteria. RyhB activity, like that of many other sRNAs, is facilitated by Hfq, and a recent survey suggested that the RyhB "targetome" is composed of 56 target mRNAs and 143 regulated genes (21). RyhB activity is part of the iron-sparing response, a regulatory principle that can be divided into three arms: control of iron-protein synthesis, Fe-S cluster biogenesis, and iron uptake.

The first RyhB target that was characterized in more detail was *sodB* (77, 78), encoding a superoxide dismutase that requires iron as a cofactor. Base-pairing of RyhB with *sodB* is facilitated by Hfq, and it is followed by immediate degradation of both RNAs by RNase E and RNase III

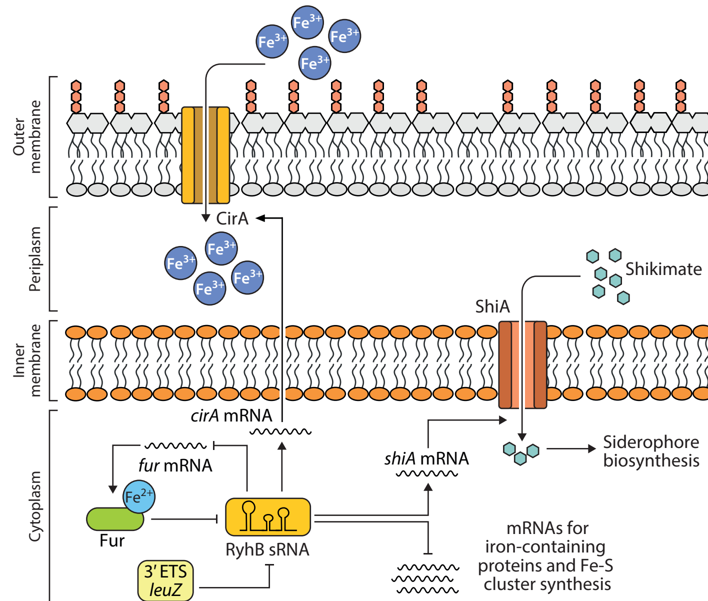

Figure 4

Control of iron metabolism by the RyhB sRNA. Under iron-rich conditions, Fur-Fe^{2+} inhibits RyhB production, allowing the use of iron in the cells. However, when iron is limited, repression by Fur is relieved and RyhB is expressed. Here, RyhB inhibits the expression of mRNAs encoding iron-binding proteins and the biogenesis of Fe-S clusters. In addition, RyhB promotes iron uptake by upregulating the expression of CirA, a transporter of Fe^{3+}-bound siderophores, and of ShiA, a shikimate transporter that is involved in siderophore synthesis. RyhB levels are also regulated by an RNA sponge that is processed from the 3′ external transcribed spacer (ETS) of the *leuZ* tRNA.

(1). Thus, downregulation of *sodB* by RyhB is part of the first arm of the response, as SodB is a nonessential iron-containing protein. RyhB-mediated control of Fe-S biogenesis, i.e., the second arm of the response, is chiefly facilitated by regulation of the *iscRSUA* mRNA that encodes the components required for Fe-S cluster assembly (144). In most cases, RyhB regulates the first gene of a polycistronic mRNA target. In the case of *iscRSUA*, RyhB binds to an intergenic region between *iscR* and *iscS*. RyhB binding to this region overlaps the translation initiation site of *iscS*, promoting the downregulation of *iscS* and the downstream genes (25). In contrast, a proximal stem-loop structure protects *iscR* mRNA from degradation, allowing IscR production. IscR inhibits transcription of the *iscRSUA* operon and thus amplifies this regulatory loop (69, 132). The third arm of the iron-sparing response is accomplished mainly by activation of two transporters: CirA, a transporter of Fe^{3+}-bound siderophores, and ShiA, a transporter of a precursor in siderophore production. Siderophores are molecules that are secreted by bacteria, bind Fe^{3+}, and help the bacteria to scavenge extracellular iron (117). The *cirA* and *shiA* mRNA are upregulated by RyhB by two separate mechanisms. Regulation of *cirA* by RyhB involves binding of Hfq to the RBS of *cirA*, which prevents ribosome binding (128). Binding of RyhB to *cirA* triggers a conformational switch in the mRNA's secondary structure, promoting the dissociation of Hfq and freeing the RBS for translation initiation. Activation of *shiA* by RyhB relies on an alternative mechanism (118). Here, the 5′ UTR of *shiA* forms a stem-loop structure that masks the RBS and represses *shiA* translation. Under iron-limiting conditions, binding of RyhB to the *shiA* 5′ UTR unlocks the inhibitory structure and enables *shiA* translation. RyhB also contributes to constant expression

of Fur during high- or low-iron conditions by binding to an upstream region of *fur* mRNA and negatively affecting its stability, creating a negative feedback loop (155). In addition, RyhB activity is inhibited by an sRNA sponge that is generated from the 3′ processed region of the *leuZ* tRNA (67).

Whereas RyhB from *E. coli* was the first characterized iron-regulated sRNA, its discovery was followed by identification of many additional iron-regulated sRNAs in various other bacteria (20, 91). These sRNAs are RyhB homologs or functional analogs, i.e., sRNAs that exert a similar physiological role in iron metabolism but do not show significant sequence similarity to RyhB. RyhB homologs in other bacteria regulate different pathways in addition to regulating iron homeostasis. For example, RyhB from *Shigella dysenteriae* regulates virulence genes (88), whereas the genome of *S. enterica* encodes two *ryhB* copies that have overlapping target sets and support survival under specific environmental conditions, e.g., replication inside macrophages (95, 108). Other pathogenic bacteria, such as *Yersinia pestis*, also encode two RyhB homologs, yet their regulatory roles in virulence are not fully understood (24). The *ryhB* gene of *Vibrio* species is longer (>200 nucleotides) in comparison to *ryhB* from *E. coli*. Despite these additional nucleotides, its target repertoire resembles that of *E. coli*, with additional targets involved in the regulation of motility, biofilm formation, and chemotaxis (83). The most thoroughly studied RyhB analogs are the PrrF sRNAs from *Pseudomonas aeruginosa*. *P. aeruginosa* encodes two PrrF sRNAs, PrrF1 and PrrF2, whose sequences are almost identical (93%) (164). The PrrF sRNAs regulate many of the same RyhB targets that have been identified in *E. coli*, but they also regulate other pathways in *P. aeruginosa* such as virulence (122) and quorum sensing (26).

Despite RyhB belonging to the most-studied Hfq-binding sRNAs, global high-throughput approaches have now revealed various unexpected RyhB targets (67, 82). For example, under iron-limiting conditions RyhB was found to interact with several genes of the flagellum regulon, suggesting RyhB could have regulatory functions beyond iron metabolism.

QUORUM SENSING

Quorum sensing is the ability of bacteria to share information about their vicinal community based on the secretion and detection of small molecules called autoinducers (96, 163). Processes controlled by quorum sensing, such as bioluminescence, secretion of virulence factors, and biofilm formation, are unproductive when undertaken by an individual bacterium but become effective when undertaken by the group. Thus, quorum sensing allows bacteria to function as multicellular organisms.

Among gram-negative bacteria, quorum sensing has been most thoroughly studied in marine *Vibrios* and controls various metabolic pathways as well as pathogenicity-related functions and involves posttranscriptional gene regulation by regulatory RNAs (141). The quorum-sensing circuits of bioluminescent *Vibrio harveyi* (a.k.a. *Vibrio campbellii*) and the major human pathogen *V. cholerae* have been systematically investigated, revealing many conserved autoinducers, receptors, and regulators (96). For example, both species produce highly similar sRNAs called Qrr (quorum regulatory RNA): four by *V. cholerae* and five by *V. harveyi* (71, 142, 146). Transcription of the Qrr sRNAs relies on the phosphorylated form of the response regulator LuxO, which receives the phosphate from the LuxU relay protein. The phosphorylation status of LuxU in turn is determined by several receptor proteins that change their activity depending on the binding of an autoinducer molecule (90) (**Figure 5**). When autoinducer concentrations are low (e.g., at low cell density), the receptors function as kinases and thus induce Qrr transcription, whereas autoinducer binding (e.g., at high cell density) converts them into phosphatases, resulting in decreased Qrr production (62, 90).

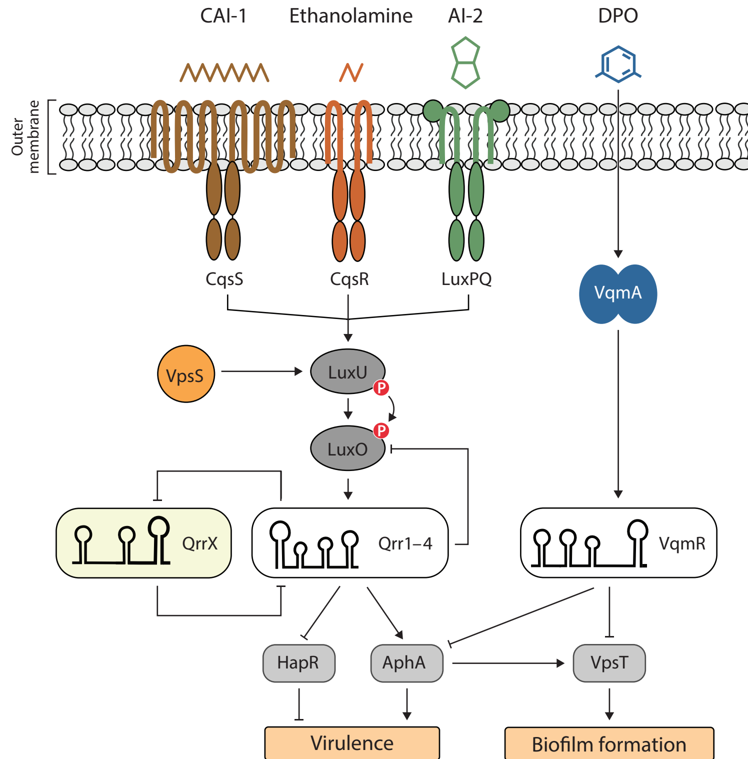

Figure 5

sRNA-mediated regulation of quorum sensing in *Vibrio cholerae*. Quorum sensing is controlled by two independent pathways involving sRNAs. The Qrr1–4 sRNAs are transcribed by phosphorylated LuxO and regulate quorum sensing, among other processes. Phosphorylation of LuxO is controlled by three membrane-associated receptors (CqsS, CqsR, and LuxPQ) and cytoplasmic VpsS that function as kinases when autoinducer concentrations are low and turn into phosphatases at high autoinducer levels. Qrr1–4 act at the posttranscriptional level to inhibit *hapR* (encoding a key regulator of high–cell density behavior), whereas Qrr2–4 induce *aphA* (encoding a key regulator of low–cell density behavior, including biofilm formation and virulence). Qrr1–4 levels are further controlled by the QrrX sponge RNA, which promotes their degradation. Independently of the Qrr-controlled quorum-sensing pathway, the VqmR sRNA, which is activated by the VqmA transcription factor together with the autoinducer DPO (3,5-dimethyl-pyrazin-2-ol), regulates biofilm formation and virulence by inhibiting *vpsT* and *aphA*, respectively.

The Qrr sRNAs are Hfq-dependent regulators and control multiple target mRNAs using a base-pairing mechanism (56, 133, 134). Among these targets, repression of *hapR* and activation of *aphA* are likely most relevant for quorum-sensing control (71, 125). Both the *hapR* (*luxR* in *V. harveyi*) and *aphA* mRNAs encode transcription factors; however, whereas HapR is a key regulator of behavior at high cell density, AphA is active at low cell density (5). Of note, many *Vibrio* species including *V. cholerae* and *V. harveyi* initiate complex regulatory programs such as biofilm formation and virulence gene expression at low cell densities, and AphA is a crucial activator of these processes (73). In *V. cholerae*, all Qrr sRNAs, i.e., Qrr1–4, inhibit the expression of *hapR*, whereas only Qrr2–4 activate the *aphA* mRNA. The reason for this difference is that Qrr1 is slightly shorter than Qrr2–4 and lacks a crucial sequence stretch that is required to interact with the *aphA* transcript (133). Base-pairing of Qrr1–4 with *hapR* sequesters the mRNA's RBS, resulting in translation repression and transcript degradation (7, 33, 71, 124). By contrast, the regulatory mechanism underlying activation of *aphA* by Qrr2–4 is not fully understood (133). In addition

to *hapR* and *aphA*, the Qrr sRNAs control the expression of several other targets, including the mRNA encoding its own transcription factor, LuxO. Base-pairing of the Qrr with the *luxO* transcript results in translation inhibition establishing a negative feedback loop that fine-tunes quorum-sensing performance (147). Of note, RNA duplex formation between the Qrrs and *luxO* does not seem to induce target degradation but rather involves a mechanism of target sequestration (33, 147).

A hallmark of nearly all quorum-sensing systems is their ability to synchronize gene expression, and in *Vibrio* species, the Qrr sRNAs are key to this process. However, the Qrrs had been documented to display a high intracellular stability (57, 134), suggesting that additional mechanisms must be at play to allow rapid transitioning between low– and high–cell density behaviors. Indeed, two regulatory principles have been documented to address this problem. In *V. harveyi*, interaction of the Qrrs with its target mRNAs has different consequences for the stability of the sRNAs. Specifically, interaction of the Qrrs with *aphA* or *luxMN* (encoding a quorum-sensing receptor) results in sRNA degradation and thus transition into high–cell density behavior (33). In contrast, *V. cholerae* (and related *Vibrio* species) produces a Qrr-specific sponge RNA, called QrrX, that base-pairs with all Qrr sRNAs and promotes their degradation by RNase E (57). Transcription of *qrrX* is controlled by QrrT, a LysR-type transcription factor that activates QrrX expression when cell density increases. Mutation of *qrrX* results in delayed quorum-sensing transition and aberrant biofilm formation, indicating that accurate control of Qrr sRNA levels is key for quorum-sensing performance in *Vibrio*.

In addition to the Qrr-controlled quorum-sensing pathway, most *Vibrio* species contain at least one other quorum-sensing system (**Figure 5**). This system is independent of LuxU and LuxO but also involves an Hfq-binding sRNA called VqmR (99). Expression of *vqmR* is induced by VqmA, an orphan LuxR-type transcriptional regulator that is activated by binding of the autoinducer molecule DPO (3,5-dimethyl-pyrazin-2-ol) (55, 166). DPO is made from L-threonine and L-alanine, and its synthesis depends on threonine dehydrogenase (Tdh) (103). The VqmR sRNA regulates multiple targets. Repression of the *vpsT* and *aphA* mRNAs has been most intensively studied. As pointed out above, AphA is crucial for various low–cell density gene expression programs including virulence. Consequently, lack of *vqmR* increases the expression of pathogenicity-related genes, whereas addition of DPO has the inverse effect (49). Similarly, DPO inhibits biofilm formation, by means of VqmR-mediated repression of *vpsT*, which encodes a central activator of biofilm formation (99, 103). AphA also activates the transcription of *vpsT* (167), indicating that together VqmR, AphA, and VpsT orchestrate a type 2 coherent feed-forward loop that links biofilm formation with DPO concentrations in *V. cholerae*. Importantly, global interaction studies and transcriptomic analysis revealed that VqmR could have dozens of additional targets in *V. cholerae* and thus might regulate quorum sensing through additional base-pairing interactions (57).

PERSPECTIVE

In the last few years, we have learnt a great deal about the various physiological roles of bacterial sRNAs and how they are integrated into cellular networks to control gene expression at the post-transcriptional level (8). Since it is impossible to summarize all this progress in one manuscript, we have here focused on four well-characterized examples from gram-negative bacteria (carbon metabolism, envelope stress, iron homeostasis, and quorum sensing). In all cases, the sRNAs affect key functions in their respective regulatory pathways and typically work together with transcription factors to match gene expression output with the relevant environmental conditions. Important drivers for these developments have been the introduction of new technologies that enabled rapid sRNA identification based on genomic and transcriptomic data and the discovery of sRNA–target RNA interactions at a genome-wide scale (52, 80).

These developments not only have implications for gram-negative model organisms that have been the focus of this review but also provide the necessary tool set to address similar questions in less-studied bacteria that have important roles in human and animal health or that are emerging pathogens. For example, transcriptomic analyses have enabled the discovery of regulatory RNAs in the human intestinal commensal *Bacteroides thetaiotaomicron* (126) and the cancer-associated, opportunistic pathogen *Fusobacterium nucleatum* (115). For the latter, it is interesting to note that functional characterization of the FoxI sRNA revealed a regulatory function that resembles the activity of the envelope stress–associated sRNAs RybB, MicA, MicL, MicV, and VrrA (**Figure 3*a***). Like the case of these sRNAs, *foxI* transcription is controlled by σ^E, and the FoxI sRNA inhibits the production of membrane proteins at the posttranscriptional level (116). However, given that the *F. nucleatum* genome does not encode an Hfq homolog, it is possible that the molecular mechanism underlying target regulation differs from previously studied examples. Thus, it will be interesting to investigate which of the general concepts developed for sRNA-mediated gene control in model organisms will also hold true in other species and where new regulatory principles can be discovered.

With respect to sRNA characterization, it is likely that as ever more RNA-RNA networks are revealed [e.g., through RNA interaction by ligation and sequencing (RIL-seq) analysis or related methods (82)], it will become feasible to predict the regulatory and physiological roles of an sRNA based on the abundance and function of its target mRNAs. Of note, a single sRNA can be part of more than one regulon and thus can help to foster interactions across regulatory networks. Similar approaches have previously been incorporated into computational target prediction tools and have helped the functional annotation of sRNAs in various organisms (165). In addition, such technologies can provide the molecular basis to map sRNA-target sites, which will facilitate our general understanding of how sRNAs select target transcripts from the pool of cellular RNAs. This information can then be harnessed to improve bioinformatics tools for target prediction, which will be particularly useful for RNA-focused studies on microorganisms where genetic manipulation is cumbersome or currently impossible to achieve.

Finally, given the recent advantages in bacterial single-cell RNA sequencing (51), future studies could provide information on sRNA-based regulation at the population versus single-cell level, possibly revealing the role of sRNAs in phenotypic heterogeneity within a bacterial population.

DISCLOSURE STATEMENT

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