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SMALL REGULATORY RNAS IN THE ENTEROBACTERIAL RESPONSE TO ENVELOPE DAMAGE AND OXIDATIVE STRESS

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Abstract

The ability of bacteria to thrive in diverse habitats and to adapt to ever-changing environmental conditions relies on the rapid and stringent modulation of gene expression. It has become evident in the past decade that small regulatory RNAs (sRNAs) are central components of networks controlling the bacterial responses to stress. Functioning at the post-transcriptional level, sRNAs base-pair with cognate mRNAs to alter translation, stability or both to either repress or activate the targeted transcripts; the RNA chaperone Hfq participates in stabilizing sRNAs and in promoting pairing between target and sRNA. In particular, sRNAs act at the heart of crucial stress responses, including those dedicated to overcoming membrane damage and oxidative stress, discussed here. The bacterial cell envelope is the outermost protective barrier against the environment, and thus is constantly monitored and remodeled. Here, we review the integration of sRNAs into the complex networks of several major envelope stress responses of Gram-negative bacteria, including the RpoE (σ^E), Cpx and Rcs regulons. Oxidative stress, caused by bacterial respiratory activity or induced by toxic molecules, can lead to significant damage to cellular components. In *E. coli* and related bacteria, sRNAs also contribute significantly to the function of the RpoS (σ^S)-dependent general stress response as well as the specific OxyR and SoxR/S-mediated responses to oxidative damage. Their activities in gene regulation and cross-talk to other stress-induced regulons are highlighted.

INTRODUCTION

One major paradigm for RNA-based regulation in both eukaryotes and prokaryotes are small regulatory RNAs (sRNAs) that pair with messenger RNAs (mRNAs), leading to changes in translation and mRNA stability. In bacteria, rather than the highly processed very short microRNAs found in eukaryotes, these sRNAs are generally on the order of 50–200 nucleotides (nt) long, and, in the Gram-negative organisms that are the major focus of this chapter, annealing of sRNAs to their target mRNAs is usually dependent on the RNA chaperone Hfq. Annealing can lead to positive regulation of translation, by remodeling

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inhibitory RNA structures or blocking access of negative regulators (for instance, RNases or the Rho transcription termination factor), or negative regulation, by inhibiting translation, recruiting ribonucleases, or both. A given sRNA can have multiple targets, and can carry out both negative and positive regulation (1–3).

A member of the conserved family of Sm and Sm-like (LSm) proteins, Hfq assembles as a stable, homo-hexameric ring which offers three principal binding sites for RNA: the proximal and distal surfaces of the ring, as well as the lateral rim. Hfq binds sRNAs on the proximal face, recognizing the uridine-stretch at the 3' end of the sRNA's Rho-independent terminator sequence. In the absence of the chaperone, almost all of these Hfq-binding sRNAs become quite unstable, and that may be sufficient to explain the loss of sRNA function in *hfq* mutants (see, for instance, (4)). In addition, Hfq binds to mRNAs, frequently but not always via its distal surface. *In vitro*, Hfq promotes pairing of sRNAs and mRNAs, suggesting that it is likely to do that as well *in vivo* (5–7). Overall, for the discussion here, the phenotypes of *hfq* mutants serve as a starting point for understanding the role of sRNA-based regulation in *E. coli* and *Salmonella*. Many, but not all of these phenotypes are now understood, and point to major roles for sRNAs in the use of alternative sigma factors and the response to stress – including envelope and oxidative damage – in bacteria.

Loss of of Hfq-dependent Regulation Leads to Low Levels of RpoS

The first description of phenotypes of a mutation in *hfq* in *E. coli* (8), including osmosensitivity and elongated cell shape, were noted as consistent with the phenotypes of mutants of *rpoS* (encoding the alternative sigma subunit of RNA polymerase, RpoS (also called σ^S). We now know that at least three sRNAs, DsrA, RprA, and ArcZ, each produced under different conditions, interact with Hfq to positively regulate RpoS translation (9) (Figure 1A). Being a major stress sigma factor, RpoS controls more than 10% of all protein-coding genes in *E. coli* (10–12); during stationary phase, the RpoS-mediated general stress response provides resistance to a variety of cell damaging conditions including, for example, oxidative stress, low pH, and high osmolarity (9). The demonstration that the *hfq* mutant was defective in the production of RpoS (13, 14) thus explained many of its phenotypes, including its sensitivity to high osmolarity and low pH.

In addition to the direct effect of sRNAs and Hfq on RpoS synthesis, a set of sRNAs are RpoS-dependent (see Figure 1A), and thus any phenotypes associated with those RNAs will also be Hfq-dependent, both for their expression and for their own function and stability. For instance, GadY, an Hfq-dependent sRNA synthesized dependent upon RpoS in *E. coli* and *Shigella*, positively regulates GadX and GadW, transcriptional activators of genes for glutamate-dependent acid resistance (15, 16), and the *E. coli*-specific SdsN represses genes involved in the metabolism of oxidized nitrogen compounds (17). More widely conserved are the RpoS-controlled sRNAs SraL and SdsR. A single target has been identified for SraL (*tig* mRNA encoding the chaperone Trigger Factor (18)), while SdsR interferes with translation of more than 20 transcripts in *E. coli* and *Salmonella* and has been implicated in the response to antibiotics and in mismatch repair upon DNA damage (19–22).

Loss of Hfq-Dependent Regulation Leads to High Levels of RpoE

A second major phenotype of *hfq* mutants was first highlighted in studies examining the transcriptional changes when Hfq was absent (23). These experiments were carried out specifically under conditions under which RpoS is not abundant, *i.e.* early exponential phase. Transcripts encoding outer membrane beta-barrel proteins were significantly over-represented, and generally were up-regulated in the *hfq* mutant, while RpoE (or σ^E, encoded by the *rpoE* gene), the alternative sigma factor that regulates outer membrane stress responses, was induced. Combined with studies by others in *Salmonella* (24), and studies of specific sRNAs and their targets (25) these observations have led to evidence that loss of sRNA down-regulation of outer membrane protein synthesis leads to an RpoE-inducing stress. Thus, *hfq* mutants express RpoE-dependent genes at a high level, discussed further below.

Specialized Sigma Factors and Hfq: Changing the Sign of Regulation

Worth noting here is that the two major phenotypes of *hfq* mutants discussed above are effects on the levels of two specialized sigma factors. Sigma factors act with core RNA polymerase to direct it to particular promoters, and thus, other than competing for core, are not themselves capable of carrying out negative regulation. Therefore, any negative regulation dependent upon a specialized sigma factor is likely indirect, by positive regulation of a negative regulator, including, in many cases, sRNAs (see Figure 1B). Thus, in the initial studies of *hfq* mutants in the RpoE response (23), many up-regulated genes have now been shown to be negatively regulated by RpoE-dependent sRNAs (26). This switch in the sign of regulation is also seen for repressors (for instance, positive regulation by the Fur repressor (27); see Figure 1B, and chapter by Chareyre and Mandin), and thus an unexpected direction of regulation should lead to examining the possible involvement of Hfq and sRNAs.

CONSTRUCTING SMALL RNA REGULATORY NETWORKS

General Principles and Expectations

For any stress, one can consider the roles of sRNAs, and what they tell us about how the stress is sensed and responded to. As shown in Figure 1A for the RpoS general stress response, sRNAs can act upstream, to regulate expression of a transcriptional regulator, or downstream, as part of the regulon. In some cases, sRNAs do both, providing feedback regulatory loops (see chapter by Brosse and Guillier). Possibly because sRNAs oftentimes work in a stoichiometric fashion (28), *i.e.* the regulatory RNA is degraded together with the mRNA it is pairing with, the promoters of sRNAs are frequently among the best regulated and most robust in a given stress regulon (see, for instance, (29)). Thus, they can also serve as excellent reporters for the stress response. The general expectations discussed here are primarily relevant to sRNAs expressed as part of a stress response (*i.e.* those expressed dependent on the transcriptional signals for the response) and are outlined here to provide some guidelines for considering the role of sRNAs within regulons.

sRNAs as guides to a stress response—What sRNAs are expressed in response to the stress (and/or are regulated by the known transcriptional regulators for that stress)? Presumably these sRNAs have effects that help in repairing or avoiding the damage from the

stress. Can that contribution to avoiding or overcoming stress be demonstrated? If not, do the sRNA targets suggest novel components of the stress response, not previously appreciated?

Are there sRNAs expressed as part of other regulons that contribute, positively or negatively, to the stress under consideration? sRNAs can provide interactions between different regulons, modulating or setting hierarchies for regulon expression.

A few clear examples of the types of sRNA functions in stress responses are noted here; some of these are discussed in more detail elsewhere in this chapter or in other chapters.

Reinforcing or helping implement the stress response (positive feedback loops)—Spot 42 sRNA synthesis is negatively regulated by cAMP/CRP and negatively regulates many operons involved in alternative carbon source use that are dependent upon cAMP (30). Thus, Spot 42 contributes to reducing the basal levels of these operons when cAMP is low (favoring efficient use of glucose/favored carbon sources).

Minimizing stress signals (negative feedback loops)—The negative feedback loop in which RpoE-dependent sRNAs (as well as others) down-regulate translation of many outer membrane proteins is discussed further below. In another, more indirect example of negative feedback, RyhB, made when iron is limiting, inhibits synthesis of non-essential iron binding proteins, thus helping to overcome the stress by increasing the availability of iron (31).

Connecting regulons/stress responses/setting hierarchies—sRNAs connect different regulons, for purposes that are not always yet well understood. In two cases noted here, sRNAs modulate the interaction between specific regulatory responses and a specialized sigma factor. For instance, the Hfq-dependent OxyS sRNA, synthesized under the control of OxyR, negatively regulates RpoS (32). Given that OxyR regulates genes involved in the response to oxidative stress, which RpoS does as well, it would seem that OxyS helps the cell to use the specific (OxyR-dependent) response rather than the general stress response under some conditions. PhoPQ, a two-component system that is activated at low Mg^{2+} concentrations and leads to the synthesis of genes regulating Mg^{2+} homeostasis as well as LPS modifications, is negatively regulated by the RpoE-dependent MicA sRNA (33).

INVOLVEMENT OF SMALL RNAS IN THE ENVELOPE STRESS RESPONSE

Cell Envelope Structure And Function In Gram-Negative Bacteria

The cell envelope represents a barrier shielding the bacterium from its environment, and allowing the selective passage of both harmful and beneficial molecules (34). In Gram-negative bacteria, the envelope is composed of two concentric membrane layers which enclose the periplasmic space containing a thin peptidoglycan (PG) cell wall (35). The inner membrane (IM) separating the cytoplasm from the periplasm is a phospholipid bilayer and the proteins associated with, or integrated in, the IM are frequently involved in key cellular processes including energy generation, signal transduction, metabolism, transport, and cell division (35). The periplasm is an aqueous cellular compartment densely packed with proteins and harbors the mesh-like PG layer which is formed from linear amino

sugar polymers cross-connected via oligo-peptide chains (36). Structural integrity of the cell envelope is ensured by the tight linkage of the PG layer to the cellular outer membrane (OM) via the lipoprotein Lpp (also referred to as Braun's lipoprotein (37)). Lpp is the most abundant protein in *E. coli* (>500.000 copies/cell; (38)), and lipids attached to the N-terminus of Lpp embed it into the OM. Concomitantly, Lpp can be covalently attached to the peptide cross-bridges of the PG layer via its carboxy-terminal end (39). In contrast to the IM, the OM is an asymmetric bilayer consisting of phospholipids in the inner, and lipopolysaccharides (LPS) in the surface-exposed leaflet (35). LPS is a complex glycolipid composed of lipid A (a glucosamine disaccharide decorated with fatty acids anchoring LPS to the membrane), an oligosaccharide core, and an extended polysaccharide chain commonly referred to as O-antigen (40). Tightly packed LPS serves as an effective permeability barrier to hydrophobic substances (41) but certain other molecules are able to cross the OM through protein transporters. Small, hydrophilic compounds can diffuse through the lumen of porins, which are highly abundant β-barrel outer membrane proteins (OMPs) that only discriminate their substrates by size (34). Gated, high-affinity uptake of ligands including siderophores, vitamins and carbohydrates is mediated by an additional class of larger integral β-barrel OM proteins. Active transport via so-called TonB-dependent receptors into the periplasmic space involves coupling to a protein complex localized in the IM (42).

The integrity of the cellular envelope is essential for bacterial survival, and consequently, its architecture and composition is tightly regulated. Bacteria have evolved a suite of stress responses which function in monitoring impairment or deficiencies of the envelope, and govern adaptation of the bacterial gene expression to alleviate stress (43). In *E. coli* and related enterobacteria, at least five major envelope stress responses coordinately function to maintain membrane homeostasis (44). While no sRNAs have been associated yet with the minor membrane stress responses coordinated via the BaeS/R or the Psp systems, the three major pathways (regulated through σ^E, the Cpx and the Rcs system, respectively) all rely on the activity of regulatory RNAs. Intriguingly, sRNAs not only function as effectors regulating individual target genes but also in mediating the extensive cross-talk and adaptation of individual stress responses.

sRNAs in the RpoE-Mediated Envelope Stress Response

Maintenance of OM homeostasis in *E. coli* and related bacteria relies on the dedicated activity of the alternative σ factor, RpoE, which is encoded by the *rpoE* gene and co-transcribed with *rseABC* from a σ^{70}-dependent promoter (45). Under nonstress conditions, RpoE is only expressed at a low basal level (38), and sequestered to the plasma membrane in an inactive state by its cognate anti-sigma factor, RseA (46) (see Figure 2A). The activity of RpoE is under complex control, and tuned to perturbations of OMP folding, the status of LPS, as well as nutrient availability and growth phase (47). The accumulation of misfolded OMPs within the periplasmic space is the most thoroughly characterized signal triggering RpoE activation (47) (Figure 2A). The C-termini of unfolded OMPs – which are inaccessible in properly assembled porins – are recognized by the periplasmic PDZ domain of a serine protease, DegS (48). The resulting conformational change initiates a protease cascade resulting in degradation of RseA, and consequent release of RpoE into the cytoplasm (47). Maximal induction of RpoE requires the integration of a second activating

signal. In response to mislocalized LPS (49), the RseB protein, which protects the RseA anti-sigma factor from cleavage by DegS, is displaced.

The RpoE-orchestrated envelope stress response possesses two parallel branches, one acting through proteins, and one mediated by sRNAs. In *E. coli*, RpoE drives transcription of ~100 genes, including genes encoding all components required to assemble and transport OMPs and LPS to the OM (29, 50, 51). However, the rate at which new OM components are synthesized may easily exceed the capacity of chaperones and transporters. While RpoE, as for all sigma factors, is intrinsically restricted to function as a transcriptional activator, it employs regulatory RNAs to function as repressors of gene expression at the post-transcriptional level.

The strongest promoters within the RpoE-regulon control RpoE itself, and two Hfq-dependent sRNAs, RybB and MicA (29, 52, 53). Both sRNAs act as global regulators of the envelope stress response, and together govern expression of >30 targets in *E. coli*. Most prominently, RybB and/or MicA repress all major OMPs, as well as several lipoproteins and transporters (26). Rapid decay of these target transcripts results in an immediate relief for the periplasmic folding machinery. In addition, the target suites of RybB and MicA also encompass genes involved in production of outer membrane vesicles, and the response regulator *phoP* (26, 33) (see below).

Mutants of *rpoE* in *E. coli* are not viable, but cells can be depleted for RpoE by overexpression of its antagonists, RseA and RseB (54, 55). The important contribution of the two regulatory RNAs, RybB and MicA, to cell homeostasis is reflected by their ability to counteract the growth and viability phenotypes associated with loss of RpoE (26).

Mechanistically, both RybB and MicA exert their regulatory activity by a variety of means. RybB employs a conserved seed region of 16 nt located at the very 5’ end of the molecule to interact with its target genes (56). Depending on the location of the base-pairing site on the target mRNA, RybB is able to interfere with ribosome association at the translational start site (57), to promote ribonuclease cleavage in the coding sequence of target mRNAs, or to interrupt structural elements within the 5’ UTR(56). In the latter case, base-pairing can abrogate the protective effect of 5’ terminal structures which have been shown to stabilize transcripts by restricting access of nucleases, including RNase E (58, 59). Similar to RybB, the first 24 nt of MicA are hyper-conserved, and involved in regulation of its target genes. In contrast to RybB, the seed region of MicA is less stringently defined, and the first 7 nt of the molecule are dispensable for regulation of approximately half of its targets (26). In the majority of cases, predicted interaction sites of MicA overlap the translation initiation region of its target transcripts; base-pairing of MicA close to the start codon for one noteworthy target, *phoP* (also see below), has been confirmed (26, 33). The regulatory mechanisms underlying the observed repression of other targets are yet to be experimentally validated (26).

Both RybB and MicA are highly conserved in numerous enterobacteria, and likely contribute to OMP homeostasis in these species (60). Albeit not conserved at the sequence level, the human pathogen *Vibrio cholerae* encodes a functional homologue of these *E.*

coli sRNAs. The 140 nt VrrA is under strict control of RpoE and represses translation of the major OMPs, OmpA and OmpT, as well as the biofilm matrix component, RbmC, and the ribosome binding protein, Vrp, in response to perturbations of the OM (61). Of note, expression of the VrrA sRNA is not affected in an *hfq* deletion mutant of *V. cholerae*, and possibly for this reason, the RNA chaperone is required for regulation of Vrp and OmpT, but not the other targets (61–64).

In *E. coli*, a third sRNA is transcribed from an RpoE-dependent promoter positioned within the *cutC* coding sequence. The 308 nt MicL (a.k.a. RyeF (53); SlrA (65)), is processed by a currently unknown cleavage mechanism to a smaller, ~80 nt transcript (MicL-S), and both isoforms associate with the Hfq chaperone (66). In stark contrast to the many targets of RybB and MicA, MicL appears to interact with only few transcripts (6), and the only experimentally confirmed target of this sRNA is currently the *lpp* gene (66). By binding to a target sequence located within the beginning of the coding sequence, MicL inhibits translation of *lpp* mRNA, and triggers accelerated degradation of the transcript. Why is repression of Lpp synthesis beneficial to the σ^E-dependent envelope stress response? As an OM lipoprotein, folding and transport of Lpp are dependent on the LolAB system. The same machinery is however also required for the installation of other lipoproteins, including BamD and LptE, which are essential to chaperone OM insertion of OMPs and LPS, respectively (35). Consequently, MicL-mediated down-regulation of Lpp may relieve envelope stress by reducing the demand on the Lol machinery, indirectly promoting the correct assembly and localization of other OM components.

sRNAs In The Cpx-Mediated Envelope Stress Response

Maintenance of OM integrity via the RpoE response is complemented by another major regulon primarily controlling homeostasis of the periplasm and the IM. The central hub of the Cpx envelope stress response is a two-component system consisting of the IM-localized histidine kinase, CpxA, and its cognate DNA-binding response regulator, CpxR (67). While the molecular characteristics of the stimulus perceived by CpxA remain to be determined, numerous environmental cues triggering the signaling cascade have been identified in the past, including alterations in IM composition (68), alkaline pH (69), and overexpression of the OM lipoprotein, NlpE (70). Recent work also reports that the Cpx system may respond to perturbations of the PG cell wall (71). Activation of CpxA results in autophosphorylation and phosphotransfer to CpxR, which consequently enables transcriptional control of the Cpx regulon comprising more than 100 genes (69) (Figure 2B). In the absence of an inducing signal, CpxA acts as a phosphatase on CpxR~P to rapidly inactivate the response regulator. Different from sigma factors, CpxR functions as both an activator and a repressor (69). It down-regulates the expression of envelope-associated, macromolecular complexes including cellular appendages (72) and respiratory complexes (73), and at the same time fosters transcription of periplasmic proteases and chaperones to alleviate the burden of protein folding (67). Another highly up-regulated gene controlled by CpxR is *cpxP*, encoding a periplasmic inhibitor of the Cpx pathway which likely exerts its negative feedback control by masking the CpxA sensory domain (74, 75).

Processing of the 3' UTR of *cpxP* transcript by the major endonuclease, RNaseE, liberates a stable, ~60 nt long mRNA fragment, termed CpxQ, which associates with the Hfq chaperone (52, 76). A transcriptomic approach in *Salmonella* revealed that CpxQ acts as a trans-encoded sRNA negatively regulating multiple targets (76). Strikingly, the CpxQ regulon is enriched for proteins localizing to the inner membrane, including those controlling the proton motive force. Employing one of two seed regions, CpxQ also represses translation of *skp* (encoding a periplasmic chaperone) and *nhaB* mRNAs (encoding a proton/sodium antiporter). Different from other chaperones, Skp is able to mistarget OMPs into the IM if the OM insertion machinery is compromised. By down-regulating *skp*, CpxQ prevents the unrestricted flux over the IM via ion-permeable pores that would result from OMPs within the IM, and thus protects cells from the collapse of the PMF. CpxQ addresses a similar problem by repression of *nhaB* as overexpression of the protein, and concomitant increase in proton uptake, results in a loss of membrane polarization. In protecting the integrity of the IM and the PMF, CpxQ appears to play a major function as a repressive arm of the Cpx response (76, 77).

Additional sRNAs have also been shown to be integrated into this complex network. The sRNAs OmrA/B, MicC and MicF sRNAs are indirectly positively regulated through cross-talk with the EnvZ/OmpR two-component system (see below). CyaR and RprA sRNAs appear to be both directly and indirectly controlled by the Cpx response, with some differences depending on whether EPEC or *E. coli* K12 was examined (78).

Expression of CyaR (formerly RyeE; (79)), a conserved, Hfq-associated sRNA, is under complex regulation by both the Crp and Cpx regulons. Crp induces CyaR under conditions when cyclic-AMP levels are high (80–82). CpxR functions as a transcriptional repressor of the *cyaR* promoter (72); CyaR in turn functions as a post-transcriptional repressor of several target genes, including the *yqaE* transcript (80). As expression of *yqaE*, encoding an IM protein of currently unknown function, is at the same time induced by CpxR~P at the transcriptional level, repression of *cyaR* by CpxR integrates the sRNA into a coherent feed-forward loop within the Cpx regulon (72, 80). The physiological importance of this regulation has not been examined; presumably, the CpxR effect on the *cyaR* promoter may not be significant under conditions when cyclic-AMP levels are high (*i.e.*, growth in poorer carbon sources), and repression of *yqaE* may not be important under those conditions.

The promoter of *rprA*, a conserved enterobacterial sRNA, is activated under conditions of high CpxR-P, and is directly bound by CpxR~P. Overexpression of RprA in turn feeds back to repress the Cpx response, indirectly via a currently undefined target (78). However, *rprA* expression is primarily controlled by an additional envelope stress response, the Rcs pathway, discussed below.

Integration of the Cpx and Rcs Envelope Stress Responses Via RprA sRNA

Damage of the surface-exposed LPS, mutations in genes required for disulfide bond formation in the periplasm, and perturbations of PG cell wall biosynthesis are all cues triggering the Rcs phosphorelay (83, 84) (Figure 2C). Signal transduction in this stress response system is more complex than in conventional two-component systems: under inducing conditions, the surface-exposed lipoprotein, RcsF, likely inactivates the periplasmic

IgaA repressor to trigger a phosphorelay. Upon activation, RcsC is autophosphorylated, and phosphotransfer via RcsD relays the signal to the cognate response regulator, RcsB. RcsB in turn controls the transcription of the Rcs stress response by binding to target promoters either as a heterodimer in cooperation with an additional DNA-binding protein (RcsA in the case of activation of colanic acid production), or as a homodimer (as is the case for the *rprA* gene). Originally identified in *E. coli* as one of the sRNAs activating *rpoS* translation (85), RprA has recently been demonstrated to post-transcriptionally modulate expression of more than 60 additional genes in *Salmonella* (86), although how many of these are direct targets remains to be explored. RprA is a substrate of RNase E, and both the full-length (107 nt) and the cleaved version of the sRNA (~50 nt) lacking the 5' end are present in the cell (86, 87). Interestingly, both variants associate with Hfq (52), and control different sets of mRNAs (86). One of the mRNA targets activated by RprA is the *Salmonella*-specific *ricI* transcript (86).

Similar to the positive regulation of *rpoS*, RprA also promotes translation of *ricI* by interference with a self-inhibitory structure within the 5' UTR of the mRNA. Of note, while only full-length RprA harbours the site required for base-pairing with *rpoS* mRNA, the *ricI* transcript is recognized via a conserved sequence stretch located downstream of the cleavage site of RprA. As expression of *ricI* is controlled by σ^S at the transcriptional level, RprA functions as the centerpiece of a post-transcriptional feed-forward loop. RicI acts as an inhibitor for conjugative transfer of the *Salmonella* virulence plasmid, pSLT, by binding to the conjugation apparatus at the cytoplasmic membrane. With regard to envelope homeostasis, the Rcs regulon might employ RprA to prevent assembly of the complex conjugation machinery when membrane integrity is compromised.

In *E. coli*, RprA is one of five currently known sRNAs (together with OmrA/B, McaS, and GcvB; (88–91)) to repress translation of *csgD*, encoding a transcriptional regulator of curli fimbriae and cellulose production (92). In addition, RprA also down-regulates expression of *ydaM*, which encodes a diguanylate cyclase involved in activating *csgD* transcription. While curli are required for efficient adhesion of bacterial cells in growing biofilms, massive synthesis of surface-exposed curli fimbriae may be detrimental to cells experiencing envelope stress (90). In addition, it has been speculated that RprA may function to balance expression of curli/cellulose and colanic acid, an additional biofilm matrix component directly controlled by the Rcs pathway (93).

Although none of the signaling components of the Cpx system has been shown to be directly controlled by RprA, overexpression of the sRNA exerts negative feed-back onto the stress response in a CpxR-dependent manner (78). Further investigation regarding the integration of the sRNA into the Cpx regulon is required, but two tempting hypotheses may explain the observed phenotype: First, a yet-to-be-identified auxiliary factor modulating CpxR activity could be under control of RprA (43). Alternatively, RprA could indirectly reduce induction of the Cpx response by contributing to stress relief. Intriguingly, one of the most up-regulated genes following pulse-overexpression of RprA in *Salmonella* is *dsbG*. Together with the CpxR-controlled *dsbB*, *dsbG* functions in disulfide bond formation within the periplasm (86, 94), and up-regulation of the gene would be consistent with the induction of the Rcs phosphorelay upon loss of DsbA (83).

Additional Pathways Mediating Envelope Homeostasis

The activity of the major envelope stress response is complemented by several additional regulatory pathways modulating membrane homeostasis and modifications. In many cases, sRNAs constitute central nodes of these systems.

The EnvZ/OmpR system is one of the most thoroughly studied two-component systems, and contributes to the maintenance of the OM by controlling expression of multiple OMPs. The environmental cues triggering activation of the sensor kinase EnvZ include increased growth temperature, acidic pH, and most importantly, increasing osmolarity (95). Phosphotransfer from activated EnvZ to its cognate response regulator, OmpR, in conditions of high osmolarity controls (among other genes) the ratio of the major OMPs, OmpC and OmpF (96). The opposite regulation of the two OMP genes by OmpR~P, *i.e.* induction of *ompC* and repression of *ompF* transcription, respectively, is further corroborated at the post-transcriptional level: While the promoter of the MicC sRNA (which represses *ompC* mRNA) is repressed by OmpR~P, the transcription factor activates production of MicF sRNA (which represses *ompF* mRNA) (97, 98).

In addition, the two homologous sRNAs OmrA and OmrB, encoded in tandem orientation on the *E. coli* chromosome, are under positive control of OmpR~P (99). Together, OmrA/OmrB repress the synthesis of the TonB-dependent receptors CirA, FecA and FepA, the OM protease OmpT, as well as the transcription factor CsgD (88, 99). In addition, OmrA/B autoregulates its own transcription by negatively regulating the *ompR-envZ* mRNA (100).

Involvement Of sRNAs In Modification Of LPS

In pathogenic bacteria, the cell surface provides numerous exposed epitopes recognized by the host's immune response after infection (101). Moreover, given its essential functions, the bacterial cell envelope is an effective target for antimicrobial peptides (AMPs). To evade the response of the host immune system, bacteria are able to tune the composition of OMPs within the OM. In addition, modifications of the LPS also contribute to the bacterial survival strategy in the presence of the host immune defense and AMPs (41).

Several sRNAs are integrated into the regulatory loops governing LPS modifications, either directly by controlling expression of modifying enzymes, or indirectly by influencing the activity of transcriptional regulators (Figure 3).

The LPS component lipid A is a common site for modifications, and can for example be subject to dephosphorylation, deacylation, or hydroxylation (102). One of the enzymes responsible for lipid A deacylation, encoded by *lpxR* in *Salmonella* and some other bacteria, including the pathogenic *E. coli* O157:H7, is post-transcriptionally repressed by MicF (103). Of note, MicF, which itself is controlled by the EnvZ-OmpR TCS, uses two RNA stretches to form base-pairing interactions both at the translation initiation site and within the coding sequence of *lpxR* mRNA.

A major regulon controlling the physiology of LPS is the PhoQ-PhoP TCS, which is activated by low levels of Mg^{2+} ions, as well as by antimicrobial peptides (104). The PhoQ-PhoP system has been extensively studied in the enteric pathogen *Salmonella* where its

integrity is essential for the infection process (105). The TCS is however widely conserved in several enterobacterial species where it is involved in the adaptation to low Mg^{2+} environments, and/or the regulation of virulence factors (104). One of the several dozen genes directly controlled via PhoQ-PhoP encodes for an sRNA, MgrR (termed Stnc560 in *Salmonella*), which was originally identified in Hfq co-immunoprecipitation experiments (53, 106). At the post-transcriptional level, the activity of MgrR is counteracted by yet another sRNA, SroC, which acts as a sponge RNA to sequester and trigger the decay of MgrR (107). SroC, processed from the *gltIJKL* mRNA encoding a glutamate/aspartate ABC transporter, was first described to directly base-pair and repress GcvB sRNA (108), where it provides a feed-forward loop regulating amino acid transport.

MgrR represses at least two mRNA targets, *ygdQ* (encoding an IM protein of unknown function), and *eptB* (*pmrC* in Salmonella) (109). The *eptB* transcript is one of the most highly deregulated genes in *hfq* mutant strains of *Salmonella* (24), and encodes a phosphoethanolamine transferase modifying the outer Kdo (3-deoxy-D-manno-octulosonic acid) unit of the LPS core (110). Thus, while directly activating other LPS–modifying enzymes, the PhoQ-PhoP TCS employs the sRNA MgrR to negatively act on *eptB* expression. EptB is barely expressed under standard laboratory growth conditions, but is transcriptionally activated by the σ^E-response, hinting at the benefit of the EptB-mediated LPS modification during OM stress. The deletion of *mgrR* from the *E. coli* chromosome, and the consequent expression of *eptB*, result in increased resistance to the AMP polymyxin B due to modification of the LPS structure (109). In addition, *eptB* mRNA is also repressed by the sRNA ArcZ, further specifying timing and degree of EptB-mediated LPS modification (111). Since ArcZ is preferentially expressed under aerobic conditions (112), the cooperative activity of both ArcZ and MgrR allow the expression of EptB only when cells encounter an Mg^{2+}/Ca^{2+}-rich, anaerobic (or microaerobic) environment (111).

Additional sRNAs mediate the crosstalk between the PhoQ-PhoP TCS and other regulons. The *phoQ* and *phoP* genes are encoded in a bicistronic operon, and the *phoQP* mRNA has been shown to be post-transcriptionally controlled by both MicA and GcvB sRNAs. However, given that *phoQ-phoP* mRNA levels are elevated in the absence of *hfq* even when *micA* and *gcvB* have been deleted from the genome, additional, yet to be identified sRNAs might contribute to the complex regulation of the TCS (113), or possibly Hfq alone can repress this mRNA (114).

MicA, induced by the σ^E-response, represses translation of the TCS by base-pairing within the translation initiation site of *phoP* (33). Repression of PhoP activity by MicA is consistent with and reinforces the activation of *eptB* (see above) under σ^E-inducing conditions. Similarly, GcvB sRNA inhibits translation initiation of *phoP* by binding a region in close proximity to the MicA pairing site (113). The post-transcriptional activity of GcvB links the PhoQ-PhoP regulon to cell metabolism, as the sRNA is mainly involved in limiting amino acid and peptide uptake under nutrient-rich conditions (115). It is intriguing, given the negative regulation of *phoP* by GcvB, that SroC provides another link between GcvB and the PhoP-dependent MgrR sRNA. With the SroC sponge acting as a competitor for mRNA targets of the different sRNAs, gene expression will not only depend on the binding affinities and relative concentrations of cognate sRNA/mRNA pairs, but also critically on

the expression level of the sponge RNA. Consequently, SroC might serve to fine-tune the coordination of post-transcriptional control via the MgrR and GcvB networks (see also the chapter by Figueroa-Bossi and Bossi).

SMALL RNAS AND THE RESPONSE TO OXIDATIVE STRESS AND DNA DAMAGE

Under aerobic growth conditions, reactive oxygen species (ROS), including, for example, superoxide and hydrogen peroxide, are generated as natural byproducts of bacterial metabolic activity (116). ROS are able to harm the cell by damaging DNA, iron-sulphur (Fe-S) clusters and other enzymes (117). Exploiting these toxic effects, one of the host defense mechanism to counteract infection with *Salmonella* and other intracellular bacteria is the production of ROS (118). In response, bacteria have evolved mechanisms to detoxify ROS, and to respond to and help the cell repair oxidative damage. As is the case for many major stress responses of Gram-negative bacteria, sRNAs are embedded in these networks (Figure 4). However, exactly what these sRNAs do is not entirely clear.

One major contributor to resistance to oxidative stress in *E. coli* and *Salmonella*, as well as other bacteria, is the general stress sigma factor, RpoS, and the genes under its control, including catalase (encoded by *katE*) required for the detoxification of hydrogen peroxide. As noted above, RpoS levels are low in *hfq* mutants because sRNAs are required to activate its translation (Figure 1A). To what extent increased sensitivity to oxidative stress of *hfq* mutants is due to the RpoS defect has not been investigated. However, ArcZ, RprA, and DsrA, the sRNAs that promote RpoS translation certainly should contribute to resistance to oxidative stress by inducing RpoS.

Aside from RpoS, microbes use additional, distinct mechanisms to detect different forms of oxidative stress. For example, the SoxR(S) and OxyR regulons mediate the response to superoxide and hydrogen peroxide stresses, respectively, in *E. coli* and related enterobacteria, and each of the systems also involves the activity of sRNAs.

sRNAs Induced by Oxidative Stress

MicF, one of the first described chromosomally encoded antisense sRNAs (98) is not only part of the OmpR-mediated envelope stress response (see above), but also integrated into the cellular program to defeat oxidative damage. Expression of MicF is induced by three homologues of the AraC family of transcription factors with overlapping activity, SoxS, Rob, and MarA, all of which help the bacteria respond to a range of toxic molecules, including antibiotics, as well as free radicals such as superoxide and nitric oxide (119). SoxS is activating upon oxidation of an Fe-S cluster in its regulator, SoxR, in response to treating cells with the superoxide-generating drug paraquat; repression of MarA by MarR is relieved in the presence of certain phenolic compounds (120), and the activity of Rob is post-transciptionally increased when bacteria encounter the iron chelators 2,2’- or 4,4’-dipyridyl (121). Under these conditions, down-regulation of OmpF by MicF contributes to increased bacterial resistance against antibiotics entering the cell through this porin. In addition, the SoxR/S regulon, and consequently MicF, are activated by nitric oxide,

produced by activated macrophages during the infection process. Deletion of *micF*, and thus loss of MicF-mediated repression of OmpF, results in similar hypersensitivity to killing of *E. coli* by murine macrophages as observed for *soxR/S* mutants (122). Complemented by its activity in repressing the LPS-modification enzyme, LpxR (see above), and the transcriptional regulator, Lrp (103, 123), MicF can be considered a bacterial virulence factor, acting by restricting the entry of a variety of harmful molecules, some of which cause oxidative stress.

One of the first Hfq-dependent sRNAs described in *E. coli* was OxyS, which, is induced as part of the OxyR regulon. Initial studies found no evidence that OxyS directly affects the resistance of bacteria to hydrogen peroxide; however, expression of the sRNA confers a protective effect against spontaneous and hydrogen peroxide-induced mutagenesis (32).

A major effect of OxyS overproduction is reduced expression of RpoS (32, 124). As noted above, RpoS contributes significantly to resistance to oxidative stress in stationary phase, e.g. via synthesis of the *katE*-encoded catalase, or the *gor*-encoded glutathione oxido reductase (125, 126). However, RpoS can also compete with RpoD for RNA polymerase, and one can imagine that the OxyS-mediated reduction of RpoS might help favor the RpoD-dependent OxyR-response to oxidative stress. Repression of RpoS once OxyR is activated might avoid redundant activation of stress genes. No direct pairing of OxyS with the *rpoS* mRNA has been detected. The current interpretation is that OxyS might compete with other sRNAs and/or the *rpoS* leader itself for binding to Hfq, thereby blocking the sRNA-dependent activation of RpoS translation (127, 128). Deletion of *oxyS* from the *E. coli* chromosome results in significantly higher intracellular levels of both hydrogen peroxide and superoxide compared to the wild-type (129). This phenotype was suggested to result from the ability of OxyS to restrict cellular respiration, and thus to reduce the burden of ROS produced during metabolic activities (129). A model in which OxyS helps to reduce the endogenous sources of the inducing stress, may be characteristic of many of the sRNA arms of stress regulons. Consistent with this idea, the best characterized OxyS target is *fhlA*, which is repressed by the sRNA through the formation of a kissing loop interaction targeting sites in both the 5' UTR and the coding sequence of the transcript (130). FhlA is a transcriptional activator of complexes involved in formate metabolism (131), whose metal cofactors likely increase cellular damage under oxidative stress conditions (132). In addition, confirmed targets of OxyS also include *wrbA* encoding an NAD(P)H:quinone oxidoreductase (133).

The ability of OxyS to function as an antimutator is the subject of a very recent study (134) (see also chapter by Altuvia, Storz and Papenfort). One of the transcripts down-regulated by OxyS is *nusG* mRNA, encoding for a highly conserved regulator of RNA polymerase (Figure 4). Together with Rho, NusG aids termination at a subset of sites, and plays an important role in silencing horizontally acquired genetic elements by inhibiting their transcription (135). One of the loci silenced by NusG and Rho is the *E. coli* cryptic prophage, *rac*, encoding amongst others the *kilR* gene (136). When NusG levels are low, *i.e.* when OxyS is active, *rac* genes are transcribed and KilR is produced. Interfering with assembly of the cell division machinery, KilR inhibits cell cycle progression, which allows the cell to facilitate damage repair. In line with this model, cells expressing OxyS and KilR

appear elongated, and the decreased rate of mutations observed in the presence of OxyS is likewise dependent on KilR (134). Repression of *nusG* by OxyS, and thus loss of silencing of cryptic prophage and other horizontally acquired elements, is reminiscent of prophage induction during the SOS response. In both cases, DNA damage in these cases may act as a signal for repressed prophages to jump ship and seek new, undamaged hosts.

RNA-based Regulation of DNA Mutagenesis

OxyS is not the only known post-transcriptional regulator of DNA damage in bacteria. Cells strictly control the rates at which mutations can occur, presumably to balance beneficial and detrimental changes of the genome. One system to limit the integration of mutations upon DNA damage is the mismatch repair (MMR), which is dedicated to the recognition and repair of mismatches in the genome. One central component of the *E. coli* MMR system is MutS, which detects and binds to DNA mismatches, thus initiating the repair process (137). While it had long been known that the cellular levels of MutS decreased in an Hfq-dependent manner when *E. coli* enters stationary growth (138, 139), the molecular principles underlying this regulation were only recently discovered. Overexpression of two sRNAs, the RpoS-dependent SdsR and the RpoS-activating ArcZ (see Figure 1A), results in significant repression of a post-transcriptional *mutS* reporter (114). More importantly however, Hfq itself binds to the 5' UTR of *mutS* mRNA independent of base-pairing sRNAs, and thereby inhibits translation of the transcript. In turn, the cell is able to react to changing environments by limiting *mutS* expression and thus raising the mutagenesis rate under both specific stress conditions (through induction of ArcZ or SdsR, respectively), as well as by titration of Hfq (through high levels of sRNAs or transcripts competing for binding).

RpoS also contributes to stationary phase mutagenesis and break repair by inducing synthesis of error-prone polymerases, the SdsR sRNA, and possibly interferes with additional mechanisms (9, 21, 22, 140). Cells lacking the sRNA GcvB were found to limit mutagenic break repair and mutagenesis in stationary phase; surprisingly, this phenotype of GcvB was entirely suppressed by loss of RpoE (141). The authors find a modest induction of RpoE in cells devoid of GcvB, and suggest the higher level of RpoE may compete with RpoS access to core polymerase. While the mode of action of GcvB and the basis for loss of RpoS activity will need further investigation, the results do point out the degree to which stress responses and the roles of sRNAs in regulating them are entangled in organisms like *E. coli*.

CONCLUSIONS AND FUTURE DIRECTIONS

sRNAs exist in essentially all bacterial stress responses, and their major roles within the regulons of specialized sigma factors has been studied extensively. In many other cases, the physiological functions of the sRNAs within a given regulon are only partially understood, but can provide new insight into what cells perceive as stress, and the pathways they can use to overcome stress or minimize intrinsic sources of stress. The recent discovery of many sRNAs encoded within the 3' UTRs of genes has expanded the identification of sRNA-dependent arms for stress responses. The sRNAs also play critical roles in cross-talk

between regulons, in setting regulatory hierarchies, and in providing feedback loops that allow rapid response to stress and rapid return to equilibrium when stresses are dealt with. Although we highlight here many examples of sRNA-mediated control that we understand, the sRNAs frequently have multiple targets for which the physiological role of the regulation remains to be understood, necessary for a full understanding of the role of the regulon.

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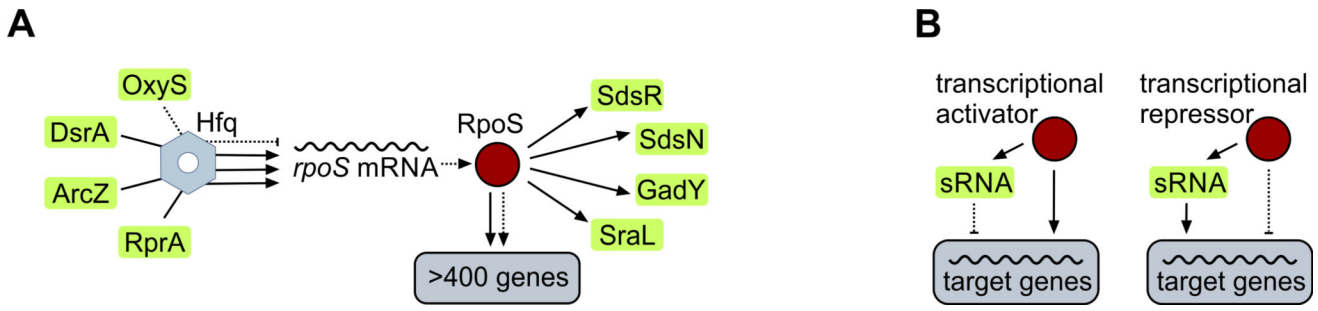

FIGURE 1.

Activity of sRNAs in the general stress response. **(A)** Together with Hfq, the sRNAs DsrA, ArcZ and RprA activate translation of the *rpoS* transcript by alleviating a self-inhibitory structure within the 5' UTR of the mRNA. The sRNA OxyS functions as an indirect, negative regulator of *rpoS* expression. The major alternative sigma factor RpoS governs the general stress response, and directly controls more than 400 genes in *E. coli* and related enterobacteria, including at least four sRNAs (SdsR, SdsN, GadY and SraL). **(B)** Transcription factors with restricted functions as activators or repressors, respectively, utilize sRNAs to facilitate opposite regulation.

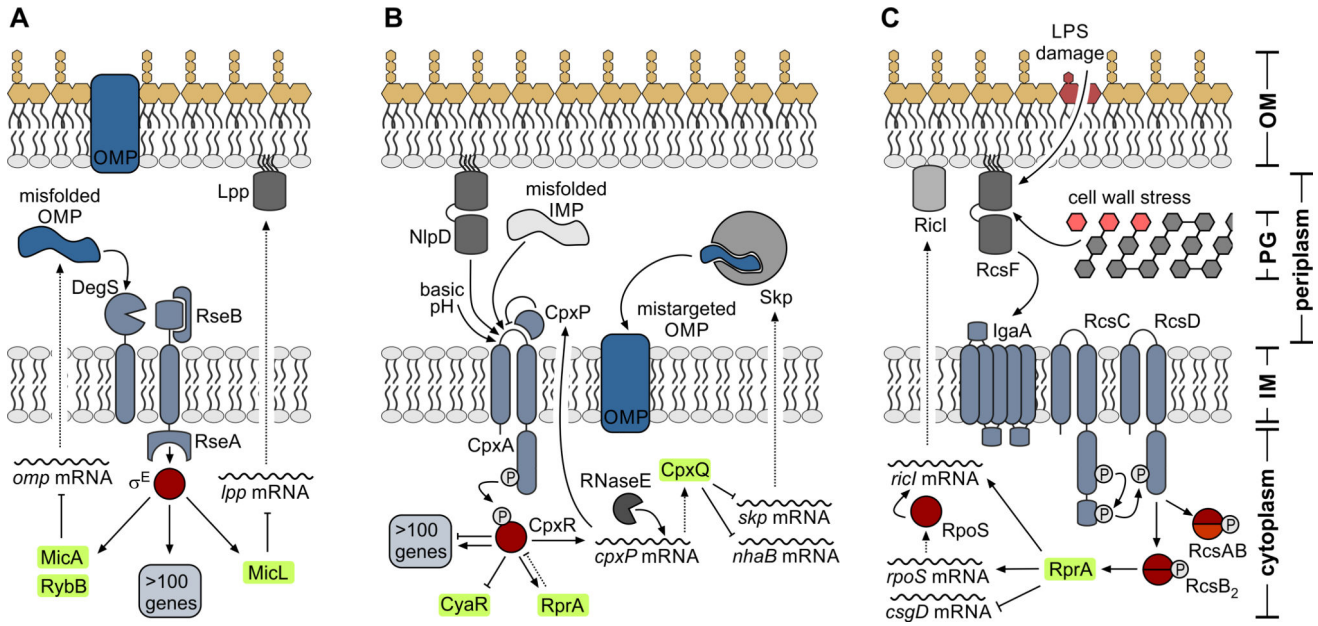

FIGURE 2.

The role of sRNAs in the major envelope stress responses. Gram-negative bacteria are diderm, with the outer membrane (OM) and inner membrane (IM) being separated by the periplasmic space containing the peptidoglycan (PG) cell wall. **(A)** OM homeostasis is regulated by the RpoE (σ^E) response. A series of proteolysis steps results in the degradation of the anti-sigma factor RseA and concomitant release of RpoE. The large regulon of the alternative sigma factor also comprises at least three sRNAs: MicA and RybB function to down-regulate the transcripts of all major OMPs to reduce the accumulation of misfolded porins within the periplasm. MicL specifically represses translation of the *lpp* mRNA. **(B)** Maintenance of the IM relies on the CpxA-CpxR TCS which amongst other targets controls expression of at least three sRNAs, CyaR, RprA and CpxQ. CpxQ is a stable fragment released by RNaseE processing from the 3' end of the *cpxP* mRNA. In association with Hfq, CpxQ functions to repress translation of several transcripts including *skp* mRNA which encodes for a periplasmic chaperone promoting the mistargeting of OMPs into the inner membrane. **(C)** The IM-associated histidine kinase RcsC, phosphotransfer protein RcsD and the response regulator RcsB constitute the core of the Rcs system. The sRNA RprA is one highly induced component of the Rcs responsem which is activated by LPS damage and perturbations of the cell wall. While acting as a negative regulator of the *csgD* mRNA, RprA also promotes translation of both the *rpoS* and the *ricI* messages. As transcription of *ricI* (encoding for an inhibitor of the conjugation machinery) is dependent on RpoS, RprA functions at the heart of a post-transcriptional feed-forward loop for RicI activity.

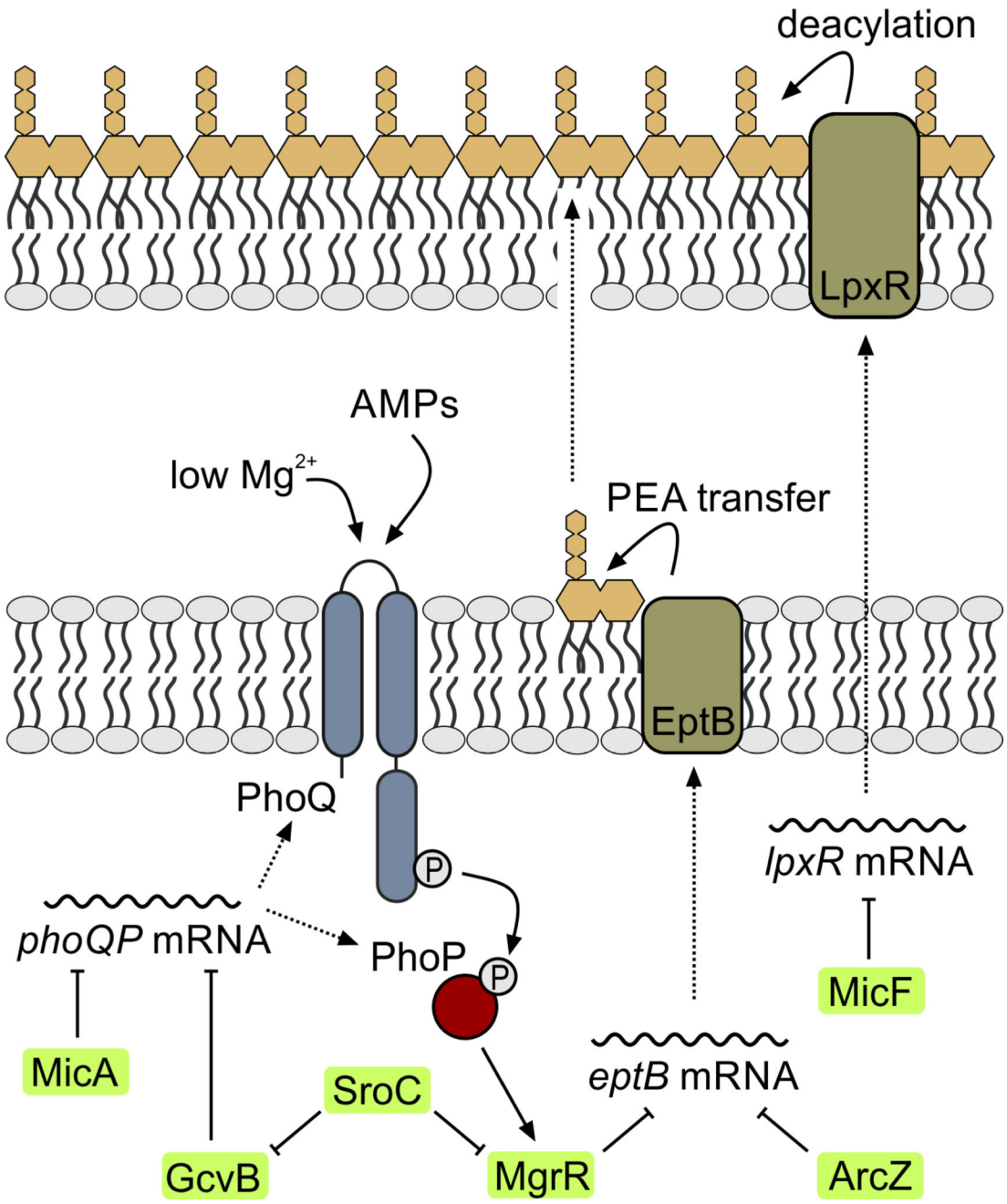

FIGURE 3.

Post-transcriptional regulation of LPS modification. The PhoPQ TCS, a major determinant of LPS modifications, is activated in response to Mg^{2+} starvation as well as by antimicrobial peptides (AMPs). Translation of the *phoPQ* bicistronic transcript is repressed by two sRNAs, MicA and GcvB. PhoPQ controls expression of MgrR which, together with ArcZ, inhibits phosphoethanolamine (PEA) addition to the LPS oligosaccharide core by EptB. Both GcvB and MgrR are regulated at the post-transcriptional level by the sRNA SroC which acts as a sponge and induces decay of its target sRNAs. Down-regulation of *lpxR* mRNA by MicF decreases lipid A deacylation.

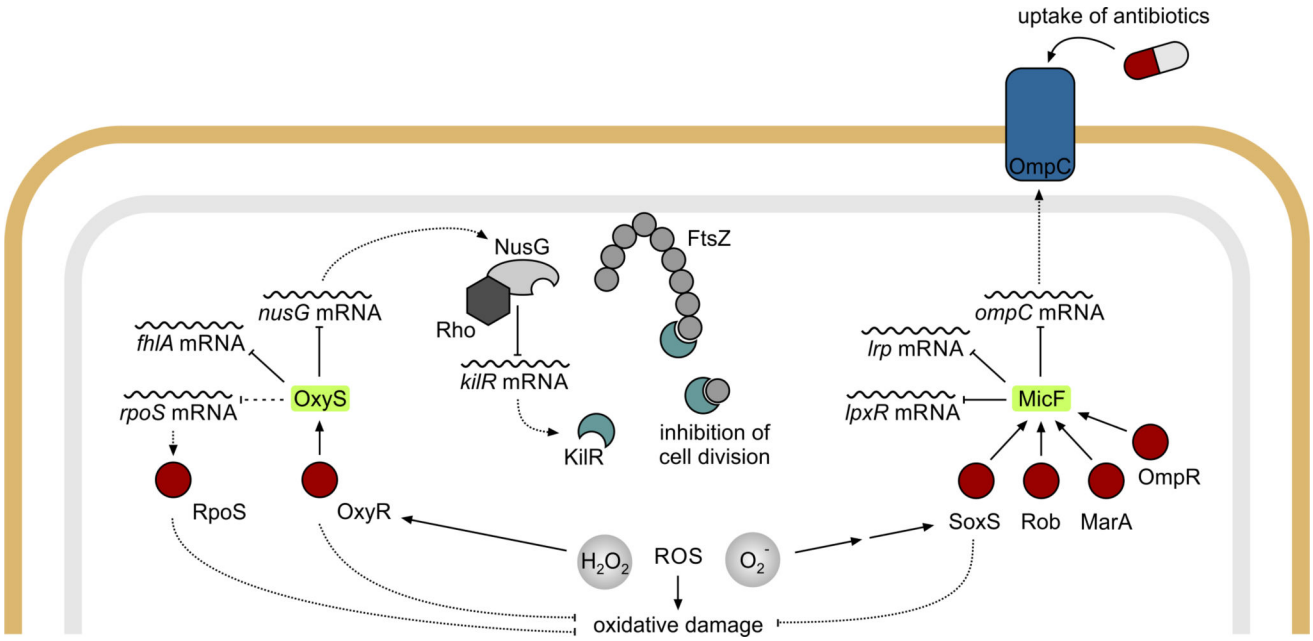

FIGURE 4.

The OxyS and MicF sRNAs are integrated into the enterobacterial response to oxidative stress. OxyS, induced by the hydrogen peroxide-responsive OxyR, down-regulates *fhlA* mRNA (encoding for a transcription factor regulating formate metabolism), and indirectly represses *rpoS* expression. In addition, OxyS-mediated repression of *nusG* results in increased expression of *kilR*, encoded in the cryptic Rac prophage. KilR sequesters FtsZ, thereby leading to inhibition of cell division and growth arrest, which allows the cell to facilitate DNA damage repair. MicF contributes to increased bacterial resistance against antibiotics of different classes by repressing the major porin OmpF. Additional targets of MicF include *lpxR* mRNA (encoding an LPS-modification enzyme), as well as *lrp* mRNA (encoding a transcriptional regulator of amino acid metabolism and transport). Expression of MicF is positively controlled by the transcription factors OmpR, MarA, Rob and SoxS, with the latter being induced in the presence of superoxide.