

## Review Article

# Impact of bacterial sRNAs in stress responses

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Bacterial life is harsh and involves numerous environmental and internal challenges that are perceived as stresses. Consequently, adequate responses to survive, cope with, and counteract stress conditions have evolved. In the last few decades, a class of small, non-coding RNAs (sRNAs) has been shown to be involved as key players in stress responses. This review will discuss — primarily from an enterobacterial perspective — selected stress response pathways that involve antisense-type sRNAs. These include themes of how bacteria deal with severe envelope stress, threats of DNA damage, problems with poisoning due to toxic sugar intermediates, issues of iron homeostasis, and nutrient limitation/starvation. The examples discussed highlight how stress relief can be achieved, and how sRNAs act mechanistically in regulatory circuits. For some cases, we will propose scenarios that may suggest why contributions from post-transcriptional control by sRNAs, rather than transcriptional control alone, appear to be a beneficial and universally selected feature.

## Introduction

In their natural habitats, bacteria are constantly exposed to stressful and ever changing environmental conditions. To cope with stress and to proliferate, bacteria have evolved intricate mechanisms to sense the surrounding milieu and to adequately respond by changing their gene expression patterns and thus phenotypes. Through major research efforts during the last two decades, we learned that small RNAs (sRNAs) play important roles as regulators of a variety of stress response pathways. Even though sRNA-mediated regulation by now is known to have an impact on virtually all metabolic and physiological processes, stress responses remain a dominant theme (for recent reviews, see refs [1–9]).

The genome-wide searches for the heterogeneous class of bacterial sRNAs go back to 2001 [10–12], at the time conducted in the enterobacterium *Escherichia coli*, and more particularly in its intergenic regions (IGRs). Subsequent work showed that such sRNAs, and those derived from other genomic loci, are present in all bacteria and archaea [1,2,13–15]. The sRNAs are (in most cases) non-coding and range from 50 to 300 nt in length. Almost all of the so far characterized sRNAs work by antisense, i.e. they base-pair to mRNA targets to regulate their translation and/or stability [4,16]. Targeting of ribosome-binding sites (RBSs) blocks initiating ribosomes and represses translation, whereas activation relies on sRNA-binding-induced structure modulation in mRNAs to render translation initiation sites available [4,17]. The effects on stability can be indirect (translationally inhibited mRNAs become ribosome-free and thus vulnerable to RNases; [18]) or direct, e.g. through coding region targeting and recruitment of RNase E [19,20]. Reciprocally, stabilization of mRNAs can also be achieved through sRNA-dependent masking of RNase E cleavage sites [21]. sRNAs can also affect transcription elongation: while sRNAs that activate translation simultaneously suppress premature transcription termination by protein Rho on the *rpoS* mRNA [22], translational inhibition of *chiP* mRNA by ChiX promotes Rho-dependent premature termination in the *chiP*–*chiQ* operon [23]. Finally, in contrast to the above-mentioned modes of action that rely on antisense, some sRNAs act by protein sequestration, exemplified by the now classical case of sRNAs that sequester the CsrA/Rsm family of global RNA-binding proteins. This class of sRNAs has been excellently reviewed elsewhere (e.g. [24]) and will not be covered here.

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Bacterial sRNAs come in two flavors: those that are expressed from the same locus as their sole target with which they share full complementarity (*cis*-encoded) and those whose targets are expressed from loci elsewhere (*trans*-encoded). The *trans*-encoded sRNAs share only partial complementarity with their targets and often use specific seed sequences to target multiple mRNAs [25,26]. Because enterobacteria have initially been the dominant models, much work concerned sRNAs that associate with Hfq, an important helper protein [27]. The roles for this homohexameric RNA chaperone are, apart from occasional effects on mRNA translation and polyadenylation, in protection of sRNAs from degradation [28] and — as the platform on which sRNAs and target mRNAs meet — in increasing the rate of sRNA–mRNA annealing (e.g. [29–31]). In contrast, *cis*-encoded sRNAs do not require Hfq for stability and regulation. Recently, the protein ProQ was shown to interact with a large set of sRNAs in *Salmonella*, most of which do not associate with Hfq [32]. ProQ affects both the stability and function of *trans*-encoded sRNAs [33,34], but — unlike Hfq — also binds many *cis*-encoded antisense sRNAs [32]. ProQ and other so far uncharacterized RNA-binding proteins will likely expand our views on how sRNAs work in enterobacteria, especially in bacteria that lack Hfq homologs.

The connection between stress and sRNAs was already suggested when expression patterns of the first candidates in *E. coli* were characterized. Examples include MicF, which is induced by environmental stresses such as high osmolarity, oxidative stress, and antibiotics [35], OxyS (oxidative stress; [36]), RyhB (iron depletion; [37]), and DsrA and RprA, which both regulate the stationary phase/stress  $\sigma$ -factor RpoS [38,39] and confer acid resistance [40,41].

Rather than providing a complete catalog of all sRNAs involved in stress responses, this review will focus on an in-depth discussion of some well-understood examples from enterobacterial systems. Whenever possible, we will also touch upon tentative reasons for *why* bacteria may have evolved, and use, sRNAs as post-transcriptional regulators, in addition to, or even instead of, transcriptional regulators.

## Where do sRNAs originate from?

Before describing some specific stress conditions in which sRNAs are important, we may ask what typical ‘sRNA genes’ are, and how they may have evolved. Based on the criteria used in the initial sRNA screens [10,11], most of the early characterized *E. coli* sRNAs shared two hallmark features: they were encoded by stand-alone genes in IGRs and displayed some degree of phylogenetic conservation. Later, less biased methods such as RNA-sequencing revealed a more diverse genomic origin for sRNAs. We now know that sRNA genes often overlap with other genes, both coding and non-coding [42–44]. For instance, the 3′-UTRs of mRNAs appear to be hotspots for sRNA expression [45]. This subset of sRNAs is either transcribed from a promoter located in the 3′ part of a coding gene or generated by ribonuclease-mediated processing of an mRNA [46].

Many sRNAs also lack conservation and may be present only in one species. This explains why they were not found in the early screens, and suggests that new sRNAs evolve rapidly and easily on an evolutionary time-scale [47]. Recent studies suggest fast initial evolution rates for young sRNAs [48], and phylogenetic analyses indicate that some mRNA target sites were present before a cognate sRNA evolved, i.e. newly emerging sRNAs might get hijacked into existing regulatory pathways [47]. Weak initial sRNA–mRNA interactions, if advantageous, would likely become fixed and improved by selective pressure. Several mechanisms that may lead to the emergence of sRNA genes have been proposed and are discussed in ref. [49].

Overall, more than a few hundred sRNAs may generally be present in most bacteria, suggesting a significant contribution to regulation. However, numbers should be taken with caution. As RNA-sequencing has become increasingly sensitive, candidate sRNAs are discovered from more and more genomic regions that confer some degree of transcription, for instance through pervasive low-level transcription. Without strong function/phenotype-based criteria, studies are needed that differentiate between transcripts with a cellular function and those that are a result of transcriptional noise or merely represent stable degradation intermediates.

## sRNAs in stress response pathways

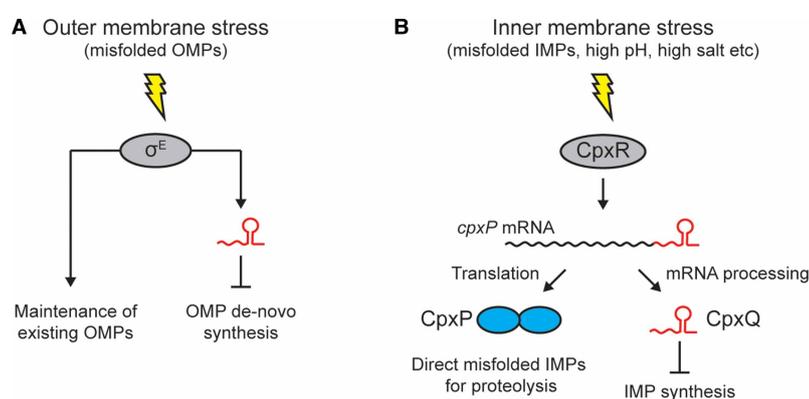
### Iron homeostasis

RyhB was found in one of the early sRNA screens in *E. coli* [11]. Expression of RyhB is induced under iron-limiting conditions when the Fur repressor dissociates from its binding site at the *ryhB* promoter [37]. The target suite of RyhB is one of the largest described so far; RyhB overexpression causes differential expression of more than 50 genes, partly within operons [50]. Many encode proteins involved in iron storage, iron–sulfur cluster biogenesis, iron-containing proteins, respiration, and siderophore biosynthesis, including its own

transcriptional regulator Fur [51]. A recent ribosome profiling study expanded the RyhB target list by numerous additional genes linked to iron metabolism [52]. While RyhB represses most target mRNAs, it activates at least two targets, *shiA* mRNA and *cirA* mRNA [53]. This involves a RyhB-dependent structural rearrangement in the *shiA* 5'-UTR that renders the RBS accessible for translation initiation [54] and, in the case of *cirA*, displacement of Hfq which otherwise inhibits *CirA* translation. In addition, RyhB was recently shown to base-pair to an external transcribed spacer (ETS) from a tRNA precursor [55]. This sequestration by the ETS was hypothesized to cancel the effect of transcriptional noise at the repressed *ryhB* promoter. Similar to *E. coli*, several other bacterial species employ sRNAs for regulation of iron homeostasis, e.g. PrrF1/2 in *Pseudomonas aeruginosa* and IsrA1 in *Synechocystis* [56,57]. What is the logic behind RyhB regulation during iron limitation? The transcription factor (TF) Fur requires iron as a cofactor to bind to DNA. At high iron concentrations, Fur represses expression of proteins needed for iron assimilation. When iron becomes depleted, Fur dissociates from its operators, leading to derepression of many genes, including *ryhB*. RyhB is now transcribed and represses target mRNAs encoding non-essential iron-using proteins. RyhB thus frees iron for essential proteins that rely on iron for their activity. The recruitment of RyhB therefore permits Fur-/iron-dependent counter-regulation of operons/genes according to opposite needs and likely permits faster recovery when iron levels are restored.

## Membrane stress

The envelope stress response (ESR) in enterobacteria, which is triggered by damage to the cell envelope, also relies on sRNA activity [58]. The ESR includes the  $\sigma^E$  and Cpx pathways that respond to the accumulation of misfolded proteins in the outer membrane or periplasm/inner membrane, respectively. As a  $\sigma$ -factor,  $\sigma^E$  can only activate, but not repress, genes. Activated genes are proteases and chaperones that degrade or re-fold misfolded outer membrane proteins (OMPs) [59]. However,  $\sigma^E$  cannot directly shut down the harmful *de novo* production of OMPs. Instead, it induces the expression of three repressor sRNAs, MicA, RybB, and MicL, whose combined effects rely on targeting and translational inhibition of mRNAs encoding OMPs and lipoproteins (Figure 1) [60–62]. The importance of this post-transcriptional arm of the  $\sigma^E$  response is underscored by its activation even in unstressed cells when these sRNA genes are deleted [60,62]. Conversely, overexpression of the sRNAs suppresses growth arrest in  $\sigma^E$ -deficient cells [61,62]. Experiments in *Salmonella* showed that all RybB-targeted OMP mRNAs, in addition to becoming translationally silenced, were subsequently degraded within a few minutes after induction of the sRNA [60]. This short time-scale suggests a benefit of using sRNAs that transcriptional control cannot provide. Let us assume that a repressor (protein) is rapidly synthesized or activated by a stress signal. Even at the fastest DNA-binding rate, this could only block *de novo* production of



**Figure 1. sRNA regulators in the envelope stress response.**

(A) The outer membrane stress response is triggered by the presence of misfolded OMPs in the periplasm. Release of  $\sigma^E$  up-regulates OMP maintenance genes. However,  $\sigma^E$  cannot directly repress gene expression, but induces three sRNAs (RybB, MicA, and MicL) to silence *de novo* synthesis of OMPs. Arrows: activation, Lines with bars: repression. (B) Inner membrane stress is sensed by the CpxAR two-component system. Phosphorylated CpxR activates expression of the dual-output *cpxP* mRNA. CpxP directs misfolded inner membrane proteins (IMPs) for degradation. The sRNA CpxQ, generated from the 3' segment of *cpxP* mRNA, targets mRNAs encoding IMPs to shut down their synthesis.

mRNAs. All mRNAs that are already present escape repression, and further synthesis of misfolded OMPs from these would continue throughout their considerable lifetime [63]. Clearly, time matters for sick cells, and the need for fast relief is likely a strong selective force. Thus, we argue that sRNAs may have been integrated into the  $\sigma^E$  response for two reasons: to confer a repression arm to the  $\sigma^E$  activator, and to enable a *rapid* shutdown of OMP synthesis and thereby to facilitate fast outer membrane stress alleviation.

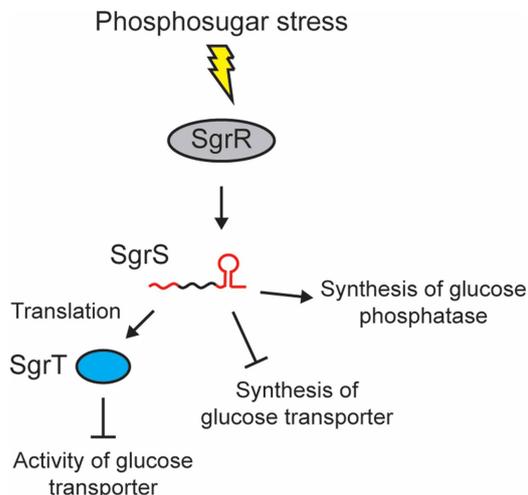
In contrast with outer membrane stress mediated through the  $\sigma^E$  response, inner membrane stress is sensed by the CpxAR two-component system that responds to external stresses and excessive accumulation of membrane proteins [64–66]. In addition, the CpxP protein directs periplasmic misfolded proteins to the DegP protease for degradation [67] and represses the Cpx response by direct interaction with CpxA [68,69]. Recently, the sensitivity to inner membrane stress in bacteria lacking *hfq* [70] was explained by the discovery of a Hfq-dependent sRNA with a crucial role in the Cpx response. The CpxQ sRNA is generated by RNase E-dependent processing of the *cpxP* mRNA and targets mRNAs encoding periplasmic and inner membrane proteins (Figure 1) [71,72]. This elegantly illustrates how one transcription unit can generate both an mRNA and an sRNA, both acting in the same pathway. One major CpxQ target is the *nhaB* mRNA, encoding a sodium–proton antiporter. CpxQ-dependent inhibition of NhaB synthesis entails the Cpx response. This reduces proton uptake and counteracts loss of proton motive force which is one of the consequences of membrane stress. Importantly, *Salmonella* cells lacking CpxQ are more sensitive to CCCP, an uncoupler, strongly indicating that expression of this single sRNA protects against inner membrane stress. In addition, CpxQ increases the amplitude of the Cpx response by regulating expression of genes from diverse processes such as type 1 fimbriae and glucose metabolism [71].

### Phosphosugar stress

A prominent example of how sRNAs are efficiently exploited for stress relief is the regulation of glucose homeostasis. When bacteria such as *E. coli* import glucose, the phosphotransferase system (PTS) converts glucose into glucose-6-phosphate (G6P) to prevent its diffusion out of the cell [73]. Since excessive intracellular G6P levels cause DNA damage and growth arrest [74,75], tight regulation of G6P accumulation is critical. This is achieved by transcriptional regulation of glucose uptake genes [73] and by the sugar stress-induced sRNA SgrS that post-transcriptionally counteracts intracellular phosphosugar accumulation in several ways (Figure 2) [76]. First, by inhibiting the translation of glucose transporter mRNAs (*ptsG* and *manXYZ*), SgrS reduces the intracellular accumulation of G6P [76–78]. Secondly, by stabilizing *yigL* mRNA, encoding the phosphatase YigL, SgrS promotes dephosphorylation of accumulated phosphosugars to enable their transport out of the cell [79]. Recently, there was a new twist to this story. In addition to its function as a base-pairing sRNA, SgrS contains an open reading frame (ORF), *sgrT*, which encodes a small hydrophobic protein that specifically interacts with, and inhibits, the activity of the glucose transporter PtsG [80,81]. Thus, expression of the dual-function gene *sgrS* counteracts phosphosugar stress by (1) inhibiting the synthesis of new glucose transporters, (2) inhibiting the activity of transporters already present, and (3) by inducing expression of a phosphatase needed for glucose efflux.

### Stress due to starvation: the biofilm case

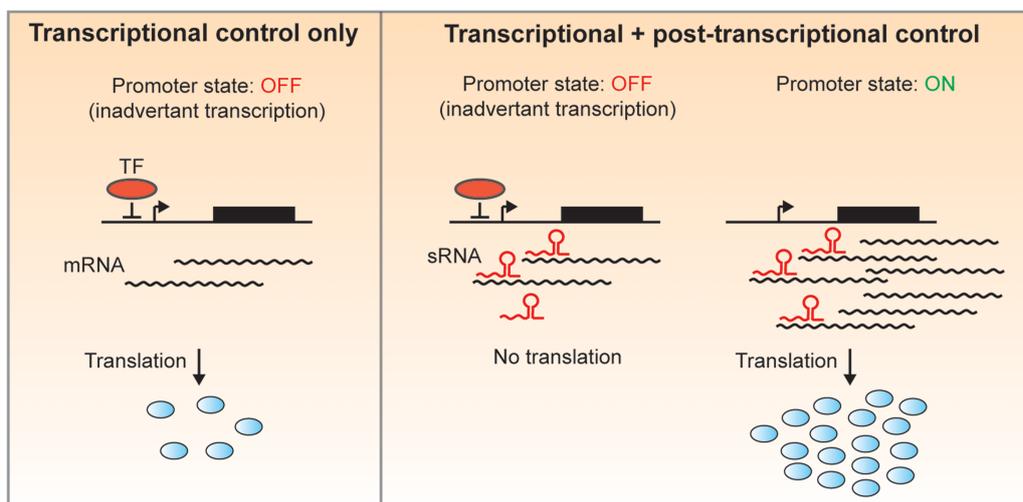
When bacteria encounter nutrient-limited environments, global gene expression profiles are profoundly altered. Several of the major transcriptional regulators responsible for this are directly regulated by sRNAs. For instance, translation of the stationary phase/stress  $\sigma$ -factor,  $\sigma^S$ , is activated by several sRNAs [38,82,83], while the global TF Lrp is subject to sRNA repression [84–86]. One bacterial response that aids survival during long-term starvation involves the formation of three-dimensional communities known as biofilms. This requires production of extracellular matrix components such as curli fibers and cellulose that mediate cell–cell and cell–surface contacts. In *E. coli*, expression of the master regulator and activator of curli fiber and cellulose production, CsgD, is inhibited by no less than seven sRNAs (OmrA, OmrB, RprA, McaS, GcvB, RydC, and RybB) [87–92]. In addition, more than 10 TFs and  $\sigma$ -factors activate or repress *csgD* transcription [93]. Why is the *csgD* gene under such extensive regulation? One reason may be that each of the many environmental signals that feed into the complex process of biofilm formation requires its own regulator [94]. In addition, expression of the biofilm components curli and cellulose on the one hand and that of the motility genes that encode flagella are inversely regulated [95]. This makes sense since these extracellular structures have directly opposite purposes, that is, to move or to adhere. Congruent with this, genes in these opposing pathways are counter-regulated by TF cross-talk.



**Figure 2. Phosphosugar stress is relieved through a dual-function sRNA.**

During its import, glucose is phosphorylated by the PTS. Excess build-up of intracellular phosphosugars triggers activation of TF SgrR, which induces the sRNA SgrS which is an antisense RNA and encodes a small protein, SgrT. To relieve phosphosugar stress, the antisense function of SgrS silences mRNAs for glucose transporters and activates a phosphatase that dephosphorylates sugars for transport out of the cell. SgrT targets and inactivates glucose transporters.

So, if the TF-dependent regulation works as described — keeping one program on and the other off — why then the apparent need for sRNAs? The reason may lie in problems inherent in transcriptional control. TFs perform poorly in keeping a silent state [96]. If the *csgD* promoter escapes repression, then curli are induced in this cell even though it is programmed to be in a motility state. This issue of noisy off-states is exacerbated by the intrinsic burstiness of transcription [97], which will generate several mRNAs, each translated into several proteins. Since synthesis of CsgD directly activates the curli genes, the post-transcriptional inhibition of *csgD* mRNA by the sRNAs prevents inadvertent curli formation and thus stabilizes the transcriptionally controlled state decisions (Figure 3).



**Figure 3. sRNAs counteract the effect of inadvertent transcription to stabilize states.**

This figure schematically shows effects on the single-cell level (see chapters on biofilm and persistence above). Intrinsic noisiness of transcription leads to inadvertent transcription events at repressed promoters. Without sRNA regulation, inadvertently expressed mRNA will be translated into many proteins and affect the phenotype of the cell. The presence of sRNAs ensures that escaping mRNAs are ‘caught’ at the post-transcriptional level, enforcing the repressed state.

## SOS response and persisters

There are other examples suggesting that sRNAs are needed to maintain transcriptionally silent states. An example that addresses toxin–antitoxin (TA) systems and persister phenotypes may illustrate this. TA systems encode toxins that mediate growth arrest, whereas the antitoxins block the translation (type 1) or the activity (type 2) of toxins [98]. Most TA systems are induced by stresses and may be part of a bet-hedging strategy in adverse conditions, for instance by inducing a persister state [99–101]. Persisters are subpopulations of cells that enter a slow- or non-growing (dormant) state in which they are tolerant but not resistant to antibiotics [102]. The best-known type 1 system, *tisB-istR1*, encodes a DNA damage-induced (SOS response) toxin, TisB, whose translation is inhibited by an antisense-type sRNA, IstR1, via an unusual mechanism that involves ‘ribosome standby’ [103,104]. Upon SOS induction, massive transcription of *tisB* mRNA out-titrates the constitutively expressed inhibitor IstR1. Consequently, TisB is produced and inserted into the inner membrane, which leads to depolarization, ATP depletion, and cessation of cell growth [105]. This, in turn, increases the frequency of persister cells [99,106], which is beneficial since it permits the survival of a subpopulation of cells (without genomic change). Conversely, the potent toxicity of TisB calls for tight repression of *tisB* expression under non-stressed conditions. Cells that enter dormancy delay their proliferation, which does not make sense if life conditions are favorable.

Here again, we see a role of an sRNA in preventing inadvertent gene expression (Figure 3). This is how it works: at the transcriptional level, expression of *tisB* is inhibited by LexA, the repressor of SOS genes [103]. At the post-transcriptional level, two RNA elements, a *cis*-acting RNA element in the *tisB* mRNA and the *trans*-acting sRNA IstR1, inhibit TisB translation [106,107]. The primary *tisB* mRNA folds into a structure that prevents translation by obscuring a ribosome standby site [104]. A processing event truncates the primary transcript to make the standby site available for 30S binding, but binding of IstR1 to the standby site inhibits TisB translation. Similar to the *csgD* case above, RNA-based regulation ensures that leaky, unwanted transcription of *tisB* mRNA (observed even under LexA-repressed conditions; [103,106]) does *not* result in toxin production. Deletion of the *cis*- and *trans*-acting inhibitory RNA elements indeed increases persister levels in unstressed cells, an undesirable outcome [106].

## Conclusions

From initially being seen as minor exceptions in a regulatory repertoire, the last two decades have confidently established sRNAs as widespread and ubiquitous key players in control of gene expression. They participate in regulation of a wide range of cellular processes and are prominently found in stress response pathways. We are now starting to understand how sRNAs are ‘born’ and how they become integrated into regulatory networks. As new functions for sRNAs are uncovered, we are also beginning to appreciate the specific beneficial features of sRNAs that are distinct from those of protein-based regulators. As hundreds or thousands of sRNAs are present in many bacteria, this raises the question: what for? Adding sRNAs as a post-transcriptional complement to TF-based transcriptional control may be part of the answer. This ‘more is better’-argument may, however, not be the whole story. Some of the examples from well-studied stress response pathways detailed above suggest that sRNAs may solve problems that TFs cannot or are less efficient at. In particular, it is clear that network motifs of regulatory circuits in which sRNAs replace TFs as nodes confer different, often advantageous, kinetic properties (e.g. [108]; for a recent review, see [109]).

In conclusion, the world of bacteria is hostile, and environmental stresses need to be met on a short time-scale. The involvement of sRNAs in essentially any stress defense strongly indicates that sRNAs have evolved because they can have kinetic properties that promote rapid stress relief, are able to counteract transcriptional noise, and stabilize physiological states. We hope that this short and certainly incomplete review of the topic may have raised some interesting points on sRNAs, their impact on stress responses, and beyond.

## Abbreviations

ESR, envelope stress response; ETS, external transcribed spacer; G6P, glucose-6-phosphate; IGRs, intergenic regions; OMPs, outer membrane proteins; PTS, phosphotransferase system; RBSs, ribosome-binding sites; sRNAs, small RNAs; TA, toxin–antitoxin; TF, transcription factor.

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## Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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