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Short Review

Small regulatory RNAs in *Vibrio cholerae*

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Abstract

Vibrio cholerae is a major human pathogen causing the diarrheal disease, cholera. Regulation of virulence in *V. cholerae* is a multifaceted process involving gene expression changes at the transcriptional and post-transcriptional level. Whereas various transcription factors have been reported to modulate virulence in *V. cholerae*, small regulatory RNAs (sRNAs) have now been established to also participate in virulence control and the regulation of virulence-associated processes, such as biofilm formation, quorum sensing, stress response, and metabolism. In most cases, these sRNAs act by base-pairing with multiple target transcripts and this process typically requires the aid of an RNA-binding protein, such as the widely conserved Hfq protein. This review article summarizes the functional roles of sRNAs in *V. cholerae*, their underlying mechanisms of gene expression control, and how sRNAs partner with transcription factors to modulate complex regulatory programs. In addition, we will discuss regulatory principles discovered in *V. cholerae* that not only apply to other *Vibrio* species, but further extend into the large field of RNA-mediated gene expression control in bacteria.

Keywords: small RNA, Hfq, base-pairing, post-transcriptional gene regulation, *Vibrio cholerae*

Main text

Vibrio cholerae is a marine pathogen that causes millions of infections every year. Cholera outbreaks are frequently associated with poor infrastructure, lack of access to sanitation, and contaminated drinking water. Yemen is currently experiencing the largest cholera epidemic in recent history with more than 2.5 million suspected cases and thousands of deaths between 2016 and 2021 according to the World Health Organization (World Health Organization. Regional Office for the Eastern 2021). In addition, cholera outbreaks have surged in Asia and Africa in 2022 and this trend might well continue over the next years due the increasing numbers of natural disasters associated with climate change (World Health Organization 2022).

The lifestyle of *V. cholerae* involves an aquatic phase where it typically forms biofilms on chitin particles, which serve as a major source of energy, carbon, and nitrogen (Lutz et al. 2013). Infection with *V. cholerae* is caused by ingestion of such particles or contaminated food, allowing the pathogen to reach the intestinal tract where it replicates and causes cholera-associated symptoms, such as diarrhea and vomiting. Once transmitted back into the aquatic environment, a new infection cycle can be initiated (Silva and Benitez 2016).

Over the past decades, numerous regulatory factors have been discovered that control pathogenesis of *V. cholerae*, that allow adaptation to changing environments (e.g. during the transmission phase), and that cause the cholera-specific disease symptoms (Harris et al. 2012). Among the latter, the cholera toxin (encoded by the *ctxAB* genes) is the most thoroughly studied virulence factor of *V. cholerae* as it is responsible for the strong diarrheal symptoms associated with cholera infections. The cholera toxin belongs to the large class of AB-type toxins of pathogenic bacteria that bind intestinal epithelial cells via the B subunit of the toxin, whereas the A subunit possesses enzymatic activity. In the case of cholera toxin, activity of the A subunit results in cAMP (cyclic adenosine monophosphate) production, which in turn causes efflux of chloride ions and water into the lumen of the small intestine causing diarrhea (Rivera-Chavez and Mekalanos 2019). The *ctxAB* genes are encoded by the CTXφ phage, which has integrated into the genomes of many *V. cholerae* strains (Faruque and Mekalanos 2012). Thus, the emergence of virulence in *V. cholerae* largely relied on horizontal gene transfer.

Control of virulence and virulence-related functions in *V. cholerae* relies on various transcription factors and post-transcriptional regulators that convert external and internal cues into appropriate gene expression changes (Hsiao and Zhu 2020). The majority of post-transcriptional regulators are small regulatory RNAs (sRNAs) that associate with the conserved RNA-binding protein, Hfq (Bardill and Hammer 2012). Hfq functions as a molecular matchmaker that facilitates base-pairing between an sRNA and its target mRNAs (Holmqvist and Vogel 2018, Kavita et al. 2018). A single sRNA typically controls multiple target transcripts (Papenfort and Vogel 2009, Papenfort and Melamed 2023), which can be either activated or repressed. Target repression by Hfq-binding often involves base-pairing of the sRNA with the ribosome binding site (RBS) of the target, which blocks ribosome binding and translation initiation (Waters and Storz 2009). However, various other mechanisms have been reported that allow target inhibition by bacterial sRNAs (Ng Kwan Lim et al. 2021). Similarly, the molecular mechanisms underlying sRNA-mediated target activation are diverse and can involve complex structural changes at the sRNA and the target mRNA level (Papenfort and Vanderpool 2015).

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Mutation of the *hfq* gene has been shown to limit the virulence of numerous microbial pathogens (Chao and Vogel 2010, Papenfort and Vogel 2010) and this is also true for *V. cholerae* (Ding et al. 2004). RNA coimmunoprecipitation experiments using Hfq as a bait revealed ~85 sRNAs interacting with Hfq in *V. cholerae* (Huber et al. 2020) and global RNA–RNA interaction studies (i.e. RIL-Seq; RNA interaction by ligation and sequencing) indicated hundreds of Hfq-mediated base-pairing events (Huber et al. 2022). Whereas many of these are likely to constitute canonical interactions between sRNAs and their mRNA targets, other types of interaction involve two sRNAs, indicating that certain sRNAs act as RNA sponges to restrict activity of other noncoding regulators (Figueroa-Bossi and Bossi 2018).

In this review article, we will discuss the various regulatory roles of sRNAs in *V. cholerae* and how they control crucial cellular processes such as quorum sensing (QS), biofilm formation and virulence, stress response, and carbon metabolism (Fig. 1). Further, we will outline open questions in the field and discuss potential roles of yet uncharacterized RNA regulators in *V. cholerae*.

Regulatory RNAs involved in quorum sensing

A crucial aspect of *V. cholerae*'s ability to cause disease is QS (Zhu et al. 2002), i.e. the process of communication among microbes (Papenfort and Bassler 2016). QS relies on the production, secretion, and detection of signaling molecules, called autoinducers (AIs) (Whiteley et al. 2017). QS controls hundreds of genes in *V. cholerae* (Papenfort et al. 2015, Ball et al. 2017, Herzog et al. 2019) and has been linked to the activity of four key signaling molecules: AI-2 [(2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate], CAI-1 [(S)-3-hydroxytridecan-4-one], ethanolamine, and DPO (3,5-dimethylpyrazin-2-ol) (Fig. 2A).

AI-2, CAI-1 and ethanolamine are detected by the membrane-bound LuxPQ, CqsS, and CqsR receptors, respectively, and all channel into the same signaling cascade (Jung et al. 2015). In the absence of AI-2, CAI-1, or ethanolamine, the receptors function as kinases that channel phosphate to the common phosphorelay protein LuxU, which further transfers the phosphate to the response regulator, LuxO (Bridges et al. 2022). Phosphorylated LuxO, in concert with the alternative sigma factor σ^N, induces the expression of four homologous sRNAs, named Qrr1-4 (quorum regulatory RNAs) (Lenz et al. 2004). Binding of a cognate AIs converts the receptors from kinases to phosphatases resulting in dephosphorylated LuxO (Boyaci et al. 2016). Thus, Qrr1-4 levels are high at low cell density when AIs are scarce and decrease with increasing cell density and AI concentrations.

The Qrr1-4 sRNAs interact with Hfq (Huber et al. 2020) and control gene expression by base-pairing with *trans*-encoded target mRNAs (Bardill et al. 2011, Shao et al. 2013, Huber et al. 2022). With respect to QS, regulation of the *hapR* and *aphA* transcripts are of major importance (Fig. 2A). The *hapR* mRNA encodes a key regulator of high cell density in *V. cholerae* (Ball et al. 2017) and base-pairing with the Qrr1-4 sRNAs results in inhibition of HapR synthesis (Lenz et al. 2004). In contrast, AphA is a central regulator of low cell density behaviors and translation of the *aphA* mRNA is induced by base-pairing with the Qrr2-4 sRNAs (Rutherford et al. 2011). Of note, Qrr1 is unable to activate *aphA* translation as it lacks a crucial base-pairing region located near the 5' end of the sRNA (Shao and Bassler 2012). In addition, the Qrrs also inhibit type VI secretion in *V. cholerae* through interaction with the first mRNA (*vca0107*) of the large type VI secretion cluster (Shao and Bassler 2014) and affect the production of the intracellular small-molecule second messenger cyclic di-GMP by activating the *vca0939* mRNA encoding a GGDEF domain-containing protein (Hammer and Bassler 2007, Zhao et al. 2013).

Whereas the interaction of the Qrr sRNAs with the *hapR* and *aphA* mRNAs have been studied intensively over the past years (Bardill and Hammer 2012), recent high-throughput identification of Hfq-assisted RNA–RNA interactions revealed several new target mRNAs (Huber et al. 2022). For example, all four Qrr sRNAs inhibit the expression of *cyaA*, encoding adenylate cyclase, which catalyzes the synthesis of cAMP. cAMP is a central regulator of carbon metabolism in many bacteria, including *V. cholerae* (Manneh-Roussel et al. 2018), and thus regulation of *cyaA* by Qrr1-4 might well connect QS and carbon metabolism. These analyses also revealed an additional base-pairing partner of Qrr1-4, which is the QrrX sponge RNA (Fig. 2A). Interaction of QrrX with the four Qrr sRNAs involves extended base-pairing and triggers RNase E-mediated degradation of the RNA duplex (Huber et al. 2022). Transcription of QrrX is induced by QrrT, a LysR-type transcriptional regulator, whose activity might well rely on binding of a yet undiscovered ligand. Given that tight control of Qrr1-4 expression is key for QS signaling in *V. cholerae* and other *Vibrios* (Svenningsen et al. 2009, Feng et al. 2015), mutation of *qrrX* and/or *qrrT* (leading to increased Qrr1-4 levels) affects QS dynamics and QS-associated behaviors, such as biofilm formation (Huber et al. 2022).

In contrast to AI-2, CAI-1, and ethanolamine, DPO signaling is independent of LuxU and LuxO and depends on the LuxR-type transcription factor VqmA and the VqmR sRNA (Papenfort et al. 2015, 2017) (Fig. 2A). DPO belongs to the family of pyrazine molecules known to be produced by bacteria, plants, and fungi (Silpe et al. 2022). VqmA is the receptor of DPO in *V. cholerae* and DPO is also detected by a VqmA homolog encoded on bacteriophage VP882 (Papenfort et al. 2017, Silpe and Bassler 2019, Wu et al. 2019, Huang et al. 2020, Duddy et al. 2021). Binding of DPO to VqmA allows transcription of the VqmR sRNA, which also binds Hfq (Papenfort et al. 2015, Huber et al. 2020). VqmR regulates multiple target mRNAs including *vpsT* (encoding a central regulator of biofilm formation, see below), as well as *aphA*, which is also a target of the Qrr2-4 sRNAs. However, whereas Qrr2-4 induce AphA production, VqmR inhibits translation initiation of the *aphA* mRNA (Herzog et al. 2019). This scheme is in line with the regulatory logic of QS in *V. cholerae* as the Qrr sRNAs are expressed at low cell density (when AI concentrations are low), while VqmR expression requires high DPO, which are typically associated with high cell densities. Additional targets of VqmR have been documented suggesting additional roles of this sRNA in motility, carbon utilization, and O-antigen synthesis (Papenfort et al. 2015, Herzog et al. 2019, Huber et al. 2022).

Taken together, post-transcriptional gene expression control and specifically the activity of Hfq-binding sRNAs are essential for signal integration and overall QS performance in *V. cholerae*. Of note, the AI synthases, the receptors, as well as the transcriptional and post-transcriptional regulators discussed in this section are often conserved among different *Vibrio* species (Nguyen and Jacq 2014). Therefore, it is possible that the RNA-based mechanisms governing QS in *V. cholerae* will also apply to other organisms.

Small RNA-mediated control of virulence and biofilm formation

The ability to form biofilms and the control of virulence factor expression are two highly interconnected processes in *V. cholerae*, and both processes receive input from QS (Silva and Benitez 2016). Specifically, AphA and HapR, which are controlled by the Qrr1-4 and VqmR sRNAs (see above section and Fig. 2A), are critical

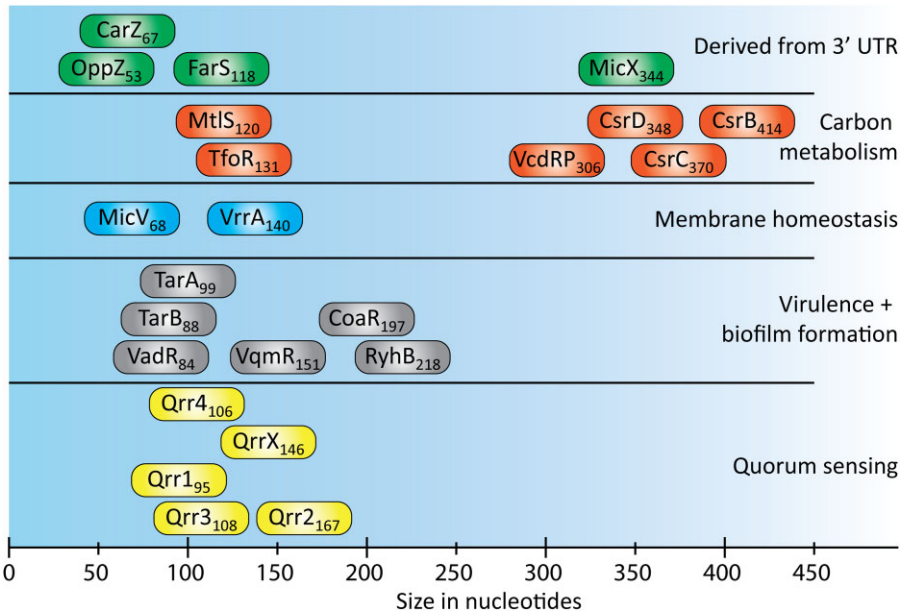

Figure 1. Size and regulatory role of sRNAs in *V. cholerae*. Functionally characterized sRNAs from *V. cholerae* have been grouped according to their size and regulatory functions. The sRNAs are shown as boxes and were color-coded regarding their physiological roles. The size of each sRNA is indicated as subscript.

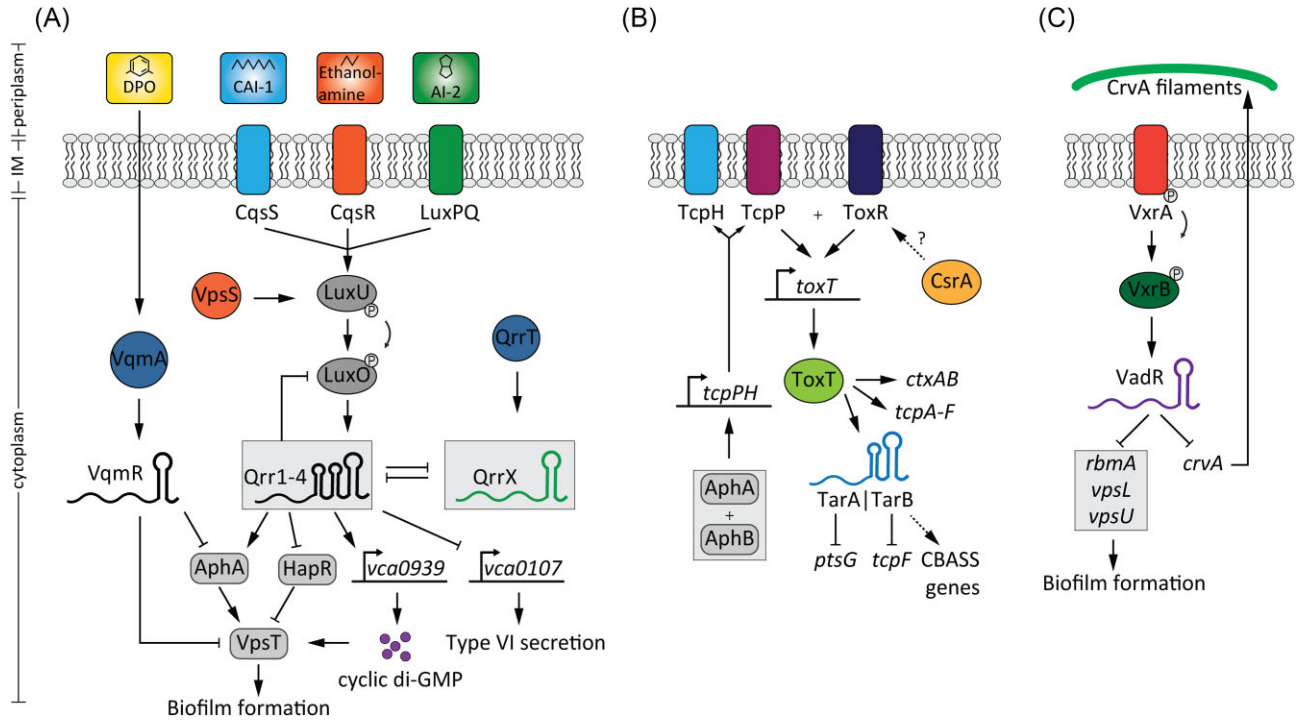

Figure 2. (A) Regulatory roles of sRNAs in QS of *V. cholerae*. At low AI concentrations (CAI-1, ethanolamine, and AI-2), the inner membrane receptors (CqsS, CqsR, and LuxPQ) act as kinases and phosphorylate LuxU. In addition, LuxU is also phosphorylated by VpsS. Phosphorylated LuxU channels the phosphate to LuxO, which activates the transcription of the Qrr sRNAs. The Qrr sRNAs destabilize the *hapR* transcript and activate the *aphA* mRNA. The levels of Qrr sRNAs is negatively controlled by the RNA sponge, QrrX, which is controlled by QrrT. The Qrr sRNAs also activate the *vca0939* mRNA, which increases cyclic di-GMP in the cell. Cyclic di-GMP binds to and activates the VpsT transcription factor, inducing biofilm formation. The Qrr sRNA also down-regulate type VI secretion by inhibiting the *vca0107* mRNA. Expression of the VqmR sRNA is controlled by the VqmA, which required binding of the DPO AI for full activity. VqmR inhibits the expression of the *vpsT* and *aphA* mRNAs. **(B)** *Vibrio cholerae* sRNAs associated with virulence gene expression. AphA in concert with AphB activates the expression of the *tcpPH* operon. TcpPH are inner membrane proteins that together with ToxR activate the *toxT* gene. ToxT activates the expression of the *ctxAB* (encoding the cholera toxin) and *tcpA-F* (encoding the toxin-coregulated pilus) operons. ToxT also activates the expression of the TarA and TarB sRNAs. TarA inhibits the expression of the *ptsG* transporter, whereas TarB controls *tcpF* and, indirectly, the production of the CBASS genes. **(C)** The VadR sRNA controls biofilm formation and cell curvature. Transcription of the *vadR* gene is activated by the VxrAB operon. VadR reduces cell curvature by inhibiting the production of CrvA protein and down-regulates biofilm formation by reducing the expression of *rbmA*, *vpsL*, and *vpsU*.

for the regulation of biofilm formation and virulence in *V. cholerae*. Here, AphA functions as activator, whereas HapR acts as a repressor of both processes (Kovacikova and Skorupski 2002a, Rutherford et al. 2011). Thus, at low cell density the genes for biofilm formation and virulence gene expression are induced, but are inhibited when the concentrations of cells and AI molecules increase.

With respect to pathogenicity, AphA acts on top of a cascade of transcription factors that ultimately lead to cholera toxin production (Fig. 2B). Together with AphB (a LysR-type transcription factor), AphA activates the transcription of the *tcpPH* operon, encoding two membrane-bound regulators (Kovacikova and Skorupski 2001). TcpP then partners with another transmembrane regulator, called ToxR, to activate the *toxT* gene (Hase and Mekalanos 1998). ToxR expression is modulated by the RNA-binding protein, CsrA, however, it is currently not clear if this process involves direct interaction of CsrA with the *toxR* transcript (Mey et al. 2015). ToxT is the main activator of *ctxAB*, and thus cholera toxin production (Weber and Klose 2011). In addition to *ctxAB*, several other genes are part of the ToxT regulon, including *tcp*, encoding a type IV bundle-forming pilus, i.e. critical for intestinal colonization (Taylor et al. 1987). ToxT also controls the expression of two Hfq-associated sRNAs, called TarA and TarB (ToxT-activated RNAs). TarA down-regulates the *ptsG* mRNA (encoding a major glucose transporter) (Richard et al. 2010) and other potential target mRNAs have recently been reported (Huber et al. 2022). TarB was identified in a genome-wide screen for ToxT-dependent genes in *V. cholerae* and is also positively regulated by the transcription factor (Bradley et al. 2011). Mutation of *tarB* has a mild positive effect on the colonization of the mouse small intestine by *V. cholerae*, which might be associated with negative regulation of the *tcpF* mRNA (encoding a secreted colonization factor) by TarB. TarB has also been reported to modulate the expression of the genes associated with the CBASS (cyclic oligonucleotide-based antiphage signaling system) bacteriophage defense system located on a genomic island called, VSP-1 (*Vibrio* seventh pandemic island-1) (Davies et al. 2012, O'Hara et al. 2022).

Another sRNA with possible roles in virulence of *V. cholerae* is CoaR (cholerae osmolarity and acidity related regulatory RNA) (Xi et al. 2020). Transcription of *coaR* is regulated by the EnvZ/OmpR two-component system and mutation of *coaR* resulted in reduced colonization of infant mouse intestines. Of note, *coaR* was previously identified as Vcr058 and the *V. cholerae* El Tor genome carries a second identical copy of this gene on the same chromosome, named Vcr061 (Papenfort et al. 2015). In addition, both sRNAs, i.e. Vcr058 and Vcr061, are associated with overlapping antisense sRNAs, called Vcr057 and Vcr060, respectively. It is, thus not clear which of these sRNAs are involved in virulence regulation and if Vcr058 and Vcr061 are both regulated by EnvZ/OmpR.

Similar to virulence gene control, regulation of biofilm formation in *V. cholerae* requires QS and also involves AphA and HapR (Hammer and Bassler 2003). Specifically, AphA induces the expression of the *vpsT* gene, encoding a transcriptional regulator that activates the genes needed for biofilm formation (Casper-Lindley and Yildiz 2004, Yang et al. 2010). In contrast, HapR down-regulates *vpsT* and inhibits biofilm formation (Srivastava et al. 2011). Thus, given that the Qrr sRNAs activate *aphA* and repress *hapR*, they also induce biofilm formation (Bardill et al. 2011) (Fig. 2A). By the same token, QrrX indirectly modulates biofilm formation by inhibiting Qrr1-4 (Huber et al. 2022). The Qrr sRNAs further facilitate biofilm formation by activating the diguanylate cyclase Vca0939, which synthesizes cyclic di-GMP (Zhao et al. 2013). Cyclic di-GMP binds to and promotes VpsT activity and thereby fosters the transcription of biofilm-associated genes (Krasteva et al. 2010).

In contrast to Qrr1-4, VqmR acts as a repressor of biofilm production via repression of two target mRNAs (Fig. 2A). First, VqmR inhibits the translation of the *vpsT* mRNA and thereby blocks biofilm formation (Papenfort et al. 2015). Second, VqmR down-regulates *aphA* (Herzog et al. 2019). AphA is a transcriptional activator of *vpsT* (Yang et al. 2010) and thus VqmR, *aphA*, and *vpsT* constitute a feed-forward loop that allows tight control of VpsT synthesis in the presence of DPO. Indeed, synthetic DPO can inhibit biofilm formation in *V. cholerae* (Papenfort et al. 2017).

Another Hfq-dependent sRNA that down-regulates biofilm formation in *V. cholerae* is VadR (VxrB activated small RNA) (Fig. 2C). However, contrary to Qrr1-4, QrrX, and VqmR, VadR expression is independent of QS. Instead, transcriptional activation of *vadR* relies on the VxrAB two-component system (Peschek et al. 2020). VxrAB signaling is triggered by cell wall damaging antibiotics and mechanical stress and promotes high-level tolerance against β-lactam antibiotics (Dörr et al. 2016, Harper et al. 2022). It also positively regulates virulence, type VI secretion, and biofilm formation (Cheng et al. 2015, Teschler et al. 2017). VadR base-pairs and inhibits the expression of several genes involved in biofilm formation, including *rbmA* (encoding a biofilm matrix protein), *vpsL*, and *vpsU* (both involved biofilm matrix synthesis). Indeed, VadR over-expression strongly inhibits biofilm formation (Peschek et al. 2020). VadR also down-regulates the translation of the *crvA* mRNA, encoding a periplasmic protein that self-assembles into filaments to induce the characteristic cell curvature of *V. cholerae* (Bartlett et al. 2017, Martin et al. 2021). Accordingly, mutation of *vadR* results in increased cell curvature, which was linked with decreased survival of *V. cholerae* upon penicillin G challenge (Peschek et al. 2020). In summary, the VadR sRNA likely acts to coordinate cell shape, antibiotic tolerance, and biofilm formation in *V. cholerae*.

Maintaining outer membrane homeostasis via σ^E-dependent small RNAs

The outer membrane (OM) of Gram-negative bacteria is a critical barrier to the outside environment and allows for the selective passage of molecules in and out of the cell (Sun et al. 2022). Transport via the OM is mediated by porins, which are abundant outer membrane proteins (OMPs). Preservation of OM homeostasis is required for bacterial survival, and thus tightly regulated by several envelope stress response systems (Mitchell and Silhavy 2019).

One of the most thoroughly studied envelope stress response system is controlled by the alternative sigma factor, σ^E (encoded by *rpoE*). In the inactive state, σ^E is sequestered at the inner membrane by RseA, its cognate antisigma factor (Saha et al. 2021) (Fig. 3). There are various stresses (e.g. misfolded OMPs) that trigger RseA proteolysis and thereby release of σ^E into the cytoplasm. Here, σ^E associates with the core RNA polymerase enzyme to regulate dozens of genes, including several sRNAs (Fröhlich and Gottesman 2018).

In *Escherichia coli* and *Salmonella enterica*, σ^E activates the expression of three Hfq-dependent sRNAs, called MicA, RybB, and MicL (Johansen et al. 2006, Papenfort et al. 2006, Thompson et al. 2007, Udekwu and Wagner 2007, Guo et al. 2014). All three sRNAs inhibit the translation of mRNAs encoding OMPs (among others), and thereby reduce the synthesis of new OMPs when the σ^E response is induced. Of note, several OMP-encoding mRNAs have been documented to be highly stable in the absence

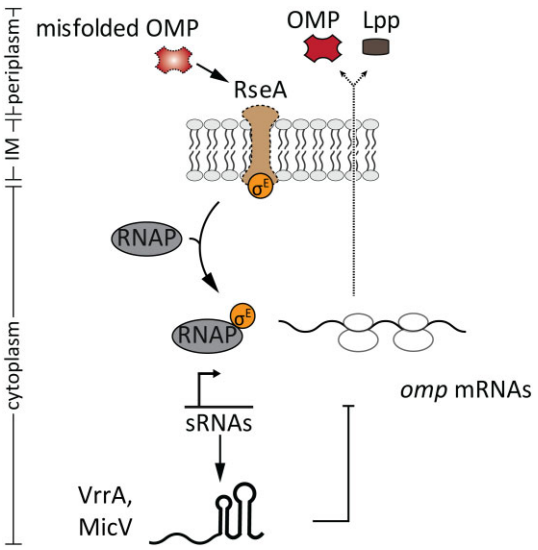

Figure 3. σ^E-dependent sRNAs control OM homeostasis in *V. cholerae*. Accumulation of misfolded OMPs triggers proteolysis of the antisigma factor, RseA, releasing σ^E into the cytoplasm. Here, σ^E associates with RNA polymerase (RNAP) to activate, among other genes, the expression of *micV* and *vrrA*. MicV and VrrA inhibit the production of several OMPs and thereby support OM homeostasis under stress conditions.

of stress, which could explain the need for post-transcriptional down-regulation of these mRNAs when the integrity of the membrane is compromised (Papenfort et al. 2006, Gogol et al. 2011).

The roles of σ^E and related stress response systems have also been studied in *V. cholerae* (DeAngelis et al. 2018). Here, deletion of the *rpoE* gene strongly attenuates virulence in an infant mouse model and has been associated with the occurrence of suppressor mutations that lower OMP production (Kovacikova and Skorupski 2002b, Davis and Waldor 2009). The σ^E regulon of *E. coli* contains ~80 genes and similar numbers have been determined for *V. cholerae* (Rhodius et al. 2006, Peschek et al. 2019). Among these are two Hfq-binding sRNAs, named VrrA and MicV (Fig. 3). VrrA was initially identified as an inhibitor of OmpA synthesis and mutation of the *vrrA* gene activated the production of OM vesicles and facilitated the colonization of infant mice (Song et al. 2008). Follow-up studies showed that VrrA also reduces the production of OmpT (Song et al. 2010), the cytoplasmic stationary phase survival factor Vrp (Sabharwal et al. 2015), and multiple other targets including the Lpp major OM lipoprotein (Peschek et al. 2019).

Transcription of both, VrrA and MicV, is directly controlled by σ^E and both share a large set of target mRNAs (Song et al. 2008, Peschek et al. 2019), many of which have to transfer into or across the inner or OM to reach their final destination. In addition, MicV and VrrA also reduce the levels of the *rpoE* mRNA, thus creating a negative feedback loop that limits their own expression under continued stress conditions (Peschek et al. 2019). This redundancy in target regulation can be explained by two highly similar base-pairing sequences in the sRNAs that mediate target mRNA recognition. Interestingly, an almost identical base-pairing sequence is also present in the RybB sRNA of *E. coli* and *Salmonella* (Papenfort et al. 2010), suggesting that sRNAs might carry conserved base-pairing domains that specify their regulatory role in the cell (Peschek et al. 2019). There are also several target mRNAs that are controlled by either MicV or VrrA. For example, regulation of the accessory colonization factor, *acfA*, is specific to VrrA, whereas *prtV*, encoding a virulence-related metalloprotease, requires only MicV.

Small RNAs regulating carbon metabolism

Bacteria can colonize various environments and have evolved numerous regulatory strategies to cope with nutrient availability in their environment. As for pathogenic bacteria, they rely on their host for nutrients supply, highlighting the role of bacterial metabolism during infection as an essential aspect to understand pathogenesis. An increasing number of sRNAs have been described to be involved in carbon metabolism (Bobrovskyy and Vanderpool 2013, Papenfort and Vogel 2014, Durica-Mitic et al. 2018), including several examples from *V. cholerae*.

Csr (carbon storage regulator) is a regulatory system that controls carbon storage and flux and is involved in biofilm formation and motility, as well as QS by modulating Qrr14 levels through LuxO (Lenz et al. 2005, Babitzke and Romeo 2007). The effector of this system, the CsrA protein, is an RNA-binding protein that interacts with GGA motifs in its target transcripts and modulates their stability and/or translation efficiency (Romeo and Babitzke 2018). In *E. coli*, CsrA controls the expression of several transcription factors involved in glycogen synthesis and catabolism, gluconeogenesis, and glycolysis. CsrA displays a wide phylogenetic distribution among eubacterial species, reflecting its extensive regulatory impact on bacterial metabolism (Sobrero and Valverde 2020). Activity of CsrA is controlled by CsrB and CsrC, two sRNAs that contain 22 and 9 CsrAbinding sites, respectively. By sequestering CsrA away from its targets, CsrB and CsrC control the metabolic status of the cell (Papenfort and Vanderpool 2015). In *V. cholerae*, three homologues sRNAs, CsrB, CsrC, and CsrD, were identified to act redundantly by titrating CsrA protein (Lenz et al. 2005).

Transcription of the *csrBCD* genes is controlled by the VarS/VarA two component system, which has documented roles in QS regulation, cell shape maintenance, and virulence factor production in *V. cholerae* (Lenz et al. 2005, Jang et al. 2010, Mey et al. 2015, Lemos Rocha et al. 2022). Although the complete set of RNAs ligands of CsrA in *V. cholerae* is currently unknown, it is interesting to note that CsrA interacts with and improves the translation of the *varA* mRNA. Increased VarA levels are likely to promote CsrBCD expression, which in turn antagonize CsrA activity (Butz et al. 2019). This feedback loop might help to limit the levels of free CsrA in the cell.

Global transcriptome analysis of *V. cholerae* cells expressing a mutated CsrA protein revealed hundreds of deregulated genes, and coimmunoprecipitation experiments showed binding of CsrA to mRNAs encoding global transcriptional regulators (Butz et al. 2021). For example, CsrA interacts with the *rpoE* and *rpoS* transcripts encoding stress-activated alternative sigma factors, as well as the mRNA encoding FlrC, which is a key regulator of flagellar gene expression (Fig. 4A).

Chitin is a major carbon and nitrogen source for *V. cholerae* in aquatic environment and is known to induce competence. Uptake and utilization of chitin requires a complex cascade of factors with many intertwining interactions (Blokesch 2012, Le Roux and Blokesch 2018) (Fig. 4B). Many of the downstream genes required for the assimilation of chitin are governed by ChiS, a chitin-sensing membrane-embedded one-component system (Klancher et al. 2020). In the presence of chitin, ChiS activates another transcription factor, called TfoS, which in turn induces the expression of TfoR, an Hfq-binding and *trans*-acting sRNA (Dalia et al. 2014, Yamamoto et al. 2014, Huber et al. 2020). TfoR then enhances the translation of *tfoX* by binding to its 5′UTR and exposing its Shine Dalgarno (SD) sequence (Yamamoto et al. 2011). Subsequently, TfoX in conjunction with CRP, a global transcription factor of carbon utilization, activates the expression of competence genes (Blokesch 2012).

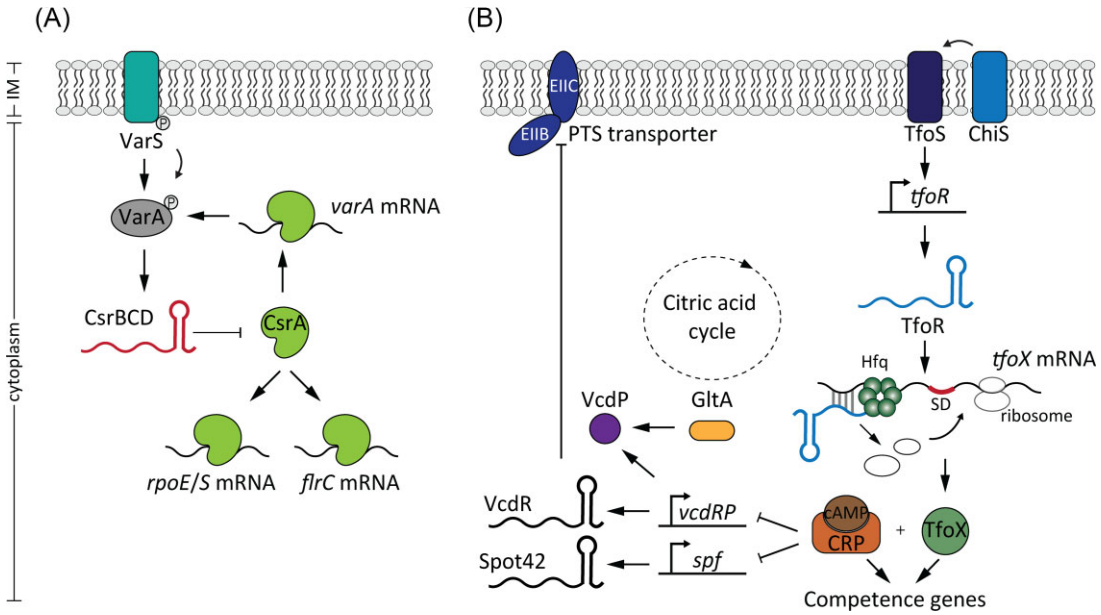

Figure 4. (A) Csr-associated regulation of gene expression. CsrA is an RNA-binding protein that interacts with various transcripts in the *V. cholerae*, including the mRNAs encoding *rpoE*, *rpoS*, and *flrC*. Activity of CsrA is antagonized by three regulatory RNAs (CsrB/C/D), whose transcription is controlled by the VarAS two-component system. **(B)** Regulation of carbon uptake and utilization by Hfq-binding sRNAs. Sensing of chitin-derived metabolites is achieved by the membrane-associated ChiS and TfoS proteins. TfoS activates the synthesis of the TfoR sRNA, which base-pairs with and activates the *tfoX* mRNA. TfoX together with the CRP transcription factor (and cAMP) induce competence gene expression in *V. cholerae*. CRP also inhibits the transcription of *spf* (encoding Spot 42) and *vcdRP*. VcdRP is a dual RNA, whose small protein component (VcdP) activates the GltA protein of the citric acid cycle, whereas VcdR functions as a base-pairing sRNA inhibiting the expression of PTS-type carbohydrate transporters.

CRP also controls the expression of two regulatory sRNAs in *V. cholerae* (Fig. 4B). Specifically, CRP inhibits the expression of the *spf* (encoding the Spot 42 sRNA) and *vcdRP* genes, the latter of which belongs to the growing class of dual RNA regulators (Venkat et al. 2021). Dual RNAs are *trans*-acting regulatory RNAs that also encode a small protein (Raina et al. 2018, Gray et al. 2022). The RNA component of VcdRP (VcdR) exerts its function by base-pairing with multiple target mRNAs using a conserved stretch of 4 cytosines at its 3′ end. Specifically, VcdR represses the expression of the PTS (phosphotransferase carbohydrate transporter) sugar transporters (PtsG, NagE and TreB), as well as the phosphor-carrier proteins PtsH and PtsI involved in glycolysis. The small protein VcdP interacts with and enhances the activity of the citrate synthase enzyme (encoded by *gltA*), the first enzyme of the citric acid cycle. Taken together, VcdRP acts as a modulator of both the citric acid cycle and glycolysis pathway, thereby maintaining a balance of the overall carbon metabolism in *V. cholerae* (Venkat et al. 2021).

Another sRNA that helps *V. cholerae* to adapt to changes in carbon source availability is MtlS. This sRNA is transcribed antisense from *mtlA* gene, encoding a mannitol transporter. MtlA is a *cis*-encoded sRNA, and thus has perfect complementarity to the 5′UTR of *mtlA* and inhibits its expression post-transcriptionally by sequestering the RBS (Chang et al. 2015). When mannitol is the sole carbon source, expression of *mtlA* is induced, which reduces MtlS levels, likely involving a mechanism of transcription interference (Zhang and Liu 2019).

Small RNAs derived from the 3′UTR of mRNAs

There are various technologies to identify small RNA regulators in bacteria (Altuvia 2007, Sharma and Vogel 2009). Among these, the development of high-throughput sequencing technologies has allowed a major breakthrough as it provided a single nucleotide resolution view on bacterial transcriptomes, which also enabled the detection and annotation of sRNAs in a wide-range of microorganisms (Barquist and Vogel 2015). In addition, computational analysis of bacterial transcriptomes revealed a large number of sRNA candidates originating from the 3′UTR of coding sequences (Hör et al. 2020, Menendez-Gil and Toledo-Arana 2020, Ponath et al. 2022).

Several 3′UTR-derived sRNAs have also been studied in *V. cholerae* and many of them interact with Hfq (Huber et al. 2020). There are two general pathways of how 3′UTR-derived sRNAs are produced: first, the sRNA gene has its own promoter, and thus its transcription is independent of the upstream gene, or second, the sRNA is expressed together with its upstream gene(s) and the full-length transcript is processed by ribonucleases, such as RNase E, to generate the mature sRNA (Ponath et al. 2022) (Fig. 5A). Both types of 3′UTR-derived sRNAs exist in *V. cholerae* and four examples (MicX, FarS, OppZ, and CarZ) have been studied in greater detail. Specifically, MicX was the first reported 3′UTR-derived sRNA and carries its own promoter, which is located in the *vca0943* (*malG*) gene (Davis and Waldor 2007, Papenfort et al. 2015). Nevertheless, MicX is also processed by RNase E giving rise to at least three sRNA isoforms that accumulate in the cell. The main target of MicX is the OMP-coding *vc0972* mRNA (Davis and Waldor 2007), however, recent RIL-Seq analysis indicated additional target mRNA candidates (Huber et al. 2022).

Similarly, only relatively few target mRNAs have been reported for FarS, OppZ, and CarZ. In contrast to MicX, FarS, OppZ, and CarZ do not have their own promoters, and thus are cotranscribed with their preceding coding genes. In the case of FarS, the upstream gene is *fabB* encoding β-ketoacyl-ACP synthase involved in fatty acid biosynthesis (Fig. 5B). Transcription of *fabB–farS* is activated by FadR, a major transcriptional regulator of fatty acid metabolism, and the transcript is processed by RNase E to release the mature FarS sRNA (Huber et al. 2020). Together with

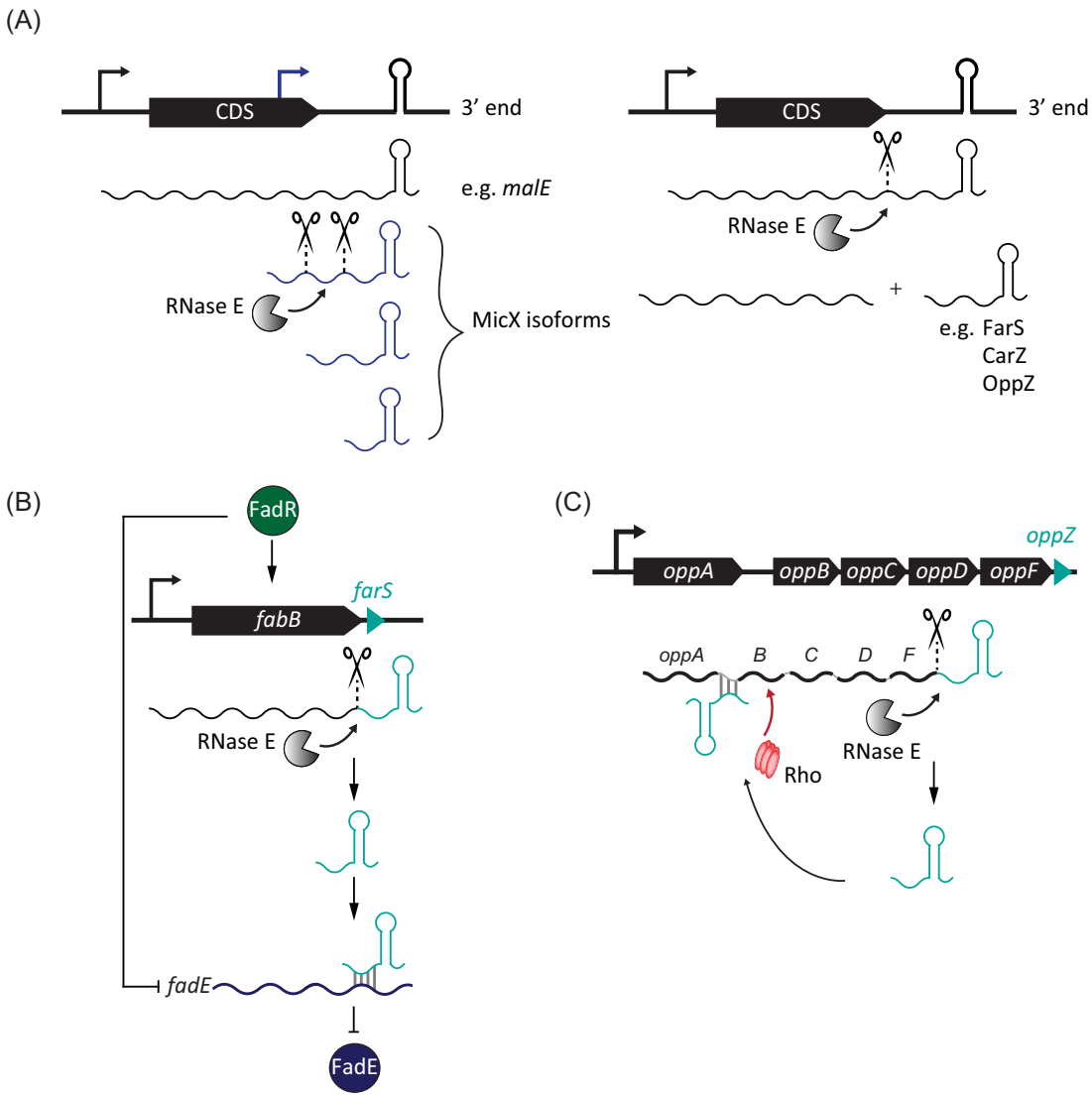

Figure 5. (A) 3′UTR-derived sRNAs in *V. cholerae*. Left: MicX is a 3′UTR-derived sRNA, i.e. produced from its own promoter. MicX is further processed by RNase E into additional isoforms. Right: Transcription of the FarS, CarZ, and OppZ sRNAs is controlled by the promoter of their upstream genes. RNase E-mediated cleavage of the respective mRNAs is required for maturation of these sRNAs. **(B)** The FarS sRNA regulates fatty acid metabolism. FarS is expressed from the promoter of its upstream gene, *fabB*, whose transcription is activated by FadR. The mature sRNA is generated by RNase E. FarS base-pairs and inhibits *fadE* whose transcription is inhibited by FadR. **(C)** Gene autoregulation by the OppZ sRNA. OppZ is produced from the 3′UTR of the *oppABCDF* operon and base-pairs with the intergenic region between the *oppA* and *oppB* mRNAs. OppZ binding inhibits *oppBCDF* expression by promoting Rho-dependent transcription termination. OppZ does not affect the *oppA* mRNA.

Hfq, FarS base-pairs with two target mRNAs, *vc1740* and *vc2231*, both of which encode FadE acyl-CoA dehydrogenase. FadE is involved in fatty acid degradation and transcription of the corresponding genes is inhibited by FadR. Thus, FadR, *fabB–farS*, and the *fadE* genes constitute a mixed feed-forward loop that modulates the transition from fatty acid degradation to biosynthesis in *V. cholerae* (Huber et al. 2020).

Regulation of genes associated with their own expression also applies to the OppZ and CarZ sRNAs. However, in contrast to FarS (and other 3′UTR-derived sRNAs), OppZ and CarZ are involved in autoregulation by base-pairing with their own polycistronic mRNAs. Specifically, OppZ and CarZ are produced from the 3′UTRs of the *oppABCDF* and *carAB* transcripts, respectively, and accumulation of the mature sRNAs requires RNase E and Hfq (Hoyos et al. 2020, Huber et al. 2020). Whereas CarZ base-pairs with the 5′UTRs of the *carAB* mRNA to inhibit CarAB translation, OppZ interacts with the intergenic region between the *oppA* and *oppB* cistrons to down-regulate OppBCDF synthesis while leaving OppA production unaffected (Hoyos et al. 2020) (Fig. 5C). Both sRNAs also regulate their own expression. Specifically, base-pairing of the sRNAs with their respective targets leads to inhibition of translation initiation, which in turn facilitates Rho-dependent transcription termination resulting in reduced sRNA synthesis (Fig. 5C). This mechanism of gene autoregulation acts at the post-transcriptional level and is independent of transcriptional regulators. Interestingly, RIL-Seq analyses in *V. cholerae* suggest that several other mRNAs contain 3′UTR-derived sRNAs that control their own translation (Huber et al. 2022) and similar cases can be found in the RIL-Seq datasets of *E. coli* and *S. enterica* (Melamed et al. 2016, Melamed et al. 2020, Matera et al. 2022), suggesting that this mechanism of gene regulation could be a common feature among 3′UTR-derived sRNAs that interact with Hfq.

Summary and outlook

The identification and characterization of sRNAs has been pioneered in model organisms such as *E. coli* and *S. enterica*, which established sRNAs as central players in nearly all areas of microbial physiology (Waters and Storz 2009, Hör et al. 2020). In *V. cholerae* and related *Vibrios*, work on regulatory RNAs for long has focused on sRNAs controlling QS- and pathogenicity-related processes (Bardill and Hammer 2012, Perez-Reytor et al. 2016). In contrast, several studies over the past few years have established equally broad roles for sRNAs in *V. cholerae* including the various aspects outlined in this article.

In addition to the numerous *Vibrio*-specific sRNAs discussed above, the genome of *V. cholerae* also carries several sRNA genes that are highly conserved and have been studied previously in other organisms. For example, the RyhB sRNA has been shown to support iron homeostasis by inhibiting the translation of mRNAs encoding nonessential iron-binding proteins under iron-limiting conditions (Chareyre and Mandin 2018). RyhB likely serves an analogous function in *V. cholerae*, but also affects biofilm formation (Davis et al. 2005). Similarly, the Spot 42 and GcvB sRNAs (Papenfort and Vogel 2011, Prasse and Schmitz 2018), involved in carbohydrate and nitrogen metabolism, respectively, are conserved and expressed in *V. cholerae* (Papenfort et al. 2015), however, no detailed analysis of their physiological roles have been performed. However, recently published data from RIL-Seq analyses suggest that their regulatory functions have been conserved as well, showing a strong enrichment for candidate target transcripts involved in carbon metabolism for Spot 42 and nitrogen metabolism for GcvB (Huber et al. 2022).

The RIL-Seq analyses also included dozens of previously characterized interactions involving well-studied sRNAs, as well as novel sRNAs that require further investigation (Huber et al. 2022). For example, Vcr043, an Hfq-binding sRNAs identified in a transcriptomic study (Papenfort et al. 2015, Huber et al. 2020), was discovered to bind to and activate the *rpoS* mRNA (Han and Lory 2021), yet it is unknown how this interaction affects RpoS regulation in *V. cholerae*. In addition, as outlined above for the CsrBCD sRNAs, not all sRNAs in *V. cholerae* interact with Hfq. For example, the FlaX sRNA is encoded downstream of the *flaA* flagellin gene and involved in motility of *V. cholerae* (Dong and Mekalanos 2012), however, the molecular underpinnings of these findings remain to be investigated.

In summary, gene regulation involving sRNAs is required for almost all aspects of *V. cholerae*'s lifestyle and physiology. Importantly, there are still dozens of annotated, yet uncharacterized sRNAs, in this organism suggesting a plethora of unexplored regulatory interactions. How these sRNAs shape gene regulation in *V. cholerae* (and possibly other related species) will require further investigations and might well involve unexpected regulatory mechanisms and additional RNA binding proteins. For instance, the global RNA binding ProQ (Holmqvist et al. 2020) is conserved in *V. cholerae*, however, its role in gene expression control has not been explored.

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