A cis-regulatory antisense RNA represses translation in *Vibrio cholerae* through extensive complementarity and proximity to the target locus

Howard Chang¹, John Michael Replogle¹, Naomi Vather¹, Maya Tsao-Wu, Ronak Mistry, and Jane M Liu*

Department of Chemistry; Pomona College; Claremont, CA USA

¹These authors contributed equally.

**Keywords:** antisense RNA, cis-acting RNA, MtlA, mannitol, post-transcriptional regulation, regulatory RNA, *Vibrio cholerae*

As with all facultative pathogens, *Vibrio cholerae* must optimize its cellular processes to adapt to different environments with varying carbon sources and to environmental stresses. More specifically, in order to metabolize mannitol, *V. cholerae* must regulate the synthesis of MtlA, a mannitol transporter protein produced exclusively in the presence of mannitol. We previously showed that a cis-acting small RNA (sRNA) expressed by *V. cholerae*, MtlS, appears to post-transcriptionally downregulate the expression of *mtlA* and is produced in the absence of mannitol. We hypothesized that since it is complementary to the 5’ untranslated region (UTR) of *mtlA* mRNA, MtlS may affect synthesis of MtlA by forming an *mtlA*-MtlS complex that blocks translation of the mRNA through occlusion of its ribosome binding site. To test this hypothesis, we used in vitro translation assays in order to examine the role MtlS plays in *mtlA* regulation and found that MtlS is sufficient to suppress translation of transcripts harboring the 5’ UTR of *mtlA*. However, in a cellular context, the 5’ UTR of *mtlA* is not sufficient for targeted repression by endogenous MtlS; additional segments from the coding region of *mtlA* play a role in the ability of the sRNA to regulate translation of *mtlA* mRNA. Additionally, proximity of transcription sites between the sRNA and mRNA significantly affects the efficacy of MtlS.

**Introduction**

With an estimated 3–5 million cases a year, cholera represents a global public health concern as well as a paradigm model for known and emerging diseases.¹⁻³ *Vibrio cholerae*, the causative agent of cholera, is a bacterium that resides mostly in the marine habitat but also thrives in the human gastrointestinal (GI) tract and in freshwater environments endemic to cholera.⁴⁻⁶ The life cycle of *V. cholerae* involves repetitive transitions between aquatic environments and the host GI tract. Genome-wide transcriptional changes occur as the bacteria transition between aquatic reservoir and host, suggesting that different sets of gene products allow *V. cholerae* to persist and thrive in environments with varying nutrients and carbon sources.⁷⁻¹⁰ This characteristic is likely key to its fitness as a facultative pathogen.¹¹ Nevertheless, the molecular and cellular components involved in the ability of *V. cholerae* to adapt to different environments and to persist in its natural environment remain to be fully defined.¹¹

In an effort to elucidate the molecular mechanisms by which *V. cholerae* adapts to changing carbon sources, we are investigating how *V. cholerae* regulates expression of *mtlA*, the gene encoding the mannitol-specific transporter, MtlA, of the phosphoenolpyruvate: sugar phosphotransferase system (PTS). Highly conserved in both Gram-negative and Gram-positive bacteria, the PTS catalyzes the uptake and concomitant phosphorylation of numerous carbohydrates.¹² In *V. cholerae*, the PTS is solely responsible for transport of several carbon sources, including mannitol.¹³,¹⁴ *V. cholerae* lacking active MtlA is unable to ferment mannitol or survive in medium containing mannitol as the sole carbon source.¹³,¹⁵,¹⁶ MtlA is up-regulated two-fold during colonization in the rabbit ileal loop, suggesting that *V. cholerae* may use MtlA and other components of the PTS to adapt appropriately to changing nutritional cues in the environment of the small intestine.¹⁶ In addition, it was recently reported that the *V. cholerae* MtlA protein is capable of activating biofilm formation independent of its role in transporting mannitol.¹⁷ In aquatic
habitats, *V. cholerae* are known to form biofilms, a feature that enhances their environmental persistence.\(^5\)\(^,\)\(^11\) It is hypothesized that mannitol may provide an extracellular cue to *V. cholerae* of hospitable environments to colonize via surface attachment and biofilm formation.\(^17\) Collectively, these findings suggest that the regulation of *mtlA* expression may have implications throughout the life cycle of *V. cholerae*, affecting both the transmission and the persistence of the pathogen.

We recently reported that in *V. cholerae*, a small RNA (sRNA), MtlS, represses the expression of *mtlA* at the post-transcriptional level.\(^16\)\(^,\)\(^18\) sRNAs are short, usually non-coding, strands of RNA that regulate gene expression, often by base pairing with mRNA targets. By binding to its mRNA target, the sRNA affects mRNA expression through a variety of mechanisms (for recent reviews see refs. 19–24). Broadly speaking, sRNAs can be classified as either cis- or trans-acting, depending on whether they are transcribed directly antisense or distal, respectively, to the genes they regulate.\(^19\)\(^,\)\(^24\) It is common for trans sRNAs to be between 50 and 300 nt long, and to share imperfect complementarity with their gene target.\(^20\) Cis sRNAs, on the other hand, can be anywhere from \(~100\) to over \(1000\) nt long, share perfect complementarity with their target mRNA, and may overlap with the 5′-end, 3′-end or middle of the gene encoded opposite the sRNA.\(^19\)\(^,\)\(^25\)\(^,\)\(^26\)

MtlS is a cis-acting sRNA. Intriguingly, a common theme among cis-acting sRNAs is that the best-characterized ones act by repressing the synthesis of a protein that is potentially toxic to the cell.\(^27\)\(^–\)\(^30\) For example, the 67 nt Sok sRNA is part of the *hok/sok* system of plasmid R1, which mediates plasmid stabilization by post-segregational killing of plasmid-free cells.\(^31\)\(^,\)\(^32\) The *hok* gene, encoding a toxic protein, is constitutively expressed from the R1 plasmid as a stable mRNA. While this mechanism is similar to that used by the SymR and Sib sRNAs, in this case, the target gene does not encode a toxin but a transporter protein.\(^21\)\(^,\)\(^22\)\(^,\)\(^23\)\(^,\)\(^24\)

Like Sok sRNA, the 77 nt SymR cis-acting sRNA of *Escherichia coli* represses the translation of a toxic protein, SymE; however, the genes encoding the sRNA and its antisense target are encoded on the chromosome rather than on a plasmid.\(^35\) SymR shares perfect complementarity with the majority of the 5′ UTR of *symE* mRNA, including the ribosome binding site (RBS).\(^33\) Furthermore, in an *E. coli ΔsymR* strain, SymE protein levels increase \(~10\)-fold, while the *symE* mRNA levels only increase \(~5\)-fold, suggesting that the sRNA regulates gene expression primarily through translational repression.\(^33\) Similarly, the *E. coli* genome contains multiple Sib sRNAs, all of which are cis-acting regulators expected to prevent translation of the highly hydrophobic and toxic Ibs proteins through sRNA:mRNA duplex formation.\(^30\) The SibC antitoxin RNA, for example, transcribed antisense to the gene encoding toxic IbsC, is 141 nt long and exhibits perfect complementarity with the entire coding sequence of *ibsC* and the majority of the *ibsC* 5′ UTR, including the RBS.\(^30\)\(^,\)\(^34\)

It has been proposed that cis sRNAs may have several advantages over their trans-acting counterparts. First, the complete and extended complementarity between the sRNA and its target can lead to very stable duplexes, allowing regulation to be independent of proteins such as the RNA chaperone protein Hfq.\(^25\) Indeed, we previously demonstrated that cis-acting MtlS sRNA represses MtlA synthesis in an Hfq-independent manner.\(^18\) In addition, the cis position should increase effective molarity that may facilitate and enhance interactions between the regulator and target molecules.\(^25\)

With regard to this current work, the cis-acting MtlS is 120 nt long and shares 71 nt of perfect complementarity with the 5′ UTR of *mtlA* (Fig. 1).\(^18\) We previously observed an inverse relationship between MtlA protein and MtlS sRNA levels in *V. cholerae*; MtlS is expressed in cells grown in all carbon sources other than mannitol, while MtlA protein is only synthesized when mannitol is present.\(^18\) We also demonstrated that MtlS sRNA represses MtlA synthesis without affecting *mtlA* mRNA levels.\(^18\) These results led us to propose a model in which the MtlS sRNA represses MtlA synthesis by binding to the 5′ UTR of the *mtlA* transcript, blocking the translation machinery from binding the mRNA. While this mechanism is similar to that used by the SymR and Sib sRNAs, in this case, the target gene does not encode a toxin but a transporter protein.

In the present study, we tested our model by assessing whether the MtlS and *mtlA* sRNAs are capable of binding to each other and whether duplex formation is sufficient for translational repression of *mtlA*. While the sense-antisense base pairing interactions for plasmid-transcribed RNAs have been extensively studied, the same cannot be said about chromosomal sense-antisense RNA pairs.\(^25\)\(^,\)\(^26\) As MtlS represents a chromosomally encoded cis-acting RNA, and is also one of the few examples of an antisense sRNA that regulates a target other than a toxin-encoding gene, understanding the molecular mechanism by which MtlS affects *mtlA* expression could provide important insights into the capabilities of cis sRNAs in bacteria. Our work shows that MtlS and

---

**Figure 1.** The *mtlA* mRNA and MtlS sRNA share 71 nt of perfect complementarity. The *mtlA* mRNA has a 75 nt long 5′ UTR, numbered -75 to 1 with respect to the start codon (bold type). The MtlS sRNA is 120 nt long. The predicted pairing between the two transcripts is shown with dashed lines.
mtlA RNAs can form a stable duplex that consequently represses the translation of mtlA mRNA. We also provide evidence that in the cellular context, MtlS is most effective at repressing mtlA mRNA translation when the sRNA is transcribed directly antisense to mtlA.

Results

MtlS binds specifically to the mtlA 5’ UTR and represses translation of downstream genes in vitro

To test directly whether MtlS binds to the mtlA mRNA, we investigated duplex formation between MtlS sRNA and the mtlA 5’ UTR RNA in vitro by gel mobility shift assays. First, a fixed concentration of biotin-labeled mtlA 5’ UTR RNA was incubated with increasing concentrations of unlabeled MtlS RNA for 30 min. Complex formation was analyzed by gel electrophoresis on a native polyacrylamide gel. We observed that MtlS is able to bind mtlA 5’ UTR RNA with an apparent K_d of ~70 nM and that the addition of unlabeled mtlA 5’ UTR RNA decreased complex formation (Fig. 2A, lane 6). As a control, we also tested binding between mtlA 5’ UTR RNA and the reverse complement of MtlS, antisense MtlS (asMtlS), which possesses no significant complementarity to the mtlA 5’ UTR. We observed no duplex formation between mtlA 5’ UTR and asMtlS (Fig. 2B). These experiments indicate that MtlS specifically binds the mtlA 5’ UTR in vitro with high affinity.

Next, we tested whether the binding of MtlS to the mtlA 5’ UTR is sufficient for repressing translation of the downstream coding sequence. Using in vitro translation assays, we first combined an mtlA transcript with increasing amounts of MtlS RNA. The mtlA transcript included the 5’ UTR of mtlA, the first 378 nt of the mtlA coding sequence, and the sequence for a Flag epitope tag immediately prior to the stop codon (mtlA’-flag). This mtlA transcript was prepared by in vitro transcription and gel purification. The resulting RNA was mixed with increasing amounts of in vitro-transcribed, gel-purified MtlS RNA. The two RNAs were incubated at 37°C for 30 min and then added to an in vitro translation reaction mixture. The reaction was allowed to proceed for 60 min at 37°C at which point the reaction was stopped and the resulting proteins were quantified through western blot analysis. We observed that the addition of MtlS to the reaction mixture reduced translation of mtlA RNA in a dose-dependent manner with an IC_{50} ~ 200 nM (Fig. 3A). We considered the effect of MtlS on mtlA mRNA translation to be specific because translation of a gfp-flag internal control RNA was largely unaffected under the same experimental conditions (Fig. 3A). Furthermore, the addition of asMtlS to the reaction mixture did not reduce mtlA’-flag translation, even when the asRNA was present in excess (Fig. 3B).

We also performed the in vitro translation assay with an RNA construct in which the 5’ UTR of mtlA was fused to the first 357 nt of the coding sequence of gfp and a C-terminal Flag epitope tag (mtlA 5’UTR-gfp'). Increasing amounts of MtlS reduced translation of the mtlA 5’UTR-gfp' transcript, while the amount of GFP synthesized from the gfp-flag internal control remained unchanged (Fig. 3C). Collectively, our in vitro data indicate that the mtlA 5’ UTR alone is sufficient for MtlS to repress the translation of downstream genes (either mtlA or gfp) in vitro.

Our proposed model suggests that occlusion of the mtlA RBS is responsible for translational repression of mtlA when MtlS sRNA is present. Thus, we expected that only the region of MtlS complementary to the RBS would be necessary to inhibit translation of mtlA mRNA. To test this, we constructed MtlS variants (Fig. 3D) and tested them using in vitro translation assays with mtlA’-flag RNA. Construct A includes only the 5’ end of MtlS (+1–45) and is expected to still bind the predicted mtlA RBS. Construct B (+1–71) served as a control in that it includes the region of MtlS predicted to bind the mtlA RBS along with the rest of the MtlS segment predicted to base pair with mtlA mRNA (Fig. 1). Initially, we observed that the full length MtlS was able to repress MtlA’-Flag synthesis in our in vitro assay, but Constructs A and B could not (data not shown). When we included denaturing and annealing steps with the RNA to improve binding, prior to adding the in vitro translation
reaction mixture, however, we consistently observed that both the full length MtlS and Construct B (+1–71) were able to repress MtlA′-Flag synthesis (Fig. 3E). The MtlS variant that included only the first 45 nt of MtlS (Construct A), however, was unable to repress MtlA′-Flag synthesis (Fig. 3E). This difference in translational repression is not due to instability of Construct A, as we observed that all 3 sRNA constructs are stable after denaturing, annealing and incubation at 37°C for 30 minutes (Fig. S1). We also tested whether Construct A is able to bind the mtlA mRNA. Our observations suggest that this shortened MtlS transcript is not efficient in binding the mtlA mRNA 5′ UTR under the in vitro translation conditions (Fig. S2). Thus, in this in vitro setting, increased pairing between MtlS and mtlA mRNA allows for a more stable duplex to form, which is necessary for translational repression of the mRNA.

**MtlS overexpression represses translation of a reporter gene in E. coli and V. cholerae**

To address whether the 5′ UTR of mtlA was also sufficient for translational repression in a cellular context, we cloned the mtlA 5′ UTR, along with the start codon, into a GFP reporter plasmid (pReporter5′UTR) and tested reporter repression by MtlS (Fig. 4). The gfp construct is transcribed from a constitutive promoter (P_LacO_1), so changes in reporter activity indicate post-transcriptional regulation.36 We first tested whether MtlS could repress translation of the mtlA 5′ UTR-gfp fusion in the absence of *Vibrio*-specific factors. Two plasmids, pReporter5′UTR and pMtlS, were introduced into *E. coli*, which lacks endogenous MtlS;16 pMtlS expresses full-length MtlS from an arabinose-inducible promoter, allowing for tight, controlled overexpression of the sRNA.18 As a control, pReporter5′UTR was also combined with a Vector plasmid, which expresses a ~250 nt long RNA that shares no extensive complementarity to mtlA. MtlS overexpression repressed mtlA 5′ UTR-gfp reporter activity by 70% in 3 separate growth conditions, as assayed by either fluorescence measurements or by western blot analysis (Figs. S2 and S3). These results support a model in which MtlS sRNA is able to repress translation of mtlA RNA through just the 5′ UTR of the messenger transcript.

We noted that a reporter plasmid containing a truncated mtlA 5′ UTR, consisting of only the last 48 of the 75 nt region, fused to the gfp coding sequence (pReporter5′Short; Fig. 4) was much less responsive to MtlS despite the fact that the MtlS and the shortened mtlA 5′ UTR were still able to bind to each other in vitro with a K_d of ~240 nM (Figs. S2B and S2C). Full length and shortened mtlA 5′ UTR constructs can both bind to MtlS during

---

**Figure 3.** In vitro translation assays of mtlA mRNA. Unless otherwise specified, both mtlA′-flag RNA (120 nM) and gfp-flag RNA (320 nM) were translated together in a reaction mixture (37°C) with varying amounts of sRNA. After 60 minutes, 1/10 of the reaction mixture was removed and subjected to western blotting using an anti-Flag antibody. (A) Increasing the amount of MtlS represses translation of mtlA′-flag mRNA, but does not affect GFP-Flag synthesis. (B) A specific sRNA is required to repress mtlA translation. In lanes 1–6, the sRNA added to the reaction mixture was antisense MtlS (asMtlS); in lane 7, the sRNA added to the reaction mixture was MtlS. (C) The 5′ UTR of mtlA is sufficient for repression of downstream gene expression in vitro. An in vitro translation assay was performed, as in (A), replacing the mtlA′-flag RNA with mtlA5′UTR-gfp′-flag (5′ UTR of mtlA fused to the first 357 nt of the gfp coding sequence; 240 nM). (D) Schematic representation of MtlS, which is a 120 nt transcript. MtlS variants synthesized in this study are Construct A (nt +1–45 of MtlS) and Construct B (nt +1–71 of MtlS). The region of MtlS expected to overlap with the predicted RBS of mtlA is denoted by a black box. (E) An MtlS transcript containing only the first 45 nt is not sufficient for translational repression of mtlA. In lane 2, MtlS RNA was added to the reaction mixture. In lanes 3 and 4, Constructs A (+1–45) and B (+1–71) were added to the reaction mixtures, respectively.
Figure 4. Schematic representation of the mtlA gene. The mtlA mRNA includes a 75 nt 5' UTR. Translational mtlA-gfp fusions (under control of the constitutive P_{Lacto-1} promoter) were constructed to include only the complete 5' UTR of mtlA (pReporter5'UTR), only a shortened 5' UTR (pReporter5'Short), or both the complete 5' UTR and the entire coding sequence of mtlA (pReporterFull). The promoter for MtlS RNA lies within the coding region of mtlA resulting in transcription of an sRNA, MtlS, antisense to the mtlA 5' UTR. MtlS shares 71 nt of perfect complementarity with the mtlA 5' UTR. pReporterFull also harbors the mts promoter and may express MtlS'; the 3' end of this transcript is unknown.

In an in vitro gel shift assay with no other RNA binding macromolecules present, but the binding affinity for Mts of the truncated mtlA 5' UTR is 3 times lower than that of the full-length mtlA 5' UTR (compare Figs. 2A and 5C). We postulate that, in a cellular context, the reduced affinity of the truncated mtlA 5' UTR for Mts may make the sRNA insufficient to out-compete the ribosome for binding the mtlA 5' UTR and prevent translation. The very low levels of repression we observe with the pReporter5'-Short construct support this hypothesis (Fig. 5B). Decreased mRNA-sRNA pairing to compete with mRNA-ribosome pairing may also explain why the 45 nt Construct A (Fig. 3D) was unable to bind the mtlA 5' UTR under in vitro translation conditions, which included ribosomes that could bind the mtlA 5' UTR and prevent the truncated Mts from binding (Fig. S2). These results suggest that in the cellular context, increased pairing between MtlS and mtlA mRNA (>45 bp) allows for more efficient translational repression of the mRNA.

To assess whether the repression of reporter activity observed upon overexpression of Mts in E. coli cells could also occur in V. cholerae, we introduced both pReporter5'UTR and pMts into V. cholerae. It is important to note that the resulting strains harbor endogenous Mts transcribed from the chromosome as well as any Mts that may be expressed from pMts. Despite this additional source of Mts and in contrast to our E. coli results, we found that the same experiment in V. cholerae resulted in only a 20–50 percent reduction in fluorescence relative to the Vector control when cells were grown in any of 3 different growth conditions (Fig. 5D).

These results are also in contrast to our previous observations that overexpression of Mts under certain conditions is sufficient to completely repress MtlA synthesis from the chromosome within 60 minutes of inducing sRNA expression. We have proposed that proteolysis of MtlA is also induced when Mts levels increase. GFP, however, is a stable protein, and we would not expect Mts to be able to repress its translation and lead to active degradation of already synthesized protein. This difference between GFP and MtlA protein stability after Mts induction may explain the discrepancies we observe in how effectively Mts knocks down MtlA synthesis versus GFP synthesis. The fact that we do observe down-regulation of gfp reporter activity in our cellular assays, however, suggests that this is an appropriate model to investigate Mts activity on mtlA expression.

With regard to the differences between Mts-mediated knockdown in E. coli vs. V. cholerae, we have observed similar overexpression levels of Mts from pMts in both E. coli and in V. cholerae (data not shown). Thus, differences in Mts levels in the different strains cannot necessarily explain our observations. Instead, these results suggest that while the mtlA 5' UTR is sufficient for 70% repression of downstream gene expression by Mts in E. coli, additional elements may be required to achieve similar repression levels in V. cholerae. Alternatively, in V. cholerae, Mts may have multiple targets that may titrate the sRNA away from the mtlA 5'UTR-gfp target (see below).

The 5' UTR of mtlA is not sufficient for reporter repression by endogenous Mts in V. cholerae

The above experiments assayed reporter activity upon overexpression of the Mts sRNA. However, to analyze the effects of natural Mts levels on reporter activity, we assayed V. cholerae cells harboring only pReporter5'UTR and grown in either sucrose/glucose or mannitol media, conditions in which the bacteria express relatively high and low levels of Mts, respectively. The normal levels of Mts in V. cholerae grown in sucrose or glucose media is low in comparison to strains that overexpress the sRNA from pMts. Surprisingly, we did not observe any repression of reporter activity in V. cholerae harboring pReporter5'UTR when the bacteria were grown in sucrose (Mts-high) medium (Fig. 6A). These results are directly in contrast to our in vitro translation data above which suggests that the 5' UTR of mtlA should be sufficient for translational repression of any downstream gene by Mts (Fig. 3C). One explanation for these results is that in the cellular context, the effective concentration of Mts is low in the vicinity of the mtlA 5'UTR-gfp transcript. Endogenous mtlA transcripts, for example, could titrate away most of the Mts, reducing the amount of sRNA available to effectively repress expression of gfp from the pReporter5'UTR plasmid. The multicopy reporter plasmid coupled with the constitutive P_{Lacto-1} promoter could also cause excess production of mtlA 5'UTR-gfp mRNA, decreasing the relative concentration of Mts compared to the chromosomal context and further exacerbating the situation.

However, we also hypothesized that additional segments within the coding sequence of mtlA may be necessary for repression of reporter activity by Mts in V. cholerae. Thus, we created a V. cholerae strain harboring a single plasmid (pReporterFull) that constitutively expresses a fusion of the mtlA 5' UTR, the
MtlS can affect downstream gene expression through the full-length 5’ UTR of mtlA. (A) Regulation of GFP activity was monitored by fluorescence assays of E. coli TOP10 cells harboring pReporter5’UTR and either Vector or pMtlS. Cells were cultured in LB medium or M9 minimal medium with the indicated carbon source (0.4%), with 0.02% arabinose to induce mtlS expression from pMtlS, at 37 °C to an OD600 of 1; relative fluorescence units (RFU) were normalized to OD600. All data points were normalized to the average RFU/OD600 of the Vector controls in each growth condition. (B) When the 5’ UTR is truncated (including only the last 48 nt of the 75 nt region), repression of reporter activity is largely abolished. GFP activity was monitored, as in (A), for E. coli TOP10 cells harboring pReporters’Short and either Vector or pMtlS. (C) The truncated mtlA 5’ UTR used in (B) can still bind MtlS. EMSA using a 5’ TYE705-labeled, shortened mtlA 5’ UTR transcript and various concentrations of MtlS. The sample run in lane 5 included an excess of unlabeled mtlA RNA (500 nM) that prevented MtlS RNA from binding labeled mtlA RNA. Positions of mtlA (open circle) or the MtlS/mtlA complex (filled circle) are indicated. (D) Regulation of GFP activity was monitored, as in (A), for V. cholerae harboring pReporters’UTR and either Vector or pMtlS. The bar graphs show the means and standard deviations from at least 3 independent samples. *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired t-test.

Figure 5. When overexpressed, MtlS can affect downstream gene expression through the full-length 5’ UTR of mtlA. (A) Regulation of GFP activity was monitored by fluorescence assays of E. coli TOP10 cells harboring pReporter5’UTR and either Vector or pMtlS. Cells were cultured in LB medium or M9 minimal medium with the indicated carbon source (0.4%), with 0.02% arabinose to induce mtlS expression from pMtlS, at 37 °C to an OD600 of 1; relative fluorescence units (RFU) were normalized to OD600. All data points were normalized to the average RFU/OD600 of the Vector controls in each growth condition. (B) When the 5’ UTR is truncated (including only the last 48 nt of the 75 nt region), repression of reporter activity is largely abolished. GFP activity was monitored, as in (A), for E. coli TOP10 cells harboring pReporters’Short and either Vector or pMtlS. (C) The truncated mtlA 5’ UTR used in (B) can still bind MtlS. EMSA using a 5’ TYE705-labeled, shortened mtlA 5’ UTR transcript and various concentrations of MtlS. The sample run in lane 5 included an excess of unlabeled mtlA RNA (500 nM) that prevented MtlS RNA from binding labeled mtlA RNA. Positions of mtlA (open circle) or the MtlS/mtlA complex (filled circle) are indicated. (D) Regulation of GFP activity was monitored, as in (A), for V. cholerae harboring pReporters’UTR and either Vector or pMtlS. The bar graphs show the means and standard deviations from at least 3 independent samples. *p < 0.05; **p < 0.01; ***p < 0.001 by unpaired t-test.

Entire mtlA coding sequence, and the coding sequence of gfp (Fig. 4). Initial fluorescence assays and western blot analysis indicated that this fusion is capable of producing full-length, fluorescent GFP (Fig. S4 and S5). When V. cholerae harboring pReporterFull grown in glucose and mannitol media, we consistently observed a significant 30% repression of gfp expression when the bacteria were grown in glucose, as compared to V. cholerae downstream gene expression by MtlS in mtlA-gfp. Consistently observed a significant 30% repression of pReporterFull were grown in glucose and mannitol media, we indicated that this fusion is capable of producing full-length, fluorescent GFP (Fig. S4 and S5). When V. cholerae harboring pReporterFull grown in glucose and mannitol media, we consistently observed a significant 30% repression of gfp expression when the bacteria were grown in glucose, as compared to V. cholerae downstream gene expression by MtlS in mtlA-gfp. Consistently observed a significant 30% repression of pReporterFull were grown in glucose and mannitol media, we indicated that this fusion is capable of producing full-length, fluorescent GFP (Fig. S4 and S5).

We tested this hypothesis by northern blot analysis of total RNA from bacteria harboring pReporterFull grown in both glucose and mannitol medium and observed no difference in mtlA-gfp RNA levels between the two growth conditions (Fig. 6B). Thus, the repression of reporter expression observed in Figure 6A (WT pFull) is not caused by differences in the abundance of the mtlA-gfp transcript in the different growth media.

Reporter activity is repressed by an antisense transcript expressed from the reporter plasmid

Previously, we mapped the precise +1 site of mtlS by sRNA-Seq,16 which, as revealed by PromoterHunter,38 is preceded by −10 and −35 elements separated by a 16-bp spacer. These putative promoter elements lie within the coding region of mtlA. Thus, we considered that inclusion of the mtlA coding sequence fused to gfp in the reporter plasmid might explain the greater translational repression of the encoded mtlA-gfp than the mtlA 5’ UTR alone, because the mtlA coding sequence enables transcription of an RNA in cis and antisense to the mtlA-gfp transcript. Hereafter, we refer to this putative transcript as MtlS* (Fig. 4). We consider MtlS* to be distinct from endogenous MtlS in that the plasmid-derived MtlS* is transcribed in close proximity to the mtlA-gfp fusion and could potentially bind more readily to the 5’ UTR of the reporter mtlA-gfp mRNA. We expect that since mtlS lacks the native transcriptional terminator of mtlS, the transcript may also include regions from the vector backbone of pReporterFull.

In order to test whether MtlS* is actually transcribed from the reporter plasmid, we used quantitative real-time PCR (qPCR) to analyze MtlS* levels in both the wild type and a ΔmtlS strain, each harboring pReporterFull. For the qPCR analysis, we used forward and reverse primers that anneal to sequences common to both MtlS and MtlS*. Thus, when analyzing RNA samples from the wild type strain, both MtlS and MtlS* are being measured. The ΔmtlS strain does not express any MtlS; however, we did observe MtlS* expression from the ΔmtlS strain harboring pReporterFull (p < 0.01, compared to ΔmtlS parent, by unpaired t-test), supporting a model in which an antisense transcript is expressed from the reporter plasmid (Fig. 7). We also attempted multiple northern blot experiments to observe MtlS* expressed from ΔmtlS bacteria harboring pReporterFull to determine the size of the transcript. However, after repeated attempts, we were unable to observe the MtlS* transcript through northern blot.
analysis. It is possible that MtlS* is a very long transcript that does not enter the gel during electrophoresis, MtlS* is a relatively unstable transcript, and/or our northern blot method is not sensitive enough to detect this particular transcript.

Surprisingly, we consistently observed that wild type V. cholerae harboring pReporters' UTR or pReporterFull, and mutant V. cholerae, lacking the genomic mtlS locus (∆mtls) and harboring pReporterFull, were cultured in M9 minimal medium with the indicated carbon source (0.4%), at 37°C to an OD600 of 10; relative fluorescence units (RFU) were normalized to OD600. All data points were normalized to the average RFU/OD600 of the manniot samples. The bar graphs show the means and standard deviations from at least 3 independent samples. n.s., p > 0.05; ***p < 0.001 by unpaired t-test. (B) Northern analysis of RNA isolated from the same WT pFull cells used for the fluorescence assay in (A). mtlA-gfp RNA was detected with biotin-labeled RNA probes specific to gfp. The lower band may represent a processed version of the mtlA-gfp RNA. rpsL RNA was detected using an IR800-dye-labeled oligonucleotide specific to rpsL and was included as a loading control. The control lane was loaded with total RNA from wild type V. cholerae lacking gfp. This blot is representative of 3 independent trials.

Figure 6. Including the mtlA coding sequence represses reporter activity in non-mannitol carbon sources. (A) Wild type (WT) V. cholerae harboring pReporters' UTR or pReporterFull, and mutant V. cholerae, lacking the genomic mtlS locus (∆mtls) and harboring pReporterFull, were cultured in M9 minimal medium with the indicated carbon source (0.4%), at 37°C to an OD600 of 10; relative fluorescence units (RFU) were normalized to OD600. All data points were normalized to the average RFU/OD600 of the manniot samples. The bar graphs show the means and standard deviations from at least 3 independent samples. n.s., p > 0.05; ***p < 0.001 by unpaired t-test. (B) Northern analysis of RNA isolated from the same WT pFull cells used for the fluorescence assay in (A). mtlA-gfp RNA was detected with biotin-labeled RNA probes specific to gfp. The lower band may represent a processed version of the mtlA-gfp RNA. rpsL RNA was detected using an IR800-dye-labeled oligonucleotide specific to rpsL and was included as a loading control. The control lane was loaded with total RNA from wild type V. cholerae lacking gfp. This blot is representative of 3 independent trials.

Figure 7. An antisense RNA is transcribed from pReporterFull. Strains of V. cholerae were cultured in M9 minimal medium with glucose (0.4%), at 37°C to an OD600 of 0.3. Expression from the mtlS promoter should be high under these conditions. qRT-PCR was used to measure the MtlS/MtlS* transcript levels of WT (wild type), ∆mtls, WT harboring pFull (pReporterFull), ∆mtls harboring pFull, and WT harboring p5'UTR (pReporter5'UTR) strains of V. cholerae. The relative abundance of MtlS/MtlS* transcript was determined as described in Materials and Methods. Shown are the means and standard deviations from at least 3 independent samples. n.s., p > 0.05; **p < 0.01; ***p < 0.001 by unpaired t-test comparing each sample to the WT sample.

MtlS* is relatively unstable (see above), then the overall levels of MtlS/MtlS* RNA would be lower in cells harboring pReporterFull, which is consistent with our observations.

In order to evaluate the contributions of the plasmid-expressed MtlS* and chromosomally-derived MtlS, we tested reporter activity in the ∆mtls strain harboring pReporterFull. The ∆mtls pReporterFull strain exhibited a similar, if not greater, reduction in fluorescence as the wild type strain harboring pReporterFull when both strains were grown in glucose versus manniot medium (compare WT pFull and ∆mtls pFull in Fig. 6A). In the ∆mtls mutants, MtlS* from the plasmid constitutes the only source of an RNA complementary to the mtlA 5' UTR (Fig. 7, compare ∆mtls and ∆mtls pFull). Therefore, these results indicate that the plasmid-derived MtlS*, transcribed in close proximity to the mtlA 5' UTR, is sufficient to repress mtlA-gfp expression.

We further reasoned that by truncating the mtlA coding sequence in pReporterFull, we could delineate the segment(s) of the mtlA coding sequence necessary for maximal expression of MtlS* and reveal potential regulatory elements of the mtlS promoter. We created truncated versions of pReporterFull that all contain the mtlA 5' UTR followed by either the first 42, 102, or 1,011 nucleotides of the mtlA coding sequence fused to the 5' end of gfp (creating pReporter42, pReporter102, or pReporter1011, respectively). These constructs were designed to incorporate only the predicted -10 and -35 elements of the predicted mtlS promoter (pReporter42), a short region beyond the
**mtlS promoter** (pReporter102), or a significant segment of **mtlA** extending beyond the **mtlS** promoter (pReporter1011).

Wild type strains harboring any of the 3 truncations exhibited some repression of reporter activity in glucose medium (Fig. 8A). However, the strains harboring pReporter102 and pReporter1011 were three-fold more effective than the strain harboring pReporter42 at repressing gfp expression. These results suggest that sequences between the +42 and +1011 nucleotides of the **mtlA** coding sequence contain important regulatory elements needed to repress translation of MtdA in *V. cholerae*. We postulated that these regulatory elements might include binding sites for transcriptional regulators that activate **mtlS** expression in the presence of glucose or the absence of mannitol. When we measured by qRT-PCR MtdS* levels expressed from Δ**mtlS** strains harboring pReporter42, pReporter102 and pReporter1011, however, we found that they all expressed similar levels of MtdS* (Fig 8B). As these measurements were taken in the Δ**mtlS** background, MtdS* is the only source of an RNA complementary to the **mtlA** 5' UTR. We therefore reason that inclusion of nt +42–1011 of the **mtlA** coding sequence affects MtdA levels not by altering the total amount of cis-encoded antisense RNA, but by providing additional elements that contribute to the overall regulation of **mtlA** expression (see Discussion).

**Effective molarity of MtdS affects translational repression of **mtlA** in *V. cholerae***

Our results above suggest that the proximal transcription of an RNA antisense to the 5' UTR of **mtlA** is more effective at repressing MtdA synthesis than the expression of a complementary RNA distal to the **mtlA** locus (Fig. 6A). However, we questioned whether increasing the amount of distally transcribed MtdS could compensate for a lack of proximity between the sRNA and the **mtlA-gfp** loci. For this analysis, we created two strains of wild type *V. cholerae* that both harbored pReporterFull. Thus, both strains are able to transcribe MtdS from the chromosome and MtdS* from the reporter plasmid. One of these strains also carried pMtdS, allowing for overexpression of the sRNA. The other strain carried the Vector control. Consistent with our observations above, when the Vector strains were grown in mannitol (MtdS/ MtdS*-low) and sucrose (MtdS/MtdS*-high) media, we observed an inverse relationship between MtdS levels and reporter activity; expression of MtdS/MtdS* was sufficient to decrease GFP fluorescence by >50% (Fig. 9A, compare “Vector” samples). Upon overexpression of MtdS from pMtdS, however, there is a significant decrease in GFP fluorescence in both sucrose and mannitol medium, compared to the Vector controls (Fig. 9A). Thus, increasing the abundance of MtdS can further reduce **mtlA-gfp** expression, even if the sRNA is expressed distal to the **mtlA-gfp** locus.

When comparing the pMtdS strain grown in mannitol (pMtdS-Mtd) vs. the Vector strain grown in sucrose (Vector-Suc), we were able to further distinguish the contributions of MtdS and MtdS* in repressing **mtlA-gfp** expression. Both of these strains are expected to produce MtdS sRNA. The Vector-Suc strain transcribes MtdS because it is grown in non-mannitol growth conditions. Harboring the MtdS-overexpression plasmid, the MtdS levels in the pMtdS-Mtd bacteria were 75-fold higher than those in the Vector-Suc bacteria (Fig. 9B). However, the Vector-Suc bacteria still had lower fluorescence levels than the pMtdS-Mtd bacteria (Fig. 9A, p < 0.05, by unpaired t-test). MtdS* is expected to be present in the Vector-Suc sample but not in the pMtdS-Mtd bacteria because expression from the **mtlS** promoter is induced in...
non-mannitol growth conditions. Thus, although increasing the amount of distally transcribed MtlS can lead to further decreases in mtlA expression, the dominant factor that affects repression of mtlA would be the proximal expression of an antisense RNA. This is likely due to the high effective molarity that a regulatory RNA would have with respect to its mRNA target when it is expressed proximal to its target locus.25,40

### Discussion

It is becoming increasingly apparent that bacteria use a variety of mechanisms for sRNA-mediated regulation of gene expression. One of the most extensively studied sRNAs is the trans-acting SgrS that negatively regulates the synthesis of PtsG, the glucose-specific transporter of the PTS in *E. coli* and *Salmonella*. Metabolic bottlenecks following sugar uptake by the PTS may cause toxic accumulation of intracellular sugar-phosphates.41 In *E. coli* and *Salmonella*, SgrS is induced upon phosphosugar stress and represses translation of *ptsG* mRNA by base pairing with the 5′ UTR of the transcript through partial complementarity and the aid of Hfq.42 Hfq can also bind to RNase E, a major endoribonuclease,43 suggesting that upon pairing with *ptsG* mRNA, SgrS and Hfq ultimately target the *ptsG* message for rapid degradation by RNase E. The physiological outcome of SgrS induction is decreased glucose transport until phosphosugar stress is alleviated.

Whereas SgrS is thought to represent a canonical model of sRNA-mediated repression of mRNA translation, involving both RBS-occlusion and targeted mRNA degradation, there are also recent examples of sRNAs that activate targets by masking RNase E cleavage sites and stabilizing the mRNA.44,45 In another example of sRNA-mediated gene activation, the trans-acting GlmY sRNA in *E. coli* acts as a molecular mimic, providing a decoy for an RNase adaptor protein to ultimately allow up-regulation of *glmS*, encoding glucosamine-6-phosphate synthase.46

With regard to cis-acting sRNAs, the best-studied class of these molecules is the anti-toxin sRNAs that are transcribed antisense to their targets, which encode proteins that are toxic to the cell.30,33,34 As with most antisense sRNAs, these cis-acting RNAs downregulate translation of their targets by base pairing with the mRNA, blocking translation, and sometimes destabilizing the transcript as well.47,48 In the present work, we investigated a cis-acting sRNA, MtlS, the target of which does not encode a toxin, but instead a transporter of the PTS in *V. cholerae*. Our in vitro data support a model in which MtlS is able to repress translation of *mtlA* mRNA by binding the 5′ UTR of the transcript. Surprisingly, however, both our in vitro data and cellular reporter assays suggest that extensive pairing (>45 bp) between MtlS and the *mtlA* 5′ UTR allows for the most efficient repression of *mtlA* translation. This was unexpected given that in numerous cases, small seed regions of an sRNA are sufficient for gene regulation. For example, although 31 nt of SgrS are capable of pairing with a 32 nt region of the *ptsG* mRNA, only a short portion (14 nt)
of SgrS is necessary for its regulatory activity; this minimal region of SgrS is complementary to the RBS of *psuG*.\textsuperscript{49} The SibC antitoxin sRNA exhibits perfect complementarity with most of the 5′ UTR and the entire coding sequence of *ibsC* mRNA.\textsuperscript{30,34} Nevertheless, a small region of SibC, just 23 nt, is sufficient for initial base pairing and subsequent target repression.\textsuperscript{34} Thus, we posit that the mechanism of MtlS-mediated regulation of *mtlA* is distinct from these prior examples and that a minor seed region for pairing the two RNAs may be insufficient to block translation of *mtlA* mRNA. Instead, an extensive duplex between the two RNAs is required for maximal repression, at least when MtlS is expressed distal to its *mtlA* mRNA target (see below).

Our results also suggest that the proximity of the *mtlA* and *mtlS* loci plays an important role in regulation of *mtlA* by the sRNA. We observed that expression of a transcript perfectly complementary to the *mtlA* 5′ UTR is capable of repressing *mtlA* translation. However, it appears that expression of the sRNA from a locus directly antisense to *mtlA* results in the most efficient and effective repression of *mtlA* expression. This leads us to predict that the proximity of the two RNAs, transcribed from the same region of the genome, may positively contribute to the rapid formation of an extensive duplex that is necessary for maximal repression of *mtlA*. This is consistent with prior suggestions that cis-acting sRNAs, due to enhanced steric proximity, may be more efficient than their trans-acting counterparts at interacting with their targets and thereby affecting gene expression.\textsuperscript{25} There is evidence in *E. coli* and *Caulobacter crescentus* that transcripts experience only limited diffusion from their sites of transcription;\textsuperscript{40} thus, expression of the sRNA proximal to the target locus may be advantageous when fast and complete regulatory responses are required.

At the same time, we recognize that the coding sequence of *mtlA* may contain additional binding sites for MtlS or that MtlA, in non-mannitol conditions, may also undergo post-translational regulation. Either or both of these models would also be consistent with our observed results. With regard to the latter scenario, we previously reported that induction of *mtlS* expression in *V. cholerae* resulted in rapid turnover of MtlA, which is normally a highly stable protein.\textsuperscript{18} We have proposed that active proteolysis may represent yet another mechanism by which MtlA levels are regulated in the cell. If the absence of mannitol and the presence of MtlS RNA does trigger rapid turnover of MtlA, one might expect the MtlA-GFP fusions to also be targets of activated proteases or protease adaptor proteins, thereby explaining the repressed reporter activity observed in our studies involving pReporterFull. We are actively investigating the post-translational regulation of MtlA.

In terms of the ability of MtlS to recognize additional regions within the *mtlA* coding sequence, it is possible that such binding events may contribute to translational repression of the mRNA in the cellular context. Although TargetRNA\textsuperscript{70} does not predict any additional MlsS-binding sites within the *mtlA* gene, RNA-Hybrid\textsuperscript{51} predicts extensive, but imperfect, base pairing between MlsS and nt +146–328 of the *mtlA* coding sequence. Thus, although in vitro binding of MlsS RNA to only the 5′ UTR of *mtlA* mRNA is sufficient to repress synthesis of MtlA, it is possible, in the cellular context, that additional pairing between MlsS and the *mtlA* coding sequence is necessary for maximal repression of *mtlA*. We are currently investigating the role of the +146–382 coding region of *mtlA* in MtlS-mediated regulation of *mtlA*.

Gaining a better understanding of how MtlS alters expression of *mtlA* will provide insight into how *V. cholerae* adapts to and thrives in a wide range of environments. We recognize that our understanding of the *mtls-mtlA* circuit remains incomplete, as we have not identified the regulator that controls expression of *mtls*. Our current observation that a plasmid containing the *mtls* promoter region results in lower Mtls/Mtls* levels supports a model in which a transcription factor, available in limiting concentrations, is involved in regulating *mtls* levels in response to carbon source availability. It is not uncommon for genes encoding transcriptional regulators to be found in proximity to the elements that they regulate.\textsuperscript{39} The gene adjacent to *mtlA*, VCA1044, however, is annotated as encoding a hypothetical 47 aa protein with no known DNA-binding motifs and is not considered a likely candidate transcriptional regulator (KEGG Database). We also previously evaluated MtlR, synthesized from the *mtl* operon, as a candidate regulator of *mtls*, but observed that it does not affect MtlS synthesis.\textsuperscript{18} The reporter constructs used in this study provide tools for further identifying the transcription factor(s) responsible for regulating *mtls* transcription and delineating its role in regulating MtlA levels in *V. cholerae*. Since this protein may be involved in the transmission, dissemination and persistence of the pathogen, uncovering the molecular mechanisms by which MtlA production is regulated will provide important insights into the *V. cholerae* life cycle.

### Materials and Methods

#### Bacterial strains, plasmids and culture conditions

All strains and primers used in this study are described in Tables S1 and S2. The *V. cholerae* strains used in this work were El Tor strain N16961 ΔtcpA and derivatives (Table S1). The tcpA mutant is highly attenuated for virulence\textsuperscript{52} and was used for safety purposes. With regard to the expression of *mtlA* and *mtls*, this mutant strain produces phenotypes identical to those of the wild type strain N16961;\textsuperscript{16,18} throughout this manuscript, “wild type” refers to the N16961 ΔtcpA strain. A detailed description of plasmid and strain construction is provided in the Supplementary Materials.

All strains were grown at 37°C with aeration at 250 rpm and 37°C in LB broth or M9 minimal medium containing 0.4% w/v carbon source. The minimal medium for *E. coli* was also supplemented with 0.1% w/v casamino acids, 0.01% w/v thiamine, 2 mM MgSO\textsubscript{4}, and 0.1 mM CaCl\textsubscript{2}. The minimal medium for *V. cholerae* was supplemented with 0.1% trace metals (5% MgSO\textsubscript{4}, 0.5% MnCl\textsubscript{2}, 0.5% FeCl\textsubscript{3}, 0.4% nitritotriacetic acid), 2 mM MgSO\textsubscript{4} and 0.1 mM CaCl\textsubscript{2}. When necessary, cultures were supplemented with 0.02% arabinose to induce the expression of genes inserted into pJML01 (Table S1). Antibiotics were used at the following concentrations: streptomycin, 50–100 μg/mL; carbenicillin, 50–100 μg/mL; chloramphenicol, 2.5–10 μg/mL.
DNA fragments

All primers used to construct DNA fragments are listed in Table S2. The mtlS and antisense mtlS DNA fragments containing either the entire mtlS gene (120 bp) or its reverse complement and preceded by a T7 promoter sequence were amplified by PCR from wild type *V. cholerae* chromosomal DNA using primer pairs LIU72/73 and T7mtlSfor/mtlSrev, respectively. In a similar manner, MtlS truncation Constructs A and B were generated using primer pairs LIU72/LIU171 and LIU72/LIU208, respectively. The *mtlA* 5’ UTR DNA fragment containing the entire *mtlA* 5’ UTR (-75 rel. to AUG through start codon, 78 bp) preceded by a T7 promoter sequence was amplified from wild type *V. cholerae* chromosomal DNA using primer pairs LIU94/93.

In order to produce the mRNA used for in vitro translation assays, a 2-step PCR was performed with chromosomal wild type *V. cholerae* DNA (primer pair LIU116/117, followed by LIU118/119), pXG-10 DNA (primer pair LIU120/121, followed by LIU118/119) or pReporter5 (primer pair LIU116/160, followed by LIU 118/119) to construct *mtlA*’-flag, *gfp*-flag and *mtlAS*’ UTR-*gfp*-flag, respectively. In each case, the primers used added a T7 transcription promoter, a Flag epitope tag, and T7 transcription terminator.

**In vitro RNA preparation**

For sRNAs, in vitro transcription reactions were performed using T7 RNA polymerase (Promega, P2075) according to the manufacturer’s instructions. Briefly, a DNA fragment was mixed in a 20 µL reaction with 500 µM rNTPs, 10 mM DTT, 1× T7 RNA polymerase buffer and 10–20 U T7 RNA polymerase. The reaction was incubated at 37°C for 2 hr and then treated with RQ1 DNase (Promega, M6101) at 37°C for 30 min. The RNA transcripts were purified on 10% TBE-urea polyacrylamide gels and eluted overnight at 37°C in buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 M ammonium acetate, 1 mM EDTA, and 0.1% SDS, followed by phenol:chloroform extraction and ethanol precipitation.

For mRNAs, in vitro transcription assays were performed using the Ribomax RNA Large Scale Production Kit (Promega, P1300), following the manufacturer’s instructions. Using 3–5 µg of template DNA in a 100 µL reaction mixture. The reaction was incubated at 37°C for 2 hr and then treated with RQ1 DNase at 37°C for 15 min. A small sample (3 µL) of the reaction was run on an 8% TBE-urea polyacrylamide gel to confirm that the correct-sized transcript had been generated. The remaining RNA was then purified using phenol:chloroform extraction and ethanol precipitation.

RNA was 3’-labeled with biotin using the RNA 3’ End Biotinylation Kit (Pierce, 20160) and following the manufacturer’s instructions. Briefly, 50 pmol of RNA was mixed in a 30 µL reaction with 1× ligase reaction buffer, 40 U RNase inhibitor, 33.3 µM biotinylated cytidine (his)phosphate, 40 U T4 RNA ligase, and 15% PEG-20,000. The reaction was incubated at 16°C for 2 hr and the biotinylated RNA was purified by phenol:chloroform extraction and ethanol precipitation.

**Electrophoretic mobility shift assay**

EMSAs were performed with labeled *mtlA* RNA in buffer containing 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 50 mM KCl, and 10 mM MgCl2 in the presence or absence of sRNA in a 20 µL reaction mixture. The samples were heated to 90°C for 5 min and then slow-cooled to 37°C (0.1°C/sec). After incubation at 37°C for 30 min, 2 μL of 10 x orange loading buffer (Licor, 927-10100) was added and the samples were loaded on a 10% TBE polyacrylamide gel. The electrophoresis was carried out at 4°C. With TYE705-labeled *mtlA* assays, the gel was directly visualized on an Odyssey Imager (Licor). With biotin-labeled *mtlA* assays, the RNA was transferred to a nylon membrane (GE Healthcare Life Sciences, RPN119B). The RNA was then visualized using a 1:10,000 dilution of IR680-conjugated streptavidin (Licor, 926-68031) in Blocking Buffer (Licor, 927-40000) with 1% SDS. Images were analyzed using Odyssey application software (Licor).

**In vitro translation assay**

Translation reactions were carried out using the PURExpress In vitro Protein Synthesis Kit (NEB, E6800S), following the manufacturer’s instructions. RNA transcripts and an RNase inhibitor (NEB, M0314S) were mixed together and incubated at 37°C for 5 minutes, slow-cooled to 37°C (0.1°C/sec), and then incubated at 37°C for 30 minutes. For assays with Constructs A and B, after the RNA transcript were mixed together, they were first heated to 85°C for 5 minutes, slow-cooled to 37°C (0.1°C/sec), and then incubated at 37°C for 30 minutes. Solutions A and B were then added to each sample to achieve final reaction volumes of 25 µL. The reaction mixtures were incubated at 37°C for 60 min. The reaction was terminated by placing the sample on ice. 2.5 µL of each reaction was used for western blotting analysis.

**Western blotting**

Total protein samples (from PURExpress reaction or from whole cells harvested from liquid cultures) were mixed 1:1 with sample buffer (250 mM Tris-HCl [pH 6.8], 10% SDS, 50% glycerol, 10% β-mercaptoethanol, 0.5% orange G). The samples were heated at 95°C for 10 min and separated on an SDS-containing polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane (Licor, 926–31090). The membranes were incubated with a dilution of primary antibody (1:2500 of rabbit anti-FLAG, AbCam, ab1162; 1:2000 of rabbit anti-GFP, AbCam, ab290; 1:10000 of mouse anti-RpoB, AbCam, ab12087) for 1 hr, followed by incubation with a 1:10000 dilution of an IR680-conjugated goat anti-rabbit and/or goat anti-mouse secondary antibody (Licor, 926–68021 and 926–32210) for 30 min. IR fluorescence was detected using Odyssey Imager (Licor) and images were analyzed using Odyssey application software (Licor).

**Fluorescence measurements**

Bacteria strains harboring *gfp* fusion plasmids were inoculated 1:100 from overnight cultures into 2 mL fresh medium in 13 mm glass test tubes. At least 3 independent overnight cultures were used throughout the study for each strain. At the indicated cell density, the bacteria were collected (1 mL, 8000 × g,
5 min), and resuspended in 1 mL PBS. Aliquots (200 μL) of each culture were transferred to a 96-well optical-bottom plate (Thermo Scientific Nunc, 165305), and relative fluorescence units (RFU; excitation 480 nm, emission 520 nm) and culture density (OD600) were measured at room temperature in a Synergy 4 instrument (Biotek). Background fluorescence and OD600 from a blank (PBS) was subtracted from each sample. The background-corrected RFU was normalized to the background-corrected OD600 and the mean RFU/OD600 of biological replicates was determined. Strains harboring plasmids bearing gfp fusions were at least 4× more fluorescent than isotopic strains harboring a control plasmid that does not express gfp (Fig. S5A). Thus, the reported RFU measurements were all above the autofluorescence of the bacteria and reflect relative cellular GFP levels. We also noted that the various growth conditions used in this study did not affect RFU of wild type GFP (Fig. S5B). Statistical analysis was performed using GraphPad Prism 5 software.

RNA isolation

For analysis of mRNA, total RNA was isolated from 1.5 mL of bacterial culture. Bacteria were first pelleted at 6000 x g for 5 min and then resuspended in 500 μL of 1× M9 salts. Lysis buffer was then added to achieve a final mixture that consisted of 40 mM sodium acetate (pH 5.0), 1% SDS and 2 mM EDTA. An equal volume of acid phenol:chloroform (Life Technologies, AM9722) was added and the mixture was incubated at 65 °C for 5 min with vortexing every minute. The samples were then centrifuged at 16000 × g for 10 min. RNA in the aqueous layer was purified by acid phenol:chloroform extraction and ethanol precipitation. For sRNA analysis, total RNA from 1.5 mL of bacterial culture was isolated using the DirectZol RNA MiniPrep Kit (Zymo, R2051). Bacteria were first pelleted at 6000 × g for 5 min and then resuspended in 350 μL of TRI Reagent. RNA was isolated following the manufacturer’s suggested protocol. For qRT-PCR analysis, DNA was removed from all samples using the TURBO DNA-free Kit (Life Technologies, AM1907), according to the manufacturer’s instructions.

Northern blotting

For mRNA analysis, total RNA (15 μg) in glyoxal loading dye was run on a 1% TBE-agarose gel and then transferred to a nylon membrane using the NorthernMax-Gly Kit (Life Technologies, AM1946). For sRNA analysis, total RNA (2 μg) in Loading Buffer II (Life Technologies, AM8546G) was run on a 10% TBE-urea polyacrylamide gel and then transferred to a nylon membrane (GE Healthcare Life Sciences, RPN119B). For all blotting, RNA was subjected to UV cross-linking after transfer, the blot was pre-hybridized in ULTRAhyb-Oligo (Life Technologies, AM9722) and then hybridized overnight at 65°C. In some cases, hybridizations were performed using DNA probes (Table S2) 5’ end labeled with an IR dye. Otherwise, northern blotting was performed with RNA probes transcribed from PCR-derived templates (Table S2) with T7 promoters by using biotin-16-UTP and T7 RNA polymerase (Promega, P2075) according to the manufacturer’s instructions (see above). All blots were washed and imaged as described in the Odyssey northern blot analysis protocol (Licor).

Quantitative real-time PCR analysis

qRT-PCR was carried out using a Stratagene MX3005P System and Brilliant II SYBR Green QRT-PCR Master Mix Kit (Agilent, 600835). The reactions contained 1× Brilliant SYBR Green QPCR Master Mix, 30 nM ROX reference dye, each primer at 100 nM (Table S2), 100 ng RNA, and 1 μL RT/RNase block enzyme mixture in a 25 μL reaction. All the reactions were carried out at the following conditions: 30 min at 50°C, 10 min at 95°C, and 40 cycles of 30 s at 95°C and 1 min at 60°C in 96-well optical reaction plates (Agilent, 401334). A dissociation curve analysis was carried out at the end of amplification to confirm PCR product specificity. Fluorescence data were collected at the end of the extension step. Technical replicates as well as no template and no RT negative controls were included and at least 3 biological replicates were studied in each case. No signals were detected in no-template controls and no-RT controls. Expression of RNA of interest was normalized to an endogenous control (4.5S RNA). Statistical analysis was performed using GraphPad Prism 5 software.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Andrew Camilli, Karl Haushalter and members of the Liu laboratory for critical readings of the manuscript. We thank the reviewers for their valuable suggestions.

Funding

This work was supported by Rose Hills Foundation Fellowships to HC and NV, Pomona College, a National Institutes of Health grant (AI090606), and a National Science Foundation Grant (CBET1258307).

Supplemental Materials

Supplemental data for this article can be accessed on the publisher’s website.

References

1. Cholera: Fact Sheet No 107. World Health Organization, 2012
2. Colwell RR. Infectious disease and environment: cholera as a paradigm for waterborne. Int Microbiol 2004; 7:285-9; PMID:15666250
3. Wilcox B, Colwell R. Emerging and reemerging infectious diseases: Biocomplexity as an interdisciplinary paradigm. EcoHealth 2005; 2:244-57; http://dx.doi.org/10.1007/s10393-005-8961-3
4. Epstein PR. Cholera and the environment: an introduction to climate change. PSR Quarterly 1992; 2:146-60
5. Reidl J, Klose KE. Vibrio cholerae and cholera: out of the water and into the host. FEMS Microbiol Rev 2002; 26:125-39; PMID:12069878; http://dx.doi.org/10.1016/S0140-6736(03)15328-7
6. Sack DA, Sack RB, Nair GB, Siddique AK. Cholera. Lancet 2004; 363:223-33; PMID:14738797; http://dx.doi.org/10.1016/S0140-6736(03)15328-7
7. Merrell DS, Hava DL, Camilli A. Identification of novel factors involved in colonization and acid tolerance of Vibrio cholerae. Mol Microbiol 2002; 43:1471-91; PMID:11952899; http://dx.doi.org/10.1046/j.1365-2958.2002.02857.x

www.tandfonline.com RNA Biology 147
8. Moorely S, Warnick PL. Identification of novel stage-specific genetic requirements through whole genome transcription profiling of Vibrio cholerae biofilm development. Mol Microbiol 2005; 57:1623-35; PMID:16153522; http://dx.doi.org/10.1111/j.1365-2958.2005.04797.x

9. Schild S, Tamayo R, Nelson EJ, Qadri F, Calderwood SB. Genes induced late in infection increase fitness of Vibrio cholerae after release into the environment. J Infect Dis 2007; 2:264-77; PMID:18005744; http://dx.doi.org/10.1086/jid. 2007.09.004

10. Xu Q, Dziejman M, Mekalanos JJ. Determination of the transcription site of Vibrio cholerae during intraintestinal growth and mucus phase in vitro. Proc Natl Acad Sci U S A 2003; 100:12869-91; PMID:12552086; http://dx.doi.org/10.1073/pnas.0307497100

11. Lutz C, Eken M, Noorani P, Sun S, McDonagh D. Environmental regulators and mechanisms of Persistence of Vibrio cholerae. Front Microbiol 2013; 4: 1-15; PMID:24379807; http://dx.doi.org/10.3389/fmicb.2013.00375

12. Xu Q, Dziejman M, Mekalanos JJ. Mechanism of Polysaccharide Production by Vibrio cholerae. J Mol Biol 2007; 367:779-90; PMID:17157805; http://dx.doi.org/10.1016/j.jmb.2007.04.024

13. Foucher J, Oms O, Lepage T, Warnick PL. Vibrio cholerae phosphoenolpyruvate-phosphotransferase system. Curr Opin Microbiol 2010; 13:18-23; PMID:2013.00375

14. Liu JM, Livny J, Lawrence MS, Kimball MD, Waldor MK. Stx2A, a widely distributed marine bacterial genus. J Bacteriol 1987; 169:598-606; PMID:21773633; http://dx.doi. org/10.1128/JB.01184-13

15. Meadow ND, Revuelta R, Chen VN, Colwell RR, Roseman S. Phosphoenolpyruvate:glycine phosphotransferase system in species of Vibrio, a widely distributed marine bacterial genus. J Bacteriol 1987; 169:598-606; PMID:21773633; http://dx.doi. org/10.1128/JB.01184-13

16. Kumar S, Smith KP, Floyd JL, Varela MF. Cloning and molecular analysis of a mannitol operon of phos- phoenolpyruvate-dependent phosphotransferase (PTS) type from Vibrio cholerae O395. Arch Microbiol 2011; 193:201-8; PMID:21420835; http://dx.doi.org/10.1007/s00203-010-0663-8

17. Liu JM, Livny J, Lawrence MS, Waldor MK, Camilli A. Experimental discovery of sRNAs in Vibrio cholerae by direct cloning, 5′/3′RNA depletion and parallel sequencing. Nucleic Acids Res 2009; 37: e16

18. Ymele-Leki P, Houot L, Watnick PI. Mannitol and the toxR regulon are essential for pathogenesis in Vibrio cholerae. Appl Environ Microbiol 2010; 76:4675-83; PMID:20378230; http://dx.doi.org/10.1128/AEM.01184-13

19. Sesto N, Wurtzel O, Archambaud C, Sorek R, Cossart P. A new class of RNA regulators. EMBO J 2013; 32:2963-79; http://dx.doi.org/10.1002/emboj.201229948

20. Georg J, Hess WR. RNA, but not protein, recognition by a bacterial Hfq-binding small RNA. Proc Natl Acad Sci U S A 2003; 100:12869-91; PMID:12552086; http://dx.doi.org/10.1073/pnas.0307497100

21. Fosso EM, Hemm MR, Storz G. Small toxic proteins and the antisense RNA that repress them. Microbiol Mol Biol Rev 2008; 72:579-89; PMID:19502351; http://dx.doi.org/10.1128/MMBR.00024-08

22. Frereille F, Unoson C, Vogel J, Wagner EGH. An antisense RNA inhibits translation by competing with standby ribosomes. Mol Cell 2007; 26:381-92; PMID:17499444; http://dx.doi.org/10.1016/j.molcel.2007.04.003

23. Fosso EM, Kawano M, Fontaine F, Kaya Y, Mendera KS, Jones KL, Ocampo A, Rudd KE, Storz G. Repression of small toxic protein synthesis by the Sib and Oha small RNAs. Mol Microbiol 2008; 70:1076-9; PMID:18104311; http://dx.doi.org/10.1111/j.1365-2958.2008.06394.x

24. Thiendl T, Berghof R, Wagner EGH, Kersters K. Mechanism of post-segregational killing by the biolys system of plastidial R1. Sok antisense RNA interacts with Hok mRNA via its 5′-end single-stranded leader and competes with the 3′-end of Hok mRNA for binding to the m6A translational initiation region. The EMBO Journal 1994; 13:1960; PMID:8168493

25. Kawano M, Aravind L, Storz G. An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin. Mol Microbiol 2007; 64:738-54; PMID:17803099; http://dx.doi.org/10.1111/j.1365-2958.2007.06394.x

26. Gopal Y, Papenfort K, Reichenbach B, Vogel J, Gieke B. Targeted decay of a regulatory small RNA by an adaptor protein for sRNAs E and counteraction by an anti-adaptor RNA. Genes Dev 2015; 29:52-64; PMID:25847961; http://dx.doi.org/10.1101/gad.210112.112

27. Kawano M. Divergently overlapping cis-encoded anti- sense RNA regulating toxin-antitoxin systems from E. coli, hok/sok, ldhL, symE/SymR. RNA Biol 2012; 9:1520-7; PMID:23131729; http://dx.doi.org/10.4161/rna.22757

28. Sesto N, Wurtzel O, Archambaud C, Sorek R, Cossart P. The exclusion: a new concept in bacterial antisense RNA-mediated gene regulation. Nat Rev Microbiol 2013; 11:75-82; PMID:23268228; http://dx.doi.org/10.1038/nrmicro2934

29. Maki K, Morita T, Ohtsuka K, Aiba H. A minimal base- pairing region of a bacterial small RNA 5′g7 required for functional repression of pstRNA. Mol Microbiol 2010; 76:782-92; PMID:20345651

30. Thijen B. TargetRNA: a tool for predicting targets of small RNA action in bacteria. Nucleic Acids Res 2008; 36:W609-13; PMID:18477632; http://dx.doi.org/10.1093/nar/gkn246

31. Rehmsmeier M, Steffen P, Hochmann M, Giegerich R. Fast and effective prediction of microRNA/target duplexes. RNA 2004; 10:1507-17; PMID:15383676; http://dx.doi.org/10.1261/rna.52486404

32. Herrington DA, Hall RH, Losonsky G, Mekalanos JJ, Taylor RK, Levine MM. Toxin, toxin–coregulated pilus, and the toxR regulon are essential for Vibrio cholerae pathogenesis in humans. J Exp Med 1988; 168:1487- 92; PMID:2902187; http://dx.doi.org/10.1084/jem.168.4.1487