RESEARCH PAPER

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The VrrA sRNA controls a stationary phase survival factor Vrp of *Vibrio cholerae*

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Abbreviations: HPF, hibernation promoting factor; IF, initiation factor; RaiA, ribosome-associated inhibitor A; RMF, ribosome modulation factor; SRA, stationary-phase-induced-ribosome associated protein; Vrp, VrrA-regulated ribosome binding protein

Small non-coding RNAs (sRNAs) are emerging regulatory elements in bacteria. The *Vibrio cholerae* sRNA VrrA has previously been shown to down-regulate outer membrane proteins (OmpA and OmpT) and biofilm matrix protein (RbmC) by base-pairing with the 5′ region of the corresponding mRNAs. In this study, we present an additional target of VrrA in *V. cholerae*, the mRNA coding for the ribosome binding protein Vrp. Vrp is homologous to ribosome-associated inhibitor A (RaiA) of *Escherichia coli* which facilitates stationary phase survival through ribosome hibernation. We show that VrrA down-regulates Vrp protein synthesis by base-pairing to the 5′ region of *vrp* mRNA and that the regulation requires the RNA chaperone protein, Hfq. We further demonstrate that Vrp is highly expressed during stationary phase growth and associates with the ribosome of *V. cholerae*. The effect of the Vrp protein in starvation survival is synergistic with that of the VC2530 protein, a homolog of the *E. coli* hibernation promoting factor HPF, suggesting a combined role for these proteins in ribosome hibernation in *V. cholerae*. Vrp and VC2530 are important for *V. cholerae* starvation survival under nutrient deficient conditions. While VC2530 is down-regulated in cells lacking *vrrA*, mutation of *vrp* results in VC2530 activation. This is the first report indicating a regulatory role for an sRNA, modulating stationary factors involved in bacterial ribosome hibernation.

Introduction

In bacteria, ribosomal fidelity plays a central role in the adaptation to environmental stresses and acts as a checkpoint for sensing shifts in temperature or nutrient levels. Ribosomes in all organisms are composed of a small and a large subunit (30S and 50S, respectively, in bacteria) that cycle through stages of association and dissociation during protein synthesis. When bacteria enter stationary phase, ribosome structure can be modified facilitating a state of hibernation.[1] The ribosome binding proteins RMF (ribosome modulation factor), HPF (hibernation promoting factor), RaiA (ribosome-associated inhibitor A) and SRA (stationary-phase-induced ribosome associated protein) are expressed in stationary phase and involved in ribosome hibernation.[2] In *E. coli*, RMF and HPF play a role in formation of 100S dimer ribosome particles. The binding of RMF causes dimerization of 70S ribosomes into 90S particles, which are further stabilized as 100S dimers upon HPF binding thus leads to "ribosome hibernation."[2] RaiA or YfiA, on the other hand is known to promote the formation of translationally inactive monomeric 70S ribosomes which consequently prevents the recycling of ribosomes for translation initiation.[3] Although RaiA and HPF share 40% amino acid sequence similarity, HPF converts 90S into 100S particles, whereas RaiA prevents RMF-dependent 90S formation.[4] Both the monomeric 70S ribosomes subunits promoted by RaiA and the 100S dimer particles promoted by HPF have been shown to be translationally inactive [3,5] that could aid longer cell survival under nutrient limited conditions.

The ribosome hibernation helps bacteria to survive under nutrient-limited conditions.[1,2] The crystal structure of the *Thermus thermophilus* 70S ribosome reveals that RaiA and HPF share a common binding site in the ribosome. In addition, the same binding site overlaps with those of all tRNA and the initiation factors IF1 and IF3, suggesting that RaiA and HPF can interfere with protein synthesis.[5] Recent studies in *Lactococcus lactis* which lacks orthologues of *rmf* and *hpf* genes, showed that RaiA (also known as YfiA) is essential for ribosome dimerization and found in 100S ribosome particles.[6]

In the past few years, it has become increasingly clear that bacterial small non-coding RNAs (sRNAs) regulate many diverse cellular processes. sRNAs mostly function as antisense regulators, affecting translation, degrading target mRNA, or binding and sequestering proteins.[7] To date, ~150 sRNAs have been predicted in *Escherichia coli* K12 by bioinformatics and experimental approaches.[7,8] Although more than 500 sRNAs were predicted for *V. cholerae*,[9] only a few of the sRNAs have been

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experimentally studied, including RyhB, which is involved in iron utilization;[10] the sRNAs (Qrr1-Qrr4, CsrB, CsrC, CsrD) that are involved in quorum sensing regulation;[11,12] the TarA and TarB sRNAs which are regulated by ToxT and involved in *V. cholerae* virulence;[13] the MicX sRNA, which regulates outer membrane proteins;[14] the IGR7 sRNA, which is involved in modulating carbon metabolism;[9] and the sRNA TfoR, which regulates natural competence in response to chitin.[15]

Previously we demonstrated that VrrA sRNA inhibited the expression of the outer membrane protein OmpA causing increased release of outer membrane vesicles.[16,17] In addition, VrrA also reduced the expression of another major outer membrane protein, OmpT.[18] Recently we have shown that VrrA plays a role in repressing the biofilm matrix protein, RbmC by base-paring to the 5′ region of *rbmC* mRNA.[19] Analogous to its RybB and MicA counterparts from *E. coli* and *Salmonella,*[20,21] transcription of *vrrA* is controlled by the alternative sigma factor E (σ^E) and *V. cholerae* cells lacking *vrrA* display increased colonization of the intestine in an infant mouse model.[16]

Many studies have been conducted to elucidate the role of RaiA in ribosome hibernation during stationary phase[6,22]. However, little is known about the regulatory factors involved in RaiA expression and the role of RaiA in bacterial physiology.

In this study we show that VrrA is the first sRNA that directly regulates Vrp (a homolog of RaiA) in an Hfq-dependent manner by base-pairing with the 5′ region of the *vrp* mRNA in *V. cholerae*. We denote the protein Vrp for VrrA-regulated ribosome binding protein of *V. cholerae* which is encoded by the *vc0706* gene locus in the NCBI data base (http://www.ncbi.nlm.nih.gov/gene/?term=vc0706). In addition to VrrA, mutation of *vc2530* (a homolog of HPF) results in upregulation of Vrp. Ribosome profile analysis of the wild-type strain of *V. cholerae* reveals that Vrp is a ribosome-associated protein present in the 30S, 70S, and 100S ribosome fraction. Under nutrient deficient conditions, cells lacking both *vrp* and *vc2530* genes exhibited reduced starvation survival compared to wild-type, however, single deletion of *vrp* or *vc2530* has no significant effect. Interestingly, while VC2530 is downregulated in cells lacking *vrrA*, mutation of *vrp* resulted in VC2530 activation. Our data provides the first evidence suggesting a role for sRNA in ribosome modulation.

Results

In silico prediction of *vrp* as a target of VrrA

In our earlier studies, we showed that VrrA targeted the translation of the outer membrane proteins OmpA, OmpT and the biofilm matrix -protein RbmC by direct binding to the 5′UTR of these target mRNAs. In order to find new targets of VrrA, we performed an *in silico* analysis using the Target RNA program.[23,24] The target predicted by the TargetRNA program gives *vrp* as a 4th hit in the list with score value of −73. For the TargetRNA program the complete sequence of VrrA was used for target search. The pairing region between VrrA and *vrp* was further authenticated using the RNA hybrid program [25] where we limit the sequence length for sRNA and target RNA by eliminating sequences corresponding to stem loops and other secondary structures predicted in VrrA. In analysis using the RNA hybrid program, we limited the VrrA sequence to an open loop single stranded stretch region from nucleotide +70 to +106 (as shown in **Fig. S1**) to pair with a target sequence in the *vrp* mRNA region from the transcriptional start site to 6 nt into the *vrp* coding region. The result obtained by the RNA hybrid program predicted that residues 72–84 of VrrA forms a 13-bp duplex with the −3 to −15 region (numbers relative to AUG start codon) of the *vrp* mRNA with a calculated free energy of −28.2 kcal/mol. This interaction would partially mask the ribosome binding site of *vrp* mRNA required for translation initiation (**Fig. 1A**).

VrrA down- regulates the expression of Vrp in an Hfq-dependent manner

To investigate the role of VrrA in Vrp expression, we determined the Vrp levels in whole cells of wild-type *V. cholerae* O1 strain A1552 and the Δ*vrrA* mutant by Western-blot analysis using a polyclonal anti-Vrp antiserum (**Fig. 1B**). The levels of Vrp in different strains were analyzed semi-quantitatively using a Fluor-S Multi-Imager (Bio-Rad) (**Fig. 1C**). Vrp expression was increased to 2.2 fold in the *vrrA* mutant (DNY7) in comparison with the wild-type *V. cholerae* strain A1552 (**Fig. 1B**, compare lanes 1 and 2; **Fig. 1C** lanes 1 and 2). Elevated Vrp expression could be restored to the wild-type level by expressing VrrA from a plasmid harboring the wild-type allele of *vrrA* (**Fig. 1B**, lane 3; **Fig. 1C** lane 3) compared to the vector control (**Fig. 1B**, lane 4; **Fig. 1C** lane 4). Further the repression of *vrp* by VrrA was also determined by Northern blot analysis (**Fig. S2A; Fig. S2B**) and result obtained is in consistent with Western blot analysis (**Fig. 1B**, lanes 1 to 4; **Fig. 1C**, lanes 1 to 4). In the strain lacking Hfq, the Vrp level are unaffected by VrrA over-expression, (**Fig. 1B**, lane 7 and 8; **Fig. 1C**, lane 7 and 8). In our earlier studies [16], we observed that the total level of VrrA was higher in the *hfq* mutant than in the wild-type *V. cholerae* strain A1552 suggesting that the Hfq protein somehow might reduce the stability, and thereby the level, of VrrA or indirectly might affect its expression. In the present study, the increased level of VrrA was unable to regulate the expression of Vrp in the *hfq* mutant, suggesting that Hfq is involved in Vrp repression by VrrA. We also observed that in the *hfq* mutant the basal level of Vrp was higher (compare lane 1 with lane 5 in **Fig. 1B**). The apparent role in Vrp repression by Hfq might not only dependent on VrrA but could also involve some other sRNA.

Vrp is a stationary phase protein

We analyzed Vrp expression during different bacterial growth phases by Western blot analysis using anti-Vrp polyclonal antiserum (**Fig. 2A**). The higher expression of Vrp was detected when the bacterial cells were harvested from an overnight culture (**Fig. 2A**, upper panel, lane 4) although only a trace amount of Vrp was detectable at an OD_{600} of 2.0 (**Fig. 2A**, upper panel, lane 3). Vrp was not detectable at an OD_{600} of 0.5 and 1 (**Fig. 2A**, upper panel, lanes 1 and 2). The quantification of Western blot data was shown in **Fig. 2B**.

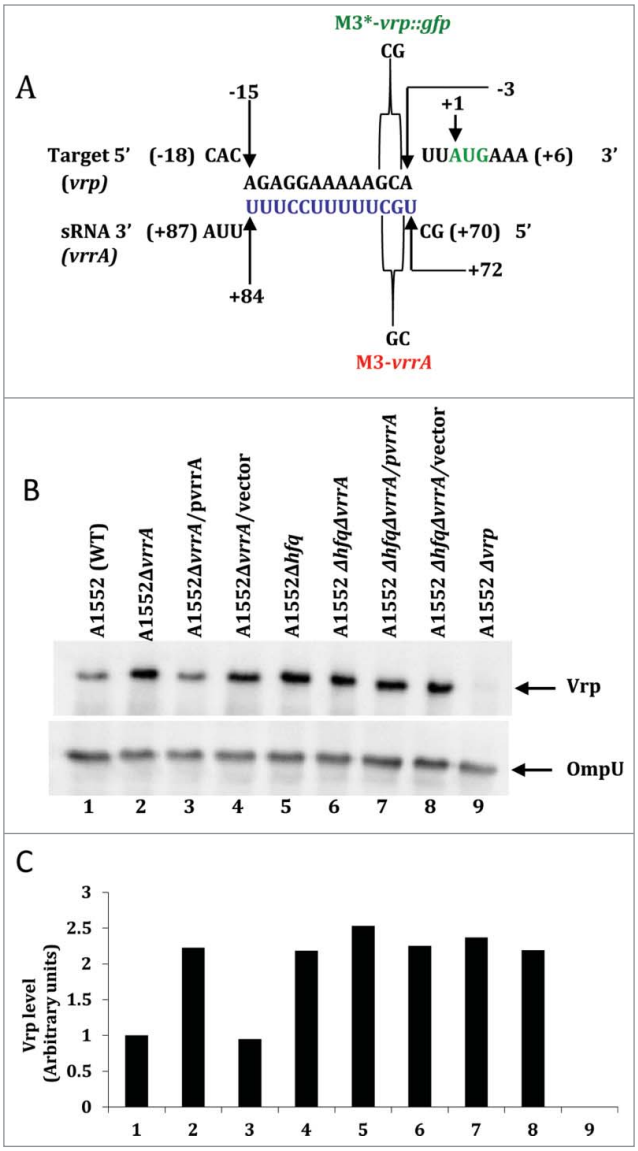

Figure 1. In silco prediction and detection of Vrp as a VrrA target. (**A**) Graphical representation of the proposed seed pair region of VrrA with the target *vrp* mRNA. VrrA binding sites were predicted using the RNAhybrid program (in blue) and shown to overlap with the ribosome binding site of *vrp*. The start codon of the *vrp* is shown in green. Compensatory base pair changes at the 5′UTR of *vrp* resulted in a new mutant, M3*-*vrp::gfp* (pDS18). (**B**) VrrA down-regulates expression of the Vrp protein. Western blot analyses of whole cell lysates collected after overnight growth from the *V. cholerae* strains: lane 1, A1552 (wild type); lane 2, DNY7 (*vrrA* mutant); lane 3, DNY11 (Δ*vrrA*/pvrrA); lane 4, DNY12 (Δ*vrrA*/vector); lane 5, DNY8 (Δ*hfq*); *lane* 6, DNY9 *(*Δ*hfq*Δ*vrrA)*; lane 7, DNY16 (Δ*hfq*Δ*vrrA*/pvrrA); lane 8, DNY17 (Δ*hfq*Δ*vrrA*/pMMB66HE); lane 9, DHS380 (Δ*vrp*) detecting Vrp and OmpU as a loading control (lower panel). (**C**) Semi-quantitative analyses of Vrp levels obtained with the same samples as in **Fig. 1B**, after SDS-PAGE and Western blot analyses of protein lysates from different derivatives of the *V. cholerae* strain A1552. The plotted data show relative levels of Vrp, with the level of the wild-type strain set to 1.0.

Vrp, a homolog of RaiA is a ribosome binding protein in *V. cholerae*

We analyzed the ribosome profile of overnight grown wild-type *V. cholerae* O1 strain A1552 on a sucrose density gradient as described in the Materials and Methods. After ultracentrifugation, different fractions were collected and optical densities at 260nm were measured using a nano-drop spectrophotometer. Ribosome profiles based on OD260nm were plotted for wild-type *V. cholerae* O1 strain A1552 (**Fig. 2C**). To determine if Vrp is a ribosome-associated protein, we used the 30S, 50S, 70S and 100S fractions for Western blot analysis. We could detect the presence of Vrp in the all the tested fractions except for 50S of the wild-type *V. cholerae* O1 strain A1552 using anti-Vrp polyclonal antiserum (**Fig. 2D**, upper panel, lanes 1, 2, 3, and 4). In this experiment, whole-cell lysate of wild-type *V. cholerae* O1 strain A1552 and the Δ*vrp* mutant were used as a positive and a negative control respectively (**Fig. 2D**, upper panel, lanes 5 and 6). As an internal control, we tested for the presence of the non-ribosome binding protein RpoS. As shown in **Fig. 2D** (lower panel, lanes 1, 2, 3, and 4) there was no RpoS protein band detected in the 30S, 50S, 70S and 100S fractions of the wild-type strain. The whole-cell lysate of wild-type *V. cholerae* O1 strain A1552 and the Δ*rpoS* mutant (**Fig 2D**, lower panel, lanes 5 and 6) were used as positive and negative controls, respectively. These findings confirm that Vrp is a ribosome binding protein in *V. cholerae*.

Expression of Vrp is up-regulated in the Δ*vc2530* mutant

In *E. coli,* there are 2 steps involved in formation of 100S particles through the formation of a 90S particle in the stationary phase of bacterial growth. YhbH (a.k.a. HPF), a homolog of *V. cholerae* VC2530 protein, converts immature 90S particles into mature 100S ribosomal particles by promoting both particle formation and stabilization. In contrast, YfiA (a.k.a. RaiA, a homolog of the Vrp) prevents 70S dimer formation. Thus, although YfiA and YhbH are highly homologous to each other, they have opposite functions in 70S dimer formation.[2,3,26] To examine the correlation between VC2530 and Vrp in *V. cholerae*, Vrp expression levels were examined in the presence or absence of VC2530 by Western blot analyses (**Fig. 3A**). The level of the Vrp protein was analyzed semi-quantitatively as shown in **Fig. 3B**. We observed an increased level of Vrp expression in the Δ*vc2530* mutant compared to the wild-type although it remains unclear whether Vrp down-regulate the expression of VC2530 directly or indirectly.

Expression of VC2530 is up-regulated in the Δ*vrp* mutant and down-regulated in the Δ*vrrA* mutant

We analyzed the expression of Flag tagged VC2530 in wild-type *V. cholerae* strain *flag-vc2530* (DHS458), Δ*vrrA-flag-vc2530* (DHS474), Δ*vrp-flag-vc2530* (DHS475) and Δ*vc2530*. Western blot analysis was performed using anti-Flag monoclonal antiserum, which detected the expected ~12-kDa protein (**Fig. 3C**) and the levels of the Flag-VC2530 protein were analyzed semi-quantitatively (**Fig. 3D**). As shown in **Fig. 3C and D**, Flag-VC2530 expression was downregulated in Δ*vrrA* and up-regulated in Δ*vrp* (1.4-fold increase compared to wild-type).

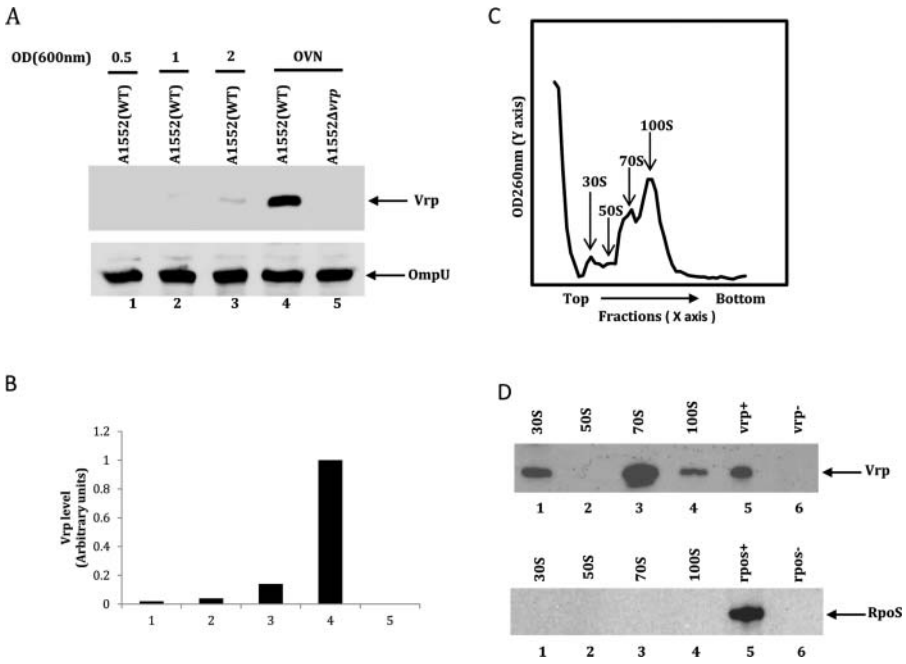

Figure 2. Vrp is ribosome binding stationary phase protein. (**A**) Growth phase dependent expression of Vrp. Bacterial whole-cell lysate samples were taken at OD 0.5, OD 1.0, OD 2.0 and overnight culture (OVN). Western blot analysis was performed for detecting Vrp and the level of OmpU was used as a loading control. (**B**) Semi-quantitative analyses of Vrp levels obtained with the Western blot analyses of same samples as in **Fig. 2A**. (**C**) Ribosome profile of overnight grown wild-type *V. cholerae* O1 strain A1552 after sucrose gradient centrifugation as described in material and methods. The Y axis represents OD260nm and the X axis represents different fractions from top to bottom (low to high density) of the sucrose gradient. Peaks containing ribosome fractions 30S, 50S, 70S and 100S are indicated. (**D**) Western blot analysis of 30S, 50S, 70S and 100S fractions of wild-type *V. cholerae* O1 strain A1552 confirms the presence of Vrp in all fractions except 50S (lane 2 compared to lanes 1,3,4). Whole-cell lysate from the *V. cholerae* strains A1552 (wild-type) was used as a positive control (lane 5); DHS380 (*vrp* mutant) was used as a negative control (lane 6). RpoS protein, a non-ribosomal binding protein, was used as an internal control (lower panel).

Impaired starvation survival of the *V. cholerae* double deletion mutant Δ*vrp*Δ*vc2530*

To determine if Vrp and VC2530 modulate the survival of *V. cholerae* under nutrient limited conditions, we performed starvation survival assays for the wild-type *V. cholerae* strain A1552, Δ*vrp*, Δ*vc2530*, Δ*vrp*Δ*vc2530* and Δ*vrrA* strains.

As shown in **Fig. 3E**, at day 0, all *V. cholerae* strains started with equal cell numbers, as determined by CFU/ml. However, at day 3 and day 4, the starvation survival of the double deletion mutant Δ*vrp*Δ*vc2530* was reduced 3.3 fold compared to the wild-type. The starvation survival of Δ*vrp* and the Δ*vc2530* single mutant were similar to that of the wild-type throughout the experiment. In addition, starvation survival of the Δ*vrrA* mutant was similar to that of the wild-type. It might be due to up-regulation of Vrp and down-regulation of VC2530 production.

VrrA targets the *vrp* mRNA at the 5′UTR

In a previous study, plasmids expressing wild-type VrrA or mutant variants of VrrA were constructed and transformed into *V. cholerae* strain Δ*vrrA* (DNY7).[18] The mutant variants M1 and M3 carry nucleotide substitutions at 73–78 and 73–74 with respect to VrrA (**Fig. 4A**), overlapping with the region predicted to form a VrrA/*vrp*-mRNA duplex. Whole-cell lysates from these strains were analyzed by Western blot to compare the production of Vrp (**Fig. 4B**). The levels of the Vrp protein were semi- quantified as shown in **Fig. 4C**. We found that the sRNA variants expressed by plasmids pTS2-M1 and pTS2-M3 were impaired in their ability to repress the expression of Vrp (**Fig. 4B**, compare lane 2, with lanes 3 and 4; **Fig. 4C**, compare lane 2, with lanes 3 and 4) while the wild-type VrrA still maintained the downregulation of Vrp expression (**Fig. 4B**, lane 2; **Fig. 4C**, lane 2).

Validation of the interaction between the VrrA and its target *vrp* by compensatory base pair changes

We used a previously developed *gfp*-based translational fusion system that allows rapid validation of a VrrA target.[27] This reporter system consists of 2 plasmids; a high-copy plasmid (pJV300) carrying the *vrrA* clone (or VrrA mutant derivatives) is co-expressed with a low copy plasmid (pXG20) carrying the 5′ UTR of wild-type *vrp* fused to *gfp* (green fluorescent protein) or M3*-*vrp::gfp*. In order to determine the transcriptional start site at the 5′UTR of *vrp* 5′ RACE was performed (**Fig. S3**). To study regulation at the seed pairing region of the M3-*vrrA* with the *vrp* mRNA, we created a mutant derivative of *vrp* at its 5′UTR termed M3*-*vrp::gfp* (**Fig. 1A**) by changing nucleotides at location −4GC to CG. Even in the absence of VrrA, the mutant M3*-*vrp::gfp* (pDS18) exhibited a reduced GFP signal compared to the WT-*vrp::gfp* (pTS32) (**Fig. S4C**, lanes 1 and 2, upper panel; **Fig. S4D**, lanes 1 and 2). It is possible that in *m3*-vrp::gfp* changing nucleotides at location −4GC to CG might affect ribosome binding efficiency. Nonetheless, the substituted nucleotide exchange mutants were used to test the regulatory effect by co-expression of the WT-*vrrA* (wild-type) or the M3-*vrrA* with that of WT-*vrp*::*gfp* (pTS32) or the mutated M3*-*vrp::gfp* (pDS18).

The compensatory M3* allele of the *vrp::gfp* can restore base pairing with M3-*vrrA* comparable to that of the WT-*vrrA* (**Fig. 4D**, lanes 5 and 6). As a loading control, GroEL was detected by Western blot analysis using anti-GroEL polyclonal antiserum (**Fig. 4D**, lower panel). **Figure 4E** shows semi-quantitative Western blot analysis of Vrp::GFP expression (normalized to GroEL) in the same samples as in **Fig. 4D**. These results demonstrate that compensatory mutations restore regulation by the corresponding variant of VrrA and disrupt regulation by WT-*vrrA*. To support the Western blot analysis of GFP in *E. coli* strains carrying different *gfp* fusion plasmids, we monitored the colony fluorescence using an agar plate-based colony fluorescence

imaging as described in the Materials and Methods. As shown in **Fig. 4F** (upper panel), the results obtained by the agar plate-based colony fluorescence imaging was in agreement with the Western-blot results in **Fig. 4D and E. Fig. 4F** (lower panel) shows the imaging of the same whole-cell colony plate under the normal light as a control.

Discussion

Bacteria can reduce the efficiency of protein synthesis during nutrient starvation or in stationary phase by converting ribosomes into translationally inactive 100S dimers[1] or 70S monomers,[26] thereby inhibiting bacterial growth. The major players in the stationary phase conversion of ribosome conformation are RMF, HPF, and RaiA (in different bacterial species),[5] but few known regulators of RMF and RaiA have been described.

In earlier studies, ppGpp was shown to be required for *rmf* transcription and it was suggested that intracellular ppGpp levels not only regulate ribosomal RNA synthesis and translational factor synthesis, but also modulate the translational activity of ribosomes during stationary phase by controlling formation of the inactive form (100S) of the 70S ribosome.[28] Recently, it was demonstrated that the bacterial metabolic regulator protein, cyclic AMP receptor protein (CRP), regulates transcriptional activation of the *rmf* gene for formation of 100S ribosome dimers in *E. coli.*[29] However, post transcriptional regulation of ribosome modulation factors had not been studied in bacteria.

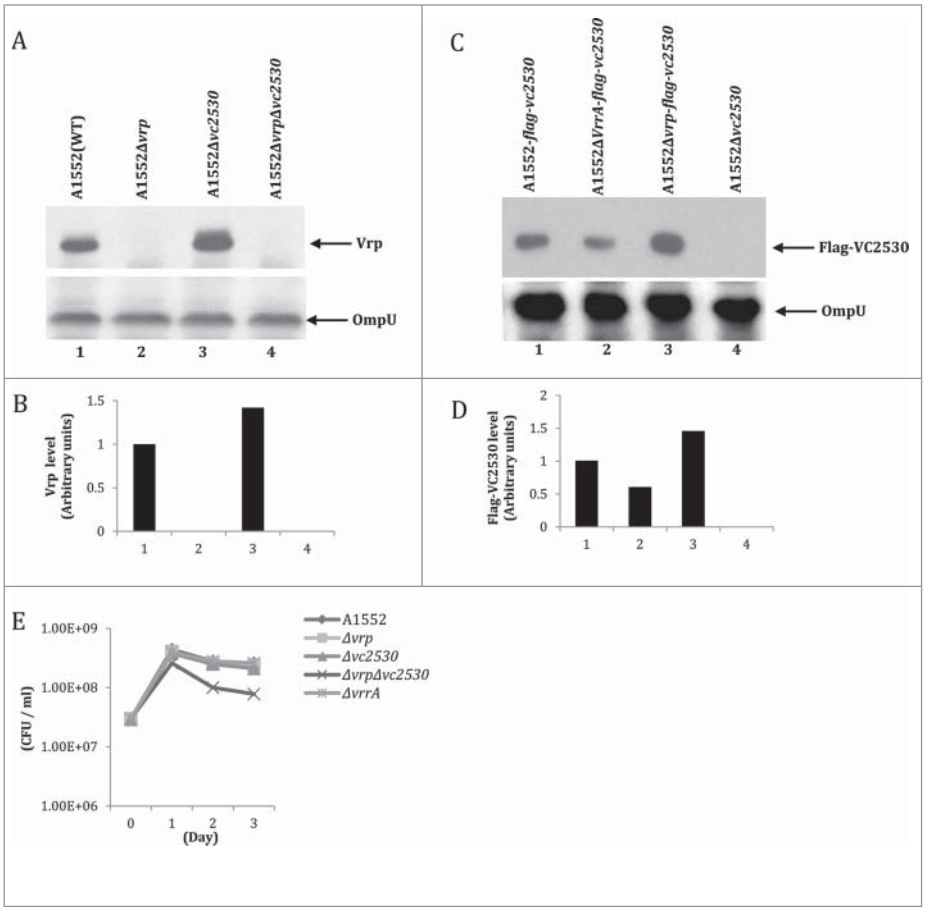

Figure 3. Vrp and VC2530 are important factors for starvation survival of *V. cholerae*.(**A**) Western blot analyses of overnight grown whole-cell lysates from the *V. cholerae* strains. Lane 1, A1552; lane 2, Δ*vrp*; lane 3, Δ*vc2530*; and lane 4, Δ*vrp* Δ*vc2530* detecting Vrp and OmpU as a loading control. (**B**) The levels of the Vrp protein in the same strains as in **Fig. 3A** were analyzed semi-quantitatively. (**C**) Western blot analyses of overnight grown whole cell lysates from the *V. cholerae* strains: lanes 1, A1552-*flag-vc2530*; lane 2, Δ*vrrA-flag-vc2530*; lane 3, Δ*vrp-flag-vc2530*; and lane 4, Δ*vc2530* detecting FLAG and OmpU as a loading control. (**D**) The levels of the Flag-VC2530 protein in the same strains as in **Fig. 3C** were analyzed semi-quantitatively. (**E**) Starvation survival assay: The survival of *V. cholerae* wild-type strain A1552 and its mutant derivatives Δ*vrp*, Δ*vc2530*, Δ*vrp*Δ*vc2530, and* Δ*vrrA,* were examined by measuring CFU/ml at 37°C in minimal media containing 0.4% glycerol. The data shown is representative of 3 independent experiments.

In this study, we show that *vrp* is the first ribosome binding protein in *Vibrio cholerae* that is a direct target of sRNA, VrrA of *V. cholerae.* VrrA targets *vrp* mRNA by base-pairing at its ribosome binding site, thus decreasing *vrp* expression. The secondary structure of VrrA predicted by RNA fold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) contains the open loop sequence from nucleotides 70 to 106 (**Fig. S1**), and part of this sequence from nucleotides 72 to 84 interacts with *vrp* mRNA. Based on this interaction, VrrA and its substituted mutant derivatives (M1 and M3) were used. Interestingly, M1 and M3 mutant derivatives of VrrA lost the ability to regulate Vrp expression.

V. cholerae can survive the extended adverse conditions of nutrient starvation, elevated salinity and decreased temperature by entering into a dormant, viable but nonculturable (VBNC) state.[30] Bacteria fail to grow on routine bacteriological media on which they would normally form colonies, but are alive and capable of renewed metabolic activity.[31] The molecular mechanism of starvation survival of *V. cholerae* is not yet largely understood although the phosphoporin protein of *V. cholerae* has been suggested to play a role in the life cycle and survival of the bacterium in the natural environment.[32]

In *E. coli*, RMF and HPF (homolog of VC2530) results in formation of 100S dimer ribosome particles while RaiA (homolog of Vrp) or YfiA, promotes the formation of translationally inactive monomeric 70S ribosomes.[2,5] The homologue of Vrp, RaiA was shown to associate preferentially with 70S ribosomes and the copy number of RaiA in the HPF deletion mutant was approximately 1.6 times higher than that of the wild-type strain.[2] In the wild-type *V. cholerae* strain A1552, we found that Vrp was associated mainly with 70S ribosomes and a lesser amount was associated with 30S and 100S ribosomes. In addition, the Vrp expression

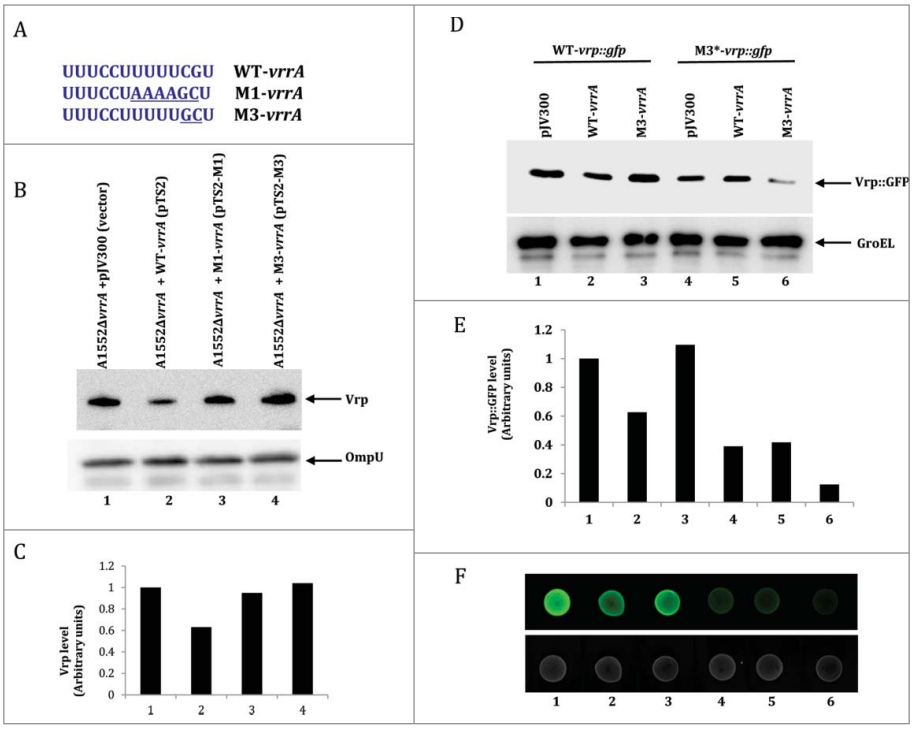

Figure 4. VrrA interact at the 5′ region of *vrp* mRNA. (**A**) The sequence of WT-*vrrA* and its mutant derivates carrying nucleotide substitutions (M1-*vrrA* and M3-*vrrA*). Substituted nucleotides are underlined. (**B**) Wild-type VrrA and its substituted nucleotide mutants derivatives (M1 and M3) were introduced into the Δ*vrrA* strain (DNY7). Western blot analyses of overnight grown strains were performed to detect the levels of Vrp and OmpU (as a loading control). (**C**) The levels of the Vrp protein in the same strains as in **Fig. 4B** were analyzed semiquantitatively. (**D**) GFP-based reporter assay of cells carrying compensatory mutations in the predicted location of base pairing with VrrA. Western blot analysis of Vrp::GFP in Top10 cells collected at OD_{600} 1.0, with plasmids carrying different *vrrA* derivatives. Lane 1, WT-*vrp::gfp* & pJV300(Vector control); lane 2, WT-*vrp::gfp* & WT-*vrrA*; lane 3, WT-*vrp::gfp* & M3-*vrrA*; lane 4, M3*-*vrp::gfp* & pJV300(VC); lane 5, M3*-*vrp::gfp* & WT-*vrrA*; lane 6, M3*-*vrp::gfp* & M3-*vrrA*. GroEL expression was used to normalize the loading amount. (**E**) Semi-quantitative Western blot analysis showing expression of Vrp::GFP for the same samples as shown in **Fig. 4D**. GroEL expression was used as reference to normalize the loaded amount. Results are representative of data obtained with 3 independent cultures. (**F**) Agar plate-based colony fluorescence imaging analysis of the same strains as in **Fig. 1D and E**. Top10 cells carrying both plasmids were grown on LB agar. The upper panel image was obtained in the fluorescence excitation at 460 nm, and light emission was recorded using a 510 nm-filter. The lower panel image shows the same plate under visible light mode.

was higher in the Δ*vc2530* mutant in comparison with the wild-type strain. However, as for the observed elevated synthesis of Vrp in the Δ*vc2530* mutant, it remains likewise enigmatic how VrrA and Vrp "down- and up-regulate" VC2530, respectively. The significance of these reciprocal changes in levels of Vrp and VC2530 in the bacterial cells is not clear as yet. The mechanism(s) behind this regulation remained to be determined. In *E. coli*, inactivation of YfiA (Vrp homolog) stimulates ribosome dimerization as the YfiA interferes with the binding of RMF and HPF to the ribosomes. The *E. coli yfiA*::Km mutant appears to be somewhat more viable in the stationary phase. The enhanced viability of this strain has been explained by increased protection of 100S ribosomes against degradation by RNA hydrolases.[33,34]

In this study, we demonstrated the role of ribosome binding proteins Vrp (homolog of RaiA) and VC2530 (homolog of HPF) in survival of *V. cholerae* under nutrient limited conditions. In *V. cholerae*, the *vrp* and *vc2530* single-deletion mutants were able to survive to an extent similar to that of the wild-type strain, probably by maintaining stabilized 70S monomeric and 100S dimeric forms of ribosomes, respectively, during stationary phase. The previous studies in *E. coli* showed that RaiA and HPF have the same binding site on the 30S subunit of the ribosome indicating that both proteins might have similar function in stabilizing ribosomes during stationary phase.[5] The expression level of Vrp was higher in the *vc2530* deletion mutant than in the wild-type; excess Vrp protein may therefore be involved in stabilization of ribosomal subunits, potentially allowing the *vc2530* deletion mutant to live as long as the wild-type. The *vrp* mutant also showed the same survival time as the wild-type under nutrient-limited conditions. In the *vrp* mutant the level of VC2530 expression is also higher than in the wild-type; excess VC2530 protein may stabilize the ribosome, thereby protecting the ribosome dimers from degradation and allowing the same survival time as for the wild-type. Deletion of both genes, *vrp* and *vc2530*, might result in a less stable ribosome, which leads to a reduction in starvation survival ability. However, the results obtained in our studies in *V. cholerae* appeared to be rather different from the findings by Ueta et al. in *E. coli* [2] where viability of the Δ*raiA*Δ*hpf* mutant in EP media was similar to that of the wild-type strain. Recently, it was shown that in *Lactobacillus lactis*, a Δ*yfiA* mutant did not display altered survival phenotype in rapidly growing cells, but the viability of the mutant was reduced when the bacterial strain was starved for carbon and energy sources for 10–20 d[6] These differences might be due to the different bacterial species and the usage of different growth conditions. From the results of the present study, we suggest that Vrp, which is under the direct regulation of VrrA, and VC2530, which is indirectly regulated by VrrA, are important for *V. cholerae* starvation survival under nutrient-limited conditions.

Materials and Methods

Bacterial strains and growth conditions

E. coli and *V. cholerae* strains used in this study are listed in **Table 1**. Bacterial strains were grown at 37°C in Luria Bertani (LB) broth or LB plates supplemented with antibiotics (where

Table 1. Bacterial strains used in this study

Bacterial strain	Description / Relevant Genotype	Source or reference
E. coli		
DH5α	λ^- φ80dlacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(r_K^- m_K^-) supE44 thi-1 gyrA relA1	[38]
SM10λpir	thi thr leu tonA lacY supE recA::RP4–2 Tc::Mu Km λpir	[39]
TOP10	F^- mcrA Δ(mrr-hsdRMS- mcrBC) φ80lacZΔM15 ΔlacX74nupG recA1 araD139Δ(ara-leu)7697 galE15 galK16rpsL(StrR) endA1 λ	Invitrogen
V. cholerae		
A1552	O1 ElTor, Inaba, Rif R	[40]
DHS 380	Δvrp derivative of A1552	This study
DHS 389	Δvrp/pBAD18	This study
DHS 391	Δvrp/pBAD18:vrp	This study
DHS 458	A1552-flag-vc2530	This study
DHS 474	A1552ΔvrrA-flag-vc2530	This study
DHS 475	A1552Δvrp-flag-vc2530	This study
A1552Δvc2530	Δvc2530	This study
DHS 415	ΔvrpΔvc2530 derivative of A1552	This study
A1552Δrpos	Δrpos	[41]
DNY7	A1552ΔvrrA	[16]
DNY8	A1552Δhfq	[16]
DNY9	A1552ΔvrrA Δhfq	[16]
DNY11	A1552ΔvrrA + pvrrA	[16]
DNY12	A1552ΔvrrA + pMMB66HE	[16]
DNY16	A1552ΔvrrA Δhfq + pvrrA	[16]
DNY17	A1552ΔvrrA Δhfq + pMMB66HE	[16]
DNY34	A1552ΔvrrA + pJV300	[18]
DNY35	A1552ΔvrrA + pTS2	[18]
DNY44	A1552ΔvrrA + pTS2-M1	[18]
DNY64	A1552ΔvrrA + pTS2-M3	[18]
DNY 88	Top10 + pJV300 + pTS32	This study
DNY 89	Top10 + pTS2 + pTS32	This study
DNY 90	Top10 + pTS2-M1 + pTS32	This study
DHS-324	Top10 + pTS2-M3 + pTS32	This study
DHS-325	Top10 + pJV300 + pTS32-M3*	This study
DHS-326	Top10 + pTS2 + pTS32-M3*	This study
DHS-327	Top10 + pTS2-M3 + pTS32-M3*	This study

appropriate) at the following concentrations: 100 μg/ml carbenicillin, 20 μg/ml chloramphenicol, 50 μg/ml rifampcin.

Primers and Plasmids

Primers and Plasmids used in this study are listed in **Tables 2 and 3** respectively.

Construction of deletion mutants

In-frame deletions were constructed using procedures that have been described previously by Vaitkevicius et al.[35]. Primer sequences are summarized in **Table 2**. Deletion of the *vrp, vc2530*, and double deletion Δ*vrp*Δ*vc2530* loci in *V. cholerae* strain A1552 resulted in DHS380, A1552Δ*vc2530* and DHS415, respectively.

Construction of chromosomal FLAG-tagged VC2530 strains

The *V. cholerae* chromosomal FLAG-tag insertion into *vc2530* locus was carried out using a slight modification of the method described earlier by Skorupski and Taylor [36]. The FLAG tag sequence (CTTGTCGTCATCGTCTTTGTAGTC) was inserted after the start codon of the *vc2530* gene, using primers DS166 and DS167 to generate a 326-bp product and, primers DS168 and DS169 to generate a 331-bp product . Both PCR products containing FLAG over-hangs were excised from an agrose gel and purified. Both fragments were mixed in equal concentrations at a 1:1 ratio used as a template for another PCR with primers DS166 and DS169, resulting in amplification of the *vc2530 gene* with a FLAG insertion (633-bp). Further, the resulting 633-bp PCR product containing the FLAG insertion was ligated into the vector pCVD442. The resulting plasmids were then integrated into the chromosome at the *vc2530* loci by homologous recombination. Following sucrose selection, positive colonies containing the *vc2530* loci with the FLAG insertion were verified by PCR using primers DS170 and DS171.

Construction of plasmids pTS32 and pDS18

WT-Vrp::*gfp* (pTS32) was constructed by following the protocol for "5′ RACE product cloning" as described by Urban and Vogel [27]. This protocol includes a 5′ RACE to determine the transcriptional start site of *vrp* and subsequent cloning into *gfp*-vector pXG20. In brief, total RNA was isolated from strain *V. cholerae* A1552 and treated with tobacco acid pyrophosphatase to enrich primary transcripts. After ligation of a 5′ end RNA adaptor, the RNA was converted to cDNA by reverse transcription

Table 2. Primers used in this study

Primer	Sequence in 5′ to 3′ direction	Restriction site	Used for construction of
vrp-2	GTTTTTGCTAGCGATTGCAGAGGTGACTTC	*NheI*	pTS32 and 5′ Race for vrp
JVO-367	ACTGACATGGAGGAGGGA		pTS32 and 5′ Race for vrp
JVO-8239	AAAACGATTATGAAAATCAACAT		pTS32-M3*
JVO-8240	TAATCGTTTTTCCTCTGTGTC		pTS32-M3*
DS125	CGCTCTAGA AACTCGAGGC TTATCAGCAG	*Xba1*	*vrp* deletion mutant
DS126	CCCATCCACTATAAACTAACA AATGCTTTTTCCTCTGTGTCA		*vrp* deletion mutant
DS127	TGTTAGTTTATAGTGGATGGG ATAGAATGATGG GAGATAGCGC		*vrp* deletion mutant
DS128	AATTCTAGA TGTATTGAGGATCGAGCTGA		*vrp* deletion mutant
DS96	GC GAATTCCATAAGGGATGACACAGAG G	*EcoR1*	pBAD18:vrp
DS77	GCTCTAGATTATTCCACTTCTTCGCTCAG	*Xba1*	pBAD18:vrp
DS166	CGCTCTAGA GAAATACATG CATACCCCGC	*Xba1*	FLAG insertion *vc2530*
DS167	CTTGTCGTCATCGTCTTTGTAGTC CATAGACTTTCCTTCTCTAGTTTAGG		FLAG insertion *vc2530*
DS168	GACTACAAAGACGATGACGACAAG CAAATCAAC ATTCAAGGCCA		FLAG insertion *vc2530*
DS169	CGCTCTAGA AGTACTTCGCTCAATTGCAT	*Xba1*	FLAG insertion *vc2530*
DS170	CGAGACACTG CTCAAAGTTG		FLAG insertion *vc2530*
DS171	CTTGTCGTCATCGTCTTTGTAGTC		FLAG insertion *vc2530*
VC2530-A	CGCTCTAGA CGTACCAATTTGCAAGAGGCA		vc2530 deletion mutant
VC2530-B	CCCATCCACTATAAACTAACA GCCTTGAATGTTGATTTGCATAG		vc2530 deletion mutant
VC2530-C	TGTTAGTTTATAGTGGATGGG CATTAATCATGCAATTGAGCG		*vc2530* deletion mutant
VC2530-D	CGCTCTAGAGGGCAGGTTATCGACACAGTA	*Xba1*	*vc2530* deletion mutant
JVO-8168	AAGGGATGACACAGAGGAA		*vrp* riboprobe
JVO-8169	GTTTTTTTAATACGACTCACTATAGGGAGGTTGAGTTTACCCTCGATATGA		*vrp* riboprobe
JVO-8106	CTGTTTCGTTTCACTTCTGAGTTC		5S rRNA Probe

with a random hexamer primer mix. Subsequently, a PCR reaction was performed using primers JVO-0367 (binding specifically to the RNA adaptor) and Vrp-2 (binding specifically to the *vrp* gene). The enriched PCR amplicon was gel extracted, digested with BseRI/NheI and subsequently cloned into a BsgI/NheI digested pXG20 backbone. The resulting plasmid pTS32 served as a template for creating and M3*-*vrp*::*gfp* (pDS18) by introducing nucleotide changes at location -4GC to CG using primer pair JVO-8239 and JVO-8240.

Anti-Vrp polyclonal antiserum preparation

The *vrp* gene was amplified by PCR using primers DS96 and DS77 and cloned into pBAD18 using *EcoR1* and *Xba1* restriction enzyme sites. Vrp expression was induced using 0.02%

Table 3. Plasmid used in this study

Original name	Plasmid Trival name	Relevant genotype/phenotype	Reference/source
pGEM-T Easy			Promega
pCR4-TOPO		TA cloning vector plasmid; Ap^r	Invitrogen
pBAD18		Cloning vector plasmid; Ap^r	[42]
pCVD442		Ap^r positive-selection suicide vector plasmid	[43]
pDS35	pBAD18:*vrp*	pBAD18 carrying *vrp*; Ap^r	This study
pXG20		Control plasmid for *gfp* fusion assays	[27]
pMMB66HE		Control plasmid	[45]
p*vrrA*		*vrrA* complementation plasmid, based on pMMB66HE	[18]
pJV300		ColE1 plasmid expressing a ~50-nt nonsense transcript derived from rrnB terminator	[27]
pTS2	WT-*vrrA*	ColE1 plasmid expressing *vrrA* from its own promoter	[18]
pTS2-M1	M1-*vrrA*	pTS2 carrying a 6-nt substitution in putative *vrp* interaction sequence, as shown in **Fig. 4A**	[18]
pTS2-M3	M3-*vrrA*	pTS2 carrying a 2-nt substitution in putative *vrp* interaction sequence,as shown in **Fig. 4A**	[18]
pTS32	WT-*vrp::gfp*	*Vibrio vrp* translational GFP fusion Plasmid(pXG20) to 16th amino acid	This study
pDS18	M3*-*vrp::gfp*	*Vibrio vrp* translational GFP fusion Plasmid(pXG20) to 16th amino acid, Point mutation in *vrp* with change at the putative VrrA interaction sequence position from GC to CG as shown in **Fig. 1A**	This study

arabinose. The Vrp protein band was excised from an SDS gel, eluted from the gel, and used to generate polyclonal rabbit antiserum (AgriSera AB, Sweden).

Western blot analysis

Bacterial cultures were collected at OD_{600} 1.0 for preparing GFP protein samples from whole-cell fractions. Overnight cultures (23 h) were used to prepare the Vrp samples. The protein samples were re-suspended in 1X sample loading buffer (50 mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100mM β-mercaptoethanol), heated at 100°C for 15 min and separated by SDS–PAGE [37]. GFP, GroEL and Vrp were detected using anti-GFP monoclonal (Roche #11814460001), anti-GroEL (Sigma #A8705) and anti-Vrp polyclonal antiserum (in this study), respectively. OmpU, a loading control, and the non ribosomal binding protein RpoS, an internal control, were detected using anti-OmpU and anti-rpoS polyclonal antiserum, respectively. Western blot detection was done using the ECL+ chemiluminescence system (GE Healthcare, United Kingdom). The levels of Vrp or GFP were analyzed semiquantitatively using a Fluor-S Multi- Imager (Bio-Rad).

Isolation of ribosome fractions by sucrose density gradient

Ribosome analysis was performed as described previously by Maki et al.[22] with minor modifications. The bacterial cells were harvested from culture an overnight culture (23 h) and centrifuged at 15000 x g for 15 min at 4°C. The pellet was suspended in buffer A (100 mM ammonium acetate, 15 mM magnesium acetate, 20 mM Tris-HCl at pH 7.4, and 6 mM 2-mercaptoethanol), and vigorously vortexed with glass beads (0.1 mm diameter, Biospec products, inc) for 5 min at 4°C. The resulting suspension was centrifuged at 15000 x g for 10 min at 4°C. The supernatant obtained was kept on ice and the pellet was re-suspended in buffer A, vortexed and centrifuged again under the same conditions. This procedure was done twice. The combined supernatants were layered on 10–40% sucrose gradients and centrifuged at 35000 r.p.m (154,693 x g) for 4 hours at 4°C in a Beckman SW 40Ti rotor. The fractions obtained after ultracentrifugation were collected and the optical densities were measured at 260 nm using a nano drop spectrophotometer (Thermo Scientific). The OD260nm values were used to plot a graph on the Y axis and the fractions from the top to bottom (low to high density) were used to plot on the X axis. The fraction containing the 30S, 50S, 70S and 100S peak was analyzed by Western blot.

5′ RACE analysis

5′ RACE was performed as previously described by Urban and Vogel[27] to determine the transcription start sites of the *vrp* gene. Total RNA isolated from the wild-type *V. cholera* strain A1552 was used to generate cDNA. Oligo Vrp-2 and JVO-0367 were used as specific primers in PCR. The PCR products were separated on a 2% agarose gel, gel-eluted and used as template for sequencing.

Gfp-based reporter assays

A *gfp*-based translational fusion system was constructed as previously described by Urban and Vogel[27]. The reporter system consists of 2 plasmids a high-copy plasmid, pJV300 carrying the *vrrA* clone (pTS2), is co-expressed with a low-copy plasmid, pXG20 carrying the 5′ UTR of *vrp* fused to *gfp* (pTS32) or pKS18. *E. coli* Top10 cells carrying both plasmids pTS2 and pTS32 variants were grown overnight and samples were prepared for Western blot analysis.

Agar plate-based colony fluorescence imaging

E. coli Top10 cells carrying *gfp* fusion plasmids were grown overnight in LB broth. The overnight grown bacterial culture was diluted 100 times and 10 μl of diluted sample was dropped onto a LB plate supplemented with the appropriate antibiotics. After overnight incubation at 37°C, the plates were photographed in a FUJI LAS-4000 image analyzer using a CCD camera with a 510 nm emission filter and excitation at 460 nm.

sRNA target analysis

The TargetRNA analysis tool (http://snowwhite.wellesley.edu/targetRNA/index2.html) was used to predict the in-silico analysis of VrrA targets. The program was used with the following parameters: removed terminator of sRNA, target confined to mRNA region from −30 to +20, hybridization seed region value of 9, and with no G-U seed pairing.

The RNA hybrid program (RNAhybrid version 2.2)[25] was used to confirm the accuracy of the predicted interaction between the VrrA and *vrp* mRNA. The query sequences used for the *vrp* mRNA included the region from the transcriptional start site to 6 nt of the *vrp* coding regions and for VrrA we used the open loop stretch nucleotide sequences (+70 to +106) as shown in **Fig. S1**.

RNA extraction and Northern blotting

RNA was isolated using Trizol extraction as previously described Song et al.[16]. The bacterial cultures were centrifuged and the supernatant was decanted. The pellet was dissolved in 1 ml Trizol reagent. The mixture was transferred to 2 ml Phase-Lock Tubes containing 400 μl chloroform, the samples were gently mixed by shaking and centrifuged (16,000 x g) for 15 min at room temperature. The supernatant containing RNA was transferred to a new tube and RNA was precipitated by addition of 2.5 volumes of isopropanol. The RNA was treated with DNase to remove any DNA contamination as described by Song et al.[16]. For Northern blot analysis, 10 μg RNA sample were resolved in a polyacrylamide or an agarose gel and transferred to a Hybond-XL membrane (GE Healthcare) by electro-blotting (1 h, 50 V, 4°C) in a tank blotter (Peqlab). Radiolabeled probes were used to visualize the required mRNA or sRNA. Northern blots were exposed to a phosphorimager screen and scanned on a StormTM phosphorimager (Molecular Dynamics, USA). Quantification was performed using Quantityone software (Roche). For *vrp* mRNA detection, Ribo-probes were prepared by PCR using primers JVO-8168 and JVO-8169, labeled with 5′P32 (α-P32-UTP), resulting in detection of 402 bp corresponding to *vrp*,

and radio labeled (γ-P32-ATP) oligo probe (JV0–8106) was used to detect the 120-bp fragment corresponding to 5S rRNA.

Bacterial starvation survival assay

Bacterial cells grown overnight in LB media were isolated and washed 3 times with a minimal medium containing 1X M9 salt (For 100 ml of 10X M9 salt: 12.8 g $Na_2HPO_4{\cdot}7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl and 1 g NH_4Cl), 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.4% glycerol. After washing, the bacterial cells were re-suspended in the same minimal media and the colony forming units of the bacterial suspension was adjusted to be 1×10^7 CFU/ml. The samples were incubated at 37°C. The survival of bacteria in this minimal media was assessed by daily CFU/ml determination.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

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

References

1. Wada A, Yamazaki Y, Fujita N, Ishihama A. Structure and probable genetic location of a "ribosome modulation factor" associated with 100S ribosomes in stationary-phase Escherichia coli cells. Proc Natl Acad Sci U S A 1990; 87:2657-61; PMID:2181444
2. Ueta M, Yoshida H, Wada C, Baba T, Mori H, Wada A. Ribosome binding proteins YhbH and YfiA have opposite functions during 100S formation in the stationary phase of Escherichia coli. Genes Cells 2005; 10:1103-12; PMID:16324148; http://dx.doi.org/10.1111/j.1365-2443.2005.00903.x
3. Agafonov DE, Kolb VA, Nazimov IV, Spirin AS. A protein residing at the subunit interface of the bacterial ribosome. Proc Natl Acad Sci U S A 1999; 96:12345-9; PMID:10535924
4. Ueta M, Ohniwa RL, Yoshida H, Maki Y, Wada C, Wada A. Role of HPF (hibernation promoting factor) in translational activity in Escherichia coli. J Biochem 2008; 143:425-33; PMID:18174192; http://dx.doi.org/10.1093/jb/mvm243
5. Polikanov YS, Blaha GM, Steitz TA. How hibernation factors RMF, HPF, and YfiA turn off protein synthesis. Science 2012; 336:915-8; PMID:22605777; http://dx.doi.org/10.1126/science.1218538
6. Puri P, Eckhardt TH, Franken LE, Fusetti F, Stuart MC, Boekema EJ, Kuipers OP, Kok J, Poolman B. Lactococcus lactis YfiA is necessary and sufficient for ribosome dimerization. Mol Microbiol 2014; 91:394-407; PMID:24279750; http://dx.doi.org/10.1111/mmi.12468
7. Majdalani N, Vanderpool CK, Gottesman S. Bacterial small RNA regulators. Crit Rev Biochem Mol Biol 2005; 40:93-113; PMID:15814430; http://dx.doi.org/10.1080/10409230590918702
8. Waters LS, Storz G. Regulatory RNAs in bacteria. Cell 2009; 136:615-28; PMID:19239884; http://dx.doi.org/10.1016/j.cell.2009.01.043
9. Liu JM, Livny J, Lawrence MS, Kimball MD, Waldor MK, Camilli A. Experimental discovery of sRNAs in Vibrio cholerae by direct cloning, 5S/tRNA depletion and parallel sequencing. Nucleic Acids Res 2009; 37:e46; PMID:19223322; http://dx.doi.org/110.1093/nar/gkp080
10. Davis BM, Quinones M, Pratt J, Ding Y, Waldor MK. Characterization of the small untranslated RNA RyhB and its regulon in Vibrio cholerae. J Bacteriol 2005; 187:4005-14; PMID:15937163; http://dx.doi.org/10.1128/JB.187.12.4005-4014.2005
11. Lenz DH, Mok KC, Lilley BN, Kulkarni RV, Wingreen NS, Bassler BL. The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in Vibrio harveyi and Vibrio cholerae. Cell 2004; 118:69-82; PMID:15242645; http://dx.doi.org/10.1016/j.cell.2004.06.009
12. Lenz DH, Miller MB, Zhu J, Kulkarni RV, Bassler BL. CsrA and three redundant small RNAs regulate quorum sensing in Vibrio cholerae. Mol Microbiol 2005; 58:1186-202; PMID:16262799; http://dx.doi.org/10.1111/j.1365-2958.2005.04902.x
13. Richard AL, Withey JH, Beyhan S, Yildiz F, DiRita VJ. The Vibrio cholerae virulence regulatory cascade controls glucose uptake through activation of TarA, a small regulatory RNA. Mol Microbiol 2010; 78:1171-81; PMID:21091503; http://dx.doi.org/10.1111/j.1365-2958.2010.07397.x
14. Davis BM, Waldor MK. RNase E-dependent processing stabilizes MicX, a Vibrio cholerae sRNA. Mol Microbiol 2007; 65:373-85; PMID:17590231; http://dx.doi.org/10.1111/j.1365-2958.2007.05796.x
15. Yamamoto S, Izumiya H, Mitobe J, Morita M, Arakawa E, Ohnishi M, Watanabe H. Identification of a chitin-induced small RNA that regulates translation of the tfoX gene, encoding a positive regulator of natural competence in Vibrio cholerae. J Bacteriol 2011; 193:1953-65; PMID:21317321; http://dx.doi.org/10.1128/JB.01340-10
16. Song T, Mika F, Lindmark B, Liu Z, Schild S, Bishop A, Zhu J, Camilli A, Johansson J, Vogel J, et al. A new Vibrio cholerae sRNA modulates colonization and affects release of outer membrane vesicles. Mol Microbiol 2008; 70:100-11; PMID:18681937; http://dx.doi.org/10.1111/j.1365-2958.2008.06392.x
17. Song T, Wai SN. A novel sRNA that modulates virulence and environmental fitness of Vibrio cholerae. RNA Biology 2009; 6:254-8; PMID:19411843; http://dx.doi.org/10.4161/rna.6.3.8371
18. Song T, Sabharwal D, Wai SN. VrrA mediates Hfq-dependent regulation of OmpT synthesis in Vibrio cholerae. J Mol Biol 2010; 400:682-8; PMID:20595045; http://dx.doi.org/10.1016/j.jmb.2010.05.061
19. Song T, Sabharwal D, Gurung JM, Cheng AT, Sjostrom AE, Yildiz FH, Uhlin BE, Wai SN. Vibrio cholerae Utilizes Direct sRNA Regulation in Expression of a Biofilm Matrix Protein. PLoS One 2014; 9:e101280; PMID:25054332; http://dx.doi.org/10.1016/10.1371/journal.pone.0101280
20. Papenfort K, Pfeiffer V, Mika F, Lucchini S, Hinton JC, Vogel J. SigmaE-dependent small RNAs of Salmonella respond to membrane stress by accelerating global omp mRNA decay. Mol Microbiol 2006; 62:1674-88; PMID:17427289; http://dx.doi.org/10.1111/j.1365-2958.2006.05524.x
21. Papenfort K, Bouvier M, Mika F, Sharma CM, Vogel J. Evidence for an autonomous 5' target recognition domain in an Hfq-associated small RNA. Proc Natl Acad Sci U S A 2010; 107:20435-40; PMID:21059903; http://dx.doi.org/10.1073/pnas.1009784107
22. Maki Y, Yoshida H, Wada A. Two proteins, YfiA and YhbH, associated with resting ribosomes in stationary phase Escherichia coli. Genes Cells 2000; 5:965-74; PMID:11168583; http://dx.doi.org/10.1046/j.1365-2443.2000.00389.x
23. Tjaden B, Goodwin SS, Opdyke JA, Guillier M, Fu DX, Gottesman S, Storz G. Target prediction for small, noncoding RNAs in bacteria. Nucleic Acids Res 2006; 34:2791-802; PMID:16717284; http://dx.doi.org/10.1093/nar/gkl356
24. Tjaden B. TargetRNA: a tool for predicting targets of small RNA action in bacteria. Nucleic Acids Res 2008; 36:W109-13; PMID:18477632; http://dx.doi.org/10.1093/nar/gkn264
25. Rehmsmeier M, Steffen P, Hochsmann 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.5248604
26. Agafonov DE, Kolb VA, Spirin AS. Ribosome-associated protein that inhibits translation at the aminoacyl-tRNA binding stage. EMBO Rep 2001; 2:399-402; PMID:11375931; http://dx.doi.org/10.1093/embo-reports/kve091
27. Urban JH, Vogel J. Translational control and target recognition by Escherichia coli small RNAs in vivo. Nucleic Acids Res 2007; 35:1018-37; PMID:17264113; http://dx.doi.org/10.1093/nar/gkl1040
28. Izutsu K, Wada A, Wada C. Expression of ribosome modulation factor (RMF) in Escherichia coli requires ppGpp. Genes Cells 2001; 6:665-76; PMID:11532026; http://dx.doi.org/10.1046/j.1365-2443.2001.00457.x
29. Shimada T, Yoshida H, Ishihama A. Involvement of cyclic AMP receptor protein in regulation of the rmf gene encoding the ribosome modulation factor in Escherichia coli. J Bacteriol 2013; 195:2212-9; PMID:23475967; http://dx.doi.org/10.1128/JB.02279-12
30. Wai SN, Mizunoe Y, Yoshida S. How Vibrio cholerae survive during starvation. FEMS Microbiol Lett 1999; 180:123-31; PMID:10556702; http://dx.doi.org/10.1111/j.1574-6968.1999.tb08786.x
31. Lowder M, Oliver JD. The use of modified GFP as a eporter for metabolic activity in pseudomonas putida. Microb Ecol 2001; 41:310-3; PMID:12032604
32. Goulart CL, Lery LM, Diniz MM, Vianez-Junior JL, Neves-Ferreira AG, Perales J, Bisch PM, von Kruger WM. Molecular analysis of VCA1008: a putative phosphoporin of Vibrio cholerae. FEMS Microbiol Lett 2009; 298:241-8; PMID:19659744

33. Zundel MA, Basturea GN, Deutscher MP. Initiation of ribosome degradation during starvation in Escherichia coli. RNA 2009; 15:977-83; PMID:19324965; http://dx.doi.org/10.1261/rna.1381309
34. Ortiz JO, Brandt F, Matias VR, Sennels L, Rappsilber J, Scheres SH, Eibauer M, Hartl FU, Baumeister W. Structure of hibernating ribosomes studied by cryoelectron tomography in vitro and in situ. J Cell Biol 2010; 190:613-21; PMID:20733057; http://dx.doi.org/10.1083/jcb.201005007
35. Vaitkevicius K, Lindmark B, Ou G, Song T, Toma C, Iwanaga M, Zhu J, Andersson A, Hammarstrom ML, Tuck S, et al. A Vibrio cholerae protease needed for killing of Caenorhabditis elegans has a role in protection from natural predator grazing. Proc Natl Acad Sci U S A 2006; 103:9280-5; PMID:16754867
36. Skorupski K, Taylor RK. Positive selection vectors for allelic exchange. Gene 1996; 169:47-52; PMID:8635748; http://dx.doi.org/10.1016/0378-1119(95)00793-8
37. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-5; PMID:5432063
38. Hanahan D. Studies on transformation of Escherichia coli with plasmids. J Mol Biol 1983; 166:557-80; PMID:6345791
39. Miller VL, Taylor RK, Mekalanos JJ. Cholera toxin transcriptional activator toxR is a transmembrane DNA binding protein. Cell 1987; 48:271-9; PMID:3802195; http://dx.doi.org/10.1016/0092-8674(87)90430-2
40. Yildiz FH, Schoolnik GK. Role of rpoS in stress survival and virulence of Vibrio cholerae. J Bacteriol 1998; 180:773-84; PMID:9473029
41. Valeru SP, Rompikuntal PK, Ishikawa T, Vaitkevicius K, Sjoling A, Dolganov N, Zhu J, Schoolnik G, Wai SN. Role of melanin pigment in expression of Vibrio cholerae virulence factors. Infect Immun 2009; 77:935-42; PMID:19103773; http://dx.doi.org/10.1128/IAI.00929-08
42. Guzman LM, Belin D, Carson MJ, Beckwith J. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol 1995; 177:4121-30; PMID:7608087
43. Donnenberg MS, Kaper JB. Construction of an eae deletion mutant of enteropathogenic Escherichia coli by using a positive-selection suicide vector. Infect Immun 1991; 59:4310-7; PMID:1937792
44. Sittka A, Pfeiffer V, Tedin K, Vogel J. The RNA chaperone Hfq is essential for the virulence of Salmonella typhimurium. Mol Microbiol 2007; 63:193-217; PMID:17163975; http://dx.doi.org/10.1111/j.1365-2958.2006.05489.x
45. Furste JP, Pansegrau W, Frank R, Blocker H, Scholz P, Bagdasarian M, Lanka E. Molecular cloning of the plasmid RP4 primase region in a multi-host-range tacP expression vector. Gene 1986; 48:119-31; PMID:3549457; http://dx.doi.org/10.1016/0378-1119(86)90358-6