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

Dharmesh Sabharwal1, Tianyan Song1, Kai Papenfort2,3, and Sun Nyunt Wai1,*

1Department of Molecular Biology, The Laboratory for Molecular Infection Medicine Sweden (MIMS), Umeå Centre for Microbial Research (UCMR), Umeå University, Umeå, Sweden; 2Institute for Molecular Infection Biology, University of Würzburg, Würzburg, Germany; 3Department of Molecular Biology, Princeton University, Princeton, NJ USA

Keywords: Hfq, Ribosome hibernation, sRNA, Vibrio cholerae, VrrA, Vrp

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 factor 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.”2,2 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,5,5 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
experimentally studied, including RyhB, which is involved in iron utilization;\textsuperscript{10} the sRNAs (Qtr1-Qtr4, CsrB, CsrC, CsrD) that are involved in quorum sensing regulation;\textsuperscript{11,12} the TarA and TarB sRNAs which are regulated by ToxT and involved in \textit{V. cholerae} virulence;\textsuperscript{13} the MicX sRNA, which regulates outer membrane proteins;\textsuperscript{14} the IGR7 sRNA, which is involved in modulating carbon metabolism;\textsuperscript{9} and the sRNA Tf0R, which regulates natural competence in response to chitin.\textsuperscript{15}

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

Many studies have been conducted to elucidate the role of RaiA in ribosome hibernation during stationary phase\textsuperscript{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 \textit{vrp} mRNA in \textit{V. cholerae}. We denote the protein Vrp for \textit{VrrA}-regulated ribosome binding protein of \textit{V. cholerae} which is encoded by the \textit{vc0706} gene locus in the NCBI gene locus database (http://www.ncbi.nlm.nih.gov/gene/?term=vc0706). In addition to \textit{VrrA}, mutation of \textit{vc2530} (a homolog of HPF) results in upregulation of Vrp. Ribosome profile analysis of the wild-type strain of \textit{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 \textit{vrrA} and \textit{vc2530} genes exhibited reduced starvation survival compared to wild-type, however, single deletion of \textit{vrrA} or \textit{vc2530} has no significant effect. Interestingly, while \textit{VC2530} is downregulated in cells lacking \textit{vrrA}, mutation of \textit{vrrA} resulted in \textit{VC2530} activation. Our data provides the first evidence suggesting a role for sRNA in ribosome modulation.

**Results**

In silico prediction of \textit{vrrA} as a target of \textit{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 \textit{in silico} analysis using the Target RNA program.\textsuperscript{25,24} The target predicted by the TargetRNA program gives \textit{vrrA} as a 4th hit in the list with score value of −73. For the TargetRNA program the complete sequence of \textit{VrrA} was used for target search. The pairing region between \textit{VrrA} and \textit{vrrA} was further authenticated using the RNA hybrid program \textsuperscript{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 \textit{VrrA}. In analysis using the RNA hybrid program, we limited the \textit{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 \textit{vrrA} mRNA region from the transcriptional start site to 6 nt into the \textit{vrrA} coding region. The result obtained by the RNA hybrid program predicted that residues 72–84 of \textit{VrrA} forms a 13-bp duplex with the −3 to −15 region (numbers relative to AUG start codon) of the \textit{vrrA} mRNA with a calculated free energy of −28.2 kcal/mol. This interaction would partially mask the ribosome binding site of \textit{vrrA} mRNA required for translation initiation (Fig. 1A).

\textbf{VrrA down-regulates the expression of Vrp in an Hfq-dependent manner}

To investigate the role of \textit{VrrA} in \textit{Vrp} expression, we determined the \textit{Vrp} levels in whole cells of wild-type \textit{V. cholerae} O1 strain A1552 and the \textit{Δhfq} mutant by Western-blot analysis using a polyclonal anti-Vrp antiserum (Fig. 1B). The levels of \textit{Vrp} in different strains were analyzed semi-quantitatively using a Fluor-S Multi-Imager (Bio-Rad) (Fig. 1C). \textit{Vrp} expression was increased to 2.2 fold in the \textit{vrrA} mutant (DNV7) in comparison with the wild-type \textit{V. cholerae} strain A1552 (Fig. 1B, compare lanes 1 and 2; Fig. 1C lanes 1 and 2). Elevated \textit{Vrp} expression could be restored to the wild-type level by expressing \textit{VrrA} from a plasmid harboring the wild-type allele of \textit{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 \textit{vrrA} by \textit{VrrA} was also determined by Northern blot analysis (Fig. S2A, S2B) and result obtained is consistent with Western blot analysis (Fig. 1B, lanes 1 to 4; Fig. 1C, lanes 1 to 4). In the strain lacking Hfq, the \textit{Vrp} level are unaffected by \textit{VrrA} over-expression, (Fig. 1B, lane 7 and 8; Fig. 1C, lane 7 and 8). In our earlier studies\textsuperscript{16,17}, we observed that the total level of \textit{VrrA} was higher in the \textit{hfq} mutant than in the wild-type \textit{V. cholerae} strain A1552 suggesting that the Hfq protein somehow might reduce the stability, and thereby the level, of \textit{VrrA} or indirectly might affect its expression. In the present study, the increased level of \textit{VrrA} was unable to regulate the expression of \textit{Vrp} in the \textit{hfq} mutant, suggesting that Hfq is involved in \textit{Vrp} repression by \textit{VrrA}. We also observed that in the \textit{hfq} mutant the basal level of \textit{Vrp} was higher (compare lane 1 with lane 5 in Fig. 1B). The apparent role in \textit{Vrp} repression by Hfq might not only dependent on \textit{VrrA} but could also involve some other sRNA.

\textbf{Vrp is a stationary phase protein}

We analyzed \textit{Vrp} expression during different bacterial growth phases by Western blot analysis using anti-Vrp polyclonal antiserum (Fig. 2A). The higher expression of \textit{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 \textit{Vrp} was detectable at an OD\textsubscript{600} of 2.0 (Fig. 2A, upper panel, lane 3). \textit{Vrp} was not detectable at an OD\textsubscript{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.
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 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 Δvrp 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 ΔrpoS 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 Δvc2530 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, YfA (a.k.a. RaiA, a homolog of the Vrp) prevents 70S dimer formation. 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 Δvc2530 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 Δvrp mutant and down-regulated in the ΔvrrA mutant

We analyzed the expression of Flag tagged VC2530 in wild-type *V. cholerae* strain flag-vc2530 (DHS458), ΔvrrA-flag-vc2530 (DHS474), Δvrp-flag-vc2530 (DHS475) and Δvc2530. 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 ΔvrrA and up-regulated in Δvrp (1.4-fold increase compared to wild-type).
Impaired starvation survival of the V. cholerae double deletion mutant ΔvrpΔvc2530

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, Δvrp, Δvc2530, ΔvrpΔvc2530 and ΔvrA 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 ΔvrpΔvc2530 was reduced 3.3 fold compared to the wild-type. The starvation survival of Δvrp and the Δvc2530 single mutant were similar to that of the wild-type throughout the experiment. In addition, starvation survival of the ΔvrA 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 ΔvrA (DNV7). 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. 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-who 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- vrA (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.
Bacteria can reduce the efficiency of protein synthesis during nutrient starvation or in stationary phase by converting ribosomes into translationally inactive 100S dimers \(^3\) or 70S monomers, \(^2\) thereby inhibiting bacterial growth. The major players in the stationary phase conversion of ribosome conformation are RMF, HPF, and RaiA (in different bacterial species), \(^3\) 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.

In this study, we show that \(vrf\) is the first ribosome binding protein in \(Vibrio cholerae\) that is a direct target of sRNA, VrrA of \(V.\ cholerae\). VrrA targets \(vrf\) mRNA by base-pairing at its ribosome binding site, thus decreasing \(vrf\) 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 \(vrf\) 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 phosphoprotein 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. \(^2\) 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
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. 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 ribosomal 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*. Where viability of the *ΔvrrAΔhpf* 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. 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.**

| Strain | Source |
|--------|--------|
| *E. coli* Top10 | ATCC 11775 |
| *V. cholerae* DNY7 | ATCC 951 |

---

**Figure 4.** VrrA interact at the 5′ region of *vrp* mRNA. (A) The sequence of WT-*vrrA* and its mutant derivatives 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 OD600 1.0, with plasmids carrying different *vrp* 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* & WT-*vrrA*; lane 5, M3*-*vrp::gfp* & WT-*vrrA*; lane 6, M3*-*vrp::gfp* & M3-*vrrA*. GroEL expression was used to normalize the loading amount. Results are representative of data obtained with 3 independent cultures. (E) Agar plate-based colony fluorescence imaging analysis of the same strains as 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. 4D 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.
appropriate) at the following concentrations: 100 μg/ml carbenicillin, 20 μg/ml chloramphenicol, 50 μg/ml rifampicin.

**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 ΔvrpΔvc2530 loci in *V. cholerae* strain A1552 resulted in DHS380, A1552Δvc2530 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 (CTTGTGCATCGTCTTTGATGTC) 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 agarose 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.
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 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 EcoRI and XbaI restriction enzyme sites. Vrp expression was induced using 0.02%

Table 2. Primers used in this study

| Primer | Sequence in 5’ to 3’ direction | Restriction site Used for construction of | 
|--------|--------------------------------|------------------------------------------|
| vrp-2  | GTTTTTGTACCGATTGCAGGAGGTGACTTC | NheI pTS32 and 5’ Race for vrp |
| JVO-367 | ACTGACATGGAGGAGGAA | pTS32-M3* |
| JVO-8239 | AAAAGGATGGAAATGAAATACAACT | pTS32-M3* |
| JVO-8240 | TAATGTTTTCCCTCTGTGTC | |
| DS125  | CGCTCTAGA AACTCGAGGC TTATCAGCAG | |
| DS126  | CCCATCCACTAAAATAAACA AATGCTTTTCTCTGTGTC | |
| DS127  | TTGATTTTATAGGAGGAGGAAATGAAATGAAATACCA | |
| DS128  | AAATTCATA TGTATGGAGGAGGAAATGAAATGAAATACCA | |
| DS96   | GC GAATTCCATA AACTCGAGGC TTATCAGCAG | |
| DS77   | GCTCTAGA TGTATGGAGGAGGAAATGAAATGAAATACCA | |
| DS166  | GGCTCTAGA TGTATGGAGGAGGAAATGAAATGAAATACCA | |
| DS167  | CCATCCACTAAAATAAACA AATGCTTTTCTCTGTGTC | |
| DS168  | CCCATCCACTAAAATAAACA AATGCTTTTCTCTGTGTC | |
| DS169  | CGCTCTAGA AACTCGAGGC TTATCAGCAG | |
| DS170  | CGAGACAGCT CCAAAGTAAATGAAATGAAATACCA | |
| DS171  | CTGTCGTACCTCTCTGTGTC | |
| VC2530-A | CCCTCTAGA CGTACAATGGGCAGAGGCA | |
| VC2530-B | CCCATCCACTAAAATAAACA AATGCTTTTCTCTGTGTC | |
| VC2530-C | CGCTCTAGA AACTCGAGGC TTATCAGCAG | |
| VC2530-D | CGCTCTAGA AACTCGAGGC TTATCAGCAG | |
| JVO-8168 | AAGGGATGACAGCAGGA | |
| JVO-8169 | GGCTCTAGA CGTACAATGGGCAGAGGCA | |
| JVO-8170 | CTGTCGTACCTCTGTGTC | |

Table 3. Plasmid used in this study

| Original name | Plasmid Trival name | Relevant genotype/phenotype | Reference/source |
|---------------|--------------------|-----------------------------|-----------------|
| pGEM-T Easy   | TA cloning vector plasmid; Ap' | | Promega |
| pCR4-TOPO     | Cloning vector plasmid; Ap' | | Invitrogen |
| pBAD18        | pBAD18 carrying vrp; Ap' | | This study |
| pCVD442       | Control plasmid for gfp fusion assays | | 27 |
| pDS35         | Control plasmid | | 45 |
| pG20          | Control plasmid | | 45 |
| pMMB66HE      | vrrA complementation plasmid, based on pMMB66HE | | 18 |
| pVJ30         | CoEl1 plasmid expressing a ~50-nt nonsense transcript derived from rrnB terminator | | 27 |
| pTS2          | CoEl1 plasmid expressing vrrA from its own promoter | | 18 |
| pTS2-M1       | pTS2 carrying a 6-nt substitution in putative vrp interaction sequence, as shown in Fig. 4A | | 18 |
| pTS2-M3       | pTS2 carrying a 2-nt substitution in putative vrp interaction sequence, as shown in Fig. 4A | | 18 |
| pTS32         | Vibrio vrp translational GFP fusion Plasmid(pXG20) to 16th amino acid | | This study |
| pDS18         | 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. 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. 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 to determine the transcription start sites of the vrp gene. Total RNA isolated from the wild-type V. cholerae 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. The reporter system consists of two 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 gfp 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) 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. 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. 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,
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 Na2HPO4·7H2O, 3 g KH2PO4, 0.5 g NaCl and 1 g NH4Cl ), 2 mM MgSO4, 0.1 mM CaCl2, 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 x 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.

Funding
This work was performed within the Umeå Centre for Microbial Research (UCMR) Linnaeus Program and was supported by grants from the Swedish Research Council, the Faculty of Medicine at Umeå University and the National Institutes of Health (NIH). KP was supported by a Post-Doctoral fellowship from the Human Frontiers Science Program (HFSP).

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 16S ribosomes in stationary phase Escherichia coli cells. Proc Natl Acad Sci U S A 1990; 87:2657-61; PMID:2181444
2. Ueta M, Yoshiha H, Wada C, Baba T, Mori H, Wada A. Ribosome binding proteins YibH and YibA have overlapping functions,leading 100S formation in the stationary phase of Escherichia coli. Genes Cells 2005; 10:1103-12; PMID:1632448; http://dx.doi.org/10.1111/j.1365-2443.2005.00903.x
3. Agafonov DE, Kolb VA, Nazimov IV, Spinin AS. A protein at the subunit interface of the bacterial ribosome. Proc Natl Acad Sci U S A 1999; 96:12345-9; PMID:10535924
4. Ueta M, Ohnishi RL, Yoshiha H, Maki Y, Wada C, Wada A. Role of HFP (hibernation promoting factor) in translational activity in Escherichia coli. J Bacteriol 2008; 190:433-25; PMID:18174192; http://dx.doi.org/10.1128/jb.00829-08
5. Polikanov YS, Blaha GM, Steitz TA. How hibernation promotes ribosome dimerization. Mol Microbiol 2014; 91:394-407; PMID:25058257.004902.x
6. 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 dso 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.03140-10
7. Song T, Mika F, Lindmark B, Liu Z, Schild S, Bishop A, Zhu J, Caro J, Johansson J, Vogel J, et al. A new Vibrio cholerae sRNA regulates colonization and affects release of outer membrane vesicles. Mol Microbiol 2008; 70:100-111; PMID:18061937; http://dx.doi.org/10.1111/j.1365-2958.2008.06352.x
8. 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
9. Song T, Sahrawal D, Wai SN. Vsa mediated 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
10. Song T, Sahrawal D, Gurung JM, Cheng AT, Sjostrom AE, Yildiz FH, Uhlén 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/10.1371/journal.pone.0101280
11. Polikanov Y, Pfeiffer V, Mika F, Lucchini S, Hinton WM. Molecular analysis of VCA1008: a putative phosporin of Vibrio cholerae. FEMS Microbiol Lett 2010; 298:241-8; PMID:19659744

www.tandfonline.com
RNA Biology
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, Marias VR, Sennis 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. Valeriu SP, Rompikuntal PK, Ishikawa T, Vairkevicius K, Sjoling A, Delganov 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. Sirika A, Pfiffer Y, 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, Pansegregaur W, Frank R, Blocher H, Scholz P, Bagdasarjan M, Lancia M. 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