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Point of View

A novel sRNA that modulates virulence and environmental fitness of *Vibrio cholerae*

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We recently described the discovery and initial functional characterization of a new sRNA, VrrA, in *Vibrio cholerae* O1 strain A1552. The VrrA homologs were found in all Vibrio strains whose genome sequences were reported at present. In this article, we summarize the multi-functional features of VrrA in *V. cholerae* pathogenesis and physiology, especially in relation to the regulation of outer membrane vesicle formation and its consequence in environmental adaptation of the bacterium. As the *vrrA* gene was not predicted by any of the previous bioinformatics-based genome-wide screenings for sRNA, we discuss the reasons and give suggestion on improving current bioinformatics tools.

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Pathogenicity and the Regulation of Virulence Gene Expression in *V. cholerae*

The acute diarrheal disease cholera remains a significant public health problem, causing more than five million cases and 200,000 deaths annually in the world. *V. cholerae* is transmitted by contaminated water and food. The primary virulence factor of toxigenic *V. cholerae* is cholera toxin (CT), encoded by the *ctxAB* operon, which resides on a filamentous phage, CTXΦ.[38] The toxin-coregulated pilus (TCP) has also been shown to be an essential colonization factor. *V. cholerae* strains lacking functional CT have been engineered for use as vaccines; however, some of these strains still exhibit residual reactogenicity (mild diarrhea and inflammatory response) in volunteers,[33] suggesting that *V. cholerae* harbors toxic factors in addition to CT. The environmental signals that promote expression of CT and other virulence factors remain largely undefined. Two membrane-localized complexes (TcpP/H and ToxR/S) have been shown to promote the transcription of *toxT*, a potent activator of CT and TCP transcription.[5]

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Bacterial sRNA and Gene Regulation

In the past few years, it has become increasingly clear that small non-coding RNAs (sRNAs) regulate many diverse cellular processes. The sRNAs are small (typically 100–300 nucleotides in length), that regulate gene expression, usually by interacting with specific mRNA targets to modulate message stability and/or accessibility to the translation machinery.[6] The first sRNAs were detected in the 1960s by chance, discovered by direct labeling as being associated with proteins on migration gels or identified after random mutations. The abundance of bacterial genome sequence data has allowed gene-finding computer programs to annotate a large number of prokaryote sequences. However, although de novo annotation programs successfully identify and map protein-coding genes, they are not designed to identify sRNA genes. Recently, the intergenic regions (IGRs) of selected bacteria and yeast genomes were systematically searched for non-coding RNA genes. These computational screenings involved a combination of criteria, including large gaps between protein-coding genes[7,17] even though in *Sulfolobus solfataricus*, 13 small RNAs (sRNAs) have been found encoded either within, or overlapping, annotated open reading frames.[9] Recently Vogel and co-workers presented a generic strategy for the systems-level analysis of the post-transcriptional regulons of RNA-binding proteins and for sRNA discovery in a wide range of bacteria by using high-throughput sequencing technology.[27,37] Several bioinformatic approaches have been developed to identify sRNA-encoding genes in IGRs of bacterial genomes by searching for the co-localization of genetic features such as predicted Rho-independent transcription terminators, promoters and transcription factor binding sites, intergenic conservation among closely related species, and/or conserved secondary structure.[30] Cyclic di-guanosine monophosphate (di-GMP) is a circular RNA dinucleotide that functions as a second messenger in bacteria to trigger physiological changes, including virulence gene expression. Recent studies show that the cyclic di-GMP in many bacterial species is sensed by a riboswitch class in messenger RNA that controls the expression of genes involved in numerous fundamental cellular processes including virulence gene expression, pilus formation and flagellum biosynthesis.[31]

sRNAs have been implicated in regulation of a wide variety of cellular processes including secretion, quorum sensing, stress responses and virulence.[6,30] In nearly all cases, genes encoding sRNAs are located far from genes encoding their mRNA targets and the sRNA:mRNA hybridization occurs over relatively short regions of imperfect complementarities. Due to such limited sRNA:mRNA complementarities, predicting the regulatory role of confirmed sRNAs through bioinformatic identification of their mRNA targets has proven very difficult.

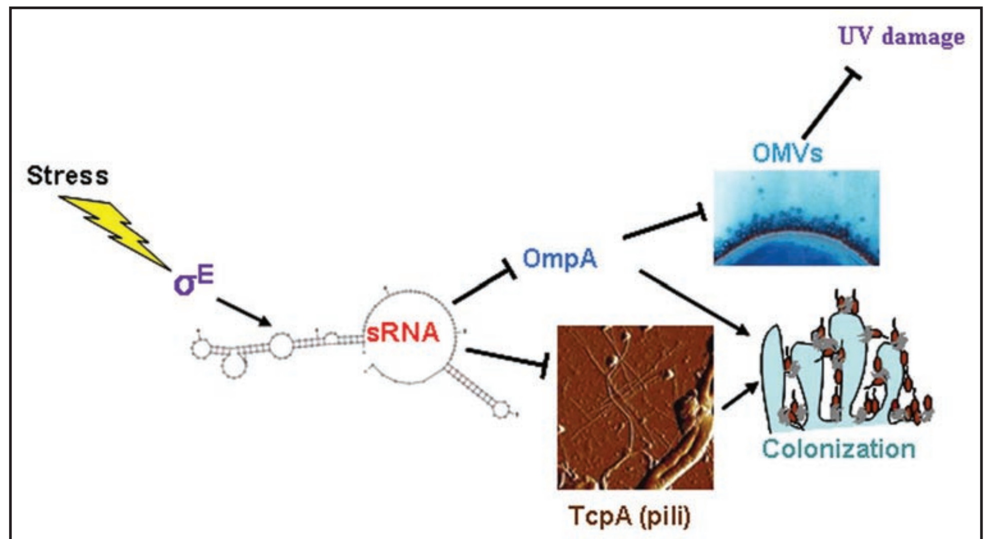

Figure 1. VrrA, a central regulatory sRNA modulating *Vibrio cholerae* colonization and environmental fitness. The *vrrA* gene is subject to Sigma E mediated regulation in response to environmental stress. The VrrA sRNA influences the major outer membrane protein OmpA synthesis and thereby the release of bacterial outer membrane vesicles (OMVs) and protection against UV irradiation. By its influence on the Toxin co-regulated pili (TcpA) synthesis, VrrA modulates the bacterial colonization in the small intestine where cholera toxin causes effects resulting in watery diarrheal disease.

Role of a Novel sRNA (VrrA) in Fitness of *V. cholerae*

In *V. cholerae*, thirty six sRNAs were predicted in the bioinformatics study by Livny et al.[18] Nine of them were experimentally studied up to now and were found to be involved in known regulatory pathways common to different bacteria but with little direct relation to virulence factors of the cholera pathogen. Recently we discovered a new abundant *V. cholerae* sRNA that we named VrrA, not previously revealed by global bioinformatics-based predictions.[29]

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A mini-Tn*5* transposon mutant library was created *V. cholerae* O strain A1552 in order to analyze the genetics of bacterial biofilm formation. Among the transposon mutants, one mutant strain was found to carry the mini-Tn*5* insertion in the intergenic region between VC1741 and VC1743. By northern blot analysis, we observed a small RNA migrating as a ~140 nt band in case of the wild type strain but it was absent in the transposon inserted mutant strain. We therefore hypothesized that there was a small RNA species encoded in this intergenic region and did 5' RACE experiments to determine the transcription start sit.[29] The transcription start site in combination with the information of a putative rho-independent terminator in the downstream region enabled us to predict the sRNA structure by the Mfold program.[29] The new sRNA was confirmed and named VrrA, for Vibrio regulatory RNA of OmpA. We observed that the *vrrA* gene and its promoter region were well conserved among Vibrios.[29]

The VrrA acts as a repressor of the synthesis of the outer membrane porin, OmpA. We observed that the *vrrA* gene is directly activated by the alternative envelope stress sigma factor, σ^E, suggesting that VrrA acts to relieve outer membrane stress by limiting the synthesis of the abundant OmpA protein. The VrrA represses *ompA* mRNA translation by base-pairing with the Shine-Dalgarno and coding region of this target. Unlike the σ^E dependent *ompA* repressor sRNAs of *E. coli* and *Salmonella typhimurium*[21,34,36] VrrA does not require the abundant RNA chaperone, Hfq, as demonstrated by in vivo and in vitro (30S toeprint) experiments.[29]

We showed that, by repressing *ompA*, VrrA positively controls the release of outer membrane vesicles (OMVs). The formation and release of OMVs have increasingly been implicated in novel pathways of envelope homeostasis and of virulence factor delivery. Our study is the first to demonstrate the control of OMV formation by a sRNA. We have assayed a *vrrA* deletion mutant in an animal model of *V. cholerae* virulence, and discovered that this sRNA affects bacterial colonization. Our analyses showed that VrrA influences the production of a major colonization factor of *V. cholerae*, the toxin co-regulated pili (Tcp).

Our findings suggest that VrrA contributes to bacterial fitness in certain stressful environments, and modulates infection of the host intestinal tract.[29] Because VrrA represses rather than promotes virulence in *V. cholerae*, attenuation of colonization ability by some means affecting VrrA expression could be considered as the basis of a strategy for therapeutic intervention in bacterial pathogenicity.[29] We aim to clarify the regulatory mechanism(s) of VrrA and thereby look for possibilities to reduce *V. cholerae* virulence. The features and implications of our findings are illustrated in the schematic representation in Figure 1.

Role of Outer Membrane Vesicles in Virulence of Bacteria

Outer membrane vesicles (OMVs) are constantly being discharged from the surface of the Gram-negative bacterial cells during growth. As the vesicles are being extruded from the surface, they may entrap some of the underlying periplasm so that they are actually small particles of Gram-negative cell wall.[3,13,39,40] Vesicles have been proposed to play a role in several virulence mechanisms: periplasmic enzyme delivery,[3,13] DNA transport,[13,42] evasion of the immune system[26] and toxin delivery.[1,10,11,12,14,15,39,40]

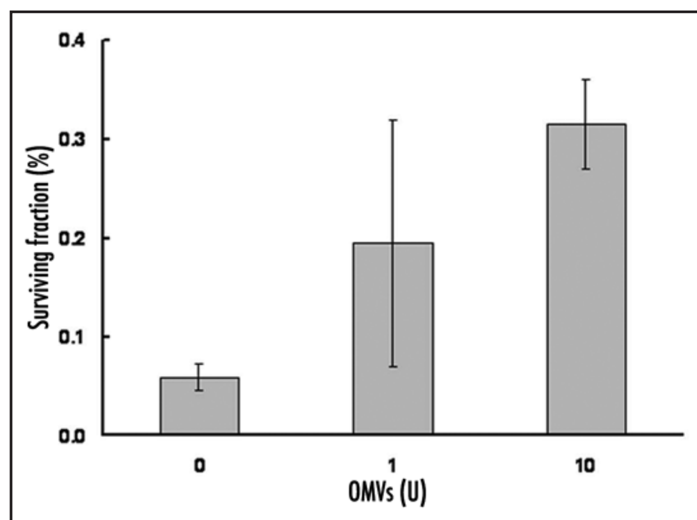

Figure 2. OMVs protect bacteria from UV irradiation. *V. cholerae* A1552 cells was mixed with different amount of OMVs followed by UV irradiation at 100 J/m^2. Each value is the mean ± standard deviation of results from three independent experiments. 3.5 ug/ml = 1 U, $p = 0.3$; 35 ug/ml = 10 U, $p < 0.05$.

Little is known yet about the mechanism(s) involved in formation of OMVs in Gram-negative bacteria. An important question is if the OMV formation as such is determined by some gene product(s) that can be identified by genetic approaches. Mutations in a few *E. coli* loci have been shown to affect the formation of OMVs. The *tol-pal* mutants are known to release more OMVs than wild type *E. coli.*[2] In our study we showed that VrrA positively controls the release of OMVs by repressing *ompA*.

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OMVs may Protect Bacteria from UV Damage

During characterization of the *ompA* mutant *V. cholerae*, we found that the *ompA* mutant bacteria better resisted killing from UV irradiation when compared with the wild-type strain (data not shown). The additional observation that the *ompA* mutant derivative released more OMVs prompted us to hypothesize that OMVs could provide some protective effect against the UV irradiation. To test this hypothesis, a UV survival assay was carried out with *V. cholerae* A1552 cells exposed to UV irradiation in the absence and presence of extra added OMVs. As shown in Figure 2, bacterial survival was about five-fold elevated in the presence of large amount of OMVs (35 μg/mL of total OMV protein). A test with ten-fold lower amount of added OMVs indicated little or no protection (Fig. 2). Since overexpression of VrrA led to more production of OMVs, and our test suggested that OMVs provided a low but detectable protection for bacteria from UV damage, it will be of interest to investigate whether UV-light and/or damage might be a signal stimulating VrrA synthesis.

The ability of bacteria to sense UV change and become UV-tolerant is important because such tolerance may enable organisms to resist irradiation in the environment, in water treatment, in shell-fish, in stages of food processing, and at locations in the domestic, commercial and hospital environment. It has also been reported that release of OMVs by Gram-negative bacteria is a novel envelope stress response.[20] Our preliminary studies indicated that VrrA sRNA level was elevated upon UV irradiation and we must consider that UV-light may be a signal for VrrA synthesis (unpublished data). We hypothesize that besides the well defined mechanisms of bacterial UV resistance, e.g., the SOS response, there is a VrrA-mediated regulatory network responding to UV irradiation in *V. cholerae*. Upon UV irradiation, VrrA would be elevated in the cell to repress the expression of OmpA, which in turn leads to more production of OMVs for protection against UV damage. The mechanism of this phenomenon is likely to be direct physical protection in which the OMVs surrounding bacteria quench the irradiation. OmpA is a β-barrel protein in the membrane and is highly conserved among Gram-negative bacteria.[4] The biological properties and functions of OmpA have been extensively studied in *E. coli.*[32] The mechanism underlying our observation of more OMV release in *ompA* mutant *V. cholerae* can possibly be explained by a role analogous to that of *E. coli* OmpA in stabilizing the cell envelope structure.[29]

V. cholerae lives in water in the environment and upon entry into the human host it starts to produce virulence factors. It is poorly understood how bacteria sense the changes from the different environments. The observation that overexpression of VrrA resulted in more OMVs production, which in turn provided stronger bacterial UV protection indicates that VrrA might play a role in the environmental survival of *V. cholerae.*

Why couldn't Genome-Wide Bioinformatics Programs Predict VrrA?

As mentioned above, bioinformatics is a powerful tool to predict sRNA genes. Several sRNAs have been identified in *V. cholerae* through genetic screens and computational methods.[16-18] Lenz et al.[16] used the following parameters in their analysis: (1) a σ54 binding site at the upstream region of the sRNA locus; (2) Rho-independent terminators (3) in IGRs (4) the sRNA must be conserved in *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. They discovered the four sRNA (Qrr1-4) in *V. cholerae.*

However, the *vrrA* gene was not predicted by any of the previous genome-wide prediction programs. Why couldn't previous genome-wide programs predict VrrA? Finding the answers to this question will certainly help to improve current sRNA prediction modules. Most of the bacterial sRNAs were found in the Intergenic Regions (IGRs), therefore bioinfomatics prediction programs restrain the sequences to be within the IGRs. In fact, many sequenced genomes, including that of *V. cholerae* N16961,[8] were incompletely annotated and large parts of the IGRs became miss-annotated. In case of the *vrrA* gene, it resides in between *V. cholerae* strain N16961 loci *vc1741* and *vc1743.*[29] The *vc1742* was miss-annotated as a coding sequence. We believe that this is one of the reasons that previous genome-wide predictions could not identify *vrrA* as a sRNA gene. The VrrA seems to be a Vibrio-specific sRNA. The other species-specific sRNAs were described in *E. coli* by a shot-gun cloning approach (RNomics),[35] in *Staphylococcus aureus* or in *Salmonella typhimurium* by microarray-based searches.[21,24,27] Massé et al.[19] have identified a role for a well conserved *E. coli* sRNA, RyhB,

in iron metabolism and potentially in defense against oxidative stress. No RyhB sequence homologs were found in *Pseudomonas aeruginosa*, despite the identification of genes positively regulated by its Fur homolog.[23] A bioinformatics approach identified two tandem sRNAs in *P. aeruginosa* that were candidates for functional homologs of RyhB. As in *E. coli* induction of these sRNAs leads to the rapid loss of mRNAs for *sodB* (superoxide dismutase), *sdh* (succinate dehydrogenase), and a gene encoding a bacterioferritin.[23,41] Thus, these sRNAs are the functional homologs of RyhB sRNA. All of these sRNA can make the difference to a species, including the virulence of pathogens.

We have experimentally proven that VrrA is under the control of the alternative sigma factor σ^E. So far, VrrA is the only sRNA found in Vibrio to be under the control of σ^E. It is most likely that there are additional σ^E-controled sRNAs in Vibrio as there are in *E. coli* and Salmonella.[25,28,22] VrrA does not require the abundant RNA chaperone, Hfq, as demonstrated by in vivo and in vitro (30S toeprint) experiments.[29]

The discovery of VrrA showed that ompA repression by sigmaE-mediated sRNAs transcends beyond species closely related to *E. coli* and Salmonella, while VrrA is no obvious homolog of those latter sRNAs. The conservation of this principle is reminiscent of RyhB sRNA and the many paralogous Fur-regulated sRNAs.[6] Integrating promoter sequences of *vrrA* in the existing prediction programs should aid in the discovery of new σ^E-controlled sRNAs in Vibrio.

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Materials and Methods

UV survival assay. OMVs used for UV survival assay were derived from *V. cholerae* A1552 as described earlier except that 0.9% NaCl was used in the final step to suspend OMVs and samples were filter-sterilized through 0.45 μm filters. OMVs derived from 5 ml overnight culture was defined to be 1 U. UV survival estimates were based on colony counts. *V. cholerae* A1552 cells were grown to $OD_{600\ nm}$ of 1.2, washed with 0.9% NaCl and ten-fold diluted in 0.9% NaCl containing different amount of OMVs as indicated. Ten ml cell suspensions were irradiated under UV at 100 J/m^2 in 8.5-cm diameter Petri dish (SARSTEDT, Germany). Irradiated cells were serially diluted and plated on LB plates under subdued lighting to avoid photoreactivation. Colony counts were performed after overnight incubation at 37°C. The surviving fraction (S/So) is expressed as the quotient of the viable count divided by the titer of bacteria without UV exposure (So).

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