A new *Vibrio cholerae* sRNA modulates colonization and affects release of outer membrane vesicles

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Summary

We discovered a new small non-coding RNA (sRNA) gene, *vrrA* of *Vibrio cholerae* O1 strain A1552. A *vrrA* mutant overproduces OmpA porin, and we demonstrate that the 140 nt VrrA RNA represses *ompA* translation by base-pairing with the 5′ region of the mRNA. The RNA chaperone Hfq is not stringently required for VrrA action, but expression of the *vrrA* gene requires the membrane stress sigma factor, σ5, suggesting that VrrA acts on *ompA* in response to periplasmic protein folding stress. We also observed that OmpA levels inversely correlated with the number of outer membrane vesicles (OMVs), and that VrrA increased OMV production comparable to loss of OmpA. VrrA is the first sRNA known to control OMV formation. Moreover, a *vrrA* mutant showed a fivefold increased ability to colonize the intestines of infant mice as compared with the wild type. There was increased expression of the main colonization factor of *V. cholerae*, the toxin co-regulated pili, in the *vrrA* mutant as monitored by immunoblot detection of the TcpA protein. VrrA overproduction caused a distinct reduction in the TcpA protein level. Our findings suggest that VrrA contributes to bacterial fitness in certain stressful environments, and modulates infection of the host intestinal tract.

Introduction

*Vibrio cholerae* is a Gram-negative bacterium that causes the acute, severe diarrhoeal disease cholera. Its natural ecosystem includes aquatic environments in endemic locations. Two factors are critical to *V. cholerae* virulence – cholera toxin (CT) and an intestinal colonization factor known as the toxin co-regulated pilus (TCP). Poorly characterized environmental cues influence the expression of CT and TCP *in vivo* (Faruque *et al*., 1998). Two sensory proteins, ToxR and TcpP, likely play a role in detection of the environmental signals, and activate the transcription of genes involved in TCP and CT expression through the expression of ToxT (Lee *et al*., 1999).

Outer membrane vesicles (OMVs) are produced by a wide variety of Gram-negative bacteria (Beveridge, 1999) including *Vibrio* species (Kondo *et al*., 1993) during their growth. They contain outer membrane proteins, lipopolysaccharides, phospholipids and, as the vesicles are being released from the surface, they entrap some of the underlying periplasm. Different hypotheses have been proposed for the function of OMVs. OMVs have been suggested to promote the adherence, the transfer of bacterial DNA and the delivery of virulence factors to bacterial or eukaryotic cells (Kuehn and Kesty, 2005; Mashburn-Warren and Whiteley, 2006). We have previously shown that OMVs contribute to the delivery of active ClyA cytotoxin, α-haemolysin and CNF1 from *Escherichia coli* to mammalian cells (Wai *et al*., 2003; Balsalobre *et al*., 2006; Kouokam *et al*., 2006). Recently, it was suggested that OMV production is a physiological consequence of Gram-negative bacteria and that OMVs are a component of the matrix of Gram-negative bacterial biofilms (Schooling and Beveridge, 2006). In their study, they found that OMVs from biofilm contained more proteolytic activity than those from planktonic cells. They speculated that OMVs could act as decoys to reduce inimical agents within biofilms before they can attack cells. OMVs are also very promising for different biotechnological applications such as the delivery of antibiotics or as efficient vaccine particles.

In contrast to the extensive research on the biological functions of OMVs, very little is known about the
mechanism and regulation of the formation of OMVs. OMV formation has been suggested to be linked to turgor pressure of the cell envelope during bacterial growth (Zhou et al., 1998). Release of OMVs is highly dependent on the envelope structure. Defects in proteins either linking the outer membrane to the peptidoglycan layer or involved in a structural network between the inner, outer membranes and the peptidoglycan layer result in the shedding of large amounts of OMVs (McBroom and Kuehn, 2007).

In the past few years, it has become increasingly clear that small non-coding RNAs (sRNAs) regulate many diverse cellular processes, including acid resistance and iron homeostasis (Majdalani et al., 2005), and the virulence of pathogens (Romby et al., 2006; Toledo-Aranas et al., 2007). A major class of sRNAs in bacteria functions by base-pairing with target mRNAs, and positively or negatively regulates translation and/or stability of these messages. This class of sRNAs usually requires the RNA chaperone Hfq as a cofactor, which facilitates the interaction between sRNAs and target mRNAs (Storz et al., 2004; Valentin-Hansen et al., 2004).

Recent systematic searches (Vogel and Sharma, 2005) revealed that E. coli expresses close to 100 sRNAs, and the total number of sRNAs in a typical enterobacterium may well range in the hundreds (Hershberg et al., 2003; Zhang et al., 2004). To date, numerous sRNAs have been predicted in V. cholerae (Livny et al., 2005), and several of these candidates have been confirmed by Northern blot analysis. Nine sRNAs have been assigned cellular functions in V. cholerae: the homologue of E. coli RyhB sRNA, which is involved in iron utilization (Davis et al., 2005; Mey et al., 2005); MicX sRNA, which negatively regulates an uncharacterized outer membrane protein (OMP) and a periplasmic component of a peptide ABC transporter (Davis and Waldor, 2007); seven sRNAs, i.e. Qrr1–Qrr4, CsrB–CsrD, which are involved in quorum-sensing regulation (Lenz et al., 2004; 2005).

Here we report on the discovery of a new sRNA in V. cholerae, to which we will refer as VrrA (Vibrio regulatory RNA of OmpA). VrrA positively regulates OMV release through downregulation of outer membrane protein OmpA. Inactivation of VrrA resulted in increased colonization of V. cholerae in the infant mouse colonization assay.

Results

Characterization of a new sRNA, VrrA, in V. cholerae

We became aware of the vrrA gene when analysing a mini-Tn5 transposon mutant (SNW6) from a library of V. cholerae El Tor O1 strain A1552 (Vaikveikivius et al., 2006), which was found to carry a mini-Tn5 insertion in the intergenic region between vc1741 and vc1743 (Fig. 1A). Inspection and sequence comparison with other Vibrio strains of the disrupted region suggested the existence of a previously unrecognized sRNA gene. We successfully validated this prediction by Northern blot analysis, which detected a ~140 nt RNA expressed from the positive strand in samples of the wild type (Fig. 4) but not of the SNW6 mutant strain (data not shown). Subsequent 5′ RACE analysis of this sRNA species (Fig. 1B) identified the transcription start site (+1) shown in Fig. 1A, which is located approximately 140 bp upstream of a putative Rhoindependent terminator downstream. The 5′ RACE analysis was performed to determine the transcription start (+1) site of the vrrA downstream gene vc1743 (Fig. S1). The +1 site of vc1743 was the same as that of VrrA. This indicates that vc1743 would be co-transcribed with vrrA. However, in the Northern blot analysis, the VrrA probe never detected the reaction band larger than 140 nt. Under the same detection condition, there was no detectable signal with a vc1743 probe (data not shown). This suggests that, although vc1743 can be co-transcribed with vrrA, the level of readthrough of the proposed terminator (Fig. 1A) is very low under the growth conditions that we used in this study. Interestingly, the V. cholerae VrrA promoter region contains a sequence that is a perfect match to the previously reported consensus of promoters recognized by the alternative sigma factor, σE (Rhodius et al., 2006; Skovierova et al., 2006). Using BLASTN searches, we identified vrrA homologues in other Vibrio species, and all of these genes show conservation of the σE binding sites in the vrrA promoter region (Fig. 1C). In order to analyse the role of RpoE in regulation of vrrA expression, we constructed an in-frame deletion rpoE mutant and tested the level of vrrA expression by Northern blot analysis. The expression of VrrA was totally abolished in the ΔrpoE mutant strain (Fig. 1D, left). Furthermore, a cloned copy of vrrA with its promoter region (plasmid pTS2) was introduced into Salmonella typhimurium strain SL1344 and its otherwise isogenic ΔrpoE mutant strain JVS-01028 (Papenfort et al., 2006), to test the σE requirement in the heterologous bacterial system. S. typhimurium carrying pTS2 expressed VrrA in a manner that was totally dependent on a functional σE (Fig. 1D, right). Taken together, our results provided conclusive genetic evidence that vrrA expression is directly controlled by the σE factor.

OmpA is downregulated by VrrA

To investigate the role of VrrA, we made comparisons using the wild-type V. cholerae strain A1552 and the vrrA deletion strain DNY7. When comparing the whole-cell protein profiles by SDS-PAGE, we noticed that a protein at 34 kDa appeared more abundant in the vrrA mutant in comparison with the wild-type strain A1552 (Fig. 2A, panel I; lanes 2 and 3). The protein was identified as the putative outer membrane porin protein OmpA by mass spectrometry analysis. The altered level of OmpA was further confirmed by Western blot analysis using anti-
Fig. 1. VrrA is conserved among vibrios and vrrA promoters contain a c^{6} consensus motif.
A. Secondary-structural prediction (Mfold) for VrrA identified in V. cholerae. Grey circles indicate the nucleotides conserved across all VrrAs listed in (C). The insert shows the genomic location of the V. cholerae vrrA gene in the vc1741-vc1743 intergenic region (note that vc1742 is a very small, 138 bp, predicted open reading frame that has no clear Shine–Dalgarno sequence and only 13 of the 46 codons are overlapping with the vrrA locus).
B. RACE mapping of 5' end of vrrA. 5' RACE was carried out as described previously (Urban and Vogel, 2007) to determine the transcription start site (+1 of vrrA). Total V. cholerae A1552 RNA was linked to a 5' adaptor RNA without or after treatment with tobacco acid pyrophosphatase (TAP) (lanes T- and T+ respectively). V. cholerae A1552 chromosomal DNA served as a control template (lane C). RT-PCR products were separated on a 2% agarose gel. The arrowhead marks the position of the strongly enhanced RT-PCR product upon TAP treatment, which corresponds to the newly initiated VrrA transcript. Cloning of the corresponding bands, followed by sequencing, identified the G residue (marked as +1 in C) as the 5' end of VrrA RNA. DNA marker sizes (lane M) are given to the left.
C. Alignment of vrrA genes identified in V. cholerae (VC), V. splendidus (VS), V. alginolyticus (VA), V. parahaemolyticus (VP), V. harveyi (VH), V. vulnificus (VV), V. shilonii (AK1), Vibrioalineae bacterium SWAT-3 (SWAT-3), Vibrio sp. MED222 (MED222) and Vibrio sp. Ex25 (EX25). Annotations for the genes flanking vrrA are VC1741/VC1743 for VC, V12B01-03703/03708 for VS, V12G01-19801/19806 for VA, V11-02639/02640 for VH, V12G01-19801/19806 for VV, V12G01-19801/19806 for AK1, V12G01-19801/19806 for SWAT-3, MED222-16406/16411 for MED222 and Ex2w-02002168/02002169 for Ex25. The putative c^{6} binding site is marked as –10 and –35, the transcription start site is labelled as +1, and the terminator is indicated by the arrow heads over the sequence. c^{6} consensus motif (Vogel and Papenfort, 2006) is shown on top. Numbering of residues follow the points represented by OD600 values. The bacteria were in the exponential growth phase between OD 600 0.2 and 2.0. We observed no growth rate difference between the wild type and mutant (data not shown). ompA and tmRNA levels were quantified as a control vector was used to transform strain DNY7, yielding strains DNY11 and DNY12 respectively. Expression of the sRNA from pvrrA was confirmed by Northern blot analysis (Fig. 4). As shown in Fig. 2A, the increased OmpA expression in the vrrA mutant carrying the vector plasmid (DNY12) was reduced in the complemented strain DNY11 (Fig. 2A, compare lanes 4 and 5). As a loading control, expression of the outer membrane protein OmpU was measured (Fig. 2A, panel III).

Absence of vrrA increases the level of ompA mRNA

Northern blot analyses were performed in order to determine and compare the relative expression levels of VrrA and ompA mRNA during growth. Our results showed that vrrA was expressed throughout growth and was stable until the stationary phase (Fig. 2B). In contrast, expression of ompA was high at the early logarithmic growth phase, but was dramatically reduced when the culture entered the late logarithmic growth phase. In other words, the ompA mRNA level decreased upon VrrA accumulation. However, in a strain lacking VrrA, expression of the ompA mRNA was maintained at a higher level throughout the exponential growth phase (Fig. 2B). Taken together, these findings strongly suggest a repressive role of VrrA for the expression of the V. cholerae ompA gene.

VrrA represses ompA mRNA translation

Many sRNAs that control OMP synthesis bind to the 5' untranslated region of the target omp mRNAs (Vogel and
VrrA directly regulates ompA mRNA by inhibiting 30S binding.

A. Interaction between VrrA and ompA mRNA, which was predicted by RNAhybrid program analysis and extended according to the toeprinting analysis (B).

B. Toeprinting analysis on ompA leader RNA (20 nM). The plus symbol ‘+’ and the minus symbol ‘−’ indicate the presence and absence, respectively, of 30S subunit (20 nM) and fMet initiator tRNA (100 nM). The ompA AUG start codon position is shown. Increasing concentrations of VrrA RNA (lanes 4 and 5: 20 and 200 nM) in the reactions inhibit 30S binding, whereas the non-specific control RNA, MicA (lane 7, 200 nM), does not inhibit the toeprint.

Papenfort, 2006). Bioinformatic predictions of the VrrA–ompA interaction with the RNAhybrid program (Rehmsmeier et al., 2004) revealed that a region of VrrA was partially complementary to nucleotides encompassing the ribosome binding site and part of the coding region of the ompA mRNA (Fig. 3A). In addition, VrrA homologues from other Vibrio species also displayed complementarity to the translation initiation region of the ompA mRNA of these strains (Fig. S2). This predicts that VrrA binds to the ribosome binding region of the ompA transcript, inhibiting ribosome entry and thus destabilizing this mRNA.

In order to assess the possibility that the regulatory function of VrrA on the ompA mRNA was direct, we performed gel-shift and RNA footprint experiments, expecting that VrrA and ompA would form a complex in vitro. Complex formation was observed and the interaction could be detected upstream and downstream of the ATG in the ompA messenger and in the complementary region in VrrA (data not shown).

To obtain direct proof of translational control, we performed toeprinting assays with ribosomal 30S subunits (Hartz et al., 1988), testing if VrrA prevented formation of the ternary translation initiation complex (mRNA/30S/ tRNAfMet) on the ompA mRNA (Fig. 3B). An ompA mRNA fragment of V. cholerae, encompassing the complete 5′ untranslated region (determined by 5′ RACE, Fig. S1) and 75 nt of the coding region, was incubated with purified 30S ribosomal subunit in the presence or absence of uncharged tRNAfMet. Subsequently cDNA was synthesized from a primer binding in the ompA mRNA coding region. This revealed the typical toeprint signal at position +14/+15 (relative to the AUG start codon of ompA mRNA). This signal was lost if the mRNA was incubated with increasing concentrations of VrrA prior to 30S binding (Fig. 3B, lanes 3–5). Instead a new toeprint pattern representing the vrrA interaction appeared (Fig. 3B, lanes 5 and 6). Salmonella MicA RNA served as a control RNA, and failed to inhibit 30S binding to the Vibrio ompA Shine–Dalgarno region (Fig. 3B, lane 7). These experiments show that VrrA specifically and directly pairs with the ompA coding region in vitro thereby inhibiting ribosome binding.

VrrA reduces the OmpA level in hfq mutant V. cholerae

To date, many of the sRNAs shown to function by base-pairing to complementary mRNA sequences appear to require involvement of the RNA chaperone protein Hfq (Brennan and Link, 2007). In order to investigate a putative involvement of Hfq for the VrrA-mediated repression of ompA, hfq mutant derivatives of the wild type and the vrrA mutant strain were constructed (DNY8 and DNY9 respectively). In the absence of Hfq, the OmpA level was still elevated by the vrrA mutation (Fig. 2A, compare lane 6 with lane 7, panels I and II). Furthermore, OmpA synthesis was still repressed by VrrA expression from plasmid, pvrA, in a strain lacking Hfq (Fig. 2A, compare lane 8 with lane 9). The Northern blot analysis of ompA mRNA showed that the transcript level was threefold higher in the Δhfq ΔvrrA double mutant than in the Δhfq single mutant (Fig. 4B, cf. lanes 6 and 7). These data indicated that the VrrA-mediated regulation of OmpA expression did occur in the absence of Hfq. Furthermore, the VrrA overexpression caused a great reduction of the ompA mRNA level both in the hfq wild-type strain DNY11 and in the hfq mutant DNY16 (Fig. 4B, lanes 4 and 8). This suggests strongly that Hfq is not essential for OmpA repression by VrrA although it is also feasible that Hfq can enhance the repression. We also observed that in the hfq mutant the basal OmpA protein level was higher (compare lane 2 with lane 6 in Fig. 2A). The apparent repression by Hfq was presumably not strictly dependent on VrrA and could also be mediated by some other sRNA or by a direct interaction of Hfq with the ompA transcript as previously proposed for E. coli (Vytvytska et al., 2000). However, RNA analysis by Northern blot hybridization showed that the total level of VrrA was slightly higher in the Δhfq

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mutant than in the wild-type strain A1552 which suggests that the Hfq protein somehow might reduce the stability, and thereby the level, of VrrA or indirectly might affect its expression (Fig. 4A, lanes 2 and 6). It has been observed in *V. cholerae* that there is an increased level of *rpoE* expression in the *hfq* mutant (Ding et al., 2004). The increased level of RpoE might promote the increased expression of VrrA in *V. cholerae*.

**VrrA promotes OMV production through repressing OmpA synthesis**

Changes in the outer membrane protein composition in Gram-negative bacteria can result in altered formation and release of OMVs (Sonntag et al., 1978). Given that OmpA is an abundant outer membrane protein in *V. cholerae*, we considered that the VrrA regulatory effect on OmpA expression might influence the production of OMVs. To test this hypothesis, we constructed an *ompA* mutant derivative and compared its production of OMVs with the wild-type strain. The result suggested that the lack of OmpA led to more production of OMVs (Fig. 5). The OMVs were visualized by electron microscopy and two different subpopulations of vesicles were observed, i.e. the smaller vesicle with an average diameter of 50 nm indicated by white arrows and larger vesicles with an average diameter of 150 nm, indicated with black arrows (Fig. 5A). The amount of OMVs released was also reflected by the amount of *V. cholerae* major outer membrane protein OmpU (Fig. 5B). As it is a major protein component of the OMVs it was used as a marker in our comparison of OMVs from the different strains. In keeping with the above results, the VrrA-overexpressing strain (*DNY11*), in which the OmpA level was repressed, produced more OMVs when compared with the wild-type strain (*DNY12*) and the *vrrA* mutant strain alone. In addition, a *ΔompA ΔvrrA* double mutant was constructed to see whether VrrA would have an impact on the OMV production in the absence of OmpA. As shown in Fig. 5A

**Fig. 4.** Northern blot analysis of VrrA (A) and *ompA* mRNA (B) levels in *V. cholerae* *ΔvrrA* and *Δhfq* mutants. Bacterial growth and RNA extraction procedures were as described in Experimental procedures. The Northern blot procedure was as described earlier (Papenfort et al., 2006). The 5S rRNA (C) was probed as internal control.

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and B, there was no significant difference in the release of OMV when the single ΔompA mutant and double ΔompA ΔvrrA mutant were compared. This is consistent with our suggestion that the VrrA effect on OMV release is occurring through OmpA protein regulation.

VrrA modulates virulence of V. cholerae

It has been shown in E. coli that OmpA protein is utilized by the bacteria for adhesion to HeLa epithelial cells and Caco-2 colonic epithelial cells (Torres and Kaper, 2003). V. cholerae OmpA shares 47.8% similarity to E. coli OmpA. To test whether OmpA has any influence on V. cholerae virulence, we used the infant mouse infection model to examine the colonization abilities of V. cholerae ompA mutant and wild-type strains. Figure 6A shows that inactivation of ompA resulted in a ~10-fold attenuation in the ability to colonize the infant mouse small intestine. As the vrrA mutant overproduced OmpA compared with the wild type (Fig. 2A), we hypothesized that this mutant would behave like the wild type or perhaps even be more virulent in the colonization assay. Indeed, the vrrA mutant showed an approximately fivefold increase in colonization ability when compared with the wild-type strain (Fig. 6A). These data suggest that OmpA is important for the colonization ability of V. cholerae, and that VrrA RNA may be considered as a regulator that modulates the virulence of V. cholerae.

We also attempted to monitor VrrA expression during V. cholerae infection of the host by quantitative RT-PCR analysis of RNA from small intestines recovered from the infant mouse infection model (see Experimental procedures for details). However, we were not able to detect the VrrA transcript from infected murine small intestinal homogenates, although VrrA was detected in RNA from in vitro samples prepared in parallel (data not shown). Although it remains to be verified, we must consider that VrrA is expressed at a rather low level in V. cholerae bacteria that are colonizing the host environment.

Earlier studies showed that TCP, a type IV pilus, is required for intestinal colonization (Thelin and Taylor, 1996). The TCP causes aggregation of V. cholerae and induces microcolony formation within the intestine. As vrrA mutant V. cholerae showed increased colonization ability, we were interested to examine whether expression of TCP was influenced by the inactivation of VrrA. We therefore cultivated V. cholerae strains in a TCP-inducing growth condition (Iwanaga and Kuyyakanond, 1987) and monitored TCP by Western blot analysis using antisera against the major subunit, TcpA (23 kDa). As shown in Fig. 6B, the TCP level was elevated in the vrrA mutant and the effect could be complemented by overexpression of VrrA from a plasmid. Thus, VrrA is either a direct or an indirect regulator of TCP and the increased ability of vrrA mutant V. cholerae in colonization could at least partially be caused by the increased production of TCP.
gested that VrrA might interact directly with the tcpA mRNA. In the tcpA mRNA 5’ region including the translation start and Shine–Dalgarno region there is good sequence complementarity to VrrA as indicated by results from a prediction using the RNAhybrid program (Fig. 6C).

**Discussion**

This study describes the discovery of a new *V. cholerae* sRNA (VrrA) that regulates expression of OmpA. VrrA appears to be a direct repressor of ompA mRNA and unlike other repressors of OmpA synthesis VrrA does not strictly require the RNA chaperone Hfq, suggesting a partly Hfq-independent pathway of ompA mRNA repression. We describe a regulatory role of VrrA in the formation and release of OMVs from *V. cholerae* and in modulation of *V. cholerae* virulence. Based on the observation of the σE consensus binding site, and direct genetic evidence for σE-dependent expression, we propose that VrrA acts as a regulator mediating σE-related stress.

OmpA is a β-barrel protein in the membrane and is highly conserved among Gram-negative bacteria (Delcour, 2002). The biological properties and functions of OmpA have been extensively studied in *E. coli* (Sugawara and Nikaido, 1992; Smith et al., 2002). The biological properties and functions of OmpA are conserved among Gram-negative bacteria (Delcour, 2002). The biological properties and functions of OmpA have been extensively studied in *E. coli* (Sugawara and Nikaido, 1992; Smith et al., 2002). Recently, the *E. coli* MicA and RseX sRNAs, and the *Salmonella* MicA and RybB sRNAs, have been demonstrated to downregulate OmpA levels by a base-pairing mechanism, and their functions are Hfq-dependent (Udekwu et al., 2005; Douchin et al., 2006; Figueroa-Bossi et al., 2006; Johansen et al., 2006; Papenfort et al., 2006; Thompson et al., 2007; Udekwu and Wagner, 2007). Importantly, the VrrA of *V. cholerae* is not a homologue of MicA, RybB or RseX, and Hfq is not strictly required for VrrA-mediated downregulation of OmpA. However, it is not ruled out that Hfq can enhance the repression of VrrA on OmpA in a similar fashion to that of the other OmpA repressors, e.g. like MicA in *E. coli* (Udekwu et al., 2005). It is noteworthy that a decrease in ompA mRNA signals upon entry into late log phase is still observed in the absence of the VrrA RNA (Fig. 2B). We do not yet understand the molecular nature of the additional regulation of ompA. The observation that *V. cholerae* OmpA expression was elevated in the hfq mutant hints at the existence of additional Hfq-dependent OmpA-regulatory sRNAs in *V. cholerae*. Alternatively, there could be a direct interaction between Hfq and the ompA mRNA as shown to occur in *E. coli* (Vytvytska et al., 2000). In addition to VrrA reported here, sRNA RyhB might also have a role in regulating OmpA in *V. cholerae*, although two research groups that characterized RyhB in *V. cholerae* reported opposite conclusions on the regulation of ompA by RyhB. Data by Davis et al. (2005) revealed that mutation of the *V. cholerae* ryhB sRNA resulted in a 1.7-fold elevation of the ompA transcript when the bacteria were grown in minimal medium supplemented with the iron chelator dipipyridyl. By comparing ompA transcript levels in wild type, ryhB mutant and hfq mutant *V. cholerae*, the authors concluded that RyhB and Hfq act in conjunction to downregulate expression of the ompA gene. However, microarray data by Mey et al. (2005) showed that ompA was 3.4-fold increased by RyhB. These data imply that OmpA regulation in *V. cholerae* is complex and that the bacteria exploit multiple regulation pathways to fine-tune OmpA expression to adapt to different growth environments.

Bacteria respond to changes in their environment by global changes in transcription. These changes in transcription are often accomplished by the induction of alternative sigma factors, which direct RNA polymerase to specific promoters, thereby inducing a set of genes called a regulon to combat the stress. In enteric bacteria one of the key pathways involved in maintaining cell envelope integrity during stress and normal growth is controlled by the alternative sigma factor σE. Previous work established that σE is essential for viability of *E. coli* (De Las Penas et al., 1997) and that it upregulates expression of ~100 protein-encoding genes that influence nearly every aspect of the cell envelope (Rhodosius et al., 2006). It has been shown in *E. coli* and *Salmonella* that the MicA and RybB sRNAs are positively and directly controlled by σE and that these sRNAs collectively act to downregulate all major and many minor OMPs under conditions of membrane stress (Figueroa-Bossi et al., 2006; Johansen et al., 2006; Papenfort et al., 2006; Thompson et al., 2007; Udekwu and Wagner, 2007). Based on the observation that the vrrA promoter includes a sequence with perfect match to the σE consensus binding site and there was no vrrA expression in the *rpoE* mutants of *V. cholerae* and *S. typhimurium*, it is evident that VrrA acts as a regulator of σE-mediated stress responses. We suggest a model (Fig. 7) that under σE-related stress conditions, typically envelope stress, VrrA is expressed to downregulate the OmpA protein level, which in turn will reduce the envelope stress by producing OMVs. This model is in line with the proposal by McBroom and Kuehn (2007), who suggested that release of OMVs by Gram-negative bacteria is a novel envelope stress response.

It is a remarkable finding that vrrA mutant *V. cholerae* showed higher ability in colonizing the infant mouse small intestine. Data presented here would be consistent with the
suggestion that the increased colonization ability was due to effects on both OmpA and TCP. We demonstrated that the interaction between VrrA and ompA mRNA is direct. Although we do not yet know the exact mechanism underlying VrrA–TCP interaction, there is possibility that VrrA regulates tcpA by directly binding to the 5′ untranslated region of tcpA, as predicted by RNAhybrid program analysis (Fig. 6C). Considering these findings, we included TCP in our model of VrrA action as summarized in Fig. 7. At the initial stage of *V. cholerae* infection, the σ^E^ level would be low and consequently also VrrA expression would be at a low level. During such a stage, the bacteria would produce OmpA, TCP and other factors that may contribute to colonization of the intestine. When bacterial numbers reach a certain level, or due to the stress from the host, the σ^E^ level elevates, which activates expression of VrrA. After synthesis, VrrA will reduce OmpA and TCP production resulting in reduced interaction with the intestinal mucosa. Concomitantly, the bacteria would produce more OMVs and thereby reduce the envelope stress (Fig. 7).

In summary, we demonstrated that VrrA positively controlled the release of OMVs by negatively controlling the expression of the outer membrane protein OmpA. Moreover, this small single regulatory RNA in *V. cholerae* may influence the bacterial colonization ability as manifested using the mouse intestine model. To the best of our knowledge, this is the first described case of a single *V. cholerae* sRNA that solitarily would affect the virulence of this bacterium. 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.

**Experimental procedures**

**Bacterial strains and growth conditions**

*Vibrio cholerae* strains are derivatives of El Tor Inaba strain A1552 (Yildiz and Schoolnik, 1998). *V. cholerae*, *E. coli* and *S. typhimurium* strains were grown at 37°C in Luria–Bertani (LB) broth supplemented, as appropriate, with carbenicillin at 100 μg ml^-1^ and chloramphenicol at 25 μg ml^-1^. For TCP expression analysis, *V. cholerae* strains were grown at inducing conditions as described previously (Iwanaga and Kuyya-kanond, 1987).

**DNA manipulations**

In-frame deletions were constructed by the procedures described previously (Vaitkevicius *et al.*, 2006). Primer sequences are summarized in Table S1. Deletion of the vrrA, ompA, hfq, tcpA and rpoE loci in *V. cholerae* strain A1552 resulted in DNY7, DNY10, DNY8, DNY51 and DNY105 respectively. vrrA deletion mutant was constructed such that 22 nucleotides upstream from and 91 nucleotides of vrrA were removed from the chromosome. Double deletion of hfq and vrrA in A1552 resulted in DNY9. Double deletion of vrrA and ompA in A1552 resulted in DNY104. A DNA fragment (304 bp) containing the vrrA gene including its putative promoter region was amplified from the A1552 genome and cloned into pMMB66HE (Furste *et al.*, 1986) at the HindIII/BamHI sites. The resulting plasmid pvrA and its vector control (pMMB66HE) were introduced by transformation into DNY7, resulting DNY11 and DNY12; and into DNY8, resulting DNY16 and DNY17 respectively.

In the pvrA construct, the vrrA gene was cloned together with its putative promoter region into the HindIII/BamHI sites in pMMB66HE and consequently the Ptac promoter in pMMB66HE was located ~100 bp upstream of vrrA's own promoter. We propose that vrrA transcription from pvrA is driven by its own promoter instead of the Ptac promoter in pMMB66HE, based on two observations. First, pvrA was not induced by Ptac inducers (e.g. IPTG) in the experiments. Second, the size of vrrA transcript in strain DNY11 (~140 nt), and we always observed a single band in the Northern blot analysis (Fig. 4).

The ColE1-based plasmid, pTS2, expressing *Vibrio vrrA* from its own promoter, was constructed based on plasmid pZEP12-luc (Lutz and Bujard, 1997). A DNA fragment of pZEP12-luc, which lacks the P_vrrA promoter region, was amplified by PCR using Phusion-polymerase (Finnzymes) and primers JVO-2512 and pLLacOB, digested with XbaI, resulting in the backbone for pTS2. The *V. cholerae* vrrA gene was PCR-amplified using primers JVO-2639 and JVO-2640. JVO-2639 binds 100 nt upstream of the +1 site of vrrA and carries a 5′ monophosphate for cloning; JVO-2640 binds 80 nt downstream of the vrrA terminator and will add an XbaI site to the PCR product. Following XbaI digestion, the product was ligated to the backbone, to yield plasmid pTS2 upon transformation.

**RNA preparation and Northern blot analysis**

RNA was prepared using Trizol according to the manufacturer's instructions (Invitrogen) and quantified on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). For Northern blotting, 20 μg of total RNA was separated on a formaldehyde:agarose gel prior to blotting as previously described (Sheehan *et al.*, 1995). The Hybond-N membrane (GE Healthcare) was subsequently hybridized with 32P-labelled gene-specific probes (Table S1). Northern blots were exposed to a phosphorimager screen and scanned on a Storm™ phosphorimager (Molecular Dynamics, USA). Quantification was performed using ImageQuant™ software (Molecular Dynamics).

**5′ RACE analysis**

5′ RACE was carried out as described previously (Urban and Vogel, 2007) to determine the transcription start sites of vrrA, ompA and vc1743. Total RNA obtained on strain *V. cholerae* A1552 was used for CDNA generation. Oligo TY2, VC2213-rev and TIS-31 (Table S1) were used as vrrA-, ompA- or vc1743-specific primers in PCR. PCR products were separated on a 2% agarose gel (Fig. 1B for vrrA and Fig. S1 for
ompA and vc1743), gel-eluted and used as template for sequencing.

Toeprinting analysis

Toeprinting reactions were carried out as described (Sharma et al., 2007) with few modifications. An unlabelled ompA mRNA fragment (0.2 pmol; 176 nt; T7 template amplified with primers JVO-2784/-2871), and 0.5 pmol of 5′-end-labelled primer JVO-2871 complementary to the ompA coding region were annealed. For inhibition analysis, 0.2 and 2 pmol of VrrA RNA (134 nt, T7 template amplified with JVO-2782/-2783) or 2 pmol of control RNA (Salmonella MicA) were added. See the figure legend of Fig. 3 for final concentrations of other components.

Isolation of OMVs

Outer membrane vesicles were isolated from culture supernatants as previously described (Wai et al., 2003).

SDS-PAGE and Western blot analysis

Protein samples were prepared from equal amount of bacteria cells after grown overnight unless otherwise indicated. The standard SDS-PAGE procedure was used (Laemmli, 1970). Gels were stained with Coomassie brilliant blue. Western blot analyses were performed as described earlier (Vaitkevicius et al., 2006), using polyclonal anti-OmpA, anti-OmpU and anti-TcpA antisera.

Electron microscopy

Procedures for electron microscopy were essentially as described earlier (Wai et al., 2003).

Infant mouse competition assay

Approximately 10^5 wild type and either vrrA or ompA mutants were inoculated intragastrically into 6-day-old CD-1 (Charles River Laboratories) mice. Mice were sacrificed after 20 h and bacteria colonizing the intestines were quantified as described previously (Gardel and Mekalanos, 1996).

Analysis of VrrA expression in the suckling mouse intestine

For analysis of gene expression in the suckling mouse intestine, mice were infected with A1552 as described (Camilli and Mekalanos, 1995) with an inoculum of around 10^5 colony-forming units (cfu) in 50 μl of LB broth. RNA was isolated from three (first experiment) or six (second experiment) separate small intestines of infected CD-1 mice 24 h post infection. RNA was isolated in parallel from an equal number of separate in vitro OD_{600} = 0.5 LB-broth cultures. RNA extraction, removal of chromosomal DNA contamination, random-primed reverse transcription and qPCR were carried out as described previously (Schild et al., 2007).

Mass spectrometry peptide sequencing

Proteins of interest were cut out from gel and analysed at Alphalyse (Denmark) for mass spectrometry.

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