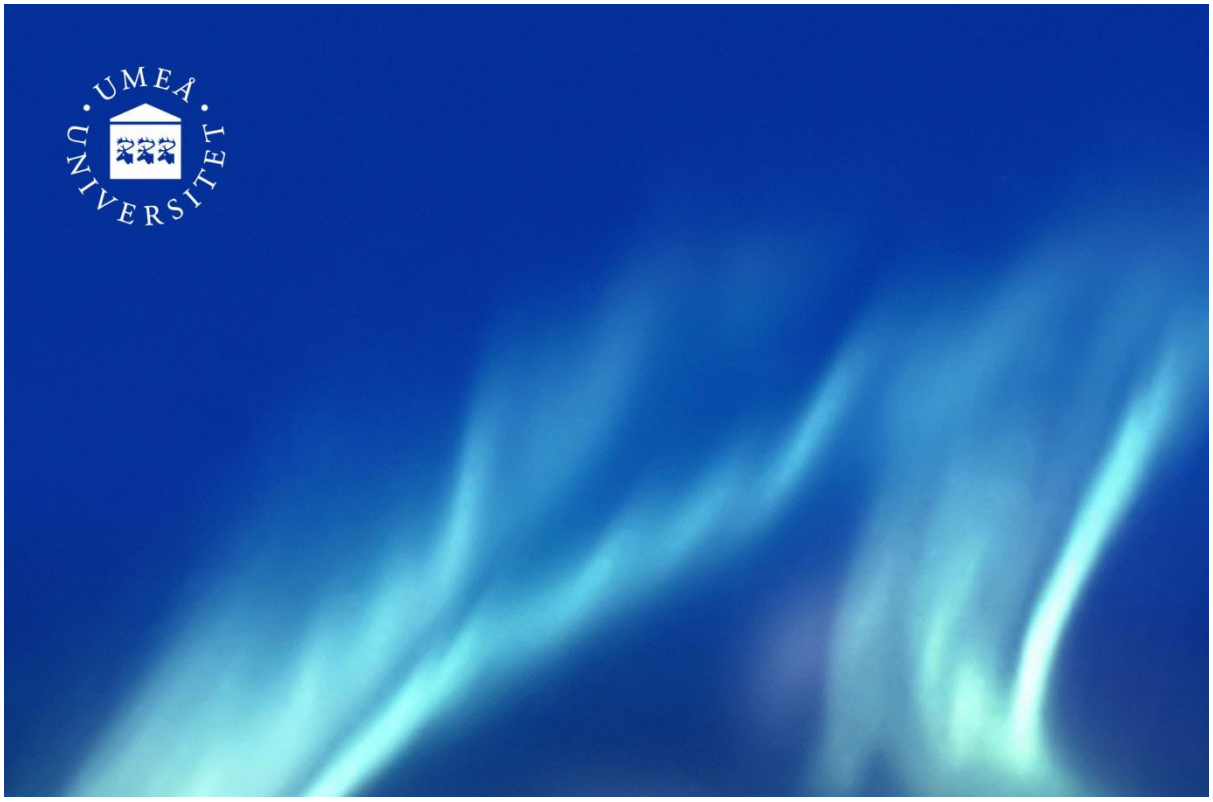

Role of *Vibrio cholera* sRNA (*VrrA*) in regulation of phosphoporin VCA1008 expression

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Abstract

Vibrio cholerae in nature often encounter growth restriction due to hyperosmolarity or other environmental stresses. Therefore, bacteria need to generate some defense mechanisms to survive against these environmental stresses. More specifically, bacteria can modulate the expression of virulence genes and/or the genes those are important for the survival of bacteria in the harsh environment. We studied the phenotypic characteristics of *V. cholerae* in high osmolarity and phosphate starvation conditions. We observed that the outer membrane protein VCA1008 was only expressed in phosphate depletion media and the expression of the type VI secretion system (T6SS) substrates, hemolysin co-regulated protein (Hcp) was somewhat reduced in the same media compared to ordinary laboratory media (LB). Therefore, we suggested that the VCA1008 might have a role in T6SS of V. *cholerae*. In addition, we observed that *V. cholerae* small RNA (*VrrA*) modulates the expression of VCA1008 under low and high phosphate cultural conditions.

Introduction

Vibrio cholerae, a Gram negative bacteria and facultative pathogen, is considered as a causal agent of cholera disease. Aquatic environment is their common habitat irrespective of saline and fresh water. There are more than 200 serogroups of *V. cholerea* but only the serotypes O1 and O139 Bengal are the causal agents of cholera disease (Shimada *et al*., 1994). However, a couple of studies (Faruque *et al*, 1998 and WHO, 2002) showed that non-O1, non-O139 (NOVC) strains can be virulent to human causing cholera-like diarrhoea.

Gram-negative bacteria possess organized cell envelopes that encompass inner and outer membranes separated by the periplasmic compartment and the peptidoglycan layer. The outer membrane serves as a barrier preventing entry of toxic substances, including antibiotics, dyes, and detergents (Nikaido, 2003; Ruiz *et al,*2006) as well as it allows nutrient acquisition and transport of molecules in and out of the cell.

The common threat to microorganisms is environmental stress or inaccessibility of nutrients in the milieu. To withstand this stress or the adaptation to a change of nutrient availability they deploy outer membrane protein (OMP) – mediated adaptation mechanisms. Recently, many researchers described that bacterial small RNAs (sRNAs) play a crucial role in the regulatory circuit of bacterial outer membrane protein expression **(**Guillier, M. and Gottesman, 2006**)**. OMPs comprise roughly 50% of the outer membrane mass and their expression is quite often regulated by the environmental condition changes, thus contributing to the bacterial survival and adaptation to different milieus (Lin *et al*, 2002; Nikaido, 2003). Reidl and Klose (2002) reported that in *V. cholerae,* OMPs play pivotal roles in bacterial physiology. With line of this notion, OpmU and OmpT (Chakrabarti *et al,*1996) porins hold distinguished channel properties whose expression is critical for bile resistance *in vitro,* virulence factor expression and intestinal colonization (Provanzano & Klose, 2000; Wibbenmeyer *et al,* 2002*;* Simonet *et al,* 2003). Xu C *et al* (2004) reported OmpW and OmpV as they are osmoregulation sensitive outer membrane proteins. The outer-membrane phosphoporin VCA1008 of *V. cholerae* was first reported as a Pi starvation-induced outer membrane protein in a classical *V. cholerae* strain (Von Kruger, 2006). Prior to this observation, Simonet (2003) revealed the involvement of VCA1008 in the virulence of *V. cholerae*. It is believed that the VCA1008 porin, associated with other outer membrane proteins, up-regulated under the phosphate depletion cultural condition, boost up the cell's ability to scavenge phosphate compounds from the environment (Lamarche *et al,* 2008). Furthermore, Osorio *et al* (2004) suggested that VCA1008 has an essential role in colonization process of *V. cholerea*. Actually, the phosphate starvation condition mimics the adverse environment of intestine where bile acts as a biological detergent and enteric bacteria respond to bile by expressing porins (Gunn, 2000; Synder & McIntosh, 2000). Recently, a study performed by Goulart *et al* (2009) underscored the involvement of VCA1008 in terms

of bile resistance and pathogenicity of *V. cholerea* suggesting that it has distinct functions in classical and El Tor *V. cholera* O1. However, the mechanism of regulation of VCA1008 is still unknown.

Recently, it is noteworthy that small non-translated bacterial RNAs are required for turning on and off virulence genes in pathogenic bacteria. The small RNAs (sRNAs) are the major regulatory molecules found in bacteria and eukaryotic cells. Most of the bacterial sRNAs in general exert their regulatory function by short base-pairing to target mRNAs. The interactions of sRNAs with target mRNAs almost exclusively results in the repression of the encoded gene, by a variety of mechanisms including translational control and induced mRNA cleavage (Storz G *et al*, 2005). Few cases of sRNA-mediated activation have been reported **(**Toledo-Arana *et al***,** 2007). We are curious about the role of sRNA in the expression of VCA1008 since functional analysis revealed that bacterial sRNAs are responsible for growth dependent outer-membrane protein expression (Udekwu *et al*, 2005).

In our earlier studies, a sRNA of *V. cholerea* (*VrrA*) was discovered and we showed that the *VrrA* modulates the expression of *V. cholerea* major outer membrane proteins OmpT and OmpA by a direct complementary base-pairing of sRNA and the target mRNAs. In this study, we analyzed if the *VrrA* has a role of VCA1008 expression under the phosphate depletion medium.

Materials and Methods

Bacterial strains and Plasmids

All bacterial strains were maintained at -80^{0}C in Luria broth with 10% glycerol. The bacterial strains, plasmids and primers used in this study are listed in Table 1 and Table 2.

Table 1. Bacterial strains and plasmids.

Starins/Plasmids	Relevant characteristics	Reference/Source
Bacteria		
E. coli DH5α	Φ80d*lacZ*ΔM15 rec*A*1*gyrA96* *Thi-1 hsdR*17 (r_k^- m_k^+) *sup*E44 *relA1deoR*Δ (lacZYA-argF)U169	Hanahan D (1983)
E. coli SM10λpir	*thi-thr leu tonA lacy supE recA::*Rp4-2 Tc::Mu Km λ pir	Miller VL and Mekalanos JJ (1988)
V. cholera A1552	O1 El Tor, Inba, Rif^r	Yildiz and Schoolnik (1998)
V. cholera A1552 Δ *vca1008*	*Δvca1008* derivative of A1552	This study
V. cholera A1552 Δ*vrrA*	*ΔvrrA* derivative of A1552	Song T *et al* (2008)
V. cholera A1552 Δ*vrrA*/pBAD18	Vector control of A1552	Song T *et al* (2008)
V. cholera A1552Δ*ompU*	*ΔompU* derivative of A1552	This study
Plasmids		
pGEM-T Easy	Cb^R TA-cloning vector plasmid	Promega
pCVD442	Cb^R positive selection suicide vector plasmid	Miller and Mekalanos (1988)
pGEM-Teasy- Δ *vca1008*	Vector plasmid with *Δ vca1008*	This study
pCVD442-Δ *vca1008*	Vector plasmid with *Δ vca1008*	This study

Table 2. Primers used in this study

Primers	Sequence	Source
VCA1008 A	5′ CGCTCTAGAGAAAGCTCAACAGGAG-3′	This study
VCA1008 B	5′-CCCATCCACTATAACTTAACACTTATCGTTGCCATTGA-3′	This study
VCA1008 C	5′-TGTTAAGTTATAGTGGATGGGGCGGATCAAATCATTGCGGGT-3′	This study
VCA1008 D	5′-CGCTCTAGACAATCGCTTGGCCAAAGAAG-3′	This study

Culture Conditions

Luria Bertoni (LB) and TG (Tris-glycerol, Kaempfer RO & Magasanik B 1967) medium were used to grow bacteria. Bacterial cells were grown at 37 °C with vigorous shaking (200 rpm) in LB containing different salts concentrations if necessary. TG media supplemented with high (TGHP) and low phosphate (TGLP) was used for the phosphate depletion and phosphate over-loaded cultural conditions. The growth of bacterial cells were measured with optical density at OD_{600} .

Starvation survival assay

Bacteria were grown in 5ml LB broth, TGHP and TGLP media in a 15ml falcon tube with shaking (200 rpm) for 12 hours at 37^{o}C, 50µl of bacterial cells were transferred into a fresh 20ml of the aforementioned media (LB, TGHP and TGLP) in a 50ml conical flask and grown at 37^{o}C with shaking (200rpm) . The survival rate was assessed by plating a serial dilution of bacterial suspensions and the colony forming unit (CFU/ml) was examined at different time points.

Polymerase chain reaction (PCR)

For a colony PCR, a standard PCR analysis protocol with a Taq DNA polymerase enzyme was used. A colony was dissolved in 10 µl MilliQ water and boiled for 5 minutes to isolate chromosomal DNA. 1 µl of the supernatant containing bacterial chromosomes was used as a template in the PCR reactions. The PCR reaction for a mutant construction, approximately 100 ng DNA was used as template and 10 pmol of forward and reverse primers along with green dye mix (New England BioLabs) and MilliQ water to a total volume of 25 µl were used for PCR reaction. A standard PCR program was initiated with a 5 minutes incubation of 95°C before 25 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 1 min were run. A final extension of 72°C for 5 minutes was carried out.

Construction of in-frame *vca1008* gene deletion mutant strain

Genomic information of *V.cholarae* O1 El Tor N16961SR strain was obtained from NCBI website (www.ncbi.gov) and used for the construction of *vca1008* gene deletion mutant strain of *V. cholarae* O1 El Tor A1552 as described below. First, two truncated regions of *vca1008* gene were amplified with primers A+B and C+D (Table 2). The amplified PCR products were purified using a Minielute® PCR purification Kit (Qiagen) and mixed as DNA templates for the second crossover PCR using the outer primers (*vca1008*-A and *vca1008*-D). The amplified PCR products were separated in a 1% agarose gel and the DNA fragment was purified using a Minielute® Gel Extraction Kit (Qiagen). The purified PCR product, lacking most of the coding sequence of the gene, was ligated into pGEM-T easy vector (Table 1) at 4 °C over night and introduced into *E. coli* DH5α cells (Table 1) by heat shock transformation for 2 min at 37 °C. After transformation, bacterial cells were streaked onto LA plates containing 100 µg/ml of carbenicillin (Cb) and 80 µg X-gal. The white colonies without β-galactosidase activity were selected. Bacterial cells were incubated overnight in 5mL LB containing 100µg/ml of Cb and plasmids were isolated using a GeneJET^{TM} Plasmid Miniprep Kit (Fermentas). The deletion fragment (2µg) were cut from the plasmid with *XbaI* restriction enzyme, separated in a 1% agarose gel, isolated using Minielute® Gel Extraction Kit (Qiagen) and ligated into a pCVD442 suicide plasmid. The plasmid pCVD442:: *ΔVCA1008* was introduced into *E. coli* SM10λ*pir* by heat shock transformation and introduced into a rifampicin-resistant *V. cholerae* O1 El Tor A1552 strain by a conjugation method. The exconjugants were selected on LB plates supplemented with 100 µg/ml of rifampicin and 100 µg/ml of Cb. After selection, bacteria were streaked onto 10% sucrose plates and incubated at 30°C. Several colonies were purified from the plates, tested for Cb sensitivity with replica

plates. Finally strains sensitive to Cb were analyzed for its deletion of *vca1008* gene by using a colony PCR.

SDS PAGE and Western Blotting

Bacteria were harvested by centrifugation at 14,000 rpm for 1 min. Pellet and supernatants were separated and supernatants were filtered through a 0.22-µm pore-size syringe (MilQpore). The pellets were re-suspended in a sample buffer. For the precipitation of supernatants, 150 µl trichloroacetic acid (TCA) was added to 500µl of culture supernatants and incubated on ice for 30 minutes. Thereafter, the resulting mixture was centrifuged at 14000 rpm for 30 minutes. The pellet was re-suspended with 40µl of 1× sampling buffer. Both pellets and supernatants samples were boiled for 10 minutes, run in a 12.5% SDS-PAGE gels and transferred onto a PVDF membrane. To detect specific protein, polyclonal antisera against Hcp, HlyA and VCA1008 were used. An ECL plus detection kit (GE health care) was used for detection for immunoreaction bands according to manufacturer's instruction.

Results and Discussions

Growth of *V. cholera* in high and low osmolarity media

In general, *Vibrio cholerae* prefers aquatic environment where the temperature, nutrients and salt concentration levels are matched with its requirements. In contrast, they confront adverse conditions while the bacteria are residing in human intestine. In our experiment, the wild type *V. cholerae* strain A1552 was grown in LB media with different concentrations of NaCl to examine the growth pattern. No significant changes of growth were observed in these growth media (data not shown). Mekalons *et al* (1988) reported that the alteration of osmolarity in a tryptone-based medium produced coordinated changes in the expression of outer membrane

proteins of *V. cholerae*. In line with this notion, they concluded that physiological and nutritional parameters induced the expression of outer membrane proteins and Tox-R regulated genes.

Role of a phosphoporin protein VCA1008 in the growth of *V. cholerae* O1 strain A1552

Most of the essential cellular components of bacteria, like nucleic acids, lipids and sugars, are phosphorylated. The phosphate balance in bacteria is controlled by the phosphate (Pi) sources from the surrounding medium. The certain bacterial outer membranes are synthesized at an increased rate during Pi starvation and Pi overloading. Since the Pi concentration is normally low in the natural habitat, bacterial cells have a strategy for the improvement of Pi uptake by modulating the expression of phosphoporin protein on the surface of the bacteria. In most of the Gram-negative bacteria, the expression of phosphoporin protein is regulated by two genes (the phoB-phoR operon), both negatively and positively. PhoR protein is a histidine protein kinase, induced in Pi starvation and is a transmembrane protein. It phosphorylates the regulator protein PhoB which is also Pi starvation-induced. The phosphorylated PhoB binds specifically to a DNA sequence of 18 nucleotides (the pho Box), which is part of the promoters of the Pho genes. The repression of *phoA* (the gene encoding alkaline phosphatase) by high Pi concentrations in the medium requires the presence of an intact Pst operon (pstS, pstC, pstA, pstB and phoU) and phoR.

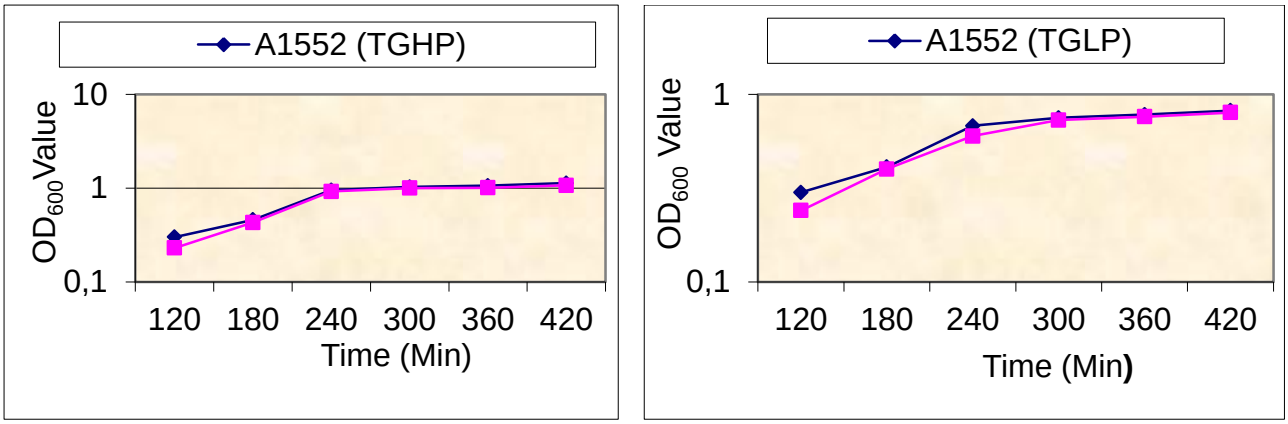

Fig. 1. The effect of Phosphate on the growth of *V. cholerae* strain A1552 and ΔVCA1008

Recently, the phophoprotein (VCA1008) was discovered in *V. cholerae* (Von Kruger *et al*., 2006). In their studies, it was shown that the VCA1008 protein has a strain-specific role in the physiology and pathogenicity of *V. cholerae* O1 strains. Disruption of *vca1008* led to a growth defect, an inability to colonize and a high susceptibility to sodium deoxycholate (DOC; the major bile compound) in the *V. cholerae* El Tor biotype strain N16961, but interestingly did not affect the classical strain O395 in the same way. Furthermore, it was described that the *vca1008* promoter activity was higher in *V. cholerae* O 1 strain N16961 cells grown under a low P(i) supply in the presence of DOC than in the absence of the detergent. In the P(i)-limited cells, *vca1008* was positively regulated by PhoB, but when DOC was added to the medium, the expression of *vca1008* was enhanced in a ToxR-dependent manner. These findings revealed a complex strain-specific interplay between ToxR and PhoB/R systems to control phoporinporin encoding genes, as well as the influence of DOC on the expression of PhoB- and ToxR-regulated genes and pathogenesis in pandemic strains of *V. cholerae*.

In order to test if VCA1008 has a role in growth of bacteria under phosphate depletion and overloading conditions, we constructed an in-frame deletion mutation in the *vca1008* gene. We analyzed the growth rate of the mutant strain in comparison with the wild type *V.*

cholerae O1 strain A1552. As shown in Fig. 1 the growth rates of Δ*vca1008* and the wild type strain both under Pi deficient (TGLP) and overloading conditions (TGHP) are similar. But it is noteworthy that both wild type and mutant strains could not reach maximum OD ~4 which was usually reached when *V. cholerae* is grown in an ordinary LB media. Both strains showed the maximum OD of 1.0 at the end of the exponential phase.

Analysis of VCA1008 expression under TGHP and TGLP conditions

In general the expression of bacterial outer membrane proteins are differentially regulated under different environmental stresses. In order to test if the VCA1008 protein is expressed under TGHP and TGLP conditions in wild type *V. cholerae* strain A1552, we performed a SDS-PAGE immunoblot analysis of whole bacterial cell lysates using polyclonal anti-VCA1008 antiserum. As shown in Fig. 2; lanes 3 and 5, the higher expression of VCA1008 was observed under both TGHP and TGLP conditions to compare with the level of expression under LB grown bacteria (Fig. 2, lane 1). The reaction band in Δ*vca1008* mutant under LB condition was a cross-reaction band with one of the major outer membrane protein OmpU of *V. cholerae* but this protein was usually not expressed under TGHP and TGLP condition (Fig. 2, lanes 4 and 6). Based on this data, we suggest that the expression of VCA1008 was under the influence of Pi in the culture media.

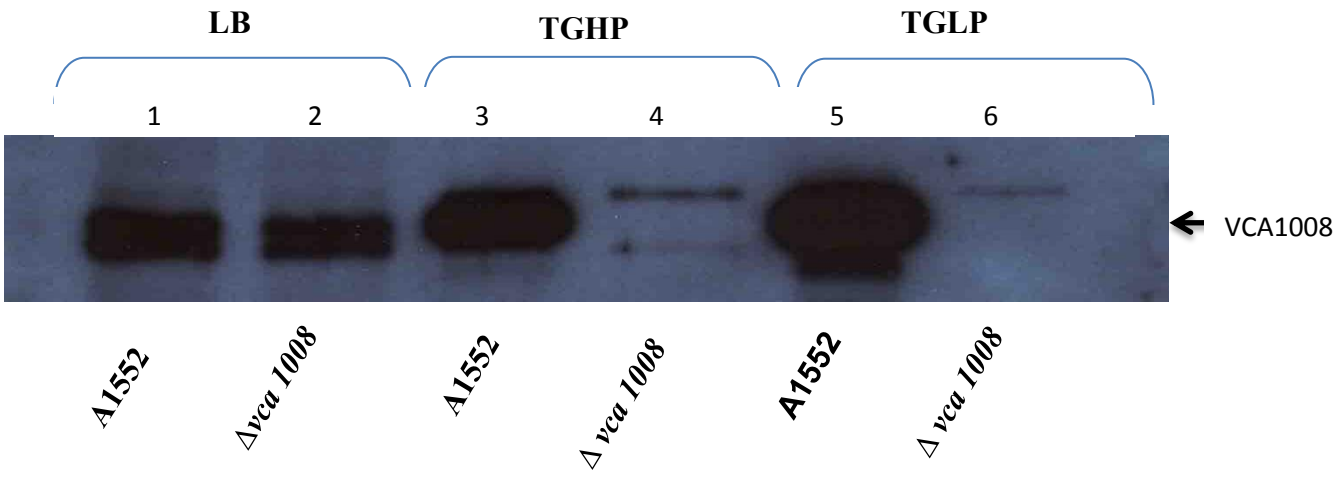

Fig. 2. The expression of VCA1008 in LB and TG media

Role of sRNA (VrrA) on expression of VCA1008

In our laboratory, we recently discovered a novel sRNA (VrrA) of *V. cholerae* O1 strain. We demonstrated that the VrrA control the expression of one of the major outer membrane protein OmpT and a peptidoglycan binding protein OmpA by direct base pairing of the target mRNAs. We asked if the VrrA also has a role in expression of VCA1008. We tested the expression levels of VCA1008 in wild type *V. cholerae* strain A1552, Δ*vrrA* (DNY7) *vrrA*/pMMB66HE::*vrrA* (DNY11) and *vrrA*/pMMB66HE (DNY12) by immunoblot analysis using anti-VCA1008 antiserum. The bacterial strains were grown in TGHP and TGLP media as described in materials and methods and the expression level of VCA1008 was tested by immunoblot analysis. As shown in Fig. 3A, when the bacterila strains were grown in TGHP media the expression of VCA1008 was not obviously different among the strains.

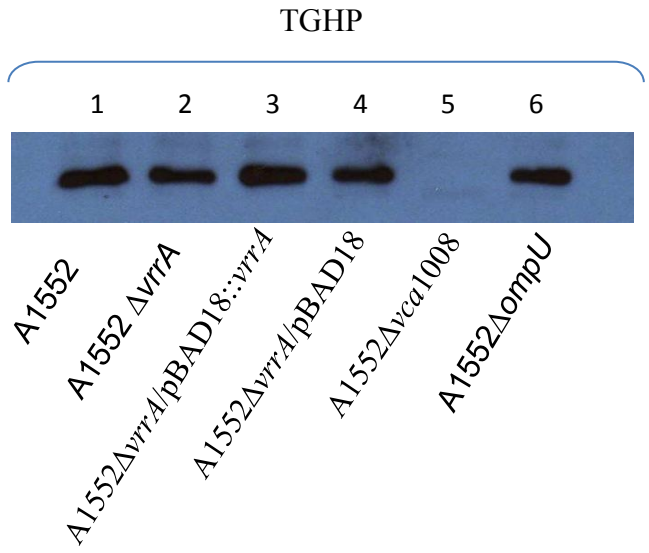

Fig. 3A. Role of sRNA (VrrA) in regulation of VCA1008 expression in TGHP (High Phosphate)

However, a higher lavel of VCA1008 expression was observed in Δ*vrrA* mutant (Fig. 3B, lane 2) to compare with the wild type strain A1552 (Fig. 3B, lane 1). Furthermore, the expression of VCA1008 was significantly reduced in the VrrA over-expressed strain (Fig. 3B, lane 3). We suggest that VrrA has a role in modulation of VCA1008 expression under low phosphate cultural condition. Currently, we are investigating how VrrA regulates the expression of VCA1008 *i. e* if the VrrA directly binds at the 5'UTR region of *vca1008* by complementary base pairing or VrrA acts on the promoter region of *pho* regulon then regultes the expression of VCA1008 indirectly.

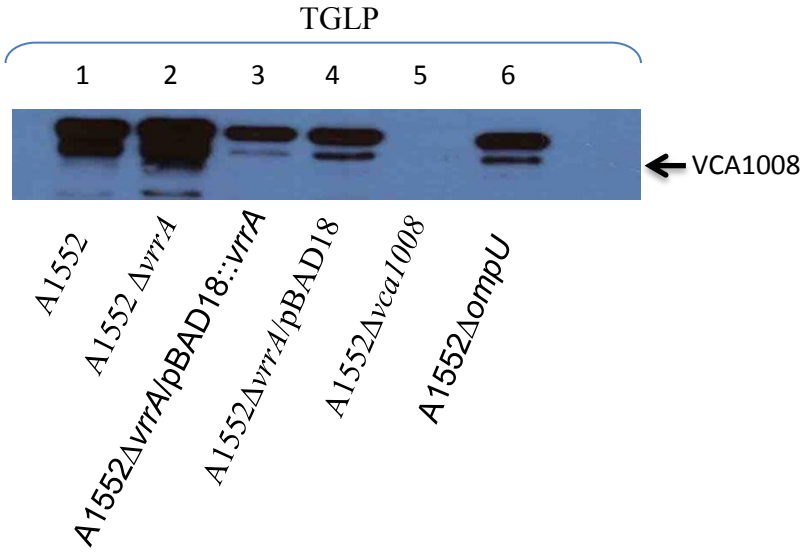

Fig. 3B. Role of sRNA (VrrA) in regulation of VCA1008 expression TGLP (Low Phosphate) media.

Analysis of role VCA1008 on type VI secretion system of V. cholerae (T6SS)

Recently a novel protein secretion system (T6SS) was discovered in Gram-negative bacterial strains (Pukatzki et al. 2006). The outer membrane lipoproteins and osmolarity regulate the expression of functional T6SS in *V. cholerae* (Ishikawa et al. 2011, manuscript under review in Infection and Immunity). In order to test if VCA1008 has a role in secretion of substrates via T6SS we used the secretion of Hcp, one of the T6SS substrates, as a marker protein to show the functional T6SS in *V. cholerae* O1 strain A1552. We analyzed the secretion of Hcp under high osmolarity in Δ*vca1008* mutant and wild type strain A1552 since in our current study, we observed that Hcp was efficiently secreted into the culture media when the bacterial strains were grown in LB containing 2% NaCl (Ishikawa et al. 2011, manuscript under review in Infection and Immunity).

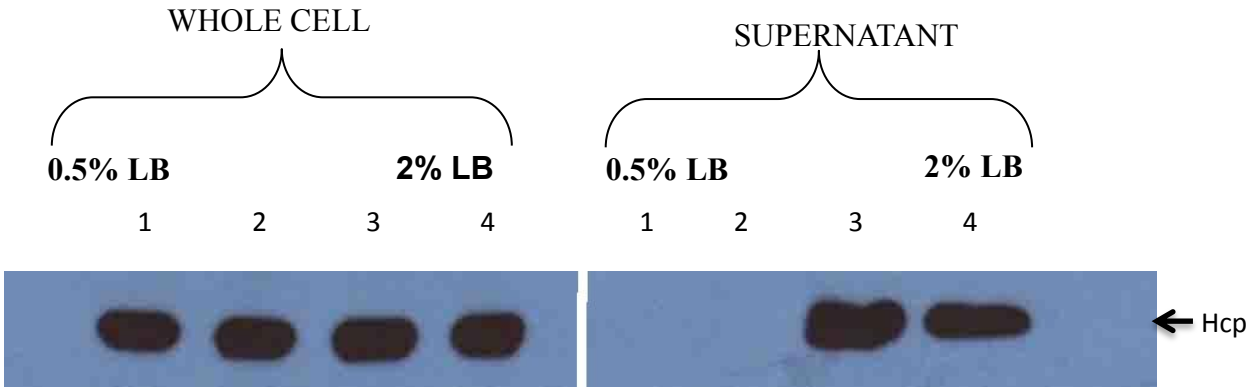

Fig. 4A. **Fig. 4B.**

Fig. 4(A). Immunoblot analysis of Hcp expression in the wild type *V. cholerae* strain A1552 and Δ*vca*1008 mutant; **Fig. 4(B).** Immunoblot analysis of Hcp secretion in the wild type *V. cholerae* strain A1552 and Δ*vca*1008 mutant.

As shown in Fig. 4A; lanes 1-4, the whole cell Hcp levels were not significantly different between the wild type and Δ*vca1008* mutant when the bacteria were grown in both LB containing 0.5% NaCl (normal osmolarity that was used for ordinary LB media) and 2% NaCl (high osmolarity condition). However, we observed that the secretion of Hcp was relatively reduced in the Δ*vca1008* mutant in comparison with wild type (Fig. 4B; lanes 3 and 4) although the secretion of Hcp was not observed in ordinary LB media (0.5% NaCl containing LB) (Fig. 4B; lanes 1 and 2). Taken together, we suggest that VCA1008 might have a role in osmolarity dependent secretion of Hcp via T6SS of V. cholerae O1 strain A1552. Currently, we are investigating how the phosphoporin protein VCA1008 plays a role for a functional T6SS in *V. cholerae*.

Conclusion:

In this project study:

1) The expression of VCA1008 porin protein was regulated by Pi concentration of the culture media.

2) We demonstrated that the expression of phosphoporin protein VCA1008 was modulated by a newly discovered sRNA(VrrA) in *V. cholerae*.

3) We suggest that the VCA1008 protein might play a role for a functional T6SS of *V. cholerae* O1 strain A1552.

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