The Hong Kong Polytechnic University

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Molecular mechanisms of antimicrobial resistance and virulence of *Vibrio parahaemolyticus*

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June 2013

Certificate of originality

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June 2013

Abstract

Vibrio parahaemolyticus is not only a clinically important foodborne pathogen worldwide but also a leading cause of foodborne illnesses in Hong Kong. My research is focused on understanding the molecular mechanisms of virulence and antimicrobial resistance in this pathogen. Through bioinformatics analysis, a novel adhesion protein that was referred to as VadF, was mined from the genomic sequence. Deletion of *vadF* gene in *V. parahaemolyticus* dramatically decreased its ability to attach to HeLa cells and its infectivity in mice model. Biochemical results showed that the N-terminal region closely linked to the transmembrane domain of VadF was responsible for the binding to N-terminal domains of fibronectin. The rest parts of VadF did not bind to fibronectin independently, but they stimulate VadF binding to fibronectin. In addition, a novel *znuA* homologue (*vpa1307*) that represents a novel subfamily of ZnuA was identified. It was shown that horizontal gene transfer is one of the most important factors that influence the virulence of *V. parahaemolyticus*. Phylogenetic analysis suggested that *vpa1307* gene in *V. parahaemolyticus* is horizontally acquired. 40% of clinical isolates possessed this gene. The expression of *vpa1307* gene was induced under

zinc limitation. Loss of function and gain of function confirmed that Vpa1307 contributes to the growth of *V. parahaemolyticus* under zinc starvation condition. Moreover, Vpa1307 also contributed to cytotoxicity against HeLa cells as well as the pathogenesis in mice. These results suggest the acquired *vpa1307* subfamily genes contribute to the fitness and virulence of *Vibrio* species.

The second direction of my research is to understand the mechanisms of antimicrobial resistance in *V. parahaemolyticus*. Fluoroquinolones are the choices for the treatment of *V. parahaemolyticus* infections. It was showed that there is an increasing trend of fluoroquinolone resistance in *V. parahaemolyticus*. However, the underlying mechanisms for the fluoroquinolone resistance in this pathogen remain to be characterized. The present work showed that a single amino acid substitution in GyrA (Ser83Ile) and ParC (Ser85Leu) contributes to the primary mechanism of fluoroquinolone resistance. Four isolates were found to carry a plasmid with a novel fluoroquinolone resistance gene, *qnrVC5*. Efflux pumps have limited role in fluoroquinolones resistance among all strains. A 1.5 kb class I integron that may contribute to trimethoprim and rifampicin resistance by the *dfrA27* and *arr3* genes was also detected in these four isolates. In the ciprofloxacin resistant *V. parahaemolyticus* strain V1, a multiple drug resistance transferable plasmid, pVP1 (about 200 kb) was

identified. pVP1 carried a novel quinolone resistance gene, *qnrVC6*. This gene conferred resistant to nalidixic acid and ciprofloxacin in *E. coli*. The genomic organizations showed that QnrVC6 is present within IS elements, which has never observed before, suggesting the *qnrVC* genes may be transferred among *Vibrio* spp. by divergent mobile genetic materials. Other drug resistance elements on pVP1 were *blaPER-1*, *aacA3*, *catB2*, *aadA1* and *dfrA1*. Transmission of pVP1 among *Vibrio* species would cause huge threat to public health and needs close monitoring.

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To my family, for always encouraging and supporting me to follow my dreams.

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Abbreviations

CBD	Gelatin and collagen binding domain
ECM	Extracellular matrix
FAP	Fibronectin attachment protein
Fn	Fibronectin
FnBP	Fibronectin binding protein
HBD	Heparin binding domain
HGT	Horizontal gene transfer
IPTG	Isopropyl β-D-1-Thiogalactopyranoside
MAM7	Multivalent adhesion molecule 7
MAPK	Mitogen-activated protein kinase
PAI	Pathogenicity island
PMQR	plasmid-mediated quinolone resistance
RT-PCR	Reverse transcription-PCR
T3SS	Type III secretion systems
TPEN	*N,N,N',N'*-Tetrakis (2-pyridylmethyl) ethylenediamine
Vp-PAI	*V. parahaemolyticus* pathogenicity island

Chapter 1 Introduction

1.1 *Vibrio parahaemolyticus* causes severe infections

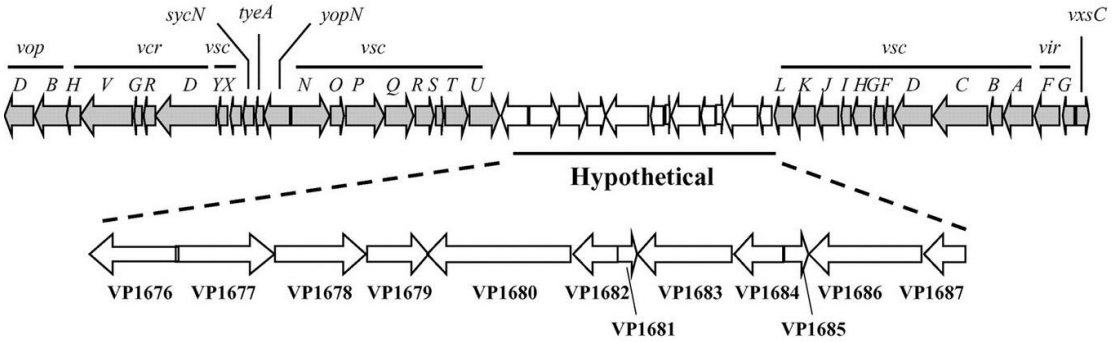
Vibrio parahaemolyticus is a motile and facultative anaerobe that inhabits estuarine and marine environments. It is responsible for acute gastroenteritis worldwide, especially in areas with high seafood consumption (Blake et al., 1980). The infections were often caused by consumption of raw or undercooked oysters in western countries. In Hong Kong, food poisoning outbreaks were frequently linked to ingestion of shrimps, a common eating habit in this city. Epidemiological data showed that in Hong Kong, this gram-negative pathogenic bacterium is the leading cause of foodborne illnesses. Around 47% of food poisoning cases are linked to this pathogen and infections are often observed in females of 20-64 years old, who are at the highest risk of getting infections (Centre for Health Protection, 2011). Even worse, this kind of infections is more dangerous to individuals with compromised immune system or those with impaired liver function (Hou et al., 2011).

1.2 *V. parahaemolyticus* produces many virulence factors

V. parahaemolyticus is recognized as the leading cause of human gastroenteritis in Hong Kong. The molecular mechanisms for its pathogenesis have evoked considerable interest since the 1990s. It has been shown that the pathogenicity of this opportunistic pathogen is highly associated with the thermostable direct hemolysin (TDH), which is a pore-forming toxin that leads to lysis of human erythrocytes, activation of calcium influx, disruption of cytoskeleton and epithelial barrier (Fabbri et al., 1999; Honda et al., 1992; Raimondi et al., 2000). A recent work has revealed that TDH can also activate caspase-1 (Ritchie et al., 2012). Unlike a variety of cellular effects caused by TDH, TDH-related hemolysin (TRH) has only been linked to hemolytic activity (Honda et al., 1988). Virulence factors other than TDH/TRH also contribute to the pathogenicity of *V. parahaemolyticus*. For example, the *tdh* mutant strain of *V. parahaemolyticus* RIMD 2210633 can still cause efficient infection (Hiyoshi et al., 2010). This bacterium possesses two chromosomes, I and II. It is interesting to note that two type III secretion systems (T3SSs), T3SS1 and T3SS2, localized on chromosome I and chromosome II, respectively (**Fig. 1**) (Makino et al., 2003). T3SS is a needle-like apparatus with two distinct translocators. This injectisome can produce a

pore in the host cell membrane. Virulence factors, termed effectors, are injected into host cells, leading to diverse cellular responses (**Fig. 2**). The components of this syringe machinery are highly conserved among diverse Gram-negative pathogens. The effectors, which are secreted by T3SS, are highly diverse (Dean, 2011; Galan and Wolf-Watz, 2006). The prevalence of T3SS1 in all *V. parahaemolyticus* strains results in autophagy, while the localization of T3SS2 on the pathogenicity island (Vp-PAI) that is more popular among clinical isolates causes gastroenteritis (Zhang and Orth, 2013). Recent evidence has proven that T3SS2 of *V. parahaemolyticus* contributes to its colonization in the distal small intestine and effective competition to protists in aquatic environment (Matz et al., 2011; Ritchie et al., 2012). Further studies have found that the effectors secreted by two T3SSs are different. VopQ, VopR, VopS and VPA0450 are secreted by T3SS1, whereas VopA, VopC, VopL, VopT, VopV and VopZ are secreted by T3SS2 (Zhang and Orth, 2013; Zhou et al., 2013a).

A



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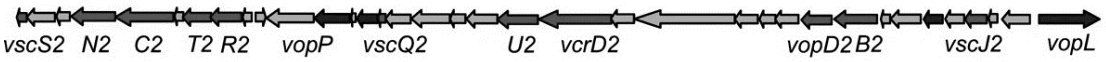


Figure 1. *V. parahaemolyticus* T3SS gene clusters. A. T3SS1 gene cluster (Ono et al., 2006). Hypothetical proteins are represented by white color arrows and grey color arrows indicate other proteins; **B.** T3SS2 gene cluster (Okada et al., 2009). Hypothetical proteins are represented by grey color arrows and black color arrows indicate regulators, effectors and apparatus proteins.

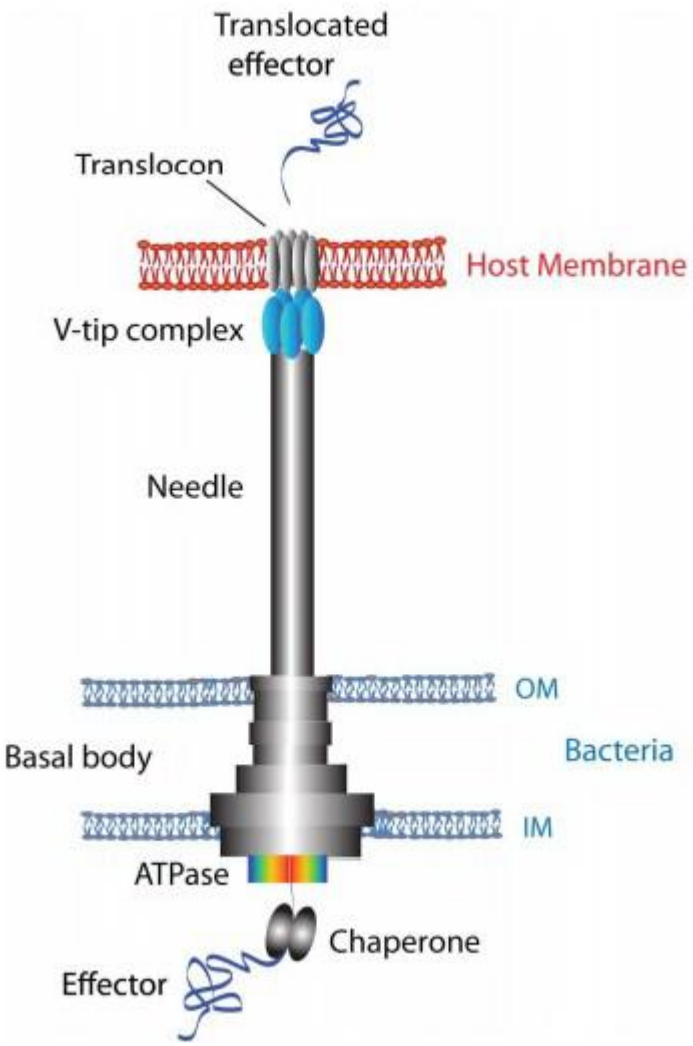


Figure 2. Model of T3SS apparatus depicting its interaction with host membrane (Sato and Frank, 2011). T3SS is a needle-like injectisome. Pathogens translocate effectors using the injectisome into host cells.

1.3 T3SS1 effectors

T3SS1 gene expression is enhanced by calcium and iron as well as ExsA (Gode-Potratz et al., 2010; Zhou et al., 2008). ExsA is an AraC family regulator. It binds to the promoter of *vp1668* and *vp1687*. ExsD represses T3SS1 by interaction with ExsA (Zhou et al., 2010). All four known effectors secreted by T3SS1 were identified by two-dimensional gel electrophoresis when *V. parahaemolyticus* was grown under the inducing condition (Ono et al., 2006). It was found that VopQ induces autophagy in a PI3-kinase independent pathway. It can cause fast conversion of LC3-I to LC3-II, which is a biomarker of autophagosome (Burdette et al., 2008; Burdette et al., 2009). Later, other researchers found that in yeast, the substrate of VopQ is the vacuolar H^+-ATPase subunit c (Matsuda et al., 2012). However, a recent study showed the actual substrate of this effector is the V_0 domain of the vacuolar H^+-ATPase instead of its subunit c. This interaction facilitates VopQ to insert into the lysosomal membrane to form a pore that permits efflux of ions and small molecules. The outward flow of ions leads to acute autophagy (Sreelatha et al., 2013). Moreover, VopQ is also a repressor of phagocytosis and an activator of JNK, p38 and mitogen-activated protein kinase (MAPK) pathways (Burdette et al., 2009; Matlawska-Wasowska et al., 2010; Shimohata et al., 2011).

Unlike VopQ, which shows no homology to the known effectors from other pathogens, VopS shows homology to Fic (filamentation induced by cyclic adenosine monophosphate) domain, of which the first histidine residue in the conserved motif HPFx[D/E]GN[G/K]R is essential for the function. VopS directly transfers adenosine 5'-monophosphate (AMP) to the conserved threonine residues on Rho GTPases. This modification inhibits Rho GTPases from activating the downstream pathways that disrupt host actin cytoskeleton (Yarbrough et al., 2009).

It is interesting to observe that VPA0450 is located on chromosome 2. This localization is totally different from that of VopQ and VopS, which are both encoded by chromosome 1. VPA0450 is an inositol polyphosphate 5-phosphatase homology protein. Biochemical experiments revealed that this effector hydrolyzes phosphatidylinositide (4,5)-bisphosphate in the host plasma membrane, causing disassociation of the actin from the binding proteins anchored on the membrane (Broberg et al., 2010). Together with VopQ and VopS, VPA0450 leads to cell lysis rapidly (**Fig. 3**). For VopR, its function and target are still unknown.

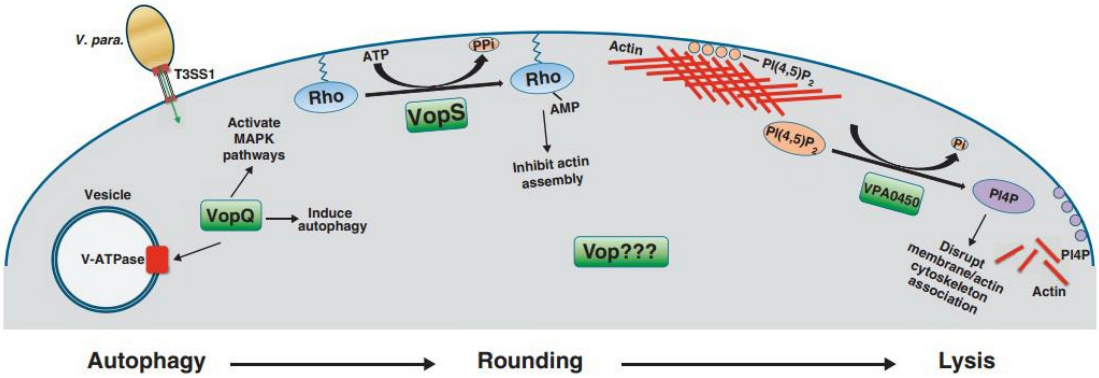


Figure 3 Model depicting the various targets of T3SS1 effectors (Zhang and Orth, 2013). VopQ induces autophagy and activates MAPK pathways; VopS directly transfers AMP to Rho GTPases and VPA0450 hydrolyzes PI(4, 5)P2, leading to cell lysis.

1.4 T3SS2 effectors

Similar to T3SS1 which can be activated by chemicals and regulators, T3SS2 is also stimulated by chemicals such as bile acids. T3SS2 is regulated by two ToxR regulators, VtrA and VtrB, encoded by two genes within the T3SS2 gene cluster. It was shown that VtrB is responsible for activation genes within T3SS2, while VtrA controls the expression of VtrB (Gotoh et al., 2010; Kodama et al., 2010).

Among the T3SS2 effectors, VopA (also called VopP) is the first to be characterized. It shares similarity with the *Yersinia* effector, YopJ. Similar to YopJ, VopA is an acetyltransferase that inhibits MAPK pathway by acetylation of the same serine and threonine residues of MKKs. Moreover, VopA acetylates an additional lysine residue on the catalytic loop of MKKs that block the binding of ATP to MKKs. The major difference between YopJ and VopA is that YopJ can block NF-κB signaling while VopA is not able to do so (Trosky et al., 2004; Trosky et al., 2007).

VopT is an ADP-ribosyltransferase homologue effector. The functions of ADP-ribosyltransferase domain in effectors ExoT and ExoS from *Pseudomonas aeruginosa* are well characterized. ExoT has only one substrate while ExoS can modify several proteins, including Ras, Rac1

and Cdc42 (Ham and Orth, 2012). In *V. parahaemolyticus*, VopT shows cytotoxicity to Caco-2 cells and inhibits the growth of yeast. Ras, a small GTPase is ADP-ribosylated by VopT (Kodama et al., 2007). However, it remains unclear whether this modification activates or inactivates Ras. Moreover, whether VopT can target other intracellular proteins requires further investigation.

It was shown that VopC, a cytotoxic necrotizing factor homologue protein, did not produce a growth defect phenotype in yeast (Kodama et al., 2007). This suggests that its targeted cellular processes are not normally rate limiting for yeast growth. The alternative method to increase the range of target detection is using the subinhibitory concentration of caspofungin to sensitize yeast to respond to the activity of effectors by perturbing MAPK pathway which is essential for maintaining cell wall integrity (Slagowski et al., 2008). A recent study has proven that VopC is a T3SS2 effector essential for bacterium invasion. VopC activates Rac and Cdc42 via deamidation of their glutamine 61 residues. Its inability to activate RhoA can well distinguish this protein from other cytotoxic necrotizing factor homologue toxins. The modification on Rac and Cdc42 keeps the active state of two Rho GTPases, which causes actin cytoskeleton rearrangement. VopC homologs are widely distributed in other *Vibrio* spp, suggesting that VopC might facilitate invasion into host cells for diverse

Vibrio spp. Meanwhile, it has been found that *V. parahaemolyticus* is an intracellular invader when T3SS2 is available (Zhang et al., 2012).

Other T3SS2 effectors also subvert the actin cytoskeleton. For example, VopL causes stress fibers in a Rho GTPase-independent pathway. It can bind directly to actin monomers with its Wiskott-Aldrich homology 2 (WH2) repeats. Further studies have shown that its C-terminal domain also plays important role in actin nucleation (Liverman et al., 2007; Namgoong et al., 2011; Yu et al., 2011).

VopV is the largest effector (1622 residues) that has been identified so far. Its binding to F-actin is dependent on the long repeat region and C-terminal domain. In infant rabbit model, the F-actin binding activity of VopV is crucial for the enterotoxicity (Hiyoshi et al., 2011; **Fig. 4**).

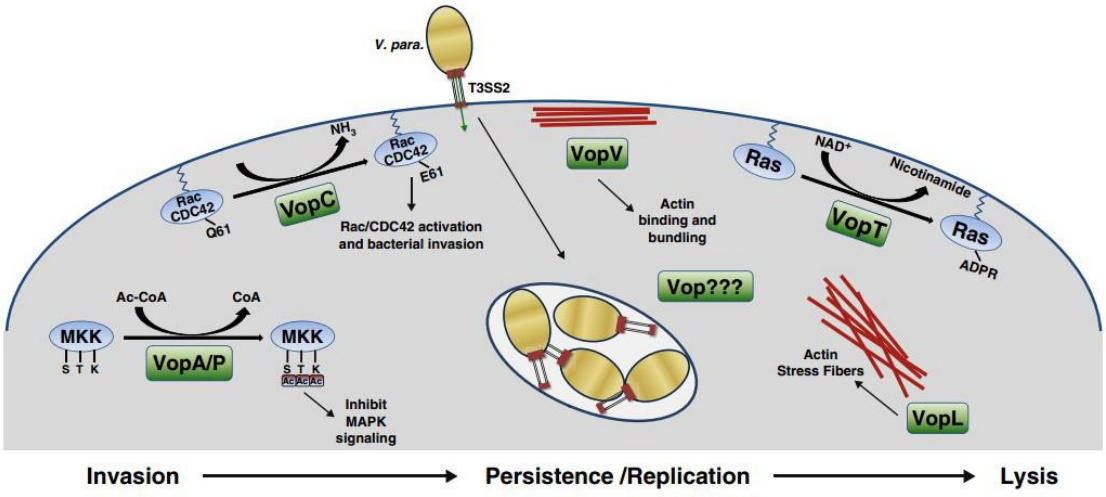


Figure 4 Model depicting the various targets of T3SS2 effectors

(Zhang and Orth, 2013). VopA acetylates residues of MKKs; VopC eamidates Rac and Cdc42; VopL binds directly to actin monomers which induces stress fibers; VopT ADP-ribosylates Ras and VopV binds to F-actin.

Recently, a novel effector, VopZ has also been found critical for the enterotoxicity caused by *V. parahaemolyticus in vivo*. It is shown that VopZ is required for blocking transforming growth factor β-activated kinase (TAK1) activation, diarrhea, tissue damage and intestinal tract colonization. It is suggested that different regions of VopZ contribute to these diverse functions. The internal region (38-62 residues) plays an important role in inhibiting TAK1 signaling, diarrhea and tissue damage (**Fig. 5**). It is intriguing to see that VopZ also manipulates actin cytoskeleton. The same internal region in VopZ contributes to the actin rearrangement. However, it is unclear how VopZ influences the actin cytoskeleton. These results only present a case that VopZ is a multifunctional effector. The cellular target of VopZ is still unclear. The preliminary result suggested that VopZ probably possesses WxxxE motif that causes cell rounding (**Appendix**, unpublished data). Which RhoGTPases are activated and whether WxxxE motif contributes to inhibit TAK1 activity need further study. Another T3SS2 secretion protein, VPA1350, has also been identified by the authors at the same time. This

protein seemed to be a structural protein or a regulator of T3SS2 since the deletion of *vpa1350* gene diminished the secretion of VopV (Zhou et al., 2013a).

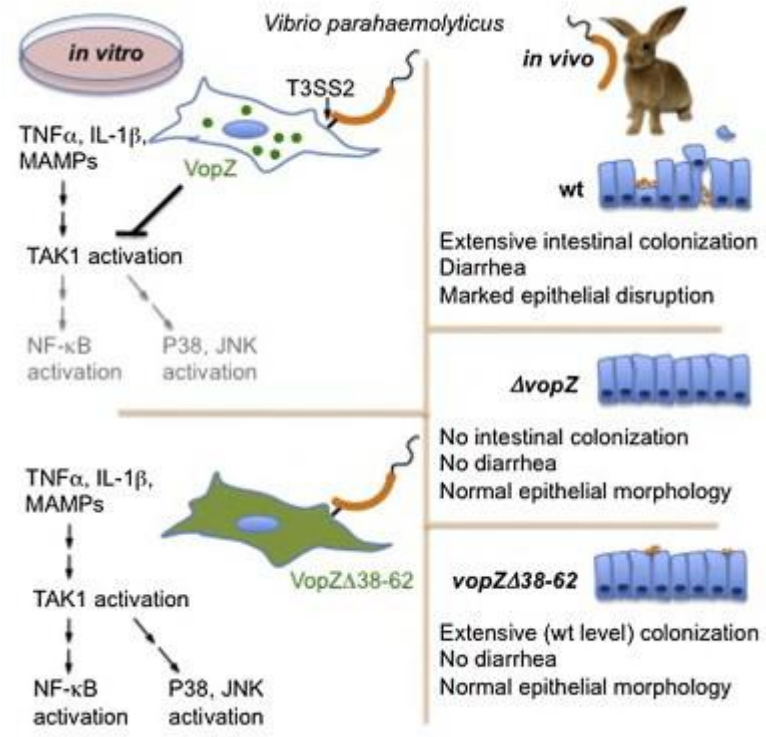


Figure 5 Model depicting the various effects of VopZ (Zhou et el., 2013a). VopZ contributes to blocking the activation of TAK1, diarrhea, tissue damage and colonization. The internal region of VopZ is required for inhibiting TAK1 signaling, diarrhea and tissue damage.

1.5 Fibronectin and fibronectin binding proteins

Mammalian fibronectin (Fn) was found to play an essential role in pathogen-host interaction. Fn is a huge, dimeric glycoprotein (≈440 kDa) usually observed in the plasma, cell surface and the extracellular matrix (ECM). It is also a versatile protein involved in cell attachment, differentiation, migration and wound repairing. Fn is composed of three types of repeats, namely type I, type II and type III repeats. Its N-terminal 30 kDa domain (heparin binding domain, HBD), which is composed of five type I repeats, binds to host cell heparin, fibrils and α5β1 integrin. The 45 KDa domain connected to HBD is a gelatin and collagen binding domain (CBD) (**Fig. 6**) (Grinnell, 1984; Pankov and Yamada, 2002). Due to the abundance and surface localization of Fn, it is not surprising that Gram-negative pathogenic bacteria utilize diverse Fn binding proteins (FnBPs) to facilitate their entry (Henderson et al., 2011).

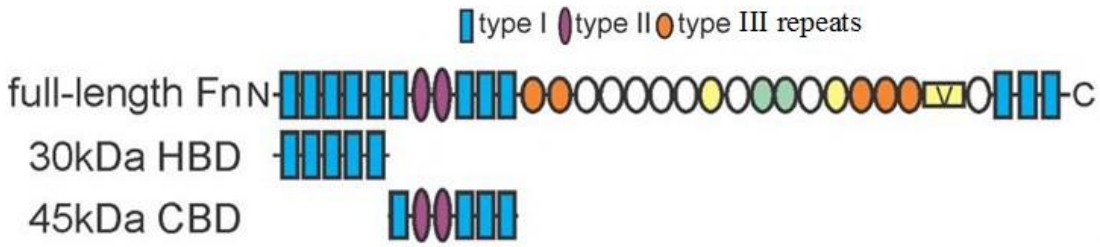


Figure 6 Model depicting the composition of Fn (Krachler and Orth., 2011). Fn is composed of type I, type II and type III repeats; HBD is composed of five type I repeats and is CBD is composed of three type I repeats and two type II repeats.

Different Gram-negative pathogens produce different numbers of FnBPs. For example, pathogenic *E. coli* strains use curli, P-fimbriae, type I fimbriae, aggregative adherence fimbriae, haemorrhagic coli pili, H6/H7 pili, Tsh autotransporter, YadA-like protein UpaG *and etc.* to bind Fn (Erdem et al., 2007; Huang et al., 2006; Kostakioti and Stathopoulos, 2004; Olsen et al., 1989; Sokurenko et al., 1994; Westerlund et al., 1991; Xicohtencatl et al., 2009; Valle et al., 2008). However, only one FnBP, DrsA and YadA were identified in *Haemophilus ducreyi* and *Y. pseudouberculosis*, respectively (Leduc et al., 2008; Tertti et al., 1992). Moreover, FnBPs bind to different regions of Fn. OpaA, a porin from *Neisseria menigitidis*, can only bind to HBD, while type I fimbriae from *E. coli* can interact with both HBD and CBD (Putten et al., 1998; Sokurenko et al., 1992). In addition, the binding to Fn is mediated by

specific regions or motifs of FnBPs. For instance, YadA uses its head region to bind to Fn while in Hap, an autotransporter in *H. influenza* uses its passenger domain to mediate the binding (Fink et al., 2002; Heise and Dersch, 2006). Furthermore, for some FnBPs, the interaction between Fn-FnBP is dependent on the phase of Fn. It is found that YadA and FimH (an FnBP in *E. coli*) only recognize the insoluble form of Fn (Sokurenko et al., 1994; Tertti et al., 1992). On the other hand, for some FnBPs, such as multivalent adhesion molecule 7 (MAM7) from *V. parahaemolyticus* bind to both soluble form and insoluble form of Fn (Krachler et al., 2011). It is probably due to the different residues that soluble and insoluble Fn exposed for FnBPs recognition. Given that some pathogens do not invade the blood plasma where the major soluble Fn is present, it is reasonable to speculate that these pathogens are not able to recognize the soluble form of Fn. Additionally, some FnBPs possess multiple binding specificities. Curli in *E. coli* can bind to lamininin in addition to Fn. Hap in *H. influenza* can recognize laminin and type IV collagen, both of which are components of ECM (Fink et al., 2002; Saldana et al., 2009). It is interesting to note that an FnBP, ComE1 in *Pasteurella multocida* can interact with double stranded DNA and its Fn binding region is the same as its DNA binding region (Mullen et al., 2008).

FnBP in *Vibrio* spp. is rarely reported. The only characterized FnBPs in *Vibrio* spp. are OmpU identified from *V. cholerae* and MAM7 from *V. parahaemolyticus*. OmpU can bind to CBD of Fn. Antibody against OmpU inhibited the attachment of *V. cholerae* to mammalian cells (Sperandio et al., 1995). A similar result was also observed in *V. vulnificus* by other authors (Goo et al., 2006). MAM7 binds to both HBD of Fn and phosphatidic acid, which enables *V. parahaemolyticus* to attach to host cells. This adhesion is also required for this pathogen to infect nematode. Given that pre-treatment with recombinant MAM7 to host cells blocks pathogens infection, MAM7 shows promising potential for vaccine development (Krachler et al., 2011; Krachler and Orth., 2011).

1.6 Horizontal gene transfer contributes to the virulence of *V. parahaemolyticus*

A bacterial pathogenic species is usually a group of diverse strains that inhabit in different environments. These strains usually display different infection abilities, which are highly correlated with their variation in the genomes. The evolutionary forces for the genomic flexibility involve gene gain, gene loss, gene duplication as well as mutations. It is known that horizontal gene transfer (HGT) greatly affects the virulence of bacterium. In *V. parahaemolyticus*, the T3SS2 gene cluster is located within Vp-PAI, implying the acquisition of T3SS2 via HGT of PAI (Okada et al., 2009; Ritchie et al., 2012). A series of recent works support that Vp-PAI is not only transferable among *V. parahaemolyticus* strains but also among *Vibrio* spp. (Caburlotto et al., 2009; Dziejman et al., 2005; Okada et al., 2010). Comparative genomic analysis of pre-pandemic and pandemic *V. parahaemolyticus* strains as well as molecular profiling studies revealed that the organization of mobile gene cassettes and PAIs were divergent in *V. parahaemolyticus* strains and suggested that emergence of pandemic strain is associated with recombination events and acquiring novel genes (Caburlotto et al., 2011; Chen et al., 2011; Garcia et al., 2012; Gavilan et al., 2013; Gennari et al., 2012;

Gonzalez-Escalona et al., 2008; Han et al., 2008; Hurley et al., 2006; Ottaviani et al., 2013; Theethakaew et al., 2013; Turner et al., 2013; Yan et al., 2011). The genetic divergence of this species also indicates that it evolves quickly in response to different pressures in the aquatic environment, which make the bacteria belonging to this species exhibit differential virulence (Caburlotto et al., 2010; Vongxay et al., 2008). Other than T3SS2 genes, other horizontally acquired genes can also contribute to the virulence. For example, a horizontally acquired gene, *vpaH* in *V. parahaemolyticus* TH3996 can significantly enhance its motility, biofilm formation and adherence (Park et al., 2005). This observation has raised considerable interest in investigating novel horizontally acquired virulence factors in *V. parahaemolyticus*.

1.7 The importance of zinc importer to microbes

Zinc is an important component for many proteins (Hantke, 2005). In *E. coli*, zinc ions are imported by two main systems, ZnuACB and ZupT, which are high-affinity and low-affinity zinc acquisition systems, respectively. The growth of *E. coli znuA* and *znuB* defective mutants is failed in Zn-restricted medium (Patzer and Kantke, 1998). For ZnuACB system, ZnuA is responsible for zinc binding, and transportation across the inner membrane with the assistance of ZnuB. ZnuC is an ATPase that provides energy for this process by ATP hydrolysis (Hantke, 2005). The expression of *znuACB* is controlled by Zur, a Fur family repressor. It is the sole repressor of zinc uptake systems in numerous bacteria species except for *Streptococcaceae* (Hantke, 2005; Llull et al., 2011). It recognizes and binds to the promoter region of *znuACB* of which the sequence is conserved to suppress its transcription when zinc is adequate in cytoplasm. On the other hand, Zur can function as a direct activator of the zinc exporter (XcZur) in phytopathogen *Xanthomonas campestris* although the promoter sequences of *znuACB* and the zinc efflux pump are quite different (Huang et al., 2008).

Intracellular pathogens require metal ions, such as zinc, ferric and copper

when they invade, survive and replicate in the host. However, zinc concentration in the host is quite low. Therefore, it is essential for pathogenic bacteria to take up zinc with a much higher efficiency within the host (Hantke, 2005). Most pathogenic bacteria take up zinc using single or multiple zinc transportation systems. For the high-affinity zinc acquisition system, ZnuACB is essential for virulence of *Brucella abortus*, *Campylobacter jejuni*, *Moraxella catarrhalis*, *Salmonella enterica* and *H. ducreyi* (Ammendola et al., 2007; Davis et al., 2009; Lewis et al., 1999; Murphy et al., 2013; Yang et al., 2006), but not for uropathogenic *E. coli*, *Proteus mirabilis* or *Y. pestis* (Desrosiers et al., 2010; Nielubowicz et al., 2010; Sabri et al., 2009). This is due to the possessing of other zinc uptake genes, such as *zupT*, encoding a low-affinity zinc acquisition protein in *E. coli* (Sabri et al., 2009).

1.8 Mechanisms of fluoroquinolone resistance in *V. parahaemolyticus*

Fluoroquinolone resistance in pathogenic bacteria is usually mediated by amino acid substitutions in DNA gyrase and topoisomerase IV genes, excessive expression efflux pumps and quinolone resistance genes on the plasmid. Mutations in *gyrA*, *gyrB*, *parC* and *parE* genes are widely reported in diverse fluoroquinolone resistant pathogens (Hooper, 1999). In fluoroquinolone resistant *Vibrionaceae*, single site mutation in *gyrA* gene is frequently detected. Single site mutation in *parC* gene was also reported in *V. anguillarm* and *V. cholerae*. However, mutations in *parC* gene from other fluoroquinolone resistant *Vibrio* spp. have not been reported so far (Chowdhury et al., 2011; Colquhoun et al., 2007; Kim et al., 2010; Kitiyodom et al., 2010; Singh et al., 2012; Srinivasan et al., 2006; Zhou et al., 2013b). In *V. parahaemolyticus*, single *gyrA* mutation together with single *parC* mutation was detected in laboratory-induced fluoroquinolone-resistant isolates (Okuda et al., 1999). However, mechanisms of fluoroquinolone resistance observed in environmental samples or clinical isolates are poorly characterized so far. This raises a question: is the mechanism for the fluoroquinolone resistance in laboratory-induced strains the same as that in the clinical and

environmental isolates? It was shown that the ciprofloxacin resistant *V. parahaemolyticus* strains isolated from farmed marine shrimps in Thailand only possess one mutation in GyrA (Kitiyodom et al., 2010). Multidrug efflux pumps, such as NorM, have also been associated with fluoroquinolone resistance in *Vibrio* spp. (Singh et al., 2006). Furthermore, Qnr-like quinolone-resistant determinants were also probably derived from *Vibrionaceae* (Poirel et al., 2005). For example, the QnrVC family genes were reported in *V. cholerae* and *qnrA1* gene was detected in *V. fluvialis* (Chowdhury et al., 2011; Fonseca et al., 2008).

Qnr proteins including QnrA, QnrB, QnrC, QnrD and QnrS five families are pentapeptide repeat proteins that contribute to low level quinolone resistance via protecting DNA gyrase and topoisomerase IV genes against quinolones (Vetting et al., 2011). Although *qnr* genes can be found both on chromosomes and plasmids, recent studies suggested that these genes are probably chromosomes origin. It was also suggested that the presence of Qnr proteins promote the emergence of higher level quinolone resistance in bacteria (Strahievitz et al., 2009). In addition to quinolone resistance, Qnr proteins likely contribute to prevent DNA damage. For example, *qnrB* genes are induced by SOS response which is a regulatory network triggered by DNA damage (Wang et al., 2009). *QnrVC* genes are the subfamily of QnrC and are first identified in *V. cholerae* (Fonseca et

al., 2008). Later, it was found that these genes are associated with SXT element or Class I integron (Kumar et al., 2011), implying the transferable ability. In *V. parahaemolyticus*, *qnrVC* gene was not detectable before.

1.9 Aims of study

The pathogenesis of *V. parahaemolyticus* is not clear and more researches are needed to gain a better understanding on the pathogenesis of this organism. Attachment is the most important initial step for infection. Zinc is always required for pathogenesis. There are increasing evidences showing that antibiotic resistance has widely spread among *V. parahaemolyticus*. However, only limited genes have been characterized for the pathogenesis and antimicrobial resistance in *V. parahaemolyticus*. In my research, I aimed to identify and characterize a novel adhesion determinant, as well as new genes associated with zinc transport and antibiotic resistance in *V. parahaemolyticus* to pave a new way for effective vaccine development.

Chapter 2 Materials and methods

2. 1 Bioinformatic analysis

Protein domain search was performed using PFAM (http://pfam.sanger.ac.uk/search). The subcellular location and the transmembrane region were predicated using TMHMM Server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), respectively. Multiple sequence alignments were determined by Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Three-dimensional (3D) structure was predicated using Swiss-model (http://swissmodel.expasy.org/) and the structural alignment was evaluated using TM-align servers (http://zhanglab.ccmb.med.umich.edu/TM-align/). Phylogenetic analysis was performed using MEGA version 5 (http://www.megasoftware.net/) after multiple alignment of the data via CLUSTAL_X. Distances were obtained using options according to Kimura's two-parameter model and clustering was performed by using the neighbour-joining method. The

topology of the neighbour-joining phylogenetic tree was evaluated by using bootstrap resampling with 1000 replications.

2. 2 Bacterial strains, plasmids and growth conditions

V. parahaemolyticus strains, *E. coli* strains and plasmids used in this study were listed in Table 1. *V. parahaemolyticus* strains were cultured in LB medium supplemented with 2.5% sodium chloride (NaCl) at 37℃. Thiosulfate-citrate-bile salts-sucrose agar (TCBS) was used to select *V. parahaemolyticus* strains. *E. coli* strains were grown in LB medium at 37℃. Plasmids pDM4 and pMMB207 were used for gene deletion and complementation experiments, respectively. Chloramphenicol (25μg/ml for *E. coli* and 5μg/ml for *V. parahaemolyticus*), kanamycin (50μg/ml for *E. coli*) and Isopropyl β-D-1-Thiogalactopyranoside (IPTG, 0.1mM for *E. coli* BL21 and 1mM for *V. parahaemolyticus*) were added to the medium if necessary. Zinc depletion was carried out with the specific zinc chelator, *N,N,N',N'*-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN, Sigma) dissolved in ethanol.

2. 3 Construction of deletion and complementary strains

The *vpa1307* gene was deleted from the *V. parahaemolyticus* strain VP3218 by homologous recombination using the suicide vector, pDM4, as described before (Liverman et al., 2007; Zhou et al., 2010). Briefly, the upstream and downstream of the *vpa1307* gene were amplified using primers vpa1307-1F/vpa1307-1R and vpa1307-2F/vpa1307-2R, respectively (Table 2). These two fragments were used as templates for the second round of PCR with primers vpa1307-1F/vpa1307-2R. The purified overlap PCR product was digested and cloned into the same digested suicide vector, pDM4. *E. coli* SY327 *λpir* carrying the recombinant plasmid, the helper plasmid pPK2013 and *V. parahaemolyticus* strain VP3218 were mixed (5:5:1, v/v/v), spun down and resuspended in 100μl LB broth, poured onto a filter on LB agar plate, and incubated overnight. The bacterium on the filter was resuspended, spread on TCBS containing 5μg/ml chloramphenicol to select transconjugants. Randomly selected transconjugants were cultured on LB agar in the presence of 5% sucrose and subjected to repeated serial passages. The knockout mutant, Δ*vpa1307* was obtained. Similar procedures were used to obtain Δ*vadF*, Δ*vcrD1* and Δ*vcrD1*Δ*vadF* using specific primers accordingly (Table 2).

To construct the complementary strain, the *vpa1307* gene with additional ribosome binding site was amplified using primers vpa1307com-F and vpa1307com-R (Zhou et al., 2010) (Table 2). PCR product was digested and cloned into the same digested pMMB207 to create pMMB207:*vpa1307*. This recombinant plasmid was transformed into *E. coli* SY327 *λpir* and then conjugated into Δ*vpa1307* with the presence of helper plasmid pPK2013 carrying *E. coli* SY327 *λpir*. Transconjugants were selected on TCBS containing 5μg/ml chloramphenicol and the strain Δ*vpa1307*::p*vpa1307* was obtained. Similar procedures were used to obtain other complementary strains, such as Δ*vcrD1*Δ*vadF*::p*vadF* and Δ*vcrD1*Δ*vadF*::p*vadFD* using specific primers accordingly (Table 2).

Site-directed mutagenesis was generated using GENEART® Site-Directed Mutagenesis kit (Invitrogen Co., NY, US) with primer pairs H69A-F/H69A-R and H148A-F/H148A-R, respectively (Table 2). Plasmid pMMB207:*vpa1307* was used as template. Successful mutations were confirmed by sequencing. Δ*vpa1307*::p*vpa1307* H69A H148A was obtained using the method described above.

2. 4 Expression and purification of recombinant proteins

Three truncated derivatives of *vadF* were amplified by PCR using primers listed in Table 2. The PCR products were digested, purified and subsequently cloned into the pET28D3 vector. Clones were confirmed by sequencing. *E. coli* BL21 (DE3) was used to overexpress histidine-tagged recombinant proteins, rVadFA, rVadFB and rVadFC at 37 ℃, 16 ℃ and 16 ℃, respectively. Proteins were purified using nickel-nitriotriacetic acid beads (Invitrogen) as previously described (Baldwin et al., 2004) and dialyzed into PBS buffer. The purified proteins were examined by SDS–PAGE analysis and their concentrations were determined by comparison with BSA standard kit (Amresco).

2. 5 Solid phase binding assay and pull down assay

One hundred μl of Fn (F2006, Sigma), HBD (F9911, Sigma) and GBD (F0162, 45-kDa, Sigma) at a concentration of 5μg/ml in 50mM Na_2CO_3, pH 9.6 were coated onto ELISA plates (IWAKI) at 4 ℃ overnight, respectively. After three washes with PBS, 1% (w/v) BSA in 50mM Na_2CO_3 (pH 9.6) was added as blocking agent (1h, room temperature). The ELISA plate was washed three times and 3xFlag-tagged recombinant VadF fragment proteins were added (1h, room temperature). After triple washes, 100μl mouse α-3x Flag-HRP antibody (diluted 1:10000, Pierce) was added and incubated at room temperature for 1h. Next, 100μl Ultra

TMB (Pierce) was added to each washed well for developing (5 min, room temperature). After quenching with 100μl H_2SO_4 (1M), absorbance was read at 450 nm. BSA was coated simultaneous as the negative control.

For the pull-down experiment, the Anti-flag M2 affinity gel (Sigma) was used according to the manufacturer's instruction.

2. 6 Cytotoxicity and cell attachment assays

HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% newborn calf serum (Invitrogen) at 37℃. HeLa cells were plated at a density of 3~6 X 10^5 per well in 12-well tissue culture plates one day before infection. 2h before infection, cells were washed several times with PBS before adding fresh DMEM (without antibiotics or serum). Overnight *V. parahaemolyticus* strains were 1:100 diluted to fresh LB broth and grown at 37℃ for 4h. Then cultures were collected, washed and resuspended in DMEM (without serum) and used to infect HeLa cells at a multiplicity of infection (MOI) of 50 cfu per cell. Plates were centrifuged at 1000rpm for 5min to facilitate bacterium attaching to monolayers. Supernatants were

collected at specific time points and the amounts of LDH released were determined using CytoTox 96 Non-Radioactive Cytotoxicity kit (Promega) following the manufacturer's instruction.

For attachment assay, similar conditions were used as mentioned above except that serum were present in DMEM (10%, v/v) and after 1h infection, cells were washed three times with PBS, lysed with 1% Triton X-100 at 37℃ for 20 min. The cell lysates were serially diluted and plated on LB agar to get the number of bound bacterial cells. Similar procedure was used to get the number of total bacterial load. Attachment rate was calculated by dividing bound bacteria to the total bacterial load.

2. 7 Growth assay and RT-PCR

Overnight *V. parahaemolyticus* strains were first cultured in LB broth to the exponential growth phase (OD600≈0.6-0.7). Then cells were 1:100 diluted with fresh LB broth with or without 35μM TPEN (less than 30μM TPEN has limited role in inhibition the growth of *V. parahaemolyticus*), respectively and grown at 37℃ with shaking (250rpm). OD600 was monitored at specific time points. Similar procedure was used in relative growth assay, except that OD600 was only monitored at 6h and 1mM

IPTG plus 5μg/ml chloramphenicol was added for complementary strains. Relative growth ratio was calculated as culture grown with TPEN to that of grown without TPEN.

To determine whether *vpa1307* was expressed in zinc limitation condition, 35μM TPEN was added to wild type log-phase *V. parahaemolyticus* culture. After induction for 30 min, 1ml culture was collected and used to extract RNA using Trizol (Invitrogen) following the manufacturer's instruction. DNA was removed from the sample with DNase (Turbo DNase, Ambion) according to the manufacturer's instruction. 0.5μg RNA was used as template using Superscript one-step RT-PCR system (Invitrogen). No TPEN added culture was used as negative control. Primers rtvpa1307-F/ rtvpa1307-R and rt16S-F/ rt16S-R were used, respectively (Table 2). Primers vpa1307-F and vpa1307-R (Table 2) were used for screening the distribution of *vpa1307* genes in *V. parahaemolyticus* clinical isolates. The *tdh* and *trh* genes were also screened.

2. 8 Murine Infection assay

Overnight *V. parahaemolyticus* strains were 1:100 diluted to fresh LB broth and grown at 37℃ for 4h. Then cultures were collected, washed and resuspended in PBS. *V. parahaemolyticus* strains (10^8 CFU) were intraperitoneally injected into 6- to 10-week-old C57BL/6 mice (n=10) as described previously (Hiyoshi et al., 2010; Pineyro et al., 2010; Whitaker et al., 2012) and mice that were alive were measured at the indicated time points. Three independent replicate experiments were performed. The animal experiments were conducted in the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention (CDC) following the guidelines and policies approved by Chinese CDC.

2.9 Isolation *V. parahaemolyticus* strains from shrimps

From January to April 2010, raw shrimp samples were obtained to isolate *V. parahaemolyticus* strains used in Chapter 5. These samples were from different markets in four different locations, namely Hong Kong Island, Hung Hom, Tsuen Wan, and Sai Kung, while *V. parahaemolyticus* strain v1 used in Chapter 6 was isolated from the shrimp purchased in Sai Kung in Hong Kong in 2011. Isolation was performed as described (Wong et al., 2012). After overnight enrichment in Alkaline Peptone Water, a loopful of inoculant was streaked and incubated on TCBS agar for 24h at 37℃.

Two typical blue-green colonies were re-streaked, purified and confirmed to be *V. parahaemolyticus* by PCR assay targeting to the *tl* gene (Wong et al., 2012).

2.10 Antimicrobial susceptibility testing

Susceptibility of *V. parahaemolyticus* to twelve antibiotics (ampicillin, cefotaxime, ceftazidime, meropenem, nalidixic acid, ciprofloxacin, norfloxacin, levofloxacin, gentamicin, chloramphenicol, tetracycline and streptomycin) was tested by agar dilution method as previously described (Wong et al., 2012). *E. coli* ATCC 25922, 35218, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 were used as quality control. For efflux pump inhibitor assay, the MICs were determined using micro-dilution method with the addition of 30 μg/ml phenylalanine arginine β-naphthylamide (PAβN) in the antibiotics. One *Salmonella enteritidis* isolate with known contribution of efflux on its fluoroquinolone resistance was used as positive control.

2.11 Molecular typing

PFGE characterization was conducted as previously described (Wong et al., 2012). Briefly, 50U of *XbaI* (New England Bio-Lab) was used to digest the agarose-embedded DNA in a water bath (4h, 37℃). A Chef Mapper electrophoresis system (Bio-Rad, Hercules, CA) was used to separate the digested fragments with pulse times of 2.16 to 63.8 s (18h, 14℃). Phage Lambda PFGE ladder (New England Biolab) was used as DNA size marker. Clonal relatedness was analyzed by Bionumerics with the use of Dice coefficient and a parameter of 0.5% for optimization and band matching tolerance.

S1-PFGE was conducted to determine the size of large plasmids. Briefly, S1 nuclease (New England Bio-Lab) was added for digesting the agarose-embedded DNA (1h, 37℃). The procedure to separate restriction fragments was the same as described above. Phage Lambda PFGE ladder (New England Biolab) was used as DNA size marker.

2.12 Quinolone resistance determine region (QRDR) mutation determination and plasmid-mediated quinolone resistance (PMQR) screening

The QRDR of *gyrA* and *parC* was amplified by PCR using primer sets (Table 3). The deductive amino acid sequence was obtained and

compared to those of wild type strain to identify the mutations. For PMQR genes, PCR assays were also used to determine the absence/presence of *qnrA, qnrB, qnrC, qnrD, qnrS, qepA, aac(6')Ib-cr* and *oqxAB* as described previously (Chen et al., 2007; Wong et al., 2013). The presence of ESBLs was determined using PCR assay targeting most of the reported β-lactamases genes as previously described (Dallenne et al., 2010). *QnrVC* genes and Class I integron were screened using primers listed in Table 3.

2.13 Conjugation

Conjugation assays were conducted as described (Wong et al., 2012) with necessary modifications. Briefly, the mixture of donor and recipient strains (5:1, v/v) was spun down and resuspended in 100μl LB broth, poured onto a filter on an LB agar plate, and incubated overnight. The bacterium on the filter was resuspended in 100μl LB broth, spread on LB plates containing ciprofloxacin (0.05, 0.1, 0.25 and 0.5μg/ml) and sodium azide (100 μg/ml) to select transconjugants.

2.14 Southern hybridization

Southern hybridization was performed using DIG High Prime DNA Labeling and Detection Starter Kit I (Roche) following the manufacturer's instructions. Briefly, chromosomal and plasmid DNA from *V. parahaemolyticus* were isolated using commercial isolation kits from Qiagen and denatured (2min boiling, 100℃) and placed on ice immediately for 5min before loading on nitrocellulose membrane. The *qnrVC5* probe was prepared by PCR amplification using primers qnrVC5 cloning F/R (table 3). 0.5μg purified PCR product was labeled using reagents from the same kit. After cross-linking, the membrane was subjected to Southern hybridization using DIG labeled *qnrVC5* probe. Positive control and negative control was PCR product of *qnrVC5* and PBS, respectively.

2.15 Plasmid sequencing and primer walking

The plasmid sequencing was performed using known plasmid pVN84 (AB200915) as template. Since the region of *qnrVC5* on the plasmid is from 2468-3127, primers were designed to amplify two other regions of the plasmid, regions (3111-7126) and region (6926-2468) using primers as shown in Table 3. After obtaining the two regions, different primers were then designed to sequence the whole sequence of these two PCR fragments (Table 3). The PCR products were sequenced and walked

toward the middle region. Region (3111-7126) was completed after 4 two-end sequences and region (6926-2468) was completed after 2 two-end sequences.

Genetic environment of resistant determinants in strain V1 was determined by primer walking approach using plasmid from transconjugant as template. Primer walking was done by step wise two-end sequencing of the plasmid outward. Sequencing service was done in Beijing Genome Institute (BGI), Shenzhen. Each sequencing step achieved about ~1.5kb reading on the plasmid. The primers were designed using the sequence obtained from the previous sequencing.

2.16 Functional test of novel *qnrVC* alleles

After decoding the sequence of the surrounding regions of *qnrVC* genes, the whole coding regions of *qnrVC5, qnrVC6* genes amplified using primers as shown in Table 3, respectively. The PCR products were purified and subsequently cloned into pCR2.1 vector (Invitrogen) as described previously (Dorter et al., 2012; Hornsey et al., 2011). The clones were confirmed by sequencing the whole region to make sure that no additional mutation was introduced into the clone. The mutation of

qnrVC was generated using GENEART® Site-Directed Mutagenesis kit (Invitrogen Co., NY, US) using primer set in Table 3. The MICs to fluoroquinolones of *E. coli* carrying novel *qnrVC* genes and derivatives were determined and compared to those of host *E. coli* strain.

Table 1. Bacteria and plasmid used in Chapters 3 and 4

Strain or plasmid	Description	Reference or source
E.coli		
SY327 λ*pir*	Δ(*lac-pro*) *argE*(Am) *rif malA recA56* λ*pir*	(Miller and Mekalanos, 1988)
DH5α	*recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1 lacZΔM15*	Lab collection
TG1	K-12 Δ(*lac-pro*) *supE thi hsdD5*/F′*traD36 proA*$^+$ *B*$^+$*lacI* ^{q} *lacZ*ΔM15	Lab collection
BL21 (DE3)	F$^-$ *ompT hsdS gal*	Lab collection
V. parahaemolyticus		
vp3218	clinical isolate, *tdh*$^+$, *t3ss1*$^+$, *t3ss2*$^+$	Dr. Edward Chan
Δ*vpa1307*	*vpa1307* gene deletion mutant	This study
Δ*vadF*	*vp1767* gene deletion mutant	This study
Δ*vcrD1*Δ*vadF*	*vp1662* and *vp1767* genes deletion mutant	This Study
Δ*vcrD1*Δ*vadF*::p*vadF*	Δ*vcrD1*Δ*vadF* complemented with *vp1767*gene	This Study
Δ*vcrD1*Δ*vadF*::p*vadFD*	Δ*vcrD1*Δ*vadF* complemented with *vp1767*gene's D fragment	This Study
Δ*vpa1307*::p*vpa1307*	Δ*vpa1307* complemented with *vpa1307*gene	This Study

Plasmids		
pDM4	Cm^r; suicide vector with an R6K origin and *sacBR* genes from *Bacillus subtilis*	(Zhou et al., 2010)
pMMB207	RSF1010 derivative, *IncQ lacI*^{q} Cm^r P*tac oriT*	(Zhou et al., 2010)
pPK2013	Km^r Tra^+ Mob^+, ColE1 replicon	(Liverman et al., 2007)
pET28D3	Km^r, 6xHistidine tag, 3xFlag tag	Dr. Joseph Barbieri

Table 2. Primers used in Chapters 3 and 4

Primer Name	Sequence or reference
Knock out *vp1767*	
vp1767-1F	CCGCTCGAGCGAATTGAGCACTTCCCATT
vp1767-1R	TTACTTTCACTAACTTTCAATCAAACTTTTATTATTAGAC
vp1767-2F	GTCTAATAATAAAAGTTTGATTGAAAGTTAGTGAAAGTAA
vp1767-2R	GCTCTAGAAACTGCCTGAGCTCGTTGTT
Knock out *vp1662*	(Zhou et al., 2009)

Knock out *vpa1307*	
vpa1307-1F	CCGCTCGAGGAGGGTTCTGACGTTGGTGT
vpa1307-1R	GTGTATTCTGTCATGATCAATTAGAACGCATGAGCACCGT
vpa1307-2F	ACGGTGCTCATGCGTTCTAATTGATCATGACAGAATACAC
vpa1307-2R	CGAGCTCACGCAAAAAGCACCATTACC
Complement *vp1767*	
vp1767com-F	CGAGCTCTAAGGAGGTAGGATAATAATGTTTGACTCTATGATA
vp1767com-R	CGGGATCCTTAGTGATGATGATGATGATGCTTAAGAGGAACGCCAG
comfragmentD-R	GCTCTAGATTAGTTATAAAAGCCGATGA
Complement *vpa1307*	
vpa1307com-F	CGAGCTCTAAGGAGGTAGGATAATATTGGGGCGCACGGTGCTC
vpa1307com-R	CGGGATCCTCAAAACTTCACAGCGCT
Expression VP1767	
VadFC-F	CGAGCTCATGCCACCAATGAGCTTGCCAG
VadFC-R	CGGGATCCTTACTTAAGAGGAACGCCA

VadFA-R	CGGGATCCTTAGTTATAAAAGCCGATGA
VadFB-F	CGAGCTCATGGTGTCTCGTATTGCGCTA
VadFB-R	CGGGATCCTTACTTAAGAGGAACGCC
Screening *vpa1307*	
vpa1307-F	TTGGGGCGCACGGTGCTCAT
vpa1307-R	TCAAAACTTCACAGCGCT
RT-PCR	
rtvpa1307-F	TACGCTGCCAGTTTTGTACG
rtvpa1307-R	GATCCGCAACTTGAACCATT
rt16S-F	GGAAGGTAGTGTAGTTAATAGC
rt16S-R	GATGTCAAGAGTAGGTAAGGT
Site-directed mutagenesis	
H69A-F	CCGATAAACAAGATCCAGCTTACGTGCAAGCTCGCC

H69A-R	GGCGAGCTTGCACGTAAGCTGGATCTTGTTTATCGG
H148A-F	GCGCATGGTAATCCGGCCGTGCAGTTTGCGG
H148A-R	CCGCAAACTGCACGGCCGGATTACCATGCGC

Table 3. Primers used in Chapters 5 and 6

Primer Name	Sequence		Primer sources
QRDR			
gyrA	F-AACGTACTAGGCAACGATTGG	R-AGAGTCGCCATCGATTGAGCCAAA	This study
parC	F-GTCGGAGCTTGGTCTTTCGGC	R-GTCGCCATACCAACCGCGATACC	This study
qnrVC detection	F-AATTTTAAGCGCTCAAACCTCCG	R-TCCTGTTGCCACGAGCATATTTT	This study
qnrVC5 cloning	F-ATGGATAAAACAGACCA	R-TTAGTCAGGAACTACTA	This study
qnrVC6 cloning	F-ATGGAAAAATCAAAGCAATT	R-TTAGTCAGGAACAATGAT	This study
Class I Integron	F-GGCATCCAAGCAGCAAGC	R-AAGCAGACTTGACCTGAT	(Kumar et al., 2011)

ICE integrase	F-GCTGGATAGGTTAAGGGCGG	R-CTCTATGGGCACTGTCCACATTG	(Xia et al., 2010)
Plasmid amplification			
Region (3111-7126)	F- TTATCTTGGGCTAGTGATGCG	R-TACCTATTTTGTCTGGTCAAT	This study
Region (6929-2468)	F- TTAATAGTAGTTCCTGACTAA	R-TATATAAAAGCTTATTAAAGC	This study
Plasmid sequencing			
Region (3111-7126) 1	F- TTATCTTGGGCTAGTGATGCG	R-TACCTATTTTGTCTGGTCAAT	This study
Region (3111-7126) 2	DF1-TGATCCAGTAGCAGCGACGG	DR1-AATACCGCCAATGCACACCT	This study
Region (3111-7126) 3	DF2-AAACTATTGAAAACTCT	DR2-GTAAGGTGAAAGAAAACATCA	This study
Region (3111-7126) 4	-	DR3-TCTTAATCGATTTTTTTGCT	This study
Region (6929-2468) 1	F-TTAATAGTAGTTCCTGACTAA	R-TATATAAAAGCTTATTAAAGC	This study
Region (6929-2468) 2	UF1-GGCGGTATGCGGAAGCT	UR1-GATTGTTTTATCGAAAGAA	This study
QnrVC site mutation	F- CTTCGAGATGCTTCATTTAAAAATTGTCAGCTTTCA		This study

R- TGAAAGCTGACAATTTTTAAATGAAGCATCTCGAAG

Chapter 3 VadF, a novel fibronectin binding adhesion produced by *V. parahaemolyticus* is essential for the pathogenesis

3.1 Introduction

Adherence to mammalian cells is the most crucial step in bacterial infection. Initial tight adhesion to the surface of host cells is the prerequisite for the subsequent effective effector protein translocation. Bacterial adherence can also subvert host actin cytoskeleton or trigger cellular signaling pathways to recruit downstream signaling proteins to the plasma membrane to facilitate subsequent pathogen invasion. Furthermore, tight attachment ensures pathogens persisting in the host niches (Carabeo, 2011; Kline et al., 2009). However, the mechanism for the cell attachment by *V. parahaemolyticus* remains largely unknown.

Besides MAM7, other FnBPs in *V. parahaemolyticus* are rarely reported. Given that pathogens usually express numerous FnBPs during infection (Henderson et al., 2011), I hypothesize that *V. parahaemolyticus* also utilizes other FnBPs in initial infection. In this study, a novel FnBP (VP1767), referred to as VadF (*V. parahaemolyticus* adhesion to fibronectin), was identified and characterized.

3.2 Results

3.2.1 Domain organization of VadF and purification recombinant proteins

In silico analysis showed that VadF is likely to be an outer membrane protein. The N-terminal region of this protein consists of a transmembrane domain followed by a fibronectin attachment protein (FAP) homologue domain (VadFA), five bacterial immunoglobulin-like group 2 domains (BIG 2) and one unknown function domain (DUF1566) (Figure 1). Multiple sequence alignment showed that VadFA is also an alanine and proline-domain that is similar to FAPs from *Mycobacterium* spp. (Fig. 2). It was shown that in *Mycobacterium* spp. FAPs are the prevalent, alanine and proline-rich proteins, which bind to Fn (Schorey et al., 1996). Thus, VadF is probably a novel FnBP.

Given the difficulty to purify the full length VadF (due to its insolubility); three truncated peptides (VadFA, VadFB and VadFC) were designed to investigate the biochemical functions of VadF (Figs. 1 and 3).

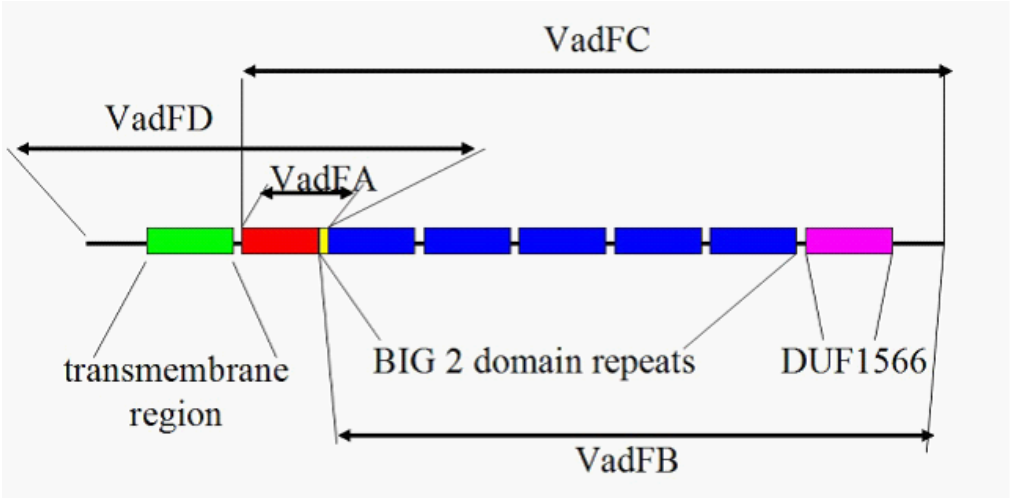


Figure 1 Schematic representation of VadF domains. The transmembrane domain, FAP homologue domain, BIG2 domain repeats and DUF1566 are represented by green, red, blue and pink color boxes (residues 14-31, 37-125, 98-522 and 625-690), respectively. The yellow part indicates the overlapped region of VadFA and the first BIG 2 domain.

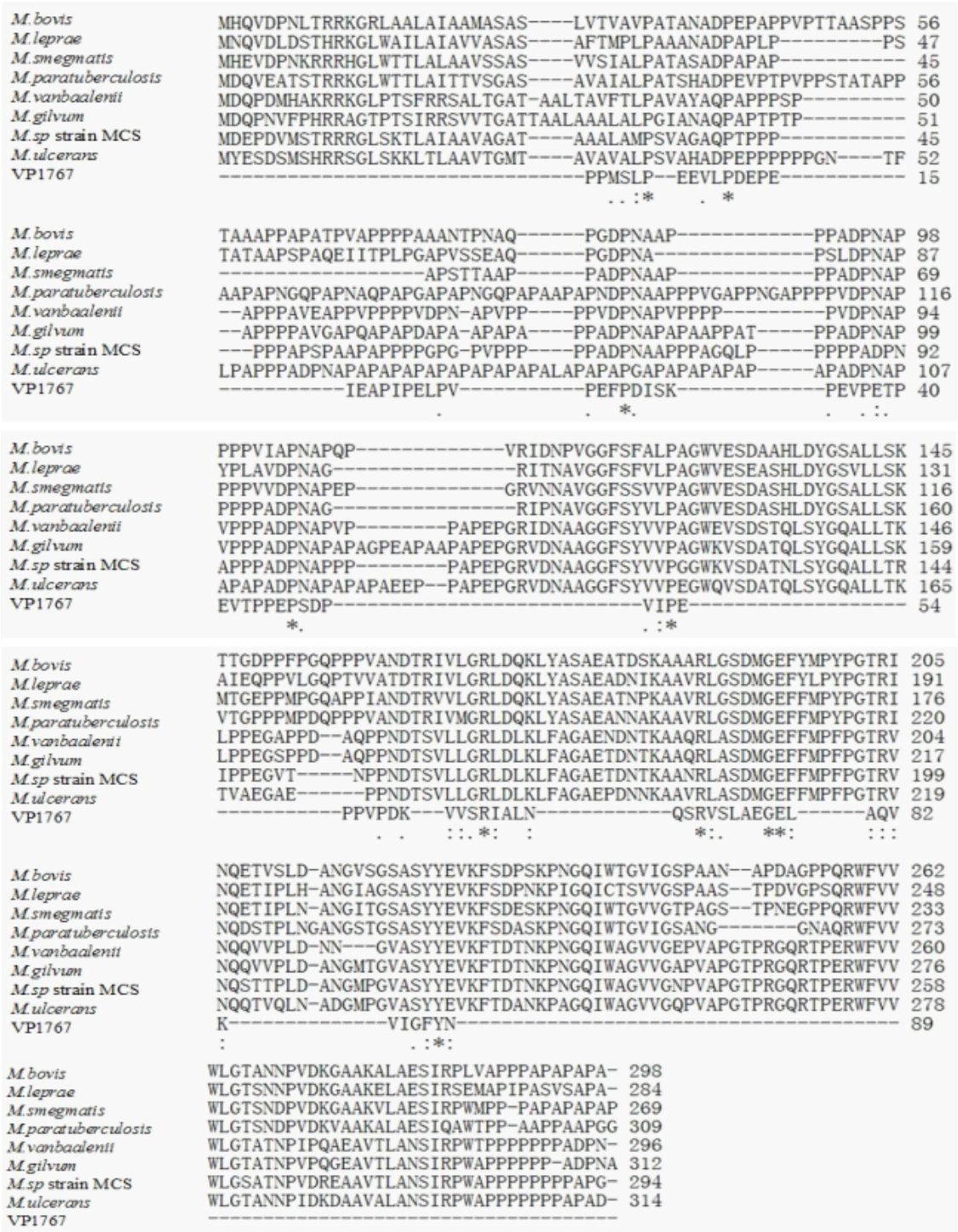


Figure 2 Multiple sequence alignment of VadFA with selected FAPs.

The amino acid sequences of FAP domains (GenBank accession No. P46842, O30620, A0PSE9, Q9F4H9, A0QYD3, Q1B881, A4TAE4 and A1T9M7 from *Mycobacterium* spp. and VP1767 from *V. parahaemolyticus*) were aligned using the CLUSTAL W2.

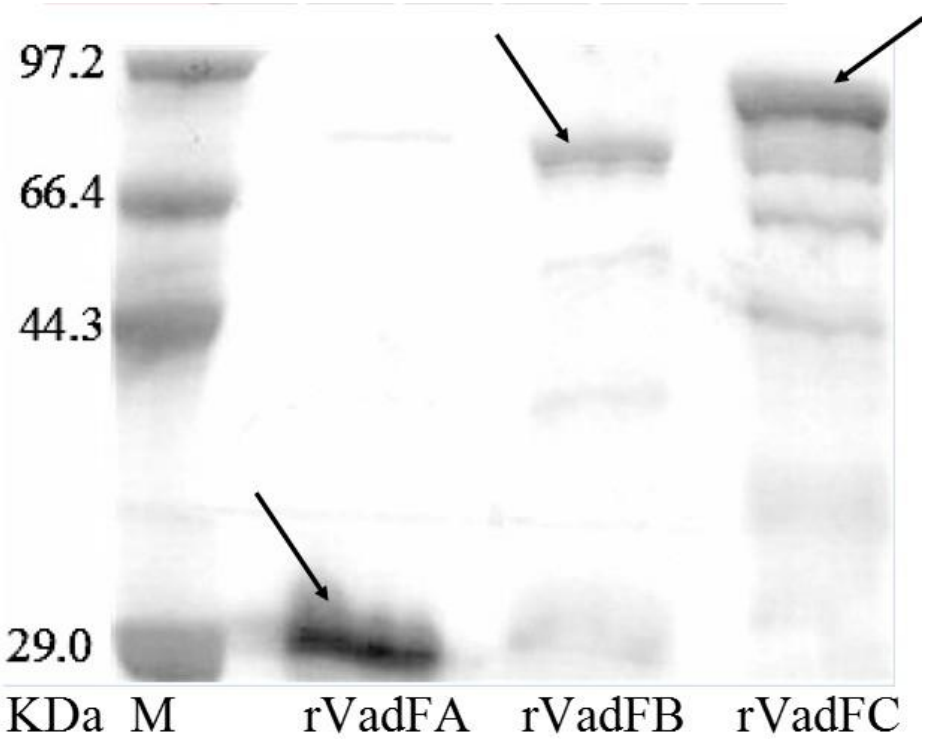


Figure 3 Purified recombinant proteins. Different fragments of VadF were expressed, purified and resolved in SDS-PAGE as indicated as arrows. M, molecular weight marker; Lane 1, rVadFA (~29 KDa); Lane 2, rVadFB (~90 KDa); Lane 3 rVadFC (~95 KDa).

3.2.2 VadF is essential for *V. parahaemolyticus* to bind to HeLa cells

To assess the role of VadF on *Vibrio parahaemolyticus* attachment to HeLa cells, *VadF* deletion mutant was constructed and tested for its binding to HeLa cells. Since wild type (WT) *V. parahaemolyticus* caused cell lysis quickly after infection, a *vcrD1* deletion (T3SS1 deficiency)

background *V. parahaemolyticus* strain, which dramatically delayed cell lysis process, was used (Burdette et al., 2008). The deletion of *vadF* in Δ*vcrD1* background led to Δ*vcrD1*Δ*vadF* dramatically decreasing (about 10-fold) the binding to HeLa cells compared to Δ*vcrD1* strain. The loss of binding to HeLa cells due to *vadF* deletion could be complemented by introducing plasmid carrying *vadF* gene. The complementary strain, Δ*vadF*Δ*vcrD1::pvadF*, showed a more than 2-fold increase of binding to HeLa cells (Fig 4). These results confirmed that VadF is an important adhesion that contributes to *V. parahaemolyticus* attachment to HeLa cells.

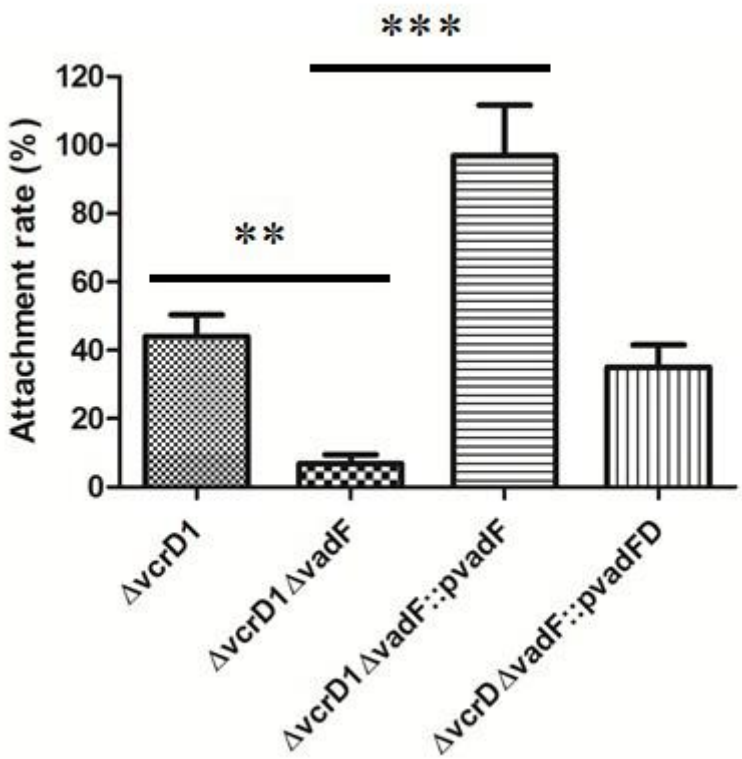


Figure 4 Attachment rate of *V. parahaemolyticus* strains to HeLa cells. Attachment rate was calculated by dividing bound bacteria to the total bacterial load. The data represent three independent experiments ± the SD. Statistical comparisons were performed with a one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests. **, $p<0.01$; ***, $p<0.001$

3.2.3 VadF is the FnBP that specifically binds to Fn

To identify the cell receptor for VadF, *in vitro* binding assay was performed. Fn was commonly used by bacteria including *V. parahaemolyticus* as adhesion receptor. In addition, previous bioinformatics analysis also has identified an Fn binding domain in VadF. The specific interaction between VadF and Fn was tested. Due to the insolubility of recombinant full length VadF, different fragments of VadF were used. Figure 4 showed that rVadFC was able to interact with Fn in a pronounced dose-dependent manner and reached a saturated status. To evaluate the region of rVadFC that contributed to its binding property, rVadFA and rVadFB fragments were tested for their binding ability to Fn. It was found that rVadFA was able to bind to Fn with less affinity than rVadFC, while rVadFB was not able to bind to Fn (Fig. 4). These results confirmed that VadF is an FnBP, whose binding is largely dependent on

its N-terminal region A, while the presence of region B could greatly enhance the binding efficiency.

To further demonstrate which domains of Fn were bound by rVadFA/rVadFC, the binding affinity of rVadFA/rVadFC to immobilized HBD and CBD was examined. As shown in Figure 5, both HBD and CBD were able to be bound by rVadFA/rVadFC. This indicated that both HBD and CBD were the targets of VadF. The binding affinity of VadF to HBD of Fn was slightly higher than that CBD of Fn.

It has been known that the some FnBPs only recognize immobilized Fn (Henderson et al., 2011). To investigate whether VadF's binding to Fn is dependent on the phase of Fn, the pull down assay was performed and it showed that rVadFC was not able to bind to soluble form of Fn (Fig. 6).

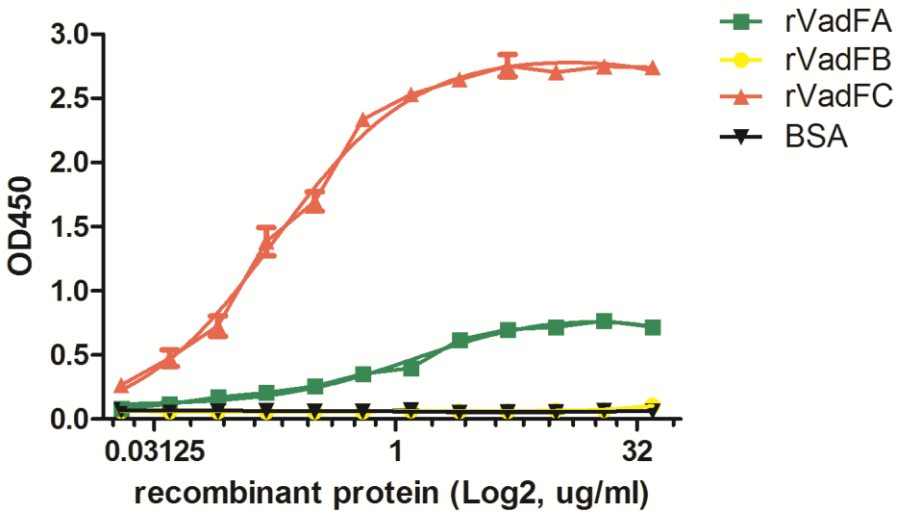


Figure 5 Binding activity of rVadFs with immobilized Fn. Different concentration of recombinant proteins (from 0.0195 to 40μg/ml) were added to each well. BSA was used as negative control. The data represent three independent experiments ± the SE

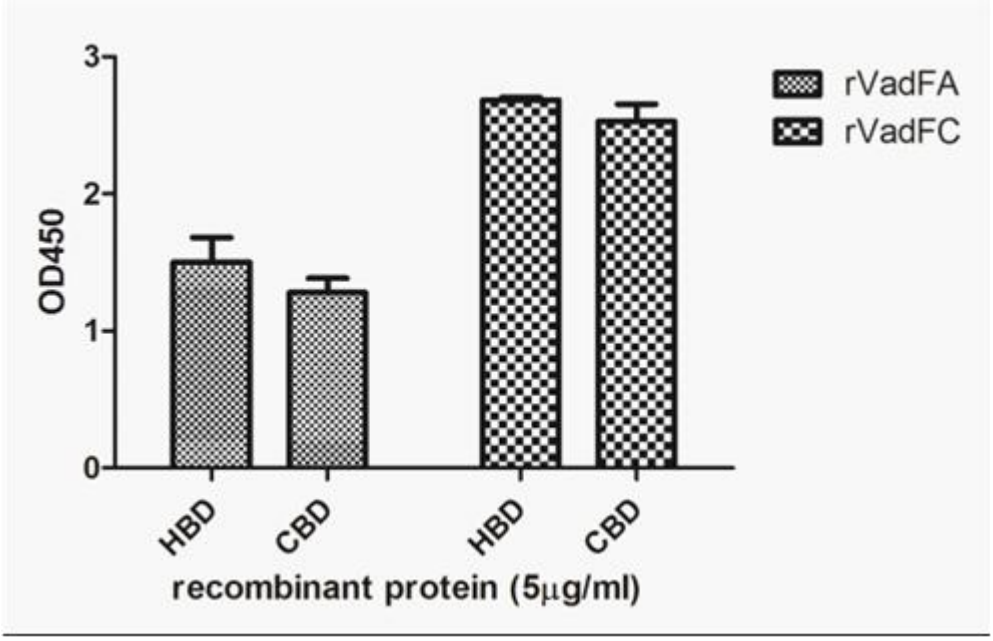


Figure 6 Interaction of rVadFs with HBD and CBD. rVadFA and rVadFC were added to each coated well, respectively. The data represent three independent experiments ± the SE

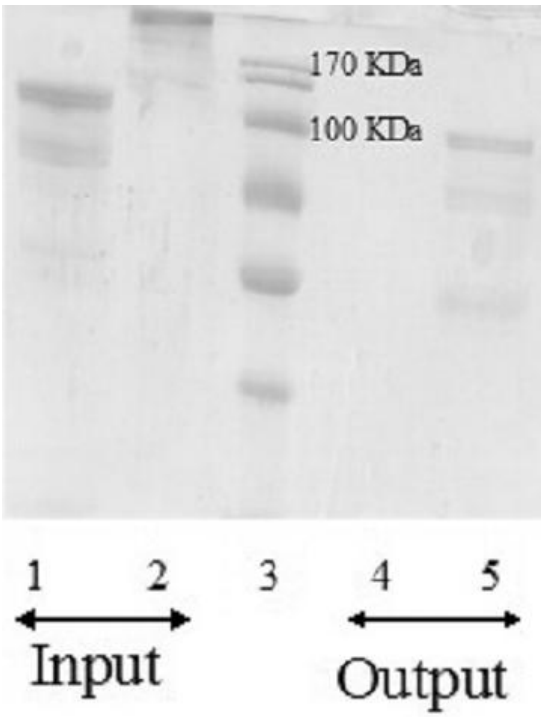


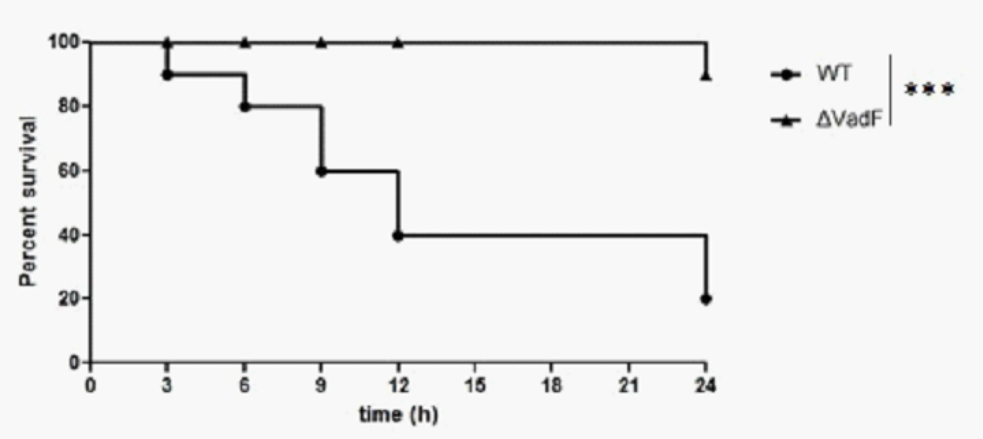
Figure 7 FLAG pull-down assay. Samples from rVadFC and Fn before addition to beads (Lanes 1 and 2, respectively, Input) and eluted from beads (Lanes 4 and 5, Output). For Lane 4, only Fn was added to beads as negative control. Lane 3 is the molecular weight marker. The SDS-PAGE gel represents two separate experiments

3.2.4 VadF is required for *V. parahaemolyticus* to colonize in mice

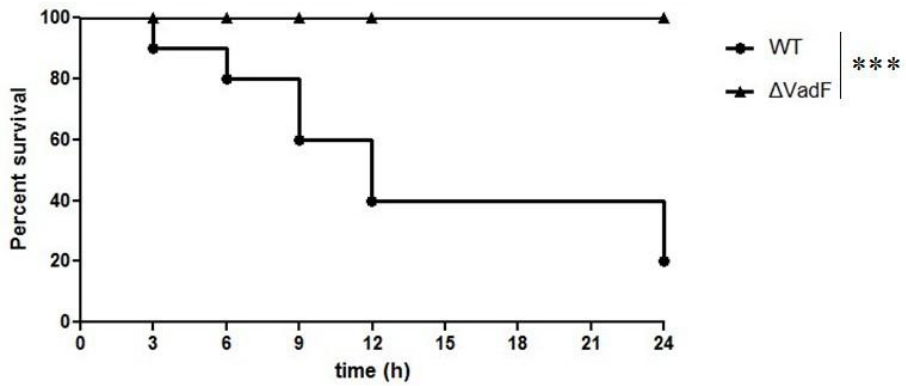
To determine the contribution of VadF to the pathogenicity of *V. parahaemolyticus*, the lethal effects of WT and its *vadF* deletion mutant on mice were evaluated in triplicates as described previously (Hiyoshi et

al., 2010; Pineyro et al., 2010; Whitaker et al., 2012). The Δ*vadF* strain was dramatically attenuated and caused less than 10% of death at 24h postinfection and the mortality rate was maintained the same after 96h of experiments, while WT *V. parahaemolyticus* strain caused death as early as 3h after infection and resulted in 80% of death in mice at 24h after infection (Fig. 8).

A



B



C

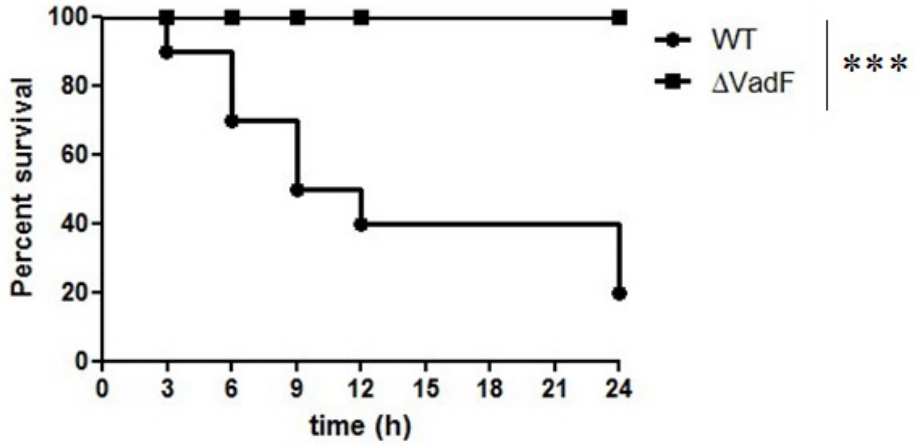


Figure 8 Survival rates of murine infected with *V. parahaemolyticus* strains. C57BL/6 mice (n=10) were infected intraperitoneally with WT or Δ*vadF* strains (10^8 CFU). Three independent replicate experiments, A, B and C, were performed. Kaplan-Meier and log rank tests were used to analyze the data (***, $P < 0.001$)

3.3 Discussion

The interactivity of pathogenic bacteria to intestine epithelial cells, resulting in their colonization, is a critical step for bacterial infection. The attachment of pathogens to eukaryotic cells can not only initiate the secretion of virulence proteins but also activate eukaryotic cellular signaling pathways and/or modulate the actin cytoskeleton to promote their entry (Carabeo, 2011; Kline et al., 2009). It was shown that Fn exposed on the intestinal epithelial cells is a primary target for many infectious pathogens (Henderson et al., 2011). Many pathogenic bacteria produce diverse FnBPs to mediate the entry process. For example, more than 10 different adhesion molecules from *E. coli* strains are capable of binding to Fn, causing the attachment of *E. coli* to hosts (Henderson et al., 2011). To date, only MAM7 has been described as an FnBP in *V. parahaemolyticus* and there may be more adhesion proteins in *V. parahaemolyticus* to facilitate the bacterial adherence to host cells. Bioinformatic approach was used to search the potential FnBPs of *V. parahaemolyticus* and a protein, encoded by *vp1767* was found. Although the N-terminal region of the protein shows low homology (E-value 0.0086, PFAM) to FAPs from *Mycobacterium* spp, it possesses multiple alanine and proline residues, a characteristic of FAPs (Schorey et al.,

1996). We hypothesized that VP1767 might be an FnBP in *V. parahaemolyticus*.

Gene deletion of *vadF* confirmed the significant role of VadF in bacterial attachment to HeLa cells. Further biochemical assays proved the specific binding of VadF to Fn, where VadFA is the major domain involved in binding to both CBD and HBD of Fn. Although VadFB was not able to bind to Fn alone, its presence could greatly enhance the binding of VadFA to both CBD and HBD of Fn, suggesting an indirect role of VadFB in Fn binding. This is also supported by the HeLa binding data. Results showed that Δ*vcrD1*Δ*vadF*::p*vadFD*, a *vadF* deletion strain complemented with *vadF*'s region D only, can only partially restore its attachment affinity. Although VadFB is mainly composed of BIG2 domains, which were shown to attach host surface ligands, such as in LigA, B and FdeC (Bodelon et al., 2013; Hamburger et al., 1999; Luo et al., 2000) and broaden the access to host receptors (Henderson et al., 2011; Nesta et al., 2012), the consistent data on solid phase binding and HeLa cell binding assays suggested that BIG2 repeats of VadF can less likely play similar roles in attaching host surface ligands.

The interaction between VadF and Fn seems different from that MAM7 and Fn, as well as those between other FnBPs and Fn. MAM7, a

previously identified FnBP in *V. parahaemolyticus* and FAPs from *Mycobacterium* spp. can only bind to HBD of Fn, while VadF can bind to both HBD and CBD of Fn (Krachler and Orth., 2011; Pasula et al., 2002). Furthermore, the RWFV motif in FAPs from *Mycobacterium* spp. was shown to contribute to the binding to Fn (Zhao et al., 1999). The lack of this motif in VadF suggested the binding mechanism of VadF to Fn differed from that of FAPs to Fn. In addition to VadF, FnBPs that bind to both CBD and HBD of Fn have been reported in type I fimbriae, LigA, LipL32 and FimA from *E. coli*, *Leptospira interrogans* and *Porphyromonas gingivalis*, respectively (Henderson et al., 2011). VadF also differed from MAM7 by that it only recognizes immobilized Fn. Considering that insoluble Fn is most common on ECM (Pankov and Yamada, 2002), *V. parahaemolyticus* might utilize VadF to colonize the specified niches. The fact that *vadF* deletion mutant of *V. parahaemolyticus* vp3218 strain was dramatically attenuated suggested the critical role of *vadF* in the pathogenesis of *V. parahaemolyticus*. Lastly, MAM7 was shown to be constitutively expressed in *V. parahaemolyticus* RIMD 2210633 (Krachler et al., 2011), while *vadF* expression was not detectable by RT-PCR (data not shown), even with the induction of bile acid and Ca^{2+}, which is the signal to induce the T3SSs expression in *V. parahaemolyticus* (Gode-Potratz et al., 2010; Gotoh et al., 2010). It is possible that the expression of *vadF* is triggered

upon direct interaction of *V. parahaemolyticus* with host cells or the expression of *vadF* requires specific signals from host niches. Further research will be needed to address these hypotheses. In conclusion, this study identified a critical adhesion gene of *V. parahaemolyticus*, which is required for its pathogenesis. The VadF is a good candidate for vaccine and therapy development for *V. parahaemolyticus* infections.

Chapter 4 Characterization of a novel zinc transporter *znuA* horizontally acquired by *V. parahaemolyticus*

4.1 Introduction

It was shown that HGT is one of the most important factors that influence the virulence of *V. parahaemolyticus*. The *vpa1307* gene is localized upstream of Vp-PAIs in both *tdh*-positive *V. parahaemolyticus* RIMD2210633 and *trh*-positive TH3996 *V. parahaemolyticus* strains. Phylogenetic analysis suggested that *vpa1307* gene in *V. parahaemolyticus* is horizontally acquired. In addition, *vpa1307* which is a novel znuA homologue represents a novel subfamily of ZnuA and in *V. parahaemolyticus*, the zinc transportation as well as its relationship with the pathogenesis is totally obstacle. The role of this gene was characterized.

4.2 Results

4.2.1 Bioinformatic analysis of *vpa1307* gene and its homologues

Since PAI is important for the virulence, we focused on genes that related to Vp-PAI (Dobrindt et al., 2004). After a close examination of Vp-PAI region from *tdh*-positive *V. parahaemolyticus* RIMD2210633, a hypothetic protein, Vpa1307 localized upstream of the Vp-PAI was found. Its homologue, which is also localized upstream of Vp-PAI, can be seen in *trh*-positive *V. parahaemolyticus* TH3996 (Fig. 1).

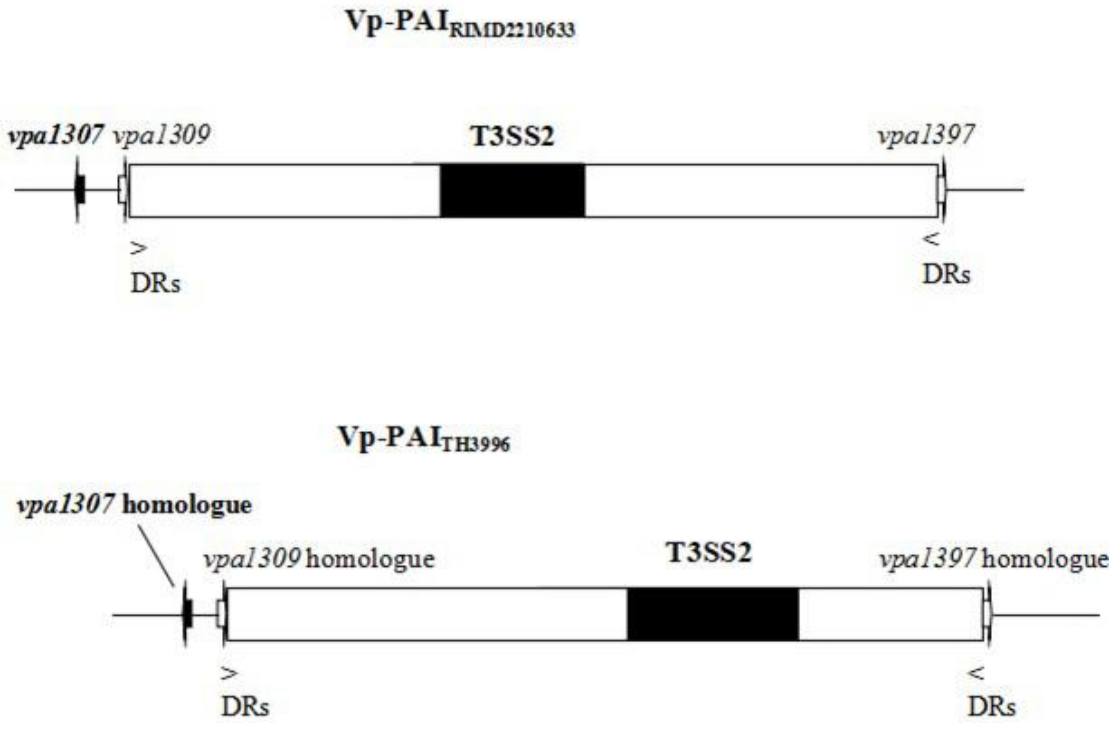


Figure 1 Schematic of *vpa1307* location in *V. parahaemolyticus* strains. Vp-PAI is flanked by direct repeats (DRs, 5'-AACTC-3'). The *vpa1307* and its homologue genes are represented by black arrows. Black boxes indicate T3SS2 gene clusters.

BLAST analysis showed that Vpa1307 shares 23% amino acid sequence identity to the zinc binding protein from *V. cholerae* O1 biovar EI Tor strain N16961. In addition, Vpa1307 and other ZnuA members possessed the same conserved three histidine residues, H69 H147 H202, that were the hallmarks of proteins affiliated with the ZnuA family (Fig. 2). It was shown that residues of H69 H147 H202 are critical for zinc binding (Banerjee et al., 2003; Ilari et al., 2011; Li and Jogl, 2007; Loisel et al., 2008; Yatsunyk et al., 2008). The 3D structure of Vpa1307 was predicted and aligned with the crystal structure of ZnuA from *Synechocystis* sp. PCC 6803. The TM score, an algorithm to calculate the structural similarity of two protein models, is 0.97, which strongly suggested Vpa1307 is likely to be a member of the ZnuA family (Fig. 3).

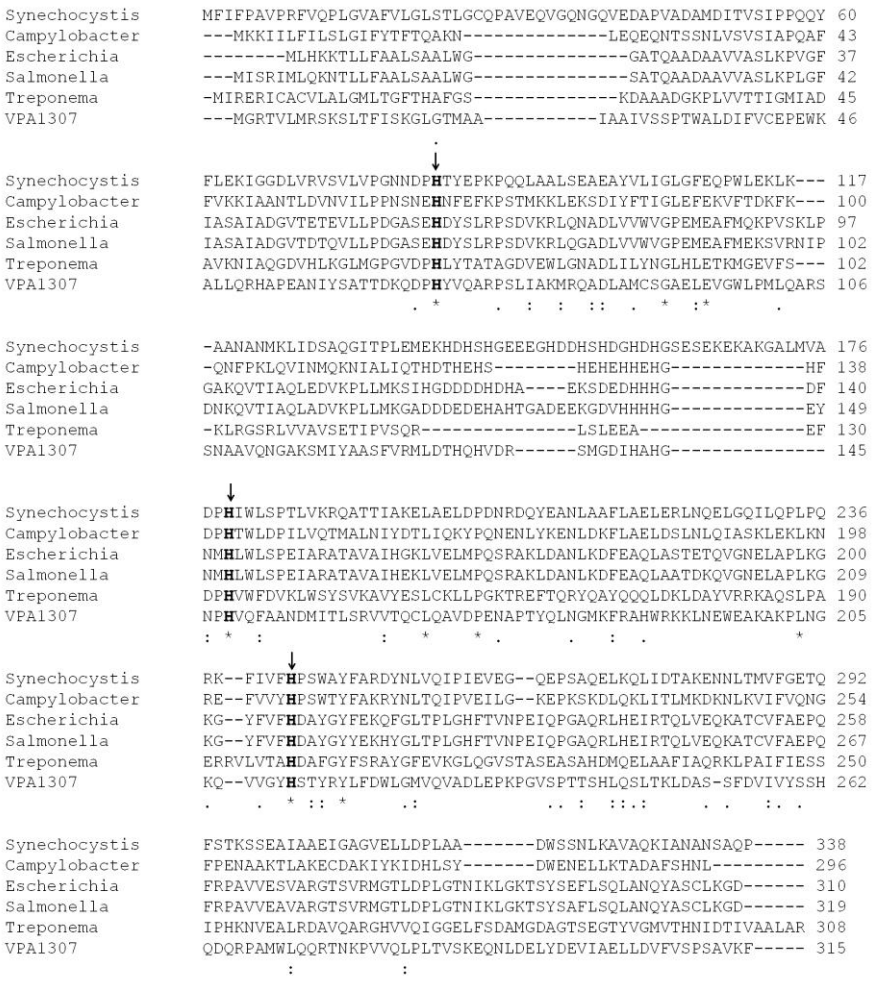


Figure 2 Multiple sequence alignment of VPA1307 and ZnuA proteins. The amino acid sequences (GenBank accession No.CAB72627, P73085, AAC74927, Q8Z5W7, AAC45725 and VPA1307 from *C. jejuni*, *Synechocystis* sp., *E. coli*, *S. enterica*, *Treponema pallidum* and *V. parahaemolyticus*, respectively) were aligned using the CLUSTAL W2. Three conserved histidine residues were indicated as black arrows.

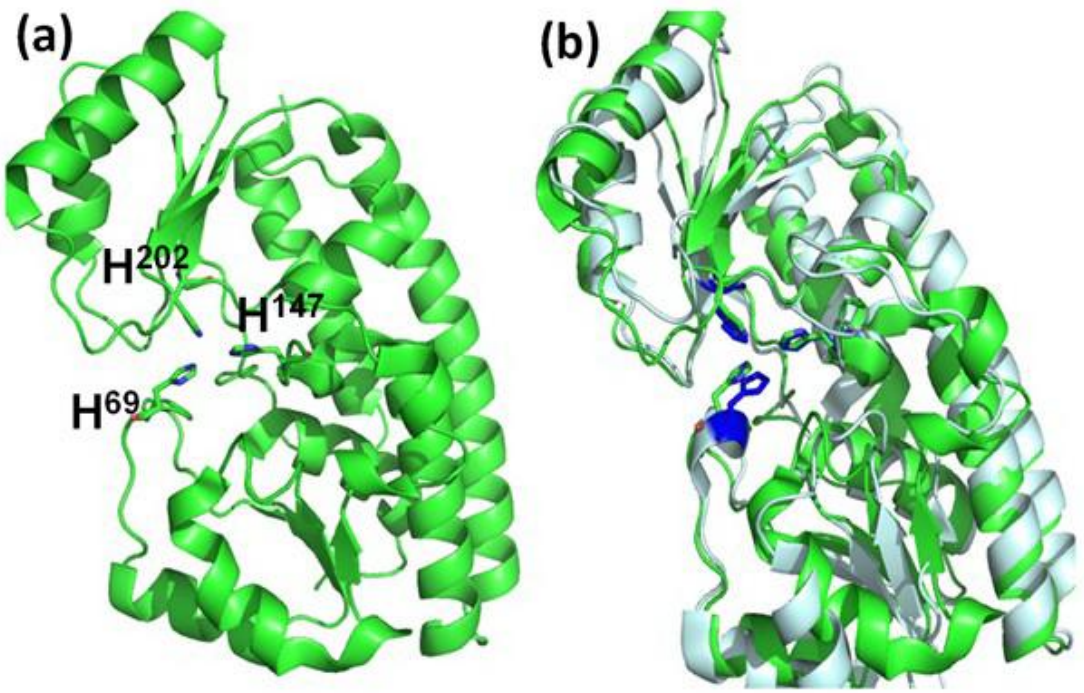


Figure 3 Structural alignments between VPA1307 and ZnuA from *Synechocystis* sp. A. VPA1307 modeled structure. Structure of VPA1307 was modeled using SWISS-MODEL program and three conserved histidine residues were labeled. B. Structural comparison of VPA1307 (green) and the crystal structure of ZnuA from *Synechocystis* sp. (PDB accession number 1PQ4) template (gray). A TM score of 0.97 was obtained over 252 aligned residues.

To explore the evolution history of Vpa1307, a phylogenetic tree was constructed (Fig. 4). The neighbor-joining phylogenetic tree showed that Vpa1307 with its four homologues from other *Vibrio* spp. fell within the lineage of ZnuA family and formed a distinct cluster within members of ZnuA from other genera. Intriguingly, the phylogenetic analysis also showed that Vpa1307 was excluded from *Vibrionaceae* clade of ZnuA,

suggesting an exogenous origin of Vpa1307 and representing a novel subfamily of ZnuA.

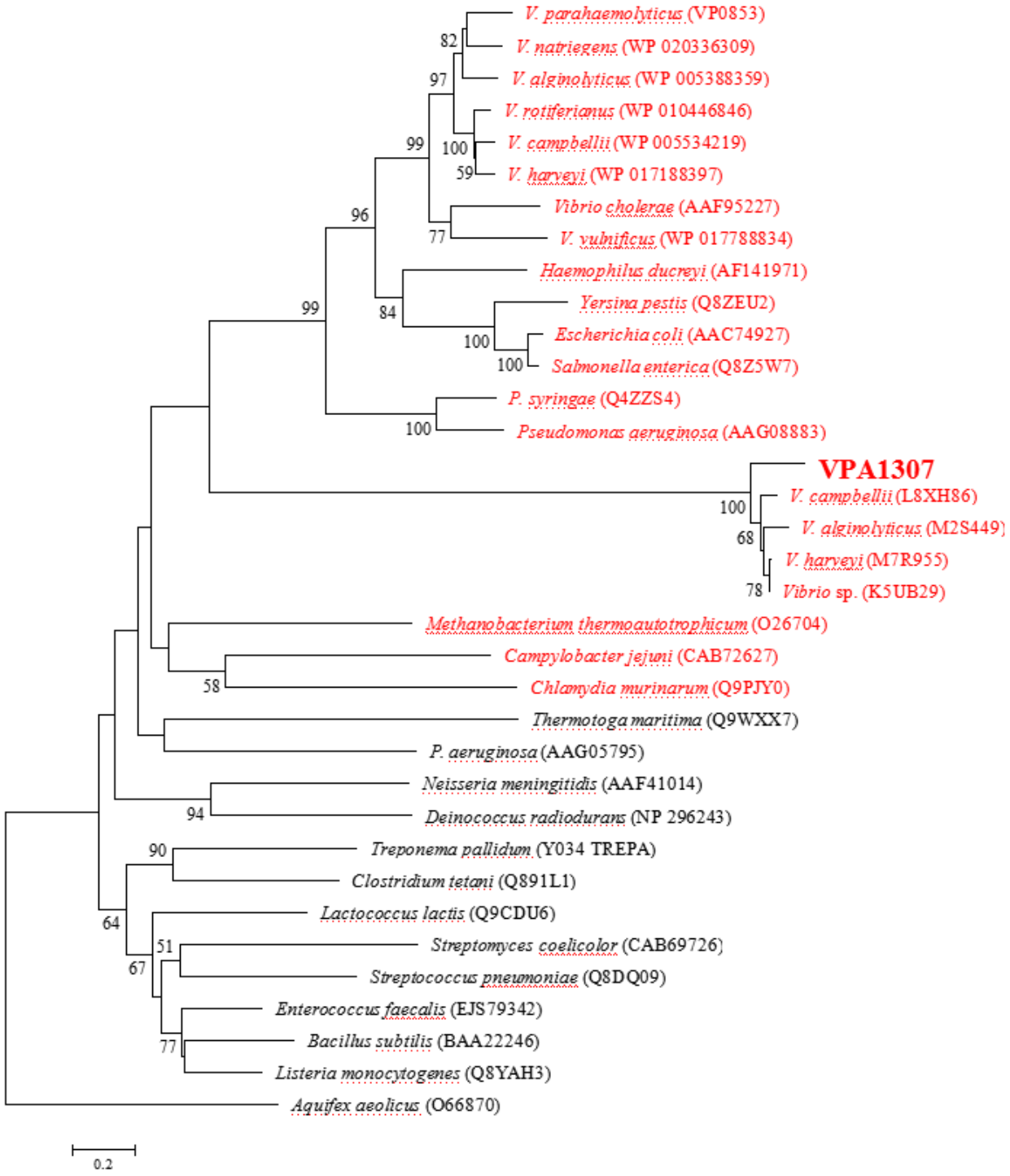


Figure 4 Neighbour-joining tree of VPA1307 and related genes. The protein sequences (inside parentheses) were obtained from NCBI (http://www.ncbi.nlm.nih.gov/) except that L8XH86, M2S449, M7R955 and K5UB29 were obtained from EBI (http://www.ebi.ac.uk/ena/data/view). ZnuA homologues were represented in red (except that of *Aquifex aeolicus*) and manganese transporters were represented in black. Bootstrap values (>50%) are shown at branch nodes. ZnuA homolog protein sequence from *Aquifex aeolicus* was used as outgroup. Bar, 0.2 difference at the amino acid level.

4.2.2 The distribution of *vpa1307* gene in *V. parahaemolyticus*

Given that *vpa1307* group genes were not prevalence in *Vibrio* spp., the spreading of this gene among *V. parahaemolyticus* strains was evaluated using PCR. Our data showed that the *vpa1307* gene was detectable in 40% of the *tdh*-positive strains while cannot be found in *tdh*- and *trh*-negative strains (Table 1).

Table 1. *vpa1307* gene distribution analysis

Strains	No. positive	Total no.	Prevalence (%)
Clinical isolates			
tdh^{+}	8	20	40
tdh^{-} *trh*$^{-}$	0	3	0

4.2.3 The expression of *vpa1307* is induced in zinc limitation condition

The expression of *vpa1307* was analyzed using RT-PCR. The results showed that *vpa1307* was not expressed under normal condition. Its transcription was induced under zinc-depleted condition (35μM TPEN added) (Fig. 5). This result indicated that the transcription of *vpa1307* would be suppressed when zinc supply is adequate.

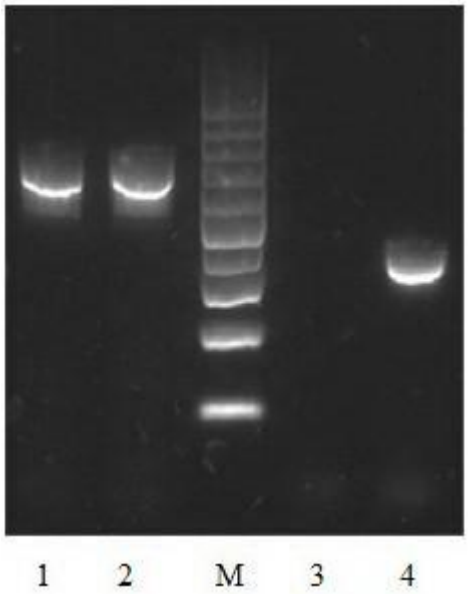


Figure 5 Detection of *vpa1307* transcript by RT-PCR in *V. parahaemolyticus*. *V. parahaemolyticus* was grown in LB and RNA was extracted after incubation with/without 35μM TPEN. 16S RNA was used as a loading control. Lane 1, 16S RNA (with TPEN); lane 2, 16S RNA (without TPEN); lane 3, *vpa1307* (without TPEN); lane 4, *vpa1307* (with TPEN); lane M, 100-bp maker (Thermo Scientific).

4.2.4 Vpa1307 contributes to *V. parahaemolyticus* growth under zinc limitation condition

The growth of the wild type (WT) strain to that of mutant strain deleted of *vpa1307* was compared. No difference in growth rate was observed when bacteria were cultivated in LB medium. However, deletion of *vpa1307* led to a growth defect of 70% in the medium containing 35 μM

TPEN, while the growth of WT was only slightly inhibited (Fig. 6). This indicated that *vpa1307* contributes to the growth of *V. parahaemolyticus* under the zinc limitation condition.

Three conserved histidine residues in the ZnuA family proteins are also present in Vpa1307. To test whether these histidine residues are essential for the function of Vpa1307, two of them, H69 and H148 were mutated to Ala. As showed in Figure 7, Δ*vpa1307*::p*vpa1307* H69A H148A had the similar relative growth rate compared with Δ*vpa1307*, while both of them were much less than that of Δ*vpa1307*::p*vpa1307*. This suggested that conserved histidine residues are essential for the function of Vpa1307, similar to those found in other ZnuA family proteins.

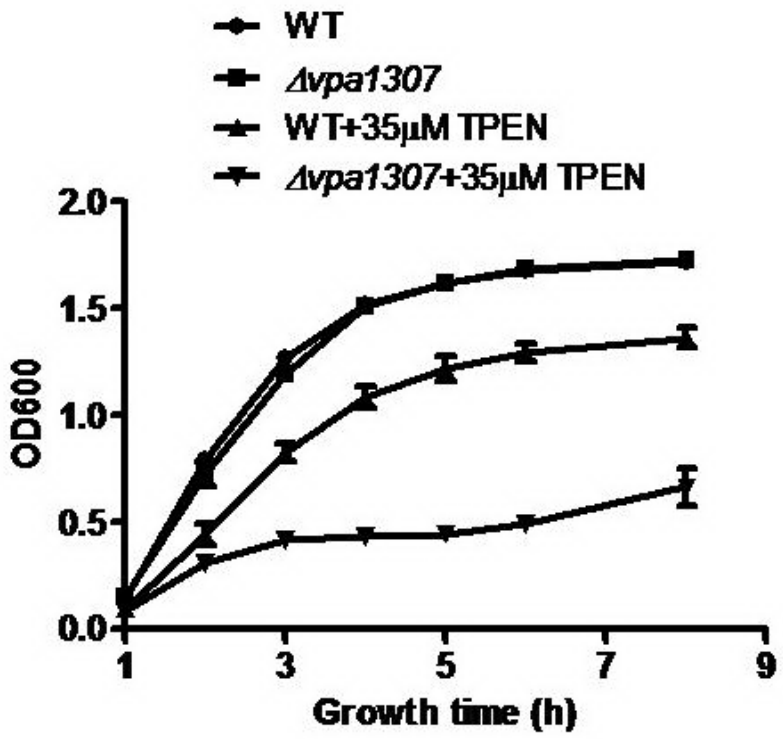


Figure 6 Growth rates of *V. parahaemolyticus* strains. *V. parahaemolyticus* strains were cultured in LB or LB supplemented with 35μM TPEN. Growth was monitored (OD600).The data represents three independent experiments ± the SD.

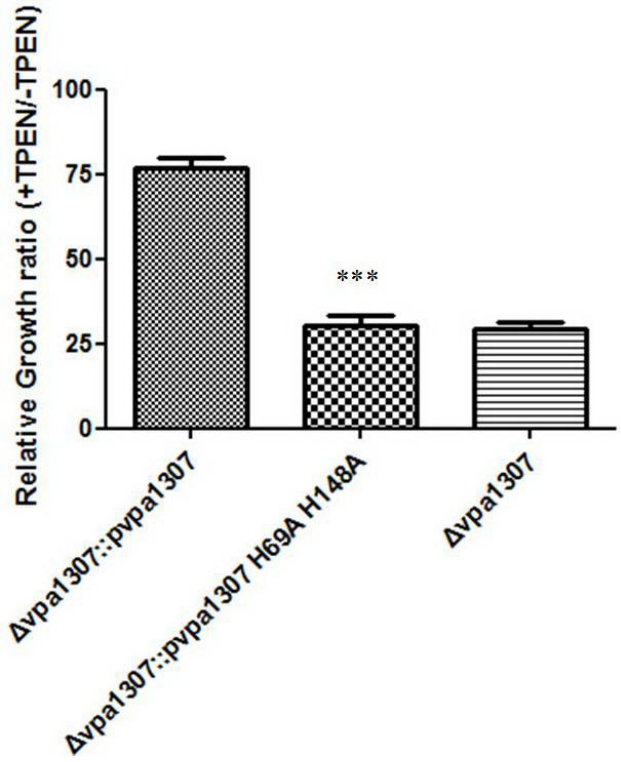


Figure 7 Relative growth rates of *V. parahaemolyticus* strains. *V. parahaemolyticus* strains were cultured in LB or LB supplemented with 35μM TPEN. Growth was monitored (OD600) at 6 h and relative growth ratio was calculated as culture grown with TPEN to that of grown without TPEN. ***, $P < 0.001$ using one-way analysis of variance (ANOVA) with Dunnett's posttest versus Δ*vpa1307*::p*vpa1307*. The data represents three independent experiments ± the SD.

4.2.5 Vpa1307 contributes to cytotoxicity against HeLa cells

Since ZnuA contributed to infect host cells in *B. abortus*, *M. catarrhalis* and *S. enterica* (Ammendola et al., 2007; Murphy et al., 2013; Yang et al., 2006), we further test whether *vpa1307* gene contributes to the virulence of *V. parahaemolyticus*. HeLa cells that maintained in serum-free DMEM were infected with Δ*vpa1307*, Δ*vpa1307*::p*vpa1307* and WT strains, respectively. LDH released from damaged cells were measured as an indicator of cytotoxicity. Comparing their cytotoxicity caused to HeLa cells, WT and complement strains caused about 70% of LDH release, whereas Δ*vpa1307* strain caused approximately 20% of LDH release after 4h infection (Fig. 8). These data indicated that Vpa1307 contributed to the cytotoxicity of *V. parahaemolyticus* strain VP3218 against HeLa cells. It also suggested that zinc acquisition is required for the infection in *V. parahaemolyticus*.

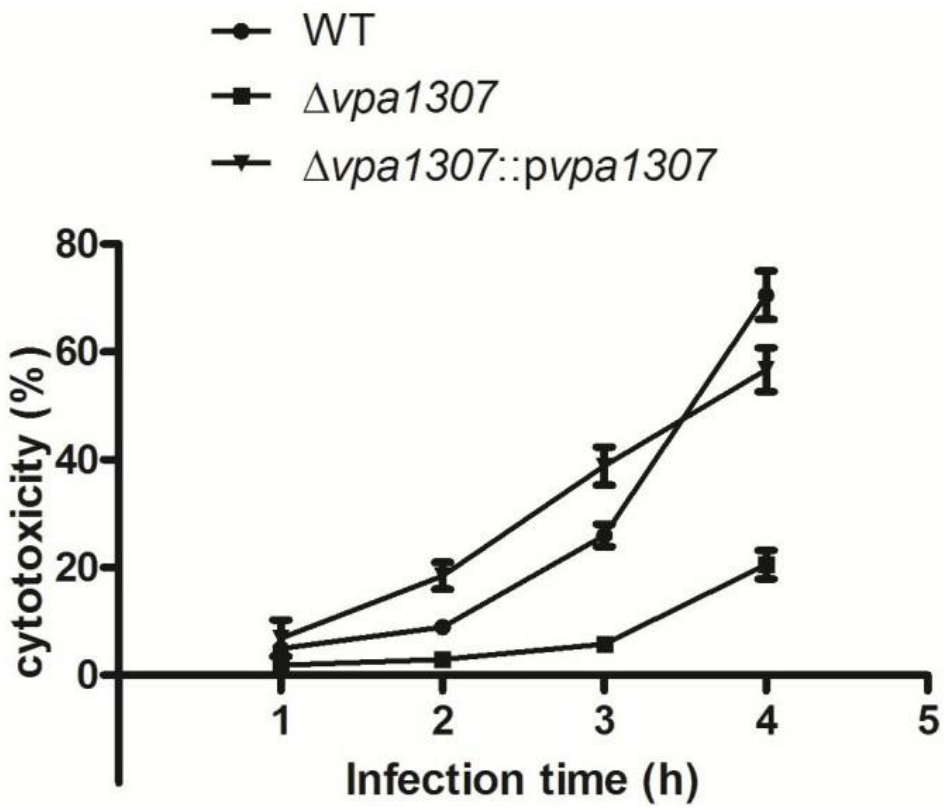


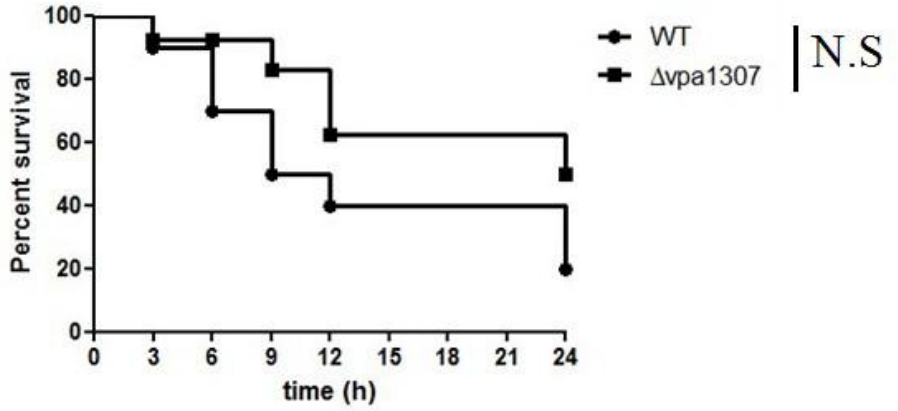
Figure 8 Cytotoxic activity of HeLa cells caused by *V. parahaemolyticus* strains. HeLa cells were infected with *V. parahaemolyticus* strains and the amounts of LDH released were measured. The data represents three independent experiments ± the SEM.

4.2.6 Vpa1307 contributes to but is not required for infection

To further evaluate the role of Vpa1307 in pathogenesis, mouse infection model was employed. As shown in Fig.9, in 24 hours of infection, the survival ratio of the WT infected mice was approximately half to one third less than that of the Δ*vpa1307* strain infected mice. This indicated

that Vpa1307 contributes to but is not essential for the virulence of *V. parahaemolyticus* strain VP3218 in mice model.

A



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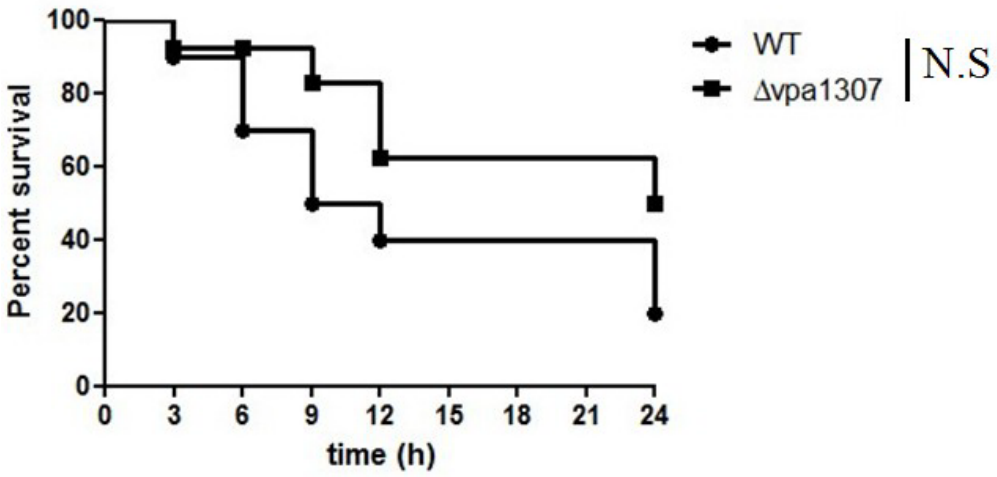

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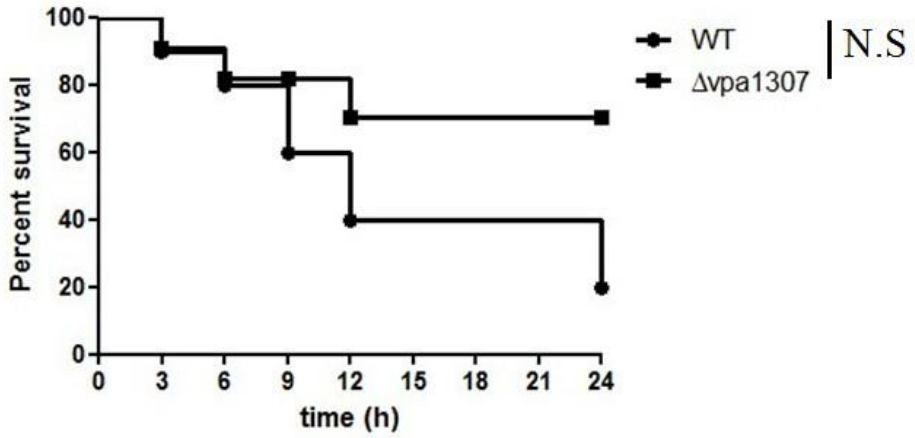

Figure 9 Survival rates of murine infected with *V. parahaemolyticus* strains. In each experiment (A, B and C), C57BL/6 mice (n=10) were infected intraperitoneally with WT or Δ*vpa1307* strains (10^8 CFU). Kaplan-Meier and log rank tests were used to analyze the data (N.S, not significant)

4.3 Discussion

In this study, a novel *znuA* homologue gene, *vpa1307* in *V. parahaemolyticus* was identified and characterized. Vpa1307 is localized upstream of Vp-PAI, and was annotated as an adhesion protein in strain RIMD2210633 (Makino et al., 2003). The results showed that *vpa1307* not only represents a novel group of ZnuA family, but also a horizontally acquired gene in *V. parahaemolyticus*.

HGT usually affects bacterial fitness to the environment and virulence. Results showed that the genes of *vpa1307* subfamily are probably to be unique horizontally acquired virulent factors. Therefore, we functional characterized *vpa1307* in *V. parahaemolyticus* strain VP3218, both *in vitro* and *in vivo*.

Similar to what was found for the *znuA* family genes; the expression of *vpa1307* was induced in zinc limitation condition and contributed to *V. parahaemolyticus* growth under zinc starvation condition. Considering that zinc concentration is low in seawater (Bruland, 1989), the acquisition of *vpa1307* gene may facilitate *V. parahaemolyticus* to persist in the marine environment and similar mechanism could be shared with other *Vibrio* species that also harbor *vpa1307* subfamily genes.

It has been shown that when more than one zinc uptake systems exist in the pathogenic bacterium, such as in uropathogenic *E. coli*, *P. mirabilis*, *Y. pestis* and *Listeria monocytogenes*, deletion only one of them did not affect their virulence *in vivo* (Corbett et al., 2012; Desrosiers et al., 2010; Nielubowicz et al., 2010; Sabri et al., 2009). Instead, the additional zinc acquiring systems contributed to the competitive advantage, such as in uropathogenic *E. coli* and *P. mirabilis* (Nielubowicz et al., 2010; Sabri et al., 2009). Given that many *V. parahaemolyticus* strains harbored more than one *znuA* homologue genes (BLAST analysis of all available *V. parahaemolyticus* genomes); it is not surprising to see that Vpa1307 is not required for the pathogenesis. It is interesting to see that Vpa1307 contributes to the cytotoxicity against HeLa cells and stimulate the pathogenesis *in vivo*. It was shown that different *V. parahaemolyticus* isolates exhibited differential virulence to host (Caburlotto et al., 2010; Vongxay et al., 2008). This is probably due to the acquisition of novel virulence genes. We found 40% of the clinical isolates possess the *vpa1307* gene. This suggested the gain of *vpa1307* gene is beneficial to this pathogen and caused a higher pathogenicity. Considering that chitin is abundant in the aquatic environments and that it has been shown to stimulate the process of natural competence and transformation (Meibom et al., 2005), it could be a great concern that *V. parahaemolyticus* may easily acquire other virulence genes that can strengthen its pathogenicity

in the environment. In conclusion, this is the first report of a horizontal acquired *znuA* gene in *V. parahaemolyticus* that contributes to the fitness and virulence of this species.

Chapter 5 Mechanisms of fluoroquinolone resistance in *V. parahaemolyticus*

5.1 Introduction

Vibrio is commonly considered as antimicrobial susceptible bacteria. However, increasing evidence found that *V. parahaemolyticus* is becoming antimicrobial-resistant, probably due to our excessive usage of antibiotics in daily lives (Han et al., 2007). *V. parahaemolyticus* from seafood and the environment is most commonly resistant to ampicillin, and it is increasingly resistant to aminoglycosides, tetracycline, sulfamethoxazole/trimethoprim, and chloramphenicol (Han et al., 2007).

A recent study in our laboratory has isolated *V. parahaemolyticus* that was resistant to Extend-Spectrum cephalosporins or fluoroquinolones. This raised huge concerns since these two classes of antibiotics, in particular fluoroquinolones, are the choices of treatment for *Vibrio* infections in human (Dutta et al., 1996; Maggi et al., 1996; Wong et al., 2012). However, the mechanism of fluoroquinolone resistance in *V.*

parahaemolyticus is obstacle. In addition, similar mechanism could be shared with other *Vibrio* spp. Thus, it is urgent to understand the mechanism of fluoroquinolone resistance in *V. parahaemolyticus*, which is essential to improving the diagnosis, treatment, and effectiveness of prevention of infections by this pathogen. In this study, the mechanism underlying fluoroquinolone resistance in *V. parahaemolyticus* isolated from shrimp samples was addressed for the first time to provide better understanding of the contribution of different mechanisms to fluoroquinolone resistance in this emerging pathogen.

5.2 Results and discussion

5.2.1 Clonal relationship of ciprofloxacin-resistant *V. parahaemolyticus*

Seven independent (isolated from different shrimp samples) ciprofloxacin-resistant *V. parahaemolyticus* (CIP MIC≧4) out of a total of 208 isolates were selected for characterizing their molecular mechanism of fluoroquinolone resistance. PFGE characterization found that the seven isolates belonged to three different patterns (A, B, C) with more than 4 bands difference and these PFGE patterns were different from the patterns identified in ESBL producing *V. parahaemolyticus* from our previous study (Wong et al., 2012) (Table 1). Within pattern C, three subtypes, C1, C2 and C3, with more than one band difference were identified. *V. parahaemolyticus* V56 and V93 had the same C3 subtype and were isolated from the same location but on different days, while V89 and V110 with C1 subtype were isolated from different locations and days (Table 1). These indicated that both clonal and non-clonal distribution of *V. parahaemolyticus* might contribute to the spread of fluoroquinolones-resistant *V. parahaemolyticus* in Hong Kong and strains with PFGE pattern C were more common than others.

5.2.2 Single GyrA and ParC mutation contributed to fluoroquinolone resistance in *V. parahaemolyticus*

All seven isolates were shown to be resistant to ampicillin, kanamycin, nalidixic acid, ciprofloxacin, norfloxacin and levofloxacin; they remained sensitive to cefotaxime, ceftazidime and meropenem. Some isolates were also resistant to streptomycin and chloramphenicol (Table 1). These antimicrobial resistance profiles were in line with previous reports that *V. parahaemolyticus* from seafood and the environment are commonly resistant to ampicillin, which is usually higher than 50%, and it is increasingly resistant to aminoglycosides, tetracycline, trimethoprim-sulfamethoxazole, and chloramphenicol (Baker et al., 2008; Chan et al., 1989; Han et a., 2007; Okoh et al., 2010; Tjaniadi et al., 2003). The data alarmed the emergence of multidrug resistant *V. parahaemolyticus* in Hong Kong. Therefore, it is of vital importance to understand the mechanisms of multidrug resistance in *V. parahaemolyticus*. Since all isolates showed fluoroquinolone resistance profile, mechanisms of fluoroquinolone resistance in these isolates were further investigated. All isolates were found to harbor the single amino acid substitution Ser83Ile in GyrA and contain one mutation in ParC, Ser85Leu.

It was shown that ciprofloxacin-resistant *V. parahaemolyticus* strains isolated from farmed marine shrimps in Thailand only possess one mutation in GyrA (Kitiyodom et al., 2010), this suggested that *gyrA* is probably the main target of ciprofloxacin in *V. parahaemolyticus* (Table 1). A single mutation in ParC, Ser85Leu has not been detected in the Thailand isolates. However, in other *Vibrio* spp., single *parC* mutation was detected in fluoroquinolone-resistant isolates. This implied that mutation in *parC* has a redundant role in fluoroquinolone resistance. The data also indicated that the mechanism between naturally occurring and laboratory-induced fluoroquinolone-resistant *V. parahaemolyticus* strains differed in that one mutation in *gyrA* may be sufficient for fluoroquinolone resistance although mutations in *parC* can enhance this trend (Okuda et al., 1999).

5.2.3 A novel *qnrVC* allele contributed to quinolone resistance in *V. parahaemolyticus*

The *qnrVC* genes including *qnrVC1*, *qnVC2* and *qnrVC3* have been reported in *V. cholerae* and shown to contribute to quinolone resistance (Fonseca et al., 2008; Kim et al., 2010; Kumar et al., 2011; Poirel et al., 2005). In this study, all isolates were negative for other PMQR genes

except that a novel *qnrVC* gene was detected in four out of seven *V. parahaemolyticus* isolates (Table 1). This was the first report on the emergence of *qnrVC* in Hong Kong. Sequence analysis of the *qnrVC* PCR products showed that the *qnrVC* gene carried by *V. parahaemolyticus* was a novel *qnrVC* allele with a substitution, Ala100Ser, compared to *qnrVC4* in *Aeromonas punctata* (Xia et al., 2010). We designated the novel *qnrVC* allele as *qnrVC5*. Conjugation experiments were conducted to examine the transmission potential of *qnrVC5* gene in all four *qnrVC5*-positive *V. parahaemolyticus* and results showed that the *qnrVC5* gene was incapable of transferring to *E. coli* J53. However, Southern hybridization of chromosomal and plasmid DNA from *qnrVC5*-positive *V. parahaemolyticus* confirmed that the *qnrVC5* gene was present on the plasmid (Fig 1).

BLAST of *qnrVC5* against GenBank identified an identical ORF from *V. cholerae* O1 plasmid pVN84 (AB200915). To confirm whether that the genetic environment of *qnrVC5* in *V. parahaemolyticus* was the same as that of *qnrVC5* on plasmid pVN84, primers targeting to different regions of pVN84 was used to amplify the adjacent genetic structure of *qnrVC5* in *V. parahaemolyticus* as described in method. PCR and sequencing results showed that the plasmid encoding *qnrVC5* from *V. parahaemolyticus* isolates was about 7.2 kb with the genetic organization

as shown in Fig 1. The plasmid identified in this study was 99% identical to pVN84 plasmid reported in *V. cholerae* isolated from Vietnam. These data suggested the *qnrVC5* on a small plasmid may spread among *Vibrio* spp. To further confirm the functionality of the novel *qnrVC5* allele, the whole coding region of *qnrVC5* was cloned into pCR2.1 vector. The MIC of *E. coli* carrying the pCR2.1-*qnrVC5* showed reduced susceptibility to ciprofloxacin (MIC=0.1) and resistance to nalidixic acid (MIC=32), confirming that this is a variant of a PMQR gene (Table 1).

5.2.4 Role of other transferable elements on *V. parahaemolyticus* antimicrobial resistance

Class I integron was rarely detected in *V. parahaemolyticus* (Ceccarelli et al., 2006; Kitiyodom et al., 2010; Taviani et al., 2008), however, all four *qnrVC5*-positive isolates were found to harbor a ~1.5 kb class I integron, whereas *qnrVC5*-negative isolates were negative for class I integron (Table 1). Sequence and BLAST analysis of this ~1.5 kb integron demonstrated that there are two antimicrobial resistance genes carried by this integron, *dfrA27* and *arr3* (Accession no. KC540632). BASLT analysis also showed these two elements are commonly observed among *E. coli, Klesbiella pneumonoae, V. cholerae* and *etc.*, suggesting that the

isolates may acquire this class I integron under the selective pressure of antimicrobials in shrimps.

It is intriguing to see that the conserved integrase of integrative conjugative elements (ICEs) of the SXT/R391 family was positive in all seven *V. parahaemolyticus* isolates. Search of all available whole genome sequences in GenBank did not identify the integrase gene in *V. parahaemolyticus* but it was also commonly present in *Vibrio* spp. (Kumar et al., 2011). This is the first report of ICEs in *V. parahaemolyticus* that showed the SXT element also spreads to a wide range of *Vibrio* spp.

5.2.5 Limited role of efflux pump in fluoroquinolone resistance in *V. parahaemolyticus*

In addition to target site mutations, multidrug efflux pumps such as NorM, VcaM, VcmA and MATE family transporters have also been shown to contribute to fluoroquinolone resistance in *Vibrio* spp. (Huda et al., 2001; Huda et al., 2003; Mohanty et al., 2012; Morita et al., 1998). In order to test the contribution of efflux pumps to fluoroquinolone resistance in *V. parahaemolyticus*, the MICs of nalidixic acid, ciprofloxacin, norfloxacin and levofloxacin were determined in the presence of the efflux pump

inhibitor, PAβN. The data showed that the presence of PAβN did not alter the MICs of these antibiotics; however the presence of this efflux pump inhibitor was found to cause ~8-16-fold reduction of MICs of these antibiotics in a *Salmonella enteritidis* control strain (data not shown), suggesting that efflux pumps might not contribute to fluoroquinolone resistance in these isolates.

5.3 Conclusion

The emergence of fluoroquinolone resistance in *V. parahaemolyticus* may cause potential public health problem in Hong Kong since fluoroquinolones are considered as choices for treatment of *V. parahaemolyticus* infections. This study first reported isolation and characterization of the fluoroquinolone-resistant *V. parahaemolyticus* from shrimp samples in Hong Kong. The mechanism of fluoroquinolone resistance in *V. parahaemolyticus* was investigated and multiple mechanisms were found. Single target mutation in GyrA and ParC may contribute primarily to the fluoroquinolone resistance in *V. parahaemolyticus*. A novel PMQR gene, *qnrVC5*, encoded on a 7.2 kb plasmid may also contribute to the development of fluoroquinolone resistance in these strains. Class I integron and integrative conjugative elements (ICEs) were also commonly present in these isolates. Both of these two transferable elements might also contribute to the development of multidrug resistance in *V. parahaemolyticus*. Efflux pumps, however, may only play an insignificant role in fluoroquinolone resistance. The understanding of the mechanisms of fluoroquinolone resistance in *V. parahaemolyticus* will provide useful information for the treatment of infections caused by *V. parahaemolyticus*.

Table 1. Characteristics of fluoroquinolone-resistant *V. parahaemolyticus* isolates and *E. coli* carrying *qnrVC5* gene

Vibrio parahaemolyticus				MICs (mg/L)									QRDR substitution		PMQR	Integron/ ICEs	
Strain	Isolation date	Isolation location*	PFGE pattern	AMP	NA	CIP	NOR	LEV	KAN	CHL	TET	TRI	GyrA	ParC	*qnr*	ICEs	Class I
V30	01/20/10	3	B	>64	>128	4	4	8	>32	<2	<2	<2	Ile83	Leu85	-	+	-
V56	02/03/10	1	C3	>64	>128	8	8	8	>32	4	<2	<2	Ile83	Leu85	-	+	-
V89	02/17/10	2	C1	>64	>128	8	8	16	>32	16	4	>32	Ile83	Leu85	*VC5*	+	+
V93	02/17/10	1	C3	>64	>128	8	8	16	>32	4	<2	<2	Ile83	Leu85	-	+	-
V110	03/03/10	4	C1	>64	>128	8	8	16	>32	16	<2	>32	Ile83	Leu85	*VC5*	+	+
V128	03/17/10	2	C2	>64	>128	8	8	16	>32	<2	<2	>32	Ile83	Leu85	*VC5*	+	+
V176	04/14/10	3	A	>64	>128	8	8	8	>32	<2	<2	>32	Ile83	Leu85	*VC5*	+	+
E. coli TG.1					2	.005	.005	.005									

E. coli TG.1 (pCR2.1)	2	.005	.005	.005
E. coli TG.1 (pCR2.1-*qnrVC5*)	32	0.1	0.1	0.25

AMP, ampicillin; NA, nalidixic acid; CIP, ciprofloxacin; NOR, norfloxacin; LEV, levofloxacin; KAN, kanamycin; CHL, chloramphenicol; TET, tetracycline; TRI, Trimethoprim. MIC data for cefotaxime, ceftazidime, meropenem were not shown in the table.

* 1, Hong Kong Island; 2,Hung Hom; 3,Tsuen Wan; 4, Sai Kung

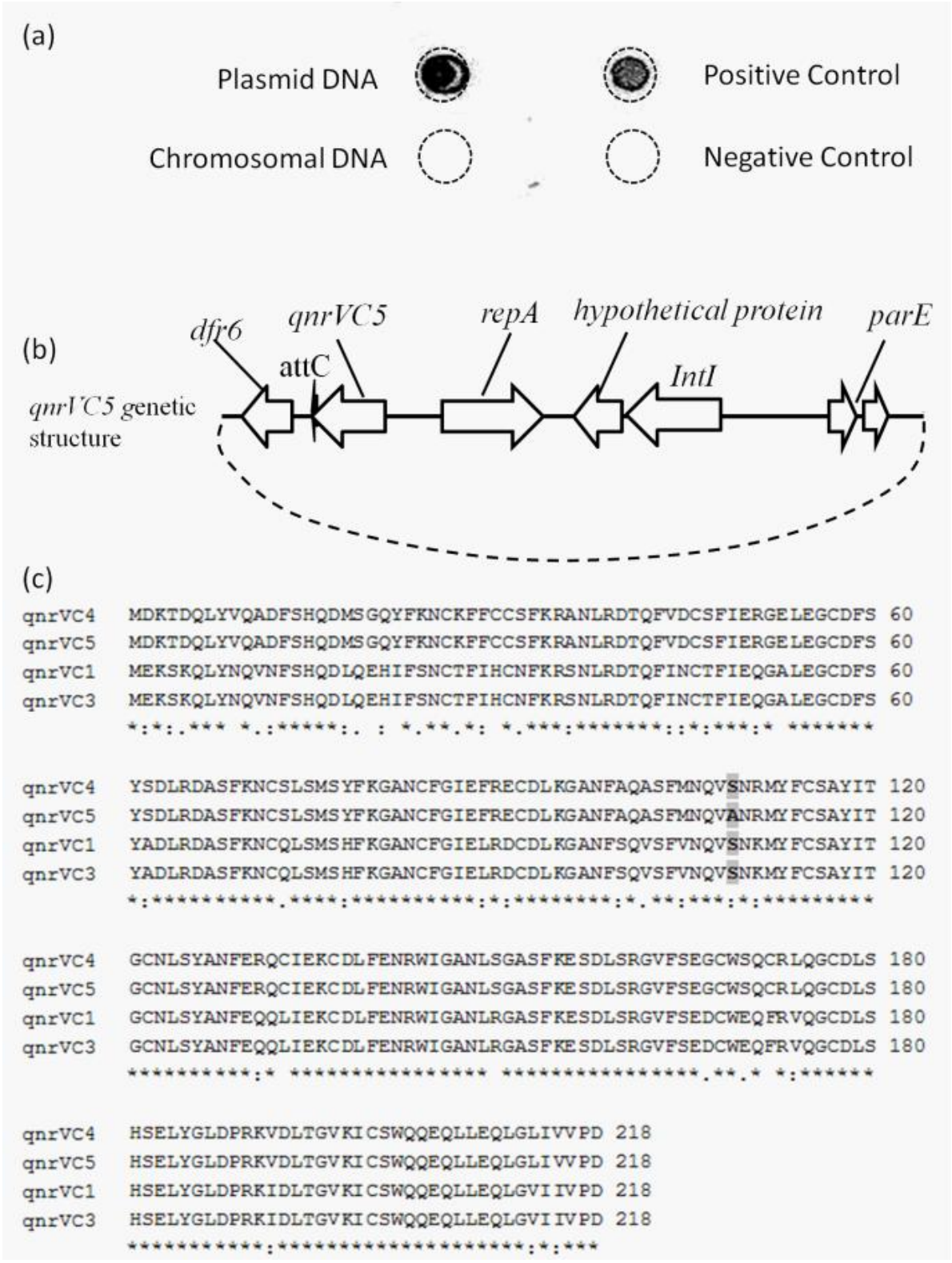

Figure 1 Southern hybridization and genetic environment of *qnrVC5*

(a) chromosomal and plasmid DNA from *V. parahaemolyticus* strains were denatured by boiling at 100 ℃ for 2min and placed on ice

immediately for 5min before loading on nitrocellulose membrane. After cross-linking, the membrane was subjected to southern hybridization using DIG labeled *qnrVC5* probe. The blot represented the hybridization results for strain V89. Positive control is PCR product of *qnrVC5*, negative control is PBS. (b) Genetic structure of ~7.2kb *qnrVC5* encoding plasmid from *V. parahaemolyticus*, which is 99% identical to the plasmid pVN84 (AB200915) from *V. cholerae* O1. (c) Amino acid sequence alignment of *qnrVC5* and known *qnrVC* variants and the $A^{100}S$ variation to other *qnrVC* genes were highlighted.

Chapter 6 Emergence of a transferable plasmid carrying a novel quinolone-resistant and an extended spectrum beta-lactamase gene in *V. parahaemolyticus*

6.1 Introduction

Resistance to extended spectrum cephalosporins was rare in *Vibrio* spp. Extended spectrum β-lactamases (ESBLs) such as TEM-63, CTX-M-3 and PER-2 have been detected in *Vibrio* spp. (Ismail et al., 2011; Petroni et al., 2002). In *V. parahaemolyticus* the only ESBL, PER-1, was reported, while the mechanisms underlying the transmission of PER-1 to *V. parahaemolyticus* are not clear (Wong et al., 2012). This study reported the identification and characterization of a multidrug-resistant plasmid mediating resistance to multiple antimicrobials including ampicillin, ceftriaxone, cefotaxime, ceftioufur, nalidixic acid, kanamycin,

chloramphenicol and streptomycin. The responsible resistance mechanisms and their genetic environments were also addressed.

6.2 Results and discussion

6.2.1 Identification of multidrug-resistant conjugative plasmid in *V. parahaemolyticus* strain V1

The *V. parahaemolyticus* strain V1 in this study was isolated from raw shrimp sample purchased from supermarket in Hong Kong in 2011. This isolate was different from the *V. parahaemolyticus* reported in our previous study (Wong et al., 2011). Antimicrobial susceptibility testing showed that this isolate was resistant to several different classes of antibiotics including ampicillin, ceftriaxone, cefotaxime, ceftiofur, nalidixic acid, kanamycin, streptomycin and chloramphenicol (Table 1). Conjugation experiment showed that all these resistance phenotypes were transferrable to *E. coli* J53 strain. Whole plasmid sequencing revealed this plasmid was more than 180 kb (data not shown). S1-PFGE confirmed that the transconjugant harbored single plasmid, with a size ~200kb (Fig. 1). The emergence of this multidrug resistant conjugative plasmid in *V. parahaemolyticus* may pose huge threat to human health in particular in areas like Hong Kong, where *V. parahaemolyticus* infections are the major causes of foodborne illnesses. This plasmid, pVP1 was further

characterized to understand the resistance determinants for these antibiotics and their transmission mechanisms.

6.2.2 Identification of resistance determinants on the conjugative plasmid

Plasmids from transconjugants were screened for potential resistance determinants such as extended spectrum β-lactamases, PMQR genes and transferable elements. Consistent with our previous findings (Wong et al., 2012), ESBL, PER-1 was detected on the plasmid. Interestingly, a *qnrVC* gene was detected on pVP1, and it might mediate the resistance to nalidixic acid. Sequence analysis of full length *qnrVC* gene revealed that this gene differed from *qnrVC*3 by one single amino acid, Asp^{71} instead of Asn^{71} in *qnrVC3*. We designated this novel *qnrVC* allele as *qnrVC*6. To determine whether *qnrVC6* contributes to nalidixic acid resistance of the conjugative plasmid, the whole encoding sequence of *qnrVC6* gene was cloned into pCR2.1. In addition, single amino acid substitution $Asp^{71}Asn$ was introduced into QnrVC6 to produce QnrVC3. After transforming pCR2.1- *qnrVC3* and pCR2.1- *qnrVC6* into *E. coli* TG1, MICs to ciprofloxacin and nalidixic acid were evaluated following the CLSI guideline. *E. coli* TG1 that carried pCR2.1- *qnrVC*3 and pCR2.1- *qnrVC*6

showed CIP MICs=0.125 and 0.25, and NAL MICs=32 and 64, respectively, a significant increase of CIP and NAL MICs compared to the host strain *E. coli* TG.1, suggesting the role of *QnrVC6* on nalidixic acid resistance and *qnrVC6* was slightly better in mediating quinolone resistance than *qnrVC3* (Table 1). Other PMQR genes, such as *qnrA*, *qnrB*, *qnrD*, *qnrS*, *aac(6')-Ib-cr*, *qepA* and *oqxAB*, were not detected on pVP1. In addition to bla_{PER-1} and *qnrVC6*, a ~3kb novel integron (Accession # KC202806) that carried several antimicrobial resistance genes, *aacA3*, *catB2*, *aadA1* and *dfrA1*, was also detected on the conjugative plasmid from the transconjugant (Fig 2a). BLAST of the integron sequence did not find any similar sequence with the organization of these four antimicrobial resistance genes, suggesting that it is a novel integron. The presence of kanamycin, streptomycin and chloramphenicol resistance phenotypes in the transconjugant suggested that these antimicrobial resistance genes could be functional.

6.2.3 Genetic environment of *blaPER-1* and *qnrVC6* on the conjugative plasmid

To further understand the transmission of bla_{PER-1} and *qnrVC6* to this multidrug resistant plasmid, primer walking approach was used to depict

the genetic structures surrounding these two resistant genes. A ~7kb fragment (Accession # KC202805) up- and downstream of bla_{PER-1} was sequenced from the plasmid. The genetic structure of bla_{PER-1} showed that the *ISCR1* and *gst* genes were present upstream and downstream of bla_{PER-1} (Fig 2b). This structure was similar to the bla_{PER-1} carried on a complex class I integron in *A. punctata* (Xia et al., 2010). However, other parts of the integron were not detected in this plasmid. A ~12kb fragment (Accession # KC202804) up- and downstream of *qnrVC6* was sequenced from the plasmid. Unlike *qnrVC3* that has been reported to be associated with SXT element or Class I integron in *V. cholerae* (Kumar et al., 2011), *qnrVC6* was located in a region with a transcription activator and a ParD/ParE toxin/antitoxin system surrounded by *IS1358a*, *ISVsa5* and three novel IS elements, designated as *ISVp1*, *ISVp2* and *ISVP3* which were members of the *IS630* family, with 87%, 67% and 74% amino acid sequence homology to *ISVch6*, *ISSpu23* and *ISVch6*, respectively (IS elements were identified by IS Finder database, http://www-is.biotoul.fr). In addition, an *attC* site was found downstream of *qnrVC6* (Fig 2c). The genetic environment of *qnrVC6* supported the transmission of this resistance gene through IS elements.

6.3 Conclusion

A *V. parahaemolyticus* strain V1 that carried a novel plasmid with multiple antimicrobial resistance genes such as bla_{PER-1}, *qnrVC6*, *aacA3*, *catB2*, *aadA1* and *dfrA1* was identified in this study. These MDR genes were most likely transmitted by transferable elements such as IS elements and integron. The emergence of this new type of conjugative plasmid mediating multiple drug resistance, in particular resistance to third generation of cephalosporin and quinolones, has raised huge public health concern. The further transmission of this plasmid in *Vibrio* species such as *V. vulnificus* and *V. cholerae* would speed up the development of MDR *Vibrio*. This would drastically compromise the efficiency of clinical treatment of severe *Vibrio* infections. Further actions will be needed to stop the transmission of this MDR plasmid in *Vibrio* spp.

Table 1. Characteristics of *V. parahaemolyticus* V1, its transconjugant and *E. coli* strains with different clones.

Strains	ESBL	PMQR	Int	AMP	CRO	CTX	TIO	MER	CIP	NAL	KAN	GEN	AMI	CHL	TET	STR
J53				16	<1	<1	2	<0.5	<0.05	8	<0.5	<4	<8	<4	<4	<4
V1	bla_{PER-1}	*qnrVC6*	Class 1	>128	64	128	128	<0.5	0.125	128	32	8	<8	64	<4	128
V1TC	bla_{PER-1}	*qnrVC6*	Class 1	>128	64	64	64	<0.5	0.125	128	32	8	<8	64	<4	128
E. coli TG.1(pCR2.1)									<0.05	4						
TG.1(pCR2.1-*qnrVC3*)									0.125	32						
TG.1(pCR2.1-*qnrVC6*)									0.25	64						

TC, Tansconjugant; AMP, Ampicillin; CRO, Ceftriaxone; CTX, Cefotaxime; TIO, Ceftioufur; MER, Meropenem; CIP, Ciprofloxacin; NAL, Nalidixic acid; KAN, Kanamycin; GEN, Gentamicin; AMI, Amikacin; CHL, Chloramphenicol; TET, Tetracycline; STR, Streptomycin; ESBL, Extended Spectrum β-Lactamase; PMQR, Plasmid Mediated Quinolone Resistance genes; Int, integron.

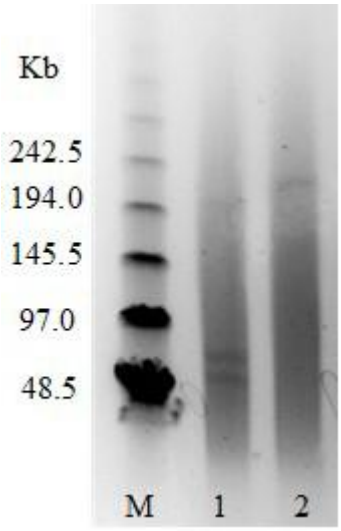

Figure 1 PFGE profile of the plasmids from strain V1 and its transconjugant. M, molecular weight maker; lane 1, plasmids extracted from strain V1; lane 2, plasmid extracted from the transconjugant

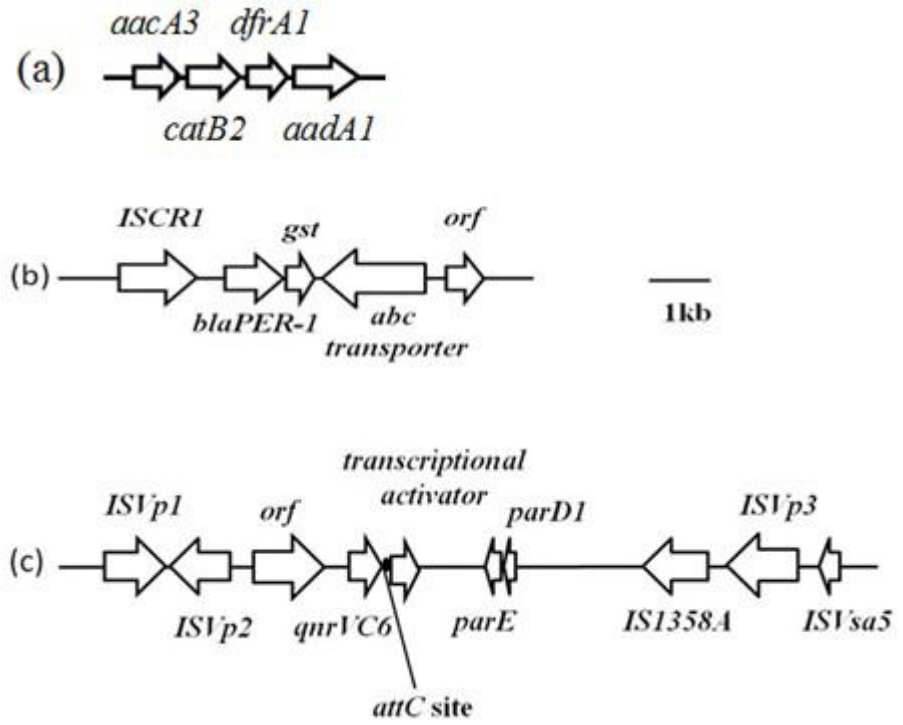

Figure 2 Genetic structures of the novel class I integron (a), *bla*$_{\text{PER-1}}$ (b) and *qnrVC6* (c). Proteins are represented by white color arrows.

Chapter 7 Conclusions and suggestions for future research

In the present study, four novel genes associated with the virulence and antibiotic resistance for *V. parahaemolyticus* were identified and characterized.

VadF, a novel virulence factor, is critical for the pathogenicity of *V. parahaemolyticus*. The data showed that VadF is a unique type of FnBP. It can bind to immobilized HBD and CBD of Fn by its N-terminal FAP homologue domain. The 5 BIG2 domains in VadF were not able to interact with Fn but played a role in enhancing its binding affinity to Fn. It would be interesting to functionally characterize the five BIG2 domains one by one. This would address a question: how many BIG2 domains are required for the full function of VadF. Additional identification of the motif that VadFA used to bind Fn will deepen our understanding of the binding mechanism. Furthermore, it will be intriguing to test whether VadF is a moonlighting protein that can not only recognize Fn but also other components of ECM, such as fibrinogen, collagen and laminin. Previous studies have identified an adhesion factor, MAM7 in *V.*

parahaemolyticus. The different features between MAM7 and VadF suggested they play different roles in the pathogenesis of *V. parahaemolyticus*. FAPs, the homolog of VadF, had the RWFV motif that is critical for its binding to Fn. However, the RWFV motif was not found in VadF. This suggested that the binding mechanism of VadF to Fn is different from that of FAPs to Fn. Based on these discoveries, we might have a great chance to develop vaccines and anti-infective agents against this pathogenic bacterium or other *Vibrio* spp. infections. A more detailed physical mapping of VadF will help us understanding the mechanism for its binding to Fn. Moreover, elucidating what regulators are involved in controlling the *vadF* expression and how they respond to different environmental conditions within the host will shed important light on the mechanisms used by *V. parahaemolyticus* to infect human beings. VadF is not only a good candidate for basic research but also an excellent candidate for vaccine development.

Vpa1307 from *V. parahaemolyticus*, a gene encoding a protein with 315 amino acids, showed low homology to ZnuA in *V. cholerae*. Phylogenetic analysis indicated that *vpa1307* would be an acquired gene through HGT and probably represent a novel subfamily of ZnuA. It was shown that the genomes of *V. parahaemolyticus* are highly flexible, leading to the divergence of the virulence. The genomic flexibility is mainly dependent

on gene gain, gene loss and homologous recombination. It helps this bacterium to adapt to different environments. The horizontally acquired *vpa1307* gene was beneficial for the growth of *V. parahaemolyticus* in zinc limited medium. Given that zinc concentration is low in seawater and host, the acquisition of *vpa1307* gene may facilitate *V. parahaemolyticus* to persist in the marine environment and host. It is interesting to see that VPA1307 contributed to the cytotoxicity against HeLa cells and stimulated the pathogenesis *in vivo*. It was shown that different *V. parahaemolyticus* isolates exhibited differential virulence to host. This is probably due to the acquisition of novel virulence genes. The PCR screening of this gene showed that 40% of clinical strains possess it, implying the gain of *vpa1307* gene is beneficial to this pathogen and caused a higher virulence.

It is worth noting that the death rate for the mutant treated mice drastically decreased after 9h inoculation. This suggests more than one zinc uptake systems may exist in the *V. parahaemolyticus* strain vp3218 that compensated the loss of *vpa1307* in the early phase of infection. It is likely that this pathogen may need to acquire more zinc for sustaining in the host after 9h, and thus VPA1307 would be helpful. More *in vivo* assays, such as the competing infection experiment, may enlighten the function of this gene.

In Chapters 5 and 6, results showed that all fluoroquinolone-resistant isolates have single amino acid substitutions in GyrA (Ser83Ile) and ParC (Ser85Leu). These point mutations probably played a major role in fluoroquinolone resistance. It was established that efflux pumps, *e.g.* NorM, VcaM, VcmA and MATE family transporters, contributed to fluoroquinolone resistance in *E. coli* KAM32, but their role in *Vibrio* spp. has not been elucidated. The results indicated that these efflux pumps play only a limited role in the primary mechanism of fluoroquinolone resistance in the *V. parahaemolyticus* Hong Kong isolates. In addition, HGT also contributed to the development of antimicrobial resistance in *V. parahaemolyticus*. Two novel *qnrVC* alleles were identified in different *V. parahaemolyticus* strains and they contributed to quinolone resistance. These two *qnrVC* genes, *qnrVC5* and *qnrVC6*, were present in different genetic environments. The *qnrVC5* gene was located in a 7 kb plasmid, flanked by *dfr6* and *repA*, whereas *qnrVC6* was present on a 200kb plasmid that was inserted by IS element. We speculated that the plasmid mediated antibiotic resistant gene transfer would be the predominant way for the spreading of antibiotic-resistant genes in *Vibrio* spp.

Appendix

Figure 1 Multiple sequence alignment of VPA1336 and its homologue proteins from *V. cholerae* strains.

The amino acid sequences (GenBank accession No. EAZ50929, EEN98474, EEO12812, WP_001084729 and VPA1336) were aligned using the CLUSTAL W2. Potential WxxxE motif was indicated as red color.

A B

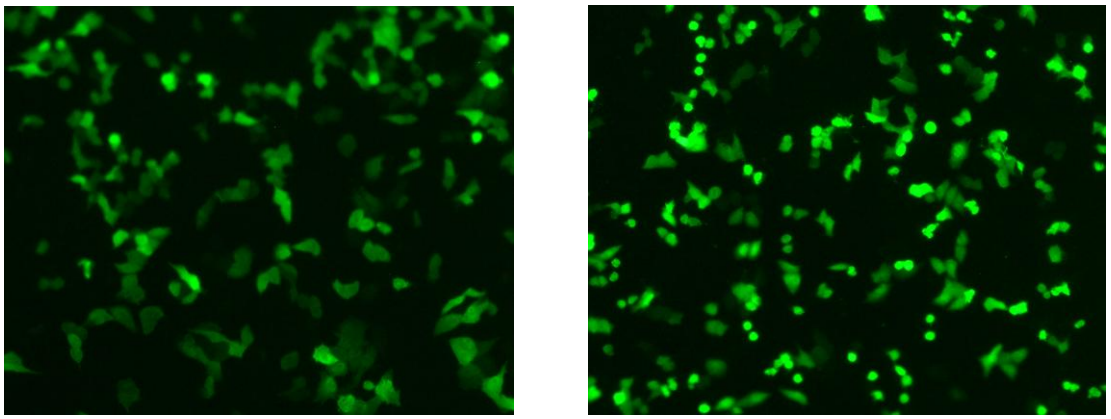

Figure 2 VAP1336 induces cell rounding.

Hela cells were transfected with peGFP-C3 (A) and peGFP-C3-VPA1336 (B), respectively. Transfected cells (green color) were visualized by the fluorescence microscope.

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