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ORIGINAL PAPER

Putative type VI secretion systems of *Vibrio parahaemolyticus* contribute to adhesion to cultured cell monolayers

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Abstract Analysis of the genome sequence of *Vibrio parahaemolyticus* reveals two IcmF family genes in putative type VI secretion system (vpT6SS) clusters in chromosomes 1 (*icmF1*) and 2 (*icmF2*). The *icmF1* gene is present in majority of clinical isolates (87.5 %), but has a low fraction (25.0 %) in environmental isolates. However, *icmF2* is contained in all strains of both clinical and environmental sources. Deletion of either *icmF1* or *hcp1* significantly reduced bacterial adhesion to Caco-2 cells or HeLa monolayers. However, the Δ*icmF2* and Δ*hcp2* mutants showed decreased adhesion only to HeLa monolayers. Western blot analysis showed that Hcp2 was present both in the supernatant and pellet samples in the wild-type strain, but only in the pellet of the Δ*icmF2* mutant, indicating that Hcp2 is a translocon of T6SS2. Although vpT6SS1 might be functional in cellular adhesion, the putative translocon Hcp1 was not detectable. Quantitative PCR revealed 10-fold and 17-fold less transcripts of *hcp1* and *icmF1* mRNA than those of *hcp2* and *icmF2* accordingly. Thus, we postulate that the putative vpT6SS systems contribute to adhesion of *V. parahaemolyticus* to host cells.

Keywords *Vibrio parahaemolyticus* · Type VI secretion system · Adhesion

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Introduction

Vibrio parahaemolyticus, a halophilic bacterium in marine environments, is the leading cause of gastroenteritis in humans in China due to consumption of raw or under-cooked seafood. The virulence factors include thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH), and type III secretion system (T3SS) (Kaper et al. 1984; Nishibuchi et al. 1986; Park et al. 2004). Most clinical isolates of *V. parahaemolyticus* exhibit Kanagawa phenomenon (KP) induced by TDH on Wagatsuma agar medium; however, only 1–3 % of the environmental isolates are KP positive (Shirai et al. 1990; Lozano-León et al. 2003; Robert-Pillot et al. 2004). TDH causes several cytotoxic effects, such as erythrocyte lysis, disruption of the microtubule cytoskeleton, and ion influx into cultured cells (Honda et al. 1988; Raimondi et al. 2000). Both T3SS1 and T3SS2 are identified as virulence factors. T3SS1 is present in all strains, contributes to cytotoxicity in HeLa cells, and induces autophagy (Park et al. 2004; Burdette et al. 2008). T3SS2, however, is found only in *tdh*- or *trh*-positive strains, and cytotoxic to Caco-2 cells and intestines (Park et al. 2004; Okada et al. 2009).

IcmF, a gene encoding the intracellular multiplication factor (IcmF), was first studied in *Legionella pneumophila* as part of the Dot/Icm cluster of genes that form type IV secretion system (T4SS) involved in host cell killing and intracellular multiplication (Purcell and Shuman 1998;

Segal et al. 1998). Recently, the IcmF family proteins were identified as innermembrane proteins of VI secretion system (T6SS) (Shrivastava and Mande 2008). T6SS is a macromolecular transenvelope machine encoded within the genomes that is only found in proteobacteria (Pukatzki et al. 2006; Mougous et al. 2006; Bingle et al. 2008; Bernard et al. 2010). The system contains 13–20 proteins (IcmF-associated homologous proteins, IAHP) coded for by the gene clusters (Boyer et al. 2009). Deletion of *icmF*-associated proteins usually does not affect expression of the translocon Hcp (hemolysin coregulated protein) but prevents its translocation (Pukatzki et al. 2006; Suarez et al. 2008).

The IAHP gene cluster of *V. cholerae* was required for secretion of Hcp and VgrG proteins and for cytotoxicity toward *Dictyostelium amoebae* and J774 macrophages (Pukatzki et al. 2006). Mougous et al. (2006) provided evidence that the apparatus was functional during chronic lung infection of *Pseudomonas aeruginosa* in cystic fibrosis patients. In *Edwardsiella tarda*, T6SS mutant strains were attenuated in blue gourami fish (Zheng and Leung 2007). T6SS has been found to be involved in increased survival within macrophages in a number of bacterial species such as *Aeromonas hydrophila* (Suarez et al. 2008), and *Salmonella enterica* (Parsons and Heffron 2005). In *Vibrio anguillarum*, T6SS was involved in resistance to stress sensing, such as low pH, ethanol, and hydrogen peroxide (Weber et al. 2009). However, bacterial T6SS are not functionally limited to eliciting diseases. They may compromise virulence or play a role in interbacterial competition (Jani and Cotter 2010).

The T6SS1 cluster in *V. parahaemolyticus* was found as divergent by microarray hybridization between pandemic strains and non-pandemic strains (Izutsu et al. 2008). About eighty percent of the *V. parahaemolyticus* pandemic strains harbored five genes (VP1390, VP1401, VP1405, VP1409, and VP1418) of T6SS1, and half of the nonpathogenic strains harbored some of the T6SS1 genes (Chao et al. 2010). However, functions of T6SS systems in *V. parahaemolyticus* remain unclear so far. We have found two *icmF* homologs in *V. parahaemolyticus*, *icmF1* present in putative T6SS1 cluster on chromosome I (vpT6SS1) and *icmF2* in putative T6SS2 cluster on chromosome II (vpT6SS2). In the present study, we attempted to examine if the putative vpT6SS systems are functional in cell adhesion and cytotoxicity by disruption of their putative structural proteins (IcmF1 and IcmF2) or effector proteins (Hcp1 and Hcp2).

Methods

Bacterial strains and plasmids

The bacterial strains and plasmids are described in Table 1. *V. parahaemolyticus* HZ is a clinical isolate from the Zhejiang Provincial Center for Disease Control and Prevention, Zhejiang, China. *E. coli* strains DH5α, BL21, and CC118λpir were used for general manipulation of plasmids, prokaryotic expression of proteins, and mobilization of plasmids into *V. parahaemolyticus*, respectively. The bacterial strains were grown at 37 °C in Luria–Bertani (LB) broth (*E. coli*) or LB broth supplemented with 3 % NaCl (*V. parahaemolyticus*). LB agar supplemented with 3 % NaCl, 10 μg/ml chloramphenicol, and 25 μg/ml polymyxin was used for screening mutant strains. The culture media were supplemented, where appropriate, with the following antibiotics: chloramphenicol (Cm, 10 μg/ml), ampicillin (Amp, 100 μg/ml), and kanamycin (Kana, 50 μg/ml).

Generation of mutant strains

In-frame gene deletion of *icmF1*, *icmF2*, *hcp1*, *hcp2*, *tdh*, *vcrD1* (encoding the T3SS1 structural protein), and *vcrD2* (encoding the T3SS2 structural protein), either alone or multiple (the triple deletion mutant DTTT void of *tdh*, *vcrD1*, and *vcrD2*, or quadruple mutants of DTTT plus additional deletion of *icmF1*, *icmF2*, *hcp1*, or *hcp2*) (Table 1), was generated by *sacB*-based allelic exchange as described previously (Park et al. 2004). Briefly, PCR amplification was performed to generate the upstream and downstream fragments of the target genes (using respective primer pairs A/B and C/D, Table S1). Overlap PCR was performed to construct a fragment with deletion of the target gene using the primer pairs A/D. The fragment was cloned into pMD18T vector (Takara) and then subcloned into an R6 K-ori suicide vector pYAK1 that contains the *sacB* gene conferring sensitivity to sucrose. The recombinant plasmid was introduced into *E. coli* CC118λpir and then mated with *V. parahaemolyticus*. The resulting mutant strains were screened using selective LB agar as specified above.

Gene complementation in mutant strains

For *icmF*1 complementation, the primer pairs picmF1-F and picmF1-R were used to amplify the entire *icmF1* ORF. The PCR product digested with *EcoR*I and *BamH*I was cloned into pMMB207. The resulting plasmid p*icmF1* was transferred into *E. coli* CC118λpir and then conjugated into *V. parahaemolyticus*. A volume of 400-μl overnight culture of *V. parahaemolyticus* containing p*icmF1* was inoculated into 5 ml of LB and incubated for 2 h with shaking at 28 °C before adding 1 mM IPTG. Protein expression was induced at 28 °C for 12 h (Zhou et al. 2008). Complementation of *icmF2*, *hcp1*, and *hcp2* was carried out in similar procedures using specific primer pairs picmF2-F/R, phcp1-F/R, and phcp2-F/R (Table S1).

Table 1 Bacterial strains and plasmids used in this study

Plasmids or strains	Description	Reference or source
Plasmids		
pMD18T	A clone vector, Ampr	Takara
pYAK1	A suicide vector with ori R6K *sacB*; Cm^r	Park et al. (2004)
pMMB207	RSF1010 derivative, *IncQ lacI q* Cmr P*tac oriT*	Zhou et al. (2008)
pET30a	PBR322 origin, PT7, *his*-tag	Novagen
E. coli		
CC118λpir	Λpir lysogen of CC118 Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*	Wang et al. (2007)
DH5α	F^- *φ80lacZΔM15 Δ(lacZYA-argF)* U169 *deoR recA1 endA1 hsdR17 phoA supE44* λ^- *thi-1 gyrA96 relA1*	Invitrogen
BL21		Novagen
V. parahaemolyticus		
HZ	Wild type, clinical strain, Cm^s	This study
Δ*icmF1*	Strain HZ with in-frame deletion of *icmF1*	This study
Δ*icmF2*	Strain HZ with in-frame deletion of *icmF2*	This study
Δ*hcp1*	Strain HZ with in-frame deletion of *hcp1*	This study
Δ*hcp2*	Strain HZ with in-frame deletion of *hcp2*	This study
DTTT	Strain HZ with in-frame deletion of *tdh*, *vcrD1*, and *vcrD2*	This study
DTTT-Δ*icmF1*	Strain DTTT with in-frame deletion of *icmF1*	This study
DTTT-Δ*icmF2*	Strain DTTT with in-frame deletion of *icmF2*	This study
DTTT-Δ*hcp1*	Strain DTTT with in-frame deletion of *hcp1*	This study
DTTT-Δ*hcp2*	Strain DTTT with in-frame deletion of *hcp2*	This study
Δ*icmF1*-p*icmF1*	Δ*icmF1*: pMMB207-*icmF1*, Cm^r	This study
Δ*icmF2*-p*icmF2*	Δ*icmF2*: pMMB207-*icmF2*, Cm^r	This study
Δ*hcp1*-p*hcp1*	Δ*hcp1*: pMMB207-*hcp1*, Cm^r	This study
Δ*hcp2*-p*hcp2*	Δ*hcp2*: pMMB207-*hcp2*, Cm^r	This study
DTTT-Δ*icmF1*-p*icmF1*	DTTT-Δ*icmF1*: pMMB207-*icmF1*, Cm^r	This study
DTTT-Δ*icmF2*-p*icmF2*	DTTT-Δ*icmF2*: pMMB207-*icmF2*, Cm^r	This study
DTTT-Δ*hcp1*-p*hcp1*	DTTT-Δ*hcp1*: pMMB207-*hcp1*, Cm^r	This study
DTTT-Δ*hcp2*-p*hcp2*	DTTT-Δ*hcp2*: pMMB207-*hcp2*, Cm^r	This study

Antibody production

The PCR products harboring *hcp1* and *hcp2* were amplified with primer pairs hcp1-F/R and hcp2-F/R and cloned into expression vector pET30a(+). Protein expression was induced in *E. coli* BL21 with IPTG. The bacterial pellet was washed twice with phosphate buffered saline (PBS) at pH 7.2 and sonicated intermittently for 15 min. His-tagged proteins were purified with nickel column (Bio-Rad). Polyclonal antibodies to Hcp1 and Hcp2 were raised by immunizing female New Zealand white rabbits (Park et al. 2004). Animal use was approved by Zhejiang University Committee for Experimental Animals and Animal Welfare.

Western blot analysis

Secreted proteins from the parent and mutant *V. parahaemolyticus* strains were prepared from supernatant samples of bacterial cultures grown for 16 h at 28 °C in LB broth. Supernatant samples were passed through a 0.22-μm pore-size syringe filter and precipitated by adding trichloroacetic acid to a final concentration of 10 % (vol/vol). The proteins were collected by centrifugation at 15,000 g for 30 min at 4 °C. The precipitates were solubilized in 40-μl 0.1 M NaOH, and 10 μl of 5 × SDS-PAGE loading buffer was added prior to SDS-PAGE with 10 % polyacrylamide. For separation of vpT6SS proteins associated with the bacterial cells, *V. parahaemolyticus* cultures were pelleted by centrifugation, and the pellets were resuspended in PBS (100 mg wet weight per ml). A volume of 160 μl was then mixed with 10 μl of 5 × SDS-PAGE loading buffer, and the mixtures were heat-treated for 5 min in a boiling water-bath to release proteins from the bacterial cells before SDS-PAGE.

Proteins on the gels were electro-transferred onto an Immobilon-P membrane (Millipore). The membranes were blocked with 5 % skim milk in tris-buffered saline (20 mM

Tris, 137 mM NaCl, pH 7.6) containing 0.05 % Tween 20 and probed with anti-Hcp1 or anti-Hcp2 polyclonal antibodies for 1 h at room temperature. The blots were then probed with anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Life Sciences), and developed by the SuperSignala West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer's instruction.

Adhesion assay

HeLa cell monolayers were grown to about 80 % confluency in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % new-born calf serum at 37 °C and 5 % CO_2. Caco-2 cell monolayers were grown to about 80 % confluency in RPMI-1640 medium with 20 % NBCS at 37 °C and 5 % CO_2. Overnight cultures of *V. parahaemolyticus* HZ or its mutants (with or without complementation) were subcultured in LB broth for further incubation at 37 °C for 3 h. The cultures were pelleted by centrifugation and re-suspended in DMEM or RPMI-1640 and adjusted to OD_{600} at 0.20 ± 0.02 (about 10^8 CFU/ml) as stock inocula. Cell monolayers were infected at multiplicity of infection (MOI) of 1:100. After incubation at 37 °C and 5 % CO_2 for 1 h, the monolayers were washed twice with PBS and lysed with 0.01 % Triton-X 100. Serial 10-fold dilutions were made from the lysates and bacteria adhered to the cells were counted on LB agar plates. Percent adherence was calculated as bacterial cells adhered/bacterial cells added into the well × 100. The inoculum sizes of the strains or mutants for all experiments ranged from 2.3 to 3.9 × 10^7 CFU/well. Adhesion of the wild-type strain HZ was initialized as 100 % for comparison with its mutants with or without complementation. When testing the effect of anti-Hcp1 polyclonal antibody on adherence, the anti-Hcp1 serum and control serum (pooled from 3 rabbits not immunized with Hcp1) were added into the adhesion cultures at the final concentration of 1:400.

Cytotoxicity assay

HeLa or Caco-2 cells and cultures of *V. parahaemolyticus* or its mutants were prepared as for the adhesion assay above. The cell monolayers were inoculated with the bacteria at MOI of 1:100 in 500 µl DMEM or RPMI-1640, and incubated at 37 °C and 5 % CO_2 for 2–4 h. The culture supernatants were collected for lactate dehydrogenase activity (LDH) using CytoTox 96 non-radioactive cytotoxicity assay (Promega).

Real-time PCR

To compare transcription levels of *hcp1*, *hcp2*, *icmF1*, and *icmF2* in the wild-type strain, overnight culture of *V. parahaemolyticus* HZ was subcultured in LB broth at 37 °C for 6 h. Total RNA extraction kit (Shanghai Sangon, China) was used to extract mRNA for cDNA synthesis using random primers (Takara). Real-time PCR was performed in a total volume of 20 µl:10 µl of SYBR qPCR Mix (TOYOBO), 0.8 µl of each primer (QF/R-hcp1 and QF/R-hcp2, Table S1) (final concentration 400 nM), 2 µl of cDNA, and 7.2 µl of Milli-Q water. Transcription levels were quantified by linear regression of the C_t values against DNA copies of recombinant pET30a vectors containing *hcp1*, *hcp2*, *icmF1*, and *icmF2* which were calculated (http://www.uri.edu/research/gsc/resources/cndna.html) and run simultaneously with the test samples.

Statistical analysis

The experiments for adhesion assays were repeated three times and all experiments were performed in triplicate wells. Data were expressed as mean ± SD. Student's *t* test was used to extract statistical differences.

Results

icmF1 has higher frequency in clinical *V. parahaemolyticus* strains than non-clinical ones

Bioinformatic analysis reveals two IcmF family genes in *V. parahaemolyticus*, *icmF1* on chromosome 1 and *icmF2* on chromosome 2. PCR typing showed that *icmF1* has different distributions, 90.9 % (30/33) positive in clinical strains, but 25 % (127/507) in non-clinical strains (Table 2), suggesting that *icmF*-related T6SS1 is possibly functional in the pathogenesis of *V. parahaemoyticus*. However, *icmF2* exists in all *V. parahaemolyticus* strains. Gene typing showed that virulence genes *tdh*, *trh*, and *vcrD2* (representing T3SS2, Park et al. 2004) are more prevalent in clinical strains than in non-clinical ones (Table 2). The *icmF1* gene is present in all T3SS2-positive strains.

The putative vpT6SS1 and vpT6SS2 are involved in adhesion to cultured cells

The Δ*icmF1* and Δ*icmF2* mutant strains showed similar growth patterns to their parent strain in vitro (data not shown). In Caco-2 cell monolayers, the *icmF1* mutant had about 53 % lower adhesion than the parent strain HZ (Fig. 1a). In HeLa cells, both Δ*icmF1* and Δ*icmF2* mutants had significantly lower levels of adhesion than strain HZ (Fig. 1b). Since several different *V. parahaemolyticus* cytotoxic factors, including TDH, T3SS1, and T3SS2, can disrupt mammalian cell structures (Nishibuchi and Kaper

Table 2 Distribution of virulence or putative virulence factors in *V. parahaemolyticus* isolates from clinical or non-clinical (seafood and aquatic environments) sources in southeastern China

Source	Total	*icmF1*	*icmF2*	*tdh*	*trh*	*vcrD2*
Clinical strains	33	30 (90.9 %)	33 (100 %)	33 (100 %)	3 (9.09 %)	29 (87.9 %)
Non-clinical strain	507	127 (25.0 %)	507 (100 %)	50 (9.9 %)	99 (19.5 %)	6 (1.2 %)

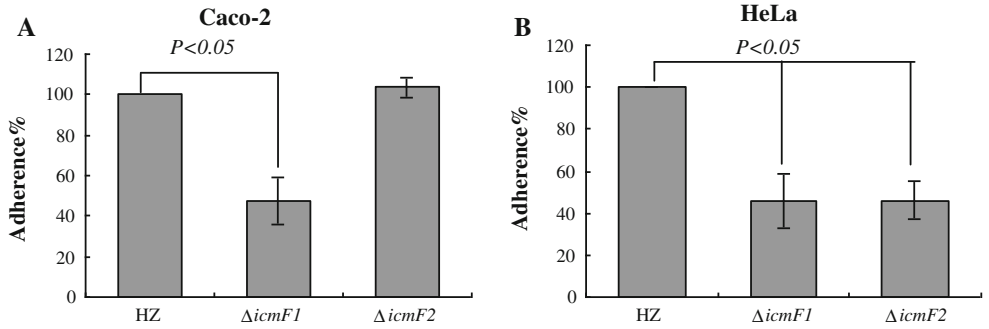

Fig. 1 Adhesion to Caco-2 and HeLa cell monolayers of *V. parahaemolyicus* strain HZ and its mutant strains Δ*icmF1* and Δ*icmF2*. Adhesion of the wild-type strain HZ (added at 2.2–2.8 $\times$ 10^7 CFU to the cell monolayers with post-washing recovery at 0.95–1.75 $\times$ 10^7 CFU for different experiments) was initialized to 100 %. Deletion of *icmF1* reduced bacterial adhesion to both Caco-2 and HeLa cells, while deletion of *icmF2* affected adhesion only to HeLa cells

1995; Park et al. 2004; Okada et al. 2009), we further examined the effect on adhesion contributed by individual icmF1, icmF2, Hcp1, or Hcp2 in the absence of cytotoxic components by making quadruple mutants from the triple deletion mutant void of *tdh*, *vcrD1*, and *vcrD2* (named DTTT, Table 1) with additional deletion of *icmF1*, *icmF*, *hcp1*, or *hcp2* (Table 1). In Caco-2 cell monolayers, deletion of either *icmF1* or *hcp1* had about 64–68 % lower adhesion than DTTT (Fig. 2a). In HeLa cell monolayers, Δ*icmF1*, Δ*icmF2*, Δ*hcp1*, and Δ*hcp2* mutants had significantly lower levels of adhesion than their parent strain DTTT (ranging from 51 to 63 %) (Fig. 2b). Such impaired adhesion was restorable by complementation of the relative genes (Fig. 2). These results suggest that both vpT6SS1 and vpT6SS2 are involved in adhesion to HeLa cells, but only T6SS1 is involved in adhesion to Caco-2 cells.

IcmF2 constitutes the structure of functional vpT6SS2, and Hcp2 is likely the adhesion molecule upon translocation

A canonical T6SS consists of several structural proteins, the innermembrane protein IcmF, two translocon proteins (usually Hcp family protein and VgrG family protein), and some secretion proteins (Cascales 2008). Deletion of structural genes usually prevents protein translocation and secretion (Pukatzki et al. 2006). Genome analysis of *V. parahaemolyticus* shows that *icmF1* and *icmF2* are embedded in two putative T6SS clusters. Therefore, we

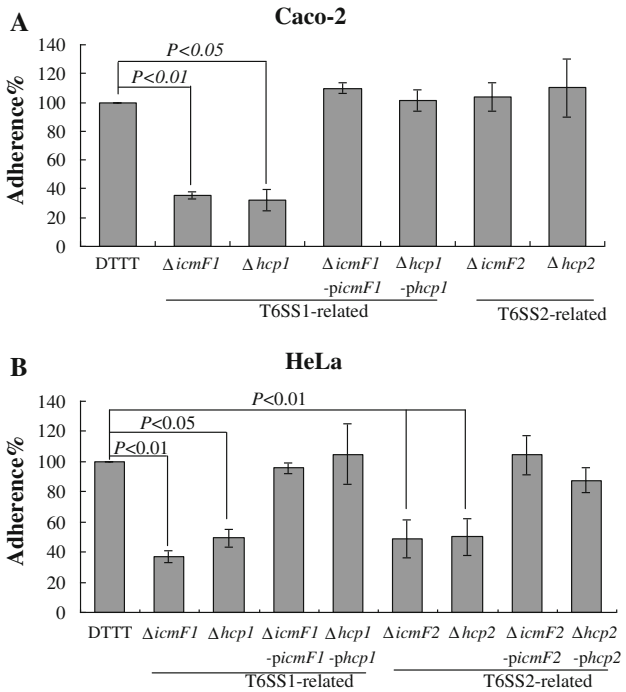

Fig. 2 Adhesion to Caco-2 and HeLa cell monolayers of the triple deletion mutant (DTTT, void of *tdh* as well as T3SS1 and T3SS2 structural protein genes *vcrD1* and *vcrD2*) and its isogenic mutants with additional deletion of *icmF1*, *icmF2*, *hcp1*, or *hcp2*. Reduced adhesion to the cell monolayers due to deletion of T6SS1- or T6SS2-related *icmF* or *hcp* genes was recoverable by genetic complementation. Adhesion of the triple DTTT mutant strain (added at 2.3–3.9 $\times$ 10^7 CFU to the cell monolayers with post-washing recovery at 1.3–2.13 $\times$ 10^7 CFU for different experiments) was initialized to 100 %

examined the putative translocon proteins Hcp1 and Hcp2 in respective clusters in pellets and supernatants of Δ*icmF1* and Δ*icmF2* mutant strains using Hcp1 and Hcp2 polyclonal antibodies. Figure 3 shows that Hcp2 could be detected both in pellet and supernatant from the wild-type strain HZ, but only in the pellet in the Δ*icmF2* mutant (Fig. 3a). However, the supernatant Hcp2 was detectable upon complementation of *icmF2* in the Δ*icmF2* mutant. These results indicate that IcmF2 is the component of the functional T6SS2 for efficient translocation of Hcp2.

Hcp1 has low transcription and expression

Because Hcp1 was not detectable using the enhanced chemiluminescence imaging, we suspected that *hcp1* might have low expression in *V. parahaemolyticus*. We introduced the recombinant expression plasmid p*hcp1* into the wild-type strain HZ or its Δ*icmF1* mutant to see if extrachromosomal expressed Hcp1 could be detectable. Hcp1 was detected only in the pellet samples, but not in supernatants, of the strain HZ and Δ*icmF1* mutant carrying p*hcp1*(Fig. 4b). No target bands were developed either in the pellet or supernatant samples from the corresponding strains containing the control vector pMMB207 (Fig. 4). We further supposed that low expression of Hcp1 might be due to low transcription of *hcp1*. Real-time PCR showed that the transcriptional level of *icmF1* was about 10-fold lower than *icmF2* (4.90×10^3 vs. 4.84×10^4 cDNA copies). The *hcp1* gene had 2.41×10^6 copies, about 17-fold lower than that of *hcp2* (4.22×10^7 copies) (Fig. 4c, d). These results strongly suggest that the putative T6SS1 genes had lower expression and transcription than their counterparts of T6SS2 in *V. parahaemolyticus*. However, Hcp1 did contribute to adhesion to cultured cells, as shown in Fig. 2 as well as by adhesion inhibition assay using anti-Hcp1 antibody, about 50 % lower recovery of the mutant strain DTTT in cultured cells treated with anti-Hcp1 antibody than that using control serum (Fig. 4e), suggesting

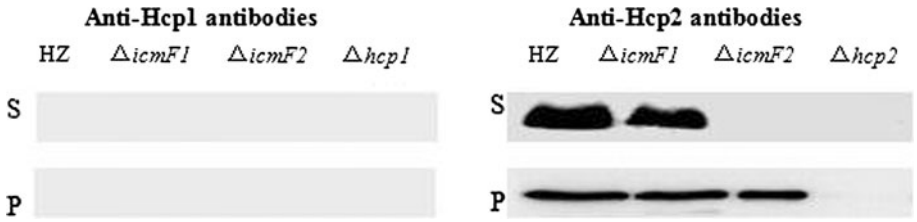

Fig. 3 Western blot analysis of Hcp1 and Hcp2 expression in *V. parahaemolyticus* clinical strain HZ and its mutant strains Δ*icmF1*, Δ*icmF2*, Δ*hcp1*, and Δ*hcp2*. *S* supernatant and *P* pellet

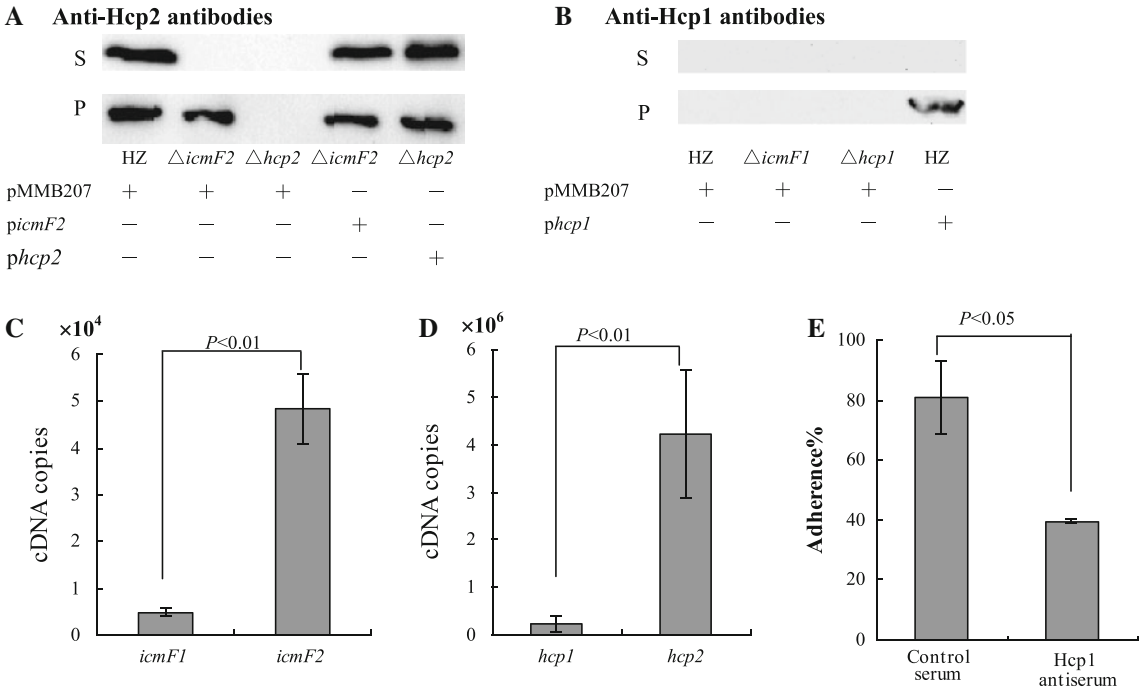

Fig. 4 Expression analysis of T6SS1- and T6SS2-related genes by Western blot (**a**–**b**) and real-time PCR (**c**–**d**, shown as cDNA copies of each target gene), and effect of anti-Hcp1 antibodies on adhesion (**e**). *S* supernatant, *P* pellet

Table 3 Cytotoxicity of *V. parahaemolyticus* clinical strain HZ and its mutants to Caco-2 and HeLa cell monolayers

Strains	Caco-2			HeLa		
	Cytotoxicity %	*P* to HZ	*P* to DTTT	Cytotoxicity %	*P* to HZ	*P* to DTTT
HZ	74.08 ± 11.12			82.28 ± 9.27		
△*icmF*1	74.76 ± 5.91	0.9301		70.65 ± 8.70	0.1882	
△*icmF*2	72.15 ± 16.50	0.8744		87.48 ± 16.15	0.6538	
△*hcp*1	77.85 ± 7.24	0.5818		76.99 ± 0.80	0.1668	
△*hcp*2	74.61 ± 8.51	0.9577		84.33 ± 6.83	0.6992	
DTTT	2.62 ± 1.46	0.0004		18.85 ± 4.93	0.0005	
DTTT-△*icmF1*	3.46 ± 1.67	0.0004	0.5635	6.10 ± 6.10	0.0003	0.0480
DTTT-△*icmF2*	3.42 ± 3.50	0.0005	0.7445	15.82 ± 3.55	0.0003	0.4365

The experiment was repeated three times and all experiments were performed in triplicate. Data were expressed as mean ± SD. "*P* to HZ" indicates statistical differences between the wild-type strain HZ and its deletion mutants, and "*P* to DTTT", statistical differences between the triple deletion mutant (simultaneous deletion of *tdh*, *vcrD1*, and *vcrD2*) and its *icmF1* or *icmF2* deletion mutants

that Hcp1 might be activated once *V. parahaemolyticus* attached to cultured cells by an unknown mechanism.

The putative vpT6SS1 and vpT6SS2 do not mediate cytotoxicity

To examine if putative vpT6SSs are involved in cytotoxic effect on eukaryotic cells, deletion mutants of *icmF1*, *icmF2*, *hcp1*, or *hcp2* were constructed from the parent strain HZ or DTTT. Single deletion of *icmF1*, *icmF2*, *hcp1*, or *hcp2* did not cause significant changes of LDH release from both Caco-2 and HeLa cell monolayers, as compared to their parent strain HZ (Table 3). Deletion of *vcrD1* and *vcrD2* almost abolished the cytotoxic effect of *V. parahaemolyticus* on Caco-2 cells, and also on HeLa cells, but to a lesser extent. Further deletion of T6SS-related genes from the DTTT triple deletion mutant did not have apparent effect on cytotoxicity.

Discussion

Early studies linked gastroenteritis to the presence of *tdh*, *trh*, T3SS1, and/or T3SS2, which caused general cytotoxicity or enterotoxicity (Alam et al. 2002; Yeung and Boor 2004; Park et al. 2004; Okada et al. 2009). Here, we provide evidence that the putative vpT6SS systems contribute to adhesion of *V. parahaemolyticus* to host cells most probably by the translocon proteins Hcp1 and Hcp2, or other unknown factors which were translocated likely by the T6SS structural IcmF family proteins.

The *icmF* family protein, though first found as a component of T4SS in *L. pneumophila* (Sexton and Vogel 2002), has been found as the component of T6SS in several bacterial species such as *V. cholerae*, *P. aeruginosa*, *A. hydrophilia* (Pukatzki et al. 2006; Mougous et al. 2006; Suarez et al. 2008) and participates in bacterial pathogenicity: adhesion to epithelial cells, cytotoxicity, resistance to phagocytosis, and replication inside the host cell (Zheng and Leung 2007; Suarez et al. 2008; Mougous et al. 2006; Bingle et al. 2008; Jani and Cotter 2010; Chow and Mazmanian 2010). By domain blast in EMBL-EBL (http://www.ebi.ac.uk/Tools/pfa/iprscan/), VP1408 and VPA1039 in *V. parahaemolyticus* were found as the T6SS IcmF family proteins and have an *icmF*-related domain at position 489–755 aa of IcmF1 (VP1408) and position 502–805 aa of IcmF2 (VP1039). VP1393 (Hcp1) and VPA1027 (Hcp2) belong to the *hcp1*-like superfamily and have a DUF796 domain (position 6–156aa of Hcp1, 6–134aa of Hcp2) defined as virulence factors and secretion apparatus (Bingle et al. 2008; Boyer et al. 2009).

The Hcp family proteins are considered as effectors of T6SS in other bacterial species (Boyer et al. 2009; Cascales 2008; Jani and Cotter 2010). Here, we show that T6SS2 is an active secretion system and Hcp2 is the translocon of T6SS2. Intriguing to us is that we were not able to detect the presence of Hcp1 in the supernatant or pellet samples of the wild-type strain HZ, although T6SS1 was functional. Real-time PCR and Western blot showed that genes of T6SS1 were in a low transcriptional and expressional level (probably below 3 ng/ml, a level detectable with our enhanced chemiluminescence imaging). Low expression of *hcp1* was also seen in other bacterial species. *V. cholerae* strain N16961 has the VAS cluster (T6SS cluster) genes almost identical to *V. cholerae* strain V52. However, Hcp was not detected in its supernatant or pellets, as contrast to that in the strain V52 (Pukatzki et al. 2006). Recently, LuxO and TsrA were found to regulate T6SS in *V. cholerae* and their deletion led to increased Hcp expression as well as induction of T6SS-dependent fecal diarrhea and intestinal inflammation in infant rabbits (Zheng et al. 2010). In *P. aeruginosa*, HSI-1(T6SS) was believed to be involved in

the course of cystic fibrosis. However, Hcp1 could only be detectable in the mutant strain with deletion of *retS*, a global regulator, but not in the wild-type PAO1 in vitro (Mougous et al. 2006). Therefore, it is possible that vpT6SS1 could be negatively regulated by an unknown factor and might be expressed at higher level in vivo.

However, T6SS systems are not the only factors contributing to adhesion of *V. parahaemolyticus* to host cells. A recent study shows that multivalent adhesion molecule 7 (MAM7) is involved in adhesion of *V. parahaemolyticus* to host cells by forming complex with fibronectin as the host cell ligand (Krachler and Orth 2011).

Adherence is a prerequisite event for bacterial infection. Attachment to epithelial cells is dependent on surface characteristics of both the bacteria and the host cells (Sakarya et al. 2010). We have found apparent differences between vpT6SS1 and vpT6SS2 in adhesion to the cell lines used, with the former being involved in adhesion to both Caco-2 and HeLa cells and the latter to HeLa cells only. This phenomenon might be due to differences between Caco-2 and HeLa cells in their surface characteristics such as receptors interacting with vpT6SS. Since Caco-2 is an intestinal epithelial cell line, T6SS1 may play an important role in enteric infection of *V. parahaemolyticus*.

In conclusion, we reveal that vpT6SS systems, though not involved in cytotoxicity as is the case with other bacterial T6SS (Pukatzki et al. 2006; Suarez et al. 2008), are functional for adhesion to host cells most probably via their Hcp2 and/or Hcp1. Because cytotoxic T3SS2 co-exists with vpT6SS1, both targeting on enterocytes (Park et al. 2004), we postulate that *V. parahaemolyticus* has developed an effective strategy of having both secretion systems functionally cooperative, T6SS1 playing its role in adhesion, the first step of infection, and the effectors exported by T3SS2 inducing enterocytotoxicity.

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