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Variation of genomic islands and flanking fragments in *Vibrio parahaemolyticus* isolates from environmental and clinical sources in Taiwan

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ABSTRACT

Vibrio parahaemolyticus is a halophilic foodborne pathogenic bacterium that causes gastroenteritis; it has become an issue of global concern since the emergence and spread of pandemic O3:K6 strains. This study evaluated the role of *Vibrio* pathogenicity island (VPaI)-associated fragments in the genetic variation and grouping of this pathogen. Distribution of some VPaI fragments and flanking fragments (VPaI-1, VPaI-4, VPaI-5, VPaI-6 and VPaI-7) was determined in a total of 53 *V. parahaemolyticus* isolates from environmental and clinical sources in Taiwan, and supported by the sequences of seven fragments of VPaI-4 and its flanking fragment VP2145. As determined from the distribution of these VPaI-associated fragments, the clinical pandemic isolates were closely related in a single cluster; the clinical nonpandemic isolates were grouped into several clusters, while the environmental isolates were comparatively highly diversified. The profiles of virulence-associated genes of environmental pathogenic isolates varied, and were closer to those of clinical nonpandemic isolates than those of pandemic isolates. Isolates with atypical profiles of the VPaI-associated fragments and virulence-associated genes were identified. Sequences of VP2145 exhibited a close phylogenetic relationship among these local isolates, which were distinct from most *V. parahaemolyticus* strains from other geographic regions. This investigation demonstrated the application of VPaI-associated fragments in studying the genetic variation and clustering of *V. parahaemolyticus* isolates from different sources.

1. Introduction

Vibrio parahaemolyticus, a marine Gram-negative halophilic bacterium, is a prevalent foodborne pathogen in many Asian countries. It has become a globally concerned etiologic agent since the emergence and spread of the pandemic O3:K6 strains after 1996 (Han et al., 2008; Wong et al., 2000). Clinical isolates of *V. parahaemolyticus* typically produce a thermostable direct hemolysin (TDH) and exhibit β-hemolysis on Wagatsuma agar which is known as the Kanagawa-phenomenon (KP). Presence of TDH-related hemolysin (TRH) encoded by *trh* has been identified in some clinical and environmental KP-negative *V. parahaemolyticus* isolates (DePaola et al., 2003; Parvathi et al., 2006), and *trh* and urease genes are in close proximity on chromosome (Park et al., 2000; Parvathi et al., 2006).

Two type III secretion system (T3SS) gene clusters have been identified in *V. parahaemolyticus* (Hiyoshi et al., 2010). T3SS1 is present in all *V. parahaemolyticus* isolates (Jones et al., 2012; Tsai et al., 2013), subtype T3SS2α is typically found with the *tdh* gene and T3SS2β is found with the *trh* gene (Noriea et al., 2010). Some *V. parahaemolyticus* strains also have two type VI secretion systems (T6SSs), whereas T6SS2 is found in all isolates, while T6SS1 is mostly associated with clinical isolates (Boyd et al., 2008; Salomon et al., 2013).

The pandemic O3:K6 strains are genetically distant from other O3:K6 strains isolated before 1996 as evidenced by pulsed-field gel electrophoresis (PFGE) analysis and other molecular typing methods (Kam et al., 2008; Kimura et al., 2008; Matsumoto et al., 2000; Wong et al., 2007; Wong et al., 2000). Meanwhile, clinical strains genetically close to the pandemic O3:K6 strains, such as strains with O4:K68, O1:KUT, O1:K25 or O6:K18 serotypes, were isolated in several countries (Chowdhury et al., 2000a; Chowdhury et al., 2000b; Chowdhury et al., 2004; Wong et al., 2005). Also, the pandemic strains have been identified in the environments of Southern Chile (Fenzalida et al., 2006) and Alaska (McLaughlin et al., 2005) and the low water temperatures in these regions are commonly not suitable for the growth of this pathogen. This information suggests the high genetic variation and adaptation of *V. parahaemolyticus* in its different environmental niches.

Investigations of the genomic islands of *V. parahaemolyticus* may demonstrate their significant role in microbial evolution by their acquisition of novel biological traits through horizontal gene transfer (Hacker and Carniel, 2001). Genomic islands are compositionally

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Table 1
Virulence-associated gene and marker gene profiles of *V. parahaemolyticus* strains examined in this study.

Group[a]	No of Isolate	Isolate	Isolation year	*tlh*[b]	tdh	trh	ureC	toxR	MTase	orf8	vcrD1	vopD	vp1680	vopT	vcrD2	vopD2	vopC	vopP	vopB2
CP	3	08-1196, 09-1157, KX-V231[c]	2008, 2009, 1996	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+
CP	1	2007113	2007	+	+	−	−	+	+	−	+	+	+	+	+	−	+	+	+
CP	1	08-888	2008	+	+	−	−	+	+	+	+	+	+	+	+	−	+	+	+
CP	1	09-1298	2009	+	+	−	−	+	−	+	+	+	+	+	+	+	+	+	+
CP	1	08-1313	2008	+	+	−	−	+	+	+	+	+	+	+	−	−	+	+	+
CN	3	2006024, 2008002, 2008003	2006, 2008	+	+	−	−	+	−	−	+	+	+	+	+	−	+	+	+
CN	1	2007111	2007	+	−	−	−	+	−	−	+	+	+	−	−	−	−	−	−
CN	1	2007122	2007	+	+	−	−	+	+	+	+	+	+	+	+	−	+	+	+
CN	1	2007060	2007	+	+	−	−	+	−	−	+	+	+	+	+	+	+	+	+
CN	1	48215[c]	1991	+	+	+	+	+	−	−	+	+	+	−	+	+	−	−	−
EP	1	SW090307-6	2009	+	−	−	−	+	−	−	+	+	+	−	−	−	−	−	−
EP	1	DO091211-3	2009	+	+	+	+	+	−	−	+	+	+	−	−	−	−	−	−
EP	1	BC100620-2	2010	+	+	−	−	+	−	−	+	+	+	+	−	−	+	+	+
EP	1	SCS1112-1	2011	+	+	−	−	+	−	−	+	+	+	−	+	+	−	+	−
EP	1	SCS1112-2	2011	+	+	−	−	+	−	−	+	+	+	−	+	+	+	+	+
EN	5	YAF1206-2, SCF1206-3, SCF1206-6, SCW1206-2, SCW1206-11	2012	+	−	−	−	+	+	−	+	+	+	−	−	−	−	−	−
EN	31	List[d]	2011, 2012	+	−	−	−	+	−	−	+	+	+	−	−	−	−	−	−

[a] Environmental and clinical isolates were grouped into environmental nonpathogenic (EN), environmental pathogenic (EP), clinical nonpandemic (CN) and clinical pandemic (CP) groups. Strain 48215 (O4:K12), a clinical strain that was isolated in Washington, USA, and strain KX-V231 (O3:K6), a clinical pandemic strain that was isolated in Thailand, were used as references. The clinical nonpandemic isolates vary in their serogroups, which were 2007111 (O4:K4), 2007122 (O1:K25), 2006024 (O4:K8), 2007060 (O3:K29), 2008002 (O8:K41) and 2008003 (O9:K44).

[b] Examined genetic markers include common virulence genes (Tsai et al., 2013), the marker of pandemic strains (orf8), T3SS1 (*vcrD1*, *vopD*, *vp1680*) and T3SS2α (*vopT*, *vcrD2*, *vopD2*, *vopC*, *vopP*, *vopB2*).

[c] Strains KX-V231 and 48215 were used as references.

[d] YAF1206-1, YAS1206-16, YAW1206-3, YAW1206-5, SCS1206-4, XLF1206-2, XLF1206-7, XLS1206-2, XLS1206-3, XLW1206-7, XLW1206-10, YAW1203-1, XLS1203-3, YAS1203-2, YAS1203-5, YAW1112-1, YAS1112-4, SCW1112-2, XLW1112-5, YAS1109-2, YAS1109-10, YAF1109-11, SCW1109-1, SCS1109-4, SCS1109-13, XLS1109-1, XLS1109-15, XLF1109-2, XLF1109-5, XLW1109-3, XLW1109-15.

biased from their host genome with respect to G + C content, dinucleotide frequency and codon usage patterns (Dobrindt et al., 2004; Hacker and Carniel, 2001). The genomic islands of *V. parahaemolyticus* are commonly known as *Vibrio* pathogenicity islands (VPaIs) because some of them encode virulence-associated factors. Seven VPaIs are identified in the pandemic *V. parahaemolyticus* RIMD2210633 genome sequence; they are VPaI-1 (VP0380–VP0403), VPaI-2 (VP0635–VP0643), VPaI-3 (VP1071-VP1094), VPaI-4 (VP2131-VP2144), VPaI-5 (VP2900-VP2910), VPaI-6 (VPA1253-VPA1270) and VPaI-7 (VPA1312-VPA1398) (Boyd et al., 2008; Hurley et al., 2006). VPaI-7, an 81 kb region of chromosome 2, encodes T3SS-2, two copies of the *tdh* gene, a cytotoxic necrotizing factor, an exoenzyme T gene and five transposase genes (Makino et al., 2003), whereas VPaI-7 or the *tdh*-pathogenicity island are closely associated with pandemic and other virulent strains of *V. parahaemolyticus* (Chao et al., 2009; Ronholm et al., 2016). VPaI-4 and VPaI-6 encode putative virulence genes (M protein, hydrolases, cytotoxin integrase, colicins) (Hurley et al., 2006). A putative virulence-associated DNA methyltransferase (MTase) gene is present in the VPaI-I of most pandemic isolates of *V. parahaemolyticus* (Wang et al., 2006). Overall, VPaI-1, VPaI-4, VPaI-5, VPaI-6 and VPaI-7 are often present in the pandemic isolates of *V. parahaemolyticus* (Boyd et al., 2008; Chao et al., 2009; Hurley et al., 2006).

We previously demonstrated that the MTase gene, a gene of VPaI-I, and some other virulence-associated genes are present in a few of the environmental isolates (Tey et al., 2015a; Tsai et al., 2013). Some of the marker genes of VPaI-2 and VPaI-3 were detected in 14–100% of the environmental and the clinical isolates (Tey et al., 2015a), suggesting that fragments of the VPaIs may be distributed among *V. parahaemolyticus* isolates from various environmental niches. These results show that the environmental strains have virulence potential (Li et al., 2014), and the horizontal transfer of these virulence-associated genes in clinical and environmental isolates deeply influence the evolution of this species. Mobile genetic elements, like VPaIs, may play a significant role in the genetic variation of *V. parahaemolyticus* in the environment. Nevertheless, studies of the VPaIs of *V. parahaemolyticus* have tended to concentrate on clinical isolates, such that only a few environmental nonpathogenic or pathogenic isolates have been examined (Hurley et al., 2006).

We recently isolated several pathogenic isolates from aquaculture environments in Taiwan and found that the compositions of their virulence-associated genes varied (Tey et al., 2015a; Tsai et al., 2013). Accordingly, we are able to characterize the different groups of local *V. parahaemolyticus* isolates, including environmental nonpathogenic (EN), environmental pathogenic (EP), clinical nonpandemic (CN) and clinical pandemic (CP) groups, by the distributions of the fragments of VPaI-1, VPaI-4, VPaI-5, VPaI-6 and VPaI-7 and the sequences of eight VPaI-4-associated fragments in some selected isolates. This investigation facilitates the understanding of VPaIs in the genetic variation of this pathogen and evaluates the application of VPaIs in the grouping of *V. parahaemolyticus* isolates.

2. Materials and methods

2.1. Bacterial isolates and cultivation

A total of 53 local *V. parahaemolyticus* isolates were examined in this study, comprising 41 environmental isolates from aquaculture specimens, obtained in 2009–2012 (Tey et al., 2015b; Yu et al., 2012), and 12 clinical isolates from stool specimens during outbreaks from 2006 to 2009, obtained from the Center for Disease Control, Ministry of Health and Welfare, Taiwan (Table 1). These isolates were grouped into four groups by their sources and phenotypic features. The environmental pathogenic group (EP) consisted of five environmental isolates which were positive in KP, while the environmental nonpathogenic group (EN) consisted of 36 environmental isolates without any of the virulence-associated factors. The clinical nonpandemic group (CN)