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Characterization and pathogenicity of acute hepatopancreatic necrosis disease natural mutants, *pir*AB$_{vp}$ (-) *V. parahaemolyticus*, and *pir*AB$_{vp}$ (+) *V. campbellii* strains

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ABSTRACT

Two *Vibrio parahaemolyticus* virulence genes, *pir*A$_{vp}$ and *pir*B$_{vp}$, are known to encode a binary *photorhabdus* insect-related (Pir) toxin that causes acute hepatopancreatic necrosis disease (AHPND) in shrimp. These genes are flanked with repeats of a mobile element (insertion sequence) in a large plasmid. This insertion sequence is closely (92%) related to the known insertion sequence ISVal1. The *pir*AB$_{vp}$ genes and the flanking ISVal1 forms a 5535-bp composite transposon, designated Tn6264. There are *pir*AB$_{vp}$ gene deletions in some strains of *V. parahaemolyticus*. During 2013–2016, we found 2 types of *pir*AB$_{vp}$ deletion mutants from AHPND-affected farms. The type I mutants included 3 strains with deletions (4.4-kb or 6.0-kb) of entire *pir*AB$_{vp}$ genes and the downstream ISVal1, and these mutants were named *pir*AB$_{vp}$(-). The type II mutants included 3 strains with smaller deletions (1.5-kb or 1.6-kb) including a *pir*A$_{vp}$ gene and a partial *pir*B$_{vp}$ gene, and were named *pir*A$_{vp}$(-). In laboratory bioassays, these were not pathogenic to shrimp confirming that both *pir*A$_{vp}$ and *pir*B$_{vp}$ are required for AHPND pathogenicity. During 2016, we also isolated 4 *V. campbellii* strains carrying *pir*AB$_{vp}$ genes from diseased shrimp. These *V. campbellii* stains were found, through laboratory bioassays and histological evaluation, to cause AHPND.

1. Introduction

Acute hepatopancreatic necrosis disease (AHPND, also known as early mortality syndrome, EMS) is a bacterial disease that has caused severe mortalities (up to 100%) in populations of farmed shrimp *Penaeus vannamei* and *P. monodon*. The disease has led to significant economic losses to the shrimp aquaculture industry. Clinical signs of the disease include an empty gastrointestinal tract, milky appearance of the stomach, whitish atrophied hepatopancreas, lethargy, and a soft exoskeleton (Leano and Mohan, 2012). AHPND was first found in China in 2009 and is emerging elsewhere throughout SE Asia including Thailand, Vietnam, Malaysia and the Philippines (Flegel, 2012; Leano and Mohan, 2012; Lightner et al., 2012; Leobert et al., 2015). In 2013, this disease was seen in Mexico (Soto-Rodriguez et al., 2015) and it had spread to Latin America between 2013 and 2015 (Han et al., 2015a).

The causative agent of AHPND was determined to be the unique strains of the *Vibrio parahaemolyticus* (Tran et al., 2013). A binary toxin, PirAB$_{vp}$, that is homologous with the *Photorhabdus* insect-related (Pir) toxin, was shown to be the virulence factor of this disease (Han et al., 2015b; Lee et al., 2015; Sirikharin et al., 2015). Their corresponding genes, named as *pir*AB$_{vp}$ are located in a large plasmid (69–70 kb) in AHPND *V. parahaemolyticus* strains (Yang et al., 2014; Han et al., 2015b). In these plasmids, *pir*AB$_{vp}$ genes are located within a 3.5-kb fragment flanked with inverted repeats of an insertion sequence.

Insertion sequences (ISs) are the simplest form of mobile elements and known to encode transposase involved in insertion and deletion events (Chandler and Mahillon, 2002). These are usually found in the flanking sequences of virulence factor- and antibiotic resistance-coding genes (Kleckner, 1981). In *Vibrio* species, several virulence-related genes, such as cholera toxin (*ctx*) gene and thermostable direct hemolysin (*tdh*) gene, were found to be flanked by ISs (Kaper et al., 1995; Kamruzzaman et al., 2008). In this study, we analyzed an IS element that is located upstream and downstream of the toxin genes, *pir*AB$_{vp}$ in *V. parahaemolyticus* causing AHPND. This IS may be relevant to the deletion mutants of *pir*AB$_{vp}$ isolated from the AHPND-affected shrimp. From 6 *V. parahaemolyticus* deletion mutants, we determined the locations of toxin genes deletions within the plasmids. In addition, we found 4 *V. campbellii* strains from the diseased shrimp that contained *pir*AB$_{vp}$ genes. The pathogenicities of AHPND *V. parahaemolyticus* mutants with *pir*AB$_{vp}$ gene deletions and *pir*AB$_{vp}$-carrying *V. campbellii* strains were evaluated through laboratory infection and subsequent histological examination in *P. vannamei* shrimp.

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2. Materials and methods

2.1. Sequence analysis of IS transposon among AHPND V. parahaemolyticus strains

The IS transposon carrying *pir*AB$_{vp}$ genes located in the plasmid pVPA3-1 (GenBank KM067908) was analyzed in the AHPND-pathogenic strain of *Vibrio parahaemolyticus* 13-028/A3 (JOKE01000000). IS was analyzed by the IS Finder website (http://www-is.biotoul.fr). The functional analyses were also conducted using Blastp at NCBI; the searches for proteins' motifs and domains were carried out using InterProScan at EMBL-EBI websites.

This IS sequence was compared among the whole genome sequence of AHPND-pathogenic strains of *V. parahaemolyticus*, available in the Genbank: M0605 (Mexico, JALL00000000), TUMSAT_DE1_S1, TUMSAT_DE2_S2, TUMSAT_D06_S3 (Thailand, BAVF01000000, BAVG01000000, BAVH01000000), NCKU_TV_3HP, NCKU_TV_5HP (Thailand, JPKS00000000, JPKT00000000) and NCKU_CV_CHN (China, JPKU00000000) using the BLAST program at NCBI website.

2.2. AHPND natural mutants and V. campbellii strains

Six natural mutants strains of AHPND *V. parahaemolyticus* (mutant type I: 13-511/A2, 14-188/7 and 14-231/32.1, and mutant type II: 16-900/1, 16-901/1, and S2) and 4 AHPND *V. campbellii* strains (16-902/1, 16-903/1, 16-904/1 and 16-905/1) were isolated from either stomachs of diseased shrimp or sediment samples from AHPND-affected farms in Vietnam, Thailand, Latin America during 2013–2016 (Table 1). Bacterial identifications were carried out using 16S rRNA sequencing (Weisburg et al., 1991) and *Vibrio*-specific PCR assays targeting *toxR* gene (Kim et al., 1999) and *hly* gene (Haldar et al., 2010).

These bacteria were grown on TSB+ (Tryptic soy broth plus 2% NaCl) at 28–29 °C with gentle shaking (100 rpm) and further tested for AHPND PCR assays with primers targeting *pir*AB$_{vp}$ (Han et al., 2015b) and the upstream of the *pir*A$_{vp}$ (Nunan et al., 2014).

2.3. PCR analysis in regions adjacent to pirAB$_{vp}$ genes

Eight primer sets were designed from the AHPND plasmid pVPA3-1 (GenBank KM067908) (Table 2, Fig. 1A); 2 primer sets (F1/R1 and F2/R2) targeted the region upstream of the *pir*A$_{vp}$, 1 primer set (F3/R3) targeted the *pir*AB$_{vp}$, and 5 primer sets (F4/R4 to F8/R8) targeted the region downstream of *pir*B$_{vp}$. Amplifications were performed using PuReTaq Ready-To-Go PCR beads (GE Healthcare) with the following parameters: initiation denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 7 min. PCR was carried out with template DNA extracted from 6 *V. parahaemolyticus* and 4 *V. campbellii* strains. Amplified PCR products were sent for DNA sequencing at the University Arizona sequencing facility, and the deletion regions were compared to the pVPA3-1 using the BLAST program at NCBI website.

Table 2
PCR primers and their sequences designed from the AHPND plasmid pVPA3-1 (GenBank KM067908).

Primer	Nucleotide sequence (5′-3′)	pVPA3-1
89F	GTCGCTACTGTCTAGCTGAA	13,419–13,438
89R	ACGGCAAGACTTAGTGTACC	13,889–13,908
F1	GGTATCAATGCAGTTTCATTGAGG	15,014–15,037
R1	CTGATGGGAAGCGAAGAGTC	15,586–15,605
F2	GCCGCTCAGATCGTAGTTTC	16,046–16,065
R2	CACAACATCGCCAGCTTTTA	16,777–16,796
F3	GTGGGGAGCTTACCATTCAA	17,340–17,359
R3	ATGCACATCAGAATCGGTGA	18,099–18,118
F4	TACGCAACAAAGCCTTCACA	20,567–20,586
R4	CCTTCGTAGGTCTGCTGGAA	21,311–21,330
F5	GAAAGGCGCTTCCTCCTAGT	21,930–21,949
R5	CGTAGAGCCTCACCGCTATC	22,708–22,727
F6	GTCGGCCCGTTATCAGTTTA	23,294–23,313
R6	GCGATAATCTCATCGGCATT	24,056–24,075
F7	TTGTAGCTGGAGGCGCTATT	24,387–24,406
R7	GTGACGGCTGGAACGATACT	25,113–25,132
F8	TCGTTCGATTTGCTTGTGAG	25,684–25,703
R8	GTTGGGTGCTCTTGGTGTTT	26,368–26,387

2.4. AHPND pathogenicity bioassays and histopathology

We determined pathogenicity of 6 mutant *V. parahaemolyticus* strains and AHPND *V. campbellii* strain by shrimp bioassays through feeding or immersion methods, and at least 2 replicate tanks were used for each bioassay.

In bioassay no. 1, 3 *V. parahaemolyticus* strains (mutant type I) 13-511/A2, 14-188/7 and 14-231/32.1 were tested. Three 90-L tanks were filled with artificial seawater (salinity of 25 ppt and temperature at 28 °C), and specific-pathogen free (SPF) *P. vannamei* ($N = 20$, weights: 0.5–1.0 g) were stocked in each tank. For the infection, each strain was

Table 1
Pathogenicity, origin and year of collection of *Vibrio parahaemolyticus* and *Vibrio campbellii* strains associated with acute hepatopancreatic necrosis disease (AHPND).

Strain	Origin (year)	VpPirA[a]	VpPirB[b]	89F/R[c]	Pathogenicity[d]
AHPND Mutant type I					
13-511/A2	Mexico (2013)	Not detected	Not detected	Pos	Non-pathogenic
14-188/7	Vietnam (2014)	Not detected	Not detected	Pos	Non-pathogenic
14-231/32.1	Mexico (2014)	Not detected	Not detected	Pos	Non-pathogenic
AHPND Mutant type II					
16-900/1	Latin America (2016)	Not detected	Pos	Pos	Non-pathogenic
16-901/1	Latin America (2016)	Not detected	Pos	Pos	Non-pathogenic
S2	Thailand (2015)	Not detected	Pos	Pos	Non-pathogenic
AHPND V. campbellii					
16-902/1	Latin America (2016)	Pos	Pos	Pos	Not determined
16-903/1	Latin America (2016)	Pos	Pos	Pos	Not determined
16-904/1	Latin America (2016)	Pos	Pos	Pos	Pathogenic
16-905/1	Latin America (2016)	Pos	Pos	Pos	Not determined
AHPND V. parahaemolyticus					
13-028/A3[e]	Vietnam (2013)	Pos	Pos	Pos	Pathogenic
C3	Thailand (2015)	Pos	Pos	Pos	Pathogenic

[a] Pos: a positive reaction by PCR targeting the pirAvp genes (Han et al., 2015b).
[b] Pos: a positive reaction by PCR targeting the pirBvp genes (Han et al., 2015b).
[c] Pos: a positive reaction by PCR targeting the plasmid sequences (ORF14/15) (Nunan et al., 2014).
[d] AHPND pathogenicity: determined by laboratory infections and/or histological examination.
[e] AHPND positive control (Tran et al., 2013).