Mechanism study of diversity in AHPND-causing *Vibrio* pathogens driven by T4SS-mediated transfer of pVA1-type plasmid

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Abstract: Acute hepatopancreatic necrosis disease (AHPND) has amounted to over US$7 billion annually in the global shrimp industry, originally shown to be caused by virulent strains of *Vibrio parahaemolyticus*. Accumulating evidences of AHPND caused by *Vibrio* species other than *V. parahaemolyticus* have been reported. The increasing number of *Vibrio* species that cause the disease makes prevention and control more difficult. However, it is unclear the mechanism of diversity in AHPND-causing *Vibrio* pathogens. Here, comparative genome analysis show that almost all AHPND-causing *Vibrio* species harbor the same virulence plasmid (pVA1-type plasmid). We characterized the genetic makeup of pVA1-type plasmid and a new type IV secretion system (T4SS), which is involved in conjugation, was found in pVA1-type plasmid. We established the plasmid conjugation model and investigated the plasmid transfer frequencies among *Vibrio* species. Combining with the infection experiments, high throughput sequencing, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, we showed that conjugative transfer of the pVA1-type plasmid resulted in the formation of new AHPND-causing *Vibrio*. Then, we determined the functions of T4SS essential components through the use of knockout and complementation strains in the conjugation assays. These results demonstrate that genes of T4SS are involved in the transfer of pVA1-type plasmids from pathogenic to non-pathogenic strains of *Vibrio*. Our study provides timely information for better understanding of the causes of the diversity of AHPND-causing *Vibrio* bacteria and provide a novel insight into an issue of major concern for the aquaculture sector globally.

Introduction

Acute hepatopancreatic necrosis disease (AHPND, also known as early mortality syndrome, EMS) is a recently emergent farmed penaeid shrimp bacterial disease. AHPND was first identified in Vietnam and China in 2010 and it is currently causing severe losses of over US$50 billion in the global shrimp industry.

The pathogen of AHPND is *Vibrio* bacteria, including *Vibrio parahaemolyticus*, *V. harveyi*, *V. owensii*, *V. campbellii and V. punensis*. These AHPND-causing *Vibrio* species harbor the same virulent plasmid (pVA1-type plasmid) with genes encoding homologues of the *Photorhabdus* insect-related (Pir) binary toxin PirAB. The pVA1-type plasmid carries a set of genes related to transfer, suggesting that it has the ability to mobilize and transfer from one *Vibrio* cell to another. This set of genes, including conjugative transfer genes, mobilization genes and *pndA* PSK system.

The conjugative genes on the pVA1-type plasmid were annotated to be components of type IV secretion systems (T4SSs), which are versatile assemblages that promote genetic exchange and/or effector translocation. Conjugation transfer mediated by T4SS is one of the important mechanisms of gene horizontal transfer.

In this work, we demonstrate conclusively, through conjugation experiments, that pVA1-type plasmid can be transferred from AHPND-*V. parahaemolyticus* to *V. campbellii*. Our results also demonstrate that genes of T4SS are involved in the transfer of pVA1-type plasmids from pathogenic to non-pathogenic strains of *Vibrio*. The study provides timely information for better understanding of the mechanism of the diversity in AHPND-causing *Vibrio* pathogens.

Materials and methods

Conjugation Experiments with DNase I

Conjugation experiments were carried out using a protocol described by Dong et al. in 2019. The experimental group was donor *Vp*2S01-*Cm*^{r} and recipient *Vc*LMB29.

High-throughput sequencing

To determine if strain *Vc*LMB29-pVPGX1 acquired the pVPGX1-*Cm*^{r}, the bacterial genome was sequenced and compared with that of recipient strain *Vc*LMB29.

Challenge Bioassays

To determine AHPND pathogenicity of the transconjugant *Vc*LMB29-pVPGX1, a challenge study was undertaken. We used three *Vibrio* strains in the study, including *Vp*2S01-*Cm*^{r} (positive), *Vc*LMB29-pVPGX1 (target), and *Vc*LMB29 (negative).

RT-PCR and SDS-PAGE

The strain *Vc*LMB29-pVPGX1 grown to a mid-logarithmichase was pelleted. Total RNA was extracted, and analyzed for the expression of *pirA* and *pirB* in shrimp by RT-PCR using primers VpPirA-284F/R and VpPirB-392F/R. The extracellular extracts from the cultured bacteria were analyzed for the presence of PirA and PirB proteins in an SDS-PAGE.

Construction of knockout mutants of *trbE* and *mobB*

we constructed deletion strains *Vp*2S01-Δ*trbE* and *Vp*2S01-Δ*mobB* with homologous recombination.

Complement of *trbE* and *mobB* genes

The plasmid pRK415 carrying *trbE* was used to construct the complement KO mutant *Vp*2S01-Δ*trbE* and *Vp*2S01-Δ*mobB* and the complements are named as *Vp*2S01::*trbE* and *Vp*2S01::*mobB*.

Results

Conjugation and sequence analysis

In conjugation experiments, filter mating protocol was used to transfer the pVPGX1-*Cm*^{r} plasmid from Vp_{AHPND} strain *Vp*2S01-*Cm*^{r} (donor) to *V. campbellii* strain *Vc*LMB29 (recipient). The result of sequence showed that the *Vc*LMB29-pVPGX1 contains a large (70,053bp, named as pVCON1) plasmid. The pVCON1 displayed a 100% identity to pVPGX1-*Cm*^{r} from donor *Vp*2S01-*Cm*^{r} (Fig. 1).

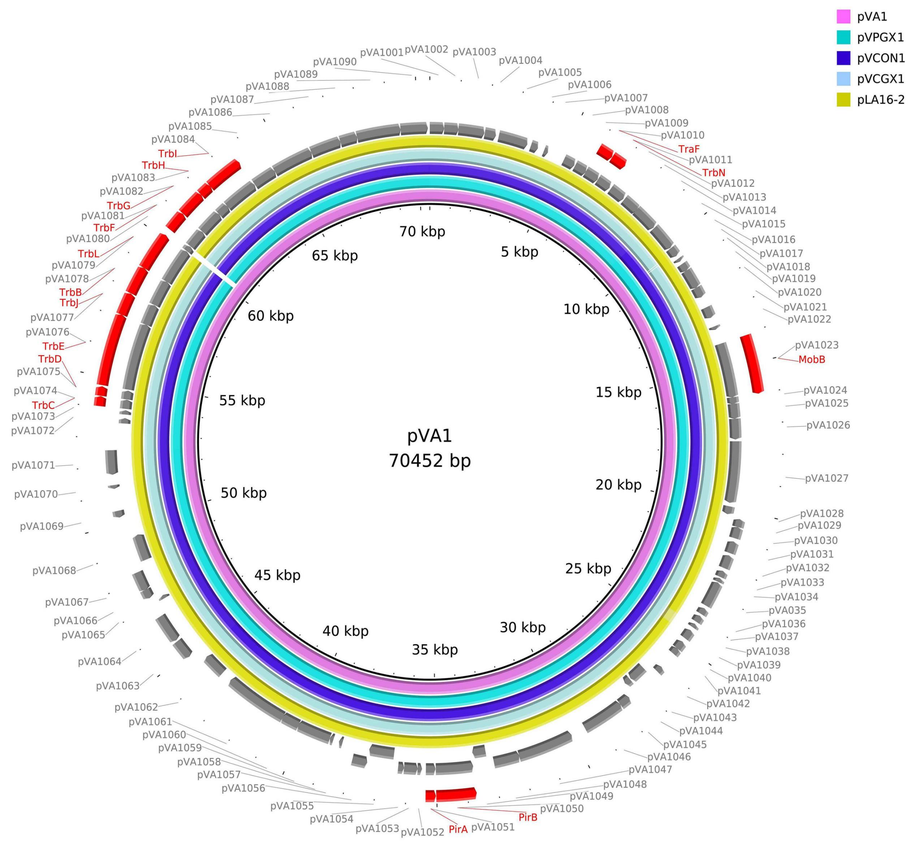

Fig 1. Comparative sequences analysis of pVA1-type plasmids. The reference sequences of pVA1-type plasmids downloaded from NCBI were used for comparative plasmid sequences analysis. The tracks from inside to outside represent the pVA1-type plasmid sequences of pVA1 (KP324996), pVPGX1 (CP020036), pVCON1 (MH890610), pVCGX1 (CP020078), pLA16-2 (CP021148), ORF (+) of pVA1, ORF (–) of pVA1, and marked genes, ORF numbers

Ability of *Vc*LMB29-pVPGX1 to cause AHPND in *P. vannamei*

From the laboratory bioassay, *P. vannamei* exposed *to Vc*LMB29-pVPGX1 or *Vp*2S01-*Cm*^{r} showed typical gross signs of AHPND within 6 h, displaying a pale and atrophied hepatopancreas (HP), and an empty stomach (ST) and midgut (MG) (Fig. 2A); all the exposed shrimp died within 24 h (Fig.2B). The shrimp in the negative control and blank control groups had a normal size HP , dark orange in color, and a full ST and MG.

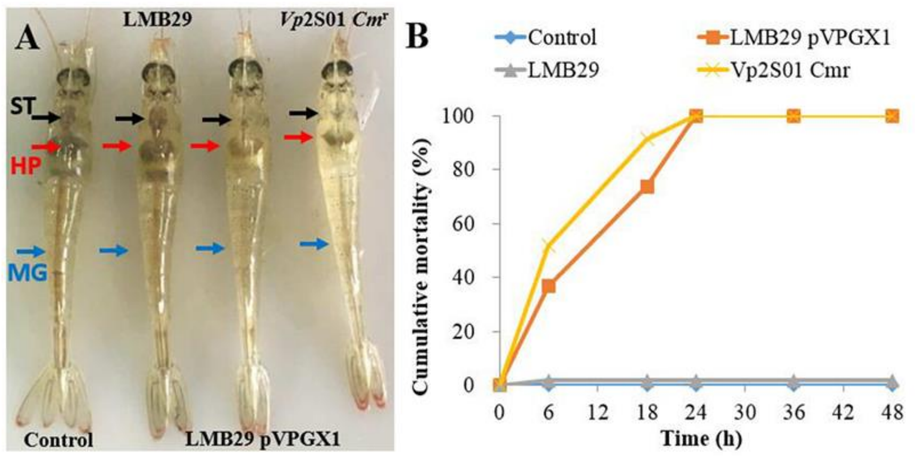

Fig 2. Gross signs and mortality of *Penaeus vannamei* exposed to AHPND-bacteria. (A) Gross signs of AHPND-affected shrimp. (B) Cumulative mortality of shrimp infected with *Vc*LMB29-pVPGX1. shrimp were exposed to *Vibrio* bacteria through immersion infection.

Expression of *pirAB* genes

The bands of both amplificated products appeared at expected sizes of 284 bp from *pirA* and 392 bp from *pirB* in the sample of the shrimp infected with *Vc*LMB29-pVPGX1 (Fig. 3A, lanes 2 and 6). There were no amplification products from the healthy shrimp and the shrimp infected with non-AHPND-causing *V. campbellii* strain *Vc*LMB29 (Fig. 3A, lanes 1 and 5). Furthermore, the result of SDS-PAGE showed that 2 bands, 17 kDa (predicted size of PirA) and 50 kDa (predicted size of PirB), were observed in the sample from strain *Vc*LMB29-pVPGX1 (Fig. 3B, lane 2) and strain *Vp*2S01 (as the positive control, Fig. 3B, lane 3).

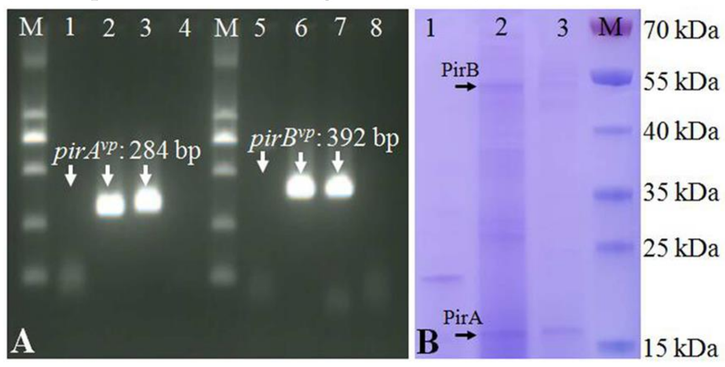

Fig 3. Expression of *pirAB* genes in *Vibrio campbellii* and *V . parahaemolyticus*. (A) RT -PCR detection of *pirA* and *pirB* expression. (B) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of PirA and PirB.

T4SS-mediated pVA1-type plasmid transfer

Based on all the experimental results, we found that the conjugative transfer efficiency between the mutant *Vp*2S01-*Cm*^{r} and the receptor strain *Vc*LMB29 was 10^{-9}~10^{-8}. And the efficiency of mutant *Vp*2S01-Δ*trbE* or *Vp*2S01-Δ*mobB* were 0. Moreover, the efficiency of complement *Vp*2S01::*trbE* and *Vp*2S01::*mobB* were both 10^{-9} (Fig. 4).

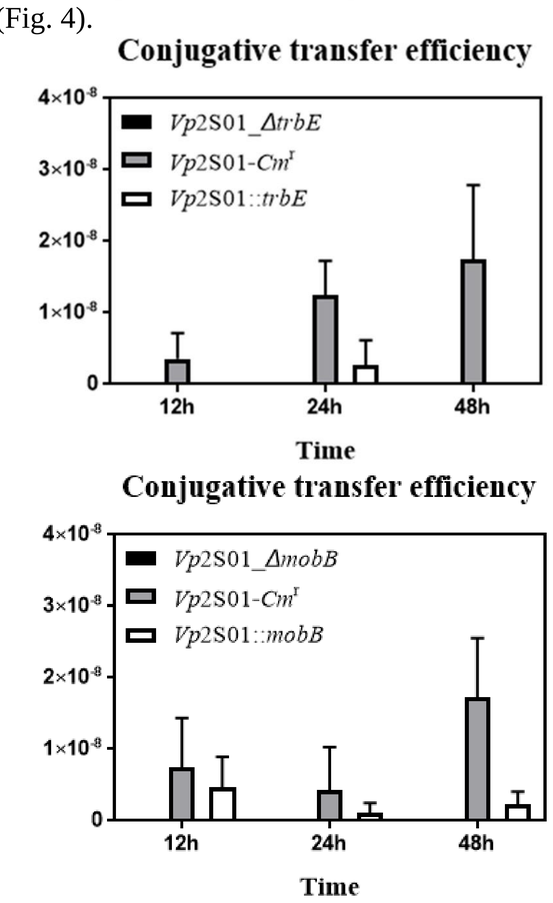

Fig 4. Conjugative transfer efficiency of knockout and complementation strains.

Conclusion

In the current study, the transfer of the pVA1-type plasmid from *Vp2S01-Cm*^{r} to *V. campbellii* was demonstrated through conjugation experiments. All of these results suggested the possible horizontal transfer of pVA1-type plasmids from AHPND-*Vibrio* to non-pathogenic bacteria.

In addition, we determined the functions of T4SS essential components through the use of knockout and complementation strains in the conjugation assays. These results demonstrate that genes of T4SS are involved in the transfer of pVA1-type plasmids from pathogenic to non-pathogenic strains of *Vibrio*.

Acknowledgements

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