Effects of salinity and temperature on the growth and expression of *pirAB* toxin genes of AHPND-causing *Vibrio parahaemolyticus*

ผลของความเค็มและอุณหภูมิต่อการเจริญและการแสดงออกของยีนสร้างสารพิษเพอร์เอบีของ วิบริโอ พาราฮีโมไลติคัส ที่เป็นสาเหตุของโรคตายด่วนในกุ้ง

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ABSTRACT

Acute hepatopancreatic necrosis disease (AHPND) has been listed as a serious infectious disease in farmed shrimp which cause by a specific strain of marine bacterium, *Vibrio parahaemolyticus* (Vp_{AHPND}). The virulence of Vp_{AHPND} is related to PirAB binary toxin which damaged shrimp hepatopancreas. The objective of this study was to examine the effects of temperature (25, 30, and 35 °C) and salinity (1, 3, and 6% NaCl) on the expression of PirAB toxin genes of Vp_{AHPND} PSU5591 previously isolated from AHPND-infected shrimp by quantitative RT-PCR. The results showed that at 35°C, increase in salinity from 1 to 6% significantly up-regulated the expression of both *pirA* and *pirB* ($P < 0.05$). At normal temperature (30°C), increasing in salinity from 1 to 3% was also up-regulated the expression of both genes ($P < 0.05$). This study provides useful information in pathogen adaptation to climate change and could benefit to shrimp aquaculture industries for set up management strategies to control AHPND.

บทคัดย่อ

โรคตับและตับอ่อนตายเฉียบพลัน (acute hepatopancreatic necrosis disease; AHPND) เป็นโรคติดเชื้อรุนแรงในกุ้งเพาะเลี้ยง มีสาเหตุจาก *Vibrio parahaemolyticus* สายพันธุ์ Vp_{AHPND} ซึ่งเป็นแบคทีเรียที่พบทั่วไปในในทะเล ปัจจัยก่อโรคของเชื้อ คือ สารพิษ PirAB ที่ทำให้เกิดการทำลายตับและตับอ่อนของกุ้ง การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของอุณหภูมิ (25, 30 และ 35 องศาเซลเซียส) และความเค็ม (NaCl ร้อยละ 1, 3 และ 6) ต่อการแสดงออกของสารพิษ PirAB ใน Vp_{AHPND} PSU5591 ที่แยกได้จากกุ้งติดเชื้อโดยวิธี quantitative RT-PCR ผลการศึกษาพบว่าที่อุณหภูมิปกติ (30 องศาเซลเซียส) ความเค็มที่เพิ่มขึ้นจากร้อยละ 1 เป็น ร้อยละ 3 และที่อุณหภูมิ 35 องศาเซลเซียส ความเค็มของเกลือที่เพิ่มขึ้นจากร้อยละ 1 เป็น ร้อยละ 6 ทำให้ยีน *pirA* และ *pirB* มีการแสดงออกสูงขึ้นอย่างมีนัยสำคัญ ($P < 0.05$) การศึกษานี้ได้ให้ข้อมูลสำคัญเกี่ยวกับการปรับตัวของเชื้อก่อโรคต่อการเปลี่ยนแปลงสภาพภูมิอากาศ และเป็นประโยชน์ต่ออุตสาหกรรมการเพาะเลี้ยงกุ้งในการจัดเตรียมแนวทางการจัดการควบคุมโรค AHPND

Keywords: *Vibrio parahaemolyticus*, PirAB, Climate change

คำสำคัญ: วิบริโอ พาราฮีโมไลติคัส สารพิษเพอร์เอบี การเปลี่ยนแปลงสภาพภูมิอากาศ

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Introduction

Vibrio parahaemolyticus is a Gram-negative, rod-shaped, halophilic bacterium belonging to the Family Vibrionaceae which can normally be found as free-living organism in marine environments or associated with aquatic animal (Davis and Sizemore, 1982). The specific strain or Vp_{AHPND} can cause acute hepatopancreatic necrosis disease (AHPND), originally known as an early mortality syndrome (EMS), in farmed shrimp. This disease affects the important commercial shrimp species, including black tiger prawn (*Peneus monodo*n) and white leg shrimp (*Litopeneus vannamei*) in post larvae stage and often causes up to 100% mortality (Tran et al., 2013). AHPND has led to significant economic losses to shrimp farming industries worldwide, especially in Southeast Asian countries (Babu et al., 2021).

The binary PirAB toxin are recognized as essential virulence factors associated with Vp_{AHPND} pathogenesis and have lethal effect on shrimp hepatopancreas (Mai et al., 2020). These PirAB toxins of Vp_{AHPND} are encoded by *pir*-like toxin genes *pirA* and *pirB* genes, respectively and are located on a 63- to 70-kb plasmid, named pVA1 (Li et al., 2017). The *pirAB* genes are showed to be homologs to the insecticidal *pirAB* genes of *Photorhabdus* sp. However, the pVA1 plasmid was also found to contain genes involved in gene transfer, including a cluster of conjugative transfer genes, and mobilization genes which suggests that pVA1 plasmid and/or some of its genetic elements are potentially able to transfer to other bacteria (Duchaud et al., 2003).

Many studies report that not only the immune systems of aquatic animals, but also the virulence of many marine pathogens is modulated by environmental factors, especially the global climate change (Cohen et al., 2018). Rising in ocean temperatures affect the evaporation of ocean water and then led to increase the salinity. Change in the expression of virulence-related genes have been reported in aquatic pathogens, including fish pathogens (*Yersinia ruckeri*, *Flavobacterium psychrophilum*, *Lactococcus garvieae*, *Aeromonas hydrophila*, *Edwardsiella tarda*, *Aliivibrio salmonicida*), shrimp pathogen *V. harveyi*, and coral pathogen *V. coralliilyticus* (Guijarro et al., 2015; Montánchez et al., 2019; Kimes et al., 2012). For Vp_{AHPND}, temperatures variation patterns has been shown to affect the specific growth rate and protein production of AHPND (Chirapongsatonkul et al., 2018). The effects of NaCl concentration and temperature on expression of *pirA* and Vp_{AHPND} virulence have been demonstrated in AHPND strain isolated from shrimp farm in Mexico (López-Cervantes et al., 2021). To the best of our knowledge, few studies have been reported on the expression of AHPND toxins. The results obtained in this study could provide the important information related to prevention and control of Vp_{AHPND} infection in farmed shrimp.

Objective of the study

The aim of this study was to examine the effects of cultivation temperature and salinity on the expression of *pirAB* genes expression of AHPND-causing *Vibrio parahaemolyticus*.

Materials and methods

Bacterial isolates and Growth conditions

In this study, Vp_{AHPND} PSU5591 isolated from AHPND-infected shrimp was recovered from previously preserved glycerol stock cultures stored at -80°C. (Kongrueng et al., 2014). It was grown on Tryptic Soy Agar (TSA) supplemented with 1% NaCl.

Growth curve assay

Vp_{AHPND} PSU5591 was cultured in TSA supplemented with 1% NaCl and incubated at 30°C for 16-18 hours. Then, individual colonies were transferred to 2 ml of Luria-Bertani (LB) broth containing either 1, 2, or 3% NaCl. The cultures were individually incubated at either 25, 30, and 35°C (combination with salinity) with agitation at 150 rpm for 18 hours. The optical density (OD_{600}) was measured at 0, 2, 4, 6, 8, 10, and 12 hours after incubation using a microplate reader (LUMIstar Omega, BMG Labtech, Germany) to follow bacterial growth.

Determination of *pirAB* gene expression by RT-PCR

Total RNA extraction

Vp_{AHPND} PSU 5591 was cultured for 18 hours under the combination of 3 temperatures and 3 salinities as describe above. The cultures were incubated with agitation at 150 rpm for 18 hours. RNA was extracted using the Quick-RNA Miniprep Plus kit (Zymo Research, USA) using the protocol described by manufacturer. The concentration and quantification of RNA were measured with MaestroNano spectrophotometer (Maestrogen, Taiwan). All extracted RNA were stored at -80°C.

RT-qPCR assay for *pirAB* gene expression

The extracted RNA was treated with DnaseI (Solis BioDyne, Estonia) and used to produce cDNA using the FIREScript RT cDNA Synthesis Kit (Solis BioDyne, Estonia) according to the manufacturer's protocol. The conditions and components for reverse transcription are shown in Table 1. The concentration of cDNA was examined using MaestroNano spectrophotometer (Maestrogen, Taiwan). RT-qPCR was performed on the cDNA using primers specific to *pirAB* toxin genes (Table 2). The 16s rRNA gene was used as a reference gene. Conditions and components of RT-qPCR is shown in Table 3. The relative expression amount of *pirAB* genes was calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (Ct_{pir,treatment} - Ct_{16S,treatment}) - (Ct_{pir,control} - Ct_{16S,control})$ (Livak and Schmittgen, 2001). Each treatment was run in three biological replicates.

Statistical analysis

The data were analyzed by one-way ANOVA using SPSS Statistics v. 17 (IBM, NY, USA). The $P < 0.05$ indicated the statistical significance.

Table 1. Components and conditions of the reverse transcription.

Component	Volume (µl)	Condition
DI water	to 20	25ºC 10 min
5µM oligo(dt) primer	1	37ºC 20 min
dNTP mix (2 µmol/µl)	0.5	85ºC 5 min
10X RT Reaction buffer DTT	2	
10U FIREScript Reverse transcriptase	1	
1U RiboGrip Rnase inhibitor	0.5	
RNA (1 µg/µl)	x	
Total volume	20	

Table 2. Primers used for RT-qPCR analysis of *pir*AB gene expression.

Primers	Sequence (5'-3')	*Reference*
16S rRNA	F: TATCCTTGTTTGCCAGCGAG	Ma et al., 2015
	R: CTACGACGCACTTTTTTGGGA	
*pir*A	F: TGAAACTGACTATTCTCACGATTG	Cruz-Flores et al., 2019
	R: TGATAGGTGTATGTTTGCTGTC	
*pir*B	F: TCACGGCTTTGAACATATGC	
	R: CATCTTCCGTACCTGTAGCA	

Table 3. Components and conditions for RT-qPCR analysis.

Component	Volume (µl)	Condition	Cycle
DI water	to 20	95ºC 60s	
Luna Universal qPCR Master mix	10	95ºC 15s	
2µM Primer-F (0.4 µM)	0.5	60ºC 30s	40
2µM Primer-R (0.4 µM)	0.5	72ºC 20s	
cDNA (10 ng)	x		
Total volume	20		

Results

Growth curve

In this study, the growth of Vp_{AHPND} PSU 5591 was measured at the combination of different temperatures (25, 30 and 35°C) and salinities (1, 3, 6% NaCl). The strain was able to grow well at all tested conditions as indicated by the OD_{600} between 1.5-2.3 at peak growth **(Fig. 1-3)**. The highest cell densities were observed in the strain grown at 30°C, for 8 to 12 hours. For all temperatures, the strain appeared to grow fast at 6% NaCl than other salinities tested in this study **(Fig. 2)**. At 35°C, the strain reached stationary phase (6 to 8 h) faster than the strain grown in lower temperatures **(Fig. 3)**.

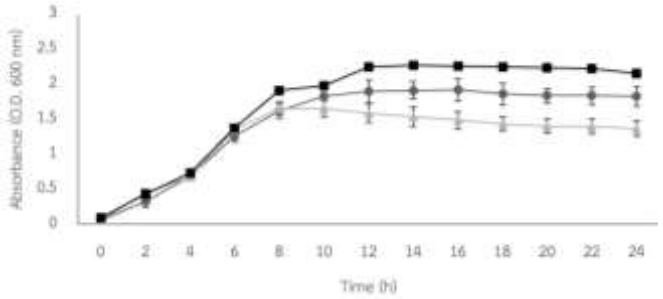

Figure 1. Growth kinetics of Vp_{AHPND} PSU 5591 under temperatures at 25°C in the presence of 1%NaCl (□), 3% NaCl (□), and 6% NaCl (■).

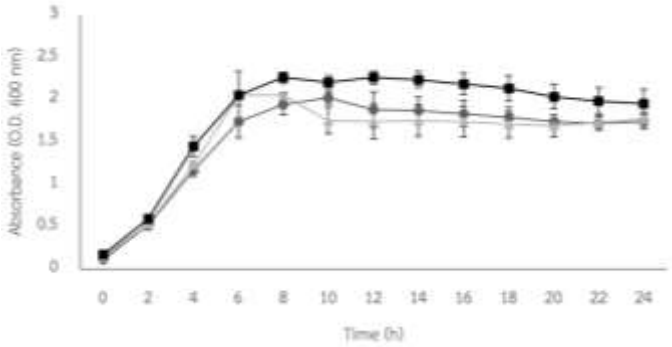

Figure 2. Growth kinetics of Vp_{AHPND} PSU 5591 under temperatures at 30°C in the presence of 1%NaCl (□), 3% NaCl (□), and 6% NaCl (■).

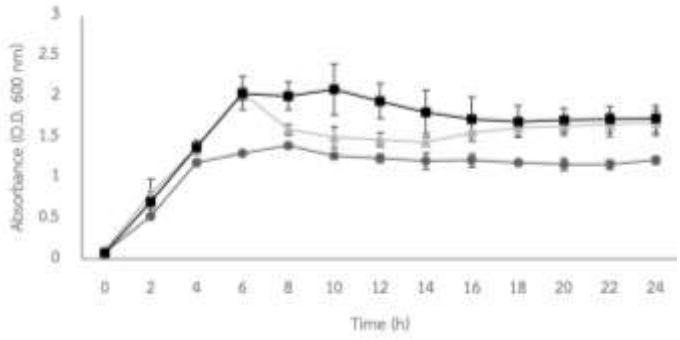

Figure 3. Growth kinetics of Vp_{AHPND} PSU 5591 under temperatures at 35°C in the presence of 1%NaCl (□), 3% NaCl (□), and 6% NaCl (■).

Determination of *pirAB* gene expression by RT-PCR

In this study, the expression of PirAB toxin genes of VP_{AHPND} PSU 5591 grown at differences cultivation temperatures and salinities were investigated by real-time RT-PCR. RNA from cells harvested at 18 hours of growth were extracted and used for analysis. No significant differences in PirAB expression were found among cells grown in all conditions at 25°C compared with control **(Fig.4A)**. The expression of *pirA* (2.57 fold) and *pirB* (2.81 fold) was non-significantly up regulated for cells grown in the presence of 6% NaCl at 35°C when compared with the cells grown in normal condition (1% NaCl, 30°C) **(Fig.4C)**. However, significant up-regulation of both *pirA* and *pirB* expression were found among cells grown in the presence of 3% NaCl at 30°C **(Fig.4B)** and 6% NaCl at 35°C **(Fig.4C)** in comparison with cells grown in the presence of 1% NaCl.

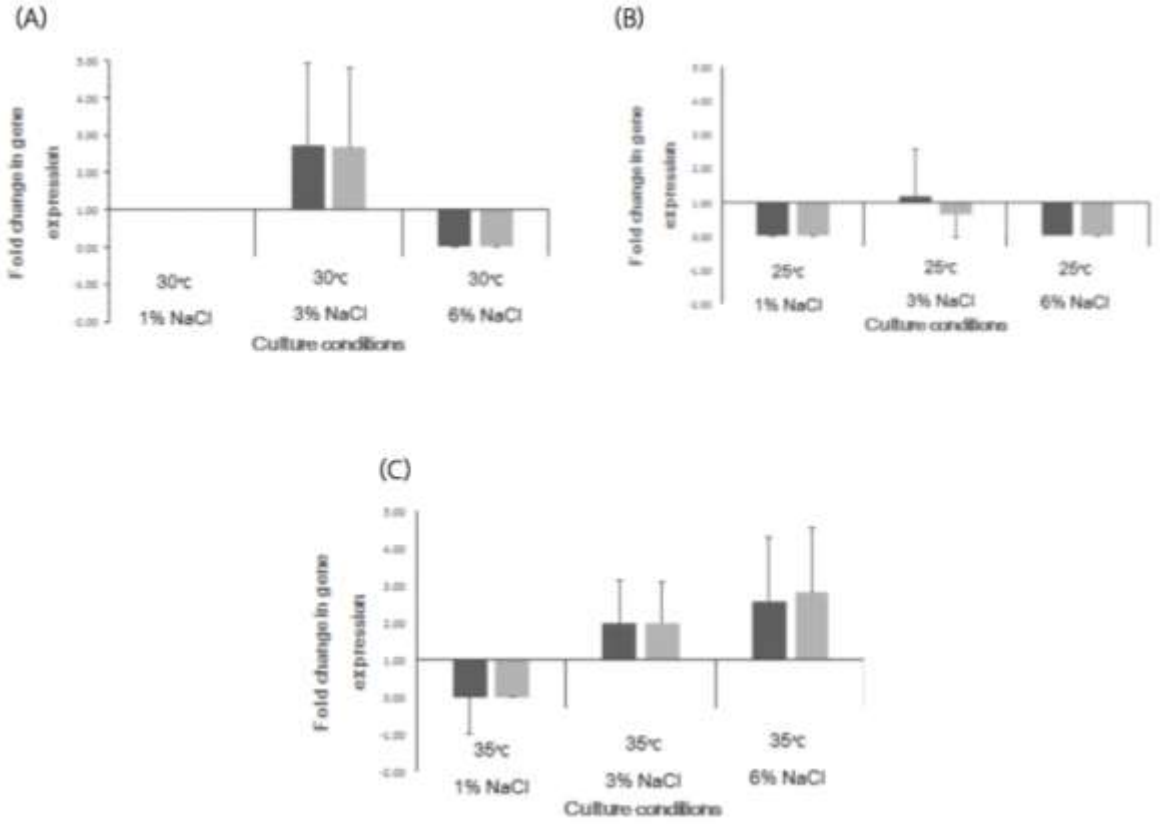

Figure 4. Expression of PirAB toxin genes of VP_{AHPND} PSU 5591**.** Bacterial cultures were grown in Luria-Bertani (LB) broth containing either 1, 2, or 3% NaCl and incubated at either (A) 25°C, (B) 30°C, or (C) 35°C. The data represent fold change of PirAB expression in VP_{AHPND} grown in each condition compared to the VP_{AHPND} control which was grown in the presence of 1% NaCl at 30°C. The experiments were done in triplicate and each bars represents the mean with standard deviations (SD).

Discussion and Conclusions

This study examined the effect of environmental factors, including temperatures and salinity, on growth and expression of PirAB toxin gene of Vp_{AHPND} PSU 5591. This strain was isolated from farmed shrimp infected with AHPND. Recently, the horizontal transfer of a pVA1-type plasmid among *Vibrio* spp. still not well known (Dong et al., 2019). Previous research has shown that *V. parahaemolyticus* strains cannot grow at low temperature (10°C) but can grow at high NaCl concentrations (9%) (Fujikawa et al., 2009).

The result of this study growth kinetics of Vp_{AHPND}, at high temperature (35 °C) show bacterial has entered a stationary phase faster than the strain grown in lower temperatures (25 °C and 30 °C). The expression of *pirA* and *pirB* were found significant up-regulation in the presence of 3% NaCl at 30°C and 6% NaCl at 35°C in comparison with cells grown in the presence of 1% NaCl. Temperature can markedly influence bacterial development and some virulence-related capacities (Larsen et al. 2004). In 2004 Cheng reported the incubation of *V. parahaemolyticus* under optimal conditions containing 2.5–3.5% NaCl significantly enhanced its virulence for *H. diversicolor supertexta* (Cheng et al., 2004).

Previous study reported about the variation in temperatures and salinity could affect the expression of Vp_{AHPND} toxins. The PirA expression was found to be higher in the cells grown in the presence of 3.5% NaCl followed by 3.0% NaCl, and less expression at 2.0% NaCl which suggested to be result from the role of Na^+ in signal transduction pathway (López-Cervantes et al., 2021). In addition, the authors also reported those temperatures could also influence bacterial adaptation and some virulence-related capacities. The results of this study were correlate with previous report.

In conclusion, the results of this study found that Vp_{AHPND} was found to be more virulence in cells grown under high temperature and salinity as *pirAB* were up regulated. Thus, to reduce to losses caused by AHPND infection in shrimp aquaculture, temperatures and salinity should be regularly monitored and controlled. In addition, more virulence study in shrimp model is needed in order to confirm the *in vitro* data.

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