



Genome Characterization and Comparison of Early Mortality Syndrome Causing *Vibrio parahaemolyticus* *pirAB*^{VP}- Mutant From Thailand With *V. parahaemolyticus* *pirAB*^{VP}+ AHPND Isolates

Nalumon Thadtapong^{1,2}, Marvin Bryan Segundo Salinas³, Varodom Charoensawan^{4,5,6}, Vanvimon Saksmerprom^{2,7} and Soraya Chaturongakul^{1,6*}

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*Correspondence:

Soraya Chaturongakul
soraya.cha@mahidol.ac.th

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¹ Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand, ² Center of Excellence for Shrimp Molecular Biology and Biotechnology, Faculty of Science, Mahidol University, Bangkok, Thailand, ³ Department of Morphophysiology and Pharmacology, College of Veterinary Science and Medicine, Central Luzon State University, Muñoz, Philippines, ⁴ Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand, ⁵ Integrative Computational BioScience (ICBS) Center, Mahidol University, Nakhon Pathom, Thailand, ⁶ Systems Biology of Diseases Research Unit, Faculty of Science, Mahidol University, Bangkok, Thailand, ⁷ National Center of Genetic Engineering and Biotechnology (BIOTEC), Pathumthani, Thailand

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BACKGROUND

Early mortality syndrome (EMS) has emerged as a devastating disease in shrimp production aquaculture since it was firstly discovered in China (2009), and onward in Malaysia (2011), Thailand (2012), and Mexico (2013) (Thitamadee et al., 2016). This disease leads to 100% shrimp mortality within 30 days after the infection and thus, economic loss of over USD 1 billion per annum worldwide (de Schryver et al., 2014; Zorriehzahra and Banaederakhshan, 2015). *Penaeus monodon* and *P. vannamei* shrimps in the post larva stage are the hosts of the EMS causative Gram-negative bacterium, *V. parahaemolyticus* (Flegel, 2012).

V. parahaemolyticus is a Gram-negative bacterium inhabiting in marine and estuarine environments (Tran et al., 2013). The same bacterium is also one of human foodborne pathogens that cause gastroenteritis after consumption of contaminated seafood (Letchumanan et al., 2014). Toxins of *V. parahaemolyticus* acting as porins in enterocyte plasma membrane and interrupting ion flux in intestine are thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH). TDH and TRH associate with *V. parahaemolyticus* hemolytic and cytotoxicity activities in the host. Another hemolysin is thermolabile hemolysin (TLH), which also causes red blood cell lysis (Wang et al., 2015). Moreover, asymptomatic infection or presence of pathogenic strains in shrimp farms or marine environments can be a reservoir for human infection.

Nowadays, EMS-causing *V. parahaemolyticus* isolates have been classified into two groups based on their histological signs: acute hepatopancreatic necrosis syndrome (AHPND) and non-AHPND (Joshi et al., 2014). AHPND *V. parahaemolyticus*, e.g., the 5HP strain, carries a 69-kb plasmid named pVA1 that contains *V. parahaemolyticus* *Photobacterium*, insect-related (Pir) toxin-like genes, *pirA*^{VP} and *pirB*^{VP} (Lee et al., 2015; Lin et al., 2017). These PirAB^{VP} toxins damage the

hepatopancreatic cells by necrosis, called sloughing cells. The shrimps infected by the AHPND *V. parahaemolyticus* normally completely die within 30 days. Gross signs of infected shrimps by AHPND strains show empty stomach and midgut, and pale hepatopancreas (Zorriehzahra and Banaederakhshan, 2015). On the other hand, the non-AHPND strains, e.g., the 2HP strain, cannot produce PirAB^{VP} toxins (Joshi et al., 2014). Infected shrimps by the non-AHPND strains still show empty stomach and pale hepatopancreas. Hepatopancreas tubule epithelia show thinness and numerous vacuoles in E-cell or collapsed cells. In immersion challenge testing, 2HP causes 75% of shrimps to die within 96 h-post infection; in contrast, the AHPND isolates (1D, 3HP, 5HP, and “China”) cause 100% mortality in shrimp in the same period of time (Joshi et al., 2014). 2HP may maintain other virulence factors that causes collapsed lesion in shrimp. Recently, we learned that the non-AHPND *V. parahaemolyticus* 2HP carries three plasmids: 183-kb pVP2HP, 65-kb pVP2HPa, and 50-kb pVP2HPb (Theethakaew et al., 2017). The 183-kb pVP2HP shares genetic element of combined 69-kb pVPE61a (containing *pirA*^{VP} and *pirB*^{VP} genes) and 118-kb pVPE61b, but *pirA*^{VP} and *pirB*^{VP} genes are absent (Theethakaew et al., 2017). However, virulence factors in non-AHPND *V. parahaemolyticus* that cause collapsed lesion and lead to EMS pathogenesis are still unclear. The virulence factor candidates in the non-AHPND *V. parahaemolyticus* 2HP might be maintained in its chromosomes (I and II). In order to provide better insights into the virulence factors in the non-AHPND *V. parahaemolyticus*, for the first time to the best of our knowledge, we performed whole genome sequencing of *V. parahaemolyticus* 2HP, and comprehensively compare with the existing genomes of the AHPND *V. parahaemolyticus* isolates (3HP, 5HP, and China), as well as non-pathogenic *V. parahaemolyticus* S02 (Yang et al., 2014). The virulence genes and pathogenicity islands were predicted in order to explore putative factors that might be involved in non-AHPND EMS pathogenesis. In addition to the pathogenic protein-coding genes, non-coding RNAs (ncRNAs) are one of the factors regulating many processes of cell and affect protein expression or activity (Gottesman and Storz, 2011). Some ncRNAs involve in regulation of virulence factors and pathogenesis (Huang et al., 2015; Wang et al., 2016; Pappesch et al., 2017). Thus, we also predicted ncRNAs from the newly sequenced non-AHPND *V. parahaemolyticus* 2HP to investigate its involvement in EMS pathogenesis. In addition, antibiotic resistant genes were predicted which suggested the incident of drug resistance in the shrimp pathogen. The genome sequences of non-AHPND and AHPND isolates from Thailand and other Asian countries were compared to elucidate common and unique mechanisms of pathogenesis in EMS-causing *V. parahaemolyticus*.

DATA DESCRIPTION

Bacterial Strain, Culture Conditions, and Genomic DNA Extraction

V. parahaemolyticus 2HP was cultured in tryptic soy broth (TSB) supplemented with 1.5% NaCl. The bacterial culture was

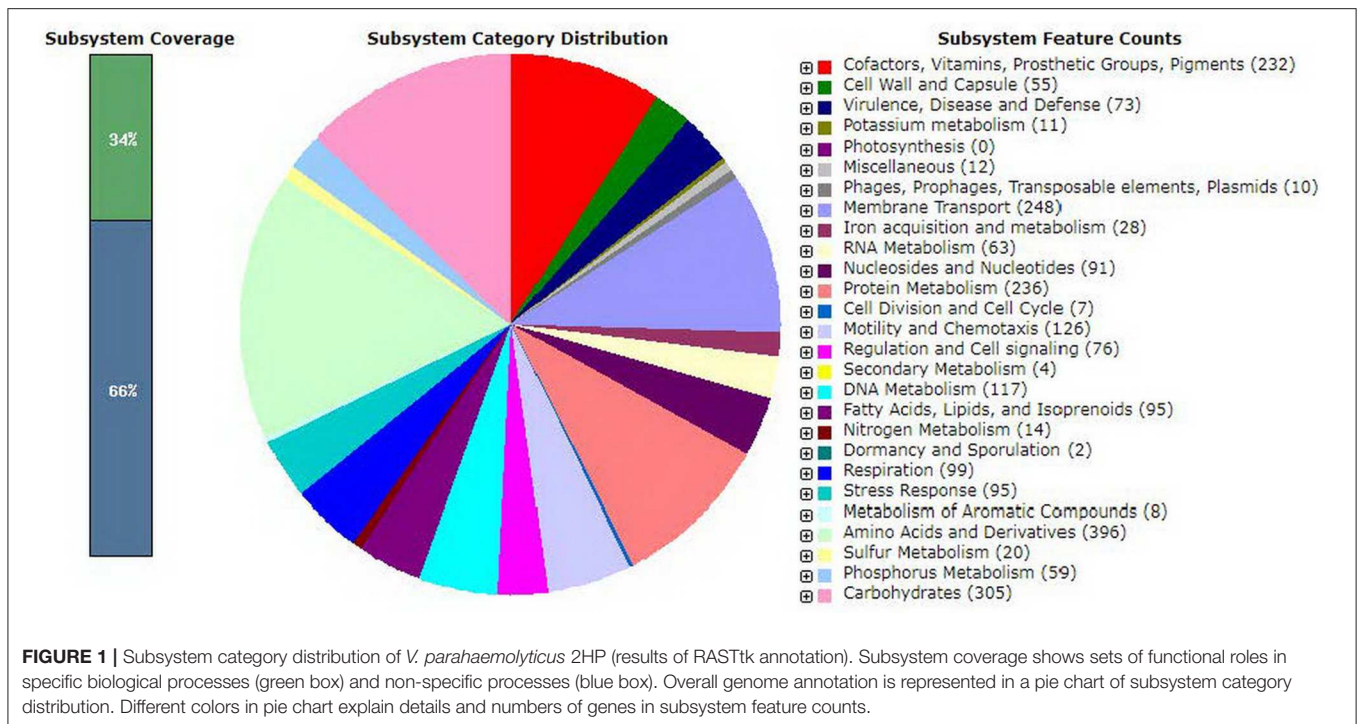
incubated at 30°C and shaken at 200 rpm overnight. Then, bacterial cells were collected by centrifugation. Genomic DNA of *V. parahaemolyticus* 2HP was extracted by the QIAamp DNA Micro kit (Qiagen, Hilden, Germany). Quantity and quality of extracted genomic DNA was measured by the Denovix spectrophotometer (Denovix, DE, USA) and 1.5% agarose gel electrophoresis.

Genomic Sequencing and Annotation

Whole genome sequencing library of genomic DNA was prepared by the QIAseq FX DNA library kit (Qiagen, Hilden, Germany) and the library was sequenced on the Illumina HiSeq 2500 platform (Illumina, CA, USA). Sequences were trimmed and assembled *de novo* by the CLC genomic workbench version 11.0.1 (CLC bio, Denmark). *De novo* assembled contigs were evaluated for quality, and genome size was estimated against *V. parahaemolyticus* RIMD2210633 (Makino et al., 2003) used as the reference genome using QUASt (Gurevich et al., 2013). Non-coding RNA, rRNA, and tRNA were predicted using Infernal (Kolbe and Eddy, 2011), RNAMmer (Lagesen et al., 2007) and Aragorn (Laslett and Canback, 2004) available from the PROKKA pipeline (Seemann, 2014) via the Galaxy server (Afgan et al., 2018). Genes and virulence factors were predicted using Rapid Annotation using Subsystem Technology tool kit (RASTtk) (Brettin et al., 2015). Pathogenicity islands (PAIs) and mobile elements were predicted using VRprofile 2.0 (Li et al., 2018a). HMMer E-value at 0.0001 is set as a cut-off. Minimum number of the significant hits for each prophage-like region was 5. Other strains of AHPND and non-pathogenic *V. parahaemolyticus* were selected to analyze and against 2HP as well. The *V. parahaemolyticus* 3HP, 5HP, and China isolates were all AHPND strains, obtained from GenBank using the accession numbers JPKS00000000, JPKT00000000, and JPKU00000000, respectively (Yang et al., 2014). The *V. parahaemolyticus* S02 (accession number JPKV00000000) was a non-pathogenic strain used in this comparison (Yang et al., 2014). Antibiotic resistant genes were predicted by ResFinder 3.1 (Zankari et al., 2012) and PATRIC 3.5.39 (Wattam et al., 2017). The comparative genomics were performed by protein family sorter program with PATRIC genus-specific families (PLfams) as a filter in the PATRIC 3.5.39 server (Wattam et al., 2017). This program uses k-mer-based functional assessment to categorize the protein family formation and differentiates the cluster of protein families across different genomes using Markov Cluster algorithm (MCL), MCL inflation value = 3 as a cut-off (Davis et al., 2016). The comparison of *zot* and *ace* nucleotide sequences were performed by Multiple Sequence Alignment, EMBL-EBI (Madeira et al., 2019) and BLASTN, NCBI (Johnson et al., 2008).

Genome Characteristics of *V. parahaemolyticus* Strain 2HP

The genome assembly of *V. parahaemolyticus* 2HP is consisted of 5,501,309 bp in 194 contigs. There are 97 RNAs i.e., 4 rRNA, and 93 tRNA. The G+C content of this assembled genome is 45.2%. The quality assessment of draft genome from QUASt showed that N50 (representing the contig length that longer or equal length contigs presents half of the bases of the assembly) and L50



(representing the minimum number of contigs that present half of the bases of the assembly) were 183,055 bp and 10, respectively. Two chromosomes of 2HP were estimated to be 2,996,005 and 1,724,740 bp for chromosome I and II, respectively. RASTtk analysis showed that, from the whole genome, the number of coding sequences was 5,424 and the number of functional subsystems was 406 (Figure 1).

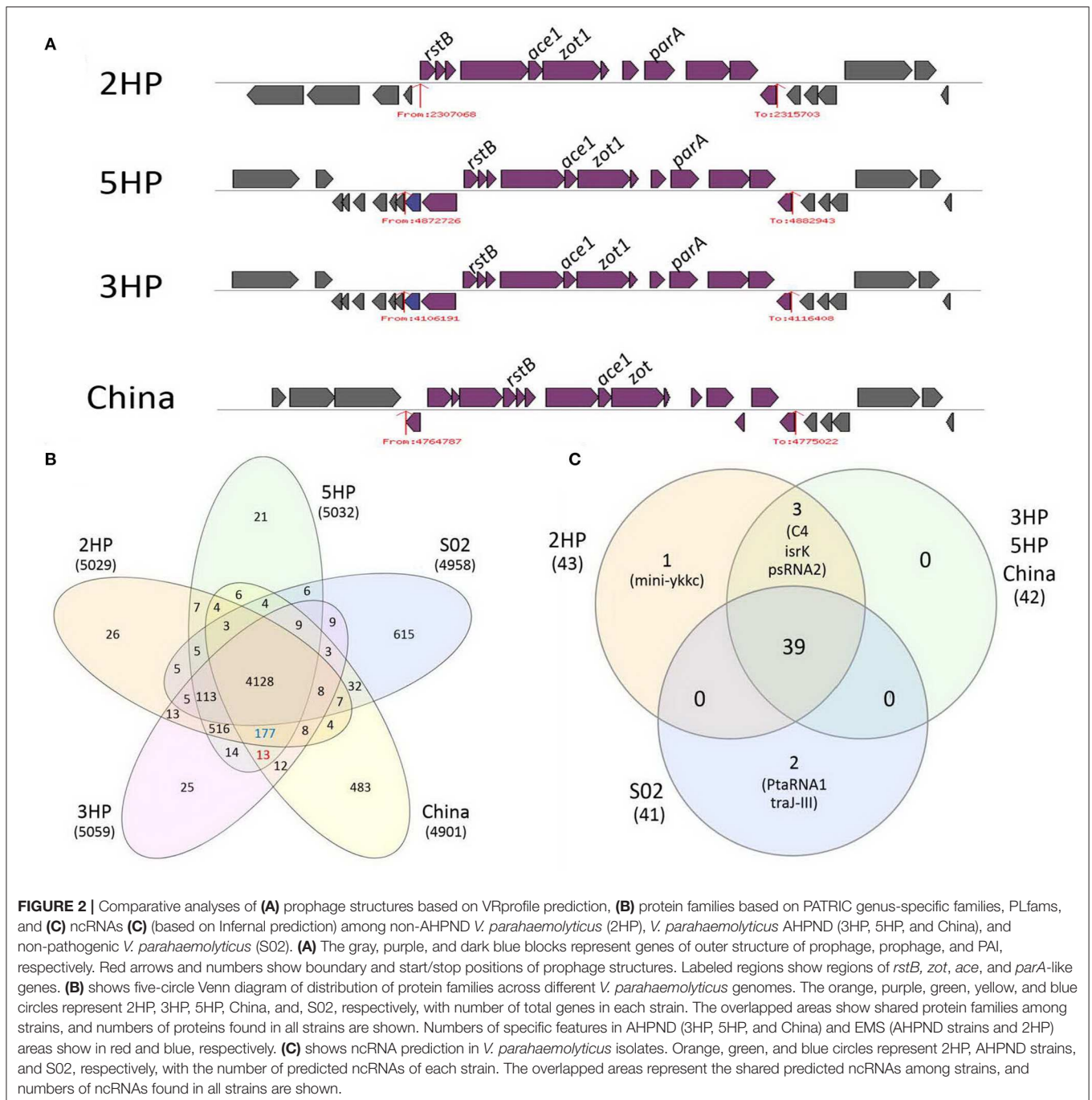
Virulence and Antibiotic Resistance Genes in *V. parahaemolyticus* Strain 2HP

From the RASTtk analysis, we found four encoding homologs of *V. cholerae* toxins, which are also similar to the *V. parahaemolyticus* AHPND strains (Yang et al., 2014). Six toxin genes are two zona occluden toxins (*zot*), two accessory cholera toxins (*ace*), the gene encoding transcriptional activator ToxR (*toxR*), and the gene encoding transmembrane regulatory protein ToxS (*toxS*). 2HP carries two different sizes of putative *ace*, 345 and 333 bp, renamed putative *ace1* and *ace2*, respectively. 3HP and 5HP also have two types of putative *ace*, but the China strain has only one putative *ace1* (345 bp). The 2HP *zot* is also consisted of two types with different sizes, 1,401 and 1,425 bp, called putative *zot1* and *zot2*, respectively. Similar with *ace*, 3HP and 5HP carry two types of *zot*. A unique gene found only in the China strain is a putative *zot* (1,386 bp), which has a different size when compared to the Thai isolates. The *zot1* and *ace1* genes are maintained on chromosome I while *zot2* and *ace2* are carried on chromosome II. We also found only one type of hemolysin in the current draft genome, the thermolabile hemolysin or *tlh* gene. These toxin genes might play a role in the EMS pathogenesis in shrimps. In addition, the presence of *zot* and *ace* genes, which are virulence factors in clinical

field (Perez-Reytor et al., 2018), indicates that this 2HP strain could cause disease in human, despite lacking thermostable directed hemolysin (*tdh*) and TDH-related hemolysin (*trh*) genes (Castillo et al., 2018). Presence of *zot1*, *zot2*, *ace1*, and *ace2* genes was confirmed by PCR (Figures S1–S3).

VRprofile predicted 19 mobile elements and secretion systems. We discovered one integrative and conjugation element (ICE), 12 insertion sequence elements, one type-3 secretion system, one type-4 secretion system, two type-6 secretion systems, and two prophages. Interestingly, one of two prophages in 2HP contains *zot1* and *ace1* (Figure 2A). This prophage contains 12 encoding genes for RstB phage-related integrase, seven hypothetical proteins, accessory cholera toxin 1, zona occluden toxin 1, putative plasmid partitioning protein Soj or ParA-like protein, and bacteriophage f237 repressor 1 (Table S1).

In addition to the virulence genes and putative PAIs described, RASTtk also annotated 34 genes that are putatively responsible for resistance to antibiotics and toxic compounds: four genes for resistance to fluoroquinolones, four genes for multidrug resistance efflux pumps, one gene for resistance to chromium compounds, 11 genes for cobalt-zinc-cadmium resistance, 14 genes for copper homeostasis (Table S5). The genome analysis on ResFinder 3.1 (% identity threshold and minimized length were 80 and 60% as cut-offs) and PATRIC (based on database of National Database of Antibiotic Resistant Organisms) found presence of one beta-lactam resistant gene, beta-lactamase CARB-type (*blaCARB-41*), and two tetracycline resistant genes, *tet-34* and *tet-35*. In aquaculture, tetracycline is one of common drugs for the treatment of bacterial diseases, e.g., vibriosis (Neela et al., 2007), and beta-lactam drugs (such as ampicillin and penicillin) have been applied in human and animal treatment,



(Fernandes et al., 2013). Our finding supports the significance of antimicrobial resistance gene surveillance in aquaculture.

Comparisons of Pathogenicity Islands Prediction With *V. parahaemolyticus* AHPND Isolates

We next investigated the common and unique PAIs in our newly sequence non-AHPND 2HP and other existing EMS *V. parahaemolyticus* AHPND 3HP, 5HP, China, and the reference

S02 strains. We found the consistency of predicted prophage in the EMS-causing strains on chromosome I, but not in the non-pathogenic strain (Figure 2A and Table S1). Prophage carrying *zot1* and *ace1* genes was found in 3HP and 5HP strains, while China strain was presented with *zot* and *ace1* in prophage. Those prophage structures are closely related to pre-CTX prophage (lacking *ctxAB* in CTX prophage) (Pant et al., 2019). In *V. mimicus*, putative pre-CTX prophage was found in a clinical strain MB451, not in an environmental strain VM223 (Hasan et al., 2010). The putative pre-CTX prophage is

consistently found in EMS strains and might be involved with EMS pathogenesis, in addition to *pirAB^{VP}*.

Non-coding RNA Prediction and Comparison

The prediction of ncRNAs by the Infernal program (Kolbe and Eddy, 2011) showed 43 putative ncRNAs in 2HP, 42 in AHPND strains, and 41 in S02 (Figure 2C). We found that the C4, IsrK, and psRNA2 ncRNAs are present only in the EMS-causing strains (2HP, 3HP, 5HP, and China), but not in S02. C4 targets incoming bacteriophage and/or interact with bacteriophage transcription (Weiss et al., 2016) while IsrK interferes with production of AntQ, an antitermination protein for transcription (Hershko-Shalev et al., 2016). The function of psRNA2; however, is still unclear (Gvakharia et al., 2010). For unique ncRNAs, mini-ykkc ncRNA, a class II guanidine riboswitch (Sherlock et al., 2017), is only found in 2HP, while PtaRNA1 and TraJ-II ncRNAs are found only in S02. PtaRNA1 is an antisense antitoxin ncRNA in XCV2162-ptRNA1 system belonging to class I toxin-antitoxin system (Findeiss et al., 2010). TraJ-II is found in 5'-UTR of *traJ* gene and functions in bacterial conjugation process (Li et al., 2018b). We validated the presence of predicted ncRNA in 2HP by RT-qPCR. Spot42, RyhB, and mini-ykkc were chosen as representatives and their transcript levels shown in (Figure S4) confirmed expression of the selected ncRNAs.

Comparative Pangenomic Analyses of EMS-Causing *V. parahaemolyticus* Isolates

Not only virulence factors lead to pathogenesis, but some genes known to be involved in the bacterial fitness, which are specific to each strain, may also play crucial roles in disease establishment (Diard and Hardt, 2017). Hence, 2HP may carry some features, in addition to *zot* and *ace*, which overlap with AHPND strains or features unique to 2HP which lead to EMS in shrimp. To investigate the strain-specific features and unique features in each group of AHPND and EMS, we selected 2HP, 3HP, 5HP, China, and S02 for pan-genomic analysis by the protein family sorter program in PATRIC (Figure 2B). The number of unique genes in 2HP, 3HP, 5HP, China, and S02 are 26, 25, 21, 483, and 615 genes, respectively (Table S4). The 2HP-specific genes include one encoding a transposase and 25 encode hypothetical proteins.

Pangenome (all genes from all genomes) and core genomes (present in all the genomes) contained 6,287 and 4,128 genes, respectively. Accessory genome (genes present in specific strains) in AHPND (3HP, 5HP, and China) and EMS strains (AHPND strains and 2HP) contained 13 and 177 genes, respectively (Tables S2, S3). AHPND-specific genes are *pirA^{VP}*, *pirB^{VP}*, one gene encoding peptidase M20, and 10 hypothetical genes. EMS strain-specific genes compose of many genes encoding functional proteins in methylation and acetylation e.g., S-adenosylmethionine (SAM)-dependent methyltransferase, acetyltransferase, histone acetyltransferase HPA2. The *rsbW* gene, which encodes a serine kinase RsbW, is specific to the EMS groups. Free RsbW phosphorylates

SypA leading to regulation of biofilm formation (Morris and Visick, 2010). The ORFs for capsular polysaccharide biosynthesis protein and YjbG polysaccharide synthesis-related protein, important in capsule formation and adherence to surface (Ferrieres et al., 2007; Broberg et al., 2011), are also EMS-specific.

In terms of key virulence factors, nucleotide sequences of accessory cholera toxin and zona occluden toxin in China strain are different from those of EMS strains from Thailand (2HP, 3HP, and 5HP). The *zot* and *ace1* from China strain are closely related to *zot* and *ace* in *V. parahaemolyticus* RIMD2210633 (clinical strain). The *zot* gene from China strain shows 97.62% similarity to RIMD2210633 strain while the *zot1* and *zot2* genes from Thai isolates share 50.73 and 51.06% nucleotide similarity, respectively, to RIMD2210633 strain. The *ace1* gene from China strain shows 99.13% nucleotide similarity to RIMD2210633 strain while *ace1* and *ace2* genes of Thai isolates show 49.86 and 47.17% nucleotide similarity, respectively, to RIMD2210633. Therefore, *zot1*, *zot2*, *ace1* (Thai), and *ace2* genes are considered members of 2HP-3HP-5HP accessory genome which are different from those of RIMD2210633 and China strains.

In summary, we have for the first time sequenced the non-AHPND 2HP *V. parahaemolyticus* and comprehensively annotated the newly sequence genome. Our comparative genomic analysis revealed the “conserved” virulence genes and prophage commonly found in both pathogenic AHPND and non-AHPND strains. In *V. cholerae*, *Zot* increases permeability of epithelial barrier by disassembling the intercellular tight junctions, and *Ace* increases ion transport and water secretion (Perez-Reytor et al., 2018). Sequences of *zot* and *ace* in 2HP, 3HP, and 5HP are different from those of clinical strain RIMD2210633. Therefore, the activities of *Zot* and *Ace* might be more specific to shrimp than human. However, *zot* and *ace* in the China strain are closely similar to those of RIMD2210633. *Zot* and *Ace* in this China strain might affect both human and shrimp. Both toxins in non-AHPND and AHPND strains might have similar functions in shrimp, similar to other hepatopancreatic toxins such as to *PirAB^{VP}* toxins (Prachumwat et al., 2019). Prophage carrying *zot* and *ace* might be necessary for EMS pathogenesis in both AHPND and non-AHPND. Moreover, this prophage may be used as biomarker for diagnosis of EMS-causing *V. parahaemolyticus*. Not only virulence genes and prophage, but ncRNA also showed specific patterns among the EMS strains. We also identified conserved ncRNAs in EMS strains involved in transcription process. These might be transcriptional factors for enhancement of EMS pathogenesis. From comparative genomic analysis, we found that most of the strains-specific genes were hypothetical genes. Interestingly, we found ORFs which encode regulatory protein and capsule formation proteins in EMS group. This finding suggests that EMS strains, AHPND and non-AHPND, carry the special features that S02 lacks. These genes might enhance EMS strains to induce the virulence and pathogenesis. The 2HP-specific genes contain a lot of hypothetical genes. 2HP may maintain some unique features that involve in EMS mechanism

of the non-AHPND strain. The functions of these gene are still unknown. Further investigation is required to identify mechanisms of pathogenesis in AHPND and non-AHPND strain for EMS.

DATA AVAILABILITY STATEMENT

The genome data of *V. parahaemolyticus* 2HP from this study had been submitted to DDBJ/EMBL/GenBank under the accession number SRKW00000000. The version of genome sequence described in this work is the first version, SRKW01000000.

AUTHOR CONTRIBUTIONS

NT performed the experiments, analyzed the data analysis, and prepared the manuscript. MS assisted with antibiotic resistant gene prediction. VC, VS, and SC designed the study and prepared the manuscript. All authors proofread the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2020.00290/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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