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DETECTION AND SEQUENCE ANALYSIS OF SIDEROPHORE BIOSYNTHETIC GENE *PvsD* FROM *Vibrio harveyi* VH1

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Abstract

Numerous studies have been conducted to investigate siderophore genes in *Vibrio* species. here are many researches has been done to learn about the siderophore genes in *Vibrio* species. Therefore, it is crucial to search the siderophore virulence associated gene in the opportunistic pathogen from *Vibrio harveyi*, to predict the potential genes contributing to its pathogenicity. This study attempts to characterize the siderophore associated gene from *V. harveyi*. The objectives are to identify and amplify the siderophore associated gene from *V. harveyi* and to characterize the gene, based on the molecular characterization and bioinformatic analysis. The results showed that the siderophore associated gene from *V. harveyi* VH1 belongs to a part of Vibrioferrin gene cluster, which is the biosynthetic of *PvsD* gene. In conclusion, the siderophores produced by *V. harveyi* VH1 belong to the vibrioferrin siderophores gene, which is responsible to help this species to accumulate iron from the environment.

INTRODUCTION

As aquaculture has grown and demand for the food security has increased, the incidence of infection by pathogenic bacteria has also severed in this industry. Infectious illnesses and mortality in wild fish populations and fish farmed in restricted circumstances are mostly caused by bacterial infections [1]. A range of disease problems, including vibriosis, poses a severe danger to the industry [2]. In the genus *Vibrio* from the Vibrionaceae family, *V. harveyi* can be considered as a high influential pathogen that can infect a wide range of marine species, specifically fishes. Infectivity studies conducted in a lab using *V. harveyi* cultivated from diseased fish showed pathogenicity [3].

Vibriosis caused by *Vibrio spp.* infection, is among the most common diseases in many fish hatcheries species and is a significant contributor of death in fish production services across the globe [4]. Some symptoms from *V. harveyi* that cause vibriosis are anorexia, darkening of the entire fish, small hemorrhaging wounds on its mouth or skin surface, tail as well as fin rot, localised necrotic lesions in the muscle, enlarged gut, and ocular opacity [5]. Virulence associated genes are responsible for the virulence factors that bacteria acquire to adhere to eukaryotes and degrade them [6]. Identifying related types or lineage of pathogens that are already expected to cause disease might help researchers better understand the cyclical population dynamics involved in the establishment of such virulent microorganisms [7].

Siderophores are small molecules synthesized and secreted by bacteria and fungi to remove iron and considered as virulence associated protein that led to pathogenicity. Several siderophore systems representative of Vibrionaceae are known and well understood. The siderophore biosynthetic gene clusters for *Vibrio vulnificus* generate an unquantified catechol siderophore, utilising, in part, identical genes as for vulnibactin, *Vibrio cholerae* produces the catechol siderophore vibriobactin using proteins encoded by vibABCDEFH. In response to low iron availability, *Vibrio*

parahaemolyticus synthesizes and secretes a polyhydroxycarboxylate-type siderophore vibrioferrin which is composed of 1 mol each of 2-ketoglutaric acid, L-alanine, ethanolamine, and citric acid [8].

Hence, there are many molecular techniques such as real-time PCR and DNA sequencing, have been used to detect, identify, and characterise microorganisms for epidemiological purposes [9]. However, the siderophores associated gene in *V. harveyi* is still understudied. Thus, these studies aimed to identify the virulence associated siderophore gene from pathogenic *V. harveyi*, which was previously isolated from diseased tiger grouper [10].

MATERIALS AND METHODS

Preparation and Growth of *Vibrio harveyi* VH1

The raw material, which was isolated *Vibrio harveyi* from previous study, was stored as glycerol stocks at -20°C [10]. The culture was grown in two ways: using TCBS agar and TSB+NaCl broth culture. For the TCBS agar it was observed after 24 hours of incubation. As for the TSB+NaCl broth culture, 100 µL from the isolated *V. harveyi* was mixed with TSB+NaCl and used within 24 hours for DNA extraction.

Isolation of Genomic DNA

For the DNA extraction, the sample used was from the fresh broth culture, which included TSB+NaCl. The DNA extraction was done using Thermo Scientific GeneJET Genomic DNA Purification Kit, following procedures described by the kit [11]. The isolated genomic DNA (5 µl) was then analysed using 1% of agarose gel electrophoresis.

Polymerase Chain Reaction

The specific primers were designated for this study: the forward primer F-Vhv (CAGGCAATGCAAATAGCGTTC) and the reverse primer R-Vhv (GGGCATACTTTCCATCCGAC). The primers were designated based on previously studied for Vibrrioferrin biosynthesis protein *PvsD* gene (Genebank: DQ201184.2) with estimation length of 1,829 bp [12]. The analysis of primers designed was done using OligoAnalyzer tool (https://sg.idtdna.com/). The polymerase chain reaction for the amplification of siderophore associated gene was done using the Thermo Scientific DreamTaq Green PCR Master Mix (2X) [13]. The PCR mixture consisted of 10µl template DNA, 25µl DreamTaq Green PCR Master Mix (2X), 1µM of each primer and 11µl of nuclease-free water. PCR amplification was performed at initial denaturation 95°C for 1 minutes, followed by 30 cycles consisting of denaturation at 95°C for 30 seconds, annealing temperature at 56°C for 30 seconds and extension at 72°C for 1 minute. In the last cycle, the final extension was at 72°C for 5 minutes. The PCR amplicon was examined using gel electrophoresis prior sent to Apical Scientific Sdn Bhd for purification and further sequencing using Sanger sequencing method.

Gel Electrophoresis

The gel electrophoresis procedure was done using the 100ml of 1X TAE buffer mixed with 1g of agarose powder. The gel was run for 60 minutes at 90V. The DNA bands on the gel were observed under UV transluminator and compared to the Vivantis 1kb DNA ladder.

Characterization of Siderophore Associated Gene from *Vibrio harveyi*

Phylogenetic tree analysis and homology sequence analysis were used to analyse the homologous sequence. Phylogenetic trees were created using the Neighbor-joining algorithm of the MEGA 11 software. Five gene sequences were retrieved from the homology sequence analysis via Genbank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

RESULTS AND DISCUSSION

Figure 1 shows the band for the PCR product of *V. harveyi*. The observed band for *V. harveyi* was approximately 1.8kb (Figure 1). Based on DNA contig analysis, a total 1,780 bp was successfully obtained for the putative siderophores gene in *V. harveyi* VH1. As shown in Table 1, the blast similarity ranging from around 95% to 96% between *V. harveyi* with other *V. parahaemolyticus* and *V. alginolyticus* species. Also, most of the obtained results show that the studied siderophore associated gene from *V. harveyi* is related to the siderophore biosynthetic gene *PvsD*.

In addition, the multiple sequence alignment result proves that the studied siderophore associated gene from *V. harveyi* is related to other *Vibrio* species. The results from this analysis were further used to construct a phylogenetic tree. As shown in Figure 2, the studied siderophore associated gene from *V. harveyi* shares a common origin with the other species. Also, the closest related species are *V. parahaemolyticus* strain XMO116 and *V. parahaemolyticus* strain XMM117. Both of the species' sequences are from the siderophore biosynthesis protein *PvsD*. This protein was found associated with one of the subunits that build vibrioferrin.

Table 1. Homology sequence similarity of studied siderophore associated gene from *Vibrio harveyi*

Strain	Description	Similarity (%)	Accession
***Vibrio diabolicus* strain FDAARGOS_96**	Siderophore biosynthesis protein *PvsD*	95.58	CP14095.1
***Vibrio alginolyticus* strain FA2**	Alkene reductase	95.67	CP042450.1
***Vibrio neocaledonicus* strain CG302-2**	Siderophore biosynthesis protein *PvsD*	95.67	CP032214.1
***Vibrio parahaemolyticus* strain FDAARGOS_115**	Siderophore biosynthesis protein *PvsD*	95.51	CP014047.2
***Vibrio antiquarius* strain EX25**	Vibrioferrin amide bond forming protein *PvsD*	95.45	CP001806.1

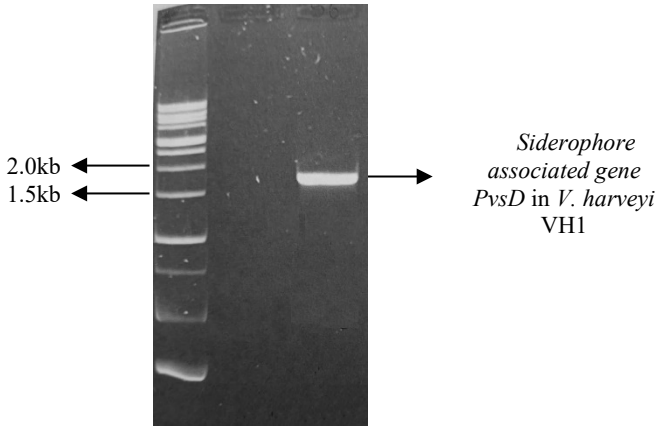

Figure 1. Amplicon for PCR amplification of siderophore associated gene of *Vibrio harveyi* VH1.

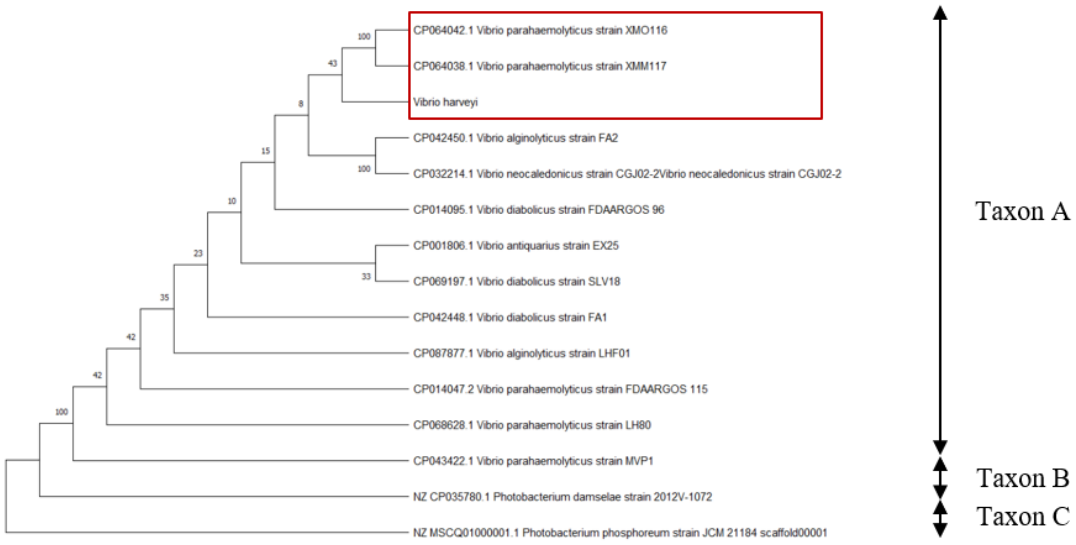

Figure 2. Phylogenetic tree of studied siderophore associated gene from *Vibrio harveyi* with other related species.

As described in Wang et al (2007), vibrioferrin is a polycistronic protein that built from a few subunits, consisting of an 11-gene cluster with two divergently transcribed, Fe^{3+} and ferric uptake regulator (Fur) regulated operons, *pvsABCDE* and *psuA-pvuABCDE*. This gene cluster is also found to share high similarity with that related to siderophore biosynthesis and transportation locus in *V. parahaemolyticus* [14]. It was also demonstrated that the siderophore biosynthesis or utilization was blocked when *pvsA* and *pvsD* of the *pvsABCDE* operon or *pvuA*, *pvuB* and *pvuE* of the *psuA-pvuABCDE* operon were single-gene in-frame mutated [12]. This further confirmed the essential roles for siderophore biosynthesis or utilization of *pvsA* and *PvsD* gene as reported in *V. alginolyticus* MVP01. Based on the finding in this study, the siderophore associated gene from *V. harveyi* is highly recommended to closely related with the *V. parahaemolyticus* strains. It can be proposed that the siderophores associated gene in *V. harveyi* can be categorised as vibrioferrin, which related to the biosynthetic gene *PvsD* of *V. parahemolyticus*.

Another research also proposed that the pathogenic *V. parahaemolyticus* able to endure form being swallowed by phagocytes using using a two-component system (TCS) [15]. According to Meng et al. (2023), TCS system perceiving the external environmental signals and transmit them to the interior to trigger the associated regulatory mechanism by using a TonB-dependent siderophore enterobactin receptor for regulating the macrophages. Thus, it can be proposed that, vibrioferrin might also related to the regulatory mechanism such as siderophore enterobactin.

CONCLUSION AND RECOMMENDATIONS

In conclusion, the siderophore associated gene from *Vibrio harveyi* VH1 was successfully identified. BLAST analysis shows 96% similarity of the derived sequence corresponding to *Vibrio parahaemolyticus*. The siderophore associated gene from *V. harveyi* VH1 was found to be a siderophore biosynthetic *PvsD* gene, a part of the subunit protein for vibrioferrin that is closely related to *V. parahaemolyticus* strains. Thus, it is proposed that this siderophore associated gene plays an important role in its pathogenicity activity. For future study, it is highly recommended to identify other biosynthesis cluster genes to further confirm whether the siderophore virulence associated genes are closely related the vibrioferrin siderophores of *V. harveyi* VH1.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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