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IDENTIFICATION OF BACTERIA ASSOCIATED WITH *Holothuria (Mertensiothuria) leucospilota* FROM PANGKOR ISLAND

FATIN NAJIHAH MUHAMMAD LUTFI*, MARYAM MOHAMED REHAN*, KAMARUL RAHIM KAMARUDIN, MOHD ZULHAFIZ CHE ZAHRI, HANINA MOHD NOOR and SALINA MAT RADZI

Food Biotechnology, Faculty of Science and Technology (FST), Universiti Sains Islam Malaysia (USIM), Bandar Baru Nilai, 71800 Nilai, Negeri Sembilan, Malaysia
**E-mail: najihahlutfi@gmail.com, maryam@usim.edu.my*

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ABSTRACT

Holothuria (Mertensiothuria) leucospilota is the most abundant sea cucumber species in Malaysia. This study aimed to identify bacteria isolated from the external and internal body parts of *H. leucospilota* collected from the coastal water of Pangkor Island, Perak, Malaysia. A total of 26 bacterial samples were isolated using streak plate method from eight body parts of two fresh *H. leucospilota* specimens and from the surrounding surface sediments and seawater. Identification of the bacterial isolates was based on microscopic examination, 16S rDNA amplification and phylogenetic analysis using the neighbour-joining method. Three genera of bacteria were identified namely *Vibrio, Bacillus*, and *Acinetobacter*. The genus *Vibrio* was found to be the main bacterial group associated with the *H. leucospilota* specimens from Pangkor Island.

Key words: *Holothuria leucospilota*, Bacteria, 16S rDNA, Pangkor Island

INTRODUCTION

The holothurians, or sea cucumbers, can be found in great numbers in the marine environment throughout the world (Ridzwan, 2007). This marine invertebrate is treasured for their health beneficial values in traditional and modern medicine. In Malaysia, sea cucumbers have been consumed as delicacies and medicine especially in the production of "gamat oil" (Bruckner, 2006). Besides benefiting humans, sea cucumbers also serve a useful role in the marine ecosystem. Sea cucumbers oblige to recycle nutrients back into the marine environment by breaking down detritus and organic matters, after which bacteria can continue the degradation process (Du *et al*., 2012).

Many marine invertebrates harbour a wide variety of microbial species (Enomoto *et al*., 2012), including sea cucumbers that live in close association with bacteria (Ogunola & Onada, 2016). Bacteria play significant roles in the digestive tracts of sea cucumbers and many other animal species. Bacteria also participate in the degradation of nutrients and stimulate immune defence of the host (Amaro *et al*., 2009; Hess *et al*., 2011; Amaro *et al*., 2012). The gut of sea cucumbers has also been shown to be inhabited by bacteria (Gao *et al.,* 2014). The abundance of bacteria flourishing the seafloor offers sea cucumbers with essential nutrients that are otherwise not available as a food source (Amaro *et al*., 2009; Gao *et al*., 2010). Sea cucumber ingested bacteria, meiofauna, decaying organic debris, inorganic components and dissolved organic matters that are accessible on surface sediments as their primary source of food (Roberts *et al*., 2001; Gao *et al*., 2010).

The most dominant sea cucumber species in the marine environment globally and in Malaysia are *Holothuria (Mertensiothuria) leucospilota* (Purcell *et al*., 2012). This sea cucumber is generally known as white threads fish in English and *bat puntil* among the Malaysians (Kamarudin *et al*., 2015). The coelomic fluid of *Holothuria leucospilota* has been shown to contain indigenous bacteria that may help them to adapt and exist in the marine environment (Lukman *et al*., 2014). The composition of bacteria in the coelomic fluid of *H. leucospilota* was also shown to be more diverse as

* To whom correspondence should be addressed.

compared to the bacteria population present in *Stichopus chloronotus*, or locally known as *gamat* species. The occurrence of microbial population in the marine ecosystem were suggested to be influenced by environmental factors such as penetration of light, feeding behaviour and the level of antimicrobial properties in the coelomic fluid of sea cucumbers (Lukman *et al*., 2014).

Studies on the host-associated microbial communities have gained increasing attention primarily due to their biotechnological potential (Taylor *et al*., 2007). However, despite the abundance of sea cucumbers particularly *H. leucospilota* in Malaysia, there are limited records concerning bacteria associated with the sea cucumber specifically those that inhabit its internal body parts. This study aimed to isolate and identify bacteria from eight body parts of *H. leucospilota* specimens collected from Pangkor Island, Perak, Malaysia, and its surrounding surface sediment and seawater. The bacterial isolates were identified through microscopic examination, and partial 16S ribosomal DNA (rDNA) amplification and sequence analysis. A phylogenetic analysis using the distance-based neighbour-joining (NJ) method was also performed to examine the genetic relationships between the bacterial isolates associated with *H. leucospilota* from Pangkor Island, Perak, Malaysia.

MATERIALS AND METHODS

Sample Collection and Bacterial Isolation

Two specimens of *H. leucospilota* were collected from Giam Island (4° 14’ 09.5"N 100° 32’ 22.4"E) and Teluk Nipah Beach (4°14’ 03.3"N 100° 32’ 41.4"E), Pangkor Island, Perak, Malaysia, and were labelled as HL and HL1. The seawater temperature was recorded at 26°C during the sampling.

Isolation of bacteria was carried out from eight body parts of each *H. leucospilota* specimen *i.e.* cuticle, coelomic fluid, cloaca, gastrointestine, respiratory tree, tentacle, cuvierian tubules, and polian vesicles; and from the surrounding surface sediments and seawater. The samples were streaked on Luria-Bertani (LB) agar (Merck, Germany) using sterile cotton swabs and incubated overnight at 26°C. Bacterial colonies with different morphologies were observed and re-streaked onto new LB agar until pure cultures or single bacterial colonies were obtained. The cultures were then preserved on nutrient agar (NA) media (Merck, Germany). After Gram staining, the pure colonies were observed under compound microscope (Olympus) with magnifications of 1000X and oil immersion.

DNA Extraction and Polymerase Chain Reaction (PCR) of 16S rDNA

Total genomic DNA was extracted using sodium hydroxide lysis technique. A loop of bacterial colony was scraped from agar media and mixed with 20 µL of 0.02M NaOH followed by incubation at 95°C for 10 min. The sample was chilled for 5 min and centrifuged (Eppendorf, Germany) at 13400 rpm for 2 min. The supernatant was used as the template DNA for PCR. Amplification of the 16S rDNA region was done using universal primers, PB36 Forward: 5’- AGA GTT TGA TCC TGG CTC AG -3’ and PB38 Reverse: 5’- GGT ACC TTG TTA CGA CTT -3’ (Lane, 1991). All PCR reactions were conducted in 25 µL volume containing 12.5 µL of PCR Master Mix (Promega), 1.3 µL of each universal primer (10 µM) and 2 µL of template DNA. The thermocycling steps were as follows: 95°C for 2 min, followed by 35 cycles at 95°C for 40 sec, 55°C for 40 sec, 72°C for 90 sec, and a final extension step at 72°C for 10 min. All PCR products were analysed on 1% (w/v) agarose gel stained with FloroSafe DNA Stain, and 1 kb DNA ladder was included as the standard DNA marker. The PCR products were sent for DNA sequencing at the First BASE Laboratories Sdn Bhd, Seri Kembangan, Selangor, Malaysia. Non-protein-coding 12S and 16S mitochondrial ribosomal RNA (rRNA) gene sequences of the HL1 specimen were deposited at GenBank, National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine (accession no.: KX768273 - KX768274).

16S rDNA Sequence Analysis

The data obtained were analysed to detect the sequence similarity in a non-redundant sequence database using Basic Local Alignment Search Tool program (BLAST) (Altschul *et al*., 1997). All the DNA sequences were aligned using the ClustalX Version 1.81. A phylogenetic tree based on the distance-based neighbour-joining (NJ) method was constructed using the Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6) software (Tamura *et al*., 2013).

RESULTS

A total of 26 bacterial samples were isolated from various body parts of the two sea cucumber specimens, seawater and sediment collected from Giam Island and Teluk Nipah Beach in Pangkor Island. The bacterial samples were morphologically analysed and categorised into bacterial genera with additional information from 16S rDNA. From the total isolates, Gram-negative bacteria that belong to

the genera *Vibrio* and *Acinetobacter* made up to 96% of the bacterial samples, while *Bacillus* is the only representative for Gram-positive bacterial group. Under microscopic examination, the bacterial representatives were observed to be short curved rods for *Vibrio* sp., round and double forms for *Acinetobacter* sp., and long curved rod for *Bacillus* sp. (Fig. 1).

PCR amplification of the 16S rDNA region resulted in a fragment length of approximately 1500 base pairs obtained for all the bacterial samples. Among the 26 PCR products, 21 showed good DNA sequencing results while five samples were excluded from analyses to avoid unreliable results due to the presence of noisy data in their nucleotide sequences. Sequence analysis of the partial 16S rDNA through BLAST identified twelve of the bacterial isolates as *Vibrio parahaemolyticus*, five isolates as *Vibrio harveyi*, two samples as *Acinetobacter schindleri*, and one bacterial isolate each of *Vibrio maritimus* and *Bacillus thuringiensis* (Table 1).

Three different bacterial species were derived from the HL specimen of Giam Island, while four bacterial species were isolated from the HL1 specimen collected from Teluk Nipah Beach including seawater and sediment. The bacteria *V.*

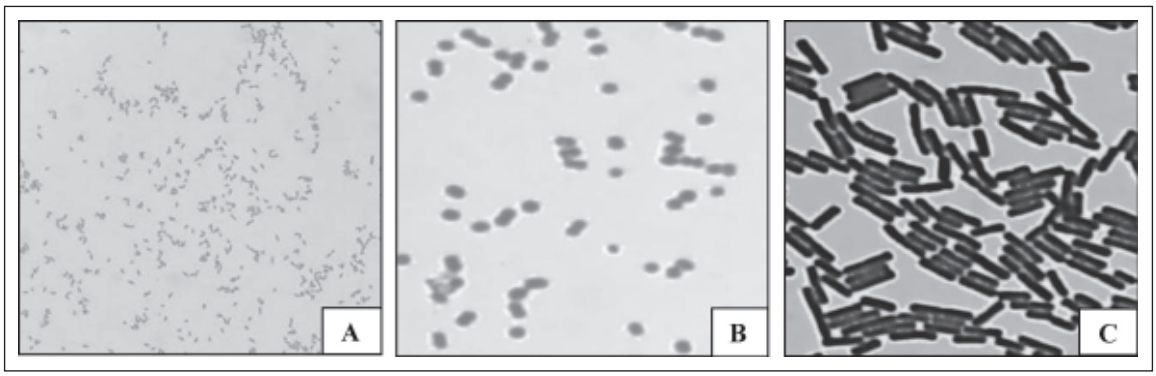

Fig. 1. Morphological characteristics of representative strain of every genus as observed under the compound microscope. (A) *Vibrio*, (B) *Acinetobacter*, and (C) *Bacillus*.

Table 1. Identification of bacterial isolates associated with the *Holothuria (Merthensiothuria) leucospilota* specimens from Pangkor Island, Perak, Malaysia based on BLAST analyses of 16S rDNA sequences

Bacterial isolates	Sources of isolates	Probable species	% ID
HL specimen			
HLw	Water	*Vibrio harveyi*	92%
HLs	Sediment	*Vibrio parahaemolyticus*	99%
HLc1	Cuticle	*Vibrio harveyi*	99%
HLcoe	Coelomic fluid	*Vibrio parahaemolyticus*	99%
HLl	Cloaca	*Vibrio parahaemolyticus*	99%
HLg1	Gastrointestine	*Vibrio parahaemolyticus*	99%
HLg2	Gastrointestine	*Vibrio harveyi*	99%
HLg3	Gastrointestine	*Vibrio maritimus*	99%
HLt1	Tentacle	*Vibrio harveyi*	99%
HL1 specimen			
HL1w	Water	*Acinetobacter schindleri*	99%
HL1s	Sediment	*Vibrio parahaemolyticus*	99%
HL1c	Cuticle	*Vibrio parahaemolyticus*	99%
HL1coe	Coelomic fluid	*Acinetobacter schindleri*	99%
HL1v	Cuvierian tubules	*Bacillus thuringiensis*	99%
HL1l2	Cloaca	*Vibrio parahaemolyticus*	99%
HL1g	Gastrointestine	*Vibrio parahaemolyticus*	99%
HL1r	Respiratory tree	*Vibrio parahaemolyticus*	99%
HL1t1	Tentacle	*Vibrio parahaemolyticus*	99%
HL1t2	Tentacle	*Vibrio parahaemolyticus*	99%
HL1p1	Polian vesicle	*Vibrio harveyi*	99%
HL1p2	Polian vesicle	*Vibrio parahaemolyticus*	99%

Note: ID – based on Identities score (Ident).

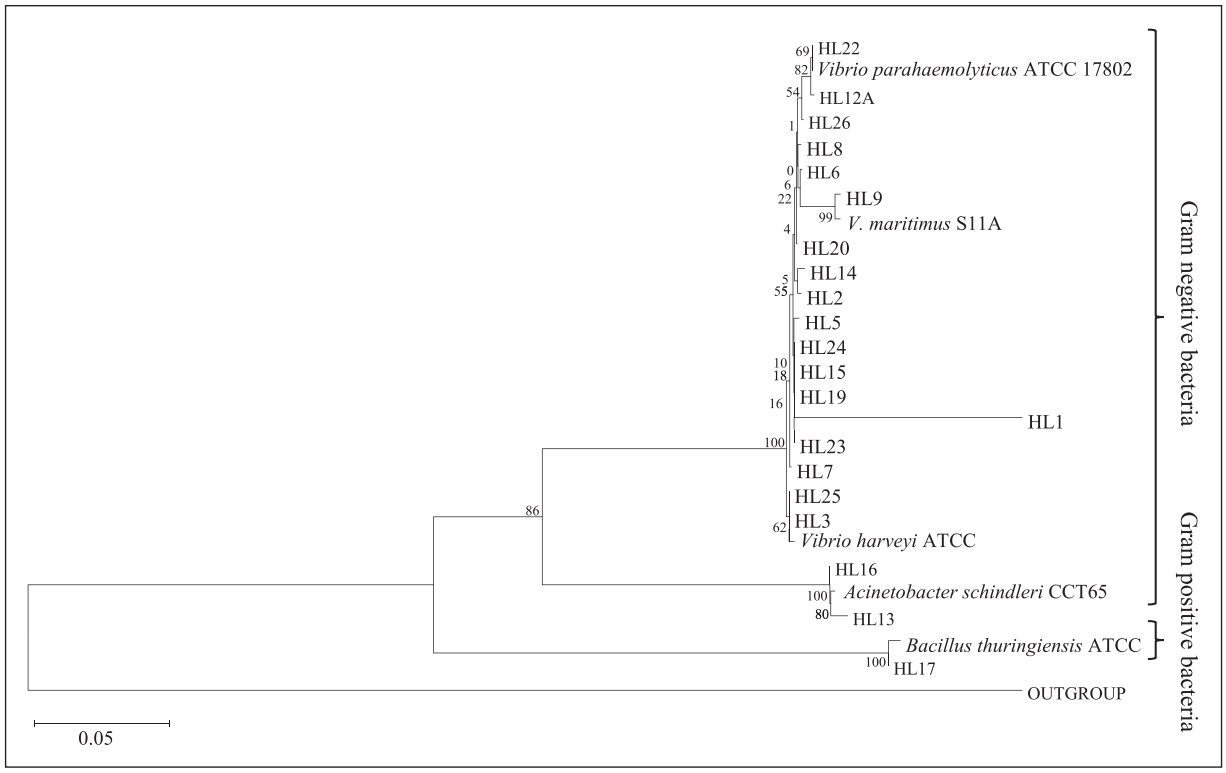

Fig. 2. Neighbour-joining (NJ) tree of 21 bacteria isolates associated with the *Holothuria (Mertensiothuria) leucospilota* specimens from Pangkor Island, Perak, Malaysia inferred from 16S rDNA gene sequences. Note: OUTGROUP - 16S rDNA sequence of *Methanosarcina barkeri* (GenBank accession no.: CP009526.1). Cluster A - family Vibrionaceae, cluster B - family Moraxellaceae, and cluster C - family Bacillaceae.

parahaemolyticus and *V. harveyi* were isolated from both *Holothurian* specimens, while *Vibrio maritimus* was present in the gastrointestine of the sea cucumber HL specimen but absent in HL1. Furthermore, *Acinetobacter schindleri* and *Bacillus thuringiensis* were found within the HL1 sea cucumber, and not from the HL specimen. The presence of *A. schindleri* in the coelomic fluid of HL1 specimen is potentially due to their presence in the surrounding seawater since the bacteria was also found in the seawater sample.

For phylogenetic analysis, a total of 21 partial sequences of 16S rDNA region were included together with a sequence of *Methanosarcina barkeri* (GenBank accession no.: CP009526.1) as the outgroup to root the distance-based NJ tree. There were 625 nucleotide bases in the final dataset following analyses using multiple alignment. A phylogenetic tree constructed using the NJ method (Saitou & Nei, 1987) is summarised in Fig. 2. The optimal NJ tree with the sum of branch length = 0.87157370 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (i.e. 1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated.

There are three primary clusters of bacteria in which Cluster A consisted of genera *Vibrio*, cluster B represented genera Acinetobacter, and cluster C comprised of genera Bacillus. Bacterial strains of *V. parahaemolyticus*, *V. harveyi*, and *V. maritimus* were grouped together in cluster A with 99% bootstrap value. Meanwhile, cluster B represented *A. schindleri* species, and the cluster was grouped together with cluster A with 89% bootstrap support. Furthermore, cluster C comprised the representative 16S rDNA sequence of genus *Bacillus*, *B. thuringiensis*. The Neighbour-joining (NJ) tree also showed a clear classification between Gram-negative bacteria (the clusters A and B) and the Gram-positive bacterium (cluster C).

DISCUSSION

A wide diversity of bacteria were known to be associated with marine life including sea cucumbers, some of which have been suggested to have

symbiotic relationships with the host and some also have the potential to produce bioactive compounds (Enomoto *et al*., 2012; Gao *et al.,* 2014; Lukman *et al*., 2014). In this study, bacteria were isolated from the i) external and internal body parts of two *H. leucospilota* specimens, ii) seawater and iii) sediment collected from two locations in Pangkor Island, Malaysia. Genetic identification of the bacterial samples suggested that *Vibrio* is the main bacterial genera associated with the two *H. leucospilota* samples. The genus *Vibrio* have been previously reported to be isolated from the sea cucumber *Apostichopus japonicas* (Enomoto *et al*., 2012; Gao *et al*., 2014; Kim *et al.,* 2017). These bacteria can be commonly recovered in the marine, estuarine, and freshwater environment worldwide (Romalde *et al*., 2014). In fact, some bacteria of the genus *Vibrio* has also been previously isolated from the coelomic fluid of a *H. leucospilota* sample collected from other location of the Malaysian seawaters, with the probable species of *Vibrio vulnificus* or *Vibrio furnissii* (Lukman *et al*., 2014).

The major bacterial species associated with both Holothurian samples in this study was *V. parahaemolyticus* with the percentage of 57%, and were isolated from different body parts of the sea cucumbers. The growth of *V. parahaemolyticus* could be affected by water salinity and temperature of the marine environments (Iida *et al*., 2006). This species was frequently found during the summer season in Europe and the United States, when the temperature is increased to 25°C and above. In the Southeast Asia, *V. parahaemolyticus* could be found all year round due to the high marine temperature between 25°C to 35°C that favour the outbreaks of this bacterium (Zulkifli *et al*., 2009). *V. parahaemolyticus* can be pathogenic to human, and it has been shown that the species isolated from cultured sea cucumber *Apostichopus japonicas* in China possess antimicrobial-resistance properties which may pose risk to public health and the environments (Jiang *et al*., 2014). Since *V. parahaemolyticus* is the prevalent bacteria associated with *H. leucospilota* in the present study, further characterization on the bacterial pathogenicity could be useful for public consumption on the sea cucumbers.

V. harveyi is the second major bacterial species found to be associated with the *H. leucospilota* specimens from Pangkor Island. This species has been extensively investigated as a luminescent bacterium (Hui & Sherkat, 2005). *V. harveyi* was also isolated from the surface of the sea cucumber *Stichopus badionotus* collected from Port Dickson beach in a previous study (Alipiah *et al*., 2016). This bacterium has been shown to have a symbiotic relationship with its host organism, where the host provides a nutrient rich environment for the growth of the bacterium and in return, the bacterial luminescent properties provide protection for the host from its predator (Lin *et al*., 2001).

Other bacterial species isolated from the *H. leucospilota* specimens in the present study are *Acinetobacter schindleri*, *Bacillus thuringiensis* and *Vibrio maritimus*. The bacterial genera of *Acinetobacter* and *Bacillus* were also previously found to be associated with sea cucumbers (Yasoda *et al*., 2006, Gao *et al*., 2014). Furthermore, *Bacillus* sp. was found in the gut of the sea cucumber *Apostichopus japonicas* from China which suggested that they can be used as candidate probiotics (Gao *et al*., 2014). However, little information about *V. maritimus* was found from previous studies that linked this bacterium with sea cucumbers. The *V. maritimus* has been isolated from *Palythoa caribaeorum,* a coral species in Brazil (Chimetto *et al*., 2011).

Further characterization of the bacterial samples isolated from *H. leucospilota* may provide better information on the interactions among bacteria and the sea cucumber host. A much deeper insight on the bacterial community associated with *H. leucospilota* from Malaysia seawaters can also be obtained by analysing more *H. leucospilota* specimens from different locations to determine and validate the bacterial species. Techniques such as massively parallel sequencing may also provide a wider information regarding the bacterial species diversity from various body parts of the sea cucumber.

CONCLUSION

In this study, 21 bacterial samples were isolated from eight body parts of *H. leucospilota* specimens, seawater and sediment from Pangkor Island, Malaysia. They were morphologically analysed and identified through sequence and phylogenetic analysis of the partial 16S rDNA. The isolates were found to be from three genera of bacteria namely *Vibrio*, *Acinetobacter*, and *Bacillus*. In particular, the bacterial species of *Vibrio parahaemolyticus*, *Vibrio harveyi*, *Acinetobacter schindleri*, *Vibrio maritimus* and *Bacillus thuringiensis* were found to be associated with *H. leucospilota* specimens from Pangkor Island, Malaysia.

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