

## Short Communication:

# Diversity of culturable epiphytic bacteria isolated from seagrass (*Halodule uninervis*) in Thailand and their preliminary antibacterial activity

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Manuscript received: 1 April 2020. Revision accepted: 6 June 2020.

**Abstract.** Boontanom P, Chantarasiri A. 2020. Short Communication: Diversity of culturable epiphytic bacteria isolated from seagrass (*Halodule uninervis*) in Thailand and their preliminary antibacterial activity. *Biodiversitas* 21: 2907-2913. Epiphytic bacteria are symbiotic bacteria that live on the surface of seagrasses. This study presents the diversity of culturable epiphytic bacteria associated with the Kuicheai seagrass (*H. uninervis*) collected from Rayong Province in Eastern Thailand. Nine epiphytic isolates were identified into four phylogenetical genera based on their 16S rRNA nucleotide sequence analyses. They are considered firmicutes in the genera of *Planomicrobium*, *Paenibacillus* and *Bacillus*, and proteobacteria in the genus of *Oceanimonas*. Three species of epiphytic bacteria preliminarily exhibited antibacterial activity against the human pathogenic *Staphylococcus aureus* using the perpendicular streak method. The knowledge obtained from this study increases understanding of the diversity of seagrass-associated bacteria in Thailand and suggests the utilization of these bacteria for further pharmaceutical applications.

**Keywords:** Diversity, epiphytic bacteria, *Halodule uninervis*, perpendicular streak, seagrass

## INTRODUCTION

The tropical marine resources along the coast of Thailand are abundant and varied. The Gulf of Thailand is a semi-enclosed tropical sea located in the Pacific Ocean which surrounded by ASEAN countries such as Thailand, Cambodia, Malaysia, and Vietnam. The gulf comprises dominant coastal ecosystems including coral reefs, mangroves, and seagrass meadows. Seagrass species have a worldwide distribution in temperate and tropical regions (Sakayaroj et al. 2010). They are a very unique and specialized group of marine flowering plants that have fully adapted to an underwater lifestyle (Hurtado-McCormick et al. 2019). Seagrass meadows are highly productive and dynamic coastal ecosystems (Mishra and Mohanraju 2018). They provide numerous important ecological services to the marine ecosystem such as carbon dioxide capture and storage, the food source of rare marine animals, habitat, and nursery of marine life, and protection of shorelines (Horinouchi et al. 2012; Ugarelli et al. 2017; Rotini et al. 2020). There have been more than one hundred seagrass studies in Thailand on various topics such as associated animals, biochemistry, conservation, distribution, ecology, management, species lists, surveys, and taxonomy. (Prathep et al. 2010). However, the data regarding seagrass-associated bacteria in Thailand remains limited.

Seagrasses harbor diverse communities of bacteria including epiphytic and endophytic bacteria symbioses with their leaves and roots. Epiphytic bacteria are non-harmful bacteria that live on the surface of various organs

of plants, while endophytic bacteria live inside the plants but have no visibly harmful effects (Tarquinio et al. 2019). The epiphytic species provide seagrasses with nitrogen fixation and nutrient cycling. They not only contribute to the seagrass ecosystem but also support other functions to herbivory and transfer of energy to higher trophic levels (Mishra and Mohanraju 2018). Cristianawati et al. (2019) studied seagrass-associated bacteria from the North Java Sea, Indonesia. Findings indicated that the endophytic bacteria were more abundant than the epiphytic bacteria. Moreover, a recent study highlighted the importance of seagrass ecosystems to the health of humans and other organisms. The study revealed that the seagrass meadows significantly reduce the relative abundance of bacterial pathogens (Lamb et al. 2017). It was believed that the seagrass-associated bacteria are involved in this antipathogenic mechanism. The previous report described some symbiotic bacteria play a protective role by releasing chemicals that protect their hosts (seagrasses) from pathogens and biofouling by other organisms (Tarquinio et al. 2019).

Many human pathogenic bacteria have been reported as antibiotic-resistant microorganisms. A large number of *Escherichia coli* strains were found to be resistant to ampicillin, with several *Staphylococcus aureus* strains resistant to methicillin (MRSA) (Cita et al. 2017). The continued emergence of pathogenic antibiotic-resistant bacteria is of worldwide concern and has led to the re-increase of mortality rates in infectious diseases. As a result, this has increased efforts to efficiently identify new

pharmaceutical compounds. Half of the drugs that are now available on the market are formulated from natural chemical products (Cita et al. 2017), while marine organisms have contributed to the discovery of novel antibiotics (Webster and Taylor 2011; Saggese et al. 2018). The marine environment is considered to be an extremely unexplored source of antimicrobial agents (Agrawal et al. 2017). Therefore, the utilization of chemicals synthesized by marine epiphytic bacteria for pharmaceutical applications is challenging.

In this study, epiphytic bacteria were isolated from the Kuicheai seagrass (*H. uninervis*) collected from the Rock Garden Beach Resort. This resort is situated in Klaeng District, Rayong Province in Eastern Thailand. The epiphytic bacteria were isolated, cultured, and genetically identified by nucleotide sequence analysis of 16S rRNA gene. The antibacterial activity of isolated epiphytic bacteria was preliminarily analyzed. The purpose of this study was to genetically identify marine epiphytic bacteria from local seagrass meadows for possible use in applied microbiology and advancing pharmaceutical researches.

## MATERIALS AND METHODS

### Study area and sampling of seagrass

Kuicheai seagrass (*H. uninervis*) was collected from the seagrass meadow offshore from the Rock Garden Beach Resort, Rayong Province, Thailand (12° 40' N, 101° 39' E) during the late winter season in January 2016 (Figure 1). The seabed of the shallow water beach frontage of this private resort is covered by over one million square meters of seagrass meadows. The Kuicheai seagrass is the

dominant seagrass species in this area (Wanna and Phongpha 2018). The seagrass samples were kept in sterilized plastic bags at 4°C and subjected to bacterial isolation within 24 hours of collection. Collected Kuicheai seagrass is shown in Figure 2. Some specimens of Kuicheai seagrasses were preserved in 10% (v/v) formalin at the Faculty of Science, Energy and Environment, King Mongkut's University of Technology North Bangkok, Thailand.



Figure 2. Kuicheai seagrasses (*H. uninervis*).



Figure 1. Location of Rock Garden Beach Resort in Rayong Province, Thailand (Source: GoogleMaps.com)

## Procedures

### Isolation and culture of marine epiphytic bacteria from seagrass samples

The isolation of marine epiphytic bacteria from seagrass samples followed the previously described method of Nugraheni et al. (2010) with slight modifications. The seagrass samples were rinsed with sterilized seawater to clean the sand particles and small fauna attached to their surfaces. The sample surfaces were then gently scraped by sterilized cotton swab and suspended in sterilized seawater. The suspension was spread plated on the surface of Zobell Marine Agar 2216 (HiMedia, India) and incubated at 27°C (the average temperature of seawater in the sampling sites) for 48 hours. The bacterial colonies were investigated in terms of morphology including shape, margin, elevation and pigmentation. Morphologically dissimilar colonies were selected and streak plated on Zobell Marine Agar 2216 to obtain pure bacterial isolates. All isolated bacteria were characterized by standard biochemical methods such as Gram staining and catalase test.

### Genetical identification of the isolated marine epiphytic bacteria

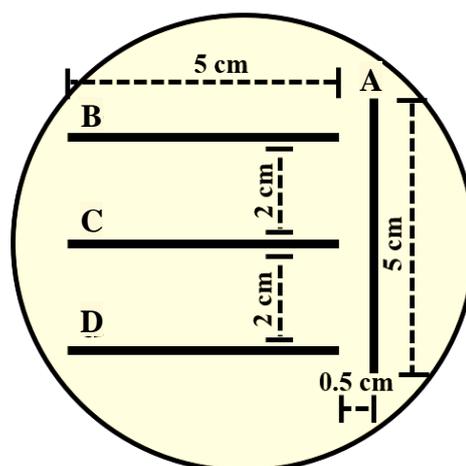
The isolated marine epiphytic bacteria were identified by 16S rRNA gene sequence analysis. The genomic DNA of each bacterial isolate was extracted by ZR Bacterial DNA MiniPrep (Zymo Research, USA) following the protocol described by Zymo Research. Polymerase chain reaction (PCR) amplification of 16S rRNA genes was performed using the OnePCR™ reaction mixture (Bio-Helix, Taiwan) with forward primer 27F (5'-AGAGTTTGTATCMTGGCTCAG-3') and reverse primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Sigma-Aldrich, Singapore). The amplification conditions were carried out following the method of Ferbiyanto et al. (2015). The PCR conditions involved a preheating step at 94°C for 4 minutes, denaturation step at 94°C for 40 seconds, annealing step at 55°C for 1 minute, extension step at 72°C for 1 minute 10 seconds and a final extension step at 72°C for 10 minutes. Amplification was performed for 35 cycles in a Mastercycler® Nexus (Eppendorf, Germany). The 1,500-bp PCR products were purified and nucleotide sequenced by using the services of Macrogen Inc. (Seoul, Korea). Nucleotide sequence similarity analysis of the resulting PCR products was performed at the National Center for Biotechnology Information (NCBI) using the BLASTn program. A phylogenetic tree was generated by the neighbor-joining (NJ) method with 100,000 bootstrap replications using the SeaView program version 4.6.4 (Gouy et al. 2010) and visualized by FigTree software version 1.4.3 (Institute of Evolutionary Biology, University of Edinburgh, UK). Four 16S rRNA gene sequences of the isolated marine epiphytic bacteria from this study were deposited in the GenBank database of NCBI under the accession numbers MT254991, MT254992, MT422059, and MT422061.

### Preliminary antibacterial activity assay of isolated marine epiphytic bacteria

Antibacterial activity assay was performed by the perpendicular streak method against human pathogenic bacteria. The isolated marine epiphytic bacteria were cultured in Zobell Marine Broth 2216 (HiMedia, India) at 27°C for 16 hours and then streak plated as a 5 cm single line on Soybean Casein Digest Agar (HiMedia, India). All agar plates were incubated at 27°C for 48 hours. The pathogenic bacteria used in this study were *Escherichia coli* strain TISTR 073 (Gram-negative bacterium), *Pseudomonas aeruginosa* strain TISTR 1287 (Gram-negative bacterium) and *Staphylococcus aureus* strain MU (Gram-positive bacterium). All pathogenic bacteria were cultured in Soybean Casein Digest Broth (HiMedia, India) at 37°C for 16 hours before use. The pathogenic bacteria were streak plated perpendicular as a 5 cm single line to the original streak of marine isolates on the previous agar plate as shown in Figure 3. All agar plates were incubated at 37°C for 48 hours. The inhibition distance was measured for each pathogenic bacterium. The capacity value of antibacterial activity was calculated as the percentage of the measured inhibition distance divided by the theoretical length of the streak line for each pathogenic bacterium (5 cm). The control plate against the same pathogenic bacteria was perpendicularly streak plated using streptomycin (Sigma-Aldrich, USA) at a final concentration of 50 µg/mL as the positive control and sterilized water was used as the negative control. Streptomycin is a useful broad-spectrum antibiotic that inhibits both Gram-positive and Gram-negative bacteria. All experiments were performed in triplicate.

### Data analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's test with a 95% confidence interval using the free statistical software, R version 3.6.1 (The R Foundation, Austria).



**Figure 3.** Streaking pattern for primary antibacterial activity assay by perpendicular streak method. A. The isolated marine epiphytic bacteria or control. B. *E. coli* strain TISTR 073. C. *P. aeruginosa* strain TISTR 1287. D. *S. aureus* strain MU

## RESULTS AND DISCUSSION

### Isolation of marine epiphytic bacteria from seagrass samples

Nine epiphytic bacterial isolates were successfully isolated from the surface of three parts of Kuicheai seagrass including leaves, stems, and roots. All the purified bacteria had a circular shape but showed dissimilarity in their margins, elevations, and pigmentations. Most isolated epiphytic bacteria were Gram-positive with catalase-positive. Bacteria were classified into four groups based on their dissimilar morphological colonies, Gram staining, and catalase test. Colony morphology and biochemical characteristics of the isolated epiphytic bacteria are shown in Table 1. All isolated epiphytic bacteria were stored as the frozen stocks in 15% (v/v) glycerol at the Faculty of Science, Energy and Environment, King Mongkut's University of Technology North Bangkok, Thailand.

### Genetical identification of the isolated marine epiphytic bacteria

The representative bacterial isolate of each bacterial group was genetically identified by the analysis of the 16S

rRNA gene. The genotypic results showed that these marine epiphytic bacteria belonged to three genera in Phylum Firmicutes as *Planomicrobium*, *Paenibacillus* and *Bacillus*, and one genus in Phylum Proteobacteria as *Oceanimonas*. The leave-associated bacterium strain PSF 142 from bacterial group 1 was closely similar to *P. okeanokoites* strain NBRC 12536 with 98.50% identity. The stem-associated bacterium strain PSS 241 from bacterial group 2 was closely similar to *O. smirnovii* strain 31-13 with 97.77% identity. The root-associated bacterium strain PSR 141 from bacterial group 3 was closely related to *Paenibacillus* sp. strain DK with 99.85% identity, and the strain PSR 243 was closely related to *B. pumilus* strain YXY-11 with 99.23% identity. A phylogenetic tree of the isolated epiphytic bacteria is shown in Figure 4. All 16S rRNA gene sequences from this study were deposited in the GenBank database of NCBI under the accession numbers MT254991, MT254992, MT422059, and MT422061, as previously mentioned in the MATERIALS AND METHODS section. The identity percentage of 16S rRNA gene sequences for the isolated epiphytic bacteria is shown in Table 3.

**Table 1.** Colony morphology and biochemical characteristics of isolated epiphytic bacteria from various parts of Kuicheai seagrass.

Bacterial isolate	Source of isolation	Shape	Margin	Elevation	Pigmentation	Gram staining	Catalase test	Bacterial group
PSF 142	Leaves	Circular	Entire	Convex	Pale orange, opaque	Positive	Positive	1
PSS 143	Stems	Circular	Entire	Convex	Pale orange, opaque	Positive	Positive	1
PSS 144	Stems	Circular	Entire	Convex	Pale orange, opaque	Positive	Positive	1
PSS 241	Stems	Circular	Entire	Convex	White, opaque	Negative	Positive	2
PSR 141	Roots	Circular	Entire	Convex	White, transparent	Positive	Positive	3
PSR 241	Roots	Circular	Irregular	Raised	Cream, opaque	Positive	Positive	4
PSR 242	Roots	Circular	Irregular	Raised	Cream, opaque	Positive	Positive	4
PSR 243	Roots	Circular	Irregular	Raised	Cream, opaque	Positive	Positive	4
PSR 245	Roots	Circular	Irregular	Raised	Cream, opaque	Positive	Positive	4

**Table 2.** Antibacterial activity of isolated epiphytic bacteria against pathogenic bacteria

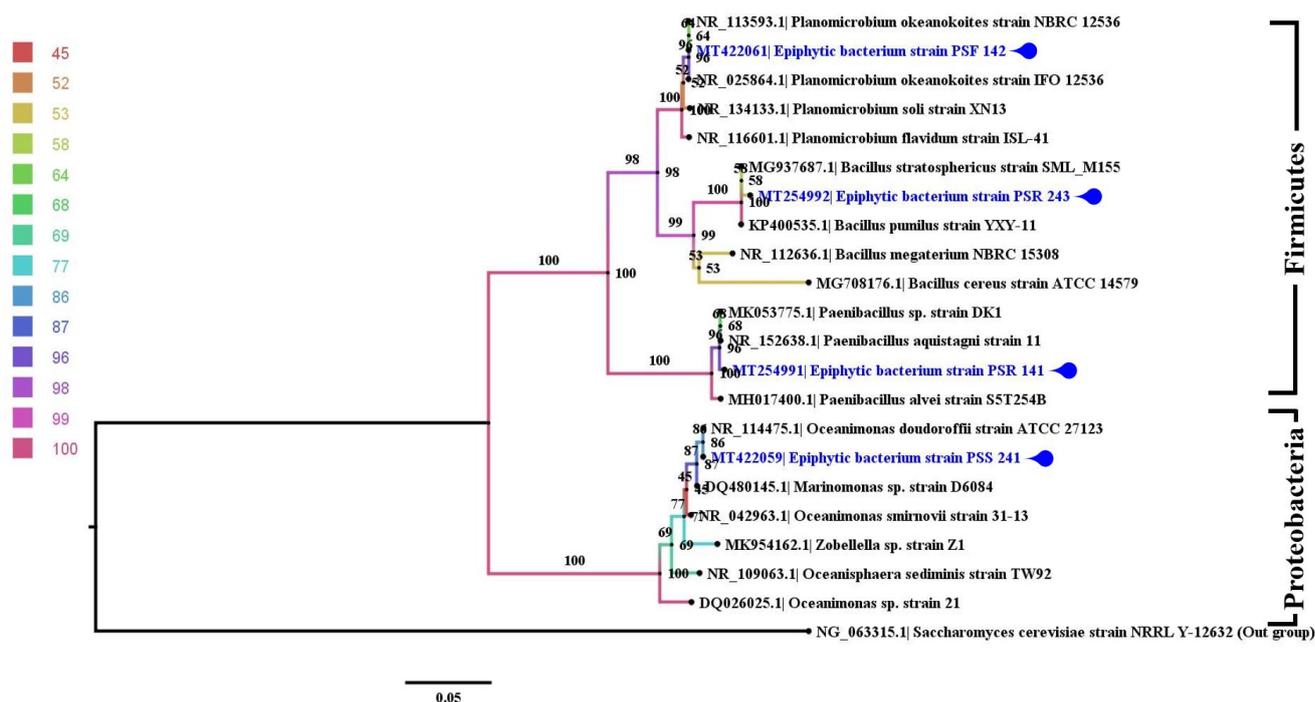
Isolated epiphytic bacteria	Capacity value of antibacterial activity (%)		
	<i>E. coli</i> strain TISTR 073	<i>P. aeruginosa</i> strain TISTR 1287	<i>S. aureus</i> strain MU
<i>P. okeanokoites</i> strain PSF 142	ND	ND	ND
<i>Oceanimonas</i> sp. strain PSS 241	No activity	No activity	15.13 ± 1.21 <sup>b</sup> ( <i>p</i> < 0.001)
<i>Paenibacillus</i> sp. strain PSR 141	No activity	No activity	10.33 ± 0.58 <sup>c</sup> ( <i>p</i> < 0.001)
<i>B. pumilus</i> strain PSR 243	No activity	No activity	5.20 ± 0.31 <sup>a</sup> ( <i>p</i> < 0.001)
Streptomycin (Positive control)	4.70 ± 1.21	3.53 ± 0.50	22.20 ± 0.72 <sup>d</sup> ( <i>p</i> < 0.001)
Sterilized water (Negative control)	No activity	No activity	No activity

Note: ND: not determined. Mean values followed by the same letter were not significantly different according to Tukey's test (*p* < 0.05) among the isolated bacteria and the positive control.

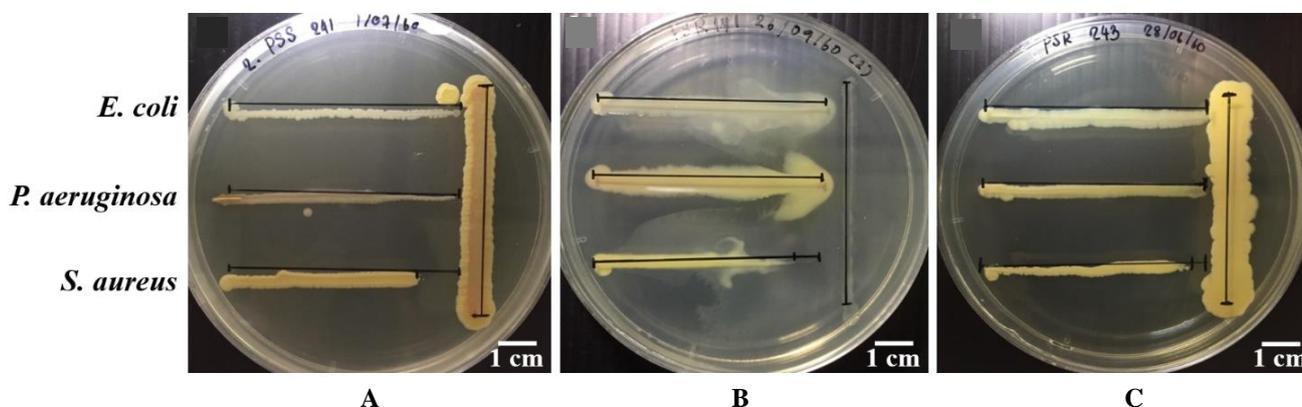
**Table 3.** Identity percentage of 16S rRNA gene sequences for the four isolated epiphytic bacteria

Bacterial isolate	Closely related bacteria	GenBank acc. no. (database)	Identity (%)*	GenBank acc. no. (deposited)
PSF 142	<i>Planomicrobium okeanokoites</i> strain NBRC 12536	NR_113593.1	98.50	MT422061
PSS 241	<i>Oceanimonas smirnovii</i> strain 31-13	NR_042963.1	97.77	MT422059
PSR 141	<i>Paenibacillus</i> sp. strain DK	MK053775.1	99.85	MT254991
PSR 243	<i>Bacillus pumilus</i> strain YXY-11	KP400535.1	99.23	MT254992

Note: \* The identity results were analyzed on March 28<sup>th</sup>, 2020.



**Figure 4.** Phylogenetic tree of 16S rRNA of the four isolated epiphytic bacteria using the neighbor-joining (NJ) method with 100,000 bootstrap replications. The phylogenetic tree was generated by the SeaView program version 4.6.4.



**Figure 5.** Antimicrobial activity assay of the isolated epiphytic bacteria against pathogenic bacteria by perpendicular streak method. A. Antimicrobial activity of *Oceanimonas* sp. strain PSS 241. B. Antimicrobial activity of *Paenibacillus* sp. strain PSR 141. C. Antimicrobial activity of *B. pumilus* strain PSR 243. All experiments were performed in triplicate

### Preliminary antibacterial activity assay of isolated marine epiphytic bacteria

Preliminary antibacterial activity assay of the isolated epiphytic bacteria against human pathogenic bacteria was performed by the perpendicular streak method. The inhibition distance near perpendicular streaking of isolated epiphytic bacteria indicated their antibiotic producing activity as shown in Figure 5. Three epiphytic bacteria were found to have antibacterial activity including *Oceanimonas* sp. strain PSS 241, *Paenibacillus* sp. strain PSR 141, and *B. pumilus* strain PSR 243 (Figure 5 and Table 2). *P. okeanokoites* strain PSF 142 could not be grown with Soybean Casein Digest Agar in this assay. Interestingly, they only showed antibacterial activity against the Gram-positive bacteria, *S. aureus* strain MU, with no antibacterial activity recorded against the Gram-negative bacteria. *Oceanimonas* sp. strain PSS 241 was found to have significantly more powerful antibacterial activity than the other isolates ( $p < 0.001$ ). However, all active isolates significantly exhibited less antibacterial activity than the positive control, streptomycin ( $p < 0.001$ ).

### Discussion

Seagrass leaves and roots possess a core of epiphytic bacteria which differ from the microbial strains present in the surrounding sediment and seawater environments (Tarquinio et al. 2019). The previous study reported that the bacterial diversity in seagrass leaves and roots showed predominant bacterial phyla belonging to Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Mishra and Mohanraju 2018; Tarquinio et al. 2019). Here, the epiphytic bacteria isolated from the Kuicheai seagrass (*H. uninervis*) collected from Rayong Province in Thailand were *Planomicrobium*, *Paenibacillus* and *Bacillus* of the Firmicutes, and *Oceanimonas* of the Proteobacteria based on their 16S rRNA nucleotide sequence analyses. The low number of bacterial strains obtained from this study may reflect the limitations in culturing techniques of marine bacteria therefore the marine bacteria are estimated at less than 2% as a pure culture (Nugraheni et al. 2010). It is also clear that a difference exists among the bacterial diversity related to the type of seagrass (Martin et al. 2018). Jiang et al. (2015) reported that *Bacillus*, *Pseudomonas*, and *Vibrio* were epiphytic bacteria on *Thalassia testudinum* and *Cymodocea rotundata* seagrasses. The isolation of marine bacteria from Indonesian seagrass (*T. hemprichii*) revealed that *B. licheniformis* strain TH8 was an isolated epiphytic bacterium (Nugraheni et al. 2010). Epiphytic bacteria that successfully colonize on the seagrass surface must be able to withstand interactions with the host defense processes and competition with other microorganisms (Egan et al. 2013).

The predominant epiphytic bacterium in this study was *B. pumilus* by 44% of nine isolated bacterial strains. They were isolated from root samples of the Kuicheai seagrass. *B. pumilus* are the ubiquitous, Gram-positive, aerobic, and endospore-forming bacteria that can be isolated from soils, plants and environmental surfaces. The presence of *B. pumilus* on the root surface can be due to the bacterial communities dwelling in the marine sediment. The

previous reported indicated that *Bacillus* species in the marine sediments favor the stability of the seagrass meadows (Mishra and Mohanraju 2018). In this study *B. pumilus* strain PSR 243 was found to exhibited slight antibacterial activity against the pathogenic *S. aureus*. Accordingly, a previous study on the isolated marine *B. pumilus* strain SF214 reported that it could produce pumilacidin which showed antibacterial activity against *S. aureus* (Saggese et al. 2018). *P. okeanokoites* and *Oceanimonas* sp. are quite rare and there are only few reports available. *P. okeanokoites* have been isolated from seafood and coastal sediments. They are Gram-positive to Gram-variable, aerobic, and non-endospore forming bacteria (Luo et al. 2014). *Oceanimonas* species are rod-shaped bacteria with flagella, Gram-negative, and aerobic (Lee et al. 2018). *Oceanimonas* sp. strain PSS 241 was found to have more antibacterial activity than any other epiphytic isolates. The chemicals and antibacterial activity produced from this bacterium should be further characterized by high-throughput technologies such as NMR spectroscopy and mass spectrometry, and assayed by the disk diffusion or the agar well diffusion techniques. *Paenibacillus* sp. strain PSR 141 was indicated as a root-associated bacterium. These species have been isolated from diverse habitats including marine environments and deep-sea surface. They are Gram-positive, facultatively anaerobic, or strictly aerobic and endospore-forming bacteria (Simon et al. 2017). *Paenibacillus* species have exhibited a broad inhibition spectrum against bacteria and pathogenic fungi by their metabolites (Grady et al. 2016). However, there is truly little information about the potential role played by epiphytic bacteria in defending seagrasses and other marine plants from the pathogens and our knowledge derived from indirect evidence such as bacterial secondary metabolites (Tarquinio et al. 2019).

In conclusion, the isolation and the genotypic identification of marine epiphytic bacteria obtained from the Kuicheai seagrass (*H. uninervis*) with preliminary antibacterial activity were reported. There were four genera of isolated epiphytic bacteria including *Planomicrobium*, *Paenibacillus*, *Bacillus* and *Oceanimonas*. *Oceanimonas* sp. strain PSS 241 was considered to be effective against the pathogenic *S. aureus* with 15% of antibacterial activity using the perpendicular streak method. Finally, further study on bacterial compound isolation and advanced bioassays are suggested.

### ACKNOWLEDGEMENTS

This research was funded by King Mongkut's University of Technology North Bangkok. Contract no. KMUTNB-61-GOV-A-72 (KMUTNB-61-GOV-03-72).

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