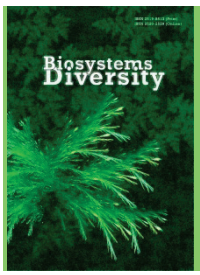

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Marine flatworm *Acanthozoon* sp.-associated bacteria with antibiotic property from the Java Sea

A. Sabdono, D. P. Wijayanti, S. Subagiyo

Diponegoro University, Semarang, Indonesia

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Diponegoro University, Semarang, Central Java, 50275, Indonesia.
Tel.: +62-24-746-00-36.
E-mail: agus_sabdono@yahoo.com

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Neglected invertebrates, marine flatworms, have attracted global research interest due to their biological and chemical potential properties. The marine flatworms (Turbellaria), Phylum Platyhelminthes, belong to the Polycladida group. There are about 3000 species of free-living flatworms that make a living by hunting and eating other animals. A marine flatworm *Acanthozoon* sp. was used in this study due to its abundant presence in the site location. *Staphylococcus epidermidis* is an opportunistic pathogenic bacterium that was previously considered a harmless skin disease bacterium. This species is now considered to be in the first rank among the causative agents of nosocomial infection, specifically in the form of infections of the urinary tract, respiratory tract, and surgical site wounds. The aims of this study were to explore the biological diversity of marine flatworm-associated bacteria with antipathogenic properties and to detect the presence of polyketide synthase (PKS) and nonribosomal peptide synthetase NRPS gene clusters through a molecular approach. Recent studies have shown that *S. epidermidis* undergoes functional changes in the pro-inflammatory peptide family so that it has functions in immune evasion and biofilm development. Therefore, the search for new antimicrobial compounds is urgently needed due to the limited choice of antibiotic use. In the preliminary screening by overlay test, 7 out of 17 (41.2%) isolates showed antibacterial activities. These isolates were reselected and their activity confirmed by using plug agar and disk-diffusion methods. The FA02, FA03, FA05, FA07, FA13, FA16, and FA17 isolates demonstrated their inhibitory activities consistently against the causative agent of nosocomial infection *S. epidermidis*. Based on the morphological and 16S rRNA partial sequencing analysis, these isolates were closely related to the genus *Virgibacillus*, *Brevibacterium*, *Alcanivorax*, and *Vibrio*. None of these seven antibacterial strains possesses PKS-I and PKS-II, except NRPS genes for *Virgibacillus salarius* strain FA02, *V. salarius* strain FA16, and *V. salarius* strain FA17. The results of this study showed that bacteria associated with marine flatworms have future potential as a source of promising natural products for the development of antibiotics.

Keywords: marine flatworms; antipathogenic property; *Staphylococcus epidermidis*; *Virgibacillus salarius*; PKS; NRPS.

Introduction

Staphylococcus epidermidis is an opportunistic pathogenic bacterium that was previously considered a harmless skin disease bacterium. This species is now considered to be the first rank among the causative agents of nosocomial infection, specifically in the form of infections in the urinary tract, respiratory tract, and surgical site wounds (Li et al., 2009; Otto, 2012; Du et al., 2013). This infection causes serious health problems in some countries due to its morbidity and mortality rates. Further, this disease is more difficult to treat after forming a biofilm and becoming a resistant bacterium to certain antibiotics (Otto, 2012). Recent studies showed that *S. epidermidis* has functions in immune evasion and biofilm development. Hence, a study on the search for new antimicrobial compounds is really urgently needed.

The marine flatworms (Turbellaria), Phylum Platyhelminthes, belong to the Polycladida group (Cuadrado et al., 2017). This comprises about 3000 species of free-living flatworms that make a living by hunting and eating other animals (Lin et al., 2017). Among them, a marine flatworm *Acanthozoon* sp. was used in this study due to its abundance at the study site. The characteristics of this species are non-parasitic, black and almost white with many small papillae with yellow to orange tips (Dixit et al., 2018) (Fig. 1).

Compared to marine sponges, ascidians, and soft corals, marine flatworms are usually overlooked in conventional biodiversity surveys and ecological studies. Recently, however, many researchers have been paying considerable attention to marine flatworms primarily due to their pharmacological importance. A large amount of research on the pharmaceutical properties of flatworms has provided useful knowledge for research on stem cells, aging, bioadhesion, and models for probing environmental changes (Lin et al., 2017).

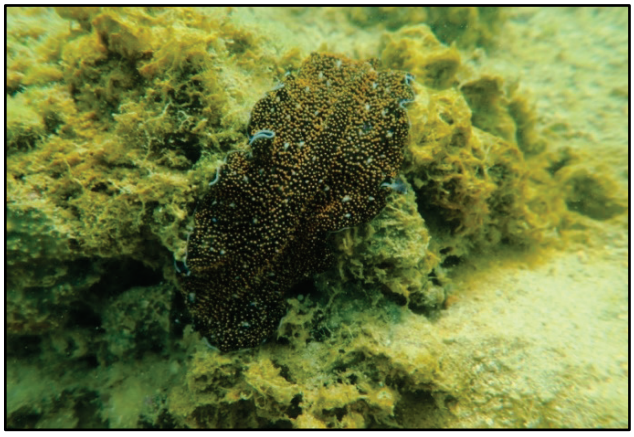

Fig. 1. In situ underwater photograph of flatworm *Acanthozoon* sp. in Awur Bay, Java Sea

In the meantime, Okabe et al. (2021) have reported successfully isolating the neurotoxin tetrodotoxin from this organism. These results demonstrated the potential of polyclads as a source of promising natural products and molecular activities. Today, it is still difficult to determine whether the active compounds produced are accumulations of food or products of bacterial symbiotics. Sadly, there is still little research on flatworm-associated bacteria in relation to the production of active compounds. Dirks et al.

(2012) showed that the genera Candidatus Riegeria symbionts could maintain the asexual reproduction and regeneration of their host flatworm *Paracatenula galateia*. Lin et al. (2017) demonstrated that *Paraplanocera* sp. flatworm-associated bacteria have antipathogenic activity against methicillin-resistant *Staphylococcus aureus* (MRSA). However, Lee et al. (2018) reported that bacteria associated with the flatworm *Dugesia japonica* can inhibit the regeneration of their host. These results showed that bacteria associated with flatworms produced antimicrobial active compounds as a chemical defense for flatworms. Thus, the objective of this study was to investigate biodiversity of marine flatworm-associated bacteria with antipathogenic properties and to detect the presence of PKS and NRPS gene clusters of active compounds.

Material and methods

Sampling and bacterial isolation. Sampling was carried out at Awur Bay (S 06°37'16.9", E 110°38'07.2"), Java Sea, Central Java, Indonesia by scuba diving (Fig. 2). Flatworms were in situ underwater photographed, sampled, placed in a ziplock, and brought to the Tropical Marine Biotechnology laboratory. Flatworm samples were identified based on their morphological appearance and compared to the book identification Reef Creature Identification (Humann & DeLoach, 2010).

Bacterial isolation and purification. The flatworms were washed with sterile seawater twice, to remove the dirt that was still attached to the flatworm body. The spread method was used to isolate flatworm bacterial strains by mashing and diluting body samples into the concentration of 10^0, 10^{-1}, 10^{-2}, and 10^{-3}. Then, 50 µL of each concentration was spread on ½ strength ZoBell 2216E marine agar medium and incubated at room temperature for 48 hours. The growth colonies were selected, and randomly picked based on morphological features, purified by making streak plates, and maintained in slant cultures at –20 ºC.

Antipathogenic assays. The soft-agar overlay technique was used for the preliminary screening of bacterially produced antipathogenic compounds of flatworm bacterial isolates (Kristiana et al., 2020). This technique consists of spotting supernatant from each flatworm isolate onto a solidified soft agar overlay that is seeded with *S. epidermidis* pathogenic test. The *S. epidermidis* pathogen, resistant to gentamicin, benzylpenicillin, ciprofloxacin, and tetracycline was obtained from Dr. Kariadi Hospital, Semarang, Indonesia. The antipathogenic test was carried out by pouring of 0.5 McFarland standard in soft agar media seeded with *S. epidermidis* into the petri dishes growing with flatworm-associated bacteria. The active isolates produce a clearing zone that was selected and reexamined using the agar plug method (Wijaya et al., 2022). Agar plugs were removed with a 6 mm diameter core from 3 x 24 hours of grown cultures of the selected bacteria from MT agar medium. The agar plugs were placed onto the Mueller Hinton Agar (MHA) plate which was previously swabbed with the *S. epidermidis*. The plates containing bacterial strains were incubated at room temperature for 24 hrs. Antipathogenic activity was indicated by an inhibition zone formation. Isolates with antimicrobial activity were selected and their activity reconfirmed again using disk-diffusion methods with slight modification (Sibero et al., 2022). This procedure is similar to that used in the agar plug-diffusion method. *S. epidermidis* was streaked onto MHA medium. The paper disk (6 mm in diameter) contains 35 µL of supernatant and was put on the agar plate culture, incubated overnight at room temperature. The clear zone formation around the disk showed the active isolate.

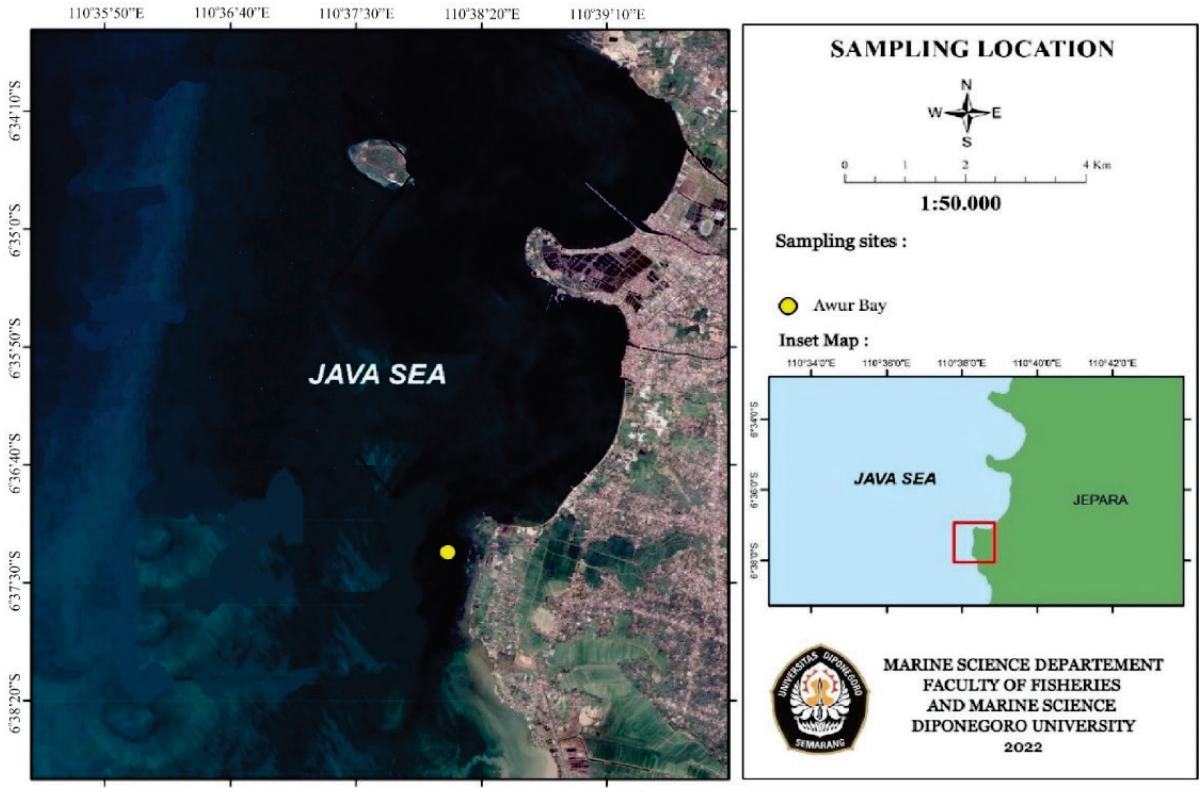

Fig. 2. Sampling site location of Awur Bay, Java Sea marked with yellow dot (S 06°37'16.9" E 110°38'07.2")

DNA extraction, amplification, sequencing, and phylogenetic-tree construction. DNA extraction and amplification were conducted according to the Kristiana et al. (2022). Genomic DNA was extracted by using a Chelex method. PCR reaction system was carried out based on recommended the GoTaq® Green Master Mix (M712) protocol. The 1% agarose gel electrophoresis and UVIDoc HD5 (UVITEC Cambridge) were used to examine and visualize the PCR products. Then, the amplification products were sent to the PT. Genetica Science (Jakarta, Indonesia) for DNA sequencing. The construction of evolutionary trees was performed by using a program for phylogenetic analysis using parsimony (PAUP), maximum likelihood, and distance methods (Swofford, 2003).

Results

Antibacterial assays. In this study, 17 flatworm-associated bacterial isolates were obtained and morphologically characterized. Most of the isolates were yellow and white in colour, small to medium in size, and round and flat in shape. Preliminary screening using the overlay method

indicated that 7 of 17 (41.2%) isolates demonstrated antibacterial activity against the pathogenic bacterium. The activity of these 7 isolates was reconfirmed using plug agar and diffused agar methods, and all these isolates, FA02, FA03, FA05, FA07, FA13, FA16, and FA17 strains, demonstrated consistent inhibition of growth of multidrug-resistant *S. epidermidis*. The diffused agar methods indicated inhibitory zones (Fig. 3, Table 1).

Polyphasic identification and phylogenetic-tree study. The BLAST analyses of the 16S rDNA sequence showed that FA02, FA03, FA05, FA07, FA13, FA16, and FA17 isolates were closely related to *Virgibacillus salarius* SA-Vb1, *Brevibacterium epidermidis* KIT32, *Alcanivorax dieselolei* strain RMR60, *Vibrio harveyi* strain 1.1, *Alcanivorax dieselolei* strain ND1-13, *Virgibacillus salarius* strain SA-Vb1, *Virgibacillus salarius* strain SA-Vb1, respectively (Table 2). To clarify the phylogenetic proximity between *Virgibacillus* and FA-02, FA-16, FA-17 isolates, *Alcanivorax* and F-05 and FA-13 isolates, *Vibrio* and FA-07, *Brevibacterium* and FA-03 isolates, 16S rRNA gene sequences from the strains presented here were aligned to establish a phylogenetic tree. The phylogenetic analysis showed that the FA-02, FA-16, and FA-17 isolates were constantly close to the *Virgibacillus salarius* strain SA-Vb1. F-05 and FA-13 isolates were closely related to *Alcanivorax dieselolei* strain RMR60. While FA-03 and FA-07 isolates were closely related to *Brevibacterium epidermidis* KIT32 and *Vibrio harveyi* strain 1.1, respectively (Fig. 4).

PKS-NRPS gene clusters detection. The amplification of PKS-l, PKS-ll, and NRPS genes is presented in Figure 5. The amplified fragments corresponding to PKS-I, PKS-II, and NRPS were 700–800 bp, 600–700 bp, and 200–300 bp, respectively. Based on the PCR results, the NRPS gene of *Virgibacillus salarius* strain FA-02, *V. salarius* strain FA-16, and *V. salarius* strain FA-17 isolates were detected. No gene clusters were present in other active strains.

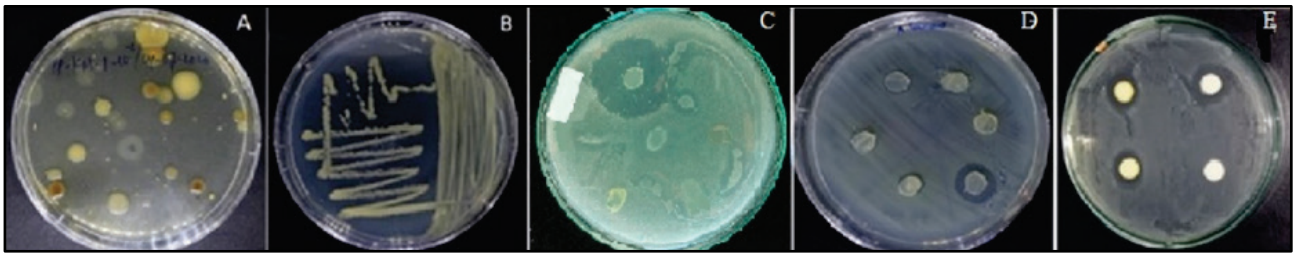

Fig. 3. Morphological characterization and antipathogenic assays of isolates:
A – bacterial isolation, *B* – purification, *C* – overlay, *D* – agar plug, and *E* – disk-diffused agar

Table 1
Morphological characterization and an antibacterial assay of bacteria isolates

Isolate	Morphological colony:					Pathogenic test			
	colour	size	shape	margin	elevation	I	II	III	inhibition, mm
FA01	yellow	small	round	spikey	flat	–	–	–	–
FA02	white	medium	round	smooth	flat	+	+	+	11.5 ± 0.2
FA03	yellow	small	round	spikey	flat	+	+	+	10.2 ± 0.2
FA04	white	medium	round	smooth	flat	–	–	–	–
FA05	white	medium	round	smooth	flat	+	+	+	10.0 ± 0.4
FA06	white	medium	round	smooth	flat	–	–	–	–
FA07	yellow	small	round	spikey	flat	+	+	+	10.5 ± 0.2
FA08	yellow	small	round	spikey	flat	–	–	–	–
FA09	white	medium	round	smooth	flat	–	–	–	–
FA10	white	medium	round	smooth	flat	–	–	–	–
FA11	yellow	small	round	spikey	flat	–	–	–	–
FA12	yellow	small	round	spikey	flat	–	–	–	–
FA13	yellow	small	round	smooth	flat	+	+	+	9.5 ± 0.6
FA14	white	medium	round	smooth	flat	–	–	–	–
FA15	yellow	small	round	spikey	flat	–	–	–	–
FA16	white	medium	round	smooth	flat	–	–	–	–
FA17	yellow	small	round	spikey	flat	–	–	–	–

Notes: I – overlay, II – agar plug, III – diffused agar, "+" – active, "–" – non-active.

Discussion

Marine flatworms, more than a thousand species, are invertebrates that belong to Platyhelminthes Turbellaria polyclad (Rawlinson, 2014). However, research on marine flatworms (Platyhelminthes) has remained neglected in Indonesia. This report is the first study on antibacterial activities from flatworm *Acanthozoon*-associated bacteria. Little is known about their diversity, biological activities, and bioactive compounds in Indonesia.

In this study, 7 of 17 (41.2%) isolates were able to inhibit the growth of the *S. epidermis* bacterial pathogen (Table 1, Fig. 3). The inhibition zone formation in active isolates confirmed the presence of synthesized metabolites with antibacterial activity. The assay results suggest an important biotechnological potential for flatworm-associated bacteria. The 16S rRNA genes revealed that the FA02, FA03, FA05, FA07, FA13, FA16, and FA17 isolates were closely related to the genus *Virgibacillus* sp. (42.85%), *Brevibacterium* sp. (14.28%), *Alcanivorax* (28.56%), and *Vibrio* (14.28%), respectively. The similarity percent of 16S rRNA genes of all the selected isolates was high (99.19–99.73%) with their reference strains (Table 2). These findings are consistent with some previous studies, such as Lin et al. (2017), which demonstrated that *Brevibacterium* sp. and *Vibrio* sp. exhibited antipathogenic activity against MRSA strain ATCC43300. Xu et al. (2015) and Zhang et al. (2016) reported that *Pseudovibrio hongkongensis* sp. nov. and *Pseudovibrio stylochi* isolated from a marine flatworm *Stylochus* sp. were capable of denitrification and fermentation. These results showed that flatworm *Acanthozoon* sp. associated culturable bacteria with anti-pathogenic compounds produced by bacteria from Phyla Firmicutes. *Virgibacillus* sp. was the most dominant genus discovered in this study. The members of this genus are widely found in many habitats and the currently described species have been mostly isolated from saline environments. During the last decade, *Virgibacillus* have been frequently reported to have antagonistic activity. Galaviz-Silva et al. (2018) reported that *Virgibacillus* bacterium isolated from Mexican coasts had antimicrobial activity against food-poisoning agents i.e., *S. aureus* and *V. parahaemolyticus*. Gupta et al. (2019) reported that the *Virgibacillus* had antagonistic activity against Gram-positive bacteria. Previous studies reported that *Virgibacillus* sp. isolated from soft coral *Sinularia* sp. (Sulistiyani et al., 2010; Kusmita et al., 2021) and hard coral *Pavona* sp. had antipathogenic activity against Multi Drug Resistant bacteria (*S. aureus*, *E. coli*, and *Enterobacter* spp.). It was concluded that *Virgibacillus* sp. offers a potential source of antibacterial compounds in particular against MDR strains.

This study showed that three isolates (42.8%), *V. salarius* strain FA02, *V. salarius* strain FA16, and *V. salarius* strain FA17, were detected for NRPS. No gene clusters were present in the other four active strains (Fig. 5). The detection of biosynthetic genes in the NRPS cluster in bacteria associated with flatworms could explain the essential functions of the isolates as antibacterial compound producers. Othoum et al. (2019) reported that *Virgibacillus* species produced polypeptides via NRPS mechanisms. Further, these biosynthetic genes of this genera are transferred horizontally. From this biosynthetic gene, several new bioactive compounds from marine microbes were discovered (Kovácsová et al., 2013, 2015; Beygmoradi & Homaei, 2017). The undetected biosynthetic gene clusters in the other active bacterial strains might have their bioactive compounds produced by the other biosynthetic pathways, such as bacteriocin, terpene, beta-lactam, or other clusters (Belknap et al., 2020). However, special attention is focused on the metabolites with antibacterial properties against bacterial pathogens that are resistant to antibiotics.

Table 2
Identification of bacteria associated with marine flatworms against tropical skin disease

Isolate	Bacterial group	Closest relative	Accession no. reference	Accession no. isolate	Homology, %
FA02	Firmicutes	*Virgibacillus salarius* strain SA-Vb1	NR041270	ON196404	99.59
FA03	Actinobacteria	*Brevibacterium epidermidis* KIT32	NR041270	ON196469	99.19
FA05	γ-proteobacteria	*Alcanivorax dieselolei* strain RMR60	MN582996	ON196502	99.65
FA07	γ-proteobacteria	*Vibrio harveyi* strain 1.1	MZ081629	ON196507	99.65
FA13	γ-proteobacteria	*Alcanivorax dieselolei* strain ND1-13	MZ234099	ON196926	99.44
FA16	Firmicutes	*Virgibacillus salarius* strain SA-Vb1	NR041270	ON197099	99.73
FA17	Firmicutes	*Virgibacillus salarius* strain SA-Vb1	NR041270	ON197100	99.45

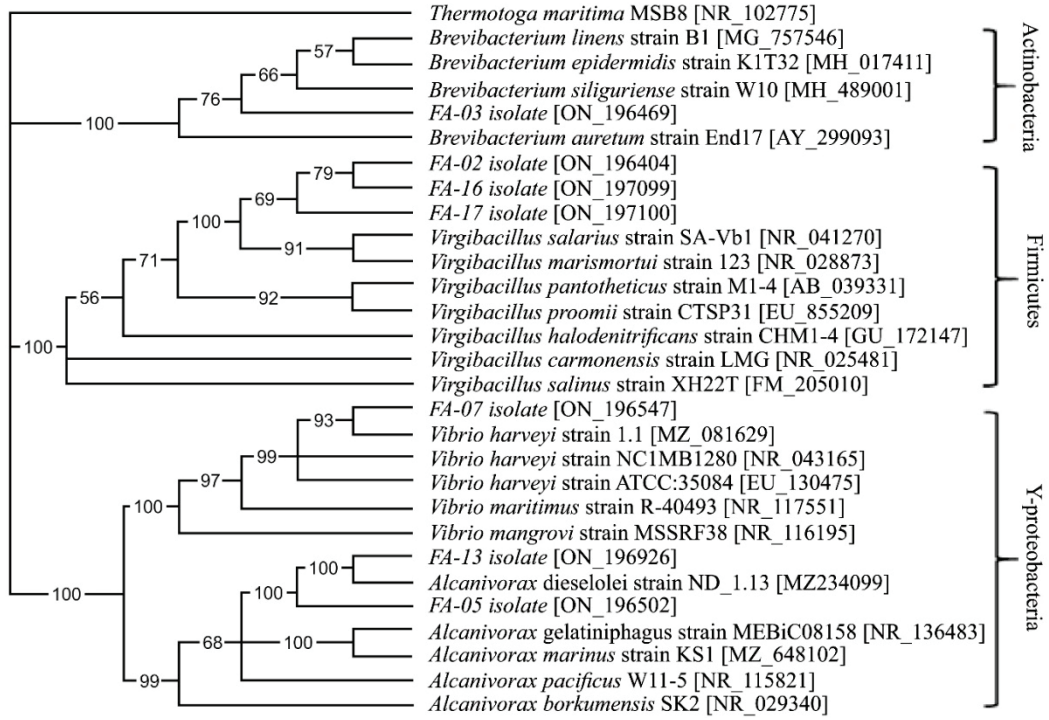

Fig. 4. Phylogenetic tree of marine flatworm-associated bacteria with antipathogenic properties against *S. epidermis*

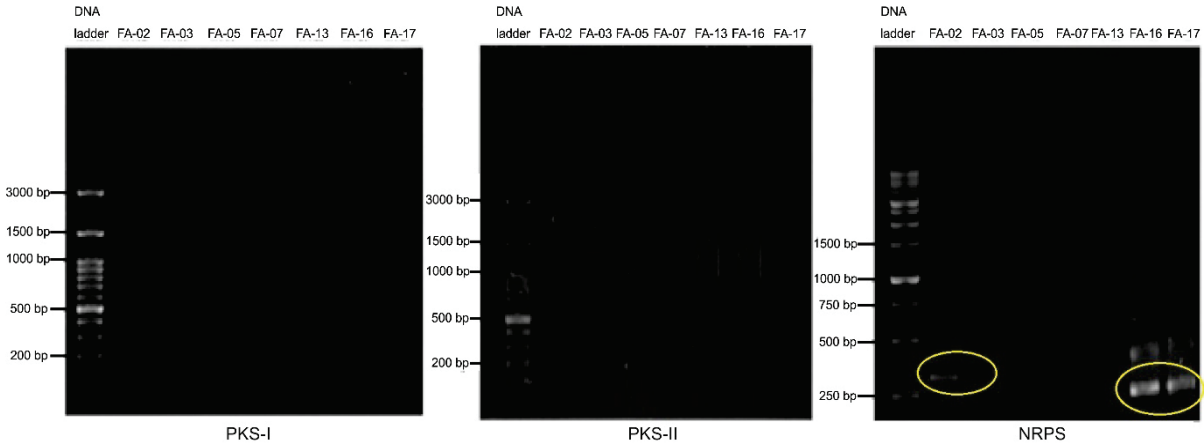

Fig. 5. PKS-I, PKS-II, and NRPS gene clusters of bacterial isolates: the detection of NRPS genes were marked with yellow ovals

In contrast to *Bacillus* sp. and *Streptomyces* sp., which are well-known as antibiotic producers (Rios-Muñiz & Evangelista-Martínez, 2022), little is known about the biosynthetic potential of the *Virgibacillus* genus. Hence, further studies are urgently needed on *V. salarius* strain FA-02, *V. salarius* strain FA-16, and *V. salarius* strain FA-17 found in this study, which might be attractive sources of such novel antimicrobial agents.

Conclusion

This study results showed that *Virgibacillus* species produce antimicrobial properties with NRPS biosynthetic genes in clusters isolated from marine flatworms. These results indicate a significant strategy for finding novel antibiotics isolated from neglected marine invertebrates. Hence, these three strains, *V. salarius* strain FA-02, *V. salarius* strain FA-16, and *V. salarius* strain FA-17 are good candidates for advanced research in separating, purifying, and structure elucidating the potentially novel compounds.

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