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Submission date: 28-Mar-2023 08:29AM (UTC+0700)

Submission ID: 2048588505

File name: 10581-Article_Text-1050715-1-10-20220414.pdf (672.24K)

Word count: 6589

Character count: 36269

Antimicrobial activity of nudibranch *Chromodoris lineolata* associated bacteria against skin diseases pathogens from Jepara Coastal Waters, Indonesia

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Manuscript received: 22 February 2022. Revision accepted: 26 March 2022.

Abstract. Wijaya AP, Sabdon A, Sibero MT, Trianto A, Radjasa OK. 2022. Antimicrobial activity of nudibranch *Chromodoris lineolata* associated bacteria against skin diseases pathogens from Jepara Coastal Waters, Indonesia. *Biodiversitas* 23: 1911-1919. The emergence of multidrug-resistant (MDR) skin pathogens has caused the treatment of skin diseases to become increasingly ineffective using by conventional antibiotics. As a source of antimicrobial compounds against MDR pathogens, marine invertebrate-associated bacteria have been widely known for their valuable biological activity. Therefore, this study aims to determine the antimicrobial activity of nudibranch *Chromodoris lineolata* associated bacteria against various skin pathogens and to detect the presence of biosynthetic gene clusters (BGC) through a molecular approach. A total of 14 nudibranch-associated bacteria were successfully isolated from Awur Bay and Panjang Island. Antimicrobial activity screening using the agar plug method indicated that three bacterial strains showed antimicrobial activity against two skin pathogens, *Propionibacterium acnes* and *Malassezia furfur*. Based on the biochemical test, these potential strains were classified into the Terrabacteria and Proteobacteria groups. Through 16S rRNA gene sequencing, potential strains were identified as *Streptomyces lateritius*, *Labrenzia marina*, and *Halomonas meridiana* through 16S rRNA gene sequencing. The results of BGC detection showed the presence of a type II polyketide synthase (PKS II) biosynthetic gene cluster in *Streptomyces lateritius* and *Labrenzia marina* strains at 600bp-650bp.

Keywords: Antimicrobial, bacteria, multi-drug resistant, nudibranch

INTRODUCTION

The rising frequency of MDR infections has caused up to 700,000 deaths globally (Wang et al. 2019). Several microorganisms, including *Propionibacterium acnes*, *Staphylococcus epidermidis*, and *Candida albicans*, have been reported as MDR skin disease pathogens (Ksiezopolska and Gabaldón 2018; Lee et al. 2018; Aoki et al. 2020). Skin disease is one of the most common diseases in developing countries, particularly in heavily populated regions (Hay et al. 2014; Karimkhani et al. 2017). It is estimated that around 20%-25% of the human population around the world is infected by fungal skin infections (Kühbacher et al. 2017). The most common treatment to cure these diseases is by applying antibiotics to the infected area (Yang et al. 2020). Several antibiotics, including clindamycin, macrolides, tetracyclines, budesonide, and fluconazole, have been extensively used (Li et al. 2016; Bienenfeld et al. 2017). Regrettably, overuse of antibiotics combined with ineffective procedures will result in the emergence of MDR skin pathogens (Chang et al. 2015; Kon 2015). The growing cases of MDR skin pathogens infection caused conventional antibiotic-based treatments for skin illnesses are becoming less efficient (Jacopin et al. 2020). The increasing number of MDR illnesses, along

with the difficulties of treating them, highlights the need for further research on novel antimicrobial agents.

Marine natural products from archipelagic countries are providing a wide range of prospective organisms as a source of novel bioactive compounds (Trianto et al. 2014; Reddy et al. 2015; Cheney et al. 2016; Böhringer et al. 2017; Winters et al. 2018; Hanif et al. 2019; Kristiana et al. 2019; Wijayanti et al. 2020). Marine invertebrates, including sponges, are considered as one of the most promising sources of novel bioactive compounds with antimicrobial properties (Trianto et al. 2014; Hanif et al. 2019). A previous study has reported several novel compounds, which is biaketide and debromoantazirine, were discovered effectively from the sponges *Dysidea* sp. in Jepara, Indonesia (Trianto et al. 2014). In contrary to sponges, nudibranch is not been widely studied besides its potential antibacterial compounds. Nudibranchs are marine invertebrates classified as gastropods from phylum Mollusca that are capable to absorb chemicals from their ingested prey and store it in their mantle. Since nudibranchs are a vulnerable species, these chemicals serve as defense mechanisms against predators. Furthermore, several studies have been conducted to investigate the potential of their bioactive properties as novel antimicrobial agents (Reddy et al. 2015; Cheney et al.

2016; Winters et al. 2018). Unfortunately, from the total of bioactive compounds recovered from Indonesia's marine environment, only 0.4% of the novel compounds recovered from phylum Mollusca (Hanif et al. 2019). A previous study showed the crude extract of nudibranch *Phyllidia varicosa* exhibited antibacterial properties against *Shigella flexneri* and *Staphylococcus aureus* (Reddy et al. 2015). However, extracting bioactive compounds straight from nudibranchs would necessitate a massive amount of nudibranchs, thereby reducing nudibranch diversity in the environment. As a result, another approach to overcoming this barrier is by utilizing nudibranch-associated bacteria as a source of novel antimicrobial compounds. Previous research found that the crude extract of bacteria from the genus *Pseudoalteromonas* associated with nudibranch exhibit antibacterial activity against methicillin-resistant *S. aureus* (MRSA) (Böhringer et al. 2017; Kristiana et al. 2019).

Therefore, this report aims to obtain and determine the antimicrobial properties of nudibranch *Chromodoris lineolata* associated bacteria from Jepara, Indonesia against various skin pathogens and detect the presence of biosynthetic gene clusters (BGC) through a molecular approach.

MATERIALS AND METHODS

Nudibranch sample collection

Scuba diving was carried out to gather nudibranch samples from two sampling sites in Awur Bay

(S 06° 37' 16.9"; E 110° 38' 07.2") and Panjang Island (S 06° 34' 33.7"; E 110° 37' 54.3"), both in Jepara, Central Java, Indonesia (Figure 1). The nudibranch photography was taken underwater at 5-10 m depth. Collected samples were placed in a ziplock bag and stored in a cool box as a source of associated bacteria (Kristiana et al. 2019). Species of nudibranchs were determined according to Sabdono et al. (2021) based on their morphological appearance.

Bacterial isolation and agar plug screening

Serial dilution was carried out to isolate associated bacteria. Each nudibranch specimen was rinsed with sterile seawater and crushed using a sterile pestle and mortar. The sample was diluted to concentrations of 10^{-0} , 10^{-1} , and 10^{-2} before being inoculated into humic vitamin agar (HVA), International Streptomyces Project (ISP) 4, and ISP 4 + Rose Bengal medium. The inoculated samples were plated evenly using the spread method and incubated at 25°C. The growing bacteria were distinguished based on morphological characteristics. To obtain pure culture, each bacteria colony was purified by the streak method into the new medium labelled as MT (Milk-Tween) containing 0.5% yeast extract, 0.5% meat extract, 0.5% soluble starch, 0.2% peptone, 0.5% glucose, 0.2% NaCl, 0.1% CaCO_3 , and 2% agar (Kristiana et al. 2020; Wijaya et al. 2020).

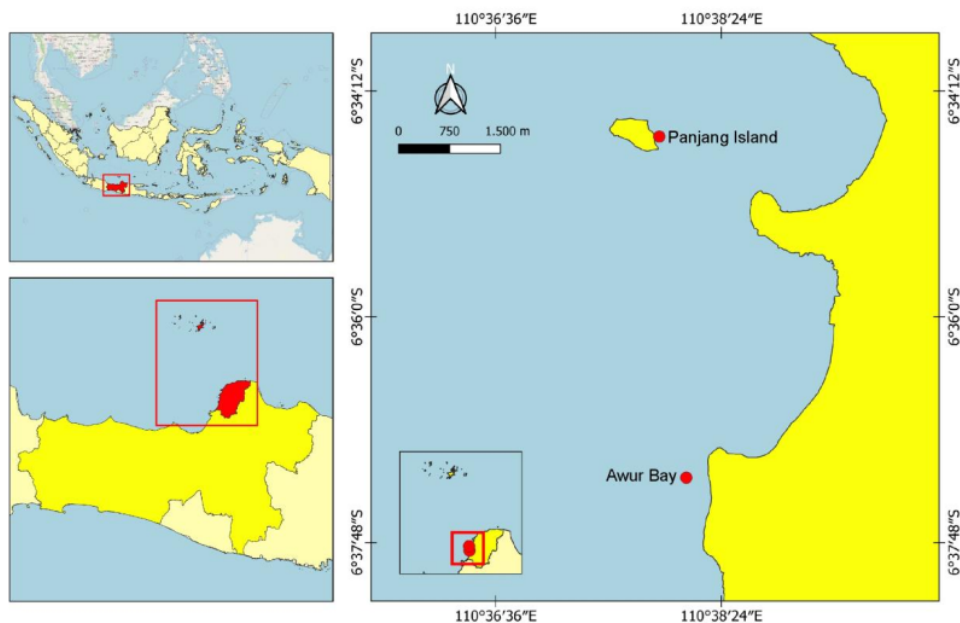


Figure 1. Location of sampling site in Jepara, Central Java, Indonesia. Site one is located in Panjang Island and site two is located in Awur Bay

The agar plug method was applied in the screening for antimicrobial activity of each isolate against several skin pathogens, including *P. acnes*, *S. aureus*, *S. epidermidis*, and *Malassezia furfur*. Pure isolates were fully streaked into MT medium and incubated for 3×24 hours at 36°C. Pathogens were refreshed on nutrient agar (NA) and potato dextrose agar (PDA) for 24 hours. Growing pathogens were then inoculated into nutrient broth (NB) and potato dextrose broth (PDB) in the test tube until they matched the McFarland 0.5 standard density. Inoculated pathogens were swabbed from a test tube into Mueller Hinton agar (MHA) medium and PDA medium. The cultivated isolates were plugged and then inoculated into a medium containing the pathogen. The test medium was incubated for 3×24 hours and documented every 24 hours. The presence of antimicrobial activity is shown by the formation of a clear zone around the agar plug (Sibero et al. 2018b).

Macroscopic characterization and biochemical tests

The form, edge, elevation, size, and color of bacterial colonies were observed macroscopically on an agar medium to characterize the morphology of potential isolates with antibacterial activity (Margarida et al. 2015). Biochemical tests are performed on gram staining, motility testing, citrate testing, indole testing, H₂S testing, and sugar fermentation testing. The gram staining test was carried out following the Himedia K001 gram staining kit procedure (Wijaya et al. 2020). The other biochemical test was conducted by inoculating each potential isolate into a test tube containing Simmons citrate agar (SCA), triple sugar iron agar (TSIA), and sulfide indole motility (SIM) medium (Talaiekhazani et al. 2015).

Salinity experiment

Using the streak method, isolates were inoculated onto nutrient agar (NA) medium with salinities of 0 and 35 ppt. The presence of bacterial growth was used to determine the results of the salinity experiment. The purpose of the salinity test is to identify whether the bacterial isolates are marine obligate or marine facultative bacteria (Wijaya et al. 2020).

Molecular identification

Molecular identification was used to determine the species of the potential isolate. DNA extraction was carried out using the zymo quick-DNA miniprep kit protocol. The 16S rRNA gene of bacteria was amplified using a polymerase chain reaction (PCR). The composition of the PCR mix consists of 1 µL of DNA template, 1 µL of primer 27F (5'AGAGTTTGATCMTGGCTCAG-3'), 1 µL of primer 1492R (5'GGTTACCTTGTTACGACTT-3'), and 9.5 µL of ddH₂O, and 12.5 µL of GoTaq®Green. PCR products were confirmed using electrophoresis and visualized with UVIDoc HD5. Detected PCR products were sequenced by Genetics Science in Jakarta, Indonesia. Bacterial species were identified through DNA barcoding based on NCBI BLAST homology. The MEGA X software was used for the construction of the

phylogenetic tree analysis (Kristiana et al. 2019; Wijaya et al. 2020; Sibero et al. 2018b; Ayuningrum et al. 2019).

Biosynthetic gene cluster detection

KS-F (5'-TSGCS TGCTTGGAYGCSATC-3') and KS-R (5'-TGGAANC CGCCGAABCCGCT-3') primers were used to identify the polyketide synthase (PKS) I gene. Meanwhile, the PKS II biosynthetic gene was identified using IIPF6 (5'-TSG CST GCT TCG AYG CSA TC-3') and IIPR6 (5'-TGG AAN CCG CCG AAB CCG CT-3') primers. Finally, the non-ribosomal peptide synthesis (NRPS) gene was identified using A2gamForward (5'-AAG GCN GGC GSB GCS TAY STG CC-3') and A3gamReverse (5'-TTG GGB IKB CCG GTS GIN CCS GAG GTG-3') primers. Amplification was carried out using the same PCR mix as explained in the previous step (Hodges et al. 2012; Sibero et al. 2018a; Sibero et al. 2019; Frederick et al. 2021; Wijaya et al. 2021).

An antimicrobial assay of crude extracts

As a seed culture, active isolates were inoculated into a test tube containing 10 mL of MT broth medium for three days. As a production culture, the seed culture was transferred into an Erlenmeyer flask containing 100 mL of MT broth medium for seven days. Both bacterial cultures were cultivated on an orbital shaker at a rate of 200 rpm and a temperature of 25°C. Metabolites were extracted using ethyl acetate with the maceration method for 1 hour. The solvent-to-media ratio was set at 1:1. The ethyl acetate phase was separated using a separatory funnel. The crude extract was evaporated using a rotary evaporator at 40°C and weighed before being placed in the freezer (Sibero et al. 2018b; Wijaya et al. 2020). The bacterial crude extract was tested against previously used skin pathogens using the disc diffusion method. The extract was diluted using dimethyl sulfoxide (DMSO) to a concentration of 1 mg/ml before being injected into sterile paper disks (6 mm Oxoid™). Paper disks were incubated for 3×24 hours in MHA and PDA medium containing each pathogen at 36°C. Amoxicillin and nystatin were used as the positive controls, while DMSO was used as the negative control. The antimicrobial properties were observed by measuring the diameter of the clear zone surrounding the paper disks (Sibero et al. 2018b; Kristiana et al. 2019; Frederick et al. 2021).

RESULTS AND DISCUSSION

Nudibranch sample collection

Nudibranchs are marine organisms that can be easily distinguished due to their unique physical traits. Most of these mollusks have a colorful body with a morphology resembling a slug without a protective exterior shell. Instead of a shell, most nudibranchs are covered in a mantle that contains a toxin stored from their prey. All species of nudibranch have a pair of antenna-like sensory organs called rhinophores on the top of their heads. This organ may be found in a variety of shapes and serves a

crucial role in species identification. In this study, the collected nudibranchs were identified based on their morphological appearance according to a previous study by Sabdono et al. (2021). A total of two nudibranchs were successfully collected from two different sampling sites, Awur Bay and Panjang Island, as representatives of nudibranchs in each area. These specimens were collected while feeding on sponges *Dysidea* sp. in shallow coral reefs at a 5-10 meter depth.

Figure 2 shows the nudibranch collected from sampling locations. A nudibranch specimen from Awur Bay was labeled as CL.3, whereas Panjang Island samples were labeled as CL.10. Even though the specimens were collected from different sampling sites, both nudibranchs shared identical morphological appearances. Each nudibranch species can be distinguished based on physical characteristics such as color and pattern. The specimens were covered in a black and yellow striped mantle, a yellow mantle edge, bushy cream-colored gills, and black-brown rhinophores covered in brown spots. According to recent research on the diversity of nudibranchs in Jepara, both of these nudibranchs were identified as *Chromodoris lineolata* (Sabdono et al. 2021). The biodiversity of nudibranchs in the environment is reported to be affected by several environmental parameters, including food, coral coverage, temperature, and substrate (Ompi et al. 2019; Sabdono et al. 2021). The environmental parameters of Awur Bay and Panjang Island were assessed, with an average temperature of 29.7°C and an average salinity of 30 ppt. A previous study by Ompi et al. (2019) found that an area with these environmental parameters was excellent habitat for nudibranchs from the Chromodorididae Family. Moreover, prior research conducted in Jepara also revealed the existence of *Dysidea* sponges in the Jepara coral reefs (Radjasa 2007). The distribution of these sponges as nudibranch prey was suspected as another factor that influenced the abundance of *Chromodoris lineolata* in the sampling sites.

Bacterial isolation and agar plug screening

Following the previous step, identified nudibranch samples went through the isolation of associated bacteria. Moreover, a total of 14 pure culture bacteria were isolated

and purified from both samples. Eight of the associated bacteria were isolated from CL.10 nudibranch, while the other six were from CL.3 nudibranch. All of these isolates were obtained from the ISP 4 medium. Unfortunately, no bacterial growth was detected in the HVA or ISP 4 + Rose Bengal medium. Bacterial growth is strongly influenced by the nutrients in the growth medium. The absence of bacterial growth was assumed to be caused by the medium's insufficient nutrients for the growth of nudibranch-associated bacteria (Fang et al. 2017; Al-Ansari et al. 2020). In the future, ISP 4 medium should be used to isolate nudibranch-associated bacteria rather than HVA and ISP 4 + Rose Bengal medium. All purified isolates were then screened for antimicrobial activities against several skin disease pathogens, including *P. acnes*, *S. epidermidis*, *S. aureus*, and *M. furfur*.

The pure culture isolates were tested for their antimicrobial activity against several skin disease pathogens. The clear zone that developed around the agar plug indicated the presence of antimicrobial activity (Sibero et al. 2018b). Table 1 shows the detailed results of the antimicrobial screening. Three isolates, CL.3.8, CL.10.5, and CL.10.11, displayed a clear zone surrounding the agar plug against two skin pathogens. Both CL.10.5 and CL.10.11 showed antifungal activity against one pathogenic fungus, namely *Malassezia furfur*. On the other hand, isolate CL.3.8 showed a broad spectrum of antimicrobial activity against *P. acnes* and *M. furfur*. Every 24 hours of observation, the diameter of the clear zone rapidly shrinks. These results indicated that the antimicrobial properties of the secondary metabolites secreted were only capable of inhibiting pathogen growth and were classified as bacteriostatic. Secondary metabolites are created by bacteria as a type of self-defense mechanism (Hodges et al. 2021). Moreover, a previous similar study by Kristiana et al. (2019) proved that nudibranch associated-bacteria from Maluku, Indonesia exhibit antibacterial properties against MDR pathogens. Aside from their potential, research into the antimicrobial properties of nudibranch-associated bacteria is still under-reported. Therefore, these findings showed the potential of nudibranch-associated bacteria secondary metabolites as prospective sources of antimicrobial compounds.

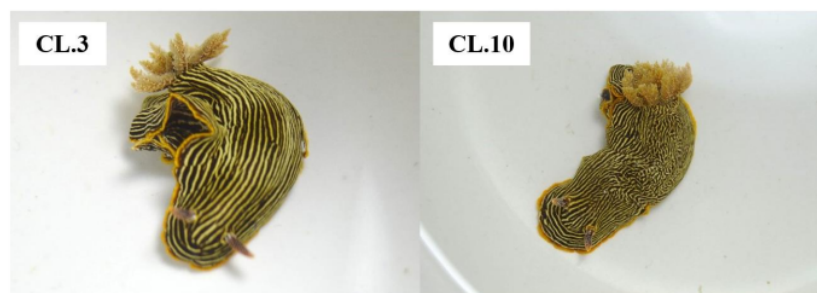


Figure 2. Nudibranch *Chromodoris lineolata* collected from Teluk Awur (left) and Panjang Island (right), Jepara, Central Java, Indonesia

Macroscopic characterization and biochemical tests

The macroscopic morphological characterization of potential isolates was evaluated based on the elevation, shape, margin, size, and color of the colony. Table 2 shows the details of the macroscopic characterization results. Gram staining revealed that CL.3.8 was categorized as gram-positive bacteria with branching streptococci-like cells. On the other hand, isolates CL.10.5 and CL.10.11 were gram-negative bacteria with rod-shaped cells. CL.3.8 features irregular shape, umbonate elevation, lobate border, and brown colonies. Based on these findings, CL.3.8 was suspected to be derived from the actinomycetes bacteria group (Aytiso and Onyango 2016). Isolate CL.10.5 features elevated, punctiform colonies, raised elevation, entire margin, and cream-colored colonies. Meanwhile, CL.10.11 features small colonies, flat elevation, entire margin, and bright cream-colored colonies. The secreted bacterial pigment usually exhibits biological activity such as antibacterial (Usman et al. 2017). Characterization results revealed that each of the potential isolates had distinct morphology traits. Due to varied macroscopic traits, it is suspected that these potential isolates come from different species.

The results of the motility test showed that CL.3.8 was classified as a non-motile bacterium. Isolates CL.10.5 and CL.10.11, on the other hand, displayed spreading growth and were classified as motile-bacteria. The H₂S and indole tests yielded negative results, indicating the isolates lacked of ability to produce H₂S and tryptophanase enzymes (Talaiekhazani et al. 2015). The citrate test of CL.3.8 showed a negative reaction. However, isolates CL.10.5 and CL.10.11 exhibited a color shift from green to blue on the test medium, indicating a positive reaction. These findings indicate that both isolates were capable of converting citrate to pyruvate and utilizing it to provide energy via the Krebs cycle. Bacteria with this ability commonly belong to the Enterobacteriaceae family (Almas et al. 2021). The fermentation test revealed that CL.3.8 and CL.10.5 were incapable of fermenting sugar. Meanwhile, a color shift in the slanted area of the TSIA medium by CL.10.11 indicated the lactose and/or sucrose fermentation. The positive results are caused by the fermentation of carbohydrates into acid products. Glucose is anaerobically fermented at the tube's bottom, while lactose and/or sucrose are aerobically fermented on the slant side of the medium (Talaiekhazani et al. 2015).

Table 1. Antimicrobial activity of nudibranch-associated bacteria against skin disease pathogens

Host species	Total isolates	Active isolates	Isolate	<i>Propionibacterium acnes</i>	<i>Staphylococcus aureus</i>	<i>Staphylococcus epidermidis</i>	<i>Malassezia furfur</i>
<i>Chromodoris lineolata</i>	6	1 (16,67%)	CL.3.1	-	-	-	-
			CL.3.2	-	-	-	-
			CL.3.3	-	-	-	-
			CL.3.5	-	-	-	-
			CL.3.7	-	-	-	-
			CL.3.8	+	-	-	+
<i>Chromodoris lineolata</i>	8	2 (25%)	CL.10.1	-	-	-	-
			CL.10.3	-	-	-	-
			CL.10.5	-	-	-	+
			CL.10.7	-	-	-	-
			CL.10.8	-	-	-	-
			CL.10.9	-	-	-	-
			CL.10.10	-	-	-	-
			CL.10.11	-	-	-	+

Note : (+) : presence of clear zone, (-) : absence of clear zone

Table 2. Macroscopic characterization and biochemical test results

Test	CL.3.8	CL.10.5	CL.10.11
Gram	Positive	Negative	Negative
Shaped	Irregular	Punctiform	Circular
Margin	Lobate	Entire	Entire
Elevation	Umbonate	Raised	Flat
Size	Large	Punctiform	Small
Color	Brown	Cream	Bright cream
Cell shape	Streptococci	Rod	Rod
Motility	-	+	+
Indole	-	-	-
H ₂ S	-	-	-
Citrate	-	+	+
Glucose	-	-	-
Lactose	-	-	+
Sucrose	-	-	+

Note : (+): Positive reaction, (-): Negative reaction

Salinity experiment

In the 0 ppt medium, isolates CL.10.5 and CL.10.11 exhibited no bacterial growth. In contrast, isolate CL.3.8 grew in both 0 and 35 ppt medium (Figure 3; Table 3). According to these findings, isolates CL.10.5 and CL.10.11 were identified as marine obligate bacteria from halophilic bacterial species. Halophilic marine microorganisms have unique mechanisms, including metabolite production, cell anatomy and morphology regulation, cation transport entity modulation, and metabolite production for survival in a rough marine environment (Hohmann 2002; Ariño et al. 2010; Nagano et al. 2010; Sharma and Sharma 2017). Isolate CL.3.8, on the other hand, is a part of the marine facultative bacteria group. Natural drainage systems and human activity are suspected to be the reasons for the broad distribution of facultative bacteria in the terrestrial and marine environment.

Molecular identification

Based on 16S rRNA gene sequencing, the three isolates CL.3.8, CL.10.5, and CL.10.11 were identified (Table 4). The isolate CL.3.8 (MZ596200) was identified as *Streptomyces lateritius* with 99.76% similarity to *S. lateritius* (NR_115438.1). Up until now, no studies have reported *S. lateritius* associated with nudibranchs. *S. lateritius* has been reported to be successfully isolated from the desert ecosystem on the Qinghai-Tibet Plateau. These bacteria were able to produce granaticin A and C from the polyketide group with antibacterial activity against *S. aureus* and *B. subtilis* (Ding et al. 2013). Isolate CL.10.5

(MZ596201) showed 98.75% similarities with *Labrenzia marina* (NR_043040.1). Previous studies have reported that bacteria from the genus *Labrenzia* have been isolated from various macroorganisms in marine ecosystems such as sponges and halophytes (Bibi et al. 2014; Park et al. 2019). Meanwhile, isolate CL.10.11 (MZ596203) has a 99.36% similarity to *Halomonas meridiana* (NR_042066.1). This bacterium is classified as a halophilic bacterium that has been reported to be commonly found in various marine environments, including deep-sea areas (Takahashi et al. 2020; Mostafa et al. 2021). The phylogenetic tree was constructed based on the BLASTn homology of 16S rRNA gene sequencing (Figure 4). The phylogenetic tree was constructed using the same branch spacing since these two species belonged to the same phylum, namely Proteobacteria. However, since *S. lateritius* belongs to the Terrabacteria group, it was not included in the same clade as the other isolates.

Table 3. Salinity experiment results of potential isolates

Isolate	Concentration		Results
	35 ppt	0 ppt	
CL.3.8	+	+	Marine facultative
CL.10.5	+	-	Marine obligate
CL.10.11	+	-	Marine obligate

Note: (+): Presence of bacterial growth; (-): Absence of bacterial growth

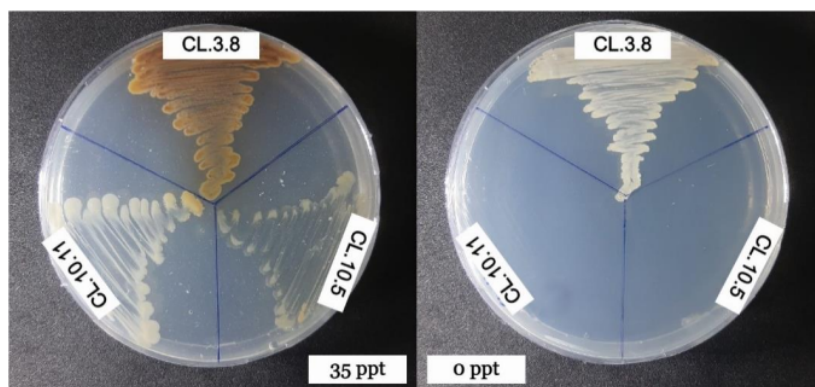


Figure 3. Potential isolates cultured on 35 ppt medium (left) and 0 ppt (right) nutrient agar medium

Table 4. Molecular identification of potential isolates

Isolates	Closest similarity	Similarity*	Acc. num. reference	Isolate acc. num.
CL.3.8	<i>Streptomyces lateritius</i>	99.76%	NR_115438.1	MZ596200
CL.10.5	<i>Labrenzia marina</i>	98.75%	NR_043040.1	MZ596201
CL.10.11	<i>Halomonas meridiana</i>	99.36%	NR_042066.1	MZ596203

Note: *According to NCBI BLAST homology

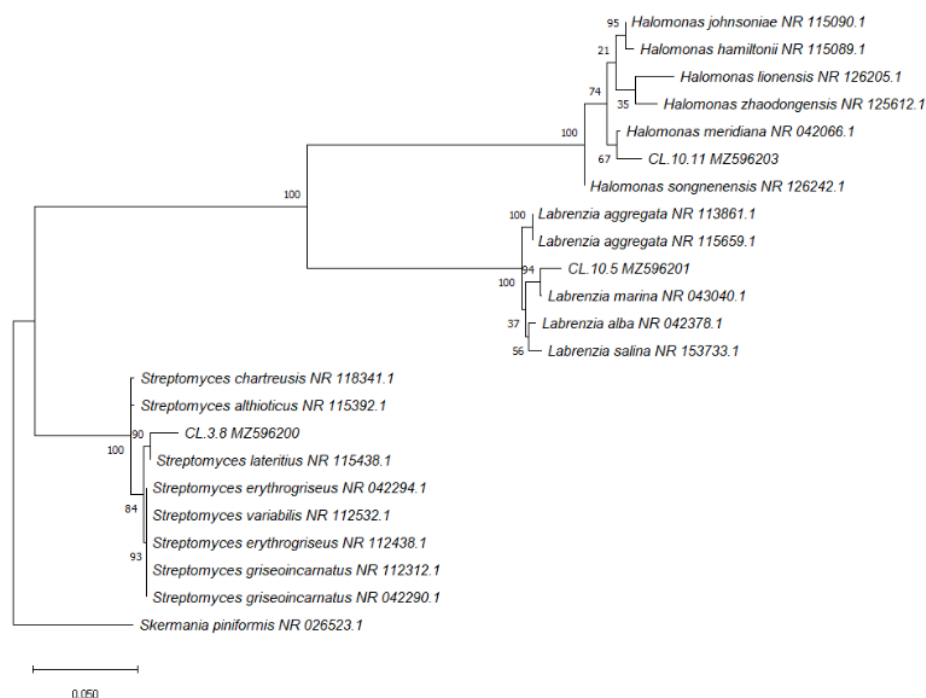


Figure 4. The phylogenetic tree of isolates CL.3.8; CL.10.5; and CL.10.11

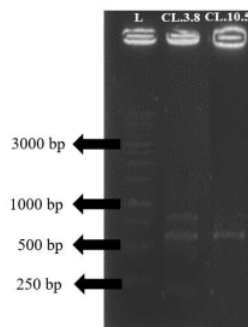


Figure 5. Detected PKS II biosynthetic gene

Biosynthetic gene cluster detection

Detection of the biosynthetic gene cluster (BGC) showed that PKS II biosynthetic genes were detected in *Streptomyces lateritius* (CL.3.8) and *Labrenzia marina* (CL.10.5) with a nitrogen base range of 600bp-650bp (Figure 5). These findings show that bioactive compounds produced by these isolates belong to the peptide or polyketide class. Unfortunately, no PKS I or NRPS biosynthesis genes were found in any of the isolates. The lack of detected biosynthetic genes in *Halomonas meridiana* (CL.10.11) was hypothesized to be caused by

the production of the antimicrobial compound through other biosynthetic pathways, such as beta-lactam, bacteriocin, terpene, PKS III, or other clusters (Belknap et al. 2020). Bacterial BGC is crucial as a biosynthetic pathway of secondary metabolites. These metabolites serve a crucial role as a bacterial defense mechanism to increase the likelihood of survival under harsh environmental conditions (Böhringer et al. 2017). In addition, several studies have proved the potential of these metabolites in industrial applications as a source of antibiotics, anti-tumor drugs, and cholesterol-lowering stimulants (Reynolds et al. 2018; Chen et al. 2019; Martinet et al. 2019).

An antimicrobial assay of crude extracts

Crude extracts from isolate CL.3.8 were tested for antibacterial activity against all four skin disease pathogens. Metabolite extraction from CL.3.8 was based on its broad spectrum of antimicrobial activity against two pathogens in agar plug screening. Regretfully, the disc diffusion test revealed that the bacterial extract did not exhibit a clear zone against all pathogens. The results showed a complete contravention to the agar plug screening results. The outcomes of this test are considered to be influenced by differences in the metabolite culture medium utilized. Agar medium was utilized in agar plug screening, whereas broth medium was used in this assay. As broth medium was suspected of being incompatible with CL.3.8 as a metabolite production medium, metabolite secretion was not maximized (Fang et al. 2017; Al-Ansari

et al. 2020). It could limit the amount of semi-polar molecules secreted by the culture. Kristiana et al. (2019) reported that an ethyl acetate extract of nudibranch-associated bacteria barely exhibited antibacterial activity against MDR pathogenic bacteria. Since MDR pathogens are capable of withstanding several antimicrobial compounds, the metabolites produced were most likely insufficient to inhibit the growth of pathogens (Kabir et al. 2012; Basak et al. 2016; Ren et al. 2020). Finally, it is highly suggested to produce bacterial metabolites by utilizing an agar medium and extracting them using additional solvents such as methanol in a future study.

In conclusion total of 14 nudibranch-associated bacteria were isolated from *Chromodoris lineolata* at Jepara, Indonesia. The result of agar plug screening showed that four isolates exhibited antimicrobial activity against *P. acnes* and *M. furfur*. These isolates, CL.3.8; CL.10.5; and CL.10.11, were closely related to *S. lateritius*, *L. marina*, and *H. meridiani*, respectively. Based on biosynthetic gene cluster detection, PKS II biosynthetic genes were detected in *S. lateritius* (CL.3.8) and *L. marina* (CL.10.5).

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial assistance from the Ministry of Education, Culture, Research, and Technology research grant program, under scheme Penelitian Dasar no. 225-61/UN7.P4.3/PP/2021 for the funding year 2021.

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