

Natural extracts derived from marine sponge-associated bacteria with diverse nonribosomal peptide synthetase genes have anticancer activities

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ABSTRACT

Marine bacteria carrying nonribosomal peptide synthetase (NRPS) genes could be a promising source of anticancer compounds. This study evaluated the anticancer activity of sponge-associated bacteria with diverse NRPS genes. Nine isolates used in this study possessed diverse NRPS genes, as detected by polymerase chain reaction (PCR) screening. 16S rRNA-based identification exhibited that all nine isolates belonged to the family of Gammaproteobacteria class and were of the genus *Pseudoalteromonas*, *Pseudomonas*, or *Serratia*. Enterobacterial repetitive intergenic consensus-PCR confirmed that all isolates were different strains. Ethyl acetate extracts of the nine bacteria showed toxicity against *Artemia salina* and median lethal concentration (LC₅₀) values of 30.94–179.41 µg/ml. Of the three bacterial isolates with the lowest LC₅₀, the STIL 37-derived extract showed the highest anticancer activity and a high selectivity index against three cancer cell lines (A549, MCF-7, and WiDr) with median inhibition concentration values of 163.26–304.51 µg/ml, but there was no effect on human mammary epithelial cells (MCF-12A), as tested by microculture tetrazolium test. According to gas chromatography-mass spectrometry analysis, hexadecanoic acid was found to be the most dominant compound in STIL 37 extract. In conclusion, all nine strains of sponge-associated bacteria, especially STIL 37, could potentially be used in the development of new anticancer drugs.

INTRODUCTION

Sponges are a known source of natural products, several of which have pharmacological functions (Gandelman *et al.*, 2014). In the last decade, ≥2,400 novel compounds have been isolated from ≥600 species of sponges. These compounds were classified as peptides, terpenoids, terpenes, and alkaloids (Mehbub *et al.*, 2014). Interestingly, many of them were reported to be synthesized by sponge-associated bacteria, which are supposed to play an important role in the host defense against environmental stress, including pathogens, parasites, and parasitoids (Flórez *et al.*, 2015). Thus, marine bacteria are expected to be a promising source for the sustainable production of new particular targeted sponge-derived bioactive compounds without causing significant damage to the environment.

The bioactive compounds produced by bacteria, such as nonribosomal peptides, may act as antibiotics (Martínez-Núñez and López, 2016), immunosuppressants (Gao *et al.*, 2012), or antitumor agents (Sato *et al.*, 2013). These compounds are synthesized by multidomain enzymes, namely, nonribosomal peptide synthetase (NRPS). The core catalytic domains of NRPS comprise a thiolation (T), adenylation (A), and condensation (C) domain and usually terminate with a thioesterase (Te) domain (Miller and Gulick, 2016). Molecular detection using primers complementing with the conserved A-domain sequence has been commonly used to discover new NRPS genes in marine bacteria (Tambadou *et al.*, 2014). This conserved domain provides molecular targets for the identification of bioactive compounds from bacteria. By employing this method, the diversity of NRPS genes in microorganisms, such as fungi (Boettger and Hertweck, 2013), bacteria (Su *et al.*, 2014), and actinomycetes (Ziemert *et al.*, 2014), has been reported.

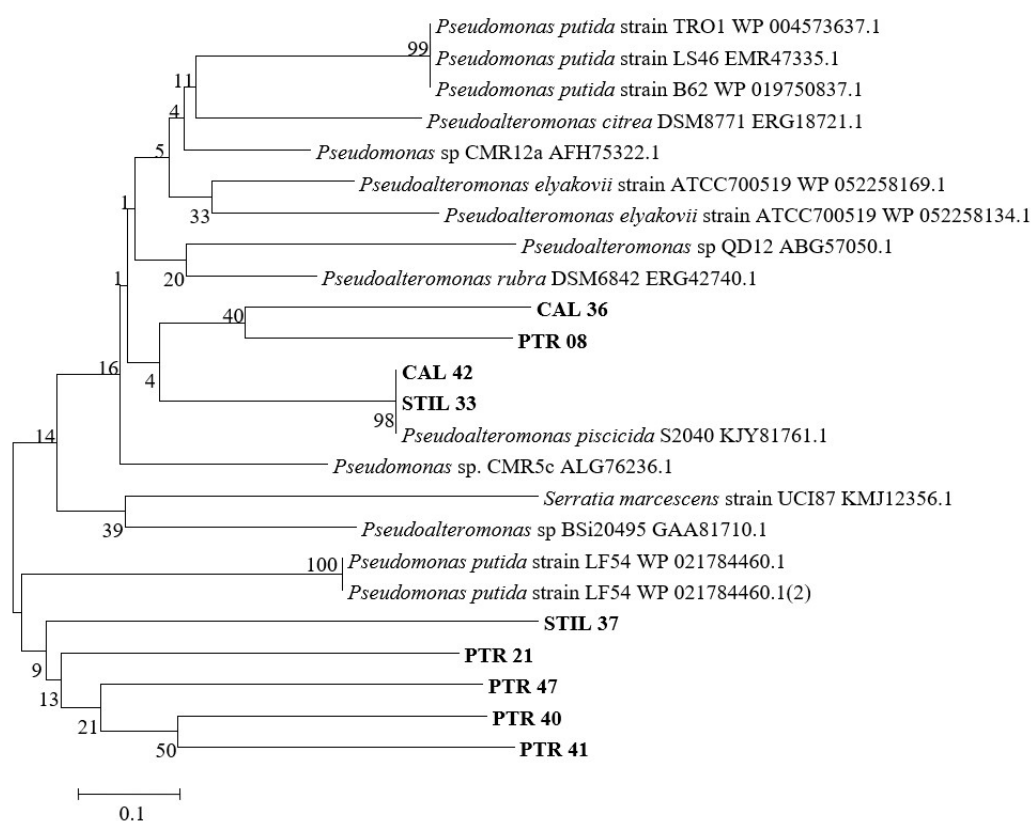
The Indonesian seas could be endowed with diverse compounds that might be a great source of components for medicinal use. However, until 2017, 732 marine natural products have been

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Table 1. Genetic materials used in this study.

No.	Isolates	Materials	References
1	CAL 36	Genomic DNA	This study
2.	CAL 42	Genomic DNA	This study
3.	PTR 08	Genomic DNA	This study
4.	PTR 21	Genomic DNA	This study
5.	PTR 40	Genomic DNA	This study
6.	PTR 41	Genomic DNA	This study
7.	PTR 47	Genomic DNA	This study
8.	STIL 33	Genomic DNA	This study
		16S-rRNA sequence	Yoghiapiscessa <i>et al.</i> , 2016
9.	STIL 37	Genomic DNA	This study
		16S-rRNA sequence	Yoghiapiscessa <i>et al.</i> , 2016

**Figure 1.** Phylogenetic tree of A-domain of NRPS genes from nine bacterial isolates to the reference strains in amino acid level. Numbers at the nodes denote the levels of bootstrap support in 1,000 time repetitions.

identified. Sponge was found as a major source of these compounds (68%). In contrast, only 0.3% of compounds are originated from bacteria, especially the actinobacteria group (Hanif *et al.*, 2019). Therefore, exploration of Indonesian marine bacteria-derived compounds is necessary, both through molecular approaches and *in vitro* studies. Although approximately 70% of Indonesian territory is the sea, investigations of NRPS genes in marine bacteria from this area are rare. Hence, we focused on the anticancer activity and biodiversity analysis of the sponge-associated bacteria in the Indonesian seas. We used polymerase chain reaction (PCR) screening with primers targeting the conserved A-domain of NRPS genes to identify nine bacteria associated with sponges that were collected from the Raja

Ampat Island in Papua and Kepulauan Seribu in Jakarta. Based on subsequent 16S-rRNA-based identification and enterobacterial repetitive intergenic consensus (ERIC)-PCR analysis, the identified bacterial isolates belonged to the Gammaproteobacteria class. We also investigated the anticancer properties of bioactive compounds that were identified from extracts of the isolated bacteria.

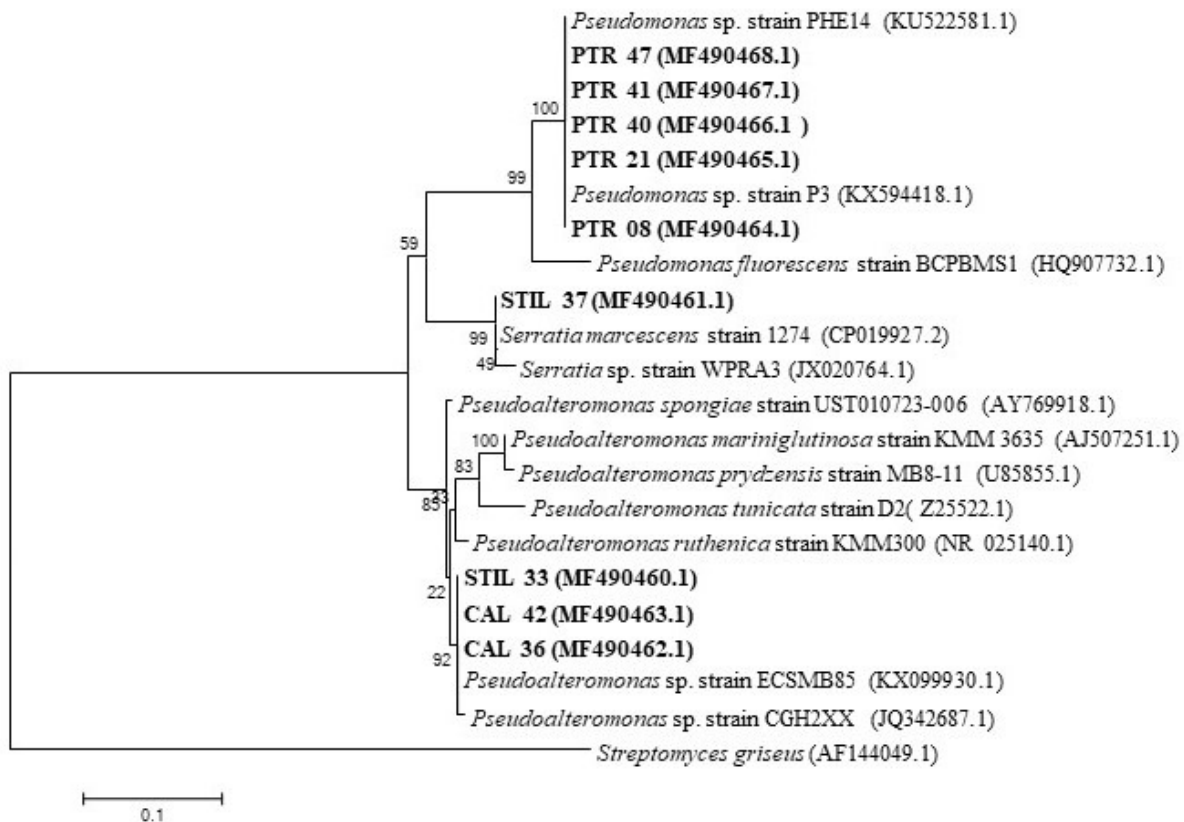
MATERIALS AND METHODS

Bacterial isolates and mammalian cells

The nine bacterial isolates used in this study were isolated in the previous study (Tokasaya, 2010). Genetic material sources

Table 2. A-domain of *NRPS* genes from sponge-associated bacteria compared with those in the GenBank database, which were obtained using BlastX.

No.	Isolates	Related species	Similarity (%)	E-value	Accession number
1.	STIL 33	<i>Pseudoalteromonas elyakovii</i> strain ATCC 700519	96	2e-37	WP052258134.1
2.	STIL 37	<i>Serratia marcescens</i> strain UCI87	89	7e-153	KMJ12356.1
3.	CAL 36	<i>Pseudoalteromonas piscicida</i> strain S2040	62	5e-96	KJY81761.1
4.	CAL 42	<i>Pseudoalteromonas elyakovii</i> strain ATCC700519	89	0.0	WP052258169.1
5.	PTR 08	<i>Pseudomonas putida</i> strain LF54	95	8e-89	WP021784460.1
6.	PTR 21	<i>Pseudomonas putida</i> strain B6-2	73	1e-59	WP019750837.1
7.	PTR 40	<i>Pseudomonas putida</i> strain TRO1	95	4e-101	WP004573637.1
8.	PTR 41	<i>Pseudomonas putida</i> strain LS46	67	2e-81	EMR47335.1
9.	PTR 47	<i>Pseudomonas putida</i> strain LF54	81	4e-61	WP021784460.1

**Figure 2.** Genetic relationship of nine bacteria isolated from sponge compared to their relative strain according to the 16S-rRNA gene.

are shown in Table 1. MCF-7 (human breast adenocarcinoma cells), A549 (human lung adenocarcinoma cells), WiDr (human colon adenocarcinoma cells), and MCF-12A (human mammary epithelial cells) were obtained from Primate Research Center, IPB University.

Genomic DNA extraction, *NRPS* genes screening, and phylogenetic analysis

Genomic DNA from bacterial cultures was isolated using a Presto™ Mini gDNA Bacteria Kit (Geneaid) following the protocol's

guidelines. Then, the A-domain of *NRPS* genes from the bacterial isolates was amplified using PCR with primers for A-domain (forward: 5' AAR DSI GGI GSI GSI TAY BIC C-3' and reverse: 5'-CKR WAI CCI CKI AIY TTI AYY TG-3') (Schirmer *et al.*, 2005). The 50 µl PCR mix consisted of 5 µl of 10 pmol forward primers, 5 µl of 10 pmol reverse primers, 25 µl GoTaq Green® Master Mix 123 (Promega), 4 µl of DNA template (100 ng/µl), and 11 µl of nuclease-free water. The PCR cycling conditions and phylogenetic tree construction were as described by Wahyudi *et al.* (2018).

Table 3. Bacterial identity based on 16S-rRNA gene sequence.

No.	Isolates (Accession number)	Related species	Similarity (%)	Accession number
1.	STIL 33 (MF490460.1)	<i>Pseudoalteromonas</i> sp. strain ECSMB85	100	KX099930.1
2.	STIL 37 (MF490461.1)	<i>Serratia marcescens</i> strain 1274	100	KX570619.1
3.	CAL 36 (MF490462.1)	<i>Pseudoalteromonas</i> sp. strain ECSMB85	100	KX099930.1
4.	CAL 42 (MF490463.1)	<i>Pseudoalteromonas</i> sp. strain ECSMB85	100	KX099930.1
5.	PTR 08 (MF490464.1)	<i>Pseudomonas</i> sp. strain P3	99	KX594418.1
6.	PTR 21 (MF490465.1)	<i>Pseudomonas</i> sp. strain P3	99	KX594418.1
7.	PTR 40 (MF490466.1)	<i>Pseudomonas</i> sp. strain P3	99	KX594418.1
8.	PTR 41 (MF490467.1)	<i>Pseudomonas</i> sp. strain PHE14	100	KU522581.1
9.	PTR 47 (MF490468.1)	<i>Pseudomonas</i> sp. strain P3	100	KX594418.1

Table 4. Toxicity of bacterial extracts on *A. salina*.

No.	Bacterial strain	LC50 ($\mu\text{g/ml}$) \pm SD*
1.	STIL 33	30.94 ^e \pm 1.03
2.	STIL 37	68.71 ^{de} \pm 6.58
3.	CAL-36	128.66 ^b \pm 36.56
4.	CAL-42	95.04 ^e \pm 20.39
5.	PTR-08	58.25 ^{def} \pm 7.13
6.	PTR-21	52.18 ^{efg} \pm 5.37
7.	PTR-40	76.62 ^{cd} \pm 16.61
8.	PTR-41	41.96 ^{fg} \pm 2.38
9.	PTR-47	179.41 ^a \pm 38.18

* Letters a–g represent significant differences between bacterial extracts according to one-way ANOVA and Duncan's multiple range test with p-values <0.05. Different letters above the number indicate that the data were significantly different.

Table 5. Median IC₅₀ value of bacterial extracts and doxorubicin against cancer and normal cell lines.

Cell line*	IC ₅₀ ($\mu\text{g/ml}$) \pm SD**			
	STIL 33	STIL 37	PTR 08	Doxorubicin
A549	593.16 ^d \pm 20.74	304.51 ^{bc} \pm 55.01	608.85 ^d \pm 32.39	32.76 ^a \pm 2.71
MCF-7	683.42 ^d \pm 37.25	186.46 ^{ab} \pm 16.66	578.85 ^d \pm 31.71	19.55 ^a \pm 7.59
WiDr	477.01 ^{cd} \pm 10.34	163.26 ^{ab} \pm 16.53	302.10 ^{bc} \pm 32.64	13.26 ^a \pm 0.41
MCF-12A	135.40 ^{ab} \pm 12.64	987.73 ^e \pm 13.31	328.429 ^{bc} \pm 16.75	28.383 ^a \pm 8.95

* A549, human lung adenocarcinoma cells; MCF7, human breast adenocarcinoma cells; WiDr, human colon adenocarcinoma cells; MCF-12A, human mammary epithelial cells.

** Letters a–e indicate significant differences between bacterial extracts according to one-way ANOVA and Duncan's multiple range test with p-values <0.05.

Means that do not share a letter are significantly different.

Table 6. The SI of bacterial extracts and doxorubicin treatment.

Extract	SI
STIL 33	0.19
STIL 37	4.02
PTR 08	0.56
Doxorubicin	2.34

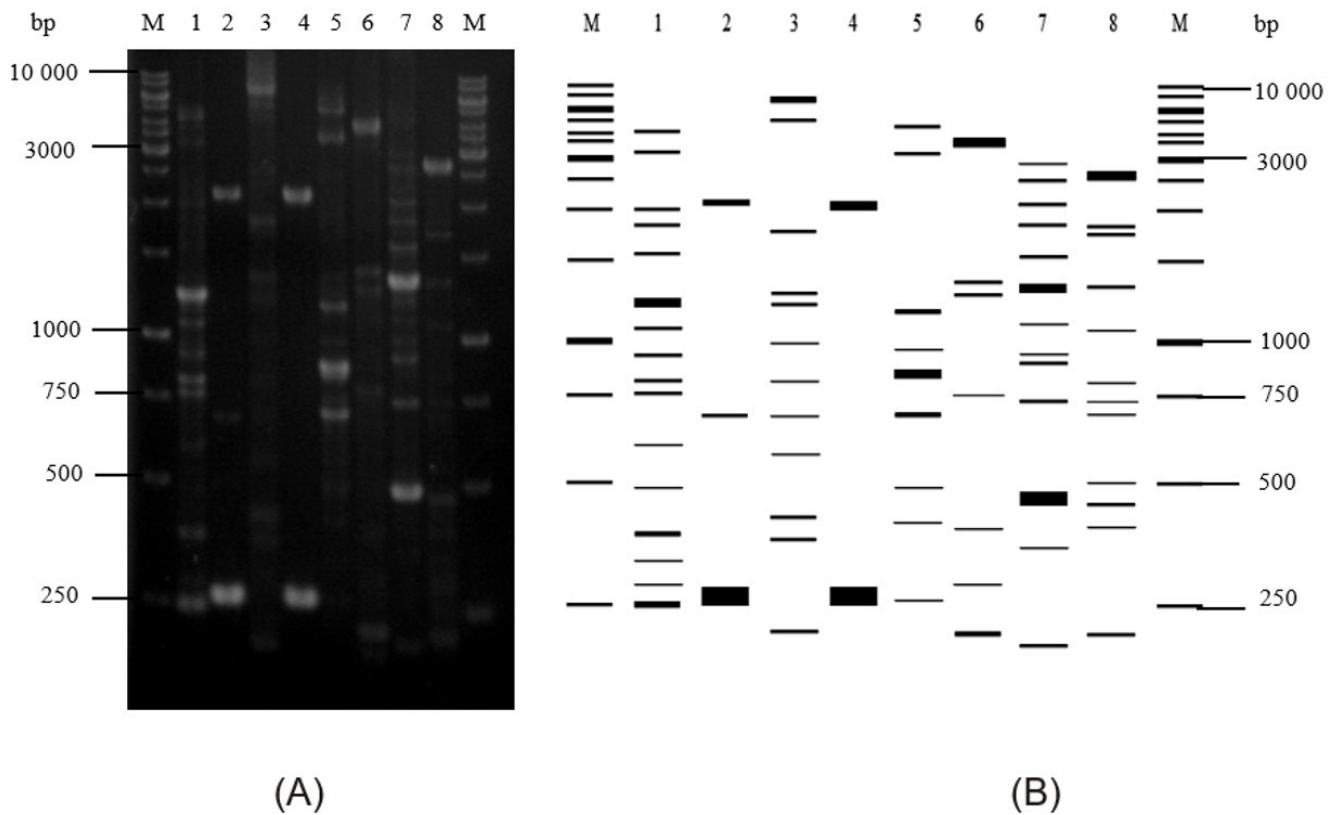


Figure 3. ERIC-PCR profiles of marine sponge-associated bacteria. (A) ERIC-PCR profiles in 1.5% agarose. (B) Illustration of ERIC-PCR profiles created by using Ms. Excel (Microsoft) and Gel Analyzer Software. M, molecular marker; lane 1, STIL 33; lane 2, CAL 42; lane 3, CAL 36; lane 4, PTR 08; lane 5, PTR 21; lane 6, PTR 40; lane 7, PTR 47; lane 8, STIL 37.

PCR amplification of 16S-rRNA genes and phylogenetic tree construction

The 16S-rRNA genes were amplified using primers 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC-3') that were developed by Marchesi *et al.* (1998) to target a conserved region of approximately 1,300 bp. The PCR mixture, cycling conditions, and phylogenetic tree construction were carried out as per Wahyudi *et al.* (2018).

Genetic profiling by ERIC-PCR

Bacterial isolates that had the same 16S-rRNA identity were then identified by ERIC-PCR methods to investigate the intraspecies diversity. The 25 μ l PCR mix consisted of 1 μ l of 25 pmol ERIC1R primers (5'-ATG TAA GCT CCT GGG GAT TCA C-3'), 1 μ l of 25 pmol ERIC2F primers (5'-AAG TAA GTG ACT GGG GTG AGC G-3') (Versalovic *et al.*, 1991), 1 μ l of DNA template (100 ng/ μ l), 12.5 μ l GoTaq Green[®] Master Mix 123 (Promega), and 9.5 μ l of nuclease-free water, which were used for amplification. The PCR conditions were as per Ayu *et al.* (2014).

Culture and extraction of bacterial metabolites

Nine bacterial isolates were inoculated into 1 l of each seawater complete (SWC) medium containing 3 ml glycerol, 1

g yeast extract, 5 g peptone, 250 ml distilled water, and 750 ml seawater. The bacterial cultures were incubated for 72 hours at 28°C with agitation at 120 rpm. Then, the bioactive compounds were extracted by adding the 1 l of bacterial culture to 1 l of ethyl acetate and shaking the mixture for 15 minutes in a separating funnel. The ethyl acetate layer was evaporated at 45°C using a rotary evaporator, and the metabolite extract was diluted in dimethyl sulfoxide.

Toxicity assay of bacterial metabolites

The toxicity effect of the bacterial extracts was assessed according to the brine shrimp lethality test (BSLT). Briefly, 20 *Artemia salina* larvae were grown in a vial, each containing 4 ml of seawater with different concentrations of bacterial extract (1,000, 750, 500, 250, 100, 10, and 0 μ g/ml) and incubated at 27°C for 24 hours under a light. Thereafter, the surviving larvae were counted. Each sample was analyzed in triplicate. The mortality percentage of each treatment was determined using the following formula:

$$\text{Mortality percentage} = \frac{\sum \text{larvae mortality} - \sum \text{untreated larvae mortality}}{\sum \text{initial number of larvae}} \times 100\%$$

The lethal concentration (LC₅₀) value was determined by converting the percentage of mortality to the probit value. The LC₅₀ was observed from the fit line by linear regression analysis (Meyer *et al.*, 1982).

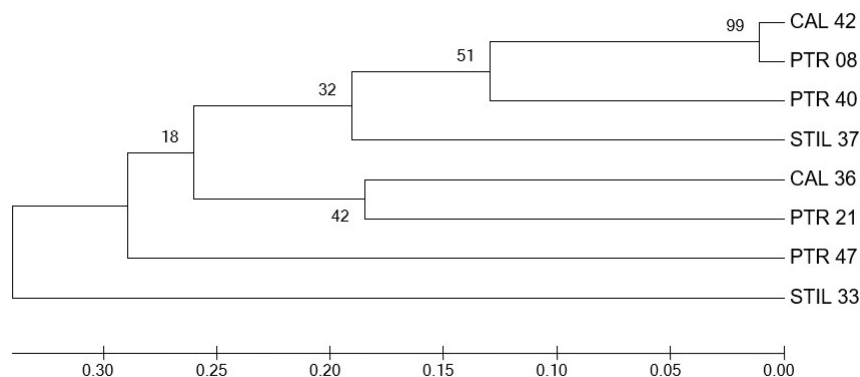


Figure 4. Phylogenetic tree constructed by binary data of ERIC-PCR profiles of nine sponge-associated bacteria using the unweighted pair group method with arithmetic mean method in MEGA 7.0.

Anticancer analysis

The microculture tetrazolium test (MTT) was used for *in vitro* assessment of the cytotoxic properties of the bacterial extracts toward normal cells (human mammary epithelial cells, MCF-12A) and cancer cells (human breast adenocarcinoma cells, MCF7; human lung adenocarcinoma cells, A549; and human colon adenocarcinoma cells, WiDr). MCF-12A and A549 were seeded in Dulbecco's Modified Eagle's Medium, and MCF-7 and WiDr were cultured in Roswell Park Memorial Institute medium added with 10% fetal bovine serum and 100 µg/ml penicillin and streptomycin. Then, 50 µl of cell suspension (5×10^3 cells/well) was seeded into 96-well plates containing 50 µl of medium 173 with different extract concentrations (800, 400, 200, 100, 50, 25, and 0 µg/ml) and incubated at 35°C in 5% CO₂ for 48 hours. All experiments were performed in triplicate, and doxorubicin was applied as a positive control. After incubation, the treated cells in each well were reacted with 10 µl of MTT in phosphate-buffered saline and incubated at 35°C for 4 hours. The formazan crystals formed were diluted in 100 µl of ethanol 96%. The absorbance was quantified via a microplate reader at 595 nm. The percentage of cancer cell proliferation inhibition was determined using the following formula:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of untreated cells} - \text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100\%$$

We performed three replicates for each MTT assay. The median inhibition concentration (IC₅₀) value was calculated by linear regression between the percentage of cell growth inhibition and the extract concentration curve.

Calculation of selectivity index (SI)

The SI of each bacterial extract against cancer and normal cells was calculated by comparing the IC₅₀ value of MCF-7 cells and MCF-12A cells. An SI value ≥ 2 indicated selective toxicity against cancer cells only. Extracts with SI value < 2 were considered toxic to both cancer and normal cells. The SI value was determined using the formula:

$$SI = \text{IC}_{50} \text{ value in normal cells} / \text{IC}_{50} \text{ value in cancer cells}$$

Gas chromatography-mass spectrometry (GC-MS) analysis

The composition of chemical compounds in STIL 37 extract was identified with GC-MS (Pyrolysis 5973 GC-MS, Agilent Technology). 0.6 µl of extract solution was injected into a column with HP-5MS column type (30 m \times 250 µm \times 0.25 µm). The early oven temperature was 80°C and constantly increased at 15°C/minute until 300°C and detained for 20 minutes. Helium gas was used as a carrier at a 1 ml/min flow rate. The injection temperature was 300°C, and the aux temperature was continued at 300°C. The result was analyzed by the GC-MS Pyrolysis program (WILLEY9THN 08. L).

Statistical analysis

The data were presented as average \pm standard deviation from three repetitions. One-way analysis of variance (ANOVA) was applied to compare the average values with 95% and 99% confidence levels. Further analysis was determined using Duncan's multiple range test, and *p*-values < 0.05 were considered statistically significant.

RESULTS

Diversity of NRPS genes in sponge-associated bacteria

Nine bacterial isolates were successfully identified as possessing NRPS genes by PCR technique using primers targeting the conserved A-domain and resulting in a band size of approximately 1,000 bp. Following a search of the National Center for Biotechnology Information's GenBank database, it was determined that these isolates possessed distinctive NRPS genes relative to those in the GenBank database. Sequence with high similarity to NRPS genes ($\geq 95\%$) was observed in 3/9 isolates, and the other six isolates showed a low similarity ($< 90\%$) to related genes in the GenBank database (Table 2). Based on the A-domain of NRPS genes, all isolates belonged to the class of Gammaproteobacteria and were from the *Pseudoalteromonas*, *Pseudomonas*, or *Serratia* genus (Fig. 1).

Bacterial identity based on 16S-rRNA and their intrastrain differentiation

We identified nine bacterial isolates with various NRPS genes based on 16S-rRNA PCR, which resulted in a PCR product of approximately 1,300 bp. All bacterial isolates were identified as

Table 7. Eight major compounds in STILL-37 ethyl acetate extract identified by GC-MS.

No.	Compounds	Peak area (%)	Retention time (minutes)	Biological activity	References
1	Hexadecanoic acid	21.85	10.441	Anticancer	Ravi and Krishnan, 2017
2	1,4-Diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane	8.77	10.363	Antibiofilm	Rajivgandhi <i>et al.</i> , 2020
3	Octadecanoic acid	7.95	11.671	Antimicrobial	Lalitha <i>et al.</i> , 2016
4	7,8,17,18-Tetrahydro-35-methoxy-1,3,21,23-tetramethyl-16H	6.85	23.489	Unknown	
5	Pyrrole[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	5.48	13.092	Anticancer	Vázquez-Rivera <i>et al.</i> , 2015
6	Cyclo(-L-Pro-L-Val)	5.33	9.569	Anticancer	Orishadipe <i>et al.</i> , 2012
7	3-Benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane	3.04	12.851	Unknown	
8	13-Docosenamido, (Z)	3.11	15.003	Unknown	

Gammaproteobacteria and included the genera of *Pseudomonas*, *Pseudoalteromonas*, and *Serratia* (Table 3). Phylogenetic tree construction using the neighbor-joining tree method also showed relatively similar grouping with strains from the GenBank database (Fig. 2). Surprisingly, four isolates (PTR 40, PTR 47, PTR 08, and PTR 21) showed the same homology with *Pseudomonas* sp. strain P3, while three isolates (STIL 33, CAL 36, and CAL 42) were homologous with *Pseudoalteromonas* sp. strain ECSMB85.

Isolates with the same closest relative strain in the GenBank database were distinguished from each other by ERIC-PCR. Each strain showed unique ERIC patterns distinctive from each other, observed as multiple fragments of DNA amplicons that were 0.20–6.0 kb in length (Fig. 3). The genetic relationship-based ERIC patterns of *Pseudomonas* and *Pseudoalteromonas* strains are shown in Figure 4.

Toxicity of bacterial crude extracts

The toxicity of the bacterial crude extracts was determined according to the LC_{50} value, and the extracts were all found to be toxic in a dose-dependent manner, with the crude extracts showing a toxicity level of 30.94–179.41 $\mu\text{g/ml}$. The STIL 33 isolate demonstrated the highest toxicity level (30.94 $\mu\text{g/ml}$), followed by PTR 41 isolate (41.96 $\mu\text{g/ml}$), PTR 21 (52.18 $\mu\text{g/ml}$), and PTR 08 (58.25 $\mu\text{g/ml}$) (Table 4).

Anticancer activity of extracts derived from sponge-associated bacteria

The three potential extracts with the lowest LC_{50} values were used for anticancer analysis against three cancer cell lines (A549, MCF-7, and WiDr) and normal cells (MCF-12A). The cytotoxic effects of the extracts were determined according to the optical density values and morphological changes in the cells after 48 hours of treatment. The anticancer activity of the extracts was determined by their IC_{50} values. All three isolates tested (STIL 33,

STIL 37, and PTR 08) exerted different anticancer activities (IC_{50} , 135.40 to 987.73 $\mu\text{g/ml}$) compared with doxorubicin as a positive control (IC_{50} , 13.26 to 32.76 $\mu\text{g/ml}$) (Table 5). The extract derived from STIL 37 demonstrated the best anticancer property, with an IC_{50} value of 163.26 $\mu\text{g/ml}$ against human colon adenocarcinoma cells (WiDr). This extract also showed a high SI (4.02), two times higher than that of doxorubicin (2.34) (Table 6).

Major compounds found in STIL 37 extract

Eight major compounds were found in STIL 37 extract as identified by GC-MS analysis, such as hexadecanoic acid (1), 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane (2), octadecanoic acid (3), 7,8,17,18-tetrahydro-35-methoxy-1,3,21,23-tetramethyl-16H (4), pyrrole[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) (5), cyclo(-L-Pro-L-Val) (6), 3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane (7), and 13-docosenamido, (Z) (8). Table 7 shows the retention time, peak area (%), and biological activities of these compounds.

DISCUSSION

NRPS genes are promising targets for the identification of bacteria capable of producing bioactive metabolites. The discovery of various *NRPS* genes in bacteria could serve as molecular evidence of the ability of bacteria to produce bioactive compounds. In this study, we identified nine bacterial isolates with diverse *NRPS* genes that belonged to either the *Pseudoalteromonas*, *Pseudomonas*, or *Serratia* genus. Bacteria carrying these genes are potentially able to synthesize bioactive compounds with pharmacological properties. Gonzalez *et al.* (2016) detected *NRPS* genes in *Pseudomonas aeruginosa* that were capable of inhibiting quorum-sensing LasR-dependent signals. *Pseudomonas* sp. isolated from Red Sea sponge that was identified as having *NRPS* genes showed antioxidant activity and cytotoxicity against hepatocellular carcinoma cells (Moneam

et al., 2017). In our investigation, six of the nine isolates showed a low similarity (<90%) to the related genes in the GenBank database, suggesting that the genes in these Indonesian sea sponge bacteria were novel. The existence of a variety of *NRPS* genes in marine bacteria indicates the presence of rich metabolite sources in the marine environment.

The identity of the nine bacteria with various *NRPS* genes was identified according to 16S-rRNA sequencing. This sequence is usually used as a genetic marker for bacterial identification. The 16S-rRNA genes are conserved in the genomes of all bacteria and suitable for molecular identification due to their small variability and their species specificity (Johnson *et al.*, 2019). All nine bacterial isolates, which belonged to either the *Pseudomonas*, *Pseudoalteromonas*, or *Serratia* genus, showed high sequence similarity ($\geq 99\%$) with their closest relatives in the GenBank database. The similarity is accepted to identify bacteria into the same species. Due to their potential to produce bioactive metabolites, these genera are of interest to the study, particularly in the microbial bioprospection field. The *Pseudomonas* genus is well known as a producer of at least 795 compounds (Isnansetyo and Kamei, 2009). Interestingly, four isolates (PTR 21, PTR 40, PTR 47, and PTR 08) were closely related to *Pseudomonas* sp. strain P3, which has the ability to degrade p-nitrophenol (Cui *et al.*, 2002). The *Pseudoalteromonas* genus shows potential because of its ability to produce antimicrobial metabolites (Offret *et al.*, 2016). Furthermore, *Serratia marcescens* strains produce prodigiosin, a red pigment that has been reported to have anticancer properties against prostate cancer and human choriocarcinoma cell lines (Li *et al.*, 2018).

Although the 16S-rRNA gene is sufficient for bioinformatics purposes, its DNA sequence cannot be utilized to determine intrastain differentiations. In our study, STIL 33, CAL 36, and CAL 42 were identified as *Pseudoalteromonas* sp. ECSMB85 and located in the same phylogenetic clade, while PTR 08, PTR 21, PTR 40, and PTR 47 were known as *Pseudomonas* sp. strain P3 and also located in the same clade, indicating that those bacteria were very closely related to each other based on their 16S-rRNA gene sequences. Therefore, another parameter is required to distinguish the isolates from each other. In this study, we used ERIC-PCR to differentiate between the genetic profiles of *Pseudomonas* and *Pseudoalteromonas* isolated from marine sponges. The ERIC-PCR approach is based on targeting a repeating and noncoding DNA consensus sequence in the intergenic region of the bacterial genome. This method has been used to type Gram-negative *Pseudomonas* (Han *et al.*, 2014) and *Pseudoalteromonas* strains (Elena *et al.*, 2002). Surprisingly, isolates identified as *Pseudomonas* sp. strain P3 (PTR 08, PTR 21, PTR 40, and PTR 47) and *Pseudoalteromonas* sp. strain ECSMB85 (STIL 33, CAL 36, and CAL 42) according to 16S-rRNA-based identification exhibited different ERIC patterns. Furthermore, it is noteworthy that all isolates used in this study were separated into different clades in the phylogenetic tree constructed from the ERIC patterns of each isolate, suggesting that these isolates might differ in strain taxa. These results indicate a high diversity among marine bacteria.

We used BSLT to screen for the toxicity of metabolites extract derived from nine sponge-associated bacteria endowed with *NRPS* genes. All extracts exhibited various toxicity levels. The LC_{50} values were <1,000 $\mu\text{g/ml}$, indicating that the extracts were biologically active. The differences in toxicity levels indicated that

the extracts from the sponge-associated bacteria were chemically diverse. Of the nine extracts tested, the STIL 33-derived extract showed the best toxicity ($LC_{50} = 30.94 \mu\text{g/ml}$). Because our previous studies had reported that three isolates (STIL 33, STIL 37, and PTR 08) showed antioxidant properties (Prastya *et al.*, 2020; Yoghiapiscessa *et al.*, 2016), we selected extracts of these isolates for anticancer analysis against carcinoma and normal cells.

Evaluation of the anticancer activity of the three revealed varying levels of growth inhibition of both normal and cancer cells, as indicated by IC_{50} values. Thus, each extract likely exerted a specific anticancer activity toward each cancer cell line. The differences observed in their anticancer activity could be due to differences in the chemical composition of the extracts and the resistance of each cancer cell line. Of the three extracts tested, STIL 37 exhibited the best anticancer properties, with an IC_{50} value of 163.26 $\mu\text{g/ml}$ against human colon adenocarcinoma cells (WiDr). Moreover, this extract also showed a high SI (4.02), two times higher than doxorubicin (2.34), suggesting that it likely had a selective action in inhibiting cancer cell proliferation. In this study, 16S-rRNA-based identification showed STIL 37 belonging to *Serratia marcescens*. In the earlier studies, prodigiosin, a red pigment produced by this species, has also been shown to significantly inhibit the proliferation of Hep2 cell lines, with an IC_{50} value of 31.25 $\mu\text{g/ml}$ (Vijayalakshmi and Jagathy, 2016). Supporting these results, a STIL 37-derived extract was also found to have cytotoxic properties against some hematopoietic cancer cell lines (Priyanto *et al.*, 2017). Therefore, it is crucial to investigate the bioactive compounds derived from STIL 37 for their potential for further exploitation for medicinal applications, mainly as an anticancer agent.

In this study, GC-MS analysis identified that STIL 37 extract was dominated by several biologically active compounds, including hexadecanoic acid, pyrrole[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), and cyclo(-L-Pro-L-Val). Those compounds might contribute to the anticancer property of this extract. A previous study demonstrated that hexadecanoic acid extracted from *Kigelia pinnata* leaves shows cytotoxic activity against human colorectal carcinoma (HCT-116) cells (Ravi and Krishnan, 2017). Other compounds, pyrrole[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), and cyclo(-L-Pro-L-Val), extracted from *Streptomyces mangrovisoli* sp. nov and *Staphylococcus* sp. strain MB30, respectively, also have been known to have anticancer activity toward A549 and HeLa cell lines (Lalitha *et al.*, 2016; Vázquez-Rivera *et al.*, 2015). Anticancer property of this extract also may be supported by other major compounds detected in this extract, such as 1,4-diaza-2,5-dioxo-3-isobutyl bicyclo[4.3.0]nonane, octadecanoic acid, 3-benzyl-1,4-diaza-2,5-dioxobicyclo[4.3.0]nonane, and 13-docosenamamide, (Z). Those dominant compounds may induce apoptotic pathways leading to a decrease in viability of cancer cells.

CONCLUSION

Our present investigation concluded that molecular screening via *NRPS* gene amplification in bacteria could be an effective approach to identifying those capable of producing bioactive compounds. Nine bacterial isolates from three different genera (*Pseudomonas*, *Pseudoalteromonas*, and *Serratia*) were capable of producing biologically active

compounds. Three isolates were shown to inhibit three carcinoma cell lines at different concentrations. The STIL 37-derived extract should be studied further using fractionation and structural elucidation. A high level of bioactivity and target cell selectivity is expected.

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CONFLICT OF INTEREST

All authors declare there is no conflict of interest related to this study.

AUTHOR CONTRIBUTIONS

All authors have contributed to the concept and experimental design, data acquisition, data analysis and interpretation, and paper writing.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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