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Diversity of Nonribosomal Peptide Synthetase Genes in the Anticancer-Producing Actinomycetes Isolated from Marine Sediment in Indonesia

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Abstract

Marine actinomycetes is a group of bacteria that is highly potential in producing novel bioactive compound. It has unique characteristics and is different from other terrestrial ones. Extreme environmental condition is suspected to lead marine actinomycetes produce different types of bioactive compound found previously. The aim of this study was to explore the presence and diversity of NRPS genes in 14 anticancer-producing actinomycetes isolated from marine sediment in Indonesia. PCR amplification and restriction fragment analysis of NRPS genes with *Hae*III from 14 marine actinomycetes were done to assess the diversity of NRPS genes. Genome mining of one species of marine actinomycetes (strain GMY01) also was employed towards this goal. The result showed that NRPS gene sequence diversity in 14 marine actinomycetes could be divided into 4 groups based on NRPS gene restriction patterns. Analysis of 16S rRNA gene sequences of representatives from each group showed that all isolates belong to genus of *Streptomyces*. Genome mining result showed that strain GMY01 harboring 10 different NRPS gene clusters that encode secondary metabolites, as pure NRPS or hybrid between NRPS and other compounds. These results indicated that marine actinomycetes having a high potential to be developed as source of anticancer drugs development.

Keyword: marine actinomycetes, non-ribosomal peptide synthetase genes diversity, *Streptomyces*, genome mining, anticancer drugs development

Introduction

Wide range of bioactive compounds metabolites were isolated and identified from soil actinomycetes. Recently, the rate of new metabolites discovery from terrestrial actinomycetes decreased, whereas the rate of re-isolation of known compounds increased (Dharmaraj, 2010). Thus, it is crucial that new group of actinomycetes from unexplored habitats to be pursued for new sources of bioactive compounds, one of which is marine actinomycetes (Magarvey *et al*., 2004).

Nonribosomal peptide synthetases (NRPS) and type I polyketide synthases (PKS-I) are biosynthetic systems involved in the synthesis of a large number of important biologically active compounds produced

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by microorganisms, among others by actinomycetes (Ayuso-Sacido and Genilloud, 2004).

Farida *et al.* (2007) isolated several marine actinomycetes from Krakal Beach, Gunung Kidul, Yogyakarta and proved the anticancer effects of the marine actinomycetes extracts. That research results also showed that the anticancer compounds from marine actinomycetes suggested maybe belong to the poliketide or non-ribosomal peptides based on the presence of NRPS or PKS gene. According to Wang *et al.* (2014), most of the screening methods performed were to explore a new bioactive compounds that focusing on the detection of NRPS or PKS genes. NRPS gene is one of the genes involved in the synthesis of a variety of important bioactive compounds called non-ribosomal peptides.

Many researchers have made isolation process of bioactive compounds from terrestrial actinomycetes, but after the elucidation process, bioactive compounds were often structurally similar to the structure of bioactive compounds that was found previously, so information about NRPS genes sequence and diversity in actinomycetes are very important to avoid the risk of re-isolation. This approach can be used as a tool for initial screening in the discovery of new bioactive compounds from actinomycetes. Referring to research conducted by Farida *et al.* (2007), although marine actinomycetes that could potentially have anti-cancer compounds and detected carry NRPS gene in the genome, but these studies have not observed NRPS genes sequence and their diversity.

The aim of this study was to explore the presence and diversity of NRPS genes in 14 marine actinomycetes. PCR amplification and restriction fragment analysis of NRPS genes from different species of marine actinomycetes were done. Genome mining of one species of marine actinomycetes (strain GMY01) also was employed towards this goal.

Material and Methods

Bacterial strains and culture conditions.

The 14 anticancer-producing actinomycetes isolated from marine sediment in Indonesia used in this study were from previously study (Farida *et al.*, 2007). All strains used in this study were grown in shaking flasks containing starch nitrate broth with filtered seawater at room temperature. All strains were stored at -80°C as suspensions of spores and hyphae in 15 % (v/v) glycerol.

Isolation of chromosomal DNA

Chromosomal DNAs were isolated by a versatile quick-prep method for genomic DNA of Gram-positive bacteria (Pospiech and Neumann, 1995), with some modifications. Bacterial culture (1 ml) grown in a starch nitrate broth shake culture were centrifuged, rinsed with TE and re-suspended in 0.4 ml TE buffer. Lysozyme was added to a concentration of 1 mg ml^{-1} and incubated at 37°C for 1 h. Then 0.1 vol 10 % SDS was added and incubated at 65°C with occasional inversion for 2 h. One-third volume 5 M NaCl and 1 vol. chloroform were added and incubated at room temperature for 0.5 h with frequent inversion. The mixture was centrifuged at 13,000 rpm for 10 min and the aqueous phase was transferred to a new tube using a blunt-ended pipette tip. Chromosomal DNA was precipitated by the addition of 1 vol. 2-propanol with gentle inversion. The DNA was transferred to a new tube, rinsed with 70 % ethanol, dried under vacuum and dissolved in a suitable volume (about 50 ml) of TE buffer.

NRPS gene PCR Amplification and Restriction Endonuclease Digestion

The NRPS gene was amplified using primers A3 (5'GCSTACSYSATSTACA CSTCSGG'3) and A7R (5'SASGTCVCCSGTS CGGTAS '3) (Ayuso-Sacido and Genilloud, 2004). Amplification of the NRPS gene was performed in T100 thermal cycler (Bio-Rad) in a total volume of 50 ml containing 30-50 ng DNA, 100 mM each primer, 10 mM dNTP, 10X buffer and 1.5 U Taq DNA polymerase

(Promega). PCR program used to follow the procedures performed by Farida et al. (2007) with modifications. PCR program included initial denaturation at 97° C for 3 min, denaturation at 95°C for 1 min, then followed by annealing at 55°C for 1 min, extension at 72°C for 3 min the whole process takes place as many as 30 cycles, the last cycle followed by a final extension at 72°C for 10 min and cooling stage at 4°C. The PCR product was visualized by 1% agarose electrophoresis. Restriction enzyme, *Hae*III, was used to digest the amplified NRPS genes fragment. The reactions were performed in final volumes of 20 µl containing at least 8 $\mu g \cdot ml^{-1}$ of NRPS genes products at 37°C for 3 h. The digestions were then analyzed by agarose gel electrophoresis (2%, w/v) for 1 h at 110 V followed by visualization of the banding patterns using a UV transilluminator (UVP Canada).

PCR Amplification and sequencing of 16S rRNA genes

The 16S rRNA gene was amplified using primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3 ') and1492 R (5'-GTT TAC CTT GTT ACG ACT T-3 ') (Jiang et al., 2007). Amplification of the 16S rRNA gene was performed in T100 thermal cycler (Bio-Rad) in a total volume of 50 ml as a described above. PCR program used to follow Jiang *et al.* (2007) with some modifications, the PCR program included initial denaturation at 97°C for 3 min, denaturation at 95°C for 1 min, then followed by annealing at 60°C for 1 min, extension at 72°C for 3 min the whole process lasted about 30 cycles, the last cycle followed by a final extension at 72° C for 10 min and cooling stage at 4°C. The PCR product was visualized by 1% agarose electrophoresis. The 16S rRNA gene was sequenced with primers designed by Chun and Goodfellow (1995).

Genome Mining* of *Streptomyces* sp. *GMY01

Previously, sequence analysis of the genome of *Streptomyces* sp. GMY01 was done with platforms Next Generation Sequencing (NGS) using 454 pyrosequencing technology (454 GS FLX) and HiSeq1000 (Illumina) (Herdini *et al.*, unpublished). Genome mining and analysis of gene cluster involved in the biosynthesis of secondary metabolites of *Streptomyces* sp. GMY01, especially gene clusters containing NRPS, were done with antiSMASH 3.0 (Medema *et al.*, 2011; Weber *et al.*, 2015) available on http://antismash.secondarymetabolites.org.

Data analysis

Data subjected from electrophoresis were analyzed using UPGMA method. The sequences of 16S rRNA determined in this study were aligned using CLUSTAL W software (Thompson *et al.*, 1994). The nucleotide similarity values were calculated from the alignment. Evolutionary trees for the datasets were inferred from the neighbour-joining method (Saitou and Nei, 1987) using MEGA version 6.0 (Tamura *et al.*, 2013). The stability of relationships was assessed by performing bootstrap analysis of neighbour-joining data based on 1,000 resampling.

Result and Discussion

Marine Sediment-derived Actinomycetes

Fourteen actinomycetes that isolated from marine sediments in Krakal beach in Indonesia and produce anticancer (Farida *et al.*, 2007) were used in this study. Based on colony morphology after 14 days of growth on solid medium starch nitrate at 30°C showed that all 14 actinomycetes diverse and different from each other (Figure 1). From the colony morphology also indicates that almost all isolates are most likely the genus *Streptomyces*. It is known that the genus of *Streptomyces* is a group of actinomycetes that generate the most bioactive compounds, including anticancer (Newman *et al.*, 2000; Doroghazi *et al*. 2014).

Diversity of Nonribosomal Peptide Synthetase Genes

Nonribosomal peptide synthetase (NRPS) gene in 14 actinomycetes from marine sediments amplified using primers,

Figure 1. Morphology of 14 anticancer-producing actinomycetes isolated from marine sediment in Indonesia after 14 days of growth on starch nitrate solid medium at 30°C. Streptomyces lavendulae was included as a comparison

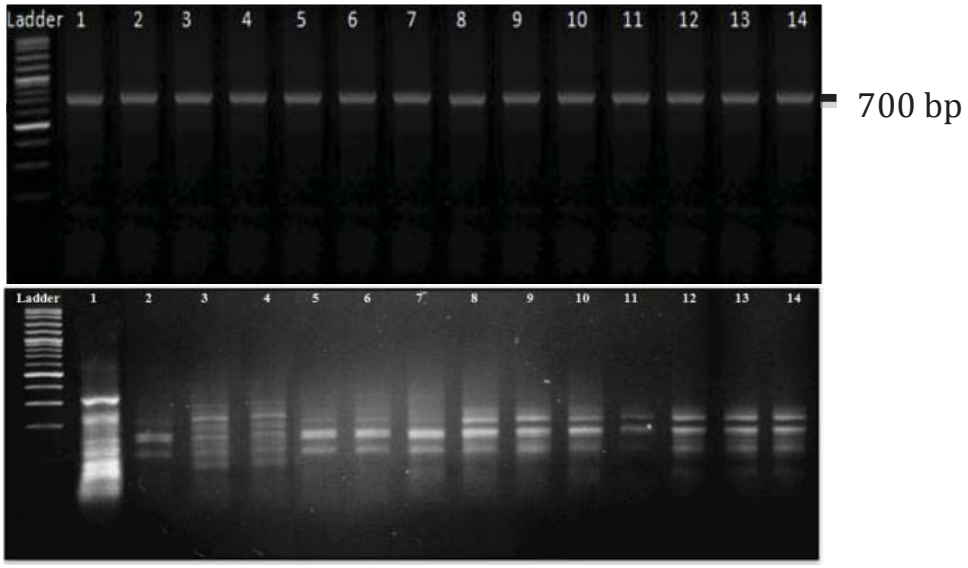

Figure 2. Visualization of NRPS gene amplification of 14 marine actinomycetes isolated by electrophoresis in 1% (w/v) agarose gel stained with etidium bromide (above), and HaeIII restriction patterns of NRPS gene 14 marine actinomycetes isolated by electrophoresis in 2% (w/v) agarose gel stained with etidium bromide (bottom). Lanes 1: GMY 1, 2: GMY 2, 3: GMY 15, 4: GMY 16, 5: GMY 23, 6: GMY 29, 7: GMY 6, 8: GMY 9, 9: GMY 4, 10: GMY 41, 11: GMY 55, 12: GMY 56, 13: GMY 57, 14: GMY 50. Using marker 100 bp DNA ladder.

A3 and A7, which had been previously developed and used by Ayuso-Sacido *et al*. (2004). Primary A3 and A7R used to strengthen adenilation NRPS gene domain with a targeted size of approximately 700-800 bp (Figure 2A). Figure 2A shows that the sizes of the PCR products of NRPS all isolates were almost identical at 700 bp. This result was in contrast with previous studies that the sizes of the PCR products of NRPS were generally varied (700-800bp) (Ayuso-Sacido and Genilloud, 2004; Ayuso-Sacido *et al.*, 2005).

To determine the diversity of NRPS genes, restriction enzyme analysis with *Hae*III enzyme was done, and the result was presented in Figure 2B. The result showed that *Hae*III could be used to classify the NRPS gene diversity. *Hae*III enzyme restriction was used to cut the NRPS genes because actinomycetes

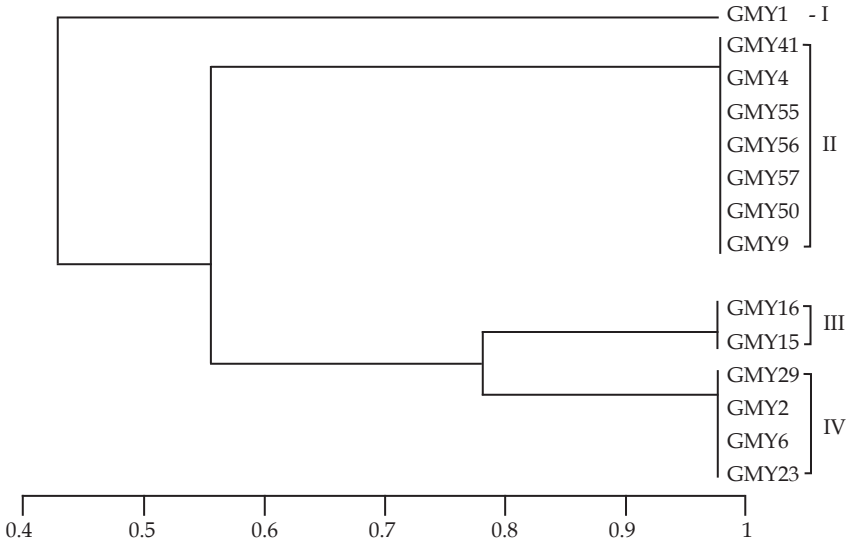

Figure 3. UPGMA dendrogram shows the clustering of 14 actinomycetes isolates generated from amplified NRPS genes restriction analysis with restriction endonuclease HaeIII, using the UPGMA algorithm and the Jaccard's coefficient. The Roman numerals I to IV represent the four clusters of NRPS obtained in the analysis. The isolates subjected to 16S rRNA gene sequencing analysis are highlighted in boldface

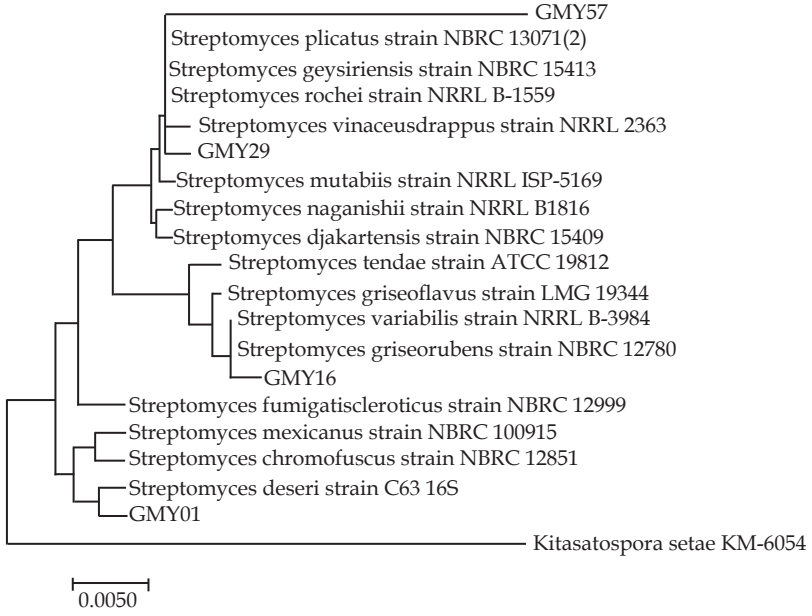

Figure 4. Neighbour-joining tree based on partial 16S rRNA gene sequences, showing relationships between GMY01, GMY16, GMY29 and GMY57 and closely related to members of the genus Streptomyces. The numbers at the nodes indicated the level of bootstrap support (%) based on a neighbour-joining analysis of 1.000 resampled datasets, only values above 50% are given.

has DNA with a high G and C bases content, the content of G and C bases in the genome of actinomycetes are high between 56-64% (Dharmaraj, 2010). *Hae*III enzyme cuts DNA at a G and C rich region. Enzyme *Hae*III restriction endonucleases are enzymes which have a tetra metric site restriction to the four bases, this enzyme cut the DNA sequences

Table 1. NRPS gene cluster of secondary metabolites from Streptomyces sp. strain GMY01 based on AntiSMASH analysis

No.	Type of gene cluster	Size (bp)	Most similar known cluster (%)	MIBiG BGC-ID
1	Nrps	43303	Stenothricin (13)	BGC0000431_c1
2	Nrps	51715	-	-
3	Nrps	91515	Eduracidin (12)	BGC0000341_c1
4	Nrps	68122	Mirubactin (50)	BGC0000392_c1
5	Nrps	62061	Scabichelin (90)	BGC0000423_c1
6	Nrps	16929	Naphthyridinomycin (21)	BGC0000394_c1
7	T1pks-Butyrolactone-Nrps	122282	Rifamycin (38)	BGC0000137_c1
8	Nrps-T1pks	29889	SGR_PTMs (50)	BGC0001043_c1
9	Bacteriocin-Nrps	51659	Griseobactin (47)	BGC0000368_c1
10	Lantipeptide-Nrps	67317	Rifamycin (9)	BGC0000136_c1

in regions that have GGCC base sequences. Other restriction enzymes can cut the NRPS gene and other genes in actinomycetes such as *Hinf*I can cut NRPS genes, PKS I and II produced a diverse banding pattern (Ayuso-Sacido *et al*., 2005), while the enzyme *Msp*I and *Taq*I can cut NRPS gene and 16S rRNA in actinomycetes (Jiang *et al*., 2007). Based on the NRPS gene cutting pattern of 14 isolates of marine actinomycetes, restriction analysis with restriction endonuclease *Hae*III of NRPS genes can be used to analyzed the diversity of NRPS gene sequence among 14 actinomycetes isolates quickly.

Cluster analysis of restriction pattern of NRPS genes using the UPGMA algorithm and the Jaccard's coefficient was showed in Figure 3. From that figure, there were four groups of actinomycetes based on NRPS genes, which have the coefficient of 1. All four groups are group 1 (GMY01); group 2 (GMY04, GMY09, GMY41, GMY55, GMY56, GMY50 and GMY57); group 3 (GMY15 and GMY16); and group 4 (GMY02, GMY06, GMY23 and GMY 29). It means that the isolated groups were expected to have high sequence similarity on adenilation domain of NRPS gene. Based on the dendogram in Figure 3, four isolates were selected for sequencing analysis of 16S rRNA gene; they were GMY01, GMY16, GMY29 and GMY57.

Neighbour-joining tree based on partial 16S rRNA gene sequences (Figure 4) showed relationships between GMY01, GMY16, GMY29 GMY57 and closely related members of the genus *Streptomyces*. Neighbour-joining tree based on partial 16S rRNA gene sequences showed that GMY01, GMY16, GMY29 and GMY57 are close related to with *S. deserti, S. griseorubens*, *S. vinaceusdrappus,* and *S. rochei* respectively. In this study, restriction fragment of NRPS gene with *Hae*III enzyme, followed by analysis of 16S rRNA gene sequences of representatives from each group have provided preliminary information that 14 anticancer-producing actinomycetes isolated from marine sediment in Indonesia (Farida *et al*., 2007) have high diversity of NRPS genes sequence and have the potential for producing other important bioactive compounds.

Genome mining of GMY01 by using AntiSMASH showed that GMY01 harboring 6 NRPS genes and 4 hybrid NRPS genes, with sequence identities to the most similar known cluster range from 9 to 90% (Table 1). This result indicated that each *Streptomyces* harbor diverse NRPS genes.

Our strategy to identify NRPS genes within 14 marine actinomycetes using two different approaches resulted in new findings. By *in silico* data mining of GMY01, we gained a valuable insight into the abundance and origin of NRPS genes present in the *Streptomyces*. Our hypothesis that *Streptomyces* from marine ecosystem is a promising sources for novel NRPS.

Conclusion

The conclusion of this study was NRPS gene sequence diversity in 14 marine actinomycetes could be divided into 4 groups based on NRPS restriction patterns. Analysis of 16S rRNA gene sequences of representatives from each group showed that all isolates belong to genus of *Streptomyces*. Genome mining result showed that strain GMY01 harboring 10 different NRPS gene clusters that encode secondary metabolites, as pure NRPS or hybrid between NRPS and other compounds. These results indicate that marine actinomycetes were having a high potential to be developed as source of anticancer drugs development.

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