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Screening Anti-MRSA Activities of Indigenous Microbes and Prediction of The Biosynthetic Gene Clusters

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Abstract. *Methicillin-Resistant Staphylococcus aureus* (MRSA) is a species of *S. Aureus*, which shows resistance to the methicillin class of antibiotics. MRSA infection poses significant health problems because it causes severe disease in a vulnerable population and also because effective antibiotics are limited. Therefore, the research to find the new anti-MRSA compound is a necessity. The genome mining approach through Biosynthetic Gene Clusters (BGC) analysis can detect the ability of microbes to produce antibiotics. Thus, the study aimed to screen potential anti-MRSA microbes and predict their BGC. This study used ten microorganisms isolated from mangrove areas in Segara Anakan Cilacap and clinical MRSA 2983 from the Faculty of Medicine, UNSOED. All samples microorganisms were cultivated in starch casein nitrate agar. DNA isolation used Quick-DNA Fungal/Bacterial Miniprep Kit, 16S rRNA gene amplification used 27F and 1492R primers, and DNA amplicons were sequenced by the Sanger sequencing method. BCG prediction of the associated genes used antiSMASH 5.1.2. The results showed that W-5A, P-6B, and W-5B isolates have anti-MRSA activities with inhibition indexes of 0.58; 0.53; and 0.47, respectively. Species identification revealed similarity of W-5A with *Streptomyces longisporoflavus* strain Moghannam M1, P-6B with *Ochrobactrum intermedium* LMG 3301, and W-5B with *Streptomyces cellulosae* strain NBRC 13027. The results of BGC analysis showed that *Streptomyces longisporoflavus* has one region involved in the synthesis of tetronasin. *Ochrobactrum intermedium* has one region involved in the synthesis ambactin. *Streptomyces cellulosae* has eight gene clusters for tiacumicin B, actinorhodin, ulleungmycin, albaflavenone, desferrioxamine B/E, stenothricin, auricin, and prejadomycin productions. These results indicate that the microbes isolated from Segara Anakan Cilacap serve as promising antibacterial producers.

1. Introduction

Secondary metabolite compounds play an essential role in the development of new antibiotics and are useful for many pharmaceuticals' purposes. Biosynthesis of secondary metabolites is encoded by Biosynthetic Gene Clusters (BGC), which is the physical grouping of cluster genes that encode all enzymes needed to produce secondary metabolites as well as pathway-specific regulatory genes [1]. In recent years, whole-genome sequence analysis has shown that microorganisms have enormous biosynthetic potential but have mainly not been investigated because large amounts of BGC in the single microbial genome are inactive. The activation of this passive BGC contributes to the discovery of new secondary metabolites. Zhang et al. [2] succeeded in activating unknown BGC for Polyketide Type 1 through metabolite engineering of *Streptomyces olivaceus* SCSIO T05 for lobophorin CR4 production. Almost all classes of lobophorin CR4 compounds have broad-spectrum antibacterial activity and anti-tumor activity.

At present, massive information about genes, including genes in secondary metabolite biosynthesis, allows the identification of BGC of a species through genome mining and predicting the chemical

structure of bioactive compounds based on gene sequence information. Genome mining is an important approach to complement bioprospection efforts because it allows researchers to survey large data sets to determine whether the genome under study contains the BGC of interest. Analysis of the presence of BGC with a genome mining approach can be carried out using the Antibiotics and Secondary Metabolites Analysis Shell (AntiSMASH) program [3]. AntiSMASH analysis of genome from Streptomyces sp. SD85 resulted in 52 BGCs for the biosynthesis of various secondary metabolites, including sceliphrolactam with unknown biosynthetic origin [4]. The genome mining approach using AntiSMASH analysis identifies the BGC of interest and allows the discovery of specific secondary metabolites more quickly than conventional methods. Hence, this approach is beneficial to accelerate the finding of new antibiotics in overcoming bacterial resistance.

Pathogenic microbes that recurrently show multi-resistance is *Methicillin-Resistant Staphylococcus aureus* (MRSA) that causes nosocomial infections. The antibiotics choices to treat MRSA infections include vancomycin, daptomycin, linezolid, and ceftaroline [5]. However, Ślusarczyk et al. [6] has reviewed the multiple incidences of MRSA resistance to ceftaroline. Therefore, research to find new anti-MRSA compound is crucial. A typical initial stage for exploring new antibiotics is to screen for bacteria that have potential as anti-MRSA. However, before applying the lengthy experimental procedures to produce the bioactive compounds, BGC analysis can be carried out to determine the gene region for the biosynthesis of specific compounds.

Indeed, the information on the existence of gene clusters for the biosynthesis of specialized metabolites in a microbe will make the production process more efficient compare to the classical approach [7]. Thus, the study was aimed to screen potential anti-MRSA microbes from Segara Anakan Cilacap and predicted their BGC. This research is essential to provide information about gene clusters from bacterial strains that can be expressed to produce new anti-MRSA compounds efficiently.

2. Materials and Methods

Microbes used in the study were isolated from the mangrove sediments of Segara Anakan Cilacap, which were K-2C, K-4B, W-1B, W-5A, W-5B, P-6A, P-6B, P-6G, P-7C, and P-7H. The initial letter code indicated the sampling location, namely Klaces (K), Kutawaru (W), and Kali Panas (P). The numeric code stated the location of the sampling point. The MRSA bacteria used was clinical isolates MRSA 2983 available from the Faculty of Medicine, UNSOED.

2.1. Cultivation of isolates

The isolates were cultivated following the procedure described by Asnani et al. [8]. The cultivation of isolates used the quadrant streak method on Starch Casein Nitrate (SCN) agar [starch, casein, KNO_3, K_2 HPO_4, $MgSO_4.7H_2O$, NaCl, $FeSO_4.7H_2O$, and Bacto agar] supplemented with nystatin (Kandistatin®) in Petri Dish. The cultures were incubated at room temperature. Observations were made on colony color, colony form, and pigment diffusion in the medium.

2.2. Screening of anti-MRSA activity

Screening of anti-MRSA activity used dual culture technique, which was a modified method of the Kirby Bauer antibiotic susceptibility test as described by Sharma & Manhas [9]. A total of 100 μL of MRSA suspension in Nutrient Broth (Merck) with standard turbidity of 0.5 McFarland units was inoculated onto Mueller Hinton Agar (Liofilchem). The culture was incubated for 10 minutes at room temperature, and then a well with a diameter of 6 mm was made. One plug (6 mm in diameter) of isolates aged 21 days in the SCN agar medium was inserted into each well; then, the culture was incubated for 18 hours at room temperature. A positive result of anti-MRSA activity was indicated by the formation of a clear zone around the agar plug. The inhibition index is calculated by the following formula.

$$I_p = \frac{a - b}{b}$$

The Ip was the inhibition index, a was the diameter of clear zone (mm), and b was di diameter of agar plug (mm). The potential microbes with anti-MRSA activity were subjected to subsequent analysis.

2.3. Morphology observations

The morphology of the isolates was observed using a stereo microscope with 30x magnification. Isolates were inoculated by the quadrant streak method on SCN agar medium and incubated for 14 days at room temperature. Macromorphological characteristics observed included colony shape, colony surface, colony size, pigmentation, colony margins, substrate mycelium, aerial mycelium, and elevation. Observation of hyphae was carried out by Heinrich Slide Culture preparations [10]. Isolates were cultivated on sterile object-glass, closed with cover glass, and then incubated at 30^{o}C for eight days. The hyphae morphology of the isolates was observed using a light microscope with 400x magnification.

2.4. DNA isolation and amplifications

The isolation of DNA followed the Instruction Manual Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). The DNA concentration was measured at λ260 nm with a nano spectrophotometer (Implen). As much as 200 ng DNA template was amplified using 2x MyTaqTM HS Red Mix (Bioline) with 1 μL of 20 μM 27F (AGAGTTTGATCMTGGCTCAG) and 1 μL of 20 μM 1492R (TACGGYTACCTTGTTTACGACTT) primers. The PCR conditions followed the procedure described by Asnani et al. [11]. The amplicon was electrophoretic on 1% agarose and visualized with UV Transilluminator (Cleaver Scientific Ltd). The molecular weights of DNA samples were determined based on the Log relationship of the DNA marker with their migration distance.

2.5. Sequencing and species identification

The amplicons obtained were bidirectional sequenced by the Sanger Sequencing method with 27F and 1492R primers. All sequences obtained were analyzed with BioEdit 7.2.5. The DNA sequences resulted from BioEdit analysis were then uploaded to GenBank through the Nucleotide Basic Local Alignment Search Tool (BLASTn) online to determine the homology of the sequences fragments. The output shows sequences based on priority, with the most homologous sequence with the sample will locate at the top row.

2.6. Biosynthetic gene clusters analysis

Analysis of the biosynthetic gene clusters used AntiSMASH 5.1.2 online tool [3]. The research used Whole Genome Shotgun deposited at GenBank from species references. All genomes were downloaded in GenBank format and entered in AntiSMASH bacterial version. The identified gene clusters were queried against the MiBIG database.

3. Results and Discussion

3.1. Screening of microbes with anti-MRSA activity

Screening for microbes with anti-MRSA activity used isolates with the aged of 21 days. This is because, at that age, the microbes are thought to release bioactive compounds that can inhibit the growth of MRSA. The screening results for anti-MRSA showed that three isolates had inhibitory activity against MRSA 2983 growth. The higher the value of the inhibition index, the higher the anti-MRSA potential of the isolate.

Table 1. The inhibition index towards MRSA

No	Isolates	Inhibition Index
1	W-5A	0.58
2	P-6B	0.53
3	W-5B	0.47
4	K-2C	n.a
5	K-4B	n.a
6	P-6B	n.a
7	P-6G	n.a
8	P-7C	n.a
9	P-7H	n.a
10	W-1B	n.a

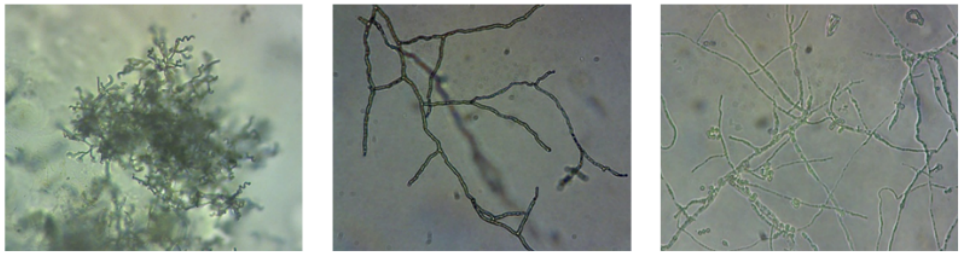
The three highest inhibition indexes were 0.58, 0.53, and 0.47 from isolates W-5A, P-6B, and W-5B, respectively (Table 1). Three isolates that have the highest inhibition index were used for further analysis.

3.2. Morphological observations

Macromorphological observations were carried out on 14 days old isolates in the SCN medium using a stereo microscope. The observations focused on colony characteristics, which were a shape, surface, size of diameter, pigmentation, margins, substrate mycelium, aerial mycelium, and elevation of the colonies (Table 2).

Table 2. Macromorphological observations of the colony

No	Characteristics	W-5A	P-6B	W-5B
1	Shape	Circular	Circular	Circular
2	Surface	Powdery	Powdery	Powdery
3	Diameter (mm)	3.8	5.2	4.3
4	Pigmentation	Light brown	Dark brown	Dark brown
5	Margins	Flat	Flat	Flat
6	Substrate mycelium	Yellow	Yellow	Yellow
7	Aerial mycelium	White	White	White
8	Elevation	*Umbonate*	*Umbonate*	*Umbonate*



(a) (b) (c)

Figure 1. The mycelium observations of isolates (a) W-5A, (b) P-6B, and (c) W-5B on Heinrich Slide Culture preparations using a light microscope with a magnification of 400x

The morphological observation of hyphae was carried out on eight days old isolates on Heinrich Slide Culture preparations using a light microscope (Figure 1). The isolates W-5A, P-6B, and W-5B had branched hyphae characters. W-5A isolates had a spiral spore chain type, while P-6B and W-5B isolates had a rectiflexibiles spore chain type.

3.3. Species identification

DNA isolation was carried out through microbial cultivation on liquid SCN, DNA extraction, DNA purification, and DNA precipitation to obtain pure DNA. DNA concentration and purity were measured at λ260 nm and λ280 nm (Table 3). The measurement results showed a DNA purity index > 1.8, which indicates that sample DNA is pure. The isolated DNA was then amplified through a polymerase chain reaction.

Table 3. The purity and concentration of DNA

Sample	λ260 (nm)	λ280 (nm)	Purity index	Concentration (µg/mL)
W-5A	0.120	0.068	1.852	56.5
P-6B	0.230	0.125	1.875	113.0
W-5B	0.076	0.043	1.892	35.0

PCR is an enzymatic synthesis process to amplify nucleotides in vitro. Target sequence amplification occurs through a series of repeated cycles consisting of three stages, namely denaturation, annealing, and extension. It is possible due to the use of a specific pair of primers which complement the ends of the target sequence. After the PCR process, electrophoresis was carried out to see the success of PCR in amplifying the isolated DNA template with the primer used.

The electrophoresis results (Figure 2) showed that there was one DNA band in each isolate sample, which indicated the success of PCR in amplifying specific DNA templates. Based on the DNA standard migration data, the relationship curve between the base pair logs and the migration distance showed the regression equation y = - 0.2044 x + 4.1189. Calculations based on the migration of sample DNA using the regression equation yielded the molecular weight of DNA isolates W-5A, P-6B, and W-5B to be 1570 bp, 1483 bp, and 1504 bp, respectively. Based on those calculation results, it suggested that all amplicons have a length of ± 1500 base pairs that correspond to the target amplicon of the 16S rRNA gene.

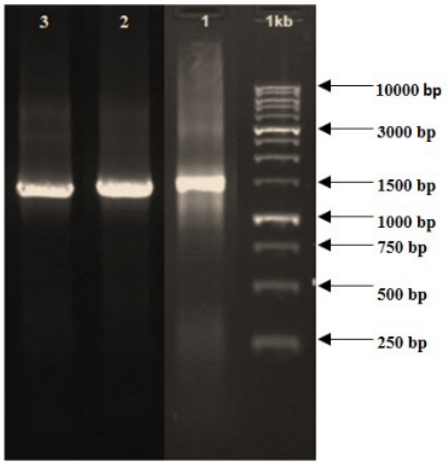


Figure 2. The bands of DNA amplicon (1) W-5A, (2) P-6B (2), and (3) W-5B. Based on the calculation of the standard DNA regression equation, the molecular weights of W-5A, P-6B, and W-5B are 1570 bp, 1483 bp, and 1504 bp, respectively, which correspond to the target amplicon 16S rRNA.

The amplicons of 16S rRNA gene fragments from isolates W-5A, P-6B, and W-5B were then sequenced to determine the DNA base sequence. The limited ability of the sequencing machine causes not all parts of the DNA base sequence are identified. Therefore, the amplicon DNA was sequenced in two directions, forward and reverse, according to the primers used, namely 27F and 1492R. The two sequencing results were then aligned using the BioEdit program to obtain a complete sequence. The complete sequences were then aligned with similar data previously published in the GeneBank via BLAST nucleotide analysis for species homology determination (Table 4).

E-value of 0.0 indicates that the isolates are homolog to the reference species in which isolates with a similarity sequence ≥97% suggests the same species. Isolate W-5A has a similarity of 99.81% with *Streptomyces longisporoflavus* strain Moghannam M1 and isolate W-5B has a similarity of 99.92% to *Streptomyces cellulosae* strain NBRC 13027. Whereas, isolate P-6B has a similarity of 99.36% with *Ochrobactrum intermedium* LMG 3301.

Table 4. Results of species identification based on the 16S rRNA gene

Isolate	Names	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc
W-5A	*Streptomyces longisporoflavus* strain Moghannam M1	1962	1962	96%	0.0	99.81%	KY657268.1
P-6B	*Ochrobactrum intermedium* LMG 3301	2547	2547	99%	0.0	99.36%	NR_026039.1
W-5B	*Streptomyces cellulosae* strain NBRC 13027	2440	2440	100%	0.0	99.92%	NR_112346.1

3.4. Biosynthetic Gene Clusters prediction

Biosynthetic Gene Clusters (BGC) are gene clusters that encode secondary metabolites. The genome mining approach to predict secondary metabolite biosynthetic pathways begins with identifying conserved biosynthetic genes. Using the antiSMASH online tool enables rapid genome identification, annotation, and analysis of BGC secondary metabolites in the genomes of bacteria and fungi [3].

The W-5A isolate identified as *Streptomyces longisporoflavus* (GenBank FJ462704.1) was predicted to have one gene cluster in Region 1.1 (Table 5). The gene cluster Type I polyketide synthase (T1PKS) involves in the biosynthesis of tetronasin from the known polyketide cluster in *S. longisporoflavus*. Tetronasin is a polyketide-polyether antibiotic which acts as an ionophore to chelate small molecular weight cations, especially sodium. Its antibiotic activity is attributed to the solubility of this complex in fat, which interferes with the selective permeation of ions throughout the lipid bilayer leading to membrane depolarization and cell death of the microbes [12].

The P-6B isolate identified as *Ochrobactrum intermedium* LMG 3301 (GenBank NZ_ACQA00000000.1) was predicted to have one gene cluster in Region 1.4 (Table 5). The gene cluster type was acyl amino acid, which involves in the biosynthesis of ambactin from the known nonribosomal peptide (NRP) cluster. Ambactin is a hexapeptide known as a bioactive compound from *Xenorhabdus miraniensis* DSM 17902 with activity against protozoa [13]. Genus Xenorhabdus produces various bioactive compounds such as antibacterial and antifungal compounds, including active compounds against insects, nematodes, protozoa, and cancer cells [14].

The W-5B isolate identified as *Streptomyces cellulosae* (GenBank NZ_JOEV00000000.1) was predicted to have 27 BGC, which involves in the biosynthesis of secondary metabolites. There are eight BGCs that encode secondary metabolites with antibacterial potential in region 5.1, 7.1, 11.2, 12.1, 14.1, 20.1, 43.1, and 71.1 (Table 5). The BGC types included T1PKS, T2PKS, PKS-lik3, NRP, linaridin, terpene, siderophore, and butyrolactone.

Region 5.1 involves the biosynthesis of tiacumicin B, which is a narrow-spectrum antibiotic inhibiting RNA synthesis. Tiacumicin B has been approved by the FDA since 2011 for the treatment of *Clostridium difficile* infection with the same cure rate as vancomycin [15]. Region 7.1 involves the biosynthesis of actinorhodin, which is a blue pigmented antibiotic that belongs to the benzoisochromanequinone (BIQ) class. Actinorhodin is well known produced by *Streptomyces coelicolor*. It has a role as a bacterial metabolite and an antimicrobial agent [16]. Region 11.2 involves the biosynthesis of ulleungmycin a cyclic hexapeptides antibiotic produced by Streptomyces sp. Ulleungmycins is active against Gram-positive strains, including antibiotic-resistant strains such as MRSA (*methicillin-resistant Staphylococcus aureus*) and QRSA (*quinolone-resistant Staphylococcus aureus*), as well as strains that are susceptible to antibiotics [17]. Region 12.1 involves in the biosynthesis of albaflavenone, which is a tricyclic sesquiterpene antibiotic produced by *Streptomyces albidoflavus*. Albaflavenone has antibacterial against *Bacillus subtilis* ATCC 6633 [18].

Table 5. The prediction of Biosynthetic Gene Clusters from AntiSMASH

Region	Position		Type	Most similar	Cluster
	From	To			
Streptomyces longisporoflavus					
Region 1.1	6101	156011	T1PKS	Tetronasin	Polyketide
Ochrobactrum intermedium					
Region 1.4	1913283	1974029	Acyl amino acids	Ambactin	NRP
Streptomyces cellulosae					
Region 5.1	359154	403368	T1PKS	Tiacumicin B	Polyktide: Modular type 1
Region 7.1	1	140226	T2PKS, NRPS, ladderane	Actinorhodin	Polyketide: type II
Region 11.2	80151	100678	Linaridin	Ulleungmycin	NRP
Region 12.1	11966	33051	Terpene	Albaflavenone	Terpene
Region 14.1	42004	53773	Siderophore	Desferrioxamin B/desferrioxamine E	Others
Region 20.1	61467	112771	PKS-like, butyrolactone	Stenothricin	NRP: Cyclic depsipeptida
Region 43.1	52257	62371	Butyrolactone	Auricin	Polyketide: Type II +
Region 71.1	1	25218	T2PKS	Prejadomycin/ Rabelomycin/ Gaudimycin C/ Gaudimycin D/ UWM6/ Gaudimycin A	Polyketide:Type II +

Region 14.1 involves the biosynthesis of desferrioxamine B/desferrioxamine E, which is a non-peptide hydroxamate siderophore group secreted by *Streptomyces pilosus*. Desferrioxiamine B is known as biological ligands for Fe ions and has been marketed under the name Desferal [19]. Region 20.1 involves biosynthesis of stenothricin. Liu et al. [20] reported that *Streptomyces roseosporus* produced at least four groups of NRPS derivative molecules, namely daptomycin, arylomycin, napsamycin, and stenothricin which are identified to have different actions. Stenothricin is active against Gram-negative and Gram-positive bacteria, including *B. subtilis*, *Staphylococcus epidermis*, *Enterococcus faecium*, *Listeria ivanovii*, *Enterobacter aerogenes* and *Acinetobacter baumannii*. Based on cytological profiling, stenothricin serves as an antibacterial by disrupting cell membranes. Region 43.1 involves the biosynthesis of auricin, which is produced by *Streptomyces aureofaciens* CCM 3239 [21]. Auricin is an antibiotic that is active against various bacterial infections of the respiratory tract. Lastly, Region 71.1 involves in prejadomycin biosynthesis, which is an aromatic polyketide produced by *Streptomyces*. *Prejadomycin* belongs to angucyclines class with various bioactivities such as antibacterial, antiviral, and inhibitory enzyme activities [22].

In this BGC analysis, most of the antibacterial compounds were the polyketide and NRP clusters. Polyketide is known as a class of bioactive compounds encoded by the PKS enzyme. PKS enzymes act like fatty acid synthase to produce various polyketides. This enzyme begins to encode polyketide by priming the initiator molecule to the catalytic residue and then creating an extender unit for chain extension. In PKS, additional modifications to the structure are possible using special enzymes, different starters and extender units, reduction and cyclization reactions producing various antibiotic compounds with antibacterial activity. Whereas, NRP is a bioactive compound encoded by the NRPS enzyme.

Nonribosomal peptides are peptides that are not synthesized at the ribosome level. The characteristic of NRP is its small size and its ability to synthesize peptides containing proteinogenic and nonproteinogenic amino acids [23].

Another gene cluster is terpenes, which are nature's largest and most structurally diverse compounds. According to Helfrich et al. [24], terpene biosynthesis is very different from PKS and NRPS biosynthesis. Five carbon units (isoterpenes) combine to make linear polyenes with branched methyl groups that form a hydrocarbon core structure in one step catalyzed by the terpene cyclase enzyme. The enzyme initiates a series of carbocation leads to cyclization and rearrangements, creating the basic hydrocarbon skeleton of the terpenes. This framework is then modified to produce a large number of terpenoid structures, which can be further modified with the addition of other building blocks such as sugars, amino acids, or fatty acids.

4. Conclusion

Three indigenous microbial from Segara Anakan Cilacap, namely isolates W-5A, P-6B, and W-5B, indicated to have anti-MRSA activity. BGC analysis using AntiSMASH from potential isolates can predict the region encoding enzymes that play a vital role in the biosynthesis of secondary metabolites, which have antibacterial activities. Future research regarding gene expressions from those specific regions will make optimum production of anti-MRSA compounds and allow for expression modification.

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