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Evaluation of antibacterial potential of mangrove sediment-derived actinomycetes

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ABSTRACT

Actinomycetes are well-known as the source of bioactive metabolites. In this work, 16 out of 118 (13.6%) isolates of mangrove sediment-derived actinomycetes showed potential antibacterial activity against at least one bacterial strain. Five extracts from isolates AMA11, AMA12 and AMA21 exhibited a broad spectrum antibacterial activity against *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* ATCC35984, methicillin-resistant *S. aureus* (MRSA) SK1, *Acinetobacter baumannii* NPRC004 and *Escherichia coli* ATCC25922. Ethyl acetate extract from the cells of AMA11 (AMA11CE) showed high activity against *S. aureus* and MRSA with the lowest minimum inhibitory concentration (MIC) of 0.5 μg ml^{-1}. At concentration of four times its MIC, AMA11CE destroyed MRSA cells as analysed by the scanning electron microscopy. In addition, AMA11CE, ethyl acetate extract from the culture broth of AMA12 (AMA12BE), AMA12CE and AMA21CE reduced violacein production in *Chromobacterium violaceum*. Furthermore, at concentrations lower than 10 μg ml^{-1}, all five extracts inhibited biofilm formation by *S. epidermidis* ATCC35984. The chemical analysis of the most active fraction from AMA11CE by GC-MS revealed the presence of 3-nitro-1,2-benzenedicarboxylic acid, hexadecanoic acid, quinoxaline-2-carboxamide and pentadecanoic acid. The 16S rDNA sequencing analysis revealed that these three potential isolates belonged to the genus *Streptomyces*. The results revealed that the actinomycetes from mangrove environment would be a good source of bioactive metabolites against pathogenic bacteria.

1. Introduction

Infections caused by antibiotic-resistant bacteria have become a major global healthcare problem in the 21st century. They require long-term and effective treatments, so the costs of diagnostics are also significantly more expensive and difficult to treat [1]. These occurrences call effective strategies for the treatment and prevention of emerging bacterial infections. Novel antibiotics seem to be an attractive goal against bacterial pathogen. With regard to the discovery for new antimicrobial agents, actinomycete species are the most important saprophytic bacteria which are able to produce diverse metabolites. Especially, members of the genus *Streptomyces* are abundant source of bioactive secondary metabolites for medical, agricultural and veterinary uses. They produce diversity of chemical structures such as polyketides, peptides, macrolides, indoles, aminoglycosides and terpenes [2]. A wide range of secondary metabolites from streptomycetes have been developed as the clinical useful drugs, including amphotericin, daptomycin, doxorubicin, rapamycin and vancomycin [3]. Most of these bioactive compounds are derived from terrestrial actinomycetes. However, the discovery rate of the new compounds from terrestrial actinomycetes has declined, whereas the rate of re-isolation of known compounds has increased [4]. Therefore, it is necessary to develop the novel and effective antibiotics to combat against life threatening diseases from diverse sources.

Mangrove environments hold a great promise for the discovery of novel bioactive compounds. They are distributed between the sea and the land in tropical and subtropical areas, which have the high organic matters to support the diversity of plants, animals and microorganisms [5]. The global mangroves are found in Asia (42%), Africa (20%), North and Central America (15%), Oceania (12%) and South America (11%) [6]. Nowadays, mangrove environments are an attractive source for the discovery of secondary metabolites. Actinomycetes isolated from

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mangrove soils and plants in China showed several activities such as antibacterial, antifungal, anti-tumor and protein tyrosine phosphate 1B inhibitory activity [7]. In India, *Streptomyces* sp. no I-1 isolated from Valapattanam mangrove ecosystem showed potential antimicrobial activity against bacterial and fungal pathogens of nosocomial origin [8]. In addition, *Streptomyces* isolates having antimicrobial activity have been isolated from mangrove sediment on the east coast of Thailand [9]. The present study deals with the isolation of actinomycetes from mangrove forest in Songkhla Province, Southern Thailand, and screening them for antimicrobial secondary metabolites against various drug-resistant bacteria. Some possible mechanisms of action including lytic, anti-biofilm, and anti-quorum sensing activities were also investigated.

2. Materials and methods

2.1. Sample collection

Five sediment samples were collected from mangrove forest in the General Prem Tinsulanonda Historical Park, Songkhla Province, Southern Thailand. Coordinates: 7°8′45″N 100°33′38″E. There is no specific permission required for this location. Each sample was collected at a depth of 5–10 cm and placed in sterile plastic bags. All samples were kept at 4 °C and transported to a laboratory at Prince of Songkla University for further study.

2.2. Isolation of actinomycetes

Sediment samples were air-dried at room temperature (RT) for 7 days and ground aseptically with pestle and mortar. Various pretreatment methods were applied to isolate actinomycetes including treatment with liquid nitrogen; 0.05% sodium dodecyl sulfate (SDS) and 5% yeast extract [10]; 1.5% phenol, dry heat [11], and rehydration and centrifugation [12]. All samples were prepared in ten-fold dilution series to 10^{-4} in sterile 50% seawater. Then, 0.1 ml from each dilution was dropped and spread onto four different selective isolation media including actinomycetes isolation agar (AIA), modified soil extract agar [13,14], humic acid vitamin agar (HV) [14] and starch nitrate agar (SN) [15] supplemented with 50 mg l^{-1} cycloheximide and 20 mg l^{-1} nalidixic acid. The plates were incubated at RT for 4 weeks. Colonies of actinomycetes were picked up and purified by streaking onto a modified soil extract agar and incubated at RT for 7-14 days. Actinomycete pure cultures were stored on International *Streptomyces* Project (ISP) 2 agar slants for working stock and in 20% glycerol at -80 °C for a long-term storage.

2.3. Cultural characterization

Morphological features of the selected actinomycetes were characterized on ISP 2 agar medium after incubation at 28 °C for 2 weeks. The color of substrate and aerial mycelium, and soluble pigment production were determined using the NBS/ISCC color system.

2.4. Molecular identification

The most active actinomycetes were identified by the 16S rDNA sequence analysis and phylogenetic study. Genomic DNA was prepared according to the modified method of Saito and Miura [16]. The actinomycete was cultured in ISP 2 medium on a rotary shaker for 3-4 days. Cells were collected and washed with TE buffer. The cell mass was suspended in 0.4 ml of TE buffer; 20 µl of lysozyme (50 µg ml^{-1}) was then added and incubated at 37 °C for 3 h; hereafter 40 µl of 10% SDS was added, tilted immediately then further incubated at 50 °C for up to 1 h. The phenol extraction of DNA was carried out by adding 0.5 ml of phenol/chloroform/isoamyl alcohol (25:24:1) and mixing well, then centrifugation at 11000 × g for 20 min. The upper phase was transferred into another 1.5 ml microcentrifuge tube. The phenol extraction was repeated, when necessary. One tenth volume of 3M sodium acetate was added to the clear solution. DNA was precipitated from the clear solution with two volumes of cold absolute ethanol. The precipitated DNA was harvested by pooling with a glass rod, dissolved in little quantity of TE buffer, and stored at -20 °C. DNA coding for 16S rDNA regions was amplified according to Trcek and Teuber [17]. The universal bacterial 16S rRNA gene primers (forward primer 20F: 5′-AGTTTGATCCTGGCTCAG-3′, positions 9–27 and reverse primer 1500R: 5′-GTTACCTTGTTACGACTT-3′, positions 1509–1492) were used. The PCR product was purified with a Geneaid PCR DNA fragments extraction kit (Geneaid, Taiwan) and sequenced by Macrogen Inc. using universal primers. The 16S rDNA sequence of the strain was assembled using BioEdit program [18] and blasted at the EzTaxon sever [19]. Multiple alignments were carried out with CLUSTAL W of the software BioEdit Sequence Alignment Editor (version 7.0.0). A phylogenetic tree was generated by neighbor-joining [20] using MEGA 6 program [21]. The robustness for individual branches was estimated by bootstrapping with 1000 replications [22]. The identification at the species, genus and family level according to the following criteria were used: (i) species identification when the sequence determined had a similarity score ≥99% with the reference sequence; (ii) a genus was assigned for similarity score < 99% and ≥95%; and (iii) a family was assigned for similarity when the score < 95% [23].

2.5. Test microorganisms

The following test microorganisms were used in this study: *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* ATCC35984, a clinical isolate of methicillin-resistant *S. aureus* (MRSA) SK1, clinical isolates of *Acinetobacter baumannii* NPRC001-005 and NPRC007, *Chromobacterium violaceum* DMST21761, *Escherichia coli* ATCC25922 and *Psuedomonas aeruginosa* ATCC27853. Bacterial strains were cultured on nutrient agar (NA) and incubated at 35 °C for 18-24 h. The suspensions of bacteria were prepared using nutrient broth (NB), and incubated on a rotary shaker (150 rpm) at 35 °C for 3-5 h. The bacterial suspensions were adjusted to equal the turbidity of 0.5 McFarland standard (10^8 CFU ml^{-1}) with sterile normal saline solution (NSS). For the broth dilution test, the bacterial suspensions were further diluted 1:200 using Mueller-Hinton broth (MHB) to obtain approximately 10^6 CFU ml^{-1}.

2.6. Antibacterial screening

Primary antibacterial screening was performed by a cross streak technique [24]. Single streak of each actinomycete was made on the center of an ISP 2 agar prepared with 50% seawater and incubated at 28 °C for 7-14 days. One loop of each test strain (10^8 CFU ml^{-1}) was streaked perpendicularly at the edge of the actinomycetes. Plates were incubated at 35 °C for 18-24 h and the inhibition zones were recorded. Control plate was maintained without inoculating actinomycetes.

2.7. Actinomycete fermentation and extraction

Actinomycetes possessing antibacterial activity were inoculated in 50 ml ISP 2 broth and incubated at 28 °C for 3 weeks. A total of 2 L culture filtrate was extracted three times with an equal volume of ethyl acetate (EtOAc) and the EtOAc phase was evaporated to dryness under reduced pressure using a rotary evaporator to obtain a broth ethyl acetate extract (BE). The actinomycete cells were soaked in 500 ml of methanol (MeOH) for 2 days. The MeOH layer was evaporated to dryness and 50 ml of water was added and then mixed with 100 ml hexane. The solution was separated by a separating funnel and the aqueous layer was extracted with EtOAc to give a cell ethyl acetate extract (CE) after evaporation of the EtOAc layer to dryness. The hexane layer was evaporated to obtain cell hexane extract (CH). Each