Research Article

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CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF STREPTOMYCES STRAINS FROM THAI MANGROVE SOILS

Sirikan Hunadanamra[1], Ancharida Akaracharanya[1] and Somboon Tanasupawat[2]*

[1]Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
[2]Department of Biochemistry and Microbiology, Faculty of Phamaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

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Abstract: Four actinomycete isolates, SAM2-1, SMP3-1, and J8-1 and J17-2 were isolated from mangrove soils collected in Samut Prakarn and Samut Songkram provinces, the inner gulf of Thailand. These isolates were identified as *Streptomyces* based on their phenotypic and chemotaxonomic characteristics. They contained L–diaminopimelic acid in cell wall. Their major menaquinone components were MK-9 (H6) and MK-9 (H8). On the screening of antimicrobial activity, they could inhibit *Staphylococcus aureus* ATCC 6538P and *Bacillus subtilis* ATCC 6633 but showed weak inhibitory activity against *Kocuria rhizophila* ATCC 9341, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and no activity against *Candida albicans* ATCC 10231. Only isolate J8-1 showed strong inhibitory activity against *Bacillus subtilis* ATCC 6633. The isolates SAM2-1, SMP3-1, J8-1 and J17-2 were closely related to *S. sundarbansensis* DSM 42019T (98.4%), *S. diastaticus* subsp. *ardesiacus* JCM 5815T (97.1%), *S. iranensis* JCM 17327T (96.4%) and *S. seoulensis* NBRC 16668T (95.3%), respectively based on 16S rRNA gene analyses.

Keywords: Actinomycetes, *Streptomyces*, Antimicrobial Activity, Mangrove Soils

INTRODUCTION

Mangroves are the wet land forests mainly found in tropical and subtropical latitudes of the world coastal area[1-2]. The environment of the mangrove ecosystem is saline, and highly rich in organic matters consistent with high sulfur and nitrogen which can be used by the living microorganisms[3]. Mangrove rhizosphere is also full of decayed organic matters originated from alluvium, with a pH value range of acid to alkaline[4]. The genus *Streptomyces* and the rare actinomycetes in genera *Micromonospora*, *Microbispora*, *Actinoplanes*, *Actinomadura* and *Pseudonocardia* isolated from mangrove sediments and from the mangrove swamps have been studied for diversity as well as antimicrobial activity [5-8]. Screening for the actinomycete species is an important aspect as there is a remarkable source for the production of diverse bioactive metabolites that possess pharmaceutically relevant biological activities[9]. In the course of our investigation of actinomycetes distributed in mangrove forests along the inner gulf of Thailand, the actinomycete isolates from soils in Samut Prakan and Samut Songkarm provinces were isolated, screened for antimicrobial activity and identified based on the phenotypic and chemotaxonomic characteristics including 16S rRNA gene sequencing.

MATERIALS AND METHODS

Isolation of actinomycetes: The mangrove soil samples collected from Samut Prakan and Samut Songkarm provinces, Thailand (Table 1) were dried both at room temperature for 1 week and at 110°C for 1 h.

Table.1: Location, pH of soil, isolate number, sequence similarity (%) and closest species

Location (Province)	pH of soil	Isolate no.	(%) Similarity	Closest species
Samut Songkram	7.5	SAM2-1	98.4	*S. sunderbansensis* DSM 42019^T
Samut Prakarn	7.8	SMP3-1	97.1	*S. diastaticus* JCM 5815^T
Samut Prakarn	6.3	J8-1	96.4	*S. iranensis* JCM 17327^T
Samut Prakarn	5.4	J17-2	95.3	*S. seoulensis* NBRC 16668^T

One gram of dried soil samples was suspended in 2.5 ml of sterile distilled water in a test tube, mixed for 30 seconds, left for 30 min, gently mixed 2-3 seconds and measureed the pH. The dried soil samples (0.5 g) were suspended in 4.5 ml of sterile distilled water and were heated at 60-65°C (15 min) to reduce non-thermotolerant microorganisms. The diluted soil suspension of 1:100 and 1:1000 (0.1 ml.) were spreaded on starch-casein nitrate agar (SCA) medium containing 15µg/ml novobiocin and 25 µg/ml nistatin and incubated at 30°C for 14 days[10]. Actinomycete colonies were pick up and further purified by streak plate technique on yeast extract-malt extract agar (ISP 2) medium[11], and incubated at 30°C for 7-14 days. The purified cultures were maintained on ISP 2 agar slant at 4°C for further studies.

***Corresponding Author:**
Dr. Somboon Tanasupawat,
Department of Biochemistry and Microbiology,
Faculty of Pharmaceutical Sciences, Chulalongkorn University,
Bangkok 10330, Thailand.

Identification methods: The phenotypic characteristics were determined as described by Shirling and Gottlieb[11] and Arai[12]. Cell wall diaminopimelic acid (DAP) isomers were determined as described by Kutzner[13]. Menaquinone system was analysed as described by Komagata and Suzuki[14]. Scanning electron microscope was used for determining the morphology of strains grew on ISP 2 agar. DNA of the strains was isolated from cells grown in Yeast extract-Malt extract broth (ISP 2) with 0.2% of glycine reported by Yamada and Komagata[15] and purified as described by Saito and Miura[16]. The 16S rDNA was amplified by PCR using primers, 8-27f and 1492r. The amplified 16S rDNA was used as templates for sequencing with Big Dye Terminator sequencing Kit (Perkin Elmer) and analyzed by AB1377 automated DNA sequencer (Perkin Elmer). The sequencing reaction for each sample was performed in DNA Thermal Cycler (Gene Amp PCR System 2400; Perkin Elmer) by using primers, 27F (5'-GTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGACCAGCC-3'). The obtained sequence was compared with all sequences from GenBank using the BLAST program. The ClustalW2 program was used for multiple alignments with selected sequences for calculating evolution distances[17] by Sea View version 4.2[18]. The phylogenetic tree was constructed using the neighbour-joining method[19]. Data were resampled with 1000 bootstrap replications. The values for sequence similarities among the most closely related strains were determined using the EzTaxon server[20].

Screening of antimicrobial activity: Primary screening of antimicrobial activities was performed on ISP 2 agar plates[21] and *S. aureus* ATCC 6538P, *B. subtilis* ATCC 6633, *K. rhizophila* ATCC 9341, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231 were used as indicator strains. All tested microorganisms were cultivated on Mueller-Hinton agar slants at 37°C for 24 h, except for the yeast strain that was cultivated on Sabouraud's dextrose agar slant at 30°C for 24h. The antimicrobial producing actinomycetes exhibited inhibitory distance against microorganisms tested. The inhibitory distances were measured and recorded. In addition, they were cultivated in ISP 2 broth on rotary shaker (200 rpm) for 4 days. Then, the selected cultures was transferred into the same medium containing calcium carbonate, and incubated for 10 days. The culture filtrate was extracted by ethyl acetate, rotary evaporator dried, and dissolved in methanol. Vacuum dried and redissolved in methanol were applied on a paper disc (0.6 cm diameter) with the amount of 1 mg/disc. The applied discs were placed on agar media spreaded over by the indicator strains using the agar disc diffusion method[22]. Streptomycin (20µg/disc) was used as positive control.

RESULTS AND DISCUSSION

Isolation and identification: The mangrove soil samples collected from Samut Prakarn and Samut Songkram provinces, Thailand. The pH of the soil samples ranged from 5.4 to 7.8. The actinomycete isolates could grow in wide range of pH. The isolate SAM2-1 was isolated from the soil collected in Samut Songkram while isolates SMP3-1, J8-1 and J17-2 were from the samples in Samut Prakarn. On the basis of their phenotypic characteristics (Tables 2 and 3), they were belonged to *Streptomyces*[23].

Table.2: Cultural characteristics of *Streptomyces* isolates

Isolate no.	Medium	Growth	Spore color	Colony color	
				Upper colony	Lower colony
SAM2-1	YMA	+++	White	Pale reddish yellow	Light reddish yellow
	TSA	++	White	White	White
	OMA	++	White	White	White
	AGA	++	White	White	White
	ISA	+++	White	White	White
SMP3-1	YMA	+++	Grayish white	Rose	Strong yellow
	TSA	+++	Gray	Rose	Rose
	OM	+++	Gray	Rose	Rose
	AGA	+++	Purplish pink	White	Pink
	ISA	+++	Vivid red purple	Vivid red purple	Vivid red purple
J8-1	YMA	+++	Yellowish grey	Yellowish grey	Brownish gold
	TSA	+	White	White	White
	OMA	+++	White	White	Yellowish white
	AGA	+	White	White	Brownish gold
	ISA	-	-	-	-
J17-2	YMA	+++	Grayish white	Yellowish white	Yellowish white
	TSA	+++	Grayish brown	Grayish brown	Pale beige
	OMA	+++	Gray	Dark brown	Black
	AGA	+	Grayish brown	Grayish brown	Brown
	ISA	+++	Grayish white	Yellowish white	Pale beige

Note: YMA, Yeast extract-Malt extract agar; TSA, Tyrosine agar; OMA, Oatmeal agar; AGA, Glycerol-asparagine agar; ISA, Inorganic salt-starch agar. +++, good; ++, moderate; +, poor; -, no growth.

Isolate SAM2-1 produced powdery colony with spiral spore chain (Fig.1), and with rectiflexibile spore chain. This isolate grew well on ISP 2 agar plate, produced white spore while the upper colony color was pale reddish yellow and the lower side was light reddish yellow. The cultural characteristics on tyrosine, oatmeal, asparagine and inorganic salt agar media are shown in Table 2.

Isolate SMP3-1 produced powdery colony with spiral spore chain (Fig.1), and with rectiflexibile spore chain. This isolate grew well on ISP 2 agar plate, produced grayish white spore while the upper colony

color was rose and the lower side was strong yellow. The cultural characteristics on tyrosine, oatmeal, asparagine and inorganic salt agar media are shown in Table 2.

Table.3: Characteristics and antibacterial activity of *Streptomyces* isolates

Characteristics	SAM2-1	SMP3-1	J8-1	J17-2
Max. NaCl (%,w/v)	5	4	2	6
Growth at pH 5-9	+	+	+	+
Growth at 45 °C	w	+	+	+
Nitrate reduction	+	+	+	+
Milk peptonization	-	-	+	+
Milk coagulation	+	-	+	-
Gelatin liquefaction	+	+	+	+
Starch hydrolysis	w	+	+	+
H_2S formation	-	-	-	-
Melanin formation	-	-	-	-
Utilization of :				
L-Arabinose	w	-	+	w
Fructose	-	-	+	-
D-Glucose	+	+	+	+
Glycerol	+	w	w	+
Mannitol	+	w	+	-
Raffinose	+	-	+	-
Rhamnose	+	-	+	+
Sucrose	-	+	w	+
D-Xylose	w	-	+	w
L-Diaminopimelic acid	+	+	+	+
Indicator strain	**Inhibitory against (mm)**			
S. aureus	24.0	15.0	25.0	25.1
B. subtilis	-	15.0	25.1	14.0
K. rhizophila	-	13.0	-	-
P. aeruginosa	10.0	-	-	-
E. coli	-	8.0	-	-

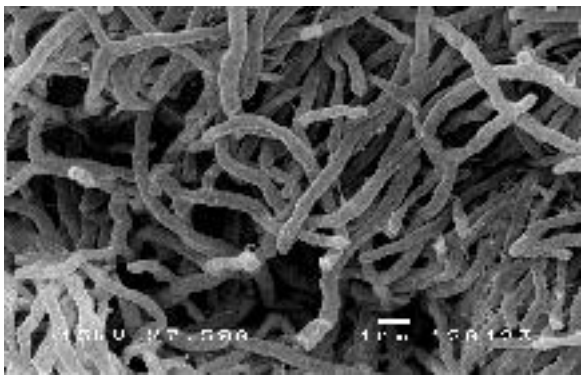
Note: +, positive; –, negative; w, weakly positive.

Isolate J8-1 produced powdery colony with spiral spore chain (Fig.1), and with rectiflexibile spore chain. This isolate grew well on ISP 2 agar plate, produced yellowish grey spore while the upper colony color was yellowish grey and the lower side was brownish gold. The cultural characteristics on tyrosine, oatmeal, asparagine and inorganic salt agar media are shown in Table 2.

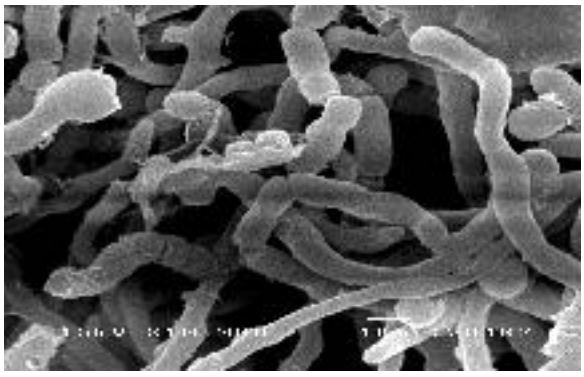
Isolate J17-2 produced powdery colony with spiral spore chain (Fig.1), and with rectiflexibile spore chain. This isolate grew well on ISP 2 agar plate, produced grayish white spore while the upper and the lower colony color were yellowish white. The cultural characteristics on tyrosine, oatmeal, asparagine and inorganic salt agar media are shown in Table 2.

a

b



c



D

Figure.1: Scanning electron micrograph of *Streptomyces* sp. SAM2-1 (a), SMP3-1 (b), J8-1(c) and J17-2 (d) grown on YMA medium (7-14 days)

The four isolates grew on ISP 2 medium containing 2% to 6% NaCl, at pH 5.0 to 9.0 and at 45°C. They

reduced nitrate, hydrolysed starch and liquefied gelatin, whereas some strains coagulated skim milk and showed milk peptonization but all did not produce H_2S, form melanin and hydrolyse chitin. All strains utilized glucose and glycerol but some did utilize L-arabinose, fructose, mannitol, rhamnose, raffinose and sucrose, as single carbon sources (Table 3). Isolates SAM2-1, SMP3-1, J8-1 and J17-2 contained L-diaminopimelic acid (L-DAP) indicating that these strains had cell wall chemotype I as described by Lechevalier & Lechevalier[24], which is the cell wall type of *Streptomyces* as described by Schleifer & Kandler[25]. The major menaquinones of strains were MK-9(H_6) (33.2-67.6%), and MK-9(H_8) (32.4%-66.8%). On the basis of 16S rRNA gene analyses, the isolates SAM2-1 (1130 bp), SMP3-1 (1070 bp), J8-1 (1088 bp) and J17-2 (1050 bp) were closely related to *Streptomyces sundarbansensis* DSM 42019^T (98.4%), *S. diastaticus* subsp. *ardesiacus* JCM 5815^T (97.1%), *S. iranensis* JCM 17327^T (96.4%) and *S. seoulensis* NBRC 16668^T (95.3%), respectively [26-28].

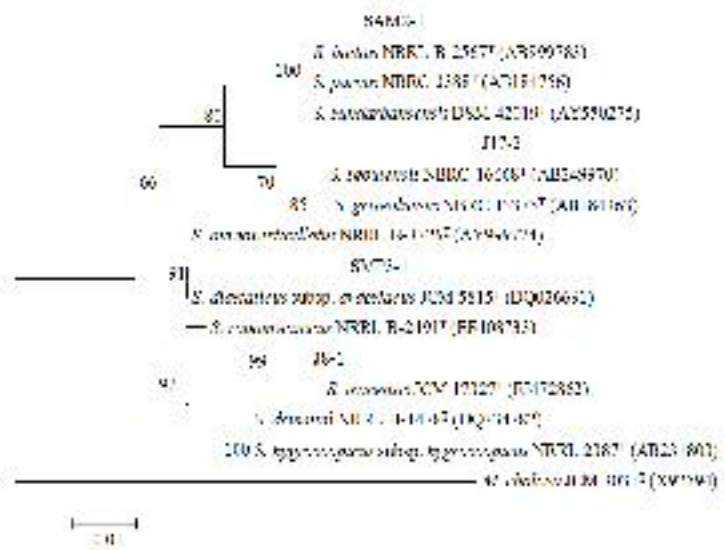


Figure.2: Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between the isolates and *Streptomyces* species. Based on 1000 resamplings, bootstrap percentages above 50% are shown. Bar, 0.01 substitutions per nucleotide position

Screening of antimicrobial activity: Four isolates showed inhibitory activity against *S. aureus* ATCC 6538P, isolates SMP3-1, J8-1 and J17-2 showed inhibitory activity against *B. subtilis* ATCC 6633, and only isolate SAM2-1 could inhibit against *P. aeruginosa* ATCC 27853 and and SMP3-1 inhibited *K. rhizophila* ATCC 9341 and *E. coli* ATCC 25922. As mentioned, the isolates could exhibit antimicrobial activities against Gram-positive bacteria and some of them could do against Gram-negative bacteria. The antimicrobial activity test by agar disc diffusion method of the isolates SAM2-1 and SMP3-1 revealed that all the culture extracts inhibited Gram positive bacteria; *S. aureus* ATCC 6538P, *B. subtilis* ATCC 16633 and *K. rhizophila* ATCC 9341 while only the culture extracts of isolate SMP3-1 inhibited *E. coli* ATCC 25922, and those of isolate SAM2-1 and SMP3-1 inhibited *P. aeruginosa* ATCC 27853. None of them inhibited *C. albicans* ATCC 10231.

Recently, the actinobacteria which identified as *S. exfoliatus*, *S. vinaceusdrappus*, *S. tendae*, *S. aureus*, *S. atriruber*, *S. olivochromogenes*, *S. malaysiensis*, *S. purpeofuscus*, *S. sparsogenes*, *S. aldersoniae*, *S. rapamycinicus* and *S. youssoufiensis* were isolated from terrestrial soils collected in Nakhon Si Thammarat, the southern part of Thailand. Most of them showed inhibitory activity against *B. subtilis* ATCC 6633 and *K. rhizophila*[27] that they were different from our *S. sundarbansensis*, *S. diastaticus*, *S. iranensis* and *S. seoulensis* strains isolated from mangrove soils. The strains in this study, however they are required to further study on DNA-DNA hybridization for clarifying their taxonomic position.

CONCLUSION

The mangrove actinomycete strains, SAM2-1 isolated from Samut Songkram and SMP3-1, J8-1 and J17-2 isolated from Samut Prakarn were identified as *Streptomyces* based on their phenotypic and chemotaxonomic characteristics including 16S rRNA gene analyses. They could inhibit Gram-positive bacteria, *S. aureus* ATCC 6538P but showed weak inhibitory activity against *K. rhizophila* ATCC 9341, Gram-negative bacteria, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853. Only isolate J8-1 showed strong inhibitory activity against *B. subtilis* ATCC 6633.

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