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Distribution and antimicrobial activity of Thai marine actinomycetes

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ABSTRACT

A total of 41 actinomycetes were isolated from marine samples collected in Thailand. On the basis of morphology, chemotaxonomy, and 16S rRNA gene sequence analysis, they were identified as *Salinispora* (13 isolates), *Micromonospora* (11 isolates), *Nocardia* (1 isolate), *Verrucosispora* (2 isolates), and *Streptomyces* (14 isolates). The antimicrobial activity screening revealed that two *Micromonospora* isolates, 12 *Salinispora* isolates and 10 *Streptomyces* isolates showed activity against *Staphylococcus aureus* ATCC 25923, *Kocuria rhizophila* ATCC 9341, *Bacillus subtilis* ATCC 6633, *Escherichia coli* NIHJ KC213, *Candida albicans* KF1, and *Mucor racemosus* IFO 4581. Based on this study, the production media and strains were the main factors that influenced the antimicrobial activity.

INTRODUCTION

Actinomycetes are Gram-positive filamentous bacteria having high mol % of the base guanine plus cytosine content in their genomes (Stackebrandt et al., 1997). They have been well known as the valuable economically importance microorganisms for a long time because of their ability to produce a large number of bioactive secondary metabolites (Berdy, 2005). In general, actinomycetes are widely distributed in terrestrial habitats, mainly in soils, organic materials, and plant materials (Goodfellow et al., 1988; Qin et al., 2012). Since they have been isolated from these habitats for a century, numerous redundant isolates were obtained. Three-quarters of the planet earth covers with the oceans which contain a huge biological diversity. Since the discovery of the true obligate marine actinomycetes in the genus Salinispora in the last decade, the ocean has been accepted for the existence of the actinomycetes. Moreover, the distribution of actinomycetes in the marine environment are largely unexplored (Bister et al., 2004; Jensen et al., 2007; Kwon et al., 2006). The aim of this study is

to isolate, identify and screen for antimicrobial activities of the marine actinomycetes isolated from sand, sediments and marine sponges collected in Thailand.

MATERIALS AND METHODS

Samples collection and isolation methods

The marine samples, including sand, sediments and marine sponges, were collected from Chumphon (Chumphon beach and Koh Khai), Chonburi (the nature education center for mangrove conservation and ecotourism, and Bangsaen beach), Phuket (Phanwa beach), Trang (Koh Rok Nork, Koh Rok Nai, and Koh Mah) and Krabi Province (mangrove forest) using scuba diving gears. They were isolated using the standard dilutionplating method. One gram of samples was suspended in 9 ml of sterile natural seawater to make 10-fold dilution series to 10⁻⁴. Each diluted suspension (0.1 ml) was spread on M1, M2 (Zhang et al., 2006) and seawater-proline media (Inahashi et al., 2011; modified with seawater) supplemented with nalidixic acid 25 μ g ml⁻¹ and cycloheximide 50 µg ml⁻¹. The plates were incubated at 28°C for 30 days. The colonies of marine actinomycetes were observed using a light microscope and were transferred to ISP2 agar plates (Shirling and Gottlieb, 1966). The purified cultures were maintained on ISP2 medium at 4°C. All isolates were preserved using freeze-drying and freezing at -80°C in 15% (v/v) glycerol solution.

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Identification methods

Phenotypic, chemotaxonomic, and genotypic characterization

The marine actinomycete isolates were identified using the phenotypic and genotypic characteristics. Morphological characteristics of the isolates were observed on the culture grown on ISP2 medium at 28°C for 14 days. Cultural characteristics were determined using 14-day cultures grown at 28°C on yeast extract-malt extract medium (ISP2 medium). The isomers of diaminopimelic acid were analyzed using the standard TLC method (Staneck and Robert, 1974).

The genomic DNA for 16S rRNA gene amplification was extracted from the cells using the method as described by Tamaoka (1994). The amplification of 16S rRNA gene was carried out using two primers 20F (5'-GAGTTTGATCCTGGCTCAG-3', positions 9-27) and 1500R (5'-GTTACCTTGTTACGACTT-3', positions 1492-1509). The PCR (Polymerase chain reaction) mixture (final volume 100 µl) contained 4 µl each of primers (10 pmol/µl), 2 µl of dNTP (10 mM), 10 µl of 10x Taq buffer, 8 µl of MgCl (25 mM), 0.5 µl of Taq DNA polymerase, 61.5 µl of dH₂O, and 10 µl of template DNA. The amplification was performed with an initial denaturation at 94°C for 3 minutes, followed by 30 cycles with denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 2 minutes, followed by the last step at 72°C for 3 minutes (Suriyachadkun et al., 2009). The PCR product was purified using the PCR purification kit (Gene aid). The sequencing of nucleotides was performed using universal primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 800R (5'-TACCAGGGTATCTAATCC-3') (Lane, 1991) (Macrogen; Seoul, Korea).

Antimicrobial activities

The culture broth library was prepared by using four different production media including 301 medium, 54 medium (2 g soluble starch, 0.5 g glycerol, 1 g defatted wheat germ, 0.3 g meat extract, 0.3 g yeast extract, 0.3 g CaCO₃, 500 ml tap water, 500 ml artificial seawater, pH 7.4–7.8), 51 medium, and yeast-dextrose (YD) broth (Sripreechasak *et al.*, 2013; 2014). The inoculum of each strain was cultured in yeast extract-dextrose broth for 4–7 days. Then, 0.1 ml of the culture was transferred to 10 ml of the screening production media and incubated in a shaking condition at 180 rpm at 30°C for 7–14 days. After incubation, 10 ml of 95% ethanol were added into the culture broth and shaked at 180 rpm for 2 hours. The extract solution was centrifuged at 3,400 rpm for 15 minutes and preserved at -20 °C. The production media without the culture was used as the negative control.

The screening of antimicrobial activities were performed using agar disc diffusion method (Qin *et al.*, 2009). Each of paper disc (8 mm) was soaked into the extract solution and air-dried. After drying, the discs were put onto the surface of the agar plate containing a tested microorganism and cooled at 4°C for 30 minutes before incubation. The bacterial plates were incubated at 37°C for 24 hours while yeast and filamentous fungus were incubated at 30°C for 48 hours. The inhibition zones (mm) were measured using a Vernier caliper. Three bacteria, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* NIHJ KB213, one yeast, *Candida albicans* KF1, and one filamentous fungi *Mucor racemosus* IFO 4581^T were used as the tested microorganisms.

RESULTS AND DISCUSSION

Identification

On the basis of 16S rRNA gene sequence analysis and morphological characteristics, the actinomycete isolates were belonged to the family *Micromonosporaceae*, *Nocardiaceae* and *Streptomycetaceae* (Table 1). They were identified as *Salinispora* (13 isolates), *Micromonospora* (11 isolates), *Nocardia* (1 isolate), *Verrucosispora* (2 isolates), and *Streptomyces* (14 isolates).

Group I, the members of this group exhibited a monomeric spore on the substrate mycelia but lacked aerial mycelia. They contained *meso*-diaminopimelic acids. They were divided into three genera based on the key morphological and chemotaxonomic characteristics and 16S rRNA gene sequence analysis including phylogenetic tree relationship.

Group IA comprised 13 isolates (KRN2-1, SPM3-5, SPM3-1, SPM3-3, SPM3-6, SPM3-7, SPM9-1, SPM9-2, SRK1-1, SRK1-2, SRK1-3, SRK2-1 and SRK2-3) (Table 1). All strains required sea water for the growth. The 16S rRNA gene analysis revealed that these isolates showed the highest similarity with *Salinispora arenicola* CNH-643^T (100% similarity). Therefore, they were identified as *S. arenicola* (Maldonado *et al.*, 2005).

Group IB consisted of 11 isolates (BS-003, BS-007, CH7-4m, CPB1-11, CPB1-21, KK1-10, PW-001, PW-002, PW-004, PW-006 and PWB-012) (Table 1). Almost all isolates showed orange to brown substrate mycelia which changed into a dark brown or black color when cultured more than 10 days. Based on BLASTn and phylogenetic analyses, these strains showed the highest 16S rRNA gene sequence similarities with type strains of the members of the genus *Micromonospora* (Kawamoto, 1989).

Group IC *Verrucosispora* comprised two isolates including KK2-1 and SPM3-8 (Table 1). The representative isolate KK2-1 produced hairy spores which born singly on the substrate mycelia. BLASTn analysis of 16S rRNA gene sequences revealed that the isolates KK2-1 and SPM3-8 were the closest similar to *Verrucosispora gifhornensis* DSM 44337^T (99.7%) and *Verrucosispora sediminis* MS426^T (99.5%), respectively. On the basis of 16S rRNA gene sequence, they were identified as *Verrucosispora* (Rheims *et al.*, 1998).

Group II consisted of one isolate, PWB-002. This isolate showed the fragmentation on the substrate mycelia and contained *meso*-diaminopimelic acid in cell-wall peptidoglycan. It showed the phylogenetic relationship within the genus *Nocardia*. Based on the fragmentation of substrate mycelia and 16S rRNA gene sequence analysis, this isolate was identified as *Nocardia* (Kageyama *et al.*, 2004).

Group III, 14 isolates including SRN1-2, LKB1-4, KK5-10, KRN1-1, PWB-011, BM2-4, CPB1-13, LKB1-6, PWB-010, PWB-016, SRN1-1, LKB1-5, LKB1-7 and LKB1-11 (Table 1). They produced extensively branch aerial and substrate mycelia. Almost all isolates produced spiral spore chains, while long straight spore chains were occasionally observed. The chemotaxonomic analysis revealed that all of these isolates contained LL-diaminopimelic acids. In addition, the 16S rRNA gene sequence analysis (ranged from 98.2% to 99.9% similarity), these isolates shared the clade within the genus *Streptomyces* (Mao *et al.*, 2007).

Isolate no.	Source	Base pair (nt)	Accession no.	Similarity (%)	Nearest relatives
1.KRN2-1	Sediment ⁶	650	LC158533	100	S. arenicola CNH-643 ^T
2.SRK2-1	Sponge ⁷	660	LC158534	100	S. arenicola CNH-643 ^{T}
3.SRK1-3	Sponge ⁷	640	LC158535	100	S. arenicola CNH-643 ^{T}
4.SPM9-1	Sponge ⁸	679	LC158536	100	S. arenicola CNH-643 ^{T}
5.SPM3-7	Sponge ⁸	639	LC158537	100	S. arenicola $CNH-643^{T}$
6.SPM9-2	Sponge ⁸	650	LC158538	100	S. arenicola CNH-643 ^{T}
7.SPM3-6	Sponge ⁸	649	LC158539	100	S. arenicola CNH-643 ^T
8.SPM3-3	Sponge ⁸	609	LC158540	100	S. arenicola CNH-643 ^T
9.SPM3-1	Sponge ⁸	630	LC158541	100	S. arenicola CNH-643 ^{T}
10.SRK1-2	Sponge ⁸	609	LC158542	100	S. arenicola CNH-643 ^T
11.SRK2-3	Sponge ⁸	600	LC158543	100	S. arenicola CNH-643 ^T
12.SRK1-1	Sponge ⁸	550	LC158544	100	S. arenicola CNH-643 ^T
13.SPM 3-5	Sponge ⁸	640	LC158545	100	S. arenicola CNH-643 ^T
14.PW-001	Sediment ¹	560	LC158516	100	M. aurantiaca ATCC 27029 ^T
15.BS-007	$Sand^2$	600	LC158517	100	M. aurantiaca ATCC 27029 ^T
16.PW-002	Sediment ¹	580	LC158518	99.66	M. aurantiaca ATCC 27029 ^T
17.BS-003	$Sand^2$	530	LC158520	99.81	M. chalcea DSM 43026 ^T
18.CH7-4m	Mangrove sediment ³	559	LC158521	99.46	M. chersina ATCC 27029 ^T
19.KK1-10	Sediment ⁴	579	LC158528	100	<i>M. marina</i> JSM $1-1^{T}$
20.CPB1-11	Sediment ⁵	549	LC158529	100	<i>M. marina</i> JSM $1-1^{T}$
21.CPB1-21	Sediment ⁵	600	LC158530	100	$M.$ marina JSM $1-1^{T}$
22.PW-004	Sediment ¹	599	LC158531	98.3	<i>M. olivasterospora</i> DSM 43868 ^T
23.PWB-012	Sand ¹	569	LC158510	99.82	<i>M. tulbaghiae</i> $TVU1^{T}$
24.PW-006	Sediment ¹	569	LC158532	100	M. tulbaghiae TVU1 ^T
25.KK2-1	Sediment ⁴	630	LC158547	99.68	V. gifhornensis DSM 44337 ^T
26.SPM3-8	Sponge ⁸	609	LC158548	99.51	V. sediminis MS426 ^T
27.PWB-002	Sand ¹	509	LC158550	99.21	N. testacea NBRC 100365 ^T
28.SRN1-2	Sponge ⁷	630	LC158551	98.25	S. gulbargensis DAS131 ^T
29.LKB1-4	Mangrove sediment9	470	LC158552	99.57	S. coelicoflavus NBRC 15399 ^T
30.KK5-10	Sediment ⁴	589	LC158554	99.46	S. coelicoflavus NBRC 15399 ^T
31.KRN1-1	Sediment ⁶	610	LC158556	99.18	S. platensis JCM 4662 ^T
32.PWB-011	Sand ¹	410	LC158558	99.02	S. olivaceoviridis NBRC 13066^{T}
33.BM2-4	Sediment ²	535	LC158560	99.07	S. radiopugnans R97 ^T
34.CPB1-13	Sediment ⁵	600	LC158561	99.17	S. radiopugnans R97 ^T
35.LKB1-6	Mangrove sediment9	560	LC158562	97.68	S. rimosus DSM 41429 ^T
36.SRN1-1	Sponge ⁶	630	LC158563	98.25	S. gulbargensis $DAS131^{T}$
37.LKB1-5	Mangrove sediment9	619	LC158564	99.84	S. sanyensis 219820 ^T
38.LKB1-7	Mangrove sediment9	509	LC158565	99.8	S. sanyensis 219820 ^T
39.PWB-010	Sand ¹	597	LC158566	98.15	S. somaliensis DSM 40738 ^T
40.PWB-016	Sand ¹	589	LC158567	98.64	S. somaliensis DSM 40738 ^T
41.LKB1-11	Mangrove sediment9	630	LC158568	99.84	S. tritolerans DAS 165^{T}

 Table 1. Isolate number, sources of isolation, length of nucleotide sequence, accession no., 16S rRNA gene sequence similarity (%) and nearest relatives.

¹Phanwa beach, Phuket; ²Bangsaen beach, Chonburi; ³The nature education center for mangrove conservation and ecotourism, Chonburi; ⁴Koh Khai, Chumphon; ⁵Chumphon beach, Chumphon; ⁶Koh Rok Nai, Trang; ⁷Koh Rok Nork, Trang; ⁸Koh Mah, Trang; and ⁹Mangrove forest, Krabi.

Antimicrobial activities

Fourteen isolates of the family *Micromonosporaceae* showed antimicrobial activities against tested microorganisms. Twelve isolates from genus *Salinispora* including the isolates SRK2-1, SRK1-3, SPM9-1, SPM3-7, SPM9-2, SPM3-6, SPM3-1, SRK1-2, SRK2-3, SRK1-1 and SPM3-5 showed antimicrobial

activities against *S. aureus* ATCC 25923, *Kocuria rhizophila* ATCC 9341 and *B. subtilis* ATCC 6633, while one isolate, KRN2-1 showed activities against *S. aureus* ATCC 25923 Tables 2 and 3. All of these isolates were identified as *S. arenicola*.

Two isolates of the genus *Micromonospora* including the isolate CH7-4m and PW-004 showed antimicrobial activities

Isolates						Inhibitio	n zone (mn	ı)				
	Cultured in 301 medium						Cultured in YD medium					
	<i>S</i> .	K	В	E	С	М	S	K	В	Ε	С	М
Salinispora												
SPM3-7	-	-	-	-	-	-	-	9.5	-	-	-	-
SPM3-1	-	-	-	-	-	-	-	8.4	-	-	-	-
SRK1-2	-	-	-	-	-	-	-	19.9	-	-	-	-
Micromonospora												
CH7-4m	9.1	16.7	22.7	-	-	-	15.7	13.3	14.8	-	-	-
PW-004	-	-	-	-	-	-	-	-	13.4	-	-	-
Streptomyces												
BM2-4	15.5	17.5	11.0	-	-	-	17.0	17.6	12.5	-	-	-
CPB1-13	14.4	14.9	9.8	-	-	-	22.9	17.2	11.6	-	-	-
PWB-010	-	9.6	8.2	-	-	-	-	10.7	10.8	11.3	-	-
PWB-016	-	11.4	8.3	-	-	-		12.0	9.0			

Table 2. Antimicrobial activities of Salinispora, Micromonospora and Streptomyces isolates when cultured in 301 and YD media.

S, Staphylococcus aureus ATCC 25923; K, Kocuria rhizophila ATCC 9341; B, Bacillus subtilis ATCC 6633; E, Escherichia coli NIHJ KC213; C, Candida albicans KF1; M, Mucor racemosus IFO 4581. -, no activity.

Table 3. Antimicrobial activities of Salinispora, Micromonospora and Streptomyces isolates when cultured in 54 and 51 media.

Isolates	Inhibition zone (mm)											
	Cultured in 54 medium					Cultured in 51 medium						
	S	K	В	Ε	С	М	S	K	В	E	С	М
Salinispora												
KRN2-1	-	-	-	-	-	-	11.7	-	-	-	-	-
SRK2-1	14.5	-	-	-	-	-	15.6	24.8	8.9	-	-	-
SRK1-3	8.7	9.5	-	-	-	-	15.6	23.8	10.2	-	-	-
SPM9-1	10.9	-	-	-	-	-	15.1	21.1	8.1	-	-	-
SPM3-7	-	-	-	-	-	-	16.8	26.2	10.2	-	-	-
SPM9-2	8.7	-	-	-	-	-	15.0	22.5	10.2	-	-	-
SPM3-6	10.0	-	-	-	-	-	17.3	22.0	12.4	-	-	-
SPM3-1	13.3	-	-	-	-	-	16.8	23.5	9.2	-	-	-
SRK1-2	17.6	-	-	-	-	-	17.9	23.6	8.7	-	-	-
SRK2-3	11.1	-	-	-	-	-	15.6	18.5	11.0	-	-	-
SRK1-1	9.6	-	-	-	-	-	13.8	20.4	8.7	-	-	-
SPM 3-5	9.2	-	-	-	-	-	15.7	23.0	10.7	-	-	-
Micromonospora												
CH7-4m	-	-	15.4	-	-	-	-	9.1	9.6	-	-	-
Streptomyces												
LKB1-4	-	-	-	-	-	-	8.5	-	-	-	-	-
LKB1-1	-	9.5	9.6	-	14.0	-	-	10.2	-	-	13.3	-
PWB-011	-	-	-	-	-	-	9.5	13.6	9.7	-	-	-
BM2-4	16.3	20.8	12.0	-	-	-	19.5	21.4	12.1	-	-	-
CPB1-13	22.7	18.6	13.3	-	-	-	15.5	17.8	12.8	-	-	-
LKB1-6	9.0	-	-	-	-	-	-	-	-	-	-	-
LKB1-5	-	9.3	13.9	-	-	-	-	-	-	-	-	-
LKB1-7	-	-	-	-	-	-	-	9.3	-	-	-	-
PWB-010	-	-	-	-	-	-	-	8.1	15.0	11.9	-	-
PWB-016	-	-	-	9.3	-	-	-	-	12.3	10.2	-	-

S, Staphylococcus aureus ATCC 25923; K, Kocuria rhizophila ATCC 9341; B, Bacillus subtilis ATCC 6633; E, Escherichia coli NIHJ KC213; C, Candida albicans KF1; M, Mucor racemosus IFO 4581. -, no activity.

against tested microorganisms. The isolate CH7-4m identified as *M. chersina* ATCC 27029^T, exhibited activities against *S. aureus* ATCC 25923, *K. rhizophila* ATCC 9341, *B. subtilis* ATCC 6633. In addition, the isolate PWB-004 identified as *Micromonospora olivasterospora*, exhibited activities against *B. subtilis* ATCC 6633.

Ten *Streptomyces* isolates showed the antimicrobial activities against tested microorganisms. However, six isolates (SRN1-2, KK5-10, KRN1-1, CH3-1, SRN1-1 and LKB1-11) did not show any antimicrobial activities when cultured in four different media. Among the active isolates, anti-Gram-positive bacteria activity could be observed in most isolates, while anti-Gram-negative bacteria, anti-yeast, and anti-mold activities were observed only 5, 8, and 4 isolates, respectively. The detailed antimicrobial activities of the *Streptomyces* isolates are shown in Tables 2 and 3. No antimicrobial activities were observed for the members of the genus *Nocardia* and *Verrucosispora*.

According to the screening of their antimicrobial activities, the good example could be observed in the members of the Salinispora group (Tables 2 and 3). No activities were observed when they were grown in 301 medium, but almost all isolates showed the activities against tested Gram-positive bacteria when they were grown in 51 medium. Besides this, the difference between strains within the same species but different isolates are other factors for the determination of the antimicrobial activity. For example, isolate SRK1-2 identified as S. arenicola showed activities in 54 and 51 media. On the basis of this study, it suggested that the screening should be determined using various different media. Moreover, the same species of the isolates does not mean the same activities will be observed. In addition, the further elucidation of bioactive compounds of S. arenicola, Micromonospora and Streptomyces isolates mentioned should be done as previously reported (Abdelmohsen et al., 2014; Supong et al., 2012).

CONCLUSION

In this study, the marine actinomycetes, S. arenicola, Micromonospora aurantiaca, Micromonospora chalcea, Micromonospora chersina, Micromonospora marina, M. olivasterospora, Micromonospora tulbaghiae, V. gifhornensis, V. sediminis, Nocardia testacea, Streptomyces coelicoflavus, Streptomyces gulbargensis, Streptomyces olivaceoviridis, Streptomyces platensis, Streptomyces radiopugnans, Streptomyces rimosus, Streptomyces sanvensis, Streptomyces somaliensis, and Streptomyces tritolerans isolates were distributed in sand, mangrove sediments, marine sediments, and marine sponges collected from Chumphon, Chonburi, Phuket, Trang, and Krabi provinces in Thailand. Micromonospora, Salinispora, and Streptomyces isolates showed antimicrobial activity against S. aureus ATCC 25923, K. rhizophila ATCC 9341, B. subtilis ATCC 6633, E. coli NIHJ KC213, C. albicans KF1, and M. racemosus IFO 4581.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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