Characterization and antimicrobial activity of Amycolatopsis strains isolated from Thai soils

Piyapat Sripairoj¹, Khanit Suwanborirux² and Somboon Tanasupawat¹*

¹Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand
²Department of Pharmaceutical Botany and Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand

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ABSTRACT

The isolation and screening of antimicrobial activity of 3 actinomycete strains isolated from soil samples collected in Chaiyaphum, Nan and Phatthalung provinces, Thailand were carried out. Strains S39-7, KC19-1 and K57-1 were belonged to the genus Amycolatopsis based on their phenotypic and chemotaxonomic characteristics. On the basis of 16S rRNA gene sequence analysis, strain S39-7 was closely related to Amycolatopsis albidoflavus KCTC 9471T (99.2%). Strains KC19-1 and K57-1 were closely related to A. kentuckyensis NRRL B-24129T with 99.3 and 99.2% similarity, respectively. All of them contained meso-diaminopimelic acid (DAP) in cell wall peptidoglycan and had MK-9 (H1) as a major menaquinones. The DNA G+C contents of the strains ranged from 67.2 to 73.4 mol%. On secondary screening of antimicrobial activity, the ethyl acetate extract of the fermentation products of strain S39-7 was active against Staphylococcus aureus ATCC 6538, Bacillus subtilis ATCC 6633, Escherichia coli ATCC 25922, Kocuria rhizophila ATCC 9341, and Pseudomonas aeruginosa ATCC 27853 while strain KC 19-1 was active against only S. aureus ATCC 6538. Strain K 57-1 was active against E. coli ATCC 25922 and K. rhizophila ATCC 9341. In addition strain S39-7 could inhibit against methicillin resistant (MRSA) S. aureus 266.

INTRODUCTION

The genus Amycolatopsis was established by Lechevalier et al., (1986) and was assigned to family Pseudonocardiaeae (Embley et al., 1988; Cross, 1994; Warwick et al., 1994). Recently, increasing interest has been shown in Amycolatopsis strains because they are a very important genus in the antibiotics industry. They produce some of the most widely used antibiotics such as rifamycin that produced from A. mediterranei (Meja et al., 1997) and vancomycin from A. orientalis (Pittenger and Brigham, 1956). In addition, vancoresmycin was produced from A. vancoresmycin (Hopmann et al., 2002), balhimycin from A. balhimycina, tolypomycin from A. tolypomycina and nogabecin from A. keratiniphila (Wink et al., 2003), and decaplanin from A. decaplanina (Wink et al., 2004). Among them, rifamycin is one of the major drugs for clinical treatment of HIV-related tuberculosis, and vancomycin is currently considered as the last line of those defense against some microorganisms that are resistant to β-lactam antibiotics (Yao et al., 2002). In the course of our investigation of actinomycete isolates from soils in Thailand, the isolation and screening of antimicrobial activity and identification of strains were determined based on the phenotypic and chemotaxonomic characteristics including 16S rRNA gene sequence analysis.

MATERIALS AND METHODS

Isolation and Characterization of the isolates

Three actinomycete strains were isolated from soil samples collected from Chaiyaphum, Nan and Phatthalung provinces, Thailand (Table 1) using starch-casein nitrate agar (Thawai et al., 2004). The phenotypic characteristics were determined by the methods described by Shirling and Gottlieb (1966) and Arai et al. (1976).

Scanning electron microscope was used for determining the morphology of strains grew on YMA (ISP medium no. 2, Yeast extract- Malt extract agar). Cell wall diaminopimelic acid (DAP) isomers were determined as described by Kutzner (1981). Menaquinone system was analysed as described by Komagata and Suzuki (1987).

* Corresponding Author
Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand. Email: Somboon.T@chula.ac.th

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16S rDNA sequence and phylogenetic analyses

DNA of the strains was isolated from cells grown in Yeast extract-Malt extract broth (YMB) with 0.2% of glycine reported by Yamada and Komagata (1970) and purified as described by Saito and Miura (1963). DNA base composition analysis was analysed by the method of Tamaoka and Komagata (1984). The complete 16S rRNA gene was amplified by PCR using primers, 8-27f and 1492r.

The amplified 16S rRNA gene was used as templates for sequencing with Big Dye Terminator sequencing Kit (Perkin Elmer) and analyzed by ABI377 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, 8-27f (5'-AGAGTTTGATC (A/C)TGCTCAG-3'), 530f (5'-GTGCGACG(A/C)GCCG-3') and 1114f (5'-GCAAAGGCGGCAACC-3'). Homology search was performed using the standard BLAST sequence similarity searching program version 2.2.1 from the web server. http://www.ncbi.nlm.nih.gov/BLAST/ against previously reported sequence at the GenBank/EMBL/DDBJ database. The sequence was multiply aligned with selected sequences obtained from GenBank/EMBL/DDBJ by using the CLUSTAL_X (Thompson et al., 1997).

The alignment was manually verified and adjusted prior to the construction of phylogenetic tree. The phylogenetic tree was constructed by using neighbor-joining (Saitou and Nei, 1987) in the MEGA program version 2.1 (Kumar et al., 2001). The confidence values of branches of the phylogenetic tree were determined using the bootstrap analyses based on 1000 resamplings (Felsensten, 1985). 16S rDNA sequence of Micromonospora chalcea JCM 3082T was used as an out group. The values for sequences similarity among the closest strains were calculated manually after pairwise alignments obtained using the CLUSTAL_X program (Thompson et al., 1997). Gaps and ambiguous nucleotides were eliminated from calculations.

Antimicrobial activity of strains

Primary screening of antimicrobial activities was performed on YMA plates (Anansiriwattana et al. (2006) against S. aureus ATCC 6538, B. subtilis ATCC 6633, E. coli ATCC 25922, P. aeruginosa ATCC 27853, Kocuria rhizophila ATCC 9341 and C. albicans ATCC 10231. 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 24 h. Secondary screening of the strains was examined by cultivating each strains into a 500-ml Erlenmeyer flask containing 250 ml of YM broth and incubated on a rotary shaker at 200 rpm, 30°C for 11 days. The culture broth was extracted with ethyl acetate (EtOAc) and concentrated under reduced pressure to yield the crude extract. The ethyl acetate extracts were tested by agar disc diffusion method (Lorian, 1991).

RESULTS AND DISCUSSION

Isolation and characterization of isolates

Three soil samples were collected from Chaiyaphum, Nan and Phatthalung provinces, Thailand. Actinomycetes were isolated and cultivated on YMA and kept in cold room at 4°C. Sources of samples, pH and strain number were shown in Table 1.

The strains S39-7, KC19-1 and K57-1 produced branched, fragmenting aerial and substrate mycelium with pink white, brownish white and yellowish white colonial color on YMA plates, respectively. They produced hyphae, spores borne in chains that are resemble to Streptomyces (Goodfellow et al., 1988; Cross, 1994) (Figure 1). The cultural characteristics of strains on YMA, tyrosine agar, oatmeal agar, glycerol-asparagine agar and inorganic salt-starch agar are shown in Table 2.

Strains S39-7, KC19-1 and K57-1 grew on YMA with 2% and 4% NaCl, at pH 7.0, 9.0 and 10 and at 28°C, whereas only strain S39-7 could grow on 6% NaCl. They grew at pH and temperature within the range as reported previously (Cross, 1994). All strains could not form melanin. The physiological and biochemical characteristics of strains S39-7, KC19-1 and K57-1 were shown in Table 3.

All strains hydrolysed esculin, produced acid from adonitol, cellubiose, dextrin, meso-erythriol, fructose, glucose, D-galactose, meso-inositol, lactose, maltose, D-mannitol, melezitose, melibiose, methyl D-glucoside, raffinose, sucrose, trehalose and xylose; and utilized fructose, glucose, glycerol, D-mannitol, raffinose, rhamnose and xylose. Variable characteristics of strains were found in gelatin and starch hydrolysis, growth at pH 5, growth on 6% NaCl, acid production from L-arabinose, rhamnose, salicin and sorbitol; utilization of L-arabinose and melibiose (Table 3).

Strains KC19-1 and K57-1 grew on YMA containing 50 µg/ml and 100 µg/ml of novobiocin whereas strain S39-7 did not grow on YMA containing 50 µg/ml and 100 µg/ml of novobisocin comparison to A. keratinophila KCTC 19104T and A. albidoflavus KCTC 9471T that were sensitive to novobiocin 100 µg/ml (data not shown). However, other Amycolatopsis species such as A. eurytherma DSM 44348T, A. palatopharyngis 1BDZT and A. rubida JCM 10871T were resistant to only on novobisocin 5 µg/ml (Huang et al., 2001; 2004; Kim et al., 2002). Therefore, the use of novobisocin in the medium for the screening of Amycolatopsis strains should be considered (Tajima et al., 2001; Takahashi and Omura, 2003).

On the basis of cell wall peptidoglycan, the strains S39-7, KC19-1 and K57-1 contained meso-diaminopimelic acid which was the same pattern as the genus Amycolatopsis. The predominant menaquinone was MK-9 (H5) and the small amounts of MK-9 (H2), MK-9 (H3) and MK-9 (H4) were found. Their DNA G+C content ranged from 67.2-73.4 mol% as reported by Lechevalier et al. (1986) (Table 3).
Phylogenetic analysis of strains S39-7, KC19-1 and K57-1 revealed that they were belonged to the genus *Amycolatopsis* (Fig. 2). The percentage of 16S rRNA gene sequence similarity of *Amycolatopsis* strains to another strains were showed in Table 1. Strain S39-7 was closely related to *A. albidoflavus* KCTC 9471T (Lee and Hah, 2001). The two organisms shared 16S rDNA similarity value of 99.2%. The strains KC19-1 and K57-1 were 99.3% related to each other and showed 99.3% and 99.2% similarity with *A. kentuckyensis* NRRL B-24129T (Labeda et al., 2003), respectively. Strain S39-7 could produce dark red soluble pigment and produced acid from raffinose but no growth at 10 °C. These characteristics could differentiate it from *A. albidoflavus* KCTC 9471T (Lee and Hah, 2001). Strain KC19-1 could produce acid from raffinose but not decompose gelatin while K57-1 could produce acid from raffinose but not from L-arabinose and did not liquefy gelatin that differentiated them from *A. kentuckyensis* NRRL B-24129T (Labeda et al., 2003).

<table>
<thead>
<tr>
<th>Location (Province)</th>
<th>pH</th>
<th>Strain no.</th>
<th>Similarity (%)</th>
<th>Closest species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phatthalung</td>
<td>7.0</td>
<td>S39-7</td>
<td>99.2</td>
<td><em>A. albidoflavus</em> KCTC 9471T</td>
</tr>
<tr>
<td>Nan</td>
<td>6.8</td>
<td>KC19-1</td>
<td>99.3</td>
<td><em>A. kentuckyensis</em> NRRL B-24129T</td>
</tr>
<tr>
<td>Chaiyaphum</td>
<td>7.5</td>
<td>K57-1</td>
<td>99.2</td>
<td><em>A. kentuckyensis</em> NRRL B-24129T</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Medium</th>
<th>Growth</th>
<th>Spore color</th>
<th>Colony color</th>
</tr>
</thead>
<tbody>
<tr>
<td>S39-7</td>
<td>YM</td>
<td>+++</td>
<td>Pinkish white</td>
<td>Vivid purplish red</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>+++</td>
<td>Pinkish white</td>
<td>Dark violet</td>
</tr>
<tr>
<td></td>
<td>Oatmeal</td>
<td>+++</td>
<td>Pinkish white</td>
<td>Deep purplish red</td>
</tr>
<tr>
<td></td>
<td>Asparagine</td>
<td>+++</td>
<td>Pinkish white</td>
<td>Dark brown</td>
</tr>
<tr>
<td></td>
<td>Inorg. salt</td>
<td>++</td>
<td>Pinkish white</td>
<td>Pinkish white</td>
</tr>
<tr>
<td>KC19-1</td>
<td>YM</td>
<td>+++</td>
<td>Brownish white</td>
<td>Pale beige</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>+++</td>
<td>Yellowish white</td>
<td>Pale yellow</td>
</tr>
<tr>
<td></td>
<td>Oatmeal</td>
<td>+++</td>
<td>Yellowish white</td>
<td>Pale yellow</td>
</tr>
<tr>
<td></td>
<td>Asparagine</td>
<td>+++</td>
<td>Brownish white</td>
<td>Pale beige</td>
</tr>
<tr>
<td></td>
<td>Inorg. salt</td>
<td>+++</td>
<td>Brownish white</td>
<td>Yellowish white</td>
</tr>
<tr>
<td>K57-1</td>
<td>YM</td>
<td>+++</td>
<td>Yellowish white</td>
<td>Pale beige</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>+++</td>
<td>Yellowish white</td>
<td>Pale yellow</td>
</tr>
<tr>
<td></td>
<td>Oatmeal</td>
<td>+++</td>
<td>Yellowish white</td>
<td>Pale beige</td>
</tr>
<tr>
<td></td>
<td>Asparagine</td>
<td>+++</td>
<td>Yellowish white</td>
<td>Yellowish white</td>
</tr>
<tr>
<td></td>
<td>Inorg. Salt</td>
<td>+++</td>
<td>Yellowish white</td>
<td>Yellowish white</td>
</tr>
</tbody>
</table>

YMA, Yeast extract- Malt extract agar; Tyrosine, Tyrosine agar; Oatmeal, Oatmeal agar (Difco); Asparagine, Glycerol-Asparagine agar; Inorg. Salt, Inorganic salt-Starch agar. ++++, good growth; ++, moderate growth.
such as known strains and species, in which a DNA homology value of about >70% plays a dominant role (Wayne et al., 1987). For further study, Amycolatopsis strains S39-7, KC19-1 and K57-1 should be hybridized with closely related type strains for proposed that they are possible new species.

**Antimicrobial activity of strains**

Strain S39-7 exhibited antimicrobial activity against S. aureus ATCC 6538, B. subtilis ATCC 6633, E. coli ATCC 25922, *K. rhizophila* ATCC 9341 and *P. aeruginosa* ATCC 27853 while Strain KC 19-1 exhibited antimicrobial activity against only *S. aureus* ATCC 6538 and strain K 57-1 exhibited antimicrobial activity against *E. coli* ATCC 25922 and *K. rhizophila* ATCC 9341. All strains did not showed activity against *C. albicans* ATCC 10231 (Table 4).

In addition strain S39-7 could inhibit against methicillin resistant *S. aureus* (MRSA) 266. The study of antimicrobial substances from Amycolatopsis strains was interesting to further studies on the fermentation, extraction, purification, and structure elucidation.

### Neighbor-joining tree showing the position of S39-7, KC19-1, K57-1 and the type strains of Amycolatopsis species based on 16S rRNA gene sequences.

![Neighbor-joining tree showing the position of S39-7, KC19-1, K57-1 and the type strains of Amycolatopsis species based on 16S rRNA gene sequences.](image-url)
CONCLUSION

The actinomycete strains, S39-7, KC19-1 and K57-1 isolated from soil collected in Phatthalung, Nan and Chaiyaphum respectively, were identified as *Amycolatopsis* based on their phenotypic and chemotaxonomic characteristics including 16S rRNA gene analyses. They could inhibit Gram-positive bacteria, *S. aureus* ATCC 6538, *B. subtilis* ATCC 6633 and *K. rhizophila* ATCC 9341 and Gram-negative bacteria, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 but showed no inhibitory activity against *C. albicans* ATCC 10231.

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