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# Characterization and screening of antimicrobial activity of *Micromonospora* strains from Thai soils

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## ABSTRACT

**Aims:** Rare actinomycete strains were isolated from mountain soils and island soil collected in Thailand. They were screened for antimicrobial activity and characterized for their secondary metabolites.
**Methodology and results:** The strains were isolated by the standard dilution technique using starch casein nitrate agar. They were identified and characterized based on the phenotypic, chemotaxonomic and genotypic characteristics. The chemotaxonomic characteristics of ten isolates coincided with those of the genus *Micromonospora*. On the basis of phylogenetic analysis using 16S rRNA gene sequences and DNA-DNA relatedness, they were divided into 6 Groups, ASC19-2-1 (Group A) was identified as *Micromonospora marina*; AL8-8 and AL10-3 (Group B) were *M. aurantiaca*; AL7-5 (Group C) was *M. chalcea*; AL3-16 and AL9-20 (Group D) were identified as *M. chokoriensis*; AL9-13 and AL9-22 (Group E) were *M. tulbaghiae*; and AL1-15-2 and AL1-16B (Group F) were *M. chersina*. On the primary screening, only the isolate AL7-5 (Group C) could inhibit *Kocuria rhizophila* ATCC 9341. This isolate produced rakicidin when cultivated on A3M, A11M and A16 media and produced compound BU4664L only on A16 medium.
**Conclusion, significance and impact of study:** The isolation and characterization of the rare actinomycetes from Thai soils will be useful for the taxonomic study and for the discovery of bioactive metabolites that are active against microorganisms.

*Keywords:* Antimicrobial activity, *Micromonospora,* rare actinomycetes, secondary metabolites, Thai soils

## INTRODUCTION

*Micromonospora* is the type genus of the family *Micromonosporaceae*. The members of this family now contains several genera, including the genera *Micromonospora*, *Actinoplanes*, *Dactylosporangium* and *Pilimelia* (Goodfellow *et al.*, 1990). *Micromonospora* strains have distinct morphological characteristics in that they produce single spores on the substrate mycelium and lack aerial mycelium. The genus *Micromonospora* is well classified on the basis of chemotaxonomic characteristics, *gyrB* and 16S rRNA gene sequence analyses including DNA-DNA relatedness (Kawamoto, 1989; Koch *et al.*, 1996; Kasai *et al*., 2000). *Micromonospora* strains are distributed widely in different environments such as in soils, water, sandstone, mangrove sediment and root nodules (Luedemann and Brodsky, 1964; Kawamoto, 1989; Hirsch *et al.*, 2004; Ara and Kudo, 2007; Garcia *et al.*, 2010; Songsumanus *et al*., 2011; Wang *et al.*, 2011).

*Micromonospora* strains produce many kinds of antibiotics such as gentamicin, micromonosporin, megalomicin, mutamicin, fortimicin, sagamicin, verdamicin, dapiramicin, clostomicin, mycinamicin, dynemicin, macquarimicin, holomicin, quinolidomicins, arisostatins A and B and anthraquinones (Glasby, 1993; Berdy, 2005; Igarashi *et al*., 2000; 2007; Omura, 2008). During an investigation of the biodiversity and antimicrobial activity screening of actinomycetes in mountain soil and island soil in Thailand, nine isolates from the genus *Micromonospora* were identified and characterized based on their phenotypic, chemotaxonomic and genotypic characteristics.

## MATERIALS AND METHODS

### Isolation methods

The actinomycete strains were isolated from mountain soils in Phukradung, Loei province, the North Eastern part of Thailand and from Nok Island, Chonburi province, the Eastern part of Thailand. The actinomycete strains were isolated by using wet heat at 70 °C for 15 min and the

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standard dilution technique on starch casein nitrate agar (Tanasupawat *et al*., 2010) supplemented with nystatin (50 mg/L)and nalidixic acid (20 mg/L). The plates were incubated at 30 °C for 14 days and then a single colony was transferred and purified on yeast extract-malt extract agar (International *Streptomyces* Project, ISP medium no. 2) as described by Shirling and Gottlieb (1966). The pH of soil samples was determined using the dried soil samples that were dried under room temperature for 7 days. One gram of dried soil was put into the test tubes containing 2.5 mL of distilled water and shaken for 2 to 3 sec before taking the pH reading.

**Identification and characterization methods**

*Phenotypic characterization*

The cell morphology was observed by scanning electron microscopy. The phenotypic properties were examined using several standard methods (Williams and Cross, 1971; Gordon *et al*., 1974; Arai, 1975). For determining the cultural characteristics, the isolates were grown on ISP 2 agar plates at 30 °C for 14 days (Shirling and Gottlieb, 1966) after that the colony colours were determined using the NBS/IBCC Colour System. Temperature, pH and NaCl concentration ranges for the growth of isolates were tested on ISP 2 agar at 30 °C for 14 days. ISP 9 agar supplemented with 1% sole carbon source was used to determine their carbon utilization.

*Chemotaxonomic characterization*

For chemotaxonomy, freeze-dried cells were collected from 4-day-old cultures grown in ISP 2 broth on a rotary shaker at 30 °C. The cell wall peptidoglycan was prepared by the method of Kawamoto *et al*. (1981). The isomer of diaminopimelic acid was determined by the TLC method of Staneck and Roberts (1974). The *N*-acyl group of the muramic acid in the peptidoglycan was analysed by spectrophotometer using the method of Uchida and Aida (1984). Isoprenoid quinones were extracted by the method of Collins *et al.* (1977) and then analysed by HPLC equipped with a Cosmosil 5C18 column (4.6 by 150 mm, Nacalai Tesque, Kyoto, Japan). Whole cell sugars were analysed according to Mikami and Ishida (1983). Methyl esters of cellular fatty acids were prepared from cells grown on ISP 2 (Yeast extract-malt extract agar), and identified by GLC according to the Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer and Kroppenstedt, 1996).

*Genotypic characterization*

Chromosomal DNA was extracted from cells grown in ISP 2 broth supplemented with 0.1% glycine (Yamada and Komagata, 1970; Tamaoka, 1994). The G + C content of the DNA was determined by HPLC (Tamaoka, 1994). DNA-DNA relatedness was determined according to Ezaki *et al.* (1989). The 16S rRNA gene was amplified by using primers 27F and 1492R as described by Nakajima *et al.* (1999). The 16S rRNA gene sequence was multialigned with selected sequences obtained from the GenBank/EMBL/DDBJ databases by the CLUSTAL_X version 1.83 program (Thompson *et al*., 1997). The aligned sequences were manually edited before constructing the phylogenetic tree by the neighbour-joining (Saitou and Nei, 1987). The branches confidence values of the phylogenetic tree were examined by bootstrap analysis (Felsenstein, 1985) based on 1,000 resamplings. Gaps and ambiguous nucleotides were deleted manually before analysing the similarity values by CLUSTAL_X (Thompson *et al*., 1997).

**Screening of antimicrobial activities and preliminary characterization of secondary metabolites**

The isolates were streaked along the diameter of ISP 2 agar medium and cultivated for 14 days at 30 °C. The test microorganisms were then streaked out perpendicular to the selected isolates about 3 mm apart from them and incubated at 30 °C for 1 day. The tested microorganisms used in this study were *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Kocuria rhizophila* ATCC 9341, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231. The clear zone was present if the isolates inhibited the growth of test microorganisms.

The selected actinomycete culture was transferred to the Bn-2 agar slant consisting (w/v) of 0.5% glucose, 0.5% soluble starch, 0.1% meat extract, 0.1% yeast extract, 0.2% NZ-case, 0.2% NaCl, 0.1% $CaCO_3$, 1.5% agar (pH 7.0) and incubated at 30 °C for 3-4 days. A loop-full of a mature slant culture was inoculated into 100 mL of the seed medium (V-22) consisting of soluble starch 1%, glucose 0.5%, NZ-case 0.3%, yeast extract 0.2%, tryptone 0.5%, $K_2HPO_4$ 0.1%, $MgSO_4.7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0) and incubated at 30 °C for 3-4 days on a rotary shaker (200 rpm). Three milliliter aliquots of the seed cultures were transferred into 500 mL flasks containing l00 mL of 3 kinds of media such as A3M: 0.5% glucose, 2% glycerol, 2% soluble starch, 1.5% Pharmamedia, 0.3% yeast extract, 1% Diaion HP-20 (Mitsubishi Chemical Co.), pH 7.0; A11M: 0.2% glucose, 2.5% soluble starch, 0.5% yeast extract, 0.5% polypeptone, 0.5% NZ-amine, 0.3% $CaCO_3$, 1% Diaion HP-20, pH 7.0; and A16: 0.2% glucose, 1.5% Pharmamedia, 0.3% $CaCO_3$, 1% Diaion HP-20, pH 7.0. Fermentation was carried out for 5-6 days at 30 °C on a rotary shaker (200 rpm). The culture broth was added with 100 mL of 1-butanol to the flask and shaken for 2 h. Mycelia were removed by centrifugation at 6,000 rpm at 4 °C for 6 min. One milliliter of supernatant was transferred to a small vial and evaporated until it dried. The extract was dissolved with 100 µL of DMSO, filtrated, and then analysed by HPLC-UV. The retention time and UV spectra of the peaks were compared by using the in-house database (Biotechnology Research Center, Toyama Prefectural University) containing about 500 reference compounds of microbial origins.

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## RESULTS AND DISCUSSION

### Identification and characterization of the isolates

Ten actinomycetes isolates, AL1-15-2, AL1-16B, AL3-16, AL7-5, AL8-8, AL9-13, AL9-20, AL9-22 and AL10-3 from mountain soils in Phukradung, Loei province, the north eastern part of Thailand and isolate ASC19-2-1 from Nok Island, Chonburi province eastern part of Thailand were isolated (Table 1). The pH of soil samples were in the range of 5.01-9.66. All isolates contained *meso*-DAP (diaminopimelic acid) and *N*-glycolyl muramic acid in the peptidoglycan. The whole cell sugars of all isolates were ribose, mannose, galactose, xylose and glucose [pattern D of Lechevalier and Lechevalier (1970)]. The chemotaxonomic characteristics of these isolates coincided with those of the genus *Micromonospora* (Kawamoto, 1989). On the basis of 16S rRNA gene sequence and phylogenetic tree analyses (Figure 1), they were divided into 6 Groups as described below.

Group A contained isolate ASC19-2-1 which produced brownish gray vegetative mycelium, well-developed and singly rough spores on substrate hyphae on ISP 2 agar plate (Figure 2 and Table 2). The isolate grew on 1.5 to 5% NaCl, at pH 5 to 8 and at 15 to 40 °C. It utilized L-arabinose, cellobiose, galactose, D-glucose, glycerol, salicin, and did weakly for D-fructose, D- lactose, sucrose and D-xylose. Milk peptonization, coagulation, gelatin liquefaction and starch hydrolysis of isolate were positive but nitrate reduction was negative and other characteristics were shown in Table 3. Major menaquinones were MK-9($H_4$) (10.1%), MK-10($H_4$) (65.0%), MK-10($H_6$) (13.1%), and MK-10($H_8$) (1.1%). Major cellular fatty acids of isolate were iso-$C_{16:0}$ (20.1%), iso-$C_{15:0}$ (18.5%), anteiso-$C_{17:0}$ (13.2%), iso-$C_{17:0}$ (10.0%), iso-$C_{17:1\omega 9c}$ (8.3%), and $C_{17:1\omega 8c}$ (6.4%) (Table 4). The 16S rRNA gene sequence and phylogenetic tree analysis (Figure 1) showed that isolate ASC19-2-1 (1493 nt) was closely related to *M. marina* JCM 12870$^T$ (99.7%), *M. aurantiaca* JCM 10878$^T$ (99.4%), *M. chalcea* JCM 3031$^T$ (99.2%), and *M. coxensis* JCM 13248 $^T$ (99.1%). DNA-DNA relatedness between isolate ASC19-2-1 and closely related species, *M. marina* JCM 12870$^T$ was 83.3%. Therefore, isolate ASC19-2-1 was identified as *M. marina* (Wayne *et al*., 1987; Tanasupawat *et al*., 2010).

Group B contained 2 isolates, AL8-8 and AL10-3. They produced brownish black vegetative mycelium, well-developed and singly smooth spores on substrate hyphae on ISP 2 agar plate (Figure 2 and Table 2). They grew on 1.5 to 4% NaCl and weakly at 5% NaCl, at pH 6 to 8 and at 20 to 40 °C but grew weakly at 15 °C. The isolates utilized L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, glycerol, inositol, raffinose, sucrose and D-xylose. The reactions of milk peptonization, gelatin liquefaction, starch hydrolysis, nitrate reduction were positive (Table 3). The isolates, AL8-8 and AL10-3 contained major menaquinones, MK-9($H_4$) (7.5, 10.7%), MK-9($H_6$) (7.6, 16.7%), MK-9($H_8$) (2.2, 4.9%), MK-10($H_4$) (27.2, 17.6%), MK-10($H_6$) (26.4, 33.3%), and MK-10($H_8$) (15.0, 13.5%). The major cellular fatty acids of isolate AL8-8 were iso-$C_{15:0}$ (6.4%), iso-$C_{16:0}$ (42.5%), iso-$C_{17:0}$ (4.1%), anteiso-$C_{17:0}$ (11.4%), iso-$C_{17:1\omega 9c}$ (6.2%), $C_{18:1\omega 9c}$ (4.3%) and 10-methyl $C_{17:0}$ (7.0%) (Table 4).

On the basis of 16S rRNA gene sequence and phylogenetic analysis (Figure 1), isolates AL8-8 (1506 nt) and AL10-3 (1501 nt) were closely related to *M. aurantiaca* JCM 10878$^T$ (99.7%), *M. marina* JCM 12870$^T$ (99.6%), *M. coxensis* JCM 13248 $^T$ (99.4%), *M. chalcea* JCM 3031$^T$ (99.1%), and *M. halophytica* JCM 3125$^T$ (99.1%). However, the DNA-DNA relatedness between isolates AL8-8 and AL10-3 and *M. aurantiaca* JCM 10878$^T$ was 100-103%. Therefore, they were identified as *M. aurantiaca* (Sveshnikova *et al*., 1969; Wayne *et al*., 1987).

**Table 1:** Sources, location, pH, isolate number, group and identification of isolates.

| Sources | Location | pH | Isolate no. | Group | Identification |
|---|---|---|---|---|---|
| Mountain soil | Phukradung, Loei | 7.00 | AL1-15-2, AL1-16B | F | *M. chercina* |
| Mountain soil | Phukradung, Loei | 5.97 | AL3-16 | D | *M. chokoriensis* |
| Mountain soil | Phukradung, Loei | 5.01 | AL7-5 | C | *M. chalcea* |
| Mountain soil | Phukradung, Loei | 9.66 | AL8-8 | B | *M. aurantiaca* |
| Mountain soil | Phukradung, Loei | 6.49 | AL9-20<br>AL9-13, AL9-22 | D<br>E | *M. chokoriensis*<br>*M. tulbaghiae* |
| Mountain soil | Phukradung, Loei | 5.02 | AL10-3 | B | *M. aurantiaca* |
| Soil | Nok Island, Chonburi | 8.88 | ASC19-2-1 | A | *M. marina* |


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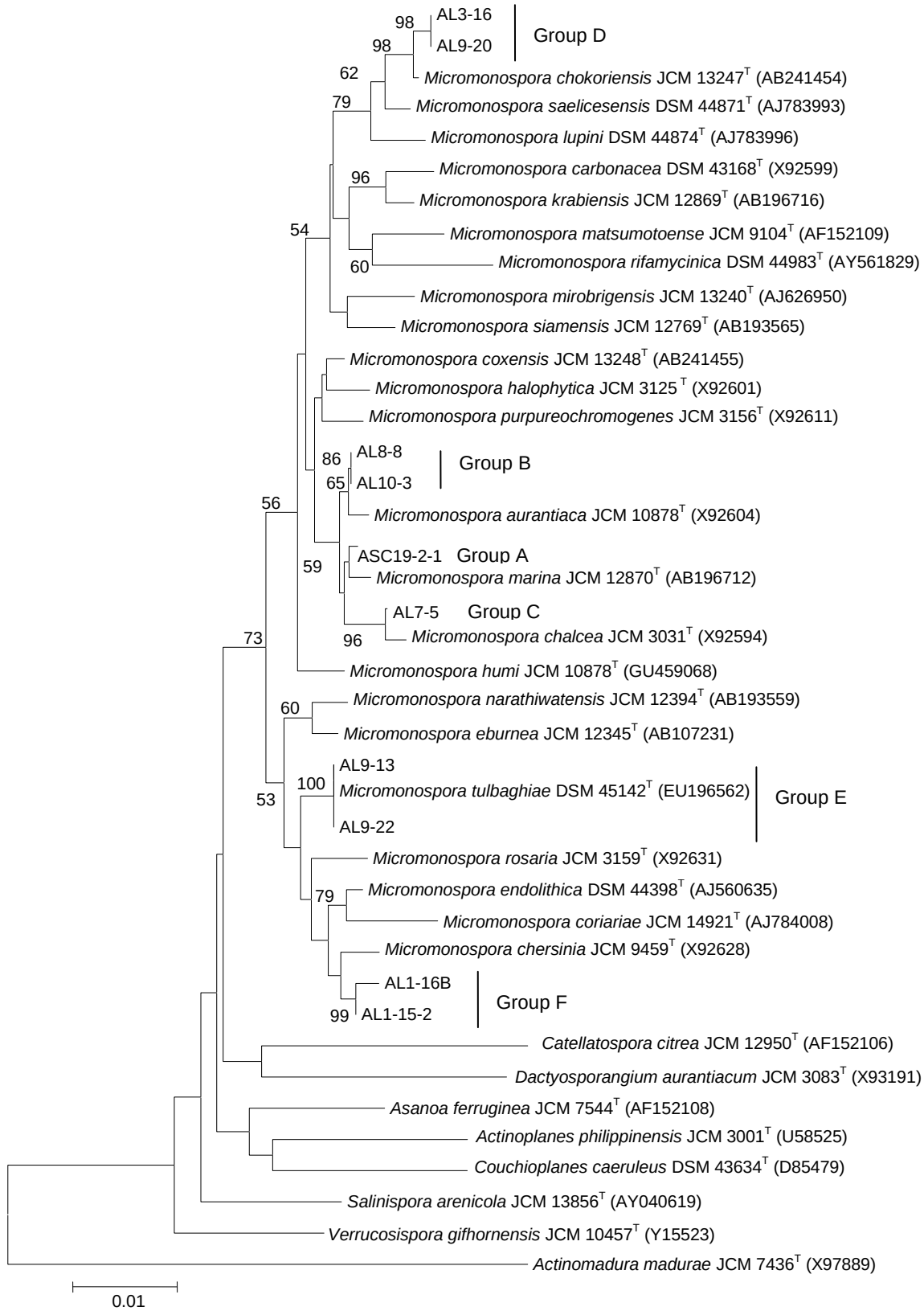


**Figure 1:** Neighbour-joining tree based on 16S rRNA gene sequences showing relationships among *Micromonospora* isolates and related *Micromonospora* species. The numbers on the branches indicate the percentage bootstrap values of 1,000 replicates; only values >50% are indicated. Bar, 0.01 substitutions per nucleotide position.


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**Table 2:** Cultural characteristics of isolates on ISP 2 at 30 °C for 14 days.

| Group/ Isolate no. | Yeast extract-malt extract agar (ISP 2) | | |
|---|---|---|---|
| | Growth | Colour of upper surface | Colour of reverse surface |
| A/ ASC19-2-1 | Abundant | Brownish gray | Brownish gray |
| B/ AL8-8 | Abundant | Brownish black | Brownish black |
| B/ AL10-3 | Abundant | Brownish black | Dark brown |
| C/ AL7-5 | Abundant | Brownish black | Brownish black |
| D/ AL3-16 | Abundant | Deep orange yellow | Strong orange yellow |
| D/ AL9-20 | Abundant | Deep orange yellow | Strong orange yellow |
| E/ AL9-13 | Abundant | Black | Black |
| E/ AL9-22 | Abundant | Black | Black |
| F/ AL1-15-2 | Abundant | Black | Black |
| F/ AL1-16B | Abundant | Black | Black |

Group C contained isolate AL7-5. The isolate produced many rough singly spores on substrate hyphae (Figure 1). The colour of the vegetative mycelium was brownish black on ISP 2 (Table 2). This isolate grew on 1.5 to 4% NaCl, on pH 6 to 8 and at 20 to 45 °C. Peptonization and coagulation of milk, gelatin liquefaction, nitrate reduction and starch hydrolysis were positive. It utilized L-arabinose, cellobiose, D-galactose, D-glucose, glycerol, lactose, D-melibiose, raffinose, sucrose, D-xylose and D-fructose (weakly) but did not utilize inositol, D-mannitol, L-rhamnose, D-ribose and salicin (Table 3). Major menaquinones were MK-9($H_4$) (19.0%), MK-9($H_6$) (6.6%), MK-10($H_4$) (42.5%), MK-10($H_6$) (17.4%) and MK-10($H_8$) (2.9%). The major cellular fatty acids of isolate were iso-$C_{15:0}$ (13.8%) iso-$C_{16:0}$ (35.6%), iso-$C_{17:0}$ (9.2%), anteiso-$C_{17:0}$(7.4%), iso-$C_{17:1\omega9c}$ (7.5%) (Table 4). On the basis of 16S rRNA gene sequence and phylogenetic analysis (Figure 1), isolate AL7-5 (1485 nt) was closely related to *M. chalcea* JCM 3031$^T$ (99.7%), *M. marina* JCM 12870$^T$ (99.3%), and *M. aurantiaca* JCM 10878$^T$ (99.1%). DNA-DNA relatedness between the strain and *M. chalcea* JCM 3031$^T$ was 92.6%. Therefore, it was identified as *M. chalcea* (Wayne *et al*., 1987; Tanasupawat *et al*., 2010).

Group D contained 2 isolates, AL3-16 and AL9-20. Isolate AL9-20 produced smooth singly spores on substrate hyphae (Figure 2). The isolates produced deep orange yellow vegetative mycelium on ISP 2 (Table 2). They grew on 1.5 to 5% NaCl, on pH 6 to 8 and at 15 to 37 °C. Gelatin liquefaction and starch hydrolysis were positive but peptonization of milk and nitrate reduction were negative. They utilized L-arabinose, D-fructose, D-glucose, lactose, D-mannitol, raffinose, sucrose and D-melibiose (weakly) but did not utilize cellobiose, D-galactose, glycerol, inositol, L-rhamnose, D-ribose, salicin and D-xylose (Table 3). The isolates AL3-16 and AL9-20 contained major menaquinones MK-9($H_4$) (8.7, 10.9%), MK-9($H_6$) (15.7, 9.7%), MK-9($H_8$) (6.2, 1.5%), MK-10($H_4$) (12.7, 30.7%), MK-10($H_6$) (33.3, 32.1%), and MK-10($H_8$) (23.4, 12.2%). The isolate AL3-16 contained iso-$C_{15:0}$ (30.5%), anteiso-$C_{15:0}$ (7.3%), anteiso-$C_{17:0}$(10.9%), and iso-$C_{17:1\omega8c}$ (9.9%) as major cellular fatty acids (Table 4). Phylogenetic analysis using 16S rRNA gene sequences revealed that strains AL3-16 (1496 nt) and AL9-20 (1482 nt) were closely related to *M. chokoriensis* JCM 13247$^T$ (99.8%) and *M. saelicesensis* JCM 44871$^T$ (99.3%) (Figure 1). On the basis of phenotypic characteristics and 16S rRNA gene sequence similarity, these isolates were identified as *M. chokoriensis* (Ara and Kudo, 2007).

Group E contained 2 isolates, AL9-13 and AL9-22. Isolate AL9-13 produced rough singly spores on substrate hyphae (Figure 2). The isolates produced black vegetative mycelium on ISP 2 (Table 2). They grew on 1.5 to 4% NaCl, at pH 5 to 8 and at 15 to 45 °C. Milk peptonization, coagulation, gelatin liquefaction, nitrate reduction, and

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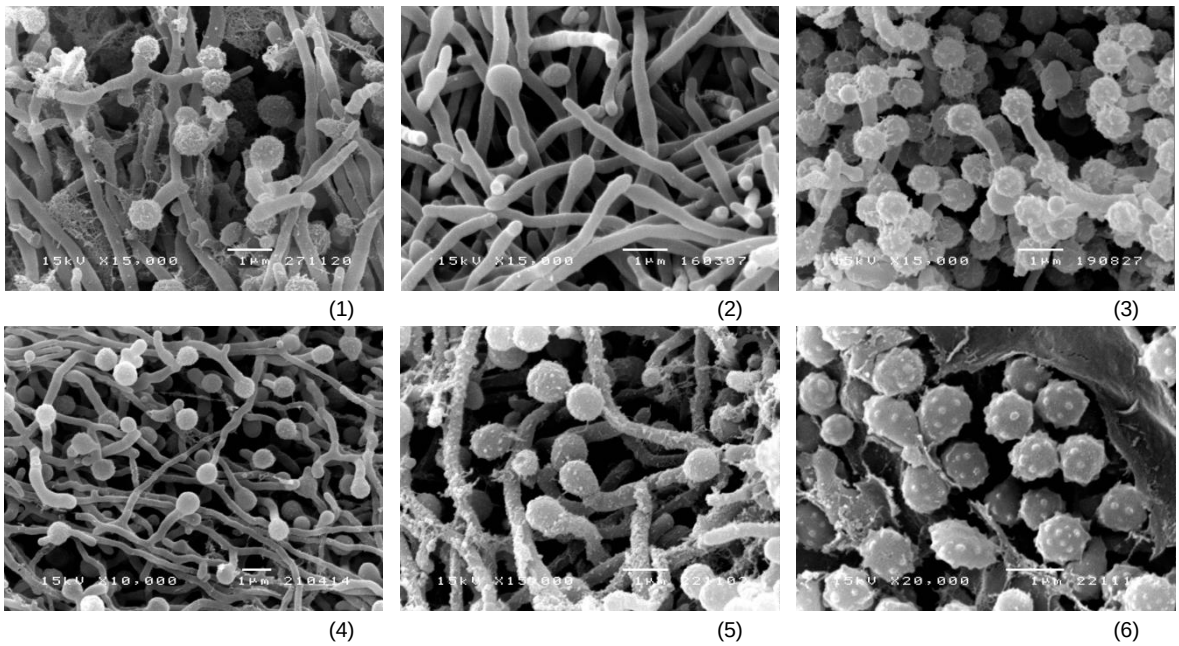


**Figure 2:** Scanning electron micrograph of *Micromonospora* isolates; 1, ASC19-2-1 (Group A); 2, AL10-3 (Group B); 3, AL7-5 (Group C); 4, AL9-20 (Group D); 5, AL9-13 (Group E) and 6, AL1-15-2 (Group F) on ISP 2 agar medium incubated at 30 °C for 14 days. Bar, 1 μm.

starch hydrolysis were positive. They utilized L-arabinose, cellobiose, D-fructose, D-glucose, salicin, sucrose and D-xylose and did weakly for D-galactose, glycerol and L-rhamnose (Table 3). The isolates AL9-13 and AL9-22 contained major menaquinones, MK-9($H_4$) (8.0, 11.8%), MK-10($H_4$) (48.2, 72.7%), and MK-10($H_6$) (25.0, 7.4%). Major cellular fatty acids of AL9-22 were iso-$C_{16:0}$ (14.9%), iso-$C_{15:0}$ (20.7%), anteiso-$C_{17:0}$ (12.1%), iso-$C_{17:0}$ (10.7%) and $C_{17:1\omega8c}$ (7.1%) (Table 4). Phylogenetic analysis using 16S rRNA gene sequences revealed that isolates AL9-13 (1460 nt) and AL9-20 (1482 nt) showed 100% similarity with *M. tulbaghiae* DSM 45142$^T$ (Figure 2). Therefore, they were identified as *M. tulbaghiae* (Kirby and Meyers, 2010).

Group F contained 2 isolates, AL1-15-2 and AL1-16B. Isolate AL1-15-2 produced warty spores on the substrate hyphae (Figure 2) and the vegetative mycelium color was black (Table 2). They grew on 1.5 to 3% NaCl, on pH 5 to 8 and at 20 to 37 °C. Milk peptonization, nitrate reduction and starch hydrolysis were negative but gelatin liquefaction was positive (Table 2). They utilized L-arabinose, cellobiose, D-galactose, D-glucose, raffinose, salicin, sucrose and D-xylose as sole carbon sources but not D-fructose, inositol, lactose, D-mannitol, D-melibiose and D-ribose (Table 3). The isolates AL1-15-2 and AL1-16B contained major menaquinones, MK-9($H_4$) (6.6, 14.2%), MK-9($H_6$) (22.2, 30.6%), MK-9($H_8$) (5.0, 14.5%), MK-10($H_4$) (9.4, 6.8%), MK-10($H_6$) (40.3, 20.2%) and MK-10($H_8$) (14.6, 13.6%). The major cellular fatty acids of AL1-15-2 were iso-$C_{16:0}$ (13.9%), iso-$C_{15:0}$ (28.3%), anteiso-$C_{17:0}$ (6.5%), iso-$C_{17:0}$ (13.0%), $C_{17:0}$ (6.3%), $C_{18:0}$ (5.1%), iso-$C_{17:1\omega9c}$ (7.2%), and $C_{17:1\omega8c}$ (5.2%) (Table 4).

Phylogenetic analysis using 16S rRNA gene sequences revealed that strains AL1-15-2 (1479 nt) and AL1-16B (1505 nt) were closely related to *M. chersina* JCM 9459$^T$ (99.5, 99.4%), *M. rosaria* JCM 3159$^T$ (99.3, 99.2%) and *M. endolithica* JCM 12677$^T$ (99.1, 99.1%) (Figure 1). The DNA G+C content of isolates, AL1-15-2 and AL1-16B were 72.1 and 72.0 mol%, respectively. The levels of DNA-DNA relatedness between strain AL1-15-2 and AL1-16B and *M. chersina* JCM 9459$^T$, the closest related species were 87.8 and 78.4%, respectively. Therefore, isolates AL1-15-2 and AL1-16B were identified as *M. chersina* (Wayne *et al*., 1987; Tomita *et al*., 1992).

**Screening of antimicrobial activities and preliminary characterization of secondary metabolites**

The results of primary screening on agar plates revealed that the nine isolates showed no antimicrobial activities against the test microorganisms mentioned above except the isolate AL7-5 that could inhibit *Kocuria rhizophila* ATCC 9341. The representative isolates of each group, ASC19-2-1, AL10-3, AL7-5, AL9-20, AL9-13 and AL1-15-2 were further cultivated in A3M, A11M and A16 broth. The fermentation broth was extracted for preliminary characterization of secondary metabolites. We found that only isolate AL7-5 which was identified as *M. chalcea* could produce rakicidin (McBrien *et al*., 1995) (Figure 3a) when cultivated on A3M, A11M and A16 and produced compound BU4664L (Figure 3b) only on A16 (Igarashi *et al*., 2005).

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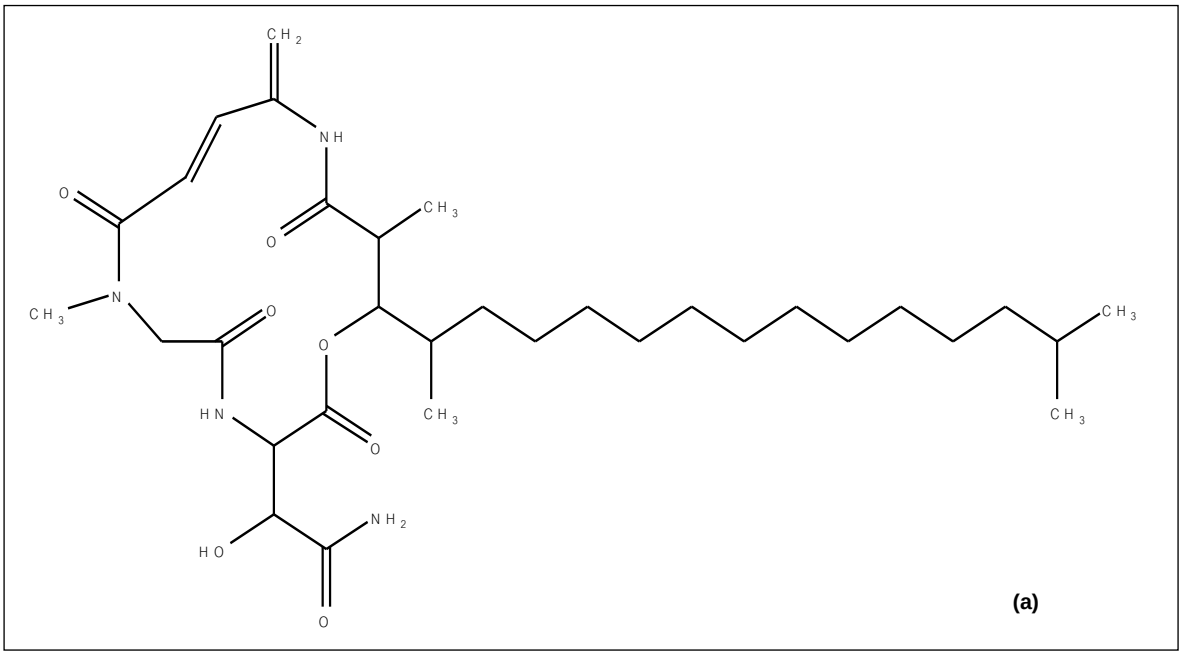


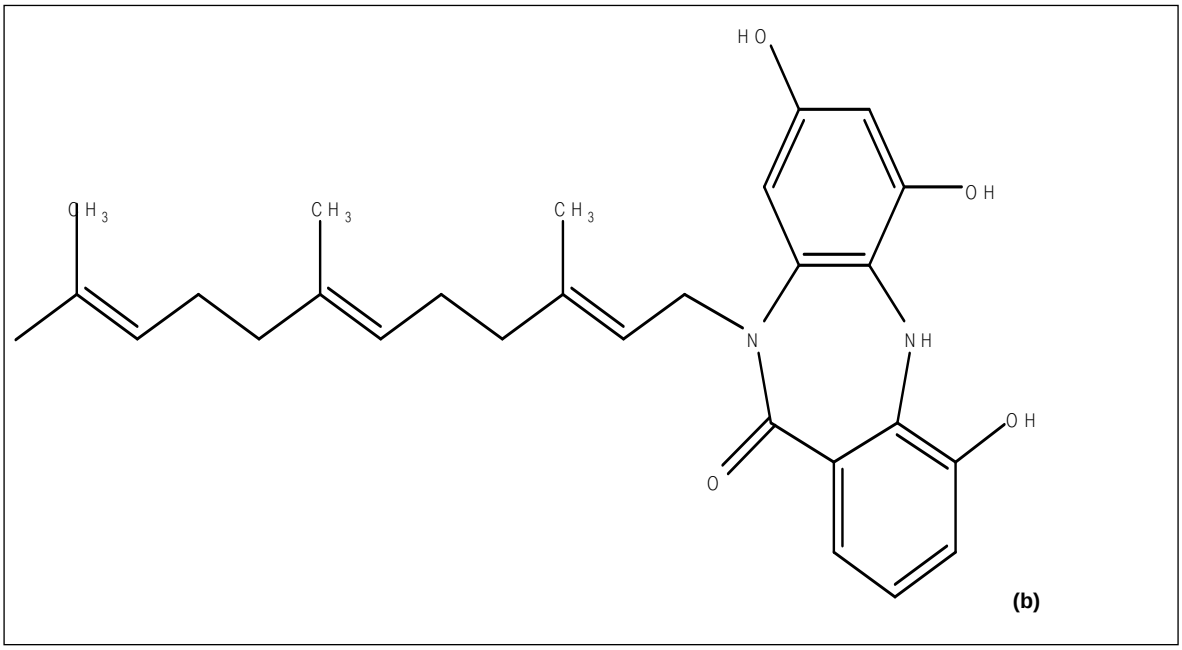


**Figure 3:** The structure of rakicidin (a) and BU4664L (b).


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**Table 3:** Differential characteristics of isolates in Group A, B, C, D, E, F and closely related *Micromonospora* species.

| Characteristics | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10[a] | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Max. NaCl (%,w/v) | 5 | 5 | 5 | 3 | 4 | 3 | 5 | 5 | 4 | 5 | 3 | 4 |
| Growth at pH 5 | + | + | + | + | - | - | - | - | + | - | + | - |
| Growth at 40 °C | + | + | + | + | + | + | - | - | + | - | - | + |
| Growth at 45 °C | - | + | - | + | + | - | - | - | + | - | - | + |
| Peptonization of milk | + | - | + | + | + | - | - | - | + | nd | - | + |
| Gelatin liquefaction | + | + | + | + | + | + | + | + | + | + | + | + |
| Nitrate reduction | - | - | + | + | + | - | - | - | + | - | - | + |
| Starch hydrolysis | + | + | + | + | + | + | + | + | + | + | - | + |
| Utilization of: | | | | | | | | | | | | |
| L-Arabinose | + | - | + | + | + | w | + | + | + | - | + | - |
| Cellobiose | + | + | + | + | + | + | - | - | + | - | + | + |
| D-Fructose | w | - | + | + | w | - | + | + | + | + | - | w |
| D-Galactose | + | + | + | + | + | + | - | - | w | nd | + | + |
| D-Glucose | + | + | + | + | + | + | + | + | + | + | + | + |
| Glycerol | + | + | w(+1) | + | + | + | - | - | w | - | +(-1) | - |
| Inositol | - | - | + | - | - | - | - | - | - | + | - | - |
| Lactose | w | - | +(-1) | w | + | + | + | + | - | - | - | + |
| D-Mannitol | - | - | - | - | - | w | + | + | - | + | - | - |
| D-Melibiose | - | - | - | - | + | + | w | w | - | - | - | + |
| Raffinose | - | - | + | - | + | + | + | + | - | - | + | + |
| L-Rhamnose | - | - | - | - | - | - | - | w | w | + | +(-1) | - |
| D-Ribose | - | - | - | - | - | - | - | - | - | + | - | - |
| Salicin | + | + | w(-1) | + | - | + | - | - | + | - | + | - |
| Sucrose | w | - | + | - | + | - | + | + | + | nd | + | + |
| D-Xylose | w | - | + | - | + | - | - | - | + | - | + | nd |

1, Group A (1 isolate); 2, *M. marina* JCM 12870$^T$; 3, Group B (2 isolates); 4, *M. aurantiaca* JCM 10878$^T$; 5, Group C; 6, *M. chalcea* JCM 3031$^T$; 7, Group D (2 isolates); 8, *M. chokoriensis* JCM 13247$^T$; 9, Group E (2 isolates); 10, *M. tulbaghiae* DSM 45142$^T$; 11, Group F (2 isolates); 12, *M. chersina* JCM 9459$^T$. nd, no data. Number in parentheses indicates the number of isolate shows positive or negative reaction. [a]Data from Kirby and Meyers (2010).


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**Table 4:** Cellular fatty acid profiles of isolates.

| Fatty acid[a] | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Saturated fatty acids | | | | | | |
| $C_{15:0}$ | tr | tr | tr | 1.7 | 0.6 | 0.5 |
| $C_{16:0}$ | 0.8 | tr | 0.9 | 3.2 | 0.7 | 3.5 |
| $C_{17:0}$ | 2.8 | 1.1 | 1.1 | 7.8 | 4.7 | 6.3 |
| $C_{18:0}$ | 1.7 | 1.3 | 3.0 | 2.0 | 1.3 | 5.1 |
| Unsaturated fatty acids | | | | | | |
| $C_{17:1}\omega$8c | 6.4 | 2.6 | 2.3 | 7.9 | 7.1 | 5.2 |
| $C_{18:1}\omega$9c | 3.4 | 4.3 | 5.4 | 4.2 | 3.2 | 1.0 |
| Branched fatty acids | | | | | | |
| iso-$C_{14:0}$ | tr | 0.9 | 0.6 | tr | tr | 0.7 |
| iso-$C_{15:0}$ | 18.5 | 6.4 | 13.8 | 30.5 | 20.7 | 28.3 |
| anteiso-$C_{15:0}$ | 6.1 | 3.0 | 2.6 | 5.4 | nd | 3.6 |
| iso-$C_{16:1}$ | 0.7 | 1.2 | 0.6 | tr | tr | 0.6 |
| iso-$C_{16:0}$ | 20.1 | 42.5 | 35.6 | 4.0 | 14.9 | 13.9 |
| iso-$C_{17:1}$ $\omega$9c | 8.3 | 6.2 | 7.5 | 4.4 | 6.8 | 7.2 |
| anteiso-$C_{17:1}$ $\omega$9c | 1.1 | 1.1 | tr | 0.6 | 0.6 | 0.8 |
| iso-$C_{17:0}$ | 10.0 | 4.1 | 9.2 | 6.1 | 10.7 | 13.0 |
| iso-$C_{18:0}$ | 0.9 | 3.4 | 2.8 | nd | 0.7 | 0.5 |
| anteiso-$C_{17:0}$ | 13.2 | 11.4 | 7.4 | 8.2 | 12.1 | 6.5 |
| 10-Methylated fatty acids | | | | | | |
| 10-Methyl $C_{17:0}$ | 1.8 | 7.0 | 2.6 | 7.3 | 4.3 | 1.1 |
| 10-Methyl $C_{18:0}$ | 0.5 | 0.8 | 1.6 | 3.0 | 1.5 | tr |
| Summed feature 3[b] | 0.9 | tr | 0.7 | tr | tr | tr |
| Summed feature 6[c] | 0.5 | tr | tr | 0.7 | 0.9 | tr |

1, ASC19-2-1; 2, AL8-8 ; 3, AL7-5 ; 4, AL3-16; 5, AL9-22 ; 6, AL1-15-2. nd, not detected. [a]Values are percentages of total fatty acids. [b]Summed feature 3 contains $C_{16:1}$ $\omega$7c/ $C_{15:0}$ iso 2-OH. [c]Summed feature 6 contains $C_{19:1}$ $\omega$11c/ $C_{19:1}\omega$9c. tr, trace less than 0.5%.

## CONCLUSION

Nine rare actinomycete strains from the genus *Micromonospora* were isolated from mountain soils in Phukradung, Loei province, the north eastern part of Thailand and they were identified as *M. aurantiaca*, *M. chalcea*, *M. chokoriensis* and *M. chersina*. One isolate from Nok Island, Chonburi province, the eastern part of Thailand was identified as *M. marina*. They were identified based on morphological, cultural, physiological and biochemical characteristics including chemotaxonomic characteristics, DNA-DNA relatedness and 16S rRNA gene analyses. Only *M. chalcea* AL7-5 could produce rakicidin and compound BU4664L and showed antibacterial activity against *K. rhizophila* ATCC 9341.

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