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An Improved Selective Isolation of Rare Actinomycetes from Forest Soil

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Various pretreatment procedures and selective media were applied to assess the optimal conditions for the isolation of rare actinomycetes from soil. Pretreatment of wet-heating for 15 min at 70°C and phenol treatment of soil suspension were the most effective methods for the isolation of those microorganisms. Hair hydrolysate vitamin agar (HHVA) was the most suitable medium for the recovery of rare actinomycetes. Thirty-five rare actinomycete strains were chosen using selective isolation approaches, then morphological and chemical properties of the isolates were determined. The isolates belonged to one of the following genus, *Micromonospora, Microbispora, Actinoplanes* and *Streptosporangium.*

***Key words*:** Pretreatment, rare actinomycetes, hair hydrolysate vitamin agar, *Micromonospora, Microbispora*

Actinomycetes are widely distributed in natural and man-made environments, and play an important role in the degradation of organic matter. They are also well known as a rich source of antibiotics and bioactive molecules, and are of considerable importance in industry. When conventional isolation techniques were applied, most of the isolates recovered on agar plates have been identified as genus *Streptomyces*, which are the dominant actinomycetes in soil (7, 12, 18). For the purpose of screening novel bioactive molecules, several factors must be considered: choice of screening source, pretreatment, selective medium, culture condition, and recognition of candidate colonies on a primary isolation plate (18).

The role of rare actinomycetes as bioactive molecule sources became apparent as these organisms provided about 25% of the antibiotics of actinomycete origin reported during 1975 to 1980 (17). Rare actinomycetes have usually been regarded as strains of actinomycetes whose isolation frequency by conventional methods is much lower than that of streptomycete strains. Consequently basic knowledge of the habitat, physiology and productivity of molecules of rare actinomycetes gradually increased. Ecologically significant properties of actinomycetes were discovered, which made the screening source expand into uncommon environments.

The aerial spores of most actinomycete genera were found to resist desiccation and show a slightly higher resistance to wet or dry heat than the corresponding vegetative hyphae. Subsequently, employing pretreatments of soil by drying and heating stimulated the isolation of rare actinomycetes (9, 18). An alternative approach was to make the isolation procedure more selective by adding chemicals such as phenol to the soil suspension (6, 19). Many actinomycetes have shown multiple resistance to wide ranges of antibiotics. Several antibiotic molecules were used in selective medium to inhibit the competing bacteria including fast-growing actinomycetes (21). Specialized growth media were developed to isolate specific actinomycete genera. Macromolecules such as casein, chitin, hair hydrolysate, and humic acid were chosen as carbon and nitrogen sources of rare actinomycetes (1, 4, 5).

Diagnosis of isolates on a primary isolation plate and recognition as a novel taxa was very important both for practical and taxonomical purposes. Until recently, bacterial systematics was based on the morphological and behavioral properties of microorganisms. Chemical information can be used at all taxonomic levels and it is likely that chemical properties will become an important part of minimal descriptions of many genera and species of actinomycetes (14, 15, 20).

In this study, we estimated the efficiency of pretreatment methods and selective isolation medium for rare actinomycete genera such as *Actinoplanes, Micromonospora, Microbispora,* and *Streptosporangium*. The isolates were characterized with chemical techniques such as fatty acid profile, major menaquinone composition, cell wall diaminopimelic acid pattern, and whole cell sugars pattern.

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Materials and Methods

Sampling and pretreatment of soil

Soil samples were collected from the humus layer of forest soil on Mt. Baekun, Chonnam, Korea. Four different pretreatment methods as described in Table 1 were carried out in the first 24 h after sampling.

Selective isolation of rare actinomycetes

Serially diluted soil suspensions were spread onto selective isolation medium, and incubated for 4 weeks at 25°C. Starch casein nitrate agar (SCA), humic acid vitamin agar (HVA), hair hydrolysate vitamin agar (HHVA), and Bennet's agar (BA) were used for the selective isolation of rare actinomycetes (1, 4, 5, 23). Preliminary designation of rare actinomycete colonies were done by microscopic observation with a long working distance microscope (Labophot; Nikon). The criteria for classification between streptomycete and rare actinomycete strains were taken from previous works (6, 10, 18, 22). Single colonies were successively transferred onto glucose yeast extract (GYE) agar and incubated until pure isolates were obtained. Spore mass and mycelium fragments of the pure isolates were stored at -30°C as glycerol (20%, v/v) suspension.

Morphology

Production of spore mass and its color, substrate mycelium color and production of diffusible pigment were detected on the 21-day old cultures on oatmeal agar (19). Spore ornamentation was observed by a scanning electron microscope.

Amino acid analysis

Chemical composition of the isolates were determined. Biomasses were obtained from liquid cultures in GYE broth medium at 28°C for 7 days, freeze dried, and kept refrigerated for further analysis. Diaminopimelic acid isomers were determined as described in Seong *et al.* (24). Acid hydrolysate of the biomass was applied on TLC plates and developed for 4 h in a solvent system containing methanol-water-10 N HCl-pyridine (80 : 26.25 : 3.75 : 10, v/v). Dry plates were sprayed with a solution of ninhydrine in acetone (0.2%, w/v).

Table 1. Recovery of microorganisms (CFU/g dry soil) on hair hydrolysate vitamin agar (HHVA) from soil

Pretreatment	Rare actinomycetes	*Streptomyces*	Other bacteria	Fungi
Control	3.2×10^4 (2.7)[b]	2.8×10^5 (23.7)	6.4×10^5 (54.1)	2.3×10^5 (19.5)
Control+Antibiotics[a]	2.6×10^4 (4.2)	2.1×10^5 (34.3)	3.4×10^5 (55.5)	3.7×10^4 (6.0)
Dry heat (100°C), 1 h	2.3×10^2 (2.5)	2.4×10^3 (25.9)	6.4×10^3 (69.0)	2.4×10^2 (2.6)
Air dry, 24 h	4.4×10^4 (2.5)	1.1×10^6 (64.3)	5.4×10^5 (31.5)	2.9×10^4 (1.7)
Wet heat (70°C), 15 min	1.3×10^5 (56.0)	1.8×10^4 (7.8)	8.4×10^4 (36.2)	-
1.5% phenol	1.6×10^5 (64.8)	6.5×10^4 (26.3)	2.2×10^4 (8.9)	-

[a]Nystatin (50 μg/ml) + Nalidixic acid (20 μg/ml).
[b]Percentage.

Fatty acid analysis

Extraction of fatty acids as their methyl esters were performed by alkaline methanolysis (11). Fatty acid methyl esters (FAMEs) were separated in a HP-1 capillary column (0.53 mm I.D., 30 m length, 2.65 μm film) in a HP 5809A gas chromatograph (Hewlett Packard) equipped with a flame ionization detector. The temperature was programmed to hold at 170°C for 1 min, then to rise by 5°C/min. Injector temperature was held at 250°C, and the detector at 300°C.

Whole cell sugar analysis

Whole cell sugars were extracted as alditol acetates (2), and were analyzed using a gas chromatograph (Hewlett Packard 5809A) fitted with a flame ionization detector. Separation was achieved using a 0.53 mm × 30 m SP 2380 (Supelco) fused silica capillary column. The temperature was programmed to hold at 160°C for 2 min, then to rise by 5°C/min. Injector temperature was held at 250°C, and the detector at 300°C.

Quinone analysis

For the extraction of menaquinone, 50 mg of dried biomass was treated with chloroform/methanol (2:1, v/v) by shaking overnight (11). The extracts were concentrated *in vacuo* using an electric aspirator, and cell debris was removed by centrifugation. The solvents were evaporated completely, and the remnants were resuspended in hexane. High performance liquid chromatographic separation of the quinones was done with an ODS Hypersil column (200 × 4.6 mm, particle size 5 μm, Hewlett Packard) and acetonitrile/tetrahydrofuran (70:30, v/v) as the mobile phase. The flow rate was 1 ml/min at 37°C, and the detector was a UV detector operated at 254 nm.

Results and Discussion

Isolation medium for rare actinomycetes

The dominance of other bacteria and fungal contamination inhibited the colonization of actinomycetes on isolation medium. When antifungal agents such as nystatin (50 μg/ml) and nalidixic acid (20 μg/ml) were supplemented into the isolation medium, the number of fungi decreased. Thus, the isolation medium was supplemented with those antibiotics in succeeding experiments.

BA and SCA supported the growth of actinomycetes

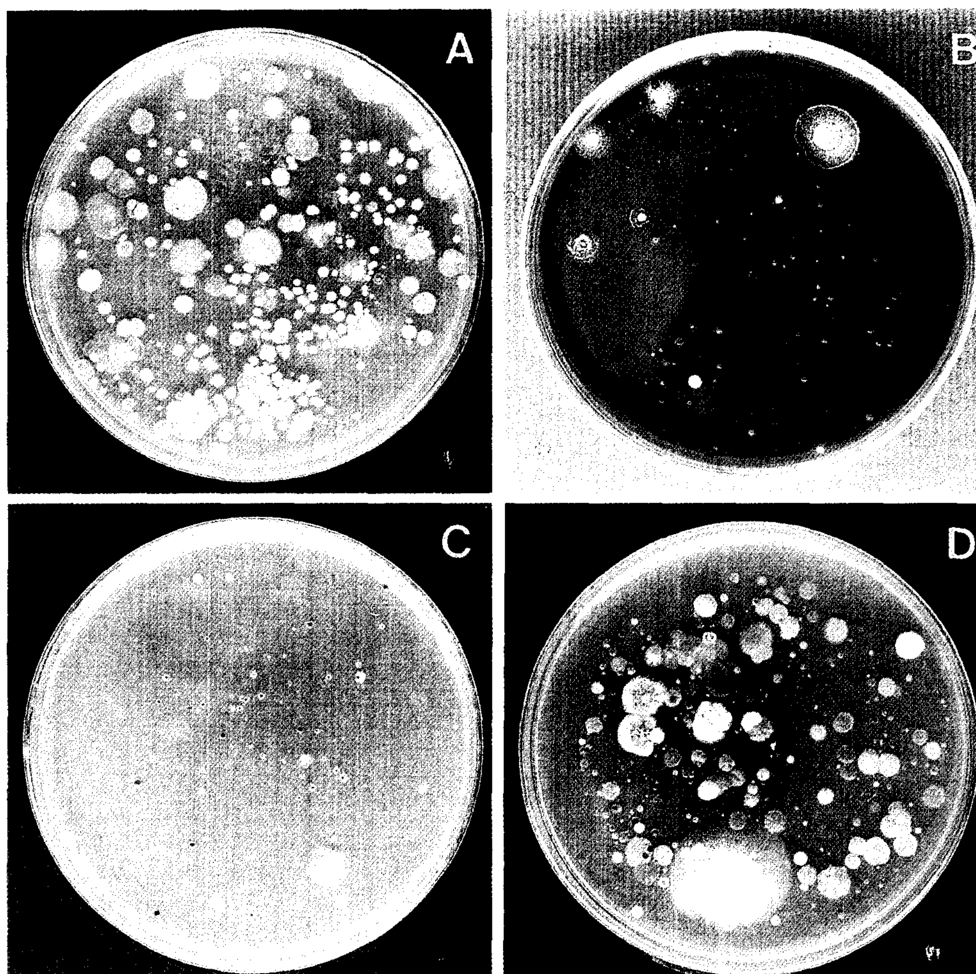

Fig. 1. Selective isolation of rare actinomycetes on starch casein nitrate agar (A), humic acid vitamin agar (B), hair hydrolysate vitamin agar (C), and Bennet's agar (D) with the pretreatment with 1.5% phenol.

including *Streptomyces* as well as fungi and yeast. The brown color of HVA made it difficult to discriminate the morphology of colonies. However, the number of *Streptomyces*, other bacteria and fungi decreased, allowing rare actinomycete colonies to dominate on HVA. Rare actinomycetes as well as *Streptomyces* grew well on HHVA. Although the growth rate of actinomycetes is low, discrimination of typical morphology of colonies was easy on HHVA. Thus, for the isolation of rare actinomycetes, HHVA is recommended (Fig. 1).

Effect of pretreatment of soil

When the forest soils were cultured without pretreatment, the number of colonies recovered was in the order of other bacteria, *Streptomyces*, fungi and non-streptomycete actinomycetes. When the soil was air-dried, other bacterial numbers decreased, and streptomycete colonies increased. All kinds of microorganisms including rare actinomycetes decreased when the soil was dried at 100°C for 1 h. Heating the soil suspension at 70°C for 15 min inhibited the fungal and bacterial colonies, thus the recovery of actinomycetes, specifically, rare actinomycetes, increased up to 50% of the total microorganisms. Phenol treatment of soil suspension lowered the number of fungi and other bacteria, but the actinomycetes were less affected, thus 65% of the colonies belonged to rare actinomycetes (Fig. 2; Table 1). This effect was also suggested in previous works (4, 6, 10).

Rare actinomycetes were preliminarily selected from HHVA by morphological examination. Categorization of the colonies as non-streptomycetes was done by naked eye observation and microscope, and the criteria for classification between streptomycete or non-streptomycete strains were derived from previous works (6, 10, 18, 22). From this preliminary selection, 39 strains were isolated and pure cultured. Morphological and chemical properties of the isolates were determined.

Morphology

Only two strains did not produce aerial spore masses on

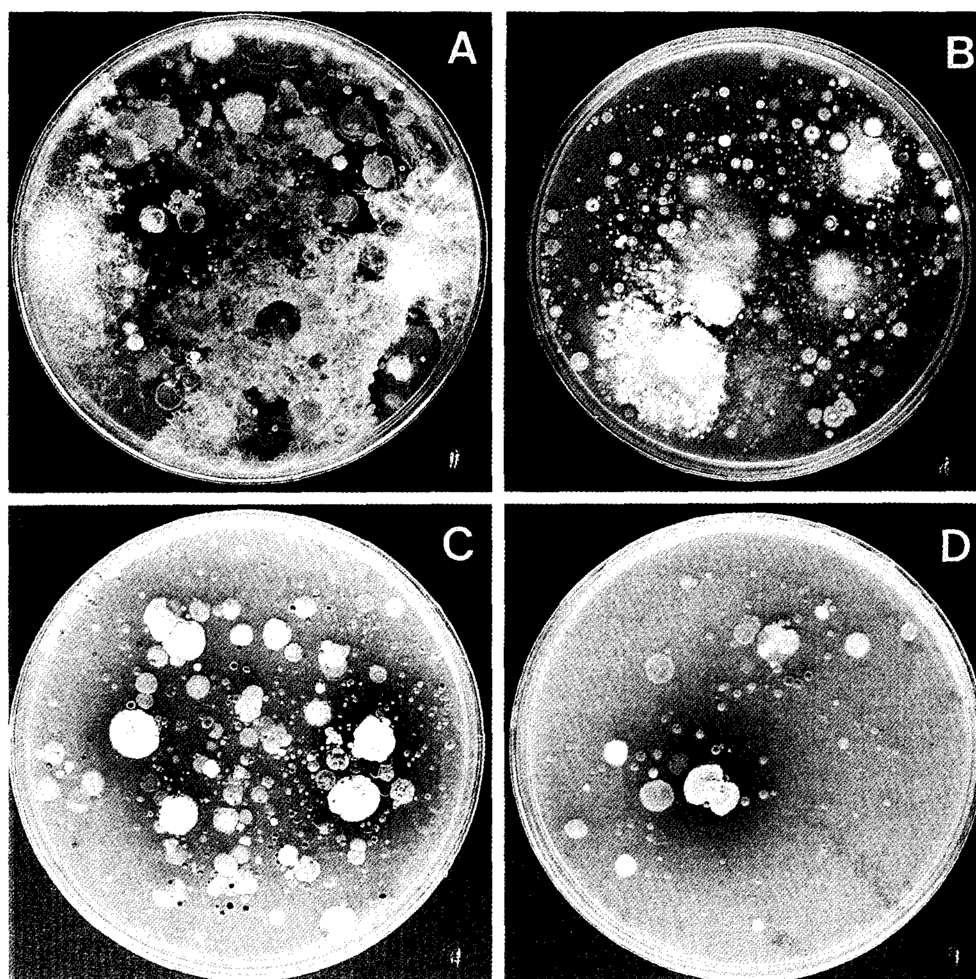

Fig. 2. Photographs showing isolation plate. A, control (10^{-3} dilution); B, air dried for 24 h and antibiotics (10^{-3} dilution); C, heat treatment (70°C, 15 min, wet) and antibiotics (10^{-2} dilution); D, 1.5% phenol and antibiotics (10^{-3} dilution).

Table 2. Identification of isolates with morphological and chemical properties

Group/genus	Wall amino acid	Whole-cell sugar pattern	Fatty acid pattern	Major menaquinone (MK)	Morphology		No. of strains
					Substrate mycelium color	Diffusible pigment	
Actinoplanes	*meso*-DAP, glycine	Ara, Xyl/Xyl	i-C16:0	-9(H_4)/ -9(H_4, H_6)	orange/yellow	-	6
Dactylosporangium	*meso*-DAP, glycine	Ara, Xyl, Rhm	a-C15:0 i-C16:0	-9(H_6, H_8)	green	green	1
Micromonospora	*meso*-DAP, glycine	Ara, Xyl/ Ara, Xyl, Rhm	i-C15:0 i-C16:0	-9(H_4)/ -10(H_4, H_6)	brown/yellow	-	9
Microbispora	*meso*-DAP	Ara, Gal, Mad	i-C16:0, 10-Me-C17:0	-9(H_0, H_2, H_4)/ -9(H_4)	pink/orange	-	6
Streptosporangium	*meso*-DAP	Ara, Gal, Mad	i-C14:0 i-C16:0 i-C17:1	-9(H_0, H_2, H_4)	white	-	5

[1]Ara, Arabinose; Xyl, Xylose; Rhm, Rhamnose; Gal, Galactose; Mad, Madurose.
[2]i-C16:0, 14-methylpentadecanoic acid; i-C15:0, 13-methyltetradecanoic acid; a-C15:0, 12-methyltetradecanoic acid; 10-Me-C17:0, 10-methylhexadecenoic acid; i-C14:0, 12-methyltriadecanoic acid; and i-C17:1, *cis*, 15-methylhexadecenoic acid.

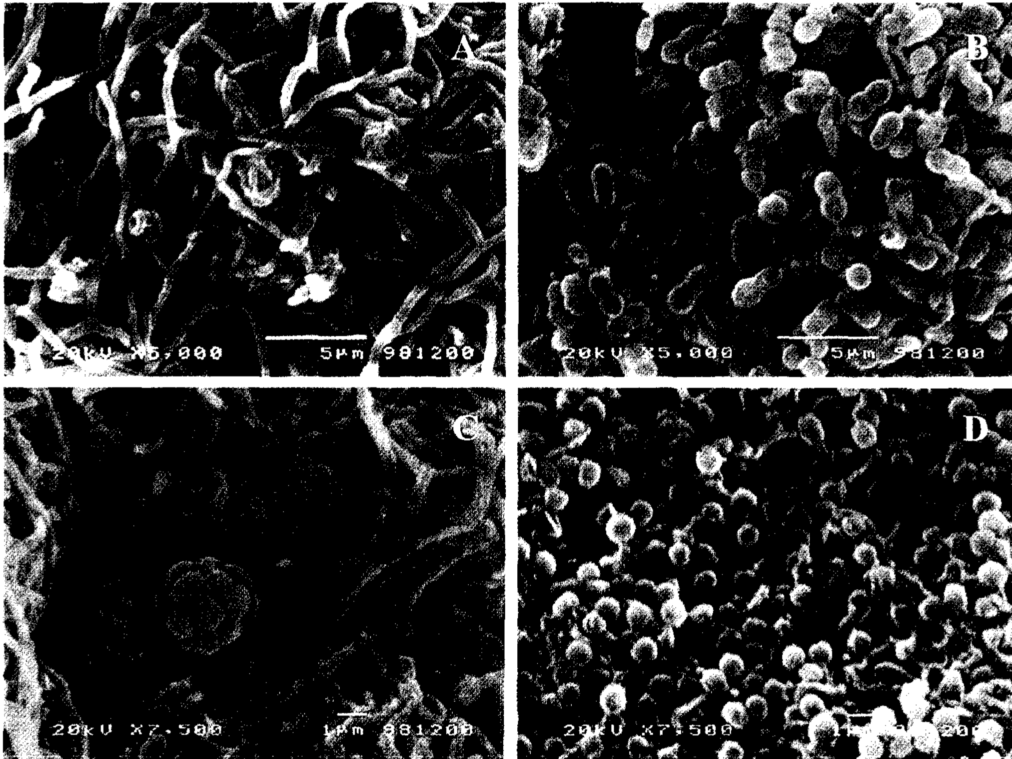

Fig. 3. Scanning electronmicrograph of spore ornamentation of representative isolates. A, *Streptosporangium* sp. (strain AO01); B, *Microbispora* sp. (strain DO05); C, *Actinoplanes* sp. (strain CN02); D, *Micromonospora* sp. (strain AM05).

oatmeal agar. Twenty-three strains produced the specific color of substrate mycelium. Only four strains produced diffusible pigments. Strain BO07 produced green pigment on oatmeal agar. Distinguished white color of mycelium was found in five strains including strain AO01. Scanning electron microscopic photographs of the spore ornamentation of the representative strains are shown in Fig. 3. Strain AO01 produced branched mycelium and globose sporangia on aerial mycelium. Sporangiospores are formed by septation of hypha within sporangium (Fig. 3A). Strain DO05 produced branched mycelium and spores in characteristic longitudinal pairs on aerial mycelium (Fig. 3B). Strain CN02 produced nonfragmenting branched mycelium. Subspherical spores are produced within spherical sporangia (Fig. 3C). Strain AM05 had scanty aerial mycelium and its spores are borne singly (Fig. 3D).

Chemical properties

Based on partial sequencing of 16S rRNA, some actinomycete groups such as actinoplanetes, maduromycetes and streptomycetes have represented distinct suprageneric groups. Chemical markers have been shown to be especially good indicators of these groups (3). A combination of chemical and morphological features was used for the recognition of rare actinomycetes.

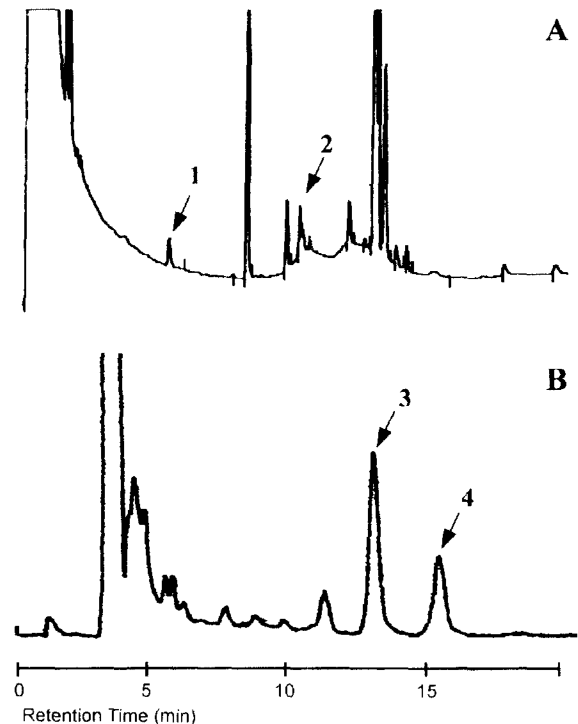

Fig. 4. Chromatogram of sugar (A) and menaquinone (B) of strain BO07 which is identified as *Dactylosporangium* sp. Arrows indicate rhamnose (1), xylose (2), MK-9 (H_6) (3), and MK-9 (H_8) (4).

Among 39 isolates which were defined as non-streptomycete actinomycetes with preliminary selection, 35 strains had *meso*-DAP, and it was found that preliminary selection on agar plate show 90% of efficiency for the discrimination as rare actinomycetes. Aspartic acid, lysine, and glycine were the main amino acids of 35 strains.

Whole cell sugar patterns of actinomycetes containing *meso*-DAP were defined on the basis of previous work (14). Twenty strains had xylose either alone or with arabinose or rhamnose, which belongs to pattern D (Fig. 4; Table 2). Eleven strains had madurose, and their sugar pattern belongs to B. Four strains containing L-DAP had no characteristic sugars (pattern C).

Whole organism methanolysates of the isolates contained fatty acids having 15 to 18 carbon chains, which are commonly found in prokaryotes (8). While *Streptomyces* had saturated *iso*-fatty acid of 15, 16 and 17 carbon numbers, and *antiiso*-fatty acids of 15 and 17 carbon numbers, the isolates had n-heaxadecanoic acid (n-C16:0) as well as 14-methylpentadecanoic acid (i-C16:0), 13-methyltetradecanoic acid (i-C15:0) and 12-methyltetradecanoic acid (a-C15:0) as the major species (Table 2). Also, *cis*-15-methylhexadecenoic acid isomers (i-C17:1), 14-methylhexadecanoic acid (a-C17:0), and 10-methyl hexadecanoic acid (10-Me-C17:0) were the attendant fatty acids of the strains (Fig. 5). Branched fatty acids were dominant in all cases, constituting 63.6% of the total fatty acids on average, and linear ones, 15.1%. The unsaturated fatty acids, mostly hexadecenoic acid isomers (C16:1) and heptadecenoic acid isomers (C17:1), accounted for 16.3% of the total.

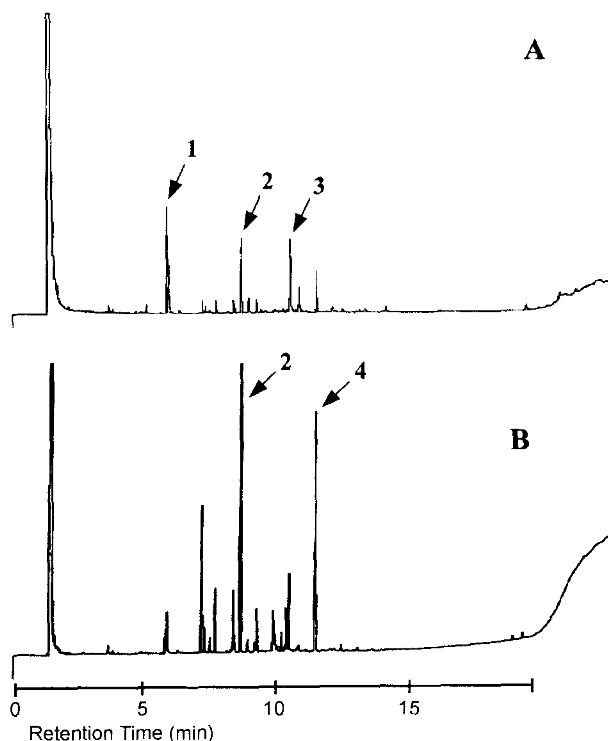

Fig. 5. Chromatogram of fatty acid of strain AO01 (A) and strain DO05 (B). Arrows indicate the i-C14:0 (1), i-C16:0 (2), i-C17:1 (3), and 10-Me-C17:0 (4).

Most of the test strains had menaquinones of 9 isoprene units, designated as MK-9 with 1 to 4 double bonds saturated, MK-9(H_2)~MK-9(H_8). MK-9(H_4) or MK-9(H_6) were dominant in all cases. Two strains had MK-10(H_4) and MK-10(H_6) (Fig. 4).

Identification of isolates

Identification of the isolates was carried out using morphological and chemical properties. Discrimination between actinoplanetes and maduromycetes was primarily done by sugar pattern (13). Diagnostic sugar of the latter is madurose, while the former is xylose (Table 2).

Main menaquinone profile was the useful criteria in actinoplanetes. *Dactylosporangium* whose manaquinone is MK-9(H_6) and MK-9(H_8) was distinguished from *Actinoplanes* and *Micromonospora* (14, 15, 16). Discrimination between *Actinoplanes* and *Micromonospora* whose sugar pattern and menaquinone profile are similar, was carried out with fatty acid profile and spore ornamentation. *Actinoplanes* had i-C16:0 as main fatty acid more than 50%. In contrast, *Micromonospora* had i-C15:0, i-C16:0, and a-C15:0 as major ones.

Because of similar sugar and main menaquinone profile of maduromycetes, discrimination between *Microbispora* and *Streptosporangium* was carried out with fatty acid profile and spore ornamentation. The former had 10-Me-C17:0 as attendant fatty acid about 14% in addition to major fatty acid i-C16:0. The latter had i-C14:0, i-C16:0 and i-C17:1 as main fatty acids in a similar ratio of 20%. Characteristic longitudinal spore pairs are found on aerial mycelium of *Microbispora*. Sporangiospores are formed by septation of hypha within sporangium of *Streptosporangium* (Fig. 3, 4).

Nine strains belonged to *Micromonospora*, six to *Microbispora*, six to *Actinoplanes*, five to *Streptosporangium*, and one to *Dactylosporangium*. Eight strains could not be identified exactly (Table 2).

References

1. Cho, S.H., C.W. Hwang, H.K. Chung and C.S. Yang. 1994. A new medium for the selective isolation of soil actinomycetes. *K. J. Appl. Microbiol. Biotechnol.* 22, 561-563.
2. Englyst, H.N. and J.H. Cummings. 1984. Simplified method for the measurement of total non-starch polysaccharides by gas-chromatography of constituent sugars as alditol acetates. *Analyst.* 101, 937-942.
3. Goodfellow, M. 1989. Suprageneric classification of actinomycetes. p. 2333-2339. *In* S.T. Williams, M.E. Sharpe, and J.G. Holt (eds.), Bergey's manual of systematic bacteriology. Williams and Wilkins, London.
4. Hayakawa, M. and H. Nonomura. 1987a. Efficacy of artificial

humic acid as a selective nutrient in HV agar used for the isolation of soil actinomycetes. *J. Ferment. Technol.* 65, 609-616.

5. Hayakawa, M. and H. Nonomura. 1987b. Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. *J. Ferment. Technol.* 65, 501-509.
6. Hayakawa, M., T. Sadaka, T. Kajiura, and H. Nonomura. 1991. New methods for the highly selective isolation of *Micromonospora* and *Microbispora*. *J. Ferment. Technol.* 72, 320-326.
7. Iwai, H. and Y. Takahashi. 1992. Selection of microbial sources of bioactive compounds. p. 281-302. *In* S. Oumra (ed.), The Search for bioactive compounds from microorganisms. Springer-Verlag, New York.
8. Kaneda, T. 1991. Iso- and anteiso-fatty acids in bacteria; biosynthesis, function, and taxonomic significance. *Microbiol. Rev.* 55, 288-302.
9. Kim, C.J., K.H. Lee, A. Shimazu, O.S. Kwon, and D.J. Park. 1995. Isolation of rare actinomycetes on various types of soil. *K. J. Appl. Microbiol. Biotechnol.* 23, 36-42.
10. Kim, C.J., K.H. Lee, O.S. Kwon, A. Shimazu, and I.D. Yoo. 1994. Selective isolation of actinomycetes by physical pretreatment of soil sample. *K. J. Appl. Microbiol. Biotechnol.* 22, 222-225.
11. Kim, S.B., M.Y. Kim, C.N. Seong, and Y.C. Hah. 1996. Lipid analysis of streptomycetes isolated from volcanic soil. *J. Microbiol.* 34, 184-191.
12. Lechevalier, H.A. and M.P. Lechevalier. 1967. Biology of actinomycetes. *Ann. Rev. Microbiol.* 21, 71-100.
13. Lechevalier, M.P., A.C. Horan and H.A. Lechevalier. 1971. Lipid composition in the classification of nocardiae and mycobacteria. *J. Bacteriol.* 105, 313-318.
14. Lechevalier, H.A. and M.P. Lechevalier. 1980. The chemotaxonomy of actinomycetes, p. 227-291. *In* A. Dietz and D.W. Thayer (eds.), Actinomycete taxonomy, Society for Industrial Microbiology, Arlington.
15. Minnikin, D.E. and M. Goodfellow. 1980. Lipid composition in the classification and identification of acid-fast bacteria, p. 189-255. *In* M. Goodfellow and R.G. Board (eds.), Microbiological classification and identification, Academic Press, London.
16. Minnikin, D.E., A.G. O'Donnell, M. Goodfellow, G. Alderson, M. Athalye, A. Schaal, and J.H. Parlett. 1984. An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J. Microbiol. Methods* 2, 233-241.
17. Nisbet, L.J. 1982. Current strategies in the search for bioactive microbial metabolites. *J. Chem. Technol. Biotechnol.* 32, 251-270.
18. Nolan, R.D. and T. Cross. 1988. Isolation and screening of actinomycetes, p. 2-8. *In* M. Goodfellow, S.T. Williams, and M. Mordarski (eds.), Actinomycetes in biotechnology. Academic Press, London.
19. Nonomura, H. 1988. Isolation, taxonomy and ecology of soil actinomycetes. *Actinomycetol.* 3, 45-54.
20. O'Donnell, A.G. 1985. Numerical analysis of chemotaxanomic data, p. 403-414. *In* M. Goodfellow, D. Jones and F.G. Priest (eds.), Computer assisted bacterial systematics, Academic Press, London.
21. Okami, Y. and K. Hotta. 1988. Search and discovery of new antibiotics, p. 39. *In* M. Goodfellow, S.T. Williams, and M. Mordarski (eds.), Actinomycetes in biotechnology. Academic Press, London.
22. Okazaki, T. 1987. Rare actinomycetes -new breed of actinomycetes. *J. Microorganism* 3, 453-461.
23. Seong, C.N. 1992. Ph. D. Thesis. Seoul National University, Seoul.
24. Seong, C.N., Y.S. Kim, K.S. Baik, S.D. Lee, Y.C. Hah, S.B. Kim, and M. Goodfellow. 1999. Mycolic acid-containing actinomycetes associated with activated sludge foam. *J. Microbiol.* 37, 66-72.
25. Shirling, E.B. and D. Gottlieb, 1966. Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* 16, 313-340.