Amycolatopsis jejuensis sp. nov. and *Amycolatopsis halotolerans* sp. nov., novel actinomycetes isolated from a natural cave

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Two actinomycete strains, designated N7-3^T and N4-6^T, were isolated from a natural cave on Jeju Island, Republic of Korea, by using a dilution method, and were subjected to physiological, chemical and molecular characterization. The nearly complete sequences of the 16S rRNA gene were aligned and compared with those of representatives of the genus *Amycolatopsis*. Phylogenetic analysis showed that the organisms belong to the family *Pseudonocardiaceae* and formed two distinct lineages within the evolutionary radius of the genus *Amycolatopsis*. The chemotaxonomic and morphological properties support their classification in the genus *Amycolatopsis*. The 16S rRNA gene sequence data revealed that the closest relatives of strains N7-3^T and N4-6^T were *Amycolatopsis sulphurea* (97.9 % similarity) and *Amycolatopsis albidoflavus* (98.7 % similarity), respectively. The combination of physiological and genetic data supported the observation that the organisms could be distinguished from each other and from established species of the genus *Amycolatopsis*. The names *Amycolatopsis jejuensis* sp. nov. and *Amycolatopsis halotolerans* sp. nov. are proposed for the two novel species, with N7-3^T (=NRRL B-24427^T=JCM 13280^T) and N4-6^T (=NRRL B-24428^T=JCM 13279^T) as the respective type strains.

During the investigation of the biodiversity of cave bacteria including actinomycetes, strains N7-3^T and N4-6^T were isolated from two different samples collected inside a natural cave. The aims of the present study were to investigate the phylogenetic position of the cave actinomycete strains based on 16S rRNA gene sequence studies and to unravel the taxonomic status of the isolates by morphological, physiological and chemotaxonomic characterization. It was evident from phenotypic and genotypic data that the organisms could be readily differentiated from each other and from established species of the genus *Amycolatopsis*.

The samples contained soil and dried bat dung collected from inside a natural cave on Jeju Island, Republic of Korea in October 2002. Serial dilution of sample suspensions were transferred onto starch casein agar [1 % soluble starch, 0.03 % casein, 0.2 % KNO₃, 0.2 % NaCl, 0.002 % CaCO₃, 0.005 % MgSO₄·7H₂O, 0.001 % FeSO₄·7H₂O and 1.8 % agar (pH 7.2)] and the agar plates were incubated for 14 days at 30 °C. The isolates were subcultured on yeast extract/malt extract agar for 7 days at 30 °C and then maintained as mycelial fragments in 20 % (v/v) glycerol at –20 °C or –70 °C. Strain N7-3^T was recovered from dried bat dung,

whereas strain N4-6^T was obtained from a soil sample. The reference strains *Amycolatopsis sulphurea* IFO 13270^T (=IMSNU 20060^T) and *Amycolatopsis albidoflavus* KCTC 9471^T (=IMSNU 22139^T) were used for comparison.

Cultural and morphological characteristics were observed by using yeast extract/malt extract agar (ISP 2 medium), oatmeal agar (ISP 3 medium) and ISP 4 medium (Shirling & Gottlieb, 1966). The degree of growth, the colour of mycelium and the presence of diffusible pigments of the organisms were recorded on all tested media after incubation for 14 days at 30 °C. The cell morphology was observed from cultures incubated on oatmeal agar for 14 days at 30 °C by using a light microscope. For electron microscopy, agar blocks with growth were fixed with 1 % osmium tetroxide, dehydrated through a graded series of ethanol and isoamyl acetate and critical-point-dried. Gold-coated specimens were observed using a Hitachi S-2460 scanning electron microscope. The organisms showed morphological properties typical for members of the genus *Amycolatopsis* in that they produced well-developed, branched aerial and vegetative hyphae, which fragmented into rod-shaped elements. The isolates showed good growth and similar colour patterns of mycelia on all tested agar media. The aerial and vegetative mycelia of strain N7-3^T were white and yellowish brown, respectively, whereas strain N4-6^T produced greyish white-coloured aerial mycelium and brown-coloured substrate mycelium. Neither of the isolates produced diffusible pigments.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains N4-6^T and N7-3^T are DQ000196 and DQ000200, respectively.

Genomic DNA was extracted and purified using the Wizard Genomic DNA Purification kit (Promega) following the manufacturer's instructions. Cloning of the 16S rRNA gene following PCR-mediated amplification from genomic DNA was carried out as previously described (Lee *et al.*, 2000a). The cloned 16S rRNA gene was sequenced using an ABI Prism BigDye Terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 3730xl; Applied Biosystems). The sequences determined in this study and reference sequences of the genus *Amycolatopsis* were aligned by using the CLUSTAL X program (Thompson *et al.*, 1997) and the alignment was manually optimized by comparison with the secondary structure of the *Escherichia coli* sequence (Brosius *et al.*, 1978). Phylogenetic trees were reconstructed using the least-squares (Fitch & Margoliash, 1967), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distances for the least-squares and neighbour-joining methods were computed by a method described by

Jukes & Cantor (1969). All of the phylogenetic analyses were performed using the programs contained in the PHYLIP package (Felsenstein, 1993). *Pseudonocardia thermophila* IMSNU 20112^T (AJ252830) was used as an outgroup taxon. The reliability of tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining data, using 1000 resamplings. The almost complete 16S rRNA gene sequences for strains N7-3^T and N4-6^T contained continuous stretches of 1514 and 1512 nt, respectively. A total of 1354 unambiguous aligned positions present in all strains between positions 72 and 1452 (*E. coli* numbering) were used for tree construction. A phylogenetic tree (Fig. 1), constructed from neighbour-joining data, showed that the organisms belong to the family *Pseudonocardiaceae* (Embley *et al.*, 1988) and form two distinct clades within the evolutionary radius of the genus *Amycolatopsis*. The other two tree-making algorithms (least-squares and maximum-parsimony methods) resulted in trees showing similar topologies (data not shown). Strain N7-3^T was closely

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences (positions 72–1452, *E. coli* equivalent) showing the phylogenetic relationships of the cave isolates within the evolutionary radius of the genus *Amycolatopsis*. Tree was constructed from Juke–Cantor distances by using the neighbour-joining method. Numbers at branching nodes are percentages of occurrence in 1000 bootstrapped trees (only values greater than 50% are indicated). Outgroup sequence used for analysis was from *Pseudonocardia thermophila* (not shown). Bar, 1 nt substitution per 100 nt.

related to *A. sulphurea* (97.9% sequence similarity), with high bootstrap support (73%). Sequence similarity values with less closely related members of the genus ranged from 94.3 to 97.0%. The phylogenetic neighbours of strain N4-6^T were *A. albidoflavus* (98.7% sequence similarity) and *A. rubida* (98.5% sequence similarity), again with high bootstrap support (93%).

For chemotaxonomic characterization, the test strains were cultivated in trypticase soy broth (Difco) for 3 days at 30 °C with shaking. The following properties were determined as described: the isomer of diaminopimelic acid (Staneck & Roberts, 1974), the acyl type of cell wall (Uchida & Aida, 1984), the sugar composition of whole-cell walls (Saddler *et al.*, 1991), respiratory menaquinone (Kroppenstedt, 1985), mycolic acid (Minnikin *et al.*, 1980), phospholipid composition (Minnikin *et al.*, 1977) and the G+C content of DNA (Mesbah *et al.*, 1989). Cellular fatty acid methyl esters were prepared by a method described previously (Minnikin, 1988) and analysed by gas chromatography with an Agilent model 6850 gas chromatograph as previously described (Lee *et al.*, 2000b). Most of the chemotaxonomic properties given in the species description were consistent with those of members of the genus *Amycolatopsis* (Lechevalier *et al.*, 1986; Henssen *et al.*, 1987; Yassin *et al.*, 1993), indicating that chemotaxonomic data also supported the phylogenetic clustering based on 16S rRNA gene sequence studies. The fatty-acid profile contained a mixture of saturated and branched-chain acids, including the major components hexadecanoic acid (C_{16:0}), octadecanoic acid (C_{18:0}), iso-hexadecanoic acid (i-C_{16:0}) and iso-pentadecanoic acid (i-C_{15:0}); with an additional hexadecanoic acid (C_{16:0}) in strain N4-6^T (Table 1). A branched, hydroxy fatty acid was also detected in extracts of the isolates and in *A. albidoflavus* IMSNU 22139^T. Strain N7-3^T differs from its phylogenetic neighbour, *A. sulphurea* IMSNU 20060^T, in that the fatty-acid profile contained minor amounts of C_{14:0}, C_{15:0}, C_{20:0}, C_{16:1}, ai-C_{15:0} and 2-OH C_{14:0} but not C_{17:1}, i-C_{17:1}, i-C_{18:0}, i-C_{18:1}, or i-C_{18:0} acids. Strain N4-6^T was readily differentiated from its closest relative, *A. albidoflavus* IMSNU 22139^T, in the presence or absence of anteiso-branched and unsaturated fatty acids.

Results of the physiological characterization are given in the species description. Catalase activity was determined with a 3% (v/v) solution of hydrogen peroxide. The production of hydrogen sulfide was detected in trypticase soy broth by using lead acetate strips. Urease activity was determined by a colour change in Bacto urea broth (Difco). Nitrate reduction and hydrolysis of casein, gelatin and starch were examined by using methods described previously (MacFaddin, 1980). Decomposition of adenine, hypoxanthine, DL-tyrosine and xanthine was examined using a method described by Gordon *et al.* (1974). The temperature range for growth was tested between 10 and 45 °C. NaCl tolerance was determined at final concentrations of 2, 3, 5, 7 and 10% (w/v). Acid production from carbohydrates and alcohols was determined by using Bacto OF (oxidation/

Table 1. Fatty-acid composition of the isolates and their phylogenetic neighbours

Strains: 1, *Amycolatopsis jejuensis* sp. nov. N7-3^T; 2, *Amycolatopsis halotolerans* sp. nov. N4-6^T; 3, *Amycolatopsis sulphurea* IMSNU 20060^T; 4, *Amycolatopsis albidoflavus* IMSNU 22139^T. Values are percentages of total fatty acids. –, Not detected.

Fatty acid	1	2	3	4
Saturated				
C _{14:0}	1.2	–	–	1.1
C _{15:0}	2.6	3.7	–	5.8
C _{16:0}	20.2	16.2	8.8	10.2
C _{17:0}	4.8	14.5	7.5	7.8
C _{18:0}	17.5	13.2	6.1	6.2
C _{20:0}	1.1	–	–	–
Unsaturated				
C _{15:1}	–	–	–	2.3
C _{16:1}	0.9	–	–	4.0
C _{17:1}	–	–	1.4	4.4
Branched				
i-C _{14:0}	2.2	4.4	1.8	9.8
i-C _{15:0}	14.3	13.0	11.2	8.7
i-C _{16:0}	14.6	17.6	19.4	26.1
i-C _{17:1}	–	–	1.7	–
i-C _{17:0}	5.0	5.0	5.3	1.6
i-C _{18:1}	–	–	7.4	–
i-C _{18:0}	–	–	1.0	–
ai-C _{15:0}	3.2	3.2	–	–
ai-C _{17:0}	3.5	3.1	17.7	–
Hydroxylated				
2-OH C _{14:0}	0.6	0.8	–	–
3-OH i-C _{15:0}	7.6	5.2	–	7.8

Table 2. Phenotypic characteristics of the isolates and their phylogenetic neighbours

Strains: 1, *A. jejuensis* sp. nov. N7-3^T; 2, *A. halotolerans* sp. nov. N4-6^T; 3, *A. sulphurea* IMSNU 20060^T; 4, *A. albidoflavus* IMSNU 22139^T. +, Positive; –, negative.

Characteristic	1	2	3	4
Acid formed from:				
Adonitol	+	+	–	+
D-Cellobiose	–	+	–	+
meso-Erythritol	–	–	–	+
D-Lactose	–	–	–	+
D-Mannitol	+	+	+	–
Melibiose	–	+	–	–
D-Trehalose	–	–	+	+
D-Xylose	–	–	–	+
Decomposition of:				
Hypoxanthine	+	+	–	+
Xanthine	–	+	–	+
Hydrolysis of starch	–	–	+	+
Growth at 37 °C	–	+	+	+
Growth in 7% NaCl	–	+	–	+

fermentation) basal medium (Difco), including each filter-sterilized compound at a final concentration of 1% (w/v). The isolates can be readily differentiated from each other and from their phylogenetic neighbours by using a range of phenotypic properties (Table 2).

The phenotypic and genotypic data supported the observation that the cave actinomycetes could be classified in two novel species of the genus *Amycolatopsis*, for which the names *Amycolatopsis jejuensis* sp. nov. and *Amycolatopsis halotolerans* sp. nov. are proposed, with N7-3^T (=NRRL B-24427^T=JCM 13280^T) and N4-6^T (=NRRL B-24428^T=JCM 13279^T) as the respective type strains.

Description of *Amycolatopsis jejuensis* sp. nov.

Amycolatopsis jejuensis (je.ju.en' sis. N.L. fem. adj. *jejuensis* of Jeju Island, Republic of Korea).

Forms well-developed, branched aerial and substrate mycelium that fragment into rod-shaped elements. The aerial mycelium is white and the vegetative mycelium is yellowish brown (ISP 2 medium). No diffusible pigment is produced. Aerobic, Gram-positive, non-acid-alcohol-fast, catalase-positive. Hydrogen sulfide is produced. Nitrate is reduced to nitrite. Exhibits weak urease activity. Growth occurs between 10 and 30 °C. Casein, gelatin, hypoxanthine and DL-tyrosine are decomposed but starch and xanthine are not decomposed. Growth occurs in the presence of 2% NaCl but not in 3% NaCl. Acid is produced from D-arabinose, D-fructose, D-galactose, D-mannose, sucrose, adonitol, *myo*-inositol and D-mannitol. No acid production is observed from L-arabinose, D-cellobiose, dextran, D-glucose, inulin, D-lactose, maltose, D-melezitose, melibiose, methyl α -D-glucoside, methyl α -D-mannoside, D-raffinose, L-rhamnose, L-ribose, salicin, L-sorbose, D-trehalose, L-xylose, D-xylose, 2,3-butanediol, dulcitol, *meso*-erythritol, glycerol, 1,2-propanediol, D-sorbitol or D-xylitol. Type IV cell wall (*meso*-diaminopimelic acid, arabinose and galactose). The acyl type of muramic acid is acetyl type. Predominant menaquinone is MK-9(H₄). Phospholipid profile contains diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol and phosphatidylmannoside. Mycolic acids are not present. Predominant fatty acids are C_{16:0} (20.2%), C_{18:0} (17.5%), i-C_{16:0} (14.6%) and i-C_{15:0} (14.3%). A branched, hydroxy fatty acid (3-OH i-C_{15:0}) is also detected. The G + C content of the DNA is 71.7 mol%.

The type strain, N7-3^T (=NRRL B-24427^T=JCM 13280^T), was isolated from dried bat dung inside a natural cave on Jeju Island, Republic of Korea.

Description of *Amycolatopsis halotolerans* sp. nov.

Amycolatopsis halotolerans (ha.lo.to'le.rans. Gr. n. *hals* salt; L. pres. part. *tolerans* tolerating, enduring; N.L. part. adj. *halotolerans* salt-tolerating).

Forms well-developed, branched aerial and substrate mycelium that fragment into rod-shaped elements. The aerial mycelium is greyish white and the vegetative mycelium is brown (ISP 2 medium). Aerobic, Gram-positive, non-acid-alcohol-fast, catalase-positive. Urease-positive. Hydrogen sulfide is produced. Nitrate is reduced to nitrite. Growth occurs between 10 and 37 °C. Growth does not occur at or above 45 °C. Hypoxanthine, DL-tyrosine and xanthine are decomposed. Casein and gelatin are hydrolysed but starch is not hydrolysed. Growth occurs in the presence of 7% NaCl but not in 10% NaCl. Acid is produced from D-cellobiose, D-fructose, D-galactose, D-mannose, melibiose, L-ribose, sucrose, L-xylose, adonitol, glycerol, *myo*-inositol and D-mannitol. No acid production is observed from L-arabinose, D-arabinose, dextran, D-glucose, inulin, D-lactose, maltose, D-melezitose, methyl α -D-glucoside, methyl α -D-mannoside, D-raffinose, L-rhamnose, salicin, L-sorbose, D-trehalose, D-xylose, 2,3-butanediol, dulcitol, *meso*-erythritol, 1,2-propanediol, D-sorbitol or D-xylitol. Type IV cell wall (*meso*-diaminopimelic acid, arabinose and galactose). The acyl type of muramic acid is acetyl type. Predominant menaquinone is MK-9(H₄), with MK-9(H₆) and MK-9(H₈) as minor components. Phospholipid profile contains phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. Mycolic acids are not present. Predominant fatty acids are i-C_{16:0} (17.6%), C_{16:0} (16.2%), C_{18:0} (13.2%) and i-C_{15:0} (13.0%). A considerable amount of a branched, hydroxy fatty acid (3-OH i-C_{15:0}) is also detected. The G + C content of the DNA is 72.5 mol%.

The type strain, N4-6^T (=NRRL B-24428^T=JCM 13279^T), was isolated from a soil sample inside a natural cave on Jeju Island, Republic of Korea.

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