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<sup>T</sup> and N4-6<sup>T</sup>, 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 Pseudonocardiaeae 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<sup>T</sup> and N4-6<sup>T</sup> 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<sup>T</sup> (= NRRL B-24427<sup>T</sup> = JCM 13280<sup>T</sup>) and N4-6<sup>T</sup> (= NRRL B-24428<sup>T</sup> = JCM 13279<sup>T</sup>) as the respective type strains.

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 % KNO<sub>3</sub>, 0·2 % NaCl, 0·002 % CaCO<sub>3</sub>, 0·005 % MgSO<sub>4</sub>·7H<sub>2</sub>O, 0·001 % FeSO<sub>4</sub>·7H<sub>2</sub>O 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<sup>T</sup> was recovered from dried bat dung, whereas strain N4-6<sup>T</sup> was obtained from a soil sample. The reference strains Amycolatopsis sulphurea IFO 13270<sup>T</sup> (= IMSNU 20060<sup>T</sup>) and Amycolatopsis albidoflavus KCTC 9471<sup>T</sup> (= IMSNU 22139<sup>T</sup>) 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<sup>T</sup> were white and yellowish brown, respectively, whereas strain N4-6<sup>T</sup> produced greyish white-coloured aerial mycelium and brown-coloured substrate mycelium. Neither of the isolates produced diffusible pigments.
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
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 N4-6T 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 (C16:0), octadecanoic acid (C18:0), iso-hexadecanoic acid (i-C16:0) and iso-pentadecanoic acid (i-C15:0); with an additional hexadecanoic acid (C16:0) in strain N4-6T (Table 1). A branched, hydroxy fatty acid was also detected in extracts of the isolates and in *A. albidoflavus* IMSNU 22139T. Strain N7-3T differs from its closest relative, *A. sulphurea* IMSNU 22139T, 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 5 % (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, D,L-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/
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-cellulbiose, D-fructose, D-galactose, D-mannose, melibiose, L-ribose, sucrose, L-xyllose, 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-xyllose, 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, diphasphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. Mycolic acids are not present. Predominant fatty acids are i-C16:0 (17-6 mol%), C16:0 (16-2 mol%), C18:0 (13-2 mol%) and i-C15:0 (13-0 mol%). A considerable amount of a branched, hydroxy fatty acid (3-OH i-C15:0) is also detected. The G+C content of the DNA is 72.5 mol%.

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

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