Nocardioides koreensis sp. nov., *Nocardioides bigeumensis* sp. nov. and *Nocardioides agariphilus* sp. nov., isolated from soil from Bigeum Island, Korea

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Three Gram-positive, coccoid to rod-shaped actinobacteria, designated strains MSL-09^T, MSL-19^T and MSL-28^T, were isolated from a soil sample collected from Bigeum Island in Korea, and were subjected to a polyphasic taxonomic analysis. All three isolates grew optimally at pH 7.5–9.0 and 28–30 °C. 16S rRNA gene analysis revealed that all three strains belonged to the genus *Nocardioides*, with strains MSL-09^T, MSL-19^T and MSL-28^T respectively showing the highest sequence similarity to *Nocardioides aquaticus* DSM 11439^T (96.25 %), *Nocardioides aquiterrae* GW-9^T (95.75 %) and *Nocardioides terrigena* DS-17^T (95.61 %).

Chemotaxonomically, they contain LL-2,6-diaminopimelic acid in the cell-wall peptidoglycan, MK-8(H₄) as the predominant menaquinone and diphosphatidylglycerol, phosphatidylglycerol and some unknown lipids as the polar lipids found in the cell wall. iso-C_{16:0} is a major fatty acid. The G + C content of the DNA was respectively 69.9, 69.3 and 69.4 mol% for strains MSL-09^T, MSL-19^T and MSL-28^T. Based on morphological, physiological, biochemical and chemotaxonomic characters presented in this study, the three strains represent novel species of the genus *Nocardioides*. The names *Nocardioides koreensis* sp. nov. (type strain MSL-09^T = KCTC 19272^T = DSM 19266^T), *Nocardioides bigeumensis* sp. nov. (type strain MSL-19^T = KCTC 19290^T = DSM 19320^T) and *Nocardioides agariphilus* sp. nov. (type strain MSL-28^T = KCTC 19276^T = DSM 19323^T) are proposed.

The genus *Nocardioides* was proposed by Prauser (1976) for Gram-positive, non-acid-fast, aerobic and mesophilic nocardioform actinomycetes that develop a mycelium that fragments into irregular rod- to coccus-like elements. Originally, the genus contained two species, *Nocardioides albus* and *Nocardioides luteus* (Prauser, 1984), and, at the time of writing, the genus harbours 23 recognized species. In this study, isolates MSL-09^T, MSL-19^T and MSL-28^T were the subject of a taxonomic investigation.

Strain MSL-09^T, MSL-19^T and MSL-28^T were isolated from a soil sample collected from Bigeum Island in Korea using R2A (1:10-diluted) medium (Difco). The isolates were routinely maintained on R2A (1:2-diluted) medium at 28 °C and maintained as glycerol suspensions (20 %, w/v) at –70 °C. Morphological, physiological, cultural and biochemical properties were examined as described by

Yoon & Park (2006). Morphological properties were examined by light microscopy (model Nikon HFX-DX) and electron microscopy (JEOL apparatus Philips SEM 515). Growth in the presence of NaCl was investigated in 2-fold-diluted R2A at various NaCl concentrations (0.5–7.0 %, w/v, at intervals of 0.5 %). The pH range for growth was determined by adjusting the pH at intervals of 0.5 pH units from pH 4.5 to 12.0. The pH was adjusted prior to sterilization by the addition of HCl or Na₂CO₃. Metabolic properties were determined using API ZYM test kits (bioMérieux) according to the manufacturer's instructions. Physiological and biochemical characteristics were examined at 28 °C and properties were recorded after 7–10 days except for the nitrate reduction test, which was recorded after 2–3 days. Other tests, including cell morphology, motility, acid production and assimilation of carbon sources, were performed as described by Kämpfer (1991). Cell biomass for DNA extraction and for analyses of cell-wall and isoprenoid quinones was obtained by cultivation at 28 °C in 2-fold-diluted R2A broth (pH 7.5). Chemotaxonomic and molecular systematic studies were performed as described by Yoon *et al.* (2005a, b, c). The isomer type of the diamino acid in the cell-wall

Abbreviation: DAP, 2,6-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains MSL-09^T, MSL-19^T and MSL-28^T are respectively EF466115, EF466114 and EF466113.

Scanning electron micrographs of cells of the novel strains are available as supplementary material with the online version of this paper.

peptidoglycan was analysed using TLC according to the method described by Komagata & Suzuki (1987). For fatty acid methyl ester analysis, cell mass of the strains was harvested from 2-fold-diluted R2A broth (pH 7.5) after incubation for 10 days at 28 °C.

Morphological, cultural, physiological and biochemical characteristics of strains MSL-09^T, MSL-19^T and MSL-28^T are given in the species descriptions and presented in Table 1. Cells are coccoid to short rods, arranged singly or in groups (Supplementary Fig. S1, available in IJSEM Online). Extraction of chromosomal DNA, amplification and purification of the 16S rRNA gene by PCR and direct

sequencing of the purified PCR product were performed as described previously (Lee *et al.*, 2000; Lee, 2006). Almost-complete sequences of the 16S rRNA genes of strains MSL-09^T (1398 nt), MSL-19^T (1398 nt) and MSL-28^T (1426 nt) were compared with those of representatives of the family *Nocardioidaceae* using the CLUSTAL_X program (Thompson *et al.*, 1997). Comparative 16S rRNA gene sequence analysis showed that strains MSL-09^T, MSL-19^T and MSL-28^T were affiliated phylogenetically to the genus *Nocardioides* (Fig. 1). In phylogenetic trees based on the neighbour-joining, maximum-parsimony (Kluge & Farris, 1969) and maximum-likelihood (Felsenstein, 1981) algorithms, strain MSL-09^T clustered with *Nocardioides*

Table 1. Differential phenotypic properties of strains MSL-09^T, MSL-19^T and MSL-28^T and related *Nocardioides* species

Taxa: 1, MSL-09^T; 2, MSL-19^T; 3, MSL-28^T; 4, *N. lentus*; 5, *N. aquaticus*; 6, *N. terrigena*. Data for reference species were taken from Collins *et al.* (1994) and Yoon *et al.* (1997, 2004). +, Positive; –, negative; w, weakly positive; v, variable (reactions in parentheses are for the type strain); ND, no data available. All organisms are positive for Gram staining and esterase lipase (C8) activity, and all are negative for ribose utilization, aesculin hydrolysis and activities of β -glucuronidase, *N*-acetyl- β -glucosaminidase and α -fucosidase.

Characteristic	1	2	3	4	5	6
Cell morphology	Short rods	Cocci	Cocci to short rods	Rods, cocci	Cocci, short rods	Rods–cocci
Cell size (μ m)	0.2–0.7 $\times$ 0.8–3.2	0.3–0.8 $\times$ 0.8–4.0	0.2–0.5 $\times$ 1.3–3.0	0.4–0.7 $\times$ 1.0–4.5	0.9–1.0 $\times$ 0.9–1.4	0.4–0.7 $\times$ 0.7–2.0
Motility	+	+	+	–	–	–
Colony colour	Cream–white	Cream	Yellow	Yellow	Dull orange	Ivory
Optimal temperature (°C)	30	28	28	28	16–26	30
Nitrate reduction	–	–	+	+	+	+
Hydrolysis of:						
Starch	+	–	–	–	w	w
Gelatin	–	–	–	+	+	ND
Tween 80	+	+	+	–	+	w
Tyrosine	+	+	–	v (+)	+	–
Catalase	–	–	–	+	+	+
Oxidase	–	–	–	w	ND	+
Carbon-source utilization						
D-Fructose	–	–	–	–	+	+
D-Galactose	–	–	–	+	–	+
<i>myo</i> -Inositol	–	–	–	–	w	–
Lactose	+	+	–	v (+)	–	+
Maltose	–	–	–	+	w	+
D-Mannitol	–	–	–	+	+	+
D-Mannose	+	+	–	–	–	–
Melibiose	+	+	+	–	w	+
Raffinose	–	–	+	+	–	–
L-Rhamnose	–	–	–	+	+	+
API ZYM test results						
Alkaline phosphatase	+	+	+	+	v	+
Esterase (C4)	+	+	+	+	w	+
Valine arylamidase	–	–	–	–	w	–
Cystine arylamidase	–	–	+	–	v	–
Trypsin	–	–	–	–	w	–
β -Galactosidase	–	–	–	–	–	+
β -Glucosidase	+	+	+	+	–	–
DNA G + C content (mol%)	69.9	69.3	69.4	74.8*	69.0*	71.5*
Isolation source	Soil	Soil	Soil	Alkaline soil*	Saline lake*	Soil*

*Data for the type strain.

Fig. 1. Phylogenetic dendrogram obtained by neighbour-joining analysis based on 16S rRNA gene sequences, showing the position of strains MSL-9^T, MSL-19^T and MSL-28^T in the genus *Nocardioideae*. Asterisks indicate branches that were also recovered using maximum-likelihood and maximum-parsimony. *Terrabacter tumescens* DSM 20308^T served as the outgroup. Numbers on branch nodes are bootstrap values expressed as percentages (1000 resamplings). Bar, 0.01 substitutions per nucleotide position.

aquaticus DSM 11439^T, MSL-19^T formed a separate lineage with *Nocardioideae aestuarii* JC2056^T and MSL-28^T formed a cluster with *Nocardioideae lentus* KSL-17^T (Fig. 1). The highest similarity values of the 16S rRNA gene sequences of strains MSL-09^T, MSL-19^T and MSL-28^T with those of recognized *Nocardioideae* species were 96.25 % (MSL-09^T, with *Nocardioideae aquaticus* DSM 11439^T), 95.75 % (MSL-19^T, with *Nocardioideae aquiterrae* GW-9^T) and 95.61 % (MSL-28^T, with *Nocardioideae terrigena* DS-17^T).

Strains MSL-09^T, MSL-19^T and MSL-28^T contained LL-2,6-diaminopimelic acid (LL-DAP) in the cell-wall peptidoglycan, the predominant menaquinone was MK-8(H₄) and the polar lipids diphosphatidylglycerol, phosphatidylglycerol and some unidentified glycolipids were detected. The fatty acid profiles comprised large amounts of branched, unsaturated, straight-chain and 10-methyl fatty acids, as detailed in the species descriptions. The chemotaxonomic characteristics were consistent with the affiliation of the strains to the genus *Nocardioideae* (Yoon *et al.*, 1997, 2004, 2005a, b, c; Yoon & Park, 2006). The DNA G+C content of strains MSL-09^T, MSL-19^T and MSL-28^T was determined by reversed-phase HPLC of nucleosides according to Mesbah *et al.* (1989) and was 69.9, 69.3 and 69.4 mol%, respectively.

Phylogenetic analyses based on 16S rRNA gene sequences and chemotaxonomic data revealed that strains MSL-09^T, MSL-19^T and MSL-28^T can be assigned to the genus *Nocardioideae* (Urzi *et al.*, 2000; Wang *et al.*, 2001) as representatives of three novel species. This conclusion is supported by a number of phenotypic differences between the isolates and their phylogenetic neighbours (Table 1), as well as chemotaxonomic differences and fatty acid

compositions. On the basis of the combination of physiological and chemotaxonomic properties and their phylogenetic distinctness, strains MSL-09^T, MSL-19^T and MSL-28^T represents novel members of the genus *Nocardioideae*, for which the names *Nocardioideae koreensis* sp. nov., *Nocardioideae bigeumensis* sp. nov. and *Nocardioideae agariphilus* sp. nov. are proposed.

Description of *Nocardioideae koreensis* sp. nov.

Nocardioideae koreensis (ko.re.en'sis. N.L. masc. adj. *koreensis* pertaining to Korea).

Cells are aerobic, non-spore-forming, Gram-positive, irregular short rods (0.2–0.7 × 0.8–3.2 µm). Colonies are circular, smooth, flat, cream to whitish in colour and 0.9–1.4 mm in diameter after 4–5 days incubation on R2A medium at 30 °C. No formation of aerial or substrate mycelium. Growth occurs at 27–37 °C but not at or below 25 °C or above 37 °C. Optimum growth at pH 7.0–8.0 and 30 °C. Growth is observed in the presence 0–5 % NaCl (w/v). Cellulose is not hydrolysed and tests for H₂S production, nitrate reduction, DNase and citrate hydrolysis are negative. Positive for starch hydrolysis. Cell-wall peptidoglycan contains LL-DAP as the diagnostic amino acid. The predominant menaquinone is MK-8(H₄) and phospholipids are diphosphatidylglycerol, phosphatidylglycerol and some unknown phospholipids. Major fatty acids are i-C_{16:0} (62.87 %), C_{18:1ω9c} (5.88 %), i-C_{14:0} (3.96 %), 10-methyl C_{17:0} (3.42 %), C_{16:0} (3.30 %), i-C_{16:1} (3.13 %), ai-C_{17:0} (1.73 %), C_{18:0} (1.57 %), C_{17:1ω8c} (1.33 %), C_{17:0} (1.20 %) and i-C_{15:0} (1.12 %). The DNA G+C content of the type strain is 69.9 mol%. Other phenotypic properties are given in Table 1.

The type strain, MSL-09^T (=KCTC 19272^T =DSM 19266^T), was isolated from a soil sample collected from Bigeum Island, Republic of Korea.

Description of *Nocardioide*s *bigeumensis* sp. nov.

*Nocardioide*s *bigeumensis* (bi.ge.um.en'sis. N.L. masc. adj. *bigeumensis* pertaining to Bigeum Island, Korea, the source of the soil sample from which the type strain was isolated).

Cells are aerobic, non-spore-forming, Gram-positive, cocci to short rods (0.3–0.8 × 0.8–4.0 µm). Cells show coccus- to rod-like appearance from early exponential phase to stationary phase. Colonies are irregular, smooth, flat, cream in colour and 1.0–2.5 mm in diameter after 4–5 days incubation on R2A medium at 28 °C. No aerial or substrate mycelium is observed. Growth occurs at 20–35 °C, with optimum growth at 28 °C. Optimum pH for growth is pH 7.5–9.0. No growth is observed in the presence of 1 % NaCl or more. Cellulose, casein, starch and urea are not hydrolysed and tests for H₂S production, nitrate reduction, DNase and citrate hydrolysis are negative. Cell-wall peptidoglycan contains LL-DAP as the diagnostic diamino acid. MK-8(H₄) is the predominant menaquinone detected and diphosphatidylglycerol and phosphatidylglycerol are the phospholipids found in the cell wall. Major fatty acids are i-C_{16:0} (38.3 %), i-C_{15:0} (13.1 %), i-C_{14:0} (9.0 %), C_{18:1ω9c} (6.25 %), 10-methyl C_{16:0} (4.8 %), C_{16:0} (4.40 %), C_{17:1ω8c} (3.9 %), C_{17:0} (3.0 %), 10-methyl C_{17:0} (1.9 %), ai-C_{15:0} (1.6 %), ai-C_{17:0} (1.2 %), C_{15:0} (1.1 %) and i-C_{16:1} (1.1 %). The DNA G+C content of the type strain is 69.3 mol%. Other phenotypic properties are given in Table 1.

The type strain, MSL-19^T (=KCTC 19290^T =DSM 19320^T), was isolated from soil from Bigeum Island, Republic of Korea.

Description of *Nocardioide*s *agariphilus* sp. nov.

*Nocardioide*s *agariphilus* (a.ga.ri.phi'lus. N.L. n. *agarum* agar; Gr. adj. *philos* loving; N.L. masc. adj. *agariphilus* agar-loving).

Cells are aerobic, non-spore-forming, Gram-positive cocci to short rods (0.2–0.5 × 1.3–3.0 µm). Cells show rod-to-coccus-like appearance (Fig. 1). Colonies are irregular, smooth, flat, white to cream in colour and 1.5–2.7 mm in diameter after 7 days incubation on R2A medium at 28 °C. Good growth is observed on agar without any addition of carbon and nitrogen sources. No aerial or substrate mycelium is formed, even after long incubation (40 days). Growth occurs at 25–37 °C but not at or below 20 °C or above 37 °C. Optimum pH for growth is 7.5. No growth in 2 % NaCl or more. Cellulose is not hydrolysed and tests for H₂S production, DNase and citrate hydrolysis are negative. Nitrate is reduced to nitrite. Cell-wall peptidoglycan contains LL-DAP as the diagnostic diamino acids. Predominant menaquinone is MK-8(H₄) and diphosphatidylglycerol and phosphatidylglycerol are the phospholipids found in the cell wall. Major fatty acids

are i-C_{16:0} (37.63 %), C_{17:1ω8c} (9.75 %), C_{17:0} (7.06 %), i-C_{17:0} (5.81 %), i-C_{17:1ω9c} (5.06 %), i-C_{15:0} (4.90 %), C_{18:1ω9c} (4.33 %), ai-C_{17:0} (3.90 %), 10-methyl C_{17:0} (2.99 %), C_{16:0} (2.55 %) and C_{18:0} (2.39 %). The DNA G+C content of the type strain is 69.4 mol%. Other phenotypic properties are given in Table 1.

The type strain, MSL-28^T (=KCTC 19276^T =DSM 19323^T), was isolated from a soil sample collected from Bigeum Island, Republic of Korea.

Acknowledgements

This work was supported by the 21C Frontier Microbial Genomics and Application Centre program of the Korean Ministry of Science & Technology (MOST) and the Korea Foundation for International Cooperation of Science & Technology (KICOS) through a grant provided by MOST in the Global Partnership Program (no. M60602000001-06E0200-00100), Republic of Korea.

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