Nocardioides alkalitolerans sp. nov., isolated from an alkaline serpentinite soil in Korea

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Four Gram-positive, rod- or coccus-shaped bacterial strains, KSL-1^T, KSL-9, KSL-10 and KSL-12, were isolated from an alkaline serpentinite soil in Korea, and their taxonomic positions were investigated in a polyphasic study. The four strains exhibited no difference in their 16S rRNA gene sequences. Phylogenetic analyses based on 16S rRNA gene sequences showed that the four strains were phylogenetically affiliated to the genus *Nocardioides*. The four strains had cell-wall peptidoglycan based on LL-diaminopimelic acid as the diamino acid, indicating wall chemotype I. The predominant menaquinone detected in the four strains was MK-8(H₄). The major fatty acid components were iso-C_{16:0}, 10-methyl-C_{18:0}, C_{18:1}ω_{9c} and C_{17:1}ω_{6c}. The DNA G + C contents were 72.4–73.6 mol%. The four strains exhibited 16S rRNA gene sequence similarity levels of 94.0–96.3 % to the type strains of *Nocardioides* species with validly published names. DNA–DNA relatedness levels between the four strains were 85–91 %. On the basis of phenotypic properties, phylogenetic distinctiveness and genotypic relatedness, strains KSL-1^T, KSL-9, KSL-10 and KSL-12 were classified in the genus *Nocardioides* as members of a novel species, *Nocardioides alkalitolerans* sp. nov. The type strain is strain KSL-1^T (=KCTC 19037^T = DSM 16699^T).

The genus *Nocardioides* was proposed by Prauser (1976) with a single species, *Nocardioides albus*, and at present it comprises 11 species with validly published names. *Nocardioides* species have been isolated from a variety of habitats, including soils (Prauser, 1976, 1984; O'Donnell *et al.*, 1982; Suzuki & Komagata, 1983), herbage (Collins *et al.*, 1994), an oil shale column (Yoon *et al.*, 1997), industrial wastewater (Yoon *et al.*, 1999), water from hypersaline lakes (Lawson *et al.*, 2000), groundwater (Yoon *et al.*, 2004) and tidal flats (Yi & Chun, 2004a, b). *N. albus*, *Nocardioides luteus*, *Nocardioides simplex* and *Nocardioides jensenii* were isolated from a variety of soils from different parts of the world (Prauser, 1976, 1984; O'Donnell *et al.*, 1982; Suzuki & Komagata, 1983). Recently, in the course of screening micro-organisms from an alkaline serpentinite soil (approximate pH 9.0–10.0) in Korea, many bacterial strains were isolated and characterized taxonomically. Of these isolates, four *Nocardioides*-like strains, KSL-1^T,

KSL-9, KSL-10 and KSL-12, are described in this paper. Accordingly, the aim of the present work was to determine the exact taxonomic positions of the four strains by a polyphasic taxonomic characterization.

Strains KSL-1^T, KSL-9, KSL-10 and KSL-12 were isolated by the standard dilution plating technique at 30 °C on tenfold-diluted nutrient agar (NA; Difco) with pH adjusted to 10.0. To investigate their morphological, physiological and biochemical characteristics, the four strains were routinely cultivated at 30 °C on twofold-diluted NA with pH adjusted to 9.0. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy (TEM). Presence of flagella was examined by TEM using cells from exponentially growing cultures. The Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. The pH range for growth was determined in twofold-diluted nutrient broth (NB; Difco) which had been adjusted to various pH values (initial pH 4.0–12.0 at intervals of 0.5 pH units). Prior to sterilization the pH of twofold-diluted NB was adjusted to various levels by the addition of Na₂CO₃ (below pH 10.5) or KOH (above pH 10.5). Growth at various temperatures (4–40 °C) was measured on twofold-diluted NA (pH 9.0). Growth at various NaCl concentrations was investigated in trypticase soy broth (Difco) lacking NaCl and in trypticase soy broth.

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The GenBank/EMBL/DDBJ numbers for the 16S rRNA gene sequences of strains KSL-1^T, KSL-9, KSL-10 and KSL-12 are AY633969–AY633972, respectively.

A full table of fatty acid compositions is available as supplementary data in IJSEM Online.

Growth under anaerobic conditions was determined after incubation in an anaerobic chamber with twofold-diluted NA (pH 9.0) that had been prepared anaerobically using nitrogen. Catalase and oxidase activities and hydrolysis of casein, gelatin, hypoxanthine, starch, Tweens 20, 40, 60 and 80, tyrosine, urea and xanthine were determined as described by Cowan & Steel (1965). Hydrolysis of aesculin and nitrate reduction were studied as described previously (Lanyi, 1987). Enzyme activity was determined by using the API ZYM system (bioMérieux). Utilization of various substrates as sole carbon and energy sources was determined as described by Shirling & Gottlieb (1966).

Cell biomass for cell-wall and isoprenoid quinone analyses and for DNA extraction was obtained from cultivation in twofold-diluted NB (pH 9.0) at 30 °C. Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC. Chromosomal DNA isolation and purification were performed according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used together with RNase A to minimize contamination with RNA. For fatty acid methyl ester (FAME) analysis, cell mass of the four strains was harvested from agar plates after incubation for 7 days on twofold-diluted NA (pH 9.0) at 30 °C. FAMES were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. The 16S rRNA gene was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2004). DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replicates for each sample. The highest and lowest values obtained in each sample were excluded, and DNA relatedness values quoted are the means of the remaining three values.

Strains KSL-1^T, KSL-9, KSL-10 and KSL-12 grew optimally at 25–30 °C and pH 7.0–9.0; they grew relatively well at pH 11.0, and weakly at pH 12.0. The four strains were similar in most phenotypic characteristics. Gelatin was hydrolysed by strains KSL-1^T, KSL-10 and KSL-12, but not by strain KSL-9. Strains KSL-9 and KSL-12 grew at pH 5.0, but the other two strains did not. Other phenotypic characteristics are shown in Table 1 or given in the species description.

Strains KSL-1^T, KSL-9, KSL-10 and KSL-12 contained LL-diaminopimelic acid as the diagnostic diamino acid in the cell wall, which is characteristic of wall chemotype I *sensu* Lechevalier & Lechevalier (1970). The four strains contained a tetrahydrogenated menaquinone with eight isoprene units [MK-8(H₄)] as the predominant isoprenoid quinone.

The four strains had similar fatty acid profiles that contained large amounts of branched, unsaturated, straight-chain and 10-methyl fatty acids; the major fatty acids were iso-C_{16:0}, 10-methyl-C_{18:0}, C_{18:1ω9c} and C_{17:1ω6c} (see supplementary table in IJSEM Online). These fatty acid profiles were generally similar to those of *Nocardioides* species described previously (Yoon *et al.*, 1997, 2004; Lawson *et al.*, 2000; Yi & Chun, 2004a). However, there were some differences in the contents of some fatty acids, particularly iso-C_{16:0} and 10-methyl-C_{18:0}, between the four strains and *Nocardioides* species, which may be caused by different cultivation conditions (Yoon *et al.*, 1997, 2004; Lawson *et al.*, 2000; Yi & Chun, 2004a, b). The DNA G + C contents of strains KSL-1^T, KSL-9, KSL-10 and KSL-12 were 72.4, 73.6, 73.2 and 73.2 mol%, respectively.

The 16S rRNA gene sequences of strains KSL-1^T, KSL-9, KSL-10 and KSL-12 determined in this study comprised 1473 nucleotides, respectively, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. The 16S rRNA gene sequences of the four strains were identical. As shown in the phylogenetic tree based on the neighbour-joining algorithm, the four strains formed a distinct evolutionary lineage within the radiation of the cluster comprising *Nocardioides* species (Fig. 1). Similar tree topology was found in the tree generated with the maximum-likelihood algorithm (data not shown). Levels of 16S rRNA gene sequence similarity between the four strains and the type strains of all *Nocardioides* species with validly published names ranged from 94.0 % (with *Nocardioides plantarum*) to 96.3 % (with *N. jensenii*). Sequence similarities to all other taxa included in the phylogenetic analysis were <94.4 % (with *Marmoricola aurantiacus* DSM 12652^T) (Fig. 1). DNA–DNA relatedness levels between strains KSL-1^T, KSL-9, KSL-10 and KSL-12 were 85–91 %, indicating that the four strains are members of the same genomic species (Wayne *et al.*, 1987).

Comparative 16S rRNA gene sequence analysis suggests the genus *Nocardioides* as a possible classification for the four strains. The chemotaxonomic data are in agreement with the result of the phylogenetic classification based on 16S rRNA gene sequences. In particular, the predominant menaquinone type and cellular fatty acid profile distinguish the four strains and *Nocardioides* species from other related genera of the family *Nocardioidaceae* (Miller *et al.*, 1991; Tamura & Yokota, 1994; Park *et al.*, 1999; Urzi *et al.*, 2000; Wang *et al.*, 2001). The four strains are similar phylogenetically and genetically as well as phenotypically. The levels of 16S rRNA gene sequence similarity are low enough to exclude the possibility of assigning the four strains to a previously described *Nocardioides* species (Stackebrandt & Goebel, 1994). The four strains are distinguishable from the other *Nocardioides* species by some phenotypic properties as shown in Table 1. Therefore, on the basis of phenotypic and chemotaxonomic data and phylogenetic distinctiveness, strains KSL-1^T, KSL-9, KSL-10 and KSL-12 should be placed in the genus *Nocardioides* as members of

Table 1. Differential phenotypic characteristics of *Nocardioides* species

Species: 1, *N. alkalitolerans* sp. nov.; 2, *N. albus*; 3, *N. luteus*; 4, *N. simplex*; 5, *N. jensenii*; 6, *N. plantarum*; 7, *Nocardioides pyridinoliticus*; 8, *Nocardioides nitrophenolicus*; 9, *Nocardioides aquaticus*; 10, *Nocardioides aquiterrae*; 11, *Nocardioides ganghwensis*; 12, *Nocardioides aestuarii*. Data taken from Collins *et al.* (1989, 1994), Lawson *et al.* (2000), Prauser (1976, 1984, 1989), Suzuki & Komagata (1983), Yoon *et al.* (1997, 1999, 2004) and Yi & Chun (2004a, b). +, Positive; –, negative; ND, not determined; w, weakly positive; v, variable; data in parentheses are for the type strain. All species are positive for Gram stain, catalase and esterase lipase (C8). All species are negative for acid-fastness, β -glucuronidase, *N*-acetyl- β -glucosaminidase and α -fucosidase.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Cell morphology	Rods, cocci	Hyphae	Hyphae	Rods, cocci	Rods, cocci	Short rods, cocci	Rods, cocci	Rods, cocci	Cocci, short rods	Rods, cocci	Rods	Rods
Cell size (μm)	0.8–1.0 $\times$ 1.5–2.0	0.5–1.0	0.5–1.0	1.0–1.2 $\times$ 1.5–6.0	0.6–0.8 $\times$ 3.0–7.0	ND	0.5–0.6 $\times$ 1.2–1.6	0.5–0.8 $\times$ 1.0–3.0	0.9–1.0 $\times$ 0.9–1.4	0.8–1.0 $\times$ 1.7–2.0	0.4–0.5 $\times$ 0.9–4.5	0.3–0.4 $\times$ 0.9–2.1
Motility	–	–	–	+	–	–	+	+	–	+	–	–
Colony colour*	Milky-white	Whitish to faintly brownish	Yellow to orange–yellow or cream	Yellowish white	Yellowish white	ND	Cream	Yellowish white	Dull orange	Cream	Ivory	Ivory
Optimal temp. ($^{\circ}\text{C}$)	25–30	28	28	26–37	ND	25	35	30	16–26	30	30	30
Nitrate reduction	+	(–)	–	(–)	+	–	+	–	+	+	+	–
Hydrolysis of:												
Aesculin	–	(w)	+	(+)	–	w	+	+	–	+	w	w
Casein	+	(+)	+	+	+	+†	+	+	+	+	+	+
Starch	–	(+)	+	(w)	–	–	+	+	w†	–	+	–
Gelatin	v (+)	(+)	+	+	+	+	+	+	+	+	+	+
Tween 80	+	(+)	+	(+)	+	+	–	+	+	+	+	+
Urea	–	(–)	–	–	+	–	–	+	–	–	–	–
Hypoxanthine	–	(+)	+	(–)	+	–	–	–	–	–	–	–
Xanthine	–	(+)	+†	(–)	+	–	+	–	–	–	w	–
Tyrosine	+	(+)	+	(+)	+	–	+	+	+	–	+	–
Utilization of:												
L-Arabinose	+	+	+	–	–	–	–	–	–	–	+	–
D-Cellobiose	+	(+)	+	(–)	–	+	+	–	–	+	+	+
D-Fructose	–	+	+†	(–)	–	+	+	+	+	+	+	+
D-Galactose	–	(+)	–	(–)	–	–	+	–	+†	+	+	+
D-Glucose	–	+	+	+	+	+†	+	+	+	+	+	+
Inositol	–	–	–	(–)	–	–	+	–	w†	–	–	–
Lactose	–	(–)	–	(–)	–	–	+†	–	–	+	+	w

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Utilization of:												
Maltose	V (–)	ND	ND	ND	ND	+	+	–	W	+	ND	ND
D-Mannitol	–	+	+	–	–	–	+†	–	+	+	+	+
D-Mannose	–	(+)	+	(–)	–	–	+†	W†	–	+	+	–
Melezitose	+	ND	ND	ND	ND	+	ND	–	–	–	ND	ND
D-Melibiose	–	ND	ND	ND	ND	ND	–	–	W	–	ND	ND
D-Raffinose	+	V (–)	–	–	–	–	–	–	–	–	+	W
L-Rhamnose	–	+	+†	–	+	+	+	+	+†	W†	–	–
D-Ribose	–	(–)	–	(–)	–	–	+	+	–	W†	–	–
Sucrose	–	V (+†)	+†	+	+	+	+	+	+	+	+	+
D-Trehalose	+	ND	ND	ND	ND	+	+	+	W	+	ND	ND
D-Xylose	–	+	+	–	–	+	+	+	W†	+	+	+
API ZYM:												
Alkaline phosphatase	+	(+)	V	(+)	W	–	+	+	V	–	+	W
Esterase (C4)	+	(+)	+	(–)	W	+	–	–	W	–	V	+
Lipase (C14)	+	(–)	–	(–)	–	W	–	–	–	–	–	–
Leucine arylamidase	–	(+)	+	(+)	+	+	+	+	+	+	+	+
Valine arylamidase	–	(–)	–	(W)	V	W	W	+	W	–	+	+
Cystine arylamidase	–	(–)	–	(W)	–	+	–	W	V	W	W	–
Trypsin	–	(+)	+	(+)	W	–	+	+	W	+	–	+
α-Chymotrypsin	–	(–)	–	(–)	–	–	–	–	–	–	–	+
Acid phosphatase	+	(–)	–	(W)	V	W	+	+	+	+	W	W
Naphthol-AS-BI-phosphohydrolase	W	(W)	V	(–)	V	+	+	+	–	+	–	W
α-Galactosidase	–	(–)	–	(–)	–	–	–	–	–	–	+	–
β-Galactosidase	–	(V)	V	(–)	–	–	–	–	–	–	+	+
α-Glucosidase	+	(+)	V	(+)	V	+	+	+	+	+	+	+
β-Glucosidase	–	(W)	–	(W)	–	+	–	W	–	W	–	–
α-Mannosidase	–	(–)	+	(–)	–	–	–	–	–	–	–	–
DNA G+C (mol%)	72.4–73.6	67	68	72–74	69	69	73	71	69	73	72	70
Isolation source	Alkaline soil	Soil	Soil	Soil	Soil	Herbage	Oil shale column	Industrial wastewater	Saline lake, Antarctica	Groundwater	Tidal flat	Tidal flat

*Differences may be caused by different cultivation conditions.

†Data from Prauser (1976, 1984), Collins *et al.* (1994), Yoon *et al.* (1997, 1999, 2004) and Lawson *et al.* (2000); result differs from that obtained by Yi & Chun (2004a).

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strains KSL-1^T, KSL-9, KSL-10 and KSL-12 and some other related taxa. Scale bar represents 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) of >50% are shown at branch points.

a novel species, for which the name *Nocardioides alkalitolerans* sp. nov. is proposed.

Description of *Nocardioides alkalitolerans* sp. nov.

Nocardioides alkalitolerans (al.ka.li.to'le.rans. Arabic article *al* the; Arabic n. *qaliy* ashes of saltwort; L. part. adj. *tolerans* tolerating; N.L. masc. part. adj. *alkalitolerans* referring to the ability to tolerate high pH).

Cells are aerobic, non-spore-forming rods (0.8–1.0 µm × 1.5–2.0 µm) in the exponential phase of growth. Cells show rod-to-coccus morphogenesis from the early exponential phase to the stationary phase. Gram-positive but Gram-variable in old cultures. Colonies are circular, smooth, glistening, convex, milky-white in colour and 0.7–1.0 mm in diameter after 7 days incubation on twofold-diluted NA (pH 9.0). Neither substrate nor aerial mycelia are formed. Optimal temperature for growth is 25–30 °C; grows at 4 and 34 °C, but not above 35 °C. Optimal pH for growth is 7.0–9.0; grows at pH 5.5 and 12.0, but growth at pH 5.0 is variable (no growth for type strain). Grows in the presence of 0–5% (w/v) NaCl. Oxidase-positive. Tweens 20, 40 and 60 are hydrolysed. Adonitol and D-sorbitol are not utilized. Cell-wall peptidoglycan contains LL-diaminopimelic acid as the diagnostic diamino acid. Predominant menaquinone is MK-8(H₄). Major fatty acids are iso-C_{16:0}, 10-methyl-C_{18:0}, C_{18:1}ω9c and C_{17:1}ω6c. DNA G+C content is 72.4–73.6 mol% (72.4 mol% for type strain) (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, KSL-1^T (= KCTC 19037^T = DSM 16699^T), was isolated from an alkaline serpentinite soil in Korea. Reference strains are KSL-9, KSL-10 and KSL-12.

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