Nocardioides kribbensis sp. nov., isolated from an alkaline soil

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Three Gram-positive, rod- or coccoid-shaped bacterial strains, KSL-2^T, KSL-5 and KSL-6, were isolated from an alkaline soil in Korea and subjected to a polyphasic taxonomical analysis. These isolates grew optimally at pH 9.0 and 30 °C. They were characterized chemotaxonomically as having cell wall peptidoglycan based on LL-2,6-diaminopimelic acid, MK-8(H₄) as the predominant menaquinone and iso-C_{16:0} as the major fatty acid. The DNA G+C content of the isolates was 73–74 mol%. Strains KSL-2^T, KSL-5 and KSL-6 were identical in their 16S rRNA gene sequences and exhibited DNA–DNA relatedness values of 88–93%. Phylogenetic trees based on 16S rRNA gene sequences showed that the three isolates fell within the evolutionary radiation encompassed by the genus *Nocardioides*. Levels of 16S rRNA gene sequence similarity between the three strains and the type strains of *Nocardioides* species ranged from 93.6% (with *Nocardioides albus*) to 97.2% (with *Nocardioides aquiterrae*). DNA–DNA relatedness levels between the three isolates and *N. aquiterrae* CJ-14^T were 8–15%. On the basis of phenotypic, phylogenetic and genetic data, strains KSL-2^T, KSL-5 and KSL-6 were classified in the genus *Nocardioides* as members of a novel species for which the name *Nocardioides kribbensis* sp. nov. is proposed, with KSL-2^T (= KCTC 19038^T = DSM 16314^T) as the type strain.

The genus *Nocardioides* was proposed by Prauser (1976) and, at the time of writing, comprises 12 species, *Nocardioides albus* (Prauser, 1976), *N. luteus* (Prauser, 1984), *N. simplex* (O'Donnell *et al.*, 1982), *N. jensenii* (Suzuki & Komagata, 1983; Collins *et al.*, 1989), *N. plantarum* (Collins *et al.*, 1994), *N. pyridinolyticus* (Yoon *et al.*, 1997), *N. nitrophenolicus* (Yoon *et al.*, 1999), *N. aquaticus* (Lawson *et al.*, 2000), *N. aquiterrae* (Yoon *et al.*, 2004), *N. ganghwensis* (Yi & Chun, 2004a), *N. aestuarii* (Yi & Chun, 2004b) and *N. alkalitolerans* (Yoon *et al.*, 2005). In this study, we describe the taxonomic characterization of three *Nocardioides*-like strains, KSL-2^T, KSL-5 and KSL-6, with cell wall peptidoglycan based on LL-2,6-diaminopimelic acid (DAP), which were isolated from an alkaline soil (approximate pH 9.0–10.0) in Korea.

Strains KSL-2^T, KSL-5 and KSL-6 were isolated by the usual dilution plating technique at 30 °C on 10 × diluted nutrient agar (NA; Difco) with pH adjusted to 10.0. 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. Gram-reaction was determined using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Growth at temperatures between 4 and 45 °C was measured on 2 × diluted NA (pH 9.0). Growth at different NaCl concentrations was investigated in trypticase soy broth (Difco) lacking NaCl and in conventional trypticase soy broth. The pH range for growth was determined in 2 × diluted nutrient broth (NB; Difco) that was adjusted to various pH values (initial pH 4.0–12.0 at intervals of 0.5 pH units). The pH of the 2 × diluted NB was adjusted prior to sterilization by the addition of Na₂CO₃ (below pH 10.5) or KOH (above pH 10.5). Growth under anaerobic conditions was determined after incubation in an anaerobic chamber with 2 × 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). Utilization of various substrates as the sole carbon and energy sources was determined according to Shirling & Gottlieb (1966). Enzyme activity was determined using the API ZYM system (bioMérieux).

Cell biomass for cell wall and isoprenoid quinone analyses and for DNA extraction was obtained from cultivation in

Abbreviation: DAP, 2,6-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KSL-2^T, KSL-5 and KSL-6 are AY835924, AY835925 and AY835926, respectively.

A table detailing the fatty acid composition of the three isolates is available as supplementary material in IJSEM Online.

Table 1. Differential phenotypic characteristics of *Nocardioides kribbensis* sp. nov. and some related *Nocardioides* species

Species: 1, *N. kribbensis* sp. nov.; 2, *N. aquaticus*; 3, *N. alkalitolerans*; 4, *N. pyridinolyticus*; 5, *N. aquiterrae*; 6, *N. plantarum*; 7, *N. ganghwensis*; 8, *N. aestuarii*. Data taken from Collins *et al.* (1989, 1994), Lawson *et al.* (2000), Yoon *et al.* (1997, 2004, 2005) and Yi & Chun (2004a, b). +, Positive reaction; –, negative reaction; ND, not determined; W, weakly positive reaction; V, variable reaction (data in parentheses are for the type strain). All species or strains are positive for Gram-stain, catalase, casein hydrolysis, esterase lipase (C8), acid phosphatase and α -glucosidase. All species or strains are negative for acid-fastness, hydrolysis of urea and hypoxanthine, α -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -fucosidase and α -mannosidase.

Characteristic	1	2	3	4	5	6	7	8
Cell morphology	Short rods, cocci	Cocci, short rods	Rods, cocci	Rods, cocci	Rods, cocci	Short rods, cocci	Rods	Rods
Motility	–	–	–	+	+	–	–	–
Optimal temp (°C)	30	16–26	25–30	35	30	25	30	30
Nitrate reduction	+	+	+	+	+	–	+	–
Hydrolysis of:								
Aesculin	+	–	–	+	+	W	W	W
Starch	–	W*	–	+	–	–	+	–
Gelatin	+	+	V (+)	+	+	+	+	+
Tween 80	+	+	+	–	+	+	+	+
Xanthine	–	–	–	+	–	–	W	–
Tyrosine	+	+	+	+	–	–	+	–
Utilization of:								
L-Arabinose	–	–	+	–	–	–	+	–
D-Cellobiose	+	–	+	+	+	+	+	+
D-Fructose	–	+	–	+	+	+	+	+
D-Galactose	+	–	–	+	+	–	+	+
D-Glucose	+	+	–	+	+	+	+	+
Inositol	–	W*	–	+	–	–	–	–
Lactose	–	–	–	+	+	–	+	W
Maltose	+	W	V (–)	+	+	+	ND	ND
D-Mannitol	+	+	–	+	+	–	+	+
D-Mannose	–	–	–	+	+	–	+	–
Melezitose	–	–	+	ND	–	+	ND	ND
D-Melibiose	–	W	–	–	–	ND	ND	ND
D-Raffinose	+	–	+	–	–	–	+	W
L-Rhamnose	+	+	–	+	W*	+	–	–
D-Ribose	+	–	–	+	W*	–	–	–
Sucrose	+	+	–	+	+	+	+	+
D-Trehalose	+	W	+	+	+	+	ND	ND
D-Xylose	–	W*	–	+	+	+	+	+
API ZYM								
Alkaline phosphatase	+	V	+	+	–	–	+	W
Esterase (C4)	+	W	+	–	–	+	V	+
Lipase (C14)	–	–	+	–	–	W	–	–
Leucine arylamidase	+	+	–	+	+	+	+	+
Valine arylamidase	–	W	–	W	–	W	+	+
Cystine arylamidase	–	V	–	–	W	+	W	–
Trypsin	–	W	–	+	+	–	–	+
α -Chymotrypsin	+	–	–	–	–	–	–	+
Naphthol-AS-BI-phosphohydrolase	+	–	W	+	+	+	–	W
β -Galactosidase	W	–	–	–	–	–	+	+
β -Glucosidase	–	–	–	–	W	+	–	–
DNA G + C content (mol%)	73–74	69	72.4–73.6	73	73	69	72	70
Isolation source	Alkaline soil	Saline lake, Antarctica	Alkaline soil	Oil shale column	Ground water	Herbage	Tidal flat	Tidal flat

*Data from Yoon *et al.* (1997, 2004, 2005) and Lawson *et al.* (2000); different result was obtained by Yi & Chun (2004a).

2 × diluted NB at 30 °C. Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC. Chromosomal DNA was isolated and purified according to Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize RNA contamination. For fatty acid methyl ester analysis, cell mass was harvested from agar plates after incubation for 5 days on 2 × diluted NA (pH 9.0) at 30 °C. The fatty acid methyl esters 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 a modified method of Tamaoka & Komagata (1984) in which 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 according to Yoon *et al.* (2004). DNA–DNA hybridization was performed fluorometrically using photobiotin-labelled DNA probes and microdilution wells (Ezaki *et al.*, 1989). Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded and the means of the remaining three values are quoted as DNA–DNA relatedness values.

Morphological, cultural, physiological and biochemical characteristics of strains KSL-2^T, KSL-5 and KSL-6 are given in the species description (see below) and are shown in Table 1. The 16S rRNA gene sequences of strains KSL-2^T, KSL-5 and KSL-6 determined in this study comprised 1476 nucleotides, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. The 16S rRNA gene sequences of the three isolates were identical. In a neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, the three strains fell within the cluster of the radiation comprising *Nocardioides* species (Fig. 1). Similar topologies were found in the tree generated with the maximum-likelihood algorithm (data not shown). Strains KSL-2^T, KSL-5 and KSL-6 exhibited 16S rRNA

gene sequence similarity values of 97.2 % to *N. aquiterrae* CJ-14^T and 93.6–96.8 % to the type strains of the other *Nocardioides* species. Sequence similarities to all other species of the family *Nocardioidaceae* included in the phylogenetic analysis were lower than 94.7 % (Fig. 1).

Chemotaxonomic data support the result of monothetic phylogenetic classification of the three strains as members of the genus *Nocardioides*. The strains contained LL-DAP as the diagnostic diamino acid in the cell wall peptidoglycan, characteristic of wall chemotype I *sensu* Lechevalier & Lechevalier (1970), and had MK-8(H₄) as the predominant menaquinone. The fatty acid profiles were characterized by a common core of straight-chain, branched, unsaturated and 10-methyl fatty acids in similar amounts; the major fatty acid was iso-C_{16:0} (42.4–42.6 %) (fatty acid profiles are presented in Supplementary Table S1 in IJSEM Online). The fatty acid profiles were similar to those of *Nocardioides* species (Yoon *et al.*, 1997, 2004; Lawson *et al.*, 2000; Yi & Chun, 2004a). The DNA G+C content of strains KSL-2^T, KSL-5 and KSL-6 was 73, 74 and 74 mol%, respectively.

DNA–DNA relatedness levels between strains KSL-2^T, KSL-5 and KSL-6 were in the range of 88–93 %, indicating that the three strains are members of the same genomic species (Wayne *et al.*, 1987). They were also almost identical in phenotypic properties. The three strains exhibited mean levels of DNA–DNA relatedness of 8–15 % to *N. aquiterrae* CJ-14^T, the closest phylogenetic neighbour, when their DNAs were used individually as labelled DNA probes for cross-hybridization. The phylogenetic distinctiveness and DNA–DNA relatedness data were sufficient to categorize strains KSL-2^T, KSL-5 and KSL-6 as a species that is distinct from the previously recognized *Nocardioides* species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). There were some differences in phenotypic characteristics between the three strains and some phylogenetically related *Nocardioides* species (Table 1). On the basis of the data presented, strains KSL-2^T, KSL-5 and KSL-6 should be

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain KSL-2^T and some other related taxa. Bar, 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) >50 % are shown at branch points.

classified in the genus *Nocardioidea* as members of a novel species, for which the name *Nocardioidea kribbensis* sp. nov. is proposed.

Description of *Nocardioidea kribbensis* sp. nov.

Nocardioidea kribbensis (kri.bben'sis. N.L. masc. adj. *kribbensis* arbitrary name formed from the acronym of the Korea Research Institute of Bioscience and Biotechnology, KRIBB, where taxonomic studies on this species were performed).

Cells are aerobic, non-spore-forming rods ($0.8\text{--}1.0 \times 1.5\text{--}2.0\text{ }\mu\text{m}$) 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, slightly convex, cream in colour and $1.0\text{--}1.5\text{ mm}$ in diameter after 6 days incubation on $2 \times$ diluted NA (pH 9.0). Neither substrate nor aerial mycelia are formed. Grows at 4 and $35\text{ }^{\circ}\text{C}$, but not at $36\text{ }^{\circ}\text{C}$. Optimal pH for growth is 9.0; growth occurs at pH 6.0 and 11.0, but not at pH 5.5. Growth occurs in the presence of 0–3% (w/v) NaCl. Oxidase-positive. Tweens 20, 40 and 60 are hydrolysed. D-Sorbitol is utilized, but adonitol is not. The cell wall peptidoglycan contains LL-DAP as the diagnostic diamino acid. The predominant menaquinone is MK-8(H_4). The major fatty acid is iso- $\text{C}_{16:0}$. The DNA G+C content is 73–74 mol% (74 mol% for type strain). Other phenotypic characteristics are given in Table 1.

The type strain, KSL-2^T (=KCTC 19038^T=DSM 16314^T), was isolated from an alkaline soil in Korea.

Acknowledgements

This work was supported by 21C Frontier program of Microbial Genomics and Applications (grant MG02-0401-001-1-0-0) from the Ministry of Science and Technology (MOST) of the Republic of Korea.

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