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

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A Gram-positive, rod- or coccus-shaped bacterial strain, KSL-104^T, was isolated from an alkaline soil from Korea and its taxonomic position was investigated by a polyphasic approach. Strain KSL-104^T grew optimally at pH 7.0–8.0 and 30 °C. It was characterized chemotaxonomically as having a cell-wall peptidoglycan type based on LL-2,6-diaminopimelic acid with MK-8(H₄) as the predominant menaquinone. The major fatty acid was iso-C_{16:0} and the major polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol. The DNA G+C content was 70.6 mol%. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences revealed that strain KSL-104^T joined the cluster comprising *Nocardioides jensenii* and *Marmoricola aurantiacus*. The cellular fatty acid profile of strain KSL-104^T was different from that of *M. aurantiacus*. Strain KSL-104^T and the type strain of *N. jensenii* exhibited a 16S rRNA gene sequence similarity value of 97.1 % and a mean DNA–DNA relatedness value of 13 %. Levels of 16S rRNA gene sequence similarity between strain KSL-104^T and the type strains of other *Nocardioides* species were in the range 94.0–96.5 %. On the basis of phenotypic, phylogenetic and genetic data, strain KSL-104^T (= KCTC 9992^T = JCM 13008^T) represents a novel species of the genus *Nocardioides*, for which the name *Nocardioides dubius* sp. nov. is proposed.

The genus *Nocardioides* was proposed by Prauser (1976) with a single species, *Nocardioides albus*, and, at the time of writing, the genus comprises 14 species with validly published names (Schippers *et al.*, 2005; Yoon *et al.*, 2005b). Recently, a bacterial strain, KSL-104^T, containing LL-2,6-diaminopimelic acid (DAP) was isolated from an alkaline soil (approximate pH 9.0–10.0) in Korea. 16S rRNA gene sequence analyses indicated that strain KSL-104^T is phylogenetically related to *Nocardioides jensenii* and *Marmoricola aurantiacus*. *N. jensenii* was proposed by the reclassification of a species that had been previously assigned to the genus *Pimelobacter* (Suzuki & Komagata, 1983; Collins *et al.*, 1989). 16S rRNA gene sequence analyses showed that *N. jensenii* forms an evolutionary lineage at the periphery of the phylogenetic radiation encompassed by the genus *Nocardioides* and joins the genus *Marmoricola* with a high bootstrap resampling value (Yoon *et al.*, 1998, 2005a, b; Urzì *et al.*, 2000; Yi & Chun, 2004a, b). The genus *Marmoricola* was described by Urzì *et al.* (2000) with a single species, *Marmoricola aurantiacus*, which was differentiated from the

genus *Nocardioides* and from *N. jensenii* by the absence of iso- and anteiso-branched fatty acids, the presence of phosphatidylinositol and the presence of only spherical cells, occurring singly, in pairs, tetrads and small clusters (Urzì *et al.*, 2000). Accordingly, the aim of the present study was to establish the exact taxonomic position of strain KSL-104^T by a polyphasic taxonomic approach.

An alkaline soil collected from Kwangchun, Korea, was used as the source for the isolation of bacterial strains. Strain KSL-104^T was isolated by the standard dilution plating technique at 30 °C on 10 × diluted nutrient agar (NA; Difco) with the pH adjusted to 10.0 using Na₂CO₃. To investigate its morphological, physiological and biochemical characteristics, strain KSL-104^T was routinely cultivated on NA at 30 °C. *N. jensenii* KCTC 9134^T, which was used as the reference strain for DNA–DNA hybridization, was obtained from the Korean Collection for Type Cultures, Taejeon, Korea. Morphological, physiological and biochemical properties were examined as described by Yoon *et al.* (2005c). Growth at various NaCl concentrations [0–8 % (w/v) at intervals of 1.0 %] was investigated in trypticase soy broth (TSB; Difco) lacking NaCl and TSB supplemented with NaCl. The pH range for growth was determined in nutrient broth (NB; Difco) that was adjusted to various pH values (initial pH 4.0–10.5 at intervals of 0.5 pH units). The pH of the NB was adjusted prior to sterilization by the

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Abbreviation: DAP, 2,6-diaminopimelic acid.

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addition of HCl or Na₂CO₃. Other physiological and biochemical tests were performed with the API 20E system (bioMérieux). Cell biomass for the analyses of cell-wall components, isoprenoid quinones, polar lipids and for DNA extraction was obtained by cultivation in NB at 30 °C. For fatty acid methyl ester analysis, cell mass of strain KSL-104^T was harvested from agar plates after incubation for 6 days on NA at 30 °C. Chemotaxonomic and molecular systematic studies were performed as described by Yoon *et al.* (2005c). Polar lipids were extracted according to Minnikin *et al.* (1984) and were identified using two-dimensional TLC followed by spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987).

The morphological, cultural, physiological and biochemical characteristics of strain KSL-104^T are given in the species description (see later) or are shown in Table 1. The 16S rRNA gene sequence of strain KSL-104^T determined in this study comprised 1473 nt, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. Comparative 16S rRNA gene sequence analyses showed that strain KSL-104^T was phylogenetically affiliated to the genera *Nocardioide*s and *Marmoricola* (Fig. 1). In the neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, strain KSL-104^T joined the cluster comprising *N. jensenii* and *M. aurantiacus* with a bootstrap resampling value of 69.4 % (Fig. 1). In trees generated with the maximum-likelihood and the maximum-parsimony algorithms, strain KSL-104^T joined the cluster comprising *Nocardioide*s species but excluding *N. jensenii* (data not shown). Strain KSL-104^T exhibited 16S rRNA gene sequence similarity levels of 97.1 % compared with the type strain of *N. jensenii* and 94.0–96.5 % with the type strains of the other *Nocardioide*s species. The similarity value between the 16S rRNA gene sequence of strain KSL-104^T and that of *M. aurantiacus* DSM 12652^T was 95.4 %.

Strain KSL-104^T contained LL-DAP as the diagnostic diamino acid in the cell-wall peptidoglycan and the predominant menaquinone was MK-8(H₄), which was the same as that found for *Nocardioide*s species and *M. aurantiacus* (Yoon *et al.*, 1997, 2004, 2005a, b; Lawson *et al.*, 2000; Urzì *et al.*, 2000; Yi & Chun, 2004a, b). Strain KSL-104^T had a cellular fatty acid profile that contained large amounts of branched, unsaturated, straight-chain and 10-methyl fatty acids. Major components (> 1 %) were branched fatty acids iso-C_{16:0} (58.5 %), iso-C_{18:0} (4.3 %), iso-C_{16:1} (3.7 %), iso-C_{17:0} (3.2 %), iso-C_{14:0} (2.5 %), anteiso-C_{17:0} (2.0 %) and iso-C_{15:0} (1.9 %), straight-chain fatty acids C_{18:0} (2.1 %) and C_{16:0} (1.2 %), unsaturated fatty acids C_{18:1ω9c} (1.7 %), C_{17:1ω8c} (1.3 %) and C_{18:1ω7c} (1.1 %) and 10-methyl fatty acids C_{17:0} (7.0 %), C_{16:0} (4.2 %) and C_{18:0} (2.7 %). This fatty acid profile was similar to those of *Nocardioide*s species (Yoon *et al.*, 1997, 2004, 2005a, b; Lawson *et al.*, 2000; Urzì *et al.*, 2000; Yi & Chun, 2004a). The major polar lipids detected in strain KSL-104^T were diphosphatidylglycerol, phosphatidylglycerol,

Table 1. Differential phenotypic characteristics of *N. dubius* sp. nov. and *N. jensenii*

Data for *N. jensenii* were taken from Collins *et al.* (1989), Suzuki & Komagata (1983) and Yi & Chun (2004a). The two species are yellowish-white, rods/cocci, are positive or weakly positive for Gram-stain, catalase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, hydrolysis of casein and gelatin and for utilization of D-glucose and sucrose. The two species are negative for acid-fastness, lipase (C14), cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-fucosidase, α-mannosidase, starch hydrolysis and utilization of L-arabinose, D-cellobiose, D-fructose, D-galactose, D-mannose, D-ribose or D-xylose. +, Positive; –, negative; W, weakly positive; V, variable reaction.

Characteristic	<i>N. dubius</i> sp. nov.	<i>N. jensenii</i>
Cell size (μm)	0.8–1.0 × 1.5–2.5	0.6–0.8 × 3.0–7.0
Motility	+	–
Oxidase	+	–
Nitrate reduction	–	+
Hydrolysis of:		
Aesculin	+	–
Hypoxanthine	–	+
Tween 80	–	+
Tyrosine	–	+
Urea	–	+
Xanthine	–	+
Utilization of:		
Acetate	–	+
Benzoate	–	+
Citrate	–	+
Salicin	+	–
Succinate	+	–
API ZYM:		
α-Glucosidase	+	V
β-Glucosidase	+	–
Acid phosphatase	+	V
Naphthol-AS-BI-phosphohydrolase	+	V
Trypsin	–	W
Valine arylamidase	–	V
DNA G + C (mol%)	70.6	68.8

*Data taken from Yi & Chun (2004a); a different result was obtained by Suzuki & Komagata (1983).

phosphatidylethanolamine and phosphatidylinositol. The DNA G + C content of strain KSL-104^T was 70.6 mol%.

Strain KSL-104^T was differentiated from *M. aurantiacus* by having a rod/coccus life cycle and by exhibiting a different fatty acid profile (Urzì *et al.*, 2000). Phosphatidylinositol was described as being a marker to differentiate the genera *Nocardioide*s and *Marmoricola* by Urzì *et al.* (2000), but this polar lipid was also reported to be present in *N. jensenii* and *Nocardioide*s *aquaticus* (Collins *et al.*, 1989; Lawson

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain KSL-104^T and some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) of >50% are shown at branch points. Bar, 0.01 substitutions per nucleotide position.

et al., 2000). There were not sufficient phenotypic, and particularly chemotaxonomic, properties to distinguish strain KSL-104^T from the genus *Nocardioides* (Yoon *et al.*, 1997, 2004, 2005a, b; Lawson *et al.*, 2000; Yi & Chun, 2004a, b). Although phosphatidylethanolamine was not found in *Nocardioides* species, inter-specific differences in polar lipid profiles have been found in many other genera as well as in the genus *Nocardioides* (Collins *et al.*, 1989; Park *et al.*, 1999; Lawson *et al.*, 2000). The chemotaxonomic properties of strain KSL-104^T were distinguishable from those of the other genera that belong to the family *Nocardioidaceae* (Miller *et al.*, 1991; Tamura & Yokota, 1994; Park *et al.*, 1999; Wang *et al.*, 2001). Accordingly, in view of the combined phenotypic and phylogenetic data, it appears to be appropriate for strain KSL-104^T to be placed in the genus *Nocardioides*. Strain KSL-104^T was distinguishable from *N. jensenii* by some phenotypic characteristics, in particular, the presence of phosphatidylethanolamine, as listed in Table 1, and the mean DNA–DNA relatedness value with *N. jensenii* KCTC 9134^T was 13%. Therefore, on the basis of the data presented, strain KSL-104^T should be classified in the genus *Nocardioides* as a representative of a novel species, for which the name *Nocardioides dubius* sp. nov. is proposed.

Description of *Nocardioides dubius* sp. nov.

Nocardioides dubius (du'bi.us. L. masc. adj. *dubius* doubtful of the taxonomic position).

Cells are aerobic, non-endospore-forming rods (0.8–1.0 × 1.5–2.5 µm) in the exponential phase of growth. Cells show rod-to-coccus morphogenesis from the early exponential phase to the stationary phase. Gram-positive. Motile by means of a single polar flagellum. Colonies are irregular, smooth, raised with erose margins, yellowish-white in colour and 2.0–3.0 mm in diameter after 3 days incubation on NA at 30 °C. Neither substrate nor aerial mycelia are formed. Growth occurs at 10 and 37 °C, but not at 4 or 38 °C. Optimal pH for growth is 7.0–8.0; growth occurs at pH 6.0 and 10.5, but not at pH 5.5. Growth

occurs in the presence of 0–5% (w/v) NaCl, with an optimum at 0% (w/v) NaCl. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are absent. H₂S and indole are not produced. Tweens 20, 40 and 60 are not hydrolysed. Pyruvate is utilized, but maltose, D-trehalose, formate and L-malate are not. The cell-wall peptidoglycan contains LL-DAP as the diagnostic diamino acid. The predominant menaquinone is MK-8(H₄). The major fatty acid is iso-C_{16:0}. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol. The DNA G + C content is 70.6 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, KSL-104^T (= KCTC 9992^T = JCM 13008^T), was isolated from an alkaline soil in Kwangchun, Korea.

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