Nocardioides daedukensis sp. nov., a halotolerant bacterium isolated from soil

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A Gram-positive, non-motile and rod- or coccoid-shaped bacterial strain, MDN22^T, was isolated from a soil sample from Korea. Strain MDN22^T grew optimally at pH 7.0–8.0, at 30 °C and in the presence of 0–0.5 % (w/v) NaCl. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain MDN22^T was phylogenetically most closely related to the genera *Nocardioides* and *Marmoricola*. In the neighbour-joining phylogenetic tree, strain MDN22^T was most closely related to *Nocardioides jensenii* KCTC 9134^T, with which it exhibited 98.3 % 16S rRNA gene sequence similarity. The strain exhibited 93.1–96.9 % and 95.3–95.9 % 16S rRNA gene sequence similarities to the type strains of other species of the genera *Nocardioides* and *Marmoricola*, respectively. The chemotaxonomic properties of strain MDN22^T were consistent with those of the genus *Nocardioides*; the cell-wall peptidoglycan type was based on LL-2,6-diaminopimelic acid, the predominant menaquinone was MK-8(H₄) and the major fatty acids were iso-C_{16:0} and C_{17:1}. The DNA G + C content was 68.7 mol%. DNA–DNA relatedness data and differential phenotypic properties suggested that strain MDN22^T could be differentiated from *N. jensenii* and *Nocardioides dubius*. On the basis of the data obtained, strain MDN22^T is considered to represent a novel species of the genus *Nocardioides*, for which the name *Nocardioides daedukensis* sp. nov., is proposed. The type strain is MDN22^T (=KCTC 19601^T=CCUG 57505^T).

The genus *Nocardioides* was first proposed by Prauser (1976) with a single species, *Nocardioides albus*. Phylogenetically, the genus forms its evolutionary lineage within the family *Nocardioideaceae* of the suborder *Propionibacterineae* (Stackebrandt *et al.*, 1997). Recently, increasing numbers of descriptions of novel species, e.g. 9 species in 2008, have enlarged the size of the genus *Nocardioides* (Euzéby, 1997). At the time of writing, the genus *Nocardioides* comprises at least 36 species with validly published names (Euzéby, 1997). In this study, we describe a halotolerant bacterial strain, MDN22^T, which was isolated from a soil sample from Korea, and establish its taxonomic position by using a polyphasic approach that included the determination of phenotypic and chemotaxonomic properties and a detailed phylogenetic investigation based on 16S rRNA gene sequences.

Strain MDN22^T was isolated by means of the standard dilution-plating technique on 10 × diluted nutrient agar (NA; Difco) at 30 °C. *Nocardioides jensenii* KCTC 9134^T and *Nocardioides dubius* KSL-104^T were used as reference strains for DNA–DNA hybridization, fatty acid analysis

and phenotypic characterization. *N. jensenii* KCTC 9134^T was obtained from the KCTC, Taejeon, Korea, and *N. dubius* KSL-104^T was obtained from our previous study (Yoon *et al.*, 2005b). The morphological, physiological and biochemical characteristics of strain MDN22^T were investigated using routine cultivation at 30 °C on NA and were determined as described by Yoon *et al.* (2005a). Cultural properties were examined as described by Yoon *et al.* (2005a): growth at various temperatures (4, 10, 20, 25, 28, 30, 35, 37, 40 and 45 °C) was measured on NA; growth with various NaCl concentrations (0, 0.5 and 1.0–10.0 %, w/v, in increments of 1 %) was investigated in trypticase soy broth prepared according to the formula of the Difco medium except that NaCl was excluded from the formula; and growth at various pH values was determined in nutrient broth (NB; Difco) adjusted to pH 4.5–10.5 in increments of 0.5 pH units by the addition of HCl or Na₂CO₃. Susceptibility to antibiotics was tested by placing antibiotic-impregnated discs on NA plates that were seeded with suspensions of the test strain. Enzyme activities and other physiological and biochemical properties were tested by using the API ZYM and API 20E systems (bioMérieux), respectively. Utilization of various substrates as sole carbon and energy sources was determined as described by Shirling & Gottlieb (1966). The morphological, cultural, physiological

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MDN22^T is FJ842646.

and biochemical characteristics of strain MDN22^T are given in Table 1 and in the species description.

Cell biomass for DNA extraction and for the analyses of cell-wall components, isoprenoid quinones and polar lipids was obtained from cultures grown in NB for 7 days at 30 °C. For cellular fatty acid analysis, cell mass of strain MDN22^T, *N. jensenii* KCTC 9134^T and *N. dubius* KSL-104^T was harvested from NA plates after cultivation for 7 days at 30 °C. Molecular systematic and chemotaxonomic properties were examined as described by Yoon *et al.* (2005a, b). Alignment of 16S rRNA gene sequences was carried out with CLUSTAL W software (Thompson *et al.*, 1994). Gaps at the 5' and 3' ends of the alignment were omitted from further analysis. Phylogenetic trees were inferred by using three tree-making algorithms in the PHYLIP package

(Felsenstein, 1993), i.e. neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods. Evolutionary distance matrices for the neighbour-joining method were calculated with the algorithm of Jukes & Cantor (1969) with the DNADIST program. The isomer of the diamino acid in the cell-wall peptidoglycan was analysed using TLC according to the method described by Komagata & Suzuki (1987). The fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). DNA–DNA hybridization was performed fluorometrically according to the procedure of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed by using five replications for

Table 1. Differential phenotypic characteristics of strain MDN22^T, *N. jensenii* and *N. dubius*

Strains: 1, MDN22^T; 2, *N. jensenii* KCTC 9134^T; 3, *N. dubius* KSL-104^T. Data are taken from Collins *et al.* (1989), Yi & Chun (2004), Yoon *et al.* (2005b) and this study. All strains are rods/cocci and give a positive result for Gram-staining, catalase, hydrolysis of casein and gelatin, activity of esterase lipase (C8) and leucine arylamidase, and for the utilization of acetate and pyruvate. All strains are negative for hydrolysis of starch, for activities of lipase (C14), valine and cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, α - and β -galactosidase, β -glucuronidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase and for the utilization of L-arabinose, cellobiose, D-fructose, D-galactose, maltose, D-mannose, D-xylose, benzoate, citrate, formate and succinate. +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3
Cell size (μ m)	0.4–0.8 $\times$ 0.8–3.0	0.6–1.0 $\times$ 2.0–7.0	0.8–1.0 $\times$ 1.5–2.5
Motility	–	–	+
Oxidase	+	–	+
Nitrate reduction	+	+	–
Hydrolysis of:			
Aesculin	+	–	+
Hypoxanthine	–	+	–
Tween 80	+	+	–
Tyrosine	–	+	–
Urea	–	+	–
Xanthine	–	+	–
Utilization of:*			
D-Glucose	–	+	+
L-Glutamate	–	+	+
L-Malate	+	+	–
Salicin	–	–	w
Sucrose	–	+	–
Trehalose	+	+	–
Enzyme activity (API ZYM)*			
Alkaline phosphatase	–	+	w
Esterase (C4)	–	+	w
Naphthol-AS-BI-phosphohydrolase	w	+	w
α -Glucosidase	–	–	+
β -Glucosidase	–	–	+
DNA G + C mol%	68.7	68.8	70.6

*Data for carbon source utilization and enzyme activity for *N. jensenii* KCTC 9134^T and *N. dubius* KSL-104^T were taken from this study.

each sample. The highest and lowest values obtained in each sample were excluded and the means of the remaining three values were quoted as DNA–DNA relatedness values.

The almost-complete 16S rRNA gene sequence of strain MDN22^T determined in this study comprised 1475 nucleotides (approx. 96 % of the *Escherichia coli* sequence). Comparative analysis of 16S rRNA gene sequences revealed that strain MDN22^T is phylogenetically most closely related to members of the genera *Nocardioides* and *Marmoricola* at the periphery of the phylogenetic radiation encompassed by the genus *Nocardioides* (Yoon *et al.*, 2005b; Lee, 2007; Dastager *et al.*, 2008). In the neighbour-joining phylogenetic tree, strain MDN22^T clustered with *N. jensenii* KCTC 9134^T with a bootstrap resampling value of 73.2 % and this cluster joined the cluster comprising species of the genus *Marmoricola* with a bootstrap resampling value of 96.0 % (Fig. 1). These relationships were also found in the tree constructed using the maximum-likelihood algorithm

(Fig. 1). Strain MDN22^T exhibited 16S rRNA gene sequence similarity values of 98.3, 96.9 and 93.1–96.2 % with *N. jensenii* KCTC 9134^T, *N. dubius* KSL-104^T and the type strains of other species of the genus *Nocardioides*, respectively, and 95.3–95.9 % with the type strains of members of the genus *Marmoricola*.

Strain MDN22^T had LL-2,6-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. The predominant isoprenoid quinone found in strain MDN22^T was menaquinone-8(H₄). The cellular fatty acid profile of strain MDN22^T is given in Table 2, together with those of *N. jensenii* KCTC 9134^T and *N. dubius* KSL-104^T, which were also analysed in this study. The fatty acid profile of strain MDN22^T was similar to those of the other species of the genus *Nocardioides* as well as *N. jensenii* KCTC 9134^T and *N. dubius* KSL-104^T in that iso-C_{16:0} was the predominant fatty acid (Yoon *et al.*, 1997, 1999; Yoon & Park, 2006; Kim *et al.*, 2009). The DNA G + C content of

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain MDN22^T, species of the genus *Nocardioides* and some other related taxa. Bootstrap values (>50 %) based on 1000 replications are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Open circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-likelihood algorithm. *Terrabacter tumescens* KCTC 9133^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Table 2. Cellular fatty acid compositions of strain MDN22^T, *N. jensenii* KCTC 9134^T, *N. dubius* KSL-104^T and three species of the genus *Marmoricola*

Strains: 1, MDN22^T; 2, *N. jensenii* KCTC 9134^T; 3, *N. dubius* KSL-104^T; 4, *M. aurantiacus* BC 361^T (data from Urzì *et al.*, 2000); 5, *M. aequoreus* SST-45^T (Lee, 2007); 6, *M. bigeumensis* MSL-05^T (Dastager *et al.*, 2008). Data for columns 1–3 were taken from this study. —, Values <1.0% or not detected; ND, no data available.

Fatty acid (%)	1	2	3	4	5	6
Straight-chain						
C _{14:0}	—	—	—	3.4	1.4	ND
C _{16:0}	0.6	2.6	1.1	41.4	35.0	4.0
C _{17:0}	1.4	1.1	—	—	2.9	ND
C _{18:0}	1.4	2.9	1.8	2.4	7.6	ND
Branched						
iso-C _{14:0}	—	—	3.0	—	—	3.3
iso-C _{15:0}	1.2	4.0	1.9	—	—	ND
iso-C _{16:0}	40.9	33.4	57.8	—	—	59.1
iso-C _{16:1}	3.5	10.9	5.1	—	—	ND
iso-C _{17:0}	—	2.1	2.4	—	—	ND
anteiso-C _{17:0}	—	1.4	1.7	—	—	6.9
iso-C _{18:0}	2.7	1.2	3.0	—	—	ND
Unsaturated						
C _{16:1}	—	—	—	14.9	6.6	ND
C _{17:1}	15.5	10.1	1.4	—	2.0	ND
C _{18:1}	7.6	4.6	2.8	33.7	40.1	ND
10-Methyl						
C _{16:0}	0.7	6.4	5.3	—	—	ND
C _{17:0}	12.2	5.5	8.6	—	—	ND
C _{18:0}	6.3	9.3	—	2.8	—	ND
Hydroxy						
C _{17:0} 3-OH	1.2	—	—	—	—	ND
Summed feature 3*	1.4	1.8	1.0	—	—	ND

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

strain MDN22^T was 68.7 mol%. The major polar lipids detected in strain MDN22^T were diphosphatidylglycerol, an unidentified phospholipid and two unidentified lipids; minor amounts of phosphatidylglycerol and phosphatidylethanolamine and an unidentified phospholipid were also detected. The polar lipid profile of strain MDN22^T was similar to those of *N. jensenii* KCTC 9134^T and *N. dubius* KSL-104^T in that diphosphatidylglycerol and phosphatidylglycerol are present in strain MDN22^T, but different in that phosphatidylinositol was absent in strain MDN22^T (Collins *et al.*, 1989; Yoon *et al.*, 2005b). The results of the chemotaxonomic analyses, showing that strain MDN22^T was similar in its properties to species of the genus *Nocardioides*, were in agreement with the result of phylogenetic analysis. Strain MDN22^T exhibited higher 16S rRNA gene sequence similarities with the type strains of *N. jensenii* and *N. dubius* than with members of the genus *Marmoricola* and its fatty acid profile was clearly

distinguishable from those of the type strains of *Marmoricola aurantiacus*, the type species of the genus, and *Marmoricola aequoreus* (Urzì *et al.*, 2000; Dastager *et al.*, 2008; Table 2). Accordingly, it is reasonable to classify strain MDN22^T as a member of the genus *Nocardioides*.

Strain MDN22^T exhibited mean DNA–DNA relatedness values of 19 and 10% with *N. jensenii* KCTC 9134^T and *N. dubius* KSL-104^T, respectively. Strain MDN22^T was distinguishable from *N. jensenii* and *N. dubius* by differences in several phenotypic characteristics as shown in Table 1. The phylogenetic distinctiveness of strain MDN22^T with respect to the other species of the genus *Nocardioides*, together with the genetic distinctiveness and differential phenotypic properties between strain MDN22^T and the two most closely phylogenetically related members of the genus *Nocardioides*, are sufficient to support the suggestion that it represents a separate species from the recognized species of the genus *Nocardioides* (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). On the basis of the data presented, strain MDN22^T represents a novel species of the genus *Nocardioides*, for which the name *Nocardioides daedukensis* sp. nov. is proposed.

Description of *Nocardioides daedukensis* sp. nov.

Nocardioides daedukensis (dae.duk.en'sis. N.L. masc. adj. *daedukensis* pertaining to Daeduk Science Park, the location of the Korea Research Institute of Bioscience and Biotechnology, where taxonomic studies of this species were performed).

Cells are aerobic, non-spore-forming, non-motile rods or cocci (0.4–0.8 × 0.8–3.0 μm). Cells show rod-to-coccus morphogenesis from the early exponential phase to the stationary phase. Gram-staining-positive, but Gram-staining-variable in old cultures. Colonies on NA are circular, raised, glistening, smooth, pale yellow in colour and 1.0–1.5 mm in diameter after incubation for 7 days at 30 °C. Neither substrate nor aerial mycelium is formed. Optimal temperature for growth is 30 °C. Growth occurs at 4 and 37 °C, but not at 40 °C. Optimal pH for growth is pH 7.0–8.0. Growth occurs at pH 6.0 and 10.0, but not at pH 5.5 or 10.5. Growth occurs in the presence of 0–9.0% (w/v) NaCl with an optimum in the presence of 0–0.5% (w/v) NaCl. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase activities are absent. H₂S and indole are not produced. Tweens 20, 40 and 60 are hydrolysed. Susceptible to the following antibiotics (μg per disc unless otherwise stated): carbenicillin (100), cephalothin (30), chloramphenicol (100), gentamicin (30), kanamycin (30), lincomycin (15), neomycin (30), oleandomycin (15), penicillin G (20 U), polymyxin B (100 U), streptomycin (50) and tetracycline (30). Resistant to the following antibiotics: ampicillin (10) and novobiocin (5). The cell-wall peptidoglycan is based on LL-2,6-diaminopimelic acid. The predominant menaquinone is MK-8(H₄). The major fatty acids (>10% of

total fatty acids) are iso-C_{16:0}, C_{17:1} and 10-methyl-C_{17:0}. The major polar lipids are diphosphatidylglycerol, an unidentified phospholipid and two unidentified lipids. Other phenotypic characteristics are given in Table 1.

The type strain, MDN22^T (=KCTC 19601^T=CCUG 57505^T), was isolated from a soil at Taejon, Korea. The DNA G+C content of the type strain is 68.7 mol% (HPLC).

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