Nocardioides caeni sp. nov., isolated from wastewater

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A Gram-positive, non-motile, rod- or coccoid-shaped bacterial strain, designated MN8^T, was isolated from sludge of domestic wastewater in Korea, and its taxonomic position was investigated by use of a polyphasic study. Strain MN8^T grew optimally at pH 6.5–7.5 and 30 °C and in the presence of 0–0.5 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain MN8^T fell within the cluster comprising *Nocardioides* species, clustering with *Nocardioides simplex* KCTC 9106^T, *Nocardioides aromaticivorans* H-1^T, *Nocardioides kongjuensis* A2-4^T and *Nocardioides nitrophenolicus* NSP41^T, with which it shared 98.4–99.0 % 16S rRNA gene sequence similarity. The chemotaxonomic properties of strain MN8^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_{18:1ω9c}. The DNA G+C content was 71.5 mol%. Strain MN8^T exhibited levels of DNA–DNA relatedness of 13–31 % to the type strains of phylogenetically related *Nocardioides* species and could be differentiated from these species based on differences in phenotypic characteristics. On the basis of the data presented, strain MN8^T is considered to represent a novel species of the genus *Nocardioides*, for which the name *Nocardioides caeni* sp. nov. is proposed. The type strain is MN8^T (=KCTC 19600^T=CCUG 57506^T).

The genus *Nocardioides*, first proposed by Prauser (1976), belongs phylogenetically to the family *Nocardioidaceae* of the suborder *Propionibacterineae* (Stackebrandt *et al.*, 1997). At the time of writing, the genus comprised at least 36 recognized species, including the recently described species *Nocardioides daphniae* (Tóth *et al.*, 2008), *N. hwasunensis* (Lee *et al.*, 2008), *N. hankookensis* (Yoon *et al.*, 2008), *N. fonticola* (Chou *et al.*, 2008), *N. salarius* (Kim *et al.*, 2008), *N. koreensis*, *N. bigeumensis* and *N. agariphilus* (Dastager *et al.*, 2008), *N. dokdonensis* (Park *et al.*, 2008), *N. basaltis* (Kim *et al.*, 2009) and *N. sediminis* (Dastager *et al.*, 2009). Here we report the taxonomic characterization of a *Nocardioides*-like bacterial strain, designated MN8^T, which was isolated from sludge of domestic wastewater.

Strain MN8^T was isolated by means of the standard dilution plating technique on nutrient agar (NA; Difco) at 30 °C.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MN8^T is FJ423551.

An extended neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain MN8^T among *Nocardioides* species and representatives of some other related taxa is available as supplementary material with the online version of this paper.

Nocardioides simplex KCTC 9106^T, *Nocardioides nitrophenolicus* KCTC 0457BP^T, *Nocardioides aromaticivorans* JCM 11674^T and *Nocardioides kongjuensis* A2-4^T were used as reference strains for DNA–DNA hybridization experiments. To investigate its morphological, physiological and biochemical characteristics, strain MN8^T was cultivated routinely on NA at 30 °C. Morphological, physiological, cultural and biochemical properties were examined as described by Yoon *et al.* (2005). Growth at various temperatures (4, 10, 20, 25, 28, 30, 35, 37, 40 and 45 °C) was measured on NA. Growth at various NaCl concentrations (0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 %, w/v) was investigated in trypticase soy broth prepared according to the formula of the Difco medium except that NaCl was excluded from the medium formula. The pH range for growth was determined in nutrient broth (NB; Difco) that was adjusted to various pH (4.5–10.5 at intervals of 0.5 pH units) by adding 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. Utilization of various substrates, enzyme activities, and other physiological and biochemical properties were tested by using the API 20E, API 20NE, API 50 CH and API ZYM systems (bioMérieux);

utilization of various substrates was determined by inoculating the API 50 CH strip with cells suspended in AUX medium (bioMérieux). Morphological, cultural, physiological and biochemical characteristics of strain MN8^T are given in the species description and in Table 1.

Cell biomass for DNA extraction and for analyses of cell-wall components and isoprenoid quinones was obtained from a culture grown in NB for 7 days at 30 °C. Molecular systematic and chemotaxonomic properties were examined as described by Yoon *et al.* (2005). Alignment of sequences

Table 1. Differential phenotypic characteristics between strain MN8^T and phylogenetically related *Nocardioides* species

Taxa: 1, strain MN8^T (*N. caeni* sp. nov.); 2, *N. kongjuensis* (data from Yoon *et al.*, 2006 and this study); 3, *N. aromaticivorans* (Kubota *et al.*, 2005; this study); 4, *N. nitrophenolicus* (Yoon *et al.*, 1999; this study); 5, *N. simplex* (Suzuki & Komagata, 1983; this study). All taxa are positive for Gram-stain, catalase, hydrolysis of casein* and gelatin*, utilization of sucrose, trehalose, gluconate, adipate and malate, and activities of alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase (weak), acid phosphatase and α -glucosidase. All are negative for nitrate reduction, hydrolysis of xanthine, utilization of glycerol, erythritol, D-arabinose, L-xylose, adonitol, galactose, sorbose, dulcitol, inositol, sorbitol, methyl α -D-mannoside, methyl α -D-glucoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, lactose, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate, 5-ketogluconate, caprate and phenylacetate, and activities of lipase (C14), α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. +, Positive reaction; –, negative reaction; w, weakly positive reaction; n, number of strains. Data in parentheses are for the type strain.

Characteristic	1	2 (n=1)	3 (n=7)	4 (n=1)	5 (n=3)
Cell morphology	Rods, cocci	Rods, cocci	Rods	Rods, cocci	Rods, cocci
Cell size (μ m)	0.3–0.7 $\times$ 0.7–2.5	0.4–0.7 $\times$ 0.8–3.0	0.5–0.7 $\times$ 1.0–2.0	0.5–0.8 $\times$ 1.0–3.0	1.0–1.2 $\times$ 1.5–6.0
Motility	–	–	–	+	+
Colony colour	Greyish yellow	Yellowish white	Milky white	Yellowish white	Yellowish white
Oxidase	+	+	–	+	(+)*
Hydrolysis of:					
Aesculin	–	–	(+)*	+	(+)*
Hypoxanthine	–	–	(+)*	–	(–)*
Starch	+	–	(–)*	+	(–)*
Tween 80	+	+	(+)*	+	(+)*
Urea	–	–	(+)*	+	(–)*
L-Tyrosine	–	–	(–)*	+	(+)*
Utilization of:†					
L-Arabinose	+	–	(+)	–	(–)
Cellobiose	+	–	(+)	–	(–)
Citrate	–	w	–	–	–
Fructose	–	+	(+)	+	(–)
Glucose	–	+	(+)	+	(+)
D-Lyxose	–	–	(w)	–	(–)
Maltose	–	–	(+)	–	(–)
Mannitol	–	–	(+)	–	(–)
Mannose	–	–	(+)	–	(–)
Methyl β -D-xyloside	–	–	(+)	–	(–)
Rhamnose	+	–	(+)	+	(–)
Ribose	–	–	(+)	+	(–)
Turanose	–	–	(+)	–	(–)
D-Xylose	–	–	(+)	+	(–)
API ZYM results†					
Esterase (C4)	–	w	(–)	–	(–)
Cystine arylamidase	w	–	(w)	w	(w)
Trypsin	–	+	(w)	w	(–)
Naphthol-AS-BI-phosphohydrolase	w	+	(w)	+	(w)
DNA G + C content (mol%)	71.5	72.1	72.0–72.4	71.4	72–74
Isolation source	Domestic wastewater	Soil	River water, sediment, soil	Industrial wastewater	Soil

*Data for the type strains of *N. aromaticivorans* and *N. simplex* are from the present study.

†Data for the type strains of the four reference species are from the present study.

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, i.e. the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods, in the PHYLIP package (Felsenstein, 1993). Evolutionary distance matrices for the neighbour-joining method were calculated with the algorithm of Jukes & Cantor (1969) with the DNADIST program. The DNA G+C content was reported as the mean of three replications. The isomer of the diamino acid in the cell-wall peptidoglycan was analysed by using TLC according to the method described by Komagata & Suzuki (1987). Fatty acid methyl ester analysis was performed once by using cell mass harvested from NA plates after incubation for 7 days at 30 °C. DNA–DNA hybridization was performed fluorometrically according to the procedure of Ezaki *et al.* (1989) by using photobiotin-labelled DNA probes and microdilution wells. Hybridization was carried out by using five replications for 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 MN8^T determined in this study comprised 1474 nt (approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence). Comparative analysis of 16S rRNA gene sequences revealed that strain MN8^T was related most closely to the genus *Nocardioideis*. In the neighbour-joining phylogenetic tree, strain MN8^T fell within the cluster comprising *Nocardioideis* species, clustering with the type strains of *N. kongjuensis*, *N. nitrophenolicus*, *N. simplex* and *N. aromaticivorans*. The clustering of strain MN8^T and these four *Nocardioideis* species was also found in the trees constructed with the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain MN8^T exhibited 98.4–99.0 % 16S rRNA gene sequence similarity to the type strains of the four *Nocardioideis* species given above and 93.0–95.8 % similarity to the type strains of other recognized *Nocardioideis* species.

Strain MN8^T had LL-2,6-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. The predominant isoprenoid quinone detected in strain MN8^T was menaquinone-8(H₄). The fatty acid profile of strain MN8^T comprised the following (each constituting >0.5 % of the total): branched fatty acids iso-C_{16:0} (40.3 %), iso-C_{17:0} (6.3 %), iso-C_{15:0} (2.9 %), iso-C_{18:0} (1.9 %), iso-C_{14:0} (1.0 %) and anteiso-C_{17:0} (0.8 %); unsaturated fatty acids C_{18:1ω9c} (16.4 %), C_{17:1ω8c} (8.6 %), C_{17:1ω6c} (8.0 %) and C_{18:1ω7c} (1.7 %); straight-chain fatty acids C_{16:0} (4.6 %), C_{17:0} (1.3 %) and C_{18:0} (1.2 %); summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH; 2.2 %); and 10-methyl C_{17:0} (0.6 %). This fatty acid profile was similar to those of recognized *Nocardioideis* species, although there were differences in the proportions of some components probably because of differences in

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain MN8^T among related *Nocardioideis* species. Bootstrap values (expressed as percentages of 1000 replications) of >50 % are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Terrabacter tumescens* KCTC 9133^T (GenBank accession number AF005023) was used as an outgroup (not shown). Bar, 0.01 substitutions per nucleotide position. The tree from which Fig. 1 was taken is available as Supplementary Fig. S1 in IJSEM Online.

cultivation and extraction conditions (Yoon *et al.*, 1997, 1999, 2006; Kubota *et al.*, 2005). The DNA G+C content of strain MN8^T was 71.5 mol%. The results obtained from chemotaxonomic analyses, showing the properties that are shared by *Nocardioideis* species, were in agreement with the result of the phylogenetic analysis, i.e. that strain MN8^T is a member of the genus *Nocardioideis*.

Strain MN8^T exhibited levels of DNA–DNA relatedness of 13–31 % to the type strains of its four closest phylogenetic relatives, namely *N. kongjuensis* (31 %), *N. nitrophenolicus* (22 %), *N. simplex* (13 %) and *N. aromaticivorans* (17 %). Strain MN8^T was distinguishable from these *Nocardioideis* species based on differences in several phenotypic characteristics (Table 1). The phylogenetic and genetic distinctiveness and differential phenotypic properties of strain MN8^T are sufficient to categorize it as a member of a species that is separate from recognized *Nocardioideis* species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). On the basis of the data presented, strain MN8^T is considered to represent a novel species of the genus *Nocardioideis*, for which the name *Nocardioideis caeni* sp. nov. is proposed.

Description of *Nocardioideis caeni* sp. nov.

Nocardioideis caeni (cae'ni. L. gen. n. *caeni* of sludge).

Cells are aerobic, non-spore-forming, non-motile rods or cocci (0.3–0.7 × 0.7–2.5 µm). Cells show rod-to-coccus morphogenesis from the early exponential phase to the stationary phase. Gram-positive but Gram-variable in old cultures. Colonies on NA are circular, raised, glistening, sticky, greyish yellow and 1.5–2.5 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 10 and 35 °C, but not at 4 or 37 °C. Optimal pH for growth is 6.5–7.5. Growth occurs at pH 6.0 and 9.5, but not at pH 5.5 or 10.0. Growth occurs in the presence of 0–1.0 % (w/v) NaCl, with optimum growth 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 (amounts per disc): carbenicillin (100 µg), cephalothin (30 µg), chloramphenicol (100 µg), gentamicin (30 µg), kanamycin (30 µg), neomycin (30 µg), novobiocin (5 µg), oleandomycin (15 µg), penicillin G (20 U), polymyxin B (100 U), streptomycin (50 µg) and tetracycline (30 µg). Resistant to the following antibiotics (amounts per disc): ampicillin (10 µg) and lincomycin (15 µg). The cell-wall peptidoglycan is based on LL-2,6-diaminopimelic acid. The predominant menaquinone is MK-8(H₄). Major fatty acids (>10 % of the total) are iso-C_{16:0} and C_{18:1ω9c}. The DNA G+C content of the type strain is 71.5 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, MN8^T (=KCTC 19600^T=CCUG 57506^T), was isolated from sludge of domestic wastewater in Korea.

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