Nocardioidea aquiterrae sp. nov., isolated from groundwater in Korea

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A bacterial strain, GW-9^T, which was isolated from groundwater in Korea, was subjected to a polyphasic taxonomic study using phenotypic characterization and phylogenetic and genetic methods. Phylogenetic analysis based on 16S rDNA sequences showed that strain GW-9^T forms an evolutionary lineage within the radiation enclosing *Nocardioidea* species and, in particular, a coherent cluster with *Nocardioidea pyridinolyticus*. The cell-wall peptidoglycan type of strain GW-9^T was based on LL-diaminopimelic acid as the diamino acid, indicating wall chemotype I. The predominant menaquinone was MK-8(H₄). Strain GW-9^T had a cellular fatty acid profile containing straight-chain, branched, unsaturated and 10-methyl fatty acids. The major fatty acid was iso-C_{16:0}. The DNA G + C content of strain GW-9^T was 73 mol%. The 16S rDNA of strain GW-9^T was 99.2 % similar to that of the type strain of *Nocardioidea pyridinolyticus* and 94.9–96.0 % similar to sequences of the type strains of other *Nocardioidea* species. Differences in phenotypic characteristics and genetic distinctiveness indicate that strain GW-9^T is separate from previously described *Nocardioidea* species. Therefore, on the basis of the data presented, a novel species of the genus *Nocardioidea*, *Nocardioidea aquiterrae* sp. nov., is proposed. The type strain is strain GW-9^T (=KCCM 41647^T=JCM 11813^T).

The first described *Nocardioidea* species were mainly isolated from various soils. *Nocardioidea albus* and *Nocardioidea luteus* were respectively isolated from soils collected in different parts of the world and soil of subtropical regions (Prauser, 1976, 1989). The type strain of *Nocardioidea simplex* was isolated from rice soil (Jensen, 1934) and atypical *N. simplex* strains were isolated from soil of the petroleum zone in Japan (Iizuka & Komagata, 1964). Subsequently, *Nocardioidea* species have been found in habitats other than soils, such as herbage (*Nocardioidea plantarum*; Collins *et al.*, 1994), an oil shale column (*Nocardioidea pyridinolyticus*; Yoon *et al.*, 1997) and industrial wastewater (*Nocardioidea nitrophenolicus*; Yoon *et al.*, 1999). Recently, *Nocardioidea aquaticus* was isolated from an aquatic rather than a terrestrial environment. It was isolated from water of the

hypersaline and meromictic Ekho Lake, located in the ice-free area of the Vestfold Hills in East Antarctica (Lawson *et al.*, 2000). These facts indicate that *Nocardioidea* species are widely distributed in nature.

In this study, an LL-2,6-diaminopimelic acid (DAP)-containing actinomycete, GW-9^T, isolated from groundwater in Korea, is described. This isolate was considered to be a *Nocardioidea*-like strain from 16S rDNA sequence comparisons, as well as the presence of LL-DAP. Accordingly, the aim of the present study was to determine the exact taxonomic status of strain GW-9^T using a combination of phenotypic properties, detailed phylogenetic analysis based on 16S rDNA sequence and genomic relatedness. On the basis of the data presented below, it is proposed that strain GW-9^T represents a novel species in the genus *Nocardioidea*, for which the name *Nocardioidea aquiterrae* sp. nov. is proposed.

Strain GW-9^T was isolated by the usual dilution plating technique on nutrient agar (NA; Difco) at 30 °C. Type strains of *Nocardioidea* species used as reference organisms for DNA–DNA hybridization were obtained from the

Published online ahead of print on 8 August 2003 as DOI 10.1099/ij.s.0.02585-0.

Abbreviation: DAP, 2,6-diaminopimelic acid.

The GenBank accession number for the 16S rDNA sequence of strain GW-9^T is AF529063.

DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and KCTC (Korean Collection for Type Cultures). Strain GW-9^T was cultivated on NA and in nutrient broth (NB; Difco) at 30 °C to investigate its morphological and physiological characteristics. Cell mass of strain GW-9^T for analyses of the cell wall and menaquinones was obtained from NB culture at 30 °C. Cell mass of strain GW-9^T and reference strains for DNA extraction was produced in NB. For fatty acid methyl ester analysis, cell mass of strain GW-9^T was obtained from agar plates after 6 days cultivation at 30 °C on NA. Cell morphology was examined by light microscopy (Nikon E600) and TEM. The presence or absence of flagella was determined using TEM with cells from exponentially growing cultures. For TEM observation, cells were negatively stained with 1% (w/v) phosphotungstic acid and, after air drying, the grids were examined with a model CM-20 TEM (Philips). Oxidase activity was determined by oxidation of 1% (w/v) *p*-aminodimethylaniline oxalate. Catalase activity was determined by bubble production in a 3% (v/v) hydrogen peroxide solution. Hydrolysis of aesculin and nitrate reduction were determined as described previously (Lanyi, 1987). Hydrolysis of casein, gelatin, hypoxanthine, starch, Tween 80, tyrosine and xanthine and urease activity were determined as described by Cowan & Steel (1965). Utilization of various substrates as sole carbon and energy sources was determined as described by Shirling & Gottlieb (1966). Growth under anaerobic conditions was determined after incubation in an anaerobic chamber with NA that had been prepared anaerobically. Growth at various temperatures (4–55 °C) was determined on NA.

The isomer type of the diamino acid in the cell-wall peptidoglycan was analysed using TLC according to the method described by Komagata & Suzuki (1987). Menaquinones were analysed as described previously (Komagata & Suzuki, 1987) using reversed-phase HPLC. For quantitative analysis of the cellular fatty acid content, a loopful of cell mass was harvested and fatty acid methyl esters were prepared and identified according to the instructions of the Microbial Identification System (MIDI). Chromosomal DNA was isolated and purified according to Yoon *et al.* (1996), with the exception that ribonuclease T1 was used together with ribonuclease A. The G + C content was determined by the method of Tamaoka & Komagata (1984). DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC.

16S rDNA was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). The PCR product was purified with a QIAquick PCR purification kit (Qiagen). Sequencing of the purified 16S rDNA was performed using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) as recommended by the manufacturer. Purified sequencing reaction mixtures were electrophoresed automatically using an Applied Biosystems model 377 automatic DNA sequencer. Alignment of 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 using three tree-making algorithms, 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 using the algorithm of Jukes & Cantor (1969) with the program DNADIST. The stability of relationships was assessed by bootstrap analysis based on 1000 resamplings of the neighbour-joining dataset using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package. 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 replications for each sample. Of the values obtained, the highest and lowest values in each sample were excluded and the means of the remaining three values were taken as DNA relatedness values.

Physiological and biochemical characteristics of strain GW-9^T are given in the species description below and are compared with those of *N. pyridinolyticus* in Table 1.

Strain GW-9^T contained LL-DAP as the diagnostic diamino acid in the cell wall, which is characteristic of wall chemotype I *sensu* Lechevalier & Lechevalier (1970). The predominant isoprenoid quinone found in strain GW-9^T was tetrahydrogenated menaquinone with eight isoprene units [MK-8(H₄)]. Strain GW-9^T had a cellular fatty acid profile containing large amounts of straight-chain, branched, unsaturated and 10-methyl fatty acids. The major fatty acid

Table 1. Differential phenotypic characteristics of strain GW-9^T and *N. pyridinolyticus*

Data for *N. pyridinolyticus* were taken from Yoon *et al.* (1997). +, Positive; –, negative; w+, weakly positive. Both species are rods or cocci, positive for motility, catalase, nitrate reduction, hydrolysis of aesculin and gelatin and utilization of D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, sucrose, D-trehalose, D-xylose and D-mannitol. Both species are negative for utilization of L-arabinose, D-melibiose, D-raffinose, stachyose, adonitol and D-sorbitol.

Characteristic	Strain GW-9 ^T	<i>N. pyridinolyticus</i>
Flagellation	Single lateral	Single polar
Utilization of:		
<i>myo</i> -Inositol	–	+
D-Ribose	w+	+
D-Melezitose	–	+
L-Rhamnose	w+	+
Optimum growth temperature (°C)	30	35
Optimum growth pH	6.0–7.0	8.0
Isolation source	Groundwater	Oil shale column

Table 2. Cellular fatty acid content of strain GW-9^T, *N. pyridinolyticus* KCTC 0074BP^T and *N. albus* KCTC 9186^T

Strains: 1, GW-9^T; 2, *N. pyridinolyticus* KCTC 0074BP^T; 3, *N. albus* KCTC 9186^T. Data for reference strains were taken from Yoon *et al.* (1997). Values are percentages of total fatty acids. Fatty acids representing less than 0.5% were omitted. ND, Not detected; TBSA, tuberculostearic acid.

Fatty acid	1	2	3
Straight-chain fatty acids			
C _{15:0}	0.4	0.4	0.7
C _{16:0}	0.5	1.2	0.7
C _{17:0}	0.5	1.2	1.5
C _{18:0}	0.3	1.0	ND
Branched fatty acids			
iso-C _{14:0}	1.5	0.5	2.2
iso-C _{15:0}	4.9	5.1	3.1
anteiso-C _{15:0}	2.4	1.4	ND
iso-C _{16:0}	57.6	47.3	53.6
iso-C _{16:1} H*	6.8	4.2	1.1
iso-C _{17:0}	0.7	1.7	1.3
anteiso-C _{17:0}	9.0	14.2	0.7
anteiso-C _{17:1} ω9c	2.4	2.1	ND
iso-C _{18:0}	0.2	0.5	0.9
Unsaturated fatty acids			
C _{17:1} ω6c	ND	ND	15.6
C _{17:1} ω8c	1.7	1.7	2.8
C _{18:1} ω9c	0.9	2.0	ND
10-Methyl fatty acids			
10-Me C _{16:0}	3.4	5.0	1.5
10-Me C _{17:0}	4.1	5.5	12.2
10-Me C _{18:0} (TBSA)	0.8	3.4	2.3
Summed feature 3†	0.7	0.8	ND

*Double bond position indicated by a capital letter is unknown.

†Contains one or both of iso-C_{15:0} 2-OH and/or C_{16:1}ω7c, which could not be separated by GLC with the MIDI system.

was iso-C_{16:0}. This cellular fatty acid profile is similar to those of *Nocardioides* species (Yoon *et al.*, 1997, 1999), particularly the type strain of *N. pyridinolyticus* (Table 2), although there are differences in the proportions of some fatty acids. The DNA G + C content of strain GW-9^T was 73 mol%.

The almost complete 16S rDNA sequence of strain GW-9^T was determined directly following PCR amplification. The sequence comprised 1484 nt, representing approximately 96% of the *Escherichia coli* 16S rRNA sequence. Phylogenetic trees based on 16S rDNA sequences showed that strain GW-9^T falls within the radiation of the cluster comprising *Nocardioides* species. In the tree based on the neighbour-joining algorithm, strain GW-9^T formed a coherent cluster with *N. pyridinolyticus* by bootstrap analysis at a confidence level of 100% (Fig. 1). The same tree topology was also found in phylogenetic trees generated with maximum-parsimony and maximum-likelihood algorithms (data not shown). The level of 16S rDNA similarity between strain GW-9^T and the type strain of *N. pyridinolyticus* was 99.2%. Strain GW-9^T exhibited 16S rDNA similarities of 94.9–96.0% with the type strains of other *Nocardioides* species with validly published names and 89.9–94.2% with members of other genera used in the phylogenetic analysis. Two independent levels of DNA–DNA relatedness between GW-9^T and *N. pyridinolyticus* KCTC 0074BP^T, its phylogenetic neighbour, were 32.5 and 28.7%, when each of their DNAs was used separately as the labelled DNA probe. Strains GW-9^T exhibited levels of DNA–DNA relatedness of 8.7–15.3% with the type strains of other *Nocardioides* species with validly published names.

16S rDNA sequence data showed that strain GW-9^T exhibited the closest phylogenetic affinity to *Nocardioides* species. Phylogenetic analysis based on 16S rDNA sequences placed strain GW-9^T within a phyletic cluster comprising *Nocardioides* species (Fig. 1). Results obtained in the chemotaxonomic analysis are consistent with the results of phylogenetic inference based on 16S rDNA sequences. The chemotaxonomic

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain GW-9^T, *Nocardioides* species and some other related taxa based on 16S rDNA sequences. Bar, 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at branch points.

data obtained from strain GW-9^T are most similar to those of members of the genus *Nocardioidea* in the family *Nocardioideaceae* (Miller *et al.*, 1991; Park *et al.*, 1999; Urzi *et al.*, 2000; Wang *et al.*, 2001). Of genera belonging to the family *Nocardioideaceae*, two genera, *Nocardioidea* and *Marmoricola*, have been known to contain MK-8(H₄) as the predominant menaquinone. However, the genus *Marmoricola* contains C_{16:0} and C_{18:1} as the major fatty acids, which distinguishes it from the genus *Nocardioidea* (Urzi *et al.*, 2000). Therefore, both phylogenetic and chemotaxonomic results clearly indicate that strain GW-9^T belongs to the genus *Nocardioidea*. Strain GW-9^T was very similar to *N. pyridinolyticus* in its morphological and physiological properties, as shown in the phylogenetic analysis. The levels of DNA–DNA relatedness, together with minor differential phenotypic properties and phylogenetic distinctiveness, reveal that strain GW-9^T is distinct from previously described *Nocardioidea* species, as well as *N. pyridinolyticus*, its closest phylogenetic neighbour (Wayne *et al.*, 1987). Therefore, on the basis of the data presented, strain GW-9^T should be placed in the genus *Nocardioidea* as a representative of a novel species, for which the name *Nocardioidea aquiterrae* sp. nov. is proposed.

Description of *Nocardioidea aquiterrae* sp. nov.

Nocardioidea aquiterrae (a.qui.ter'rae. L. n. *aqua* water; L. gen. fem. n. *terrae* of earth or ground; N.L. gen. fem. n. *aquiterrae* from/of groundwater).

Cells are aerobic, non-spore-forming, non-acid-fast rods, 0.8–1.0 × 1.7–2.0 µm in the exponential phase of growth after approximately 7 days incubation at 30 °C on NA. Motile by means of a single lateral flagellum. Cells show rod-to-coccus morphogenesis from the early exponential phase to the stationary phase. Gram-positive (Gram-variable in old cultures). Colonies are smooth, circular, convex, cream-coloured and 0.5–1.0 mm in diameter after 7 days incubation on NA. Neither substrate nor primary mycelium is formed. Optimal temperature for growth is 30 °C; grows at 15 and 42 °C, but not at 10 °C or temperatures above 43 °C. Optimal pH for growth is 6.0–7.0; no growth at pH 5.0. Oxidase-positive. Urease-negative. Casein and Tween 80 are hydrolysed. Hypoxanthine, starch, tyrosine and xanthine are not hydrolysed. The cell-wall peptidoglycan contains LL-DAP as the diagnostic diamino acid. The predominant menaquinone is MK-8(H₄). The cellular fatty acids consist of straight-chain, branched, unsaturated and 10-methyl fatty acids. The major fatty acid is iso-C_{16:0}. The DNA G + C content of the type strain is 73 mol% (determined by HPLC). Other characteristics are given in Table 1.

The type strain, strain GW-9^T (=KCCM 41647^T=JCM 11813^T), was isolated from groundwater in Korea.

Acknowledgements

This work was supported by the NRL research programme (grants M10104000294-01J000012800 and M10104000294-01J000012811) and the 21C Frontier programme 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 and by the research fund of the Probiotic Corporation of Korea.

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