Nocardioides ganghwensis sp. nov., isolated from tidal flat sediment

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A strictly aerobic, non-motile, rod-shaped actinomycete strain, named JC2055^T, was isolated from a sediment sample of getbol, the tidal flat of Korea. 16S rDNA analysis revealed that the getbol isolate belonged to the genus *Nocardioides* with the highest sequence similarity to *Nocardioides aquiterrae* GW-9^T (95.5%). The major menaquinone was MK-8(H₄) and the predominant cellular fatty acids were iso-16:0 and 17:1 ω 8c. Tuberculostearic acid was absent. The G + C content of DNA was 72 mol%. Based on the morphological, physiological, biochemical and chemotaxonomical data presented in this study, strain JC2055^T can be readily differentiated from other validly named *Nocardioides* species. The name *Nocardioides ganghwensis* sp. nov. is proposed for the isolate. The type strain is JC2055^T (=IMSNU 14028^T = KCTC 9920^T = JCM 12124^T).

Recently, in a course of study on the culturable aerobic bacterial community in getbol (Korean tidal flat), a Gram-positive bacterium that showed high 16S rDNA sequence relatedness to the genus *Nocardioides* was isolated from Ganghwa island in Korea (Yi & Chun, 2002). In this study, the getbol isolate, designated JC2055^T, together with nine *Nocardioides* type strains (Collins *et al.*, 1994; Lawson *et al.*, 2000; O'Donnell *et al.*, 1982; Prauser, 1976, 1986; Suzuki & Komagata, 1983; Yoon *et al.*, 1997, 1999, 2004) were the subject of a taxonomic investigation to elucidate the identity of the newly isolated strain.

Strain JC2055^T was isolated from a sediment sample of the getbol of Ganghwa island in Korea (37° 35' 31.9" N 126° 27' 24.5" E) using MR2A [R2A (Difco) supplemented with artificial sea salts (Sigma)]. The isolate was routinely cultured on marine agar 2216 (MA; Difco) at 30 °C and maintained as a glycerol suspension (20%, w/v) at –80 °C. The reference strains used in this study are listed in Table 1. Test strains were cultured on nutrient agar (NA; Difco) at 30 °C except for *Nocardioides aquaticus*, for which MA was used.

16S rDNA was enzymically amplified from a single colony. Primers, PCR conditions and sequencing method have

been described elsewhere (Chun & Goodfellow, 1995). The sequence of strain JC2055^T was manually aligned with representative sequences of actinomycetes obtained from GenBank. Phylogenetic trees were inferred using the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993), maximum-parsimony (Fitch, 1972) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). The resultant neighbour-joining tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 resamplings. Alignment and phylogenetic analyses were carried out using the JPHYDIT program (available at <http://imsnu.snu.ac.kr/~pbblab/jphydit>) and PAUP 4.0 (Swofford, 1998) as described previously (Chun *et al.*, 2000).

A nearly complete 16S rDNA sequence of strain JC2055^T was obtained (1374 bp). Preliminary sequence comparison with 16S rRNA gene sequences held in GenBank indicated that our isolate belonged to the genus *Nocardioides*. The newly determined sequence was then aligned manually against representatives of the family *Nocardioideaceae* using bacterial 16S rRNA secondary structure information. Domains used to construct the phylogenetic trees were the regions available for all sequences (positions 38–1431; *Escherichia coli* numbering system), excluding positions showing ambiguous alignment (positions 76–95, 199–219, 455–478, 1025–1037 and 1135–1140). On the basis of 16S rDNA similarity, the closest cultured bacterial relatives were *Nocardioides* species (93.4–95.5%), *Aeromicrobium* species (92.8–93.2%) and *Marmoricola aurantiacus* DSM 12652^T (93.1%). The highest sequence similarity was to *Nocardioides aquiterrae* (95.5%). This relationship

Published online ahead of print on 23 January 2004 as DOI 10.1099/ijs.0.03040-0.

The GenBank accession number for the 16S rDNA sequence of strain JC2055^T is AY423718.

A supplementary table showing the fatty acid compositions of strain JC2055^T and *N. plantarum* strain IMSNU 22067^T is available in IJSEM Online.

Table 1. Characteristics that differentiate strain JC2055^T from other *Nocardioide*s strains

Strains: 1, strain JC2055^T; 2, *N. albus* IMSNU 20105^T; 3, *N. aquaticus* IMSNU 13113^T; 4, *N. aquiterrae* KCCM 41647^T; 5, *N. jensenii* IMSNU 22036^T; 6, *N. luteus* IMSNU 22020^T; 7, *N. nitrophenolicus* KCTC 0457BP^T; 8, *N. plantarum* IMSNU 22067^T; 9, *N. pyridinolyticus* KCTC 0074BP^T; 10, *N. simplex* IMSNU 21329^T. +, Positive reaction; –, negative reaction; w, weakly positive; v, variable. All *Nocardioide*s strains are positive for Gram reaction, catalase, gelatin hydrolysis, esterase lipase (C8) and leucine arylamidase; negative for acid-fastness, arginine dihydrolase, acid production from glucose (API 20NE kit), indole production from tryptophan, decomposition of adenine, alginate and cellulose, α -chymotrypsin, β -glucuronidase, *N*-acetyl- β -glucosaminidase and α -fucosidase. Acetate was utilized by all strains; D-sorbitol, inulin, 2-propanol, L-ascorbate, polyethylene glycol, salicylate and thiamin were not.

Characteristics	1	2	3	4	5	6	7	8	9	10
Motility	–	–	–	+	–	–	+	–	+	+
Mycelium and spore formation*	–	+	–	–	–	+	–	–	–	–
Oxidase	–	–	–	+	–	–	–	–	–	+
β -Galactosidase	+	+	–	+	–	+	–	–	+	+
Nitrate reduction	+	–	+	+	+	–	–	–	+	–
Urease	–	–	–	–	+	–	–	–	–	–
Decomposition of:										
Casein	+	+	w	+	+	+	+	–	+	+
DNA	+	w	+	+	+	+	+	–	+	+
Aesculin	w	w	–	+	–	+	w	w	+	+
Hypoxanthine	–	+	–	–	+	+	–	–	–	–
Starch	+	+	–	+	–	+	–	–	+	w
Tween 80	+	+	+	+	+	+	–	+	–	+
Tyrosine	+	+	+	–	+	+	+	–	+	+
Xanthine	w	+	–	+	+	+	–	–	+	–
API ZYM:										
Alkaline phosphatase	+	+	v	–	w	v	+	–	+	+
Esterase (C4)	v	+	w	–	w	+	–	+	–	–
Lipase (C14)	–	–	–	–	–	–	–	w	–	–
Valine arylamidase	+	–	w	–	v	–	+	w	w	w
Cystine arylamidase	w	–	v	w	–	–	w	+	–	w
Trypsin	–	+	w	+	w	+	+	–	+	+
Acid phosphatase	w	–	+	+	v	–	+	w	+	w
Naphthol-AS-BI-phosphohydrolase	–	w	–	+	v	v	+	+	+	–
α -Galactosidase	+	–	–	–	–	–	–	–	–	–
β -Galactosidase	+	v	–	–	–	v	–	–	–	–
α -Glucosidase	+	+	+	+	v	v	+	+	+	+
β -Glucosidase	–	w	–	w	–	–	w	+	–	w
α -Mannosidase	–	–	–	–	–	+	–	–	–	–
Carbon source utilization:										
Acetamide	–	–	–	–	–	–	–	–	–	+
Benzoate	–	+	–	–	+	+	–	–	–	–
Citrate	+	+	–	w	+	+	+	+	–	+
D-Cellobiose	+	+	–	+	–	+	–	+	+	–
D-Fructose	+	+	+	+	–	–	+	+	+	–
D-Galactose	+	+	+	+	–	–	–	–	+	–
D-Glucose	+	+	+	+	+	+	+	–	+	+
D-Mannitol	+	+	+	+	–	+	–	–	–	–
D-Mannose	+	+	–	–	–	+	–	–	–	–
D-Raffinose	+	–	–	–	–	–	–	–	–	–
D-Ribose	–	–	–	–	–	–	+	–	+	–
D-Salicin	+	w	–	–	–	–	–	+	–	–
D-Xylose	+	+	+	+	–	+	+	+	+	–
Glycerol	+	w	+	–	w	–	–	+	–	–
Inositol	–	–	–	–	–	–	–	–	+	–
Lactose	+	–	–	–	–	–	–	–	–	–

Table 1. cont.

Characteristics	1	2	3	4	5	6	7	8	9	10
L-Arabinose	+	+	—	—	—	+	—	—	—	—
L-Arginine	—	—	—	—	—	—	+	+	—	+
L-Asparagine	+	W	—	+	+	—	—	—	+	+
L-Lysine	—	—	—	+	W	—	—	—	—	—
L-Ornithine	+	W	—	—	—	—	—	+	—	+
L-Rhamnose	—	+	W	—	+	—	+	+	+	—
N-Acetylglucosamine	+	W	—	—	—	+	—	—	—	—
Succinate	+	+	+	+	—	+	+	+	+	+
Sucrose	+	—	+	+	W	—	+	+	+	+
Tartrate	—	—	—	W	W	—	—	—	—	—

*Tested on agar media in this study.

between our isolate and other members of the family *Nocardioideaceae* was also evident in the phylogenetic tree (Fig. 1). Except for *Nocardioideus jensenii* DSM 20641^T, which formed a clade with *Marmoricola aurantiacus* DSM 12652^T as reported previously (Urzi *et al.*, 2000), all *Nocardioideus* species formed a monophyletic clade. Although the branching position varied to some extent depending on the tree-making algorithms, the phyletic line of strain JC2055^T was clearly placed within this *Nocardioideus* clade based on all four tree-inferring methods used in this study. Our isolate formed a clade with *Nocardioideus plantarum* NCIMB 12834^T in the neighbour-joining, Fitch–Margoliash and two of the four most parsimonious trees with a relatively low bootstrap value (67%), or a sister group to the subclade containing *Nocardioideus albus* KCTC 9186^T, *Nocardioideus luteus* KCTC 9575^T, *Nocardioideus nitrophenolicus* NSP 41^T and *Nocardioideus simplex* KCTC 9106^T in the maximum-likelihood and remaining most parsimonious trees.

Morphological and physiological tests were performed using MA (for strain JC1055^T and *N. aquaticus* IMSNU 13113^T) and NA (for other reference strains) as the basal media at 30 °C. Cellular morphology and motility were examined by scanning electron and phase-contrast

microscopy using 3-day-old cells. Formation of mycelium and spores was examined after 3 weeks on several culture media, namely MA, ISP medium no. 3 (Difco), ISP medium no. 4 (Difco), PYGV (Staley, 1968), NA and BM (basal medium; Baumann *et al.*, 1972). For our isolate and *N. aquaticus* IMSNU 13113^T, all media except MA were supplemented with half-strength artificial sea water [ASW (NaCl, 23.939 g; MgCl₂, 5.079 g; Na₂SO₄, 3.994 g; CaCl₂, 1.123 g; KCl, 0.667 g; NaHCO₃, 0.196 g; KBr, 0.098 g; H₃BO₃, 0.027 g; SrCl₂, 0.024 g; NaF, 0.003 g; distilled water, 1 l); Lyman & Fleming, 1940].

Growth under anaerobic conditions was checked in an anaerobic chamber (10 % CO₂, 10 % H₂, 80 % N₂; Sheldon Manufacturing). The growth ranges of the test strain for temperature (between 5 and 50 °C at 5 °C intervals), pH (between pH 4 and 12 at intervals of 1) and NaCl concentration [between 0 and 15 % (w/v) at 1 % intervals] were determined using synthetic ZoBell medium [ZoBell, 1941; Bacto agar (Difco) 15 g; Bacto peptone (Difco), 5 g; yeast extract (Difco), 1 g; ferric citrate, 0.1 g; sea salts, 40 g; distilled water, 1 l). Sea salts were not added to the synthetic medium when the growth range for NaCl concentration was tested. 6 M KOH and 6 M HCl were used to adjust the final pH.

Fig. 1. Neighbour-joining tree based on nearly complete 16S rDNA sequences showing relationships between strain JC2055^T and members of the genus *Nocardioideus*. Percentage bootstrap values >50% (1000 resamplings) are given at branching points and solid circles indicate that the corresponding nodes (groupings) are also recovered in Fitch–Margoliash, maximum-likelihood and maximum-parsimony trees. *Nocardia asteroides* ATCC 19247^T (GenBank accession no. Z36934) was used as an outgroup (not shown). Bar, 0.01 nt substitution per position.

Standard physiological and biochemical tests were performed as described previously (Smibert & Krieg, 1994). Acid-fastness was determined by Ziehl–Neelsen staining (Hendrickson & Krenz, 1991). Hydrolysis of alginic acid (0.5 %, w/v), casein [50 % skim milk (Difco), v/v], cellulose [0.5 % carboxymethyl cellulose (Sigma), w/v], starch (0.2 %, w/v) and Tween 80 (1 %, w/v) was tested using MA or NA as the basal medium. DNase test agar (Difco) was used to assay DNase activity. Decomposition of adenine (0.5 %, w/v), hypoxanthine (0.5 %, w/v), L-tyrosine (0.5 %, w/v) and xanthine (0.4 %, w/v) was checked using MA or NA according to Gordon *et al.* (1974). Arginine dihydrolase, β -galactosidase, nitrate reduction, urease, acid production from glucose and indole production tests were performed using the API 20NE kit (bioMérieux), and other enzymic activities were determined using the API ZYM kit (bioMérieux). Strips for our isolate and *N. aquaticus* IMSNU 13113^T were inoculated with a heavy bacterial suspension in half-strength ASW and AUX medium (bioMérieux) supplemented with 2.5 % (w/v) NaCl. Sole carbon source utilization was tested in 96-well tissue culture microplates (Falcon) as described by Gosink *et al.* (1998). BM, supplemented with 2 % (v/v) Hutner's mineral base (Cohen-Bazire *et al.*, 1957) and modified by reducing the concentration of sea salts to half-strength, was used as basal medium for all test strains. The results of morphological, biochemical and physiological tests are given in Table 1 and the species description.

Chemotaxonomic characteristics of strain JC2055^T were determined using cells grown at 30 °C for 3 days on MA or in MB (marine broth 2216; Difco). Menaquinone was isolated according to Minnikin *et al.* (1984) and analysed by HPLC (Waters) as described by Collins (1985). DNA G+C content (mol%) was determined by HPLC analysis of deoxyribonucleosides as described by Mesbah *et al.* (1989) using a reverse-phase column (Supelcosil LC-18-S; Supelco). Analysis of fatty acid methyl esters was performed by GLC according to the instructions of the Microbial Identification System (MIDI). *Nocardioide*s *plantarum* IMSNU 22067^T, grown on NA at 30 °C for 4 days, was also analysed, as its fatty acid composition has never been reported. Cell wall diamino acids were determined as described by Stanek & Roberts (1974). Our isolate contained MK-8(H₄) as the major menaquinone and LL-diaminopimelic acid as the diagnostic cell wall diamino acid. The results of chemotaxonomic analyses are given in the species description and as supplementary data in IJSEM Online. Most chemotaxonomic characters of strain JC2055^T were typical of the genus *Nocardioide*s (Collins *et al.*, 1994; Lawson *et al.*, 2000; O'Donnell *et al.*, 1982; Yoon *et al.*, 1997, 1999, 2004). In general, the cellular fatty acid composition of our isolate also resembled that of *Nocardioide*s species. However, the strain had i-16:0 (30.4 %) and 17:1 ω 8c (25.7 %) as the major fatty acids, and contained no tuberculostearic acid, which is present in other *Nocardioide*s species at 0.8–13.9 %.

On the basis of 16S rDNA phylogeny and chemotaxonomy, it is clear that our isolate belongs to the genus *Nocardioide*s. Low 16S rDNA sequence similarity values to *Nocardioide*s species (93.4–95.5 %) indicate that strain JC2055^T represents a novel genomic species in the genus (Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001). Along with the fatty acid composition, a number of morphological and physiological characteristics can be used to differentiate the strain from other *Nocardioide*s species at the phenotypic level (Table 1). Based on the polyphasic evidence it is therefore suggested that strain JC2055^T be assigned to a novel species, for which the name *Nocardioide*s *ganghwensis* sp. nov. is proposed.

Description of *Nocardioide*s *ganghwensis* sp. nov.

*Nocardioide*s *ganghwensis* (gang.hwen'sis. N.L. masc. adj., *ganghwensis* named after Ganghwa island in Korea, the geographical origin of the type strain).

Gram-positive and strictly aerobic bacterium. Oxidase-negative, catalase-positive and not acid-fast. Spores are not formed. Cells are non-motile rods and approximately 0.9–4.5 $\times$ 0.4–0.5 μ m. Substrate or aerial mycelium is not observed. Colonies on MA are ivory, circular, convex, entire, glistening, opaque and butyaceous. Colonies are approximately 1–2 mm in diameter after 3 days (on MA at 30 °C) and reach a maximum diameter of 3–4 mm after 5 days. Growth occurs in 0–8 % (w/v) NaCl with the optimum being at 0–1 %. Growth occurs at pH 6–10, with the optimum being at pH 7. Growth occurs at 10–40 °C, with the optimum being at 30 °C. Major menaquinone is MK-8(H₄). Cell wall diamino acid is LL-DAP. Major fatty acids are i-16:0 (30.4 %) and 17:1 ω 8c (25.7 %). In addition, 18:1 ω 9c (5.7 %), i-16:1 H (4.4 %), 10Me-17:0 (3.9 %), i-15:0 (2.7 %), i-17:0 (2.4 %) and 17:0 (2.0 %) are also present. Tuberculostearic acid is absent. The DNA G+C content is 72 mol%. Results of API 20NE and API ZYM kits, decomposition of high molecular mass substrates and carbon source utilization are given in Table 1. The type and only strain is JC2055^T (IMSNU 14028^T = KCTC 9920^T = JCM 12124^T). Isolated from a sediment sample of getbol (tidal flat), Ganghwa island, Korea.

Acknowledgements

We thank Professor S. T. Lee (KAIST) and Dr J. H. Yoon (KRIBB) for the gift of the type strains of *Nocardioide*s *pyridinolyticus* and *N. nitrophenolicus*, respectively. This work was supported by the 21C Frontier Microbial Genomics and Applications Center Program (grant MG02-0101-001-2-1-0) and the Strategic National R&D Program through the Genetic Resources and Information Network (grant M1-0219-00-0018), MOST, Republic of Korea.

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