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Nocardioides paucivorans sp. nov. Isolated from Soil[§]

Jae-Hyung Ahn, Jun-Muk Lim, Soo-Jin Kim, Jaekyeong Song, Soon-Wo Kwon, and Hang-Yeon Weon*

Agricultural Microbiology Division, National Academy of Agricultural Science, Rural Development Administration, Wanju-gun 565-851, Republic of Korea

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One strain, designated KIS31-44^T, was isolated from a soil sample collected from Dokdo Island, South Korea. The strain is Gram-stain-positive, aerobic, non-spore-forming and non-motile. It grows optimally at 28–30°C, at pH 7.0 and 0% NaCl. 16S rRNA gene sequence analysis showed that strain KIS31-44^T belonged to the genus *Nocardioides* and shared the highest sequence similarities with *Nocardioides aestuarii* JC2056^T (95.5%) and *Nocardioides terrae* VA15^T (95.0%). The major fatty acids of strain KIS31-44^T were $C_{17:1}$ *ω6c*, $C_{18:1}$ *ω9c*, summed feature 3 ($C_{16:1}$ *ω6c* and/or $C_{16:1}$ *ω7c*), iso-$C_{16:0}$, $C_{18:0}$ 10-methyl (TBSA), $C_{16:0}$ 2-OH, $C_{17:0}$ 10-methyl, and iso-$C_{16:1}$ H. The major isoprenoid quinone was MK-8 (H_4). The strain contained diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol as the major polar lipids. The peptidoglycan structure was A3*γ*-type with LL-diaminopimelic acid. Based on these data, the isolate represents one novel species in the genus *Nocardioides*, for which the name *Nocardioides paucivorans* sp. nov. (type strain KIS31-44^T =DSM 27142^T =KACC 17309^T) is proposed.

Keywords: *Nocardioides paucivorans*, polyphasic taxonomy, novel species, soil

Introduction

The genus *Nocardioides*, first described by Prauser (1976), is a member of the family *Nocardioidaceae*, in the class *Actinobacteria*. Since then, many new species which were isolated from diverse environments such as soil, sand, tidal flat sediment, water, cryoconite, plants, sludge, plankton, lichen, glacier, and oil shale, were validly named into the genus *Nocardioides*. At the time of writing, 73 species were on the List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.cict.fr). The genus was characterized as the presence of the LL-2,6-diaminopimelic acid (LL-Dpm) in the cell-wall peptidoglycan and the major menaquinone MK-8 (H4) (Prauser, 1976; O'Donnell *et al.*, 1982; Urzì *et al.*, 2000; Zhang *et al.*, 2009). As the major fatty acids, many species of the genus *Nocardioides* contained iso-$C_{16:0}$, $C_{17:1}$ *ω8c* or $C_{18:1}$ *ω9c*, however, some species had $C_{16:0}$, $C_{17:0}$, $C_{15:0}$, iso-$C_{15:0}$ or iso-$C_{17:0}$ (Cui *et al.*, 2009; Kim *et al.*, 2009; Zhang *et al.*, 2009, 2012; Yamamura *et al.*, 2011; Liu *et al.*, 2013).

In the course of analysis of the bacterial population in a soil sample collected from Dokdo Island, South Korea, we isolated strain KIS31-44^T, which was shown to represent a novel species of the genus *Nocardioides* on the basis of phenotypic data and phylogenetic inference.

Materials and Methods

Bacterial strains

The strain KIS31-44^T was isolated through the serial dilution technique using R2A (Becton Dickinson, USA) agar at the incubation temperature of 28°C for a week. The isolate was routinely cultivated on R2A at 28°C and stored as aqueous glycerol suspensions (20%, v/v) at -70°C.

The reference strains *Nocardioides aestuarii* JC2056^T and *Nocardioides terrae* VA15^T were grown on R2A medium at 28°C for biochemical and physiological tests.

Phylogenetic analysis

Chromosomal DNA was isolated with a Wizard Genomic DNA Purification kit (Promega, USA). 16S rRNA gene of strain KIS31-44^T was amplified using two universal primers, as described previously (Kim *et al.*, 2013). 16S rRNA gene sequence was determined at GenoTech Corp. (Korea) using sequencing primers 800R (5′-TACCAGGGTATCTAATCC-3′), 518F (5′-CCAGCAGCCGCGGTAATACG-3′) and 984F (5′-AACGCGAAGAACCTTAC-3′). The 16S rRNA gene sequence of strain KIS31-44^T was compared with those from EzTaxon (Kim *et al.*, 2012) and the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi.). 16S rRNA gene sequence similarities were calculated using the EzTaxon server. Alignment of the sequence data was performed with the SILVA Incremental Aligner (Pruesse *et al.*, 2012). Phylogenetic trees were constructed using MEGA version 5.1 (Tamura *et al.*, 2011) on the basis of the neighbor-joining (Saitou and Nei, 1987), maximum-parsimony (Kluge and Farris, 1969), and maximum-likelihood (Felsenstein, 1981) algorithms. In each case bootstrap values were calculated based on 1,000 replications.

Nucleotide sequence accession number

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain KIS31-44^T is KJ135311.

*For correspondence. E-mail: why@korea.kr; Tel.: +82-63-238-3042; Fax: +82-63-238-3834
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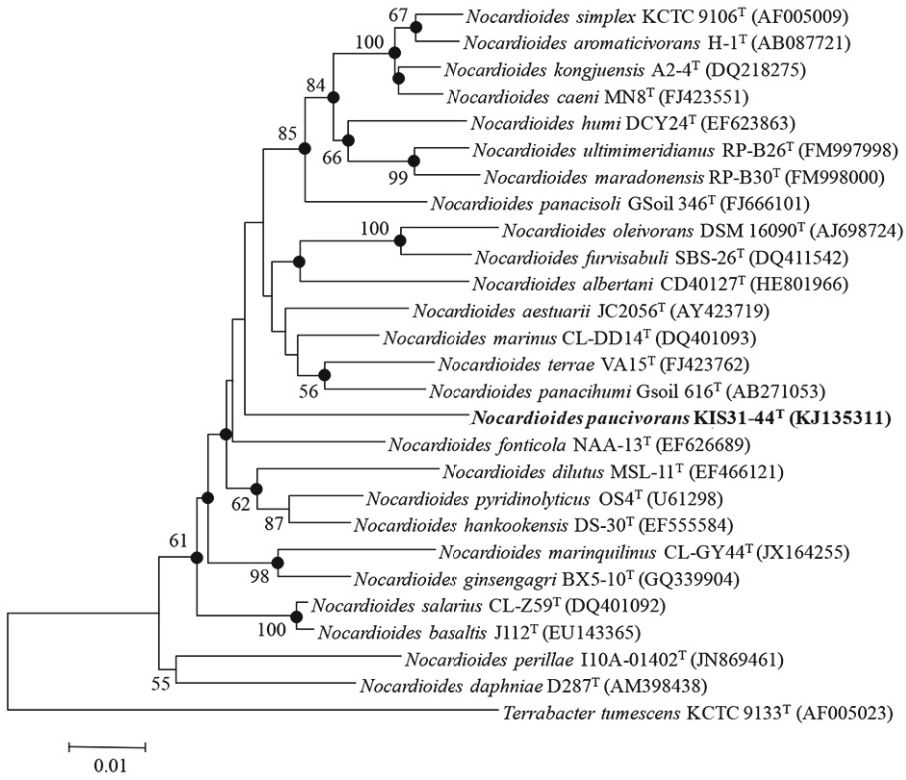

Fig. 1. Phylogenetic tree of strain KIS31-44^T and type strains of other related species of the genus *Nocardioides* based on 16S rRNA gene sequences. Distances and clustering were performed using the neighbour-joining method with the software package MEGA version 5.1. Bootstrap values (>50%) based on 1,000 replications are listed as percentages at the branching points. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. Bar, 0.01 substitutions per nucleotide position.

Determination of DNA G+C content

The G+C content was determined by the fluorometric method (Gonzalez and Saiz-Jimenez, 2002) using the CFX96 system (Bio-Rad, USA).

Morphological, physiological, and biochemical characterization

Cell morphology was observed with cells incubated at 28°C on R2A agar using transmission electron microscopy (model 912AB; Leo, Germany). Growth under anaerobic condition was determined after incubating in the BBL GasPak Anaerobic System (Difco, USA) for 14 days at 28°C. Growth at 4, 10, 15, 18, 20, 25, 28, 35, 37, and 40°C, and at pH 3.0–12.0 (at intervals of 1.0 pH units) was assessed after 5 days of incubation in R2A broth. Tolerance to NaCl concentrations from 0 to 5% (w/v) at 1% intervals was tested on R2A broth. Catalase activity was determined by bubble production in 3% (v/v) H_2O_2 solution, and oxidase activity was tested using 1% (w/v) *N,N,N′,N′*-tetramethyl *p*-phenylenediamine reagent. For Gram reaction, Difco Gram staining kit was used. Casein, CM-cellulose, hypoxanthine, starch, Tween 80, tyrosine, and xanthine hydrolyses were examined on R2A plates containing milk powder [5% (w/v)], CM-cellulose [1% (w/v)], hypoxanthine [0.5% (w/v)], starch [1% (w/v)], Tween 80 [1% (w/v)], tyrosine [0.1% (w/v)] and xanthine [0.5% (w/v)], respectively. DNase activity was determined with DNase test agar (Becton Dickinson). Biochemical tests were carried out using API 20NE, API ID 32GN and API ZYM test kits according to the protocols of the manufacturer (bioMérieux, France). Growth was checked on R2A, ISP 2, nutrient agar (NA), and trypticase soy agar (TSA) (all from Becton Dickinson).

Chemotaxonomy

For cellular fatty acid analysis, strain KIS31-44^T and reference strains were grown on R2A at 28°C for 3–5 days to the late exponential phase of growth. Fatty acid extraction was performed according to Sasser (1990). The fatty acids were analyzed by HP-6890 gas chromatograph (Hewlett Packard, USA) according to the standard protocol of the Sherlock Microbial Identification System (MIDI Sherlock version 6.10, MIDI database TSBA 6). Menaquinones and phospholipids were extracted and purified using the protocol of Minnikin *et al.* (1984). Polar lipids were examined using two-dimensional TLC. The phospholipid pattern was determined as described by Collins *et al.* (1980) using molybdophosphoric acid (for detection of all lipids), ninhydrin (lipids containing free amino groups), Zinzadze (phosphorus-containing lipids) and α-naphthol reagents (glycolipids). For peptidoglycan analysis, cells of strain KIS31-44^T were grown in shake flasks containing liquid ISP 2 medium on a rotary shaker for 4 days at 30°C. Amino acids and the isomers in cell-wall hydrolysates were analyzed as described by Hamada *et al.* (2012).

Results and Discussion

Phylogeneitc analysis

Strain KIS31-44^T revealed the highest sequence similarities

Table 1. Phenotypic comparisons among strain KIS31-44^T and the closely related *Nocardioides* members

Strain: 1, KIS31-44^T; 2, *Nocardioides aestuarii* JC2056^T; 3, *Nocardioides terrae* VA15^T. All data were obtained from this study unless otherwise mentioned. All strains are non-motile, catalase-positive and oxidase-negative. All strains are positive for aesculin hydrolysis, and assimilation of D-glucose, but negative for indole production, glucose fermentation, arginine dihydrolase, urease and assimilation of L-arabinose, D-mannose, *N*-acetylglucosamine, capric acid, adipic acid, trisodium citrate, D-ribose, inositol, itaconic acid, suberic acid, sodium malonate, lactic acid, L-alanine, potassium 5-ketogluconate, 3-hydroxybenzoic acid, L-serine, salicin, L-fucose, D-sorbitol and potassium 2-ketogluconate. All strains showed positive activities for esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase, but negative for alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, and α-fucosidase. +, Positive; W, weakly positive; -, negative; NA, not available.

Characteristics	1	2	3
Isolation source	Soil	Tidal flat sediment	Soil[b]
Cell morpholoy	Short rods or cocci	Rods[a]	Short rods or cocci[b]
Cell size (μm)	0.6–0.8 × 0.6–1.0	0.3–0.4 × 0.9–2.1[a]	0.2–0.3 × 0.3–1.0[b]
Colony color	Light yellow	Ivory[a]	Cream[b]
Temperature range (°C)	10–33	20–35[a]	16–34[b]
NaCl range (%)	0	0–8[a]	0–1[b]
Nitrate reduction	-	-	+
Hydrolysis of:			
Casein	-	+[a]	+[b]
DNA	-	+[a]	NA
Gelatin	-	+	+
Hypoxanthine	-	-[a]	NA
Tween 80	+	+[a]	-[b]
Tyrosine	-	-[a]	NA
Xanthine	-	-[a]	NA
Assimilation of:			
D-Mannitol	-	+	+
D-Maltose	-	+	+
Potassium gluconate	-	+	+
Malic acid	-	W	-
Phenylacetic acid	-	-	+
L-Rhamnose	-	-	+
D-Saccharose	-	+	+
Sodium acetate	-	W	-
Glycogen	-	W	+
D-Melibiose	-	+	-
Propionic acid	-	W	-
Valeric acid	-	+	+
L-Histidine	-	+	-
3-Hydroxybutyric acid	-	+	-
4-Hydroxybenzoic acid	-	-	+
L-Proline	-	+	+
Enzymatic activity of :			
Leucine arylamidase	-	+	+
Acid phosphatase	-	-	+
β-Galactosidase	-	+	-
α-Glucosidase	-	+	+
N-Acetyl-β-glucosaminidase	-	-	+
α-mannosidase	-	-	+
DNA G+C content (mol%)	66	70[a]	71.6[b]

[a] Data from Yi and Chun (2004); [b] Zhang *et al.* (2009).

with *Nocardioides aestuarii* JC2056^T (95.5%) and *Nocardioides terrae* VA15^T (95.0%). Neighbor-joining tree (Fig. 1) indicated that the strain was a member of the genus *Nocardioides*, forming an independent branch within the genus *Nocardioides*. Independent phylogenetic position of strain KIS31-44^T shown in neighbor-joining tree was also shown on the maximum-parsimony and maximum likelihood trees (data not shown).

Morphological, physiological, and biochemical characterization

Cells of strain KIS31-44T were non-spore-forming, non-motile, cocci or short rods (0.6–0.8 × 0.6–1.0 μm) (Supplementary data Fig. S1). Strain KIS31-44^T was Gram-stain-positive, aerobic, catalase-positive and oxidase-negative. It grew optimally at 28–30°C and at pH 7.0, and grew only in 0% NaCl. Strain KIS31-44^T can be differentiated from two

closely related species on the basis of various physiological and biochemical properties such as cell size, colony color, temperature and NaCl ranges for growth, nitrate reduction, hydrolyses of several substances, assimilation of various substances and enzymatic activities (Table 1).

Chemotaxonomy

The cellular fatty acid profiles of strain KIS31-44^T and closely related members of the genus *Nocardioides* were shown on Table 2. The major fatty acids of strain KIS31-44^T were $C_{17:1}$ *ω6c*, $C_{18:1}$ *ω9c*, summed feature 3 ($C_{16:1}$ *ω6c* and/or $C_{16:1}$ *ω7c*), iso-$C_{16:0}$, $C_{18:0}$ 10-methyl (TBSA), $C_{16:0}$ 2-OH, $C_{17:0}$ 10-methyl, and iso-$C_{16:1}$ H (Table 2). Strain KIS31-44^T had relatively large amounts of $C_{17:1}$ *ω6c*, $C_{18:1}$ *ω9c*, and summed feature 3 ($C_{16:1}$ *ω6c* and/or $C_{16:1}$ *ω7c*) and small amount of iso-$C_{16:0}$ as compared with two reference strains. The major isoprenoid quinone of strain KIS31-44^T was MK-8 (H_4) in agreement with the other members of the genus *Nocardioides* (Busse and Schumann, 1999). Strain KIS31-44^T contained diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylinositol (PI) as the major polar lipids (Supplementary data Fig. S2) which were also shown in other *Nocardioides* species such as *Nocardioides perillae*, *Nocardioides iriomotensis*, and *Nocardioides hwas* (Lee *et al*., 2008; Yamamura *et al*., 2011; Du *et al*., 2013). The peptidoglycan structure of strain KIS31-44^T was A3*γ*-type with LL-diaminopimelic acid which is characteristic for the genus *Nocardioides*. The genomic DNA G+C content of strain KIS31-44^T was 65.9 mol%.

In conclusion, based on the phenotypic, chemotaxonomic and phylogenetic data presented, strain KIS31-44^T would appear to represent one novel species of the genus *Nocardioides*, for which the name *Nocardioides paucivorans* sp. nov. is proposed.

Table 2. Fatty acids compositions of strain KIS31-44^T and type strains of the closely related *Nocardioides*

Strain: 1, KIS31-44^T; 2, *Nocardioides aestuarii* JC2056^T; 3, *Nocardioides terrae* VA15^T. -, Not detected or <0.5% of total fatty acids.

Fatty acids	1	2	3
$C_{14:0}$	0.8	-	0.5
iso-$C_{14:0}$	0.8	1.5	1.0
$C_{15:0}$ 2-OH	3.0	-	-
$C_{15:1}$ *ω6c*	2.2	0.6	-
anteiso-$C_{15:0}$	-	1.5	1.5
iso-$C_{15:0}$	-	3.5	4.9
$C_{16:0}$	3.2	0.5	2.7
$C_{16:0}$ 2-OH	6.4	-	-
iso-$C_{16:0}$	8.5	45.4	37.0
iso-$C_{16:1}$ H	5.2	3.1	2.5
$C_{17:0}$	2.6	4.5	2.2
$C_{17:0}$ 2-OH	2.0	-	-
$C_{17:0}$ 3-OH	-	2.3	-
$C_{17:0}$ 10-methyl	5.6	3.4	6.5
$C_{17:1}$ *ω6c*	14.7	-	6.6
$C_{17:1}$ *ω8c*	6.7	20.2	5.0
anteiso-$C_{17:0}$	-	3.4	4.4
anteiso-$C_{17:1}$ *ω9c*	-	0.7	1.1
iso-$C_{17:0}$	-	2.1	3.0
$C_{18:0}$	2.2	-	2.8
$C_{18:0}$ 10-methyl (TBSA)	7.8	-	4.7
$C_{18:1}$ *ω9c*	10.2	2.6	3.6
iso-$C_{18:0}$	3.9	1.1	0.5
Summed feature*			
3	8.5	0.5	1.6
4	1.6	-	-
6	1.6	1.0	0.5
9	1.2	1.2	5.8

* Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system: summed feature 3, $C_{16:1}$ *ω6c* and/or $C_{16:1}$ *ω7c*; summed feature 4, anteiso-$C_{17:1}$ B and/or iso-$C_{17:1}$ I; summed feature 6, $C_{19:1}$ *ω9c* and/or $C_{19:1}$ *ω11c*; summed feature 9, $C_{16:0}$ 10-methyl and/or iso-C17:1 *ω9c*.

Description of *Nocardioides paucivorans* sp. nov.

Nocardioides paucivorans (pau.ci.vo´rans. L. adj. *paucus* little; L. v. *vorare* to eat, to devour; L. pres. part. *vorans* eating; N.L. adj. *paucivorans* eating little, referring to the fact that the type strain utilized only glucose as sole source of carbon and energy).

Cells are Gram-stain-positive, aerobic, non-spore-forming, non-motile and cocci or short rods (0.6–0.8 × 0.6–1.0 μm). Colonies are whitish, round, convex on R2A medium. Growth occurs at 10–33°C (optimum, 28–30°C), and at pH 4.0–8.0 (optimum, 7.0). Growth did not occur in NaCl concentrations above 1%. Hydrolyzes chitin and Tween 80, but does not hydrolyze casein, CM-cellulose, DNA, hypoxanthine, starch, tyrosine and xanthine. Positive activities for aesculin hydrolysis, but negative activities for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease and gelatin hydrolysis (API 20NE test strip). Assimilates D-glucose, but does not assimilate L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, phenylacetic acid, L-rhamnose, D-ribose, inositol, D-saccharose, itaconic acid, suberic acid, sodium malonate, sodium acetate, lactic acid, L-alanine, potassium 5-ketogluconate, glycogen, 3-hydroxybenzoic acid, L-serine, salicin, D-melibiose, L-fucose, D-sorbitol, propionic acid, valeric acid, L-histidine, potassium 2-ketogluconate, 3-hydroxybutyric acid, 4-hydroxybenzoic acid and L-proline (API 20NE and API ID 32GN test strips). Positive activities for esterase (C4), esterase lipase (C8), and naphthol-AS-BI-phosphohydrolase, but negative for alkaline phosphatase, lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, *α*-chymotrypsin, acid phosphatase, *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *α*-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminidase, *α*-mannosidase, and *α*-fucosidase (API ZYM test strip). The major fatty acids (>5%) are $C_{17:1}$ *ω6c*, $C_{18:1}$ *ω9c*, summed feature 3 ($C_{16:1}$ *ω6c* and/or $C_{16:1}$ *ω7c*), iso-$C_{16:0}$, $C_{18:0}$ 10-methyl (TBSA), $C_{16:0}$ 2-OH, $C_{17:0}$ 10-methyl and iso-$C_{16:1}$ H. The predominant menaquinone is MK-8 (H_4). The main polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. The peptidoglycan contains LL-diaminopimelic acid in the cell wall and has A3*γ*-type peptidoglycan.

The type strain, KIS31-44^T (=DSM 27142^T =KACC 17309^T), was isolated from a soil sample collected from Dokdo Island, South Korea. The DNA G+C content of the type strain is 65.9 mol%.

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