Gordonia sihwensis sp. nov., a novel nitrate-reducing bacterium isolated from a wastewater-treatment bioreactor

Kwang Kyu Kim,^{1,2} Chang Soo Lee,^{1,2} Reiner M. Kroppenstedt,² Erko Stackebrandt² and Sung Taik Lee¹

Correspondence

Sung Taik Lee

e_stlee@kaist.ac.kr

¹Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Yuseong, Taejeon, Korea

²DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1b, 38124 Braunschweig, Germany

A nitrate-reducing bacterium, strain SPR2^T, was isolated from a sulphur-oxidizing, autotrophic denitrification reactor used for advanced treatment of wastewater from the lake of Sihwa, Korea. The strain was aerobic but could grow under anaerobic conditions. It was Gram-positive, exhibited rough white colonies on complex nutrient agar, produced elementary branching hyphae that fragmented into rod/coccus-like elements and showed chemotaxonomic markers that were consistent with classification in the genus *Gordonia*, i.e. meso-diaminopimelic acid, arabinose and galactose in whole-cell hydrolysates, N-glycolylmuramic acid in the peptidoglycan wall, unbranched saturated and monounsaturated fatty acids plus tuberculostearic acid (TBSA), mycolic acids that comprised 48–56 carbon atoms and MK-9(H₂) as the predominant menaquinone. The 16S rDNA sequence of strain SPR2^T showed highest similarity to *Gordonia amicalis* DSM 44461^T and *Gordonia hydrophobica* DSM 44015^T, with values of 98.2 and 97.9 %, respectively. These values were far below 99.5 % (usually found at the intraspecies level) and they were in the range that separates species at the intragenic level. The separate phylogenetic position of SPR2^T was supported by differences in fatty acid and mycolic acid compositions and in carbon utilization tests that distinguished strain SPR2^T from all known *Gordonia* species. Combined genotypic and phenotypic data show that strain SPR2^T merits recognition as a novel species within the genus *Gordonia*, for which the name *Gordonia sihwensis* sp. nov. is proposed; the type strain is SPR2^T (= DSM 44576^T = NRRL B-24155^T).

Members of the genus *Gordonia* were originally regarded as opportunistic pathogens that were isolated from sputum of humans suffering from pulmonary diseases, e.g. *Gordonia bronchialis*, *Gordonia aichiensis* and *Gordonia sputi* (Tsukamura, 1971, 1978, 1982). However, many *Gordonia* isolates are able to degrade xenobiotics, such as benzothiophene and dibenzothiophene by *Gordonia desulfuricans* and *Gordonia amicalis* (Kim *et al.*, 1999, 2000) and alkanes by *Gordonia alkanivorans* (Kummer *et al.*, 1999), whereas *Gordonia hydrophobica* and *Gordonia hirsuta* were able to remove malodorous animal emissions such as aldehydes and ketones in biofilters (Bendinger *et al.*, 1995; Klatte *et al.*, 1996). *Gordonia nitida*, which degrades 3-ethylpyridine and 3-methylpyridine, was isolated from soil (Yoon *et al.*, 2000) and *Gordonia polyisoprenivorans* was

found in fouling water of automobile tyres and metabolizes polyisoprenoids (Linos *et al.*, 1999).

The aim of this study was to clarify the taxonomic position of an organism isolated from a sulphur-oxidizing, autotrophic denitrification reactor used for advanced treatment of wastewater from the lake of Sihwa, Korea. Sulphur particles were taken from the reactor and samples from the top layers were diluted serially in 0.85 % saline. Aliquots of each serial dilution were spread on succinate/mineral agar medium [0.04 % (w/v) NH₄Cl, 0.08 % (w/v) K₂HPO₄, 0.03 % (w/v) KH₂PO₄, 0.27 % (w/v) sodium succinate hexahydrate, 0.01 % (w/v) yeast extract, 0.5 % (v/v) mineral solution, 1.5 % (w/v) Bacto Agar (Difco) (Frankel *et al.*, 1997)] and incubated in an anaerobic jar (Difco) at 28 °C, pH 7 for 7 days.

A pinpoint colony, designed SPR2^T, was isolated and subcultured on TSB agar medium [3 % (w/v) trypticase soy broth (BBL), 1.5 % (w/v) Bacto Agar (Difco)]. For

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determination of colour, morphology and biochemical reactions, strain SPR2^T was grown on solidified GYM agar medium [0.4 % (w/v) D-glucose, 0.4 % (w/v) yeast extract, 1 % (w/v) malt extract, 1.2 % (w/v) agar no. 1 (Oxoid)] at 28 °C. For biochemical tests, strain SPR2^T was harvested from GYM agar after 24 h. Morphological studies and colour examinations were carried out after 1, 3 and 7 days. For analysis of fatty acid and mycolic acid compositions, strain SPR2^T was grown on TSB agar at 28 °C for 4 days. For cell-wall and quinone analyses, cells were grown in TSB at 28 °C for 4 days on a rotary shaker, harvested by centrifugation and washed twice with distilled water. For the nitrate-reducing test, strain SPR2^T was cultivated in potassium nitrate medium [0.3 % (w/v) beef extract, 0.5 % (w/v) gelatin peptone, 0.1 % (w/v) KNO₃] under anaerobic conditions at 28 °C and pH 7.

Macroscopic and microscopic studies and staining procedures followed the instructions of Lefford (1980). Carbon source utilization and quantitative enzyme tests were performed in standard microtitration plates as described previously (Klatte, 1994; Linos *et al.*, 1999). Amino acid and sugar analyses of whole-cell hydrolysates followed the procedures of Stanek & Roberts (1974). Murein acyl type was determined by a modification of the colorimetric method of Uchida & Aida (1977). In contrast to the original procedure, whole-cell hydrolysate was neutralized by passing it through an ion-exchange column (Analytichem Bond Elut SCX). Isoprenoid quinones were extracted and purified by using the small-scale integrated procedure of Minnikin *et al.* (1984). Dried preparations were dissolved in 200 µl 2-propanol and 1–10 µl aliquots were separated by HPLC without further purification. Menaquinones were separated by HPLC on a LiChrosorb RP-18 column at 40 °C by using acetonitrile/2-propanol (65:35, v/v) as solvent (Kroppenstedt, 1982, 1985). Polar lipids were extracted, examined by two-dimensional TLC and identified by using published procedures (Minnikin *et al.*, 1977). Fatty acid methyl esters and mycolic acid trimethylsilyl ethers were prepared and analysed as described previously (Klatte *et al.*, 1994) by using the standard Microbial Identification system (MIDI) for automated GC analyses (Sasser, 1990; Kämpfer & Kroppenstedt, 1996).

Formation of nitrite in potassium nitrate medium after growth for 3 days was determined by following the method of Griess (1879). Reduction of nitrate was determined by the development of pink to deep red colour when nitrite reacted with 0.6 % (w/v) *N,N*-dimethyl- α -naphthylamine and 0.8 % (w/v) sulfanilic acid that were dissolved in 30 % (w/v) glacial acetic acid.

Extraction of genomic DNA, PCR-mediated amplification of 16S rDNA and sequencing of purified PCR product were carried out according to Rainey *et al.* (1996). Sequence reaction mixtures were electrophoresed by using a model 373A automatic sequencer (Applied Biosystems). 16S rDNA sequences were aligned with published sequences from the DSMZ database by using the ae2 editor (Maidak *et al.*, 1994)

and sequences were retrieved from EMBL. Pairwise similarities and evolutionary distances were calculated by using the methods of De Soete (1983) and Jukes & Cantor (1969), respectively. Phylogenetic dendrograms were constructed according to algorithms in the PHYLIP package (Felsenstein, 1993). Stability of branching points was evaluated by bootstrap analysis (Felsenstein, 1989). Strain designation and 16S rDNA accession numbers of reference strains are given in Fig. 1.

Strain SPR2^T produced short, elementary branching hyphae that disintegrated into rod/coccus-like elements, showing the typical rod-coccus growth cycle that is usually found among strains of the genus *Gordonia* and related taxa, such as *Rhodococcus* (Goodfellow, 1991). Aged cells tended to form aggregates. Upon cultivation on standard nutrient agar, strain SPR2^T exhibited white and rough colonies. Cells were Gram-positive, non-acid-fast and non-motile. No spores were detected.

Results of physiological tests obtained from microtitration plates revealed that strain SPR2^T was able to utilize 18 of 32 carbon sources by means of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]

Fig. 1. 16S rDNA dendrogram obtained by distance matrix (neighbour-joining) analysis, showing the position of *Gordonia sihwensis* SPR2^T among species of the genus *Gordonia*. Species of the genera *Williamsia* and *Skermania* were used to define the root. Numbers at branching points refer to bootstrap values. Bar, 5 substitutions per 100 nucleotide positions.

Table 1. Physiological properties of *Gordonia sihwensis* SPR2^T and type strains of the genus *Gordonia*

Strains: 1, *G. sihwensis* SPR2^T; 2, *G. aichiensis* DSM 43978^T; 3, *G. alkanivorans* DSM 44369^T; 4, *G. amarae* DSM 43392^T; 5, *G. amicalis* DSM 44461^T; 6, *G. bronchialis* DSM 43247^T; 7, *G. desulfuricans* DSM 44462^T; 8, *G. hirsuta* DSM 44140^T; 9, *G. hydrophobica* DSM 44015^T; 10, *Gordonia namibiensis* DSM 44568^T; 11, *G. nitida* DSM 44499^T; 12, *G. polyisoprenivorans* DSM 44302^T; 13, *G. rhizosphera* NBRC 16068^T; 14, *G. rubripertincta* DSM 43197^T; 15, *G. sputi* DSM 43896^T; 16, *Gordonia terrae* DSM 43249^T; 17, *G. westfalica* DSM 44215^T. Assimilation of auxonographic substrate was detected photometrically by means of reduction of the redox dye MTT. +, $E_{540}(\text{test}) - E_{540}(\text{control}) > 0.129$; –, $E_{540}(\text{test}) - E_{540}(\text{control}) < 0.129$; ND, not determined.

Characteristic	1	2*	3	4*	5	6	7	8	9*	10	11	12	13*	14	15*	16*	17*
Utilization of:																	
D-Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–
L-Rhamnose	–	–	–	–	+	+	–	–	+	–	+	+	+	+	–	+	–
D-Ribose	–	–	+	+	+	–	+	–	+	+	+	–	–	+	–	–	+
D-Sucrose	+	–	+	+	+	+	+	–	+	+	+	+	+	+	–	+	+
D-Turanose	+	–	+	+	+	+	+	–	–	–	+	+	+	–	–	–	–
D-Arabitol	+	–	+	+	–	–	–	–	–	+	–	+	–	+	+	+	+
<i>D</i> -Inositol	–	–	+	–	–	+	–	+	+	–	+	+	+	–	–	–	–
<i>N</i> -Acetyl-D-glucosamine	+	–	+	–	–	+	+	–	–	–	–	+	+	–	–	–	–
Glucarate	–	–	+	–	+	–	–	–	+	–	+	–	–	+	–	+	–
Gluconate	+	–	+	–	+	+	+	–	–	+	+	+	+	+	–	–	–
D-Glucosaminic acid	+	–	+	–	+	–	+	–	–	–	+	+	+	+	–	–	+
Caprate	+	+	+	ND	+	+	+	ND	–	–	–	ND	–	–	+	–	+
Citrate	+	+	+	+	+	–	+	–	+	–	+	+	+	–	+	+	+
4-Aminobutyrate	+	+	–	ND	–	–	+	ND	–	–	–	ND	–	–	–	–	–
2-Hydroxyvalerate	+	–	+	–	+	–	+	+	+	–	+	–	–	–	–	–	–
2-Oxoglutarate	+	+	+	+	+	–	+	+	+	–	+	–	+	–	+	+	+
Pimelate	+	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–
Succinate	–	–	+	–	+	+	–	–	+	+	+	–	+	–	+	–	–
Benzoate	–	–	+	–	–	–	–	–	–	–	+	+	+	+	–	+	+
3-Hydroxybenzoate	–	–	+	–	–	–	+	–	–	–	+	–	+	–	–	–	–
4-Hydroxybenzoate	–	–	+	+	+	+	+	–	–	+	+	+	+	–	–	–	–
Phenylacetate	+	–	+	–	+	+	+	–	–	–	+	–	–	+	–	–	–
Quinate	–	+	–	+	–	–	–	–	–	+	–	+	+	–	–	–	–
L-Alanine	–	+	+	–	+	+	+	–	+	+	+	+	–	+	–	–	–
L-Aspartate	–	+	+	–	+	–	–	–	–	–	+	+	–	–	–	–	+
L-Leucine	+	–	+	–	+	+	+	–	+	–	–	–	–	–	–	–	–
L-Proline	+	–	+	+	–	–	+	–	+	–	–	+	–	–	–	–	–
L-Serine	+	–	–	–	+	–	–	–	+	+	+	–	–	–	–	–	–
L-Valine	+	–	–	+	–	+	–	–	+	–	+	+	–	–	–	–	–
Putrescine	–	–	+	+	+	–	+	–	–	–	–	+	–	–	–	–	–
Tyramine	–	–	+	ND	+	–	+	ND	–	+	+	+	+	–	–	–	–
Acetamide	–	–	–	–	+	+	+	–	+	–	–	+	–	–	–	–	–
Hydrolysis of:																	
<i>p</i> -Nitrophenyl β-D-xyloside	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
<i>p</i> -Nitrophenyl phosphorylcholine	+	–	+	–	+	–	+	+	+	–	+	–	–	–	–	–	–
2-Deoxythymidine-5'- <i>p</i> -nitrophenyl phosphate	+	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–

*Data taken from Linos *et al.* (1999).

reduction test. Two of three chromogenic substrates could be hydrolysed (Table 1).

Chemotaxonomic properties of strain SPR2^T were also consistent with its classification in the genus *Gordonia* (Minnikin *et al.*, 1977; Stackebrandt *et al.*, 1988; Klatte *et al.*, 1994). Whole-cell hydrolysates of SPR2^T contained

meso-diaminopimelic acid as the only diamino acid of the peptidoglycan and arabinose plus galactose as major cell-wall sugars. As expected for the genus *Gordonia* and related taxa, the sugar moiety of the peptidoglycan was glycolated. MK-9(H₂) was the predominant menaquinone; a small amount of MK-8(H₂) was also present. The polar lipids were composed of diphosphatidylglycerol,

phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannosides and some unspecified glycolipids. The fatty acid pattern was composed of straight-chain saturated and unsaturated fatty acids plus tuberculostearic acid. Although this pattern is roughly the same in all gordoniae, there are qualitative and quantitative

species-specific and often intraspecific differences (Klatte, 1994) (Table 2). Strain SPR2^T also synthesized a homologous series of mycolic acids that ranged from 48 to 56 carbon atoms in length, with C₅₀, C₅₂ and C₅₄ as the three principal mycolic acids. This chain-length was in the range expected for *Gordonia* species (C₄₈–C₆₆). The pattern of

Table 2. Cellular fatty acid composition (%) of *Gordonia sihwensis* SPR2^T and type strains of the genus *Gordonia*

Strains: see legend to Table 1. Abbreviations: C_{18:1}, octadecenoic acid (oleic acid); TBSA, tuberculostearic acid; 10-methyl C_{17:0}, 10-methyl-heptadecanoic acid. In addition, *G. polyisoprenivorans* contains 2% C_{10:0}.

Fatty acid	1	2*	3	4*	5	6*	7	8*	9*	10	11	12*	13†	14*	15*	16*	17
C _{14:0}	1	2	4	1	2	1	2	2	1	2	3	2	3	2	2	2	2
C _{15:1}	1								2	1							
C _{15:0}	1	1	1	1	3	1			4	4	1		4	2	2	1	1
C _{16:1 cis-7}	1			3	1	11	1	1			1	1					12
C _{16:1 cis-9}	19	13	16	15	14	16	15	16	13	12	13	12	4	15		16	
C _{16:0}	39	34	28	29	29	23	26	30	27	24	33	29	27	25	44	32	32
C _{17:1 cis-9}	2	1	2	2	6	2		1	4	8	3	1	7	5	2	1	
C _{17:0}	1	1	1	1	5	1	1	1	4	7	2	1	13	3	2	1	2
10-Methyl C _{17:0}					2	7			4	2	1			1			
C _{18:1}	12	17	25	24	23	10	18	29	14	25	21	21	27	24	29	26	25
C _{18:0}	1	2	2	1	2	1	7	3	1	2	1	4	11			2	1
TBSA	22	25	20	21	13	20	30	17	26	12	19	29	3	17	15	19	17
C _{19:1}						3				1				3			

*Data from Linos *et al.* (1999).
†Data from Takeuchi & Hatano (1998).

Table 3. Mycolic acid composition (%) of *Gordonia sihwensis* SPR2^T and type strains of the genus *Gordonia*

Strains: see legend to Table 1. The main mycolic acid components are shown, designated by chain-length (no. carbon atoms).

Mycolic acid	1	2*	3	4*	5	6*	7	8*	9*	10	11	12†	13‡	14*	15*	16*	17
C ₄₈	7			5	1												
C ₄₉	4				2												
C ₅₀	20			30	7												
C ₅₁	4			3	8												
C ₅₂	28		11	40	24						12						
C ₅₃	6		2	4	17						4						
C ₅₄	22		29	18	19					3	26			4		5	5
C ₅₅	2		8		8					3	8			3		1	
C ₅₆	7		32		10		1		12	13	34			14		27	20
C ₅₇			6		3				11	11	6			11		4	7
C ₅₈			12		1		5		30	24	10	8	7	23		36	34
C ₅₉									16	14		3	5	17		3	9
C ₆₀		14				8	19	19	26	20		30	26	18	8	19	18
C ₆₁		11				21	2	10	5	7		6	10	5	12	1	5
C ₆₂		47				25	50	31		5		47	34		48	4	2
C ₆₃		11				25	3	9				1			14		
C ₆₄		17				13	20	31				5			8		

*Data from Klatte *et al.* (1994, 1996).
†Data from Linos *et al.* (1999).
‡Data from Takeuchi & Hatano (1998).

isolate SPR2^T is similar to those of *Gordonia amarae* and *G. amicalis*, which possess similar-sized mycolic acids with chain-lengths of about C₄₈–C₅₈ (Table 3).

Analysis of the almost-complete 16S rDNA sequence of strain SPR2^T (1499 nt) indicated membership of the genus *Gordonia*, the intrageneric relatedness of which ranges from 94.9 to 99.7 % sequence similarity among the type strains of species. The 16S rDNA sequence of strain SPR2^T showed highest similarity, with values of 97.9 and 98.2 %, to those of

G. hydrophobica and *G. amicalis*, respectively. Despite the overall high 16S rDNA sequence similarity between the species of this genus, strain SPR2^T occupies a distinct phylogenetic position no matter which algorithm is used for the construction of dendrograms (distance matrix or maximum-likelihood). Fig. 1 displays a neighbour-joining tree. Bootstrap values indicate that only a few branches and branch-clusters are supported by high statistical significance. Several species are significantly more closely related to other *Gordonia* type strains by 16S rDNA sequence

Table 4. Physiological, morphological and chemotaxonomic characteristics of *Gordonia sihwensis* sp. nov. and comparison with phylogenetically most closely related strains

Characteristic	<i>G. sihwensis</i> SPR2 ^T	<i>G. hydrophobica</i> DSM 44015 ^T	<i>G. amicalis</i> DSM 44461 ^T
Utilization of:			
L-Rhamnose	–	+	+
D-Ribose	–	+	+
D-Turanose	+	–	+
D-Arabitol	+	–	–
<i>i</i> -Inositol	–	+	–
<i>N</i> -Acetyl-D-glucosamine	+	–	–
Glucarate	–	+	+
Gluconate	+	–	+
D-Glucosaminic acid	+	–	+
Caprate	+	–	+
4-Aminobutyrate	+	–	–
Pimelate	+	–	+
Succinate	–	+	+
4-Hydroxybenzoate	–	–	+
Phenylacetate	+	–	+
L-Alanine	–	+	+
L-Aspartate	–	–	+
L-Proline	+	+	–
L-Valine	+	+	–
Putrescine	–	–	+
Tyramine	–	–	+
Acetamide	–	+	+
Hydrolysis of:			
<i>p</i> -Nitrophenyl β-D-xyloside	–	–	–
<i>p</i> -Nitrophenyl phosphorylcholine	+	+	+
2-Deoxythymidine-5'- <i>p</i> -nitrophenyl phosphate	+	+	–
Nitrate reduction	+	+	–
Colony morphology	Rough; white to tan	Rough; white to tan	Smooth; orange-red
Cell morphology	Rods (1.5–2.0 μm)	Long rods (2–3 μm)	Short rods (0.7–1.3 μm)
Fatty acid composition*:			
C _{15:0}	1	4	3
C _{16:1 cis-9}	19	13	14
C _{16:0}	39	27	29
C _{17:0}	1	4	5
C _{18:1}	12	14	23
TBSA	22	26	13
Mycolic acid†	48–56 (50, 52, 54)	56–61 (58, 59, 60)	48–58 (52, 53, 54)

*The six most diagnostic fatty acids were selected for comparison (composition in %). TBSA, Tuberculostearic acid.

†Chain-lengths of mycolic acids were compared. Principal components are shown in parentheses.

similarity than strain SPR2^T, e.g. those of *Gordonia westfalica*, *G. alkanivorans* and *G. nitida* (99.1–99.9%), *G. sputi* and *G. aichiensis* (99.7%) or *Gordonia rhizosphaera* and *G. bronchialis* (98.7%). Analysis of DNA–DNA relatedness between the type strains of *G. amicalis* and *Gordonia rubripertincta* (which have 99% 16S rDNA similarity) revealed only 31% DNA–DNA similarity (Kim *et al.*, 2000), whilst DNA similarity between *G. nitida* and *G. rubripertincta* (99% 16S rDNA similarity) gave a mean value of 42% (Yoon *et al.*, 2000). As these 16S rDNA similarity values are significantly higher than those found for strain SPR2^T with the type strain of any other *Gordonia* species, we conclude that DNA–DNA similarities are not needed to demonstrate convincingly that the isolate represents a novel genomic species. The rationale to describe a novel species for strain SPR2^T is justified by considering the differences observed in the chain-length of mycolic acids and physiological properties (Table 4).

Description of *Gordonia sihwensis* sp. nov.

Gordonia sihwensis (sih.wen'sis. N.L. fem. adj. *sihwensis* referring to lake Sihwa in Korea, from where the type strain was recovered).

Aerobic, Gram-positive, nitrate-reducing, non-motile actinomycete that forms rod/coccus-like elements. Colonies are white; colonies of the type strain are rough with irregular margins. Whole-cell hydrolysates contain *meso*-diaminopimelic acid, arabinose and galactose. Peptidoglycan sugars are glycolated. Predominant menaquinone is MK-9(H₂); a small amount of MK-8(H₂) is also present. Polar lipids are comprised of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides. Major fatty acids are C_{16:1} cis-9, C_{16:0}, C_{18:1} and 10-methyl C_{18:0}. Principal mycolic acids have a chain-length of 50, 52 or 54 carbon atoms. Physiological properties are indicated in Table 1.

The type strain is SPR2^T (=DSM 44576^T=NRRL B-24155^T). Isolated from a sulphur-oxidizing autotrophic denitrification reactor used for advanced treatment of wastewater from the lake of Sihwa, Korea.

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