Humicoccus flavidus gen. nov., sp. nov., isolated from soil

Jung-Hoon Yoon, So-Jung Kang, Seo-Youn Jung and Tae-Kwang Oh

Korea Research Institute of Bioscience and Biotechnology (KRIBB), PO Box 115, Yusong, Taejeon, Republic of Korea

Correspondence
Jung-Hoon Yoon
jhyoon@kribb.re.kr

A Gram-positive, non-motile, spherical, non-spore-forming bacterial strain, DS-52^T, was isolated from soil from Dokdo, Korea, and its taxonomic position was investigated by using a polyphasic approach. It grew optimally at 25 °C and pH 6.0–7.0. Strain DS-52^T had meso-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan, and galactose, mannose, xylose and rhamnose as whole-cell sugars. It contained MK-8(H₄) and MK-9(H₄) as the predominant menaquinones and anteiso-C_{15:0}, iso-C_{15:0} and C_{17:0} as major fatty acids. Major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylmethylethanolamine. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain DS-52^T is most closely related to the genus *Nakamurella* of the suborder *Frankineae*. Strain DS-52^T exhibited 16S rRNA gene sequence similarity values of 96.5 % to *Nakamurella multipartita* JCM 9543^T and 92.0–93.9 % to other members of the suborder *Frankineae*. The diagnostic diamino acid type and polar lipid profile of strain DS-52^T were the same as those of the genus *Nakamurella*. However, strain DS-52^T could be clearly distinguished from the genus *Nakamurella* by differences in predominant menaquinones, major fatty acids and cell-wall sugars. Accordingly, based on combined phenotypic, chemotaxonomic and phylogenetic data, strain DS-52^T (=KCTC 19127^T = CIP 108919^T) is proposed as the type strain of a novel species in a new genus, *Humicoccus flavidus* gen. nov., sp. nov.

The family *Microsphaeraceae* was proposed by Stackebrandt *et al.* (1997) to accommodate a single genus and single species, *Microsphaera multipartita*, which had been described by Yoshimi *et al.* (1996). *M. multipartita* is an aerobic, Gram-positive and coccoid-shaped actinomycete that belongs to the suborder *Frankineae* (Yoshimi *et al.*, 1996; Stackebrandt *et al.*, 1997). The chemical markers characterizing the genus *Microsphaera* include the presence of meso-diaminopimelic acid in the peptidoglycan, MK-8(H₄) as the predominant menaquinone, iso-C_{15:0}, iso-C_{16:0} and C_{18:1} as the major fatty acids and a DNA G+C content of 67.5 mol% (Yoshimi *et al.*, 1996). The bacterial genus name *Microsphaera* was recently proven to be illegitimate because of priority of the fungal genus name *Microsphaera* (Tao *et al.*, 2004). Accordingly, the genus name *Microsphaera* was replaced by *Nakamurella* and the family name *Microsphaeraceae* was replaced by *Nakamurellaceae* (Tao *et al.*, 2004). In this study, we describe a Gram-positive, spherical, bacterial strain, DS-52^T, which was considered to be phylogenetically related to the genus *Nakamurella* based on 16S rRNA gene sequence comparisons. The aim of the present study was to determine the

exact taxonomic position of strain DS-52^T by using a polyphasic approach.

Strain DS-52^T was isolated from soil samples collected in Dokdo (37° 14' 12" N 131° 52' 07" E), Korea, using the standard dilution plating technique at 25 °C on 10 × diluted nutrient agar (NA; Difco). *Nakamurella multipartita* DSM 44233^T, which was used as a reference strain, was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. The morphological, physiological and biochemical characteristics of strain DS-52^T were investigated using routine cultivation on NA at 25 °C. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy. The presence of flagella was determined via transmission electron microscopy using cells from exponentially growing cultures. For transmission electron microscopic observation, the cells were negatively stained with 1 % (w/v) phosphotungstic acid and, following air-drying, the grids were examined with a Philips CM-20 transmission electron microscope. The Gram reaction was determined using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth at various temperatures (4–40 °C) was measured on NA. To investigate tolerance to NaCl, trypticase soy broth was prepared according to the formula of the Difco medium and NaCl

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concentrations were varied (0, 0.5 and 1.0–7.0 %, w/v, at intervals of 1.0 %). The pH range for growth was determined in nutrient broth (NB; Difco) that was adjusted to various pH values (pH 4.5–10.5 at intervals of 0.5 pH units) prior to sterilization by adding directly HCl or Na₂CO₃. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on NA and on NA supplemented with nitrate, both of which had been prepared anaerobically using nitrogen. Catalase and oxidase activities and hydrolysis of casein, gelatin, hypoxanthine, starch, Tweens 20, 40, 60 and 80, tyrosine, urea and xanthine were determined as described by Cowan & Steel (1965). Hydrolysis of aesculin and nitrate reduction were studied as described by Lanyi (1987). Utilization of substrates as sole carbon and energy sources was tested according to the method of Kämpfer *et al.* (1991). Susceptibility to antibiotics was tested on NA plates using discs containing the following antibiotics: polymyxin B (100 U), streptomycin (50 µg), penicillin G (20 U), chloramphenicol (100 µg), ampicillin (10 µg), cephalothin (30 µg), gentamicin (30 µg), novobiocin (5 µg), tetracycline (30 µg), kanamycin (30 µg), lincomycin (15 µg), oleandomycin (15 µg), neomycin (30 µg) and carbenicillin (100 µg). Enzyme activities and other physiological properties were tested by using the API ZYM and API 20E systems (bioMérieux).

Cell biomass of strain DS-52^T for DNA extraction and for cell-wall, isoprenoid quinone and polar lipid analyses was obtained from cultures grown in NB at 25 °C. Cell mass of *N. multipartita* DSM 44233^T for cell-wall and polar lipid analyses was obtained by cultivation in trypticase soy broth (Difco) at 28 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene was amplified by PCR using two universal primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984) with a modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. The isomer type of the diamino acid in the cell-wall peptidoglycan was analysed by using TLC according to the method described by Komagata & Suzuki (1987). Whole-cell sugars were determined as described by Komagata & Suzuki (1987). Isoprenoid quinones were extracted according to the method of Komagata & Suzuki (1987) and analysed by using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. Polar lipids were extracted according to the procedures described by Minnikin *et al.* (1984) and identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). For fatty acid analysis, cell mass of strain DS-52^T was harvested from NA plates after incubation for 10 days at 25 °C, and cell mass of *N. multipartita* DSM 44233^T was harvested from NA plates

after incubation for 10 days at 28 °C. The fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990).

Table 1. Differential phenotypic characteristics of strain DS-52^T and *Nakamurella multipartita*

+, Positive reaction; –, negative reaction. Cells of the two taxa are spherical and Gram-positive. Both strains have an optimal temperature for growth of 25 °C. They are positive for growth at 10 °C, catalase, hydrolysis (data from this study for *N. multipartita*) of aesculin, starch and urea, utilization of D-glucose, D-fructose, D-galactose, D-mannose, D-xylose, sucrose and maltose, and susceptibility to streptomycin, chloramphenicol, neomycin and oleandomycin. They are negative for motility, oxidase, anaerobic growth, hydrolysis (data from this study for *N. multipartita*) of tyrosine and xanthine, utilization of acetate, and susceptibility to penicillin G, ampicillin, cephalothin and carbenicillin. Major polar lipids are diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylmethylethanolamine (data for *N. multipartita* from this study).

Characteristic	DS-52 ^T	<i>N. multipartita</i>
Cell size (µm)	0.6–1.2	0.8–3.0
Colony colour	Light-yellow	White
Maximum NaCl concentration (% w/v) for growth	4	6
Growth at:		
4 °C	+	–
35 °C	–	+
Hydrolysis of:*		
Casein	–	+
Hypoxanthine	–	+
Utilization of:		
Succinate	+	–
L-Malate	+	–
Pyruvate	–	+
L-Glutamate	–	+
Susceptibility to antibiotics*		
Polymyxin (100 U)	+	–
Gentamicin (30 µg)	+	–
Novobiocin (5 µg)	+	–
Tetracycline (30 µg)	+	–
Kanamycin (30 µg)	+	–
Lincomycin (15 µg)	+	–
Predominant menaquinone(s)	MK-8(H ₄), MK-9(H ₄)	MK-8(H ₄)
Cell-wall sugars†	Gal, Man, Xyl, Rha	Glc, Man, Xyl, Rha
DNA G + C content (mol%)	72.6	67.5

*Data for *N. multipartita* from this study.

†Data for *N. multipartita* from this study. Abbreviations: Gal, galactose; Glc, glucose; Man, mannose; Xyl, xylose; Rha, rhamnose.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain DS-52^T and some other related taxa. Bootstrap values (1000 replications) are shown as percentages at each node only if they are 50% or greater. Filled circles indicate branches also recovered in trees generated with the maximum-likelihood and maximum-parsimony algorithms. *Cellulomonas flavigena* NCIMB 8073^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Cells of strain DS-52^T were Gram-positive, non-spore-forming cocci (0.6–1.2 µm in diameter). Morphological, cultural, physiological and biochemical characteristics of strain DS-52^T are given in the genus and species descriptions or are shown in Table 1. The almost complete 16S rRNA gene sequence of strain DS-52^T, comprising 1475 nucleotides (approximately 96% of the *Escherichia coli* 16S rRNA gene sequence), was determined in this study. In the neighbour-joining tree based on 16S rRNA gene sequences, strain DS-52^T joined the phylogenetic lineage of *N. multipartita* at a bootstrap resampling value of 100% and this cluster was independent from the clade comprising other members of the suborder *Frankineae* (Fig. 1). These topologies were also found in the trees generated with the maximum-likelihood and maximum-parsimony algorithms (Fig. 1). Strain DS-52^T exhibited 16S rRNA gene sequence similarity values of 96.5% to *N. multipartita* JCM 9543^T and of 92.0–93.9% to other members of the suborder *Frankineae* used in the phylogenetic analysis. Strain DS-52^T had meso-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. The whole-cell sugars detected in strain DS-52^T were galactose, rhamnose, xylose and mannose, whereas *N. multipartita* DSM 44233^T was found to have glucose, rhamnose, xylose and mannose as whole-cell sugars. Strain DS-52^T contained similar amounts of MK-8(H₄) and MK-9(H₄) as the predominant menaquinones. Strain DS-52^T had a cellular fatty acid profile that contained large amounts of branched and straight-chain fatty acids; the major components (>10% of total fatty acids) were anteiso-C_{15:0}, iso-C_{15:0} and C_{17:0} (Table 2). Major polar lipids detected in strain DS-52^T and *N. multipartita* DSM 44233^T were diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylmethylethanolamine. The DNA G+C content of strain DS-52^T was 72.6 mol%.

16S rRNA gene sequence analyses indicated that strain DS-52^T is phylogenetically most closely related to *N. multipartita* (Fig. 1). Cell morphology, peptidoglycan diamino acid type and major polar lipids of strain DS-52^T were the same as those of *N. multipartita* (Yoshimi *et al.*, 1996; Table 1). However, strain DS-52^T showed distinct differences from *N. multipartita* in content of the predominant menaquinones and fatty acid profile. *N. multipartita* contains MK-8(H₄), at a peak area ratio of 97%, as the

predominant menaquinone (Yoshimi *et al.*, 1996); a similar result was also obtained in this study. Strain DS-52^T showed notable differences from *N. multipartita* in the proportions of anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{16:0} and C_{17:0} and in the absence of unsaturated and 10-methyl fatty acids (Table 2). There was also a slight difference in the whole-cell sugars

Table 2. Cellular fatty acid content of strain DS-52^T and *Nakamurella multipartita*

Values are percentage of total fatty acids. —, Not detected or not described. Fatty acids that represented <0.5% in the two strains were omitted.

Fatty acid	DS-52 ^T	<i>N. multipartita</i> DSM 44233 ^T
Straight-chain		
C _{14:0}	0.4	1.5
C _{15:0}	3.9	0.7
C _{16:0}	9.0	8.4
C _{17:0}	17.3	2.5
C _{18:0}	1.2	—
Branched		
iso-C _{14:0}	1.3	4.4
iso-C _{15:0}	19.9	7.5
anteiso-C _{15:0}	25.8	2.2
iso-C _{16:0}	6.6	37.8
anteiso-C _{16:0}	1.7	—
iso-C _{17:0}	4.1	6.0
anteiso-C _{17:0}	5.5	2.1
Unsaturated		
C _{17:1} ω6c	—	5.0
C _{17:1} ω8c	—	2.1
C _{18:1} ω7c	—	0.23
C _{18:1} ω9c	—	13.1
Hydroxy		
C _{16:0} 2-OH	—	0.6
10-Methyl C _{17:0}	—	2.1
Summed feature 3*	2.8	2.7

*Summed feature represents groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 3 comprised C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

between strain DS-52^T and *N. multipartita* (Table 1). Based on the data presented here, it is proposed that strain DS-52^T represents a novel species in a new genus, *Humicoccus flavidus* gen. nov., sp. nov.

Description of *Humicoccus* gen. nov.

Humicoccus (Hu.mi.coc'cus. L. n. *humus* the soil; Gr. n. *kokkos* a grain or berry; N.L. masc. n. *Humicoccus* coccus isolated from soil).

Cells are Gram-positive, non-spore-forming cocci. Strictly aerobic. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant menaquinones are MK-8(H₄) and MK-9(H₄). The cellular fatty acid profile consists of straight-chain and branched fatty acids. The type species is *Humicoccus flavidus*.

Description of *Humicoccus flavidus* sp. nov.

Humicoccus flavidus (fla'vi.dus. L. masc. adj. *flavidus* pale yellow).

Has the following properties in addition to those given for the genus. Cells are 0.6–1.2 µm in diameter. Colonies are circular, convex, smooth, glistening, light-yellow in colour and 1.0–1.8 mm in diameter after incubation for 10 days at 25 °C on NA. Growth occurs at 4 and 32 °C, but not at 33 °C. The optimal pH for growth is 6.0–7.0; growth occurs at pH 5.0 and 8.5, but not at pH 4.5 or 9.0. Growth occurs in the absence of NaCl; growth does not occur in the presence of greater than 5 % (w/v) NaCl. Growth does not occur under anaerobic conditions on NA or on NA supplemented with nitrate. Gelatin is hydrolysed, but Tweens 20, 40, 60 and 80 are not. H₂S and indole are not produced. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are absent. In assays with the API ZYM system, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase are present, but alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. D-Cellobiose, D-trehalose, L-arabinose and salicin are utilized as sole carbon and energy sources, but benzoate, citrate and formate are not. The whole-cell sugars are galactose, mannose, xylose and rhamnose. The major polar lipids are diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylmethylethanolamine. The major fatty acids (>10 % of total fatty acids) are anteiso-C_{15:0}, iso-C_{15:0} and C_{17:0}. The DNA G + C content is 72.6 mol% (HPLC). Other phenotypic properties are given in Table 1.

The type strain, DS-52^T (=KCTC 19127^T = CIP 108919^T), was isolated from soil.

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