Gordonia malaquae sp. nov., isolated from sludge of a wastewater treatment plant

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The taxonomic status of a bacterial isolate from the sludge of a wastewater treatment plant was characterized by using a polyphasic taxonomic approach. Chemotaxonomic investigations revealed the presence of cell-wall chemotype IV, short-chain mycolic acids that co-migrated with those extracted from members of the genus *Gordonia*, fatty acids C_{16:0} and C_{18:0} (found by pyrolysis gas chromatography) and a dihydrogenated menaquinone with nine isoprene units [MK-9(H₂)] as the predominant menaquinone. The genus assignment was confirmed by 16S rRNA gene sequencing. Comparative analysis of the 16S rRNA gene sequence showed that the novel isolate constitutes a hitherto unknown subline within the genus *Gordonia*, displaying 95.9 to 97.6 % gene sequence similarity to the recognized species of the genus. The novel isolate was distinguished from the type strains of phylogenetically related species by using a set of phenotypic features. The genotypic and phenotypic data show that the new strain merits classification as a novel species of the genus *Gordonia*, for which the name *Gordonia malaquae* sp. nov. is proposed. The type strain is IMMIB WWCC-22^T (=DSM 45064^T =CCUG 53555^T).

The genus *Gordonia* belongs to the suborder *Corynebacterineae* (Stackebrandt *et al.*, 1997). The genus *Gordonia* has attracted much interest in recent years for a variety of reasons. In contrast to the originally isolated strains of the genus, which were described as opportunistic pathogens in humans (Tsukamura, 1971, 1978, 1982), most members of the genus *Gordonia* described recently represent environmental isolates that play an important role in bioremediation and the biodegradation of pollutants (Bendinger *et al.*, 1995; Klatte *et al.*, 1996; Kim *et al.*, 2000; Linos *et al.*, 2002; Kageyama *et al.*, 2006; Soddell *et al.*, 2006). At the time of writing, the genus *Gordonia* includes 22 species with validly published names. The aim of this study was to clarify the taxonomic position of strain IMMIB WWCC-22^T which was isolated from the sludge of a wastewater treatment plant. Based on phylogenetic and phenotypic evidence, it is proposed that this new isolate be classified as a novel species of the genus *Gordonia*.

Strain IMMIB WWCC-22^T was isolated from the sludge of a wastewater treatment plant located in Taichung Industrial Park, Taichung city, Taiwan. The organism was cultivated

on Columbia agar supplemented with 5 % sheep blood agar and brain–heart infusion (BHI) agar to determine its morphological characteristics. Pigment production was determined by growing the strain at 27 °C for 7 days; observations were made at 24 h intervals. Air-dried smears were stained by the Gram method in order to determine the Gram stain and cell morphology. The Ziehl–Neelsen method was used to determine acid-fastness. Growth temperatures were determined by incubating the strain at 27, 37 and 42 °C. The physiological properties of the novel strain were determined by using tests to determine the hydrolysis of complex substrates as described previously (Gordon, 1966, 1967; Gordon & Mihm, 1957) as well as tests to determine carbon source utilization according to Yassin *et al.* (1995). The isomeric form of the diaminopimelic acid was determined by the method of Becker *et al.* (1964) and whole-cell sugars were determined according to Lechevalier (1968). Lipids were extracted using acid methanolysis and mycolic acids were detected with TLC as described by Minnikin *et al.* (1980); pyrolysis GC of the mycolate was performed according to Yassin *et al.* (1993a). Non-hydroxylated fatty acids were purified, identified and quantified by GC as described by Yassin (1988). Phospholipids were extracted, purified and identified as described previously (Yassin *et al.*, 1993b). Menaquinones were extracted and purified according to

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMMIB WWCC-22^T is AM406674.

Collins *et al.* (1977). Mass spectral analyses of the menaquinones were recorded as described recently by Yassin & Hupfer (2006) in positive ion mode on a Q-TOF 2 mass spectrometer (Micromass) equipped with a nanospray source.

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and the purification of PCR products were carried out using previously described procedures (Rainey *et al.*, 1996). Purified PCR products were sequenced using a *Taq* DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's protocol. A Genetic Analyzer (310 DNA; Applied Biosystems) was used for electrophoresis of the sequence reaction products. The 16S rRNA gene sequences of strain IMMIB WWCC-22^T, as well as those of the other recognized species of the genus *Gordonia* retrieved from GenBank, were added to the ARB-database (Ludwig *et al.*, 2004) and aligned using the appropriate tool from the ARB package. The resulting alignment was corrected manually and evolutionary trees were inferred using maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. An evolutionary distance matrix was calculated using the correction of Jukes & Cantor (1969). The topologies of the resultant trees were evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining data based on 1000 resamplings using the ARB package.

To establish the phylogenetic position of strain IMMIB WWCC-22^T, its 16S rRNA gene sequence was determined in this study [1489 nucleotides; 96.5% of the *Escherichia coli* sequence (Brosius *et al.*, 1978)]. A tree depicting the

phylogenetic relationship of the novel strain within the genus *Gordonia* is shown in Fig. 1. The novel strain formed a distinct subline within the genus *Gordonia*, branching proximal to the base of a subcluster of species, which includes *Gordonia hirsuta*, *Gordonia amarae*, *Gordonia sihwensis* and *Gordonia hydrophobica*. Bootstrap resampling, however, showed that the association of strain IMMIB WWCC-22^T with this subcluster of species is not statistically significant and, from the tree construction analysis, it is evident that the novel strain does not exhibit a significant affinity with any recognized species. The novel strain shared closest 16S rRNA gene sequence similarity with the type strains of *G. hydrophobica* (97.6%), *Gordonia defluvii* (97.5%), *Gordonia rubripertincta* (97.4%), *Gordonia desulfuricans*, (97.3%), *Gordonia namibiensis* (97.3%), *Gordonia alkanivorans* (97.2%), *Gordonia westfalica* (97.2%), *G. sihwensis* (97.1%) and *Gordonia amicalis* (97.1%). Lower 16S rRNA gene sequence similarities were found with the type strains of the remaining *Gordonia* species. DNA–DNA relatedness studies were not carried out between strain IMMIB WWCC-22^T and its phylogenetically closest relatives as it has already been established that representatives of other *Gordonia* species with similar 16S rRNA gene sequence similarities, for example *Gordonia araii* and *Gordonia effusa* and the type strains of *G. amarae*, *G. hydrophobica* and *G. hirsuta* (Kageyama *et al.*, 2006), share DNA–DNA relatedness values well below the 70% cut-off point recommended for the delineation of bacterial species (Wayne *et al.*, 1987). The novel strain can be distinguished from its phylogenetically closest relatives by using a combination of phenotypic properties (Table 1).

Fig. 1. Maximum-likelihood tree showing the position of *Gordonia malaquae* sp. nov. IMMIB WWCC-22^T in the genus *Gordonia*. The tree was based on comparison of 16S rRNA gene sequences that were at least 90% complete (with regard to the *E. coli* sequence). Numbers at nodes are levels of bootstrap support (%) based on analyses of 1000 resampled datasets. Solid circles indicate that the corresponding nodes (groupings) are also recovered in neighbour-joining and maximum-parsimony trees. Bar, 5.0% sequence divergence.

Table 1. Differential physiological characteristics of strain IMMIB WWCC-22^T and other members of the genus *Gordonia*

Strains: 1, *Gordonia malaquae* sp. nov. IMMIB WWCC-22^T; 2, *G. desulfuricans* DSM 44462^T; 3, *G. hirsuta* DSM 44140^T (data from Linos *et al.*, 2002); 4, *G. hydrophobica* DSM 44015^T; 5, *G. rubripertincta* DSM 43197^T; 6, *G. sihwensis* DSM 44576^T (Kim *et al.*, 2003). All strains (not determined for strain numbers 3 and 4) were positive for acetate, 2,3-butandiol, citrate, glucose, paraffin and trehalose and for hydrolysis of testosterone and urea. All strains (not determined for strain numbers 3 and 4) were negative for the utilization of adipic acid, adonitol, isoamyl alcohol, L-arabinose, cellobiose, gelatin, lactate, lactose, maltose, melezitose, raffinose, rhamnose and D-xylose and for the hydrolysis of adenine, casein, elastin, aesculin, gelatin, guanine, hypoxanthine, tyrosine and xanthine. +, Positive; –, negative; W, weakly utilized after three weeks incubation; ND, not determined.

Characteristic	1	2	3	4	5	6
Utilization as sole sources of carbon and energy:						
meso-Erythritol	W	–	ND	–	–	ND
D-Galactose	–	–	+	–	–	+
Gluconate	–	+	–	–	+	+
p-Hydroxybenzoate	–	+	–	–	–	–
myo-Inositol	–	–	+	–	–	–
Mannitol	W	–	ND	–	+	ND
1,2-Propandiol	–	–	ND	+	+	ND
Sorbitol	–	–	ND	–	+	ND
Sucrose	+	+	–	+	+	+
Utilization as sole sources of carbon and nitrogen:						
Acetamide	–	+	–	+	–	–
L-Alanine	+	+	–	+	+	–
Arginine	–	+	ND	–	–	ND
Ornithine	–	–	ND	–	+	ND
Proline	+	–	–	+	–	+
Serine	+	–	–	–	+	+

Strain IMMIB WWCC-22^T has morphological properties consistent with its assignment to the genus *Gordonia*. The organism is aerobic and forms smooth, creamy colonies on Columbia and BHI agars. The cells are rod- and coccoid-like, stain Gram-positive and are non-acid–alcohol-fast. The novel strain grows at temperatures up to 37 °C, but not at 42 °C. The physiological properties of strain IMMIB WWCC-22^T are given in detail in the species description below. The biochemical characteristics determined in this study that distinguish strain IMMIB WWCC-22^T from *G. desulfuricans* DSM 44462^T, *G. hydrophobica* DSM 44015^T and *G. rubripertincta* DSM 43197^T are presented in Table 1.

Chemotaxonomically, strain IMMIB WWCC-22^T possesses chemical markers consistent with its phylogenetic assignment to the genus *Gordonia*. The cell wall contains meso-diaminopimelic acid as well as arabinose and galactose (i.e. wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970). One-dimensional TLC of whole-cell acid methanolysates of the novel strain revealed the presence of two lipid spots on

the chromatogram. The lower spot corresponded to mycolic acids, as identified from its R_F value (0.55), and the higher spot corresponded to non-hydroxylated fatty acids. Pyrolysis GC of the purified mycolic acid methyl esters from strain IMMIB WWCC-22^T released fatty acid methyl esters of C_{16:0} (29 % of total cleavage products) and C_{18:0} (71.0 %) as pyrolysis cleavage products. GC analyses of the non-hydroxylated fatty acid methyl esters revealed the presence of dodecanoate (0.9 % of total fatty acids), tetradecanoate (5.0 %), pentadecanoate (0.9 %), *cis*-hexadecenoate (5.0 %), hexadecanoate (40.0 %), heptadecanoate (1.6 %), octadecenoate (14.4 %), octadecanoate (18.5 %), tuberculostearic acid (10-methyl octadecanoate, 13.2 %), eicosenoate (1.0 %) and eicosanoate (0.15 %) as the major cellular fatty acid methyl esters. Polar lipid analysis showed that the novel strain contains phosphatidylethanolamine, phosphatidyl-inositol, phosphatidylinositol dimannosides, phosphatidyl-glycerol and diphosphatidylglycerol as the characteristic phospholipids (i.e. phospholipid type PII *sensu* Lechevalier *et al.*, 1977). Mass spectral analysis of the main component from strain IMMIB WWCC-22^T shows a strong peak at m/z 809.5 attributable to $[M+Na]^+$ in the high mass region. This corresponds to a dihydrogenated menaquinone with nine isoprene units MK-9(H₂). The second band shows a strong peak at m/z 741.58 attributable to $[M+Na]^+$ in the high mass region. This corresponds to a dihydrogenated menaquinone with eight isoprene units, MK-8(H₂).

It is apparent from the genotypic and phenotypic data that strain IMMIB WWCC-22^T represents a novel species of the genus *Gordonia*, for which the name *Gordonia malaquae* is proposed.

Description of *Gordonia malaquae* sp. nov.

Gordonia malaquae (mal.a'quae. L. adj. *malus* bad; L. n. *aqua* water; N.L. gen. n. *malaquae* of bad water, effluent).

Forms smooth, cream coloured colonies on agar media. Cells are rod- and coccoid-like, Gram-positive and non-acid–alcohol-fast. Grows at temperatures between 22–37 °C, but not at 42 °C. Contains the salient chemotaxonomic characteristic of the genus *Gordonia*. Mycolic acids cleave on pyrolysis to release fatty acids of C_{16:0} and C_{18:0} as the major cleavage products. The fatty acid profile consists mainly of straight-chain saturated, unsaturated and 10-methyl branched fatty acids. Hydrolyses urea and testosterone, but not adenine, casein, elastin, aesculin, gelatin, guanine, hypoxanthine, tyrosine or xanthine. Assimilates acetate, 2,3-butandiol, citrate, glucose, paraffin, sucrose, trehalose and xylose as carbon sources but not adonitol, adipate, iso-amylalcohol, L-arabinose, cellobiose, meso-erythritol, galactose, gluconate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, *myo*-inositol, lactate, lactose, maltose, mannitol, melezitose, 1,2-propandiol, raffinose, rhamnose or sorbitol. Utilizes L-alanine, proline and serine as simultaneous carbon and nitrogen sources, but not acetamide, arginine, gelatin or ornithine.

The type strain, IMMIB WWCC-22^T (=DSM 45064^T=CCUG 53555^T), was isolated from sludge from a wastewater treatment plant, Taiwan.

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