Williamsia deligens sp. nov., isolated from human blood

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The taxonomic status of two bacterial strains isolated from human blood was characterized using a polyphasic 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 *Williamsia* and that produce C_{16:0} and C_{18:0} fatty acids on pyrolysis GC, and dihydrogenated menaquinone with nine isoprene units as the predominant isoprenologue. The generic assignment was confirmed by 16S rRNA gene sequencing. Comparative analysis of the 16S rRNA gene sequence showed that these isolates constitute a distinct phyletic line within the genus *Williamsia*, displaying 96.2 and 97.2 % sequence similarities to *Williamsia muralis* and *Williamsia maris*, respectively. The two isolates could be distinguished from the type strains of the latter species on the basis of several phenotypic traits. The genotypic and phenotypic data show that the strains merit classification as a novel species of *Williamsia*, for which the name *Williamsia deligens* sp. nov. is proposed, with type strain IMMIB RIV-956^T (= DSM 44902^T = CCUG 50873^T).

The genus *Williamsia* was proposed by Kämpfer *et al.* (1999) to accommodate actinomycetes with atypical cell morphology as revealed under electron microscopy that are unable to grow at 5 or at 45 °C and possess mycolic acids with carbon chain lengths of 50 to 56. Based on its mycolic acids, it seems that *Williamsia* takes an intermediate position between *Rhodococcus* (mycolic acid chain lengths of 34–45) and *Gordonia* (mycolic acid chain lengths of 54–66) (Kämpfer *et al.*, 1999). The genus *Williamsia* currently comprises two recognized species, *Williamsia muralis* isolated from indoor building material of a children's day-care centre in Finland (Kämpfer *et al.*, 1999) and *Williamsia maris* isolated from deep sediments of the Sea of Japan (Stach *et al.*, 2004). In this paper we describe two bacterial strains which were isolated from human blood. Based on phylogenetic and phenotypic data it is proposed that these strains (designated IMMIB RIV-956^T and IMMIB RIV-956FI) are similar and should be classified as representing a novel species of the genus *Williamsia*.

Isolates IMMIB RIV-956^T and IMMIB RIV-956FI were isolated from human blood. The type strains of *W. maris*

(DSM 44693^T) and *W. muralis* (DSM 44343^T) were received from the DSMZ. All strains were cultured on Columbia agar supplemented with 5 % sheep blood agar and brain heart infusion (BHI) agar to determine their morphological characteristics. Production of pigments was determined by growing the strains at 27 °C for 7 days, and observations were made at 24 h intervals. Air-dried smears at 24, 48 and 72 h intervals were stained by using the Gram's method in order to determine the Gram reaction and cell morphology. The Ziehl–Neelsen method was used to determine acid-fastness. Growth temperatures were determined by incubating the organisms at 27, 37 and 42 °C. The physiological properties of the strains were determined by using tests to determine hydrolysis of complex substrates, as described by Gordon (1966, 1967) and Gordon & Mihm (1957), as well as tests to determine carbon source utilization according to Yassin *et al.* (1995). The isomeric form of diaminopimelic acid was determined according to the methods of Becker *et al.* (1964) and whole-cell sugars were determined by the method of 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 by Yassin *et al.* (1993b). Menaquinones were extracted and purified according to the method of Collins

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains IMMIB RIV-956^T and IMMIB RIV-956FI are AJ920290 and AJ920291, respectively.

et al. (1977). Mass spectral analyses of the menaquinones were recorded in positive ion mode on a Q-TOF 2 mass spectrometer (Micromass) equipped with a nanospray source. Analytes were dissolved in acetonitrile and were injected into the mass spectrometer by glass capillaries (long type; Protona) using a capillary voltage of 950 V and a source block temperature of 80 °C. Instrument calibration was made with a mixture of sodium iodide and caesium iodide dissolved in 50 % aqueous 2-propanol. The collision energy was 35–45 eV at 0.7 bar. For the compounds under study, the major ions observed with the electrospray technique were protonated pseudo-molecular ions, $[M + Na]^+$. The identity of menaquinones was verified by observing the diagnostic ion at m/z 187, which represents the 2-methyl naphthoquinone core.

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of PCR products were carried out using the procedures described by Rainey *et al.* (1996). Purified PCR products were sequenced using a Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) as described in the manufacturer's protocol. An Applied Biosystems 310 DNA Genetic Analyzer was used for

electrophoresis of the sequence reaction products. The 16S rRNA gene sequences of *W. maris* DSM 44693^T and *W. muralis* DSM 44343^T determined in this study, as well as those of *W. maris* and *W. muralis* retrieved from GenBank, were added to the ARB database (Ludwig *et al.*, 2004) and aligned using the appropriate tool within the ARB package. The resulting alignment was corrected manually and evolutionary trees were inferred using the maximum-parsimony (Kluge & Farris, 1969), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms. An evolutionary distance matrix was calculated using the corrections 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.

The almost complete 16S rRNA gene sequences of strains IMMIB RIV-956^T and IMMIB RIV-956FI [1479 and 1478 nt, respectively; 95.9 and 95.8 %, respectively, of the *Escherichia coli* sequence (Brosius *et al.*, 1978)], *W. maris* DSM 44693^T and *W. muralis* DSM 44343^T were determined in this study. Those for the latter two strains were found to be identical to the sequences of the same strains available

Fig. 1. Neighbour-joining tree showing the position of *Williamsia deligens* IMMIB RIV-956^T within the radiation of the mycolic acid-containing taxa. The tree was based on a comparison of 16S rRNA gene sequences that were at least 90 % complete (with regard to the *E. coli* sequence). Bootstrap percentages based on 1000 resamplings are shown at nodes. Bar, 10.0 % sequence divergence.

from the public databases under accession numbers AB010909 and Y17384, respectively. Therefore, the database sequences of these two species were used in our comparative analyses. 16S rRNA gene sequence comparisons revealed clearly that isolates IMMIB RIV-956^T and IMMIB RIV-956Fl are members of the suborder *Corynebacterineae* (Stackebrandt *et al.*, 1997) and contained all signature nucleotides expected for this suborder. Furthermore, because the signature nucleotide pattern of the family *Gordoniaceae* was based on only one genus, *Gordonia*, the inclusion of novel members in this family makes it necessary for this pattern to be emended (Stackebrandt *et al.*, 1997). Including the 16S rRNA gene sequences of strains IMMIB RIV-956^T, IMMIB RIV-956Fl, *W. muralis* and *W. maris* in the phylogenetic tree of the family *Gordoniaceae* reveals that members of this family are characterized by having the following signature nucleotides at positions 661–744 (A–U), 824–876 (U–A), 825–875 (A–U), 843 (U), 1002–1038 (A–U) and 1122–1151 (G–C). Additionally, members of the genus *Williamsia* are characterized by possessing signature nucleotides at positions 293–304 (G–C), 307 (C) and 1007–1022 (G–C), whereas members of the genus *Gordonia* have A–U, U and C–G at the respective positions. However, these patterns will need to be updated as novel species are added to these genera.

The phylogenetic tree (Fig. 1) shows the position of strain IMMIB RIV-956^T within the radiation of representative

phylogenetic groups of the suborder *Corynebacterineae*. It is evident from this that strain IMMIB RIV-956^T (and strain IMMIB RIV-956Fl; data not shown) represent a distinct subline within the genus *Williamsia*. This association is supported by the results obtained using all three treeing algorithms and by very high bootstrap values. The 16S rRNA gene sequences of IMMIB RIV-956^T and IMMIB RIV-956Fl display 96.2 and 97.2 % similarities to *W. muralis* DSM 44343^T and *W. maris* DSM 44693^T, respectively. Although there is no precise correlation between the degree of 16S rRNA gene sequence divergence and species delineation, it is generally recognized that divergence values of 3 % or more are significant (Stackebrandt & Goebel, 1994). The observed divergence of 3.0 % between isolates IMMIB RIV-956^T and IMMIB RIV-956Fl and *W. maris* DSM 44693^T and *W. muralis* DSM 44343^T is consistent with separate species status.

Strains IMMIB RIV-956^T and IMMIB RIV-956Fl have morphological properties consistent with their assignment to the genus *Williamsia*. They are aerobic organisms which form smooth, orange-red-pigmented colonies on Columbia agar supplemented with 5 % sheep blood. Cells are rod- and coccoid-like, stain Gram-positive and are not acid-fast. The organisms are able to grow at 27 and 37 °C but not at 42 °C. The physiological properties of strains IMMIB RIV-956^T and IMMIB RIV-956Fl are given in detail in the species

Table 1. Differential physiological characteristics of *Williamsia deligens* sp. nov., *W. maris* DSM 44693^T and *W. muralis* DSM 44343^T

Strains IMMIB RIV-956^T and IMMIB RIV-956Fl (*W. deligens* sp. nov.) gave identical results. All the strains were positive for acetate, L-alanine, 2,3-butanediol, citrate, glucose, mannitol, paraffin, sucrose, D-sorbitol, trehalose, D-xylose and urea. All strains were negative for utilization of acetamide, adipic acid, cellobiose, isoamyl alcohol, L-arabinose, lactose, melezitose, ornithine, proline, raffinose and serine and for hydrolysis of adenine, casein, elastin, aesculin, gelatin, guanine, hypoxanthine, tyrosine and xanthine.

Characteristic	<i>W. deligens</i>	<i>W. maris</i>	<i>W. muralis</i>
Hydrolysis of testosterone	–	–	+
Utilization as sole sources of carbon and energy:			
Adonitol	–	–	+
L-Arabinose	–	–	+
meso-Erythritol	–	–	+
Galactose	–	+	–
Gluconate	–	–	+
Lactate	–	+	+
myo-Inositol	–	+	–
Maltose	+	+	–
Rhamnose	–	–	+
1,2-Propandiol	–	+	+
m-Hydroxybenzoate	–	–	+
p-Hydroxybenzoate	–	+	+
Utilization of acetamide as sole source of carbon and nitrogen	W*	–	–

*Utilized weakly after 3 weeks incubation.

description below. Biochemical characteristics used to differentiate the new isolates from *W. maris* DSM 44693^T and *W. muralis* DSM 44343^T as determined in this study are given in Table 1.

Chemotaxonomically, strains IMMIB RIV-956^T and IMMIB RIV-956Fl possess chemical markers which support their assignment to the genus *Williamsia*. Their cell walls contain *meso*-diaminopimelic acid as well as arabinose and galactose (i.e. cell-wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970). One-dimensional TLC of whole-cell acid methanolysates of the organisms revealed the presence of two lipid spots on the chromatogram. The lower spot corresponded to mycolic acids as identified by 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 strains IMMIB RIV-956^T and IMMIB RIV-956Fl released fatty acid methyl esters of $C_{16:0}$ (41.3 % of the total cleavage products) and $C_{18:0}$ (58.6 %) as pyrolysis cleavage products. GC analyses of the non-hydroxylated fatty acid methyl esters revealed the presence of tetradecanoate (2.6 % of the total fatty acids), *cis*-hexadecenoate (1.6 %), hexadecanoate (14.5 %), 10-methyl hexadecanoate (0.5 %), octadecenoate (5.2 %), octadecanoate (18.3 %), tuberculostearic acid (10-methyl octadecanoate, 16.9 %), eicosanoate (1.2 %), docosenoate (0.3 %), docosanoate (16.6 %), tetracosenoate (2.1 %) and tetracosanoate (19.9 %) as the major cellular fatty acid methyl esters. Polar lipid analysis showed that the organisms contain phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol and diphosphatidylglycerol as the characteristic phospholipids (i.e. phospholipid type PII *sensu* Lechevalier *et al.*, 1977). Mass spectral analysis of the main components from strains IMMIB RIV-956^T and IMMIB RIV-956Fl revealed a strong peak at m/z 809.63 attributable to $[M+Na]^+$ in the high-mass region. This corresponds to a dihydrogenated menaquinone with nine isoprene units, MK-9(H_2). The second band shows a strong peak at m/z 741.63 attributable to $[M+Na]^+$ in the high-mass region. This corresponds to a dihydrogenated menaquinone with eight isoprene units, MK-8(H_2).

It is apparent from the genotypic and phenotypic data that strains IMMIB RIV-956^T and IMMIB RIV-956Fl are similar and represent a novel species of the genus *Williamsia*, for which the name *Williamsia deligens* sp. nov. is proposed.

Description of *Williamsia deligens* sp. nov.

Williamsia deligens (de.li' gens. L. part. adj. *deligens* choosy, referring to the preference of carbon source).

Forms smooth, orange- to orange-red-pigmented colonies on agar media. Cells are rod- and coccoid-like, Gram-positive and not acid-fast. It grows over a temperature range 22–37 °C, but not at 42 °C. Shows the salient chemotaxonomic characteristics of the genus *Williamsia*. Its mycolic acids are cleaved on pyrolysis to release fatty acids of $C_{16:0}$ and $C_{18:0}$ as the major products. The fatty acid profile

mainly consists of straight-chain saturated, unsaturated and 10-methyl-branched components. Hydrolyses urea, but not adenine, casein, elastin, aesculin, gelatin, guanine, hypoxanthine, testosterone, tyrosine or xanthine. Assimilates acetate, 2,3-butanediol, citrate, glucose, maltose, mannitol, paraffin, sucrose, sorbitol, trehalose and xylose as carbon sources but not adonitol, adipate, isoamyl alcohol, L-arabinose, cellobiose, *meso*-erythritol, galactose, gluconate, *m*-hydroxybenzoate, *p*-hydroxybenzoate, *myo*-inositol, lactate, lactose, melezitose, 1,2-propandiol, raffinose or rhamnose. Utilizes L-alanine but not acetamide, arginine, gelatin, ornithine, proline or serine as simultaneous carbon and nitrogen sources.

The type strain, IMMIB RIV-956^T (= DSM 44902^T = CCUG 50873^T), was isolated from human blood.

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