

Micrococcus flavus sp. nov., isolated from activated sludge in a bioreactor

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Bacterial strain LW4^T was isolated from activated sludge of a wastewater-treatment bioreactor. Cells of strain LW4^T were Gram-positive cocci, with a diameter of 0.7–1.0 µm. Colonies produced on LB agar plates were yellow, smooth, circular and 0.5–1.5 mm in diameter. Strain LW4^T was aerobic and grew over the temperature range 26–34 °C and pH range 5–9, with optimal growth at 30.5–31.5 °C and pH 6.0–6.2. The cell-wall peptidoglycan of strain LW4^T contained amino acid residues of lysine, glutamic acid, alanine, glycine and aspartic acid. The most abundant cellular fatty acids of strain LW4^T were anteiso-C_{15:0} (32.15 %) and iso-C_{15:0} (31.65 %). Major respiratory quinones were MK-8(H₂) (57.3 %) and MK-7(H₂) (32.9 %). The DNA G + C content was 71.4 mol% (*T_m*). 16S rRNA gene sequence analysis indicated that strain LW4^T was phylogenetically related to members of the genus *Micrococcus*, with similarities ranging from 96.5 to 97.3 %. Levels of DNA–DNA relatedness of strain LW4^T to *Micrococcus luteus* DSM 20030^T, *Micrococcus lylae* DSM 20315^T and *Micrococcus antarcticus* AS 1.2372^T were 55, 48 and 36 %, respectively. Based on these results, it is concluded that strain LW4^T represents a novel species of the genus *Micrococcus*, for which the name *Micrococcus flavus* sp. nov. is proposed. The type strain is strain LW4^T (= CGMCC 1.5361^T = JCM 14000^T).

Since the first description of the genus *Micrococcus* by Cohn (1872), several emendations to this description have been made based on utilization of glucose, G + C content of the genomic DNA and phylogenetic analysis of the 16S rRNA gene (detailed in Wieser *et al.*, 2002). Although the genus comprises only three recognized species at the time of writing, *Micrococcus luteus*, *Micrococcus lylae* (Stackebrandt *et al.*, 1995; Kloos *et al.*, 1974) and *Micrococcus antarcticus* (Liu *et al.*, 2000), the isolation and detection of *Micrococcus*-like bacteria from activated sludge have been reported repeatedly (Painter, 1983; Kataoka *et al.*, 1996; Wieser *et al.*, 2002). In this study, we report the isolation from activated sludge and the identification of a novel *Micrococcus*-like isolate, strain LW4^T.

Bacterial strain LW4^T was isolated from activated sludge of a sequential batch reactor treating mixed wastewater of various nitroaromatic compounds (nitrobenzene, nitrophenol, 2,4-dinitrophenol) and aniline. The reactor had been operated for 1 year at the time when the sludge was sampled,

and the performance of the reactor was highly efficient (removal rates ~99 %) in removing all nitroaromatic compounds and aniline. The sludge sample was suspended in sterile water by using vigorous vortexing, and a portion of the suspension was spread directly on LB agar plates. The plates were incubated at 30 °C for about 1 week. Single colonies on the plates were picked up and bacterial strain LW4^T was obtained by repeatedly streaking the culture on new plates from a single colony.

Routine cultivation was conducted at 30 °C with LB media. Gram reactions were determined according to the method described by Gerhardt *et al.* (1994). Cell motility and morphology were examined by transmission electron microscopy and scanning electron microscopy (see Supplementary Fig. S1 in IJSEM Online). Growth temperature range was determined with a TN3F temperature-gradient incubator (Advantec). Catalase and oxidase activities, the Voges–Proskauer reaction, aerobic production of acids from carbohydrates, carbon source utilization and other biochemical characterization were performed according to the methods of Barrow & Feltham (1993) and Wieser *et al.* (2002).

Cells of strain LW4^T were Gram-positive cocci, with a diameter range of 0.7–1.0 µm. Flagella were not observed.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Micrococcus flavus* LW4^T is DQ491453.

A scanning electron micrograph of cells of strain LW4^T and a table detailing its cellular fatty acid composition are available as supplementary material in IJSEM Online.

Colonies were yellow, smooth, circular and 0.5–1.5 mm in diameter after 3 days cultivation on LB agar. Strain LW4^T was aerobic and grew over the temperature range 26–34 °C and pH range 5–9. Optimal growth was observed at 30.5–31.5 °C and pH 6.0–6.2. Strain LW4^T was not able to use nitrobenzene, nitrophenol, 2,4-dinitrophenol or aniline for growth. Additional physiological and biochemical properties of strain LW4^T are provided in the species description below.

Biomass for chemotaxonomic analyses was cultivated according to Stackebrandt *et al.* (1995) and at 30 °C for 24 h. Cell-wall analysis was performed according to the method described by Hasegawa *et al.* (1983). Cellular fatty acids were extracted, methylated and analysed by using the Sherlock Microbial Identification System following the manufacturer's instructions. Menaquinones were extracted and purified according to the method of Collins (1985) and were analysed by HPLC (Wu *et al.*, 1989), with a previously characterized mixture of various menaquinones and ubiquinones (Hu *et al.*, 2001) as a reference. The results indicated that the cell-wall peptidoglycan of strain LW4^T contained lysine, glutamic acid, alanine, glycine and aspartic acid. The most abundant cellular fatty acids of strain LW4^T were anteiso-C_{15:0} (32.15 %) and iso-C_{15:0} (31.65 %), which were also found to be the dominant cellular fatty acids of other members of the genus *Micrococcus* (Wieser *et al.*, 2002). However, a significant amount of iso-C_{15:1} was detected in strain LW4^T (19.21 %), compared with only trace amounts in other *Micrococcus* species (Wieser *et al.*, 2002). Detailed information on the cellular fatty acid composition of strain LW4^T is provided in Supplementary Table S1. Strain LW4^T had MK-8(H₂) (57.3 %) as the major respiratory quinone, plus a significant amount of MK-7(H₂) (32.9 %).

DNA base composition was determined by thermal denaturation (Marmur & Doty, 1962), with *Escherichia coli* K-12 as reference. The DNA G + C content of strain LW4^T was 71.4 mol%.

The nearly complete 16S rRNA gene of strain LW4^T (1403 bp) was amplified and sequenced as described by Zhang *et al.* (2003). Alignments of 16S rRNA gene sequences

were performed with the CLUSTAL_X program, version 1.64b (Thompson *et al.*, 1997). A neighbour-joining phylogenetic tree (Fig. 1) was constructed based on evolutionary distances that were calculated with the Kimura two-parameter model. Alignment positions with insertions or deletions were excluded from the calculations. 16S rRNA gene sequence analysis indicated that strain LW4^T was phylogenetically related to members of the genus *Micrococcus*, with similarities ranging from 96.5 to 97.3 %. The phylogenetic tree (Fig. 1) also indicated that strain LW4^T clustered with *Micrococcus* species and that this cluster was strongly supported (100 %).

Based on the above phenotypic and phylogenetic studies, it is clear that strain LW4^T represents a member of the genus *Micrococcus*. Strain LW4^T showed a range of phenotypic characteristics that differentiated it from recognized *Micrococcus* species (Table 1), such as the ability to reduce nitrate, assimilation of various carbon resources and major respiratory quinones. To distinguish strain LW4^T from other *Micrococcus* species further, levels of DNA–DNA relatedness of strain LW4^T to the type species of recognized *Micrococcus* species were determined by using the method described by Huß *et al.* (1983). Levels of DNA–DNA relatedness of strain LW4^T to *M. luteus* DSM 20030^T, *M. lylae* DSM 20315^T and *M. antarcticus* AS 1.2372^T were 55, 48 and 36 %, respectively.

Based on the results presented here, it is concluded that strain LW4^T represents a novel species of the genus *Micrococcus*, for which the name *Micrococcus flavus* sp. nov. is proposed.

Description of *Micrococcus flavus* sp. nov.

Micrococcus flavus (fla'vus. L. masc. adj. *flavus* yellow, pertaining to the yellow colour of the colonies).

Cells are spherical, 0.7–1.0 µm in diameter and non-motile. Gram-positive, aerobic and heterotrophic. Colonies are yellow, smooth and circular with entire margins. Optimal growth occurs at 30.5–31.5 °C and pH 6–6.2. Catalase- and oxidase-positive. Starch is hydrolysed. Negative for the Voges–Proskauer reaction, lipase, reduction of nitrate and

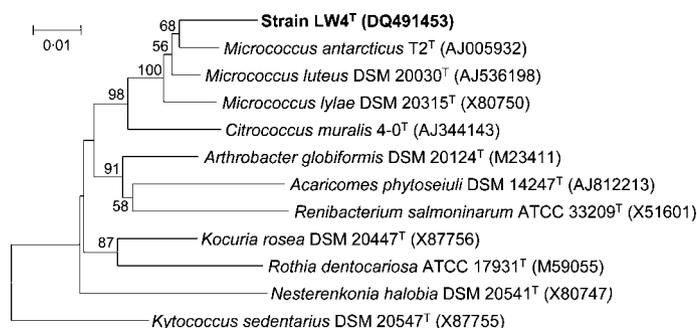


Fig. 1. Phylogenetic tree constructed with the neighbour-joining method according to 16S rRNA gene sequence evolutionary distance among strain LW4^T and the type strains of recognized members of the genus *Micrococcus* and type species of the family *Micrococcaceae*. *Kytococcus sedentarius* DSM 20547^T was used as an outgroup. GenBank accession numbers are given in parentheses. Numbers represent confidence levels (values greater than 50 % are shown) from 1000 replicate bootstrap samplings. Bar, evolutionary distance (K_{nuc}) of 0.01.

Table 1. Phenotypic characteristics that differentiate strain LW4^T from related *Micrococcus* speciesData for reference species were taken from Liu *et al.* (2000) and Wieser *et al.* (2002).

Characteristic	Strain LW4 ^T	<i>M. luteus</i>	<i>M. lylae</i>	<i>M. antarcticus</i>
Optimal growth temperature (°C)	31	37	37	16.8
Nitrate reduction	–	–	–	+
Voges–Proskauer reaction	–	–	–	+
Major quinone(s)	MK-8(H ₂), MK-7(H ₂)	MK-8, MK-8(H ₂)	MK-8(H ₂)	MK-8, MK-8(H ₂)
Hydrolysis of:				
Tween 80	–	–	–	+
Starch	+	–	–	+
Assimilation of:				
D-Mannose	–	+	–	–
L-Malate	–	–	–	+
D-Trehalose	+	–	+	–
DNA G+C content (mol%)	71.4	70.0*	69†	66.4†

*Value for strain Ballarat determined by Wieser *et al.* (2002).†Values for the type strains determined by Liu *et al.* (2000).

utilization of citric acid. Gelatin is not hydrolysed. No acid production from carbohydrates. Glycerol, trehalose and dextrin are used as carbon sources, but D-arabinose, fructose, mannitol, rhamnose, melibiose, xylitol, malic acid, L-glutamic acid, L-lactic acid, nitrilosides, L-arabinose, cellobiose, D-lactose, D-glucose, inositol, maltose, D-mannose, D-melibiose, raffinose, D-ribose, salicin and sorbitol are not. The predominant menaquinones are MK-8(H₂) and MK-7(H₂). The major cellular fatty acids are anteiso-C_{15:0} (32.15%) and iso-C_{15:0} (31.65%). The G+C content of the DNA is 71.4 mol% (T_m).

The type strain, LW4^T (=CGMCC 1.5361^T=JCM 14000^T), was isolated from activated sludge in a bioreactor.

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