Massilia flava sp. nov., isolated from soil

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A Gram-stain-negative, rod-shaped bacterium, designated strain Y9^T, was isolated from a soil sample collected in Ningxia Province in China and was characterized to determine its taxonomic position. Strain Y9^T contained Q-8 as the predominant ubiquinone. Major fatty acid components were summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and C_{16:0}. The major polar lipids were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The G+C content of the genomic DNA of strain Y9^T was 68.7 mol%. A phylogenetic tree based on 16S rRNA gene sequences showed that the strain fell within the evolutionary radiation encompassed by the genus *Massilia*. Levels of 16S rRNA gene sequence similarity between strain Y9^T and the type strains of recognized *Massilia* species ranged from 95.2 to 98.2 %, the highest values being with *Massilia albidiflava* 45^T (98.2 %) and *Massilia lutea* 101^T (98.0 %). However, levels of DNA–DNA relatedness between strain Y9^T and *M. albidiflava* KCTC 12343^T and *M. lutea* KCTC 12345^T were 37 and 26 %, respectively. Strain Y9^T was clearly differentiated from its nearest phylogenetic relatives in the genus *Massilia* based on phenotypic, chemotaxonomic and phylogenetic properties. Therefore, strain Y9^T is considered to represent a novel species of the genus *Massilia*, for which the name *Massilia flava* sp. nov. is proposed. The type strain is Y9^T (=CGMCC 1.10685^T =KCTC 23585^T).

The genus *Massilia* was proposed by La Scola *et al.* (1998) with the description of *Massilia timonae*, isolated from the blood of an immunocompromised patient with cerebellar lesions. Subsequently, the genus *Naxibacter* was described by Xu *et al.* (2005) on the basis of differential phenotypic characteristics from closely related genera. However, differences between *Massilia* and *Naxibacter* were not genus-specific. Furthermore, the two genera shared identical chemotaxonomic properties. Consequently, Kämpfer *et al.* (2011) proposed the transfer of all *Naxibacter* species to the genus *Massilia*. At the time of writing, the genus *Massilia* thus comprises 16 recognized species, isolated from air, soil and water (La Scola *et al.*, 1998; Xu *et al.*, 2005; Gallego *et al.*, 2006; Kämpfer *et al.*, 2008, 2011; Zhang *et al.*, 2006; Zul *et al.*, 2008; Weon *et al.*, 2008, 2009, 2010). Members of the genus *Massilia* are Gram-negative and have relatively high DNA G+C contents. Chemotaxonomically, members of this genus contain summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and C_{16:0} as major fatty acids and Q-8 as the predominant isoprenoid quinone.

In this study, we describe a *Massilia*-like soil isolate, designated strain Y9^T. The organism was considered to be

Massilia-like on the basis of preliminary 16S rRNA gene sequence analysis. Accordingly, the aim of present work was to elucidate the taxonomic position of strain Y9^T, based on a combination of phenotypic, genetic and chemotaxonomic analyses.

Strain Y9^T was originally isolated from a soil sample from Ningxia Province in China. For isolation, the soil sample (1 g) was suspended in distilled water (100 ml) and spread on plates of ISP2 medium after serial dilution. Plates were incubated at 28 °C for 3 days. Single colonies were purified by transferring them onto new plates and were incubated again on the same medium. ISP2 medium contained the following (l⁻¹): 4 g yeast extract, 10 g malt extract, 4 g glucose and 20 g agar. The medium was adjusted to pH 7.5 with 1 mM NaOH. Purified colonies were tentatively identified from partial sequences of the 16S rRNA gene. Strain Y9^T was maintained on ISP2 agar at 4 °C and as glycerol suspensions (20 %, v/v) at –20 °C.

Cellular morphological characteristics of strain Y9^T were examined by light microscopy (BH-2; Olympus) after 24 h growth on ISP2 medium at 28 °C. Colonial properties were examined after 3 days of growth at 28 °C on ISP2 medium. Phenotypic characteristics such as Gram staining, catalase and oxidase activities and hydrolysis of casein, Tween 80 and starch were performed by using the methods of Smibert & Krieg (1994). Growth at 4, 10, 28, 37, 40, 45 and

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Y9^T is HM777013.

A supplementary figure is available with the online version of this paper.

55 °C was measured on ISP2 medium. The pH range (pH 4–10 at intervals of 0.5 pH units) for growth and the requirement for NaCl [0, 1, 2, 3, 5 and 7 % (w/v)] were determined as described by Xu *et al.* (2005). The commercially available API test kits (bioMérieux) were used to determine the biochemical properties, utilization of carbohydrates and enzyme activities of the novel strain. Tests were generally performed according to the manufacturer's instructions. The API ZYM tests were read after 4 h incubation at 37 °C and the other API tests were read after 72 h at 28 °C.

Strain Y9^T was a Gram-stain-negative, non-spore-forming bacterium. Cells were rod-shaped (0.6–0.8 × 1.5–2.5 µm) with round ends. Colonies were circular to irregular, convex, yellow and 1–3 mm in diameter after 3 days of cultivation at 28 °C on ISP2 medium. Strain Y9^T grew optimally at 28–30 °C (range 10–45 °C) and did not grow in the presence of more than 1 % (w/v) NaCl. Growth was

observed at pH 6.5–8.5 (optimally at pH 7.0–7.5). Catalase activity and oxidase activity were present. The phenotypic properties of strain Y9^T are summarized and compared with those of related species of the genus *Massilia* in Table 1 and are given in the species description.

Biomass for chemotaxonomic studies was obtained after incubation at 28 °C for 3 days in shake flasks of tryptone soy broth (TSB; Oxoid). Ubiquinones were extracted and purified according to the methods of Collins *et al.* (1987); purified ubiquinones were identified by reversed-phase HPLC (Wu *et al.*, 1989). Polar lipids were extracted according to the method of Minnikin *et al.* (1979) and polar lipid profiles were analysed according to Collins & Jones (1980). Genomic DNA of strain Y9^T for G + C content determination was prepared according to the method of Marmur & Doty (1962). Whole-cell fatty acids were analysed by using the standard Microbial Identification System (MIDI) (Sasser, 1990; Kämpfer & Kroppenstedt,

Table 1. Differential characteristics between strain Y9^T and the type strains of closely related species of the genus *Massilia*

Strains: 1, Y9^T; 2, *M. aerilata* KACC 12505^T; 3, *M. albidiflava* KCTC 12343^T; 4, *M. aurea* DSM 18055^T; 5, *M. brevitalea* DSM 18925^T; 6, *M. dura* KCTC 12342^T; 7, *M. lutea* KCTC 12345^T; 8, *M. niabensis* DSM 21312^T; 9, *M. niastensis* DSM 21313^T; 10, *M. plicata* KCTC 12344^T; 11, *M. timonae* DSM 16850^T; 12, *M. suwonensis* KACC 12635^T. Data for reference strains were taken from Zul *et al.* (2008) (*M. brevitalea* DSM 18925^T), Weon *et al.* (2009) (*M. niabensis* DSM 21312^T and *M. niastensis* DSM 21313^T), Weon *et al.* (2010) (*M. suwonensis* KACC 12635^T) and Weon *et al.* (2008) (remaining strains) unless otherwise indicated. The reported data were obtained on the basis of the same and/or comparable conditions. In API 20NE tests, all strains are negative for indole production, glucose fermentation, arginine dihydrolase and assimilation of D-mannitol. In API ZYM tests, all strains are positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, acid phosphatase and esterase (C4), but negative for lipase (C14), N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. w, Weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Nitrate reduction	+	+	+	–	+	–	–	+	–	–	–	–
Enzyme activities												
Oxidase	+	+	+ ^{a*}	W ^b	–	+ ^a	+ ^a	+	+	– ^a	+ ^c	+
Catalase	+	+	+	+	W	+	+	+	+	+	+	ND
Urease	–	+	–	–	–	–	–	–	–	–	–	–
α-Galactosidase	–	–	+	–	–	+	–	–	–	+	–	–
α-Glucosidase	+	+ ^e	+ ^a	+ ^b	+	+ ^a	+ ^a	+	–	– ^a	– ^b	ND
β-Glucosidase	–	–	+	–	–	–	–	–	–	–	W	–
β-Glucuronidase	–	–	–	–	–	–	–	+	–	–	–	–
β-Galactosidase	+	–	+	+ ^d	–	+	+ ^a	+	+	+ ^a	ND	–
Hydrolysis of:												
Casein	–	+	+ ^a	+ ^b	+	+ ^a	+ ^a	–	–	+ ^a	ND	+
Starch	+	–	+ ^a	+ ^b	+	– ^a	+ ^a	+	W	+ ^a	+ ^c	–
Gelatin liquefaction	W	+	+	+	–	+	+	–	+	+	+	+
Utilization of:												
D-Glucose	+	+	+	+	+	+	–	–	+	+	+	–
Sucrose	+	–	+ ^a	– ^b	–	+ ^a	+ ^a	–	–	+ ^a	– ^d	–
Maltose	+	+	ND	+ ^b	+	– ^a	ND	–	+	– ^a	+ ^d	+
L-Rhamnose	+	–	– ^a	+ ^b	–	– ^a	– ^a	–	+	– ^a	+ ^d	–
D-Mannose	+	+	+	+	–	+	+	–	+	+	+	–
L-Arabinose	+	+	+	–	–	+	+	–	+	+	+	–
DNA G + C content (mol%)	68.7	68.9	65.3	66.0	65.3	65.9	63.3	67.8	66.6	65.1	62–67†	67.8

*Data taken from: *a*, Zhang *et al.* (2006); *b*, Gallego *et al.* (2006); *c*, Lindquist *et al.* (2003); *d*, Zul *et al.* (2008).

†Range of values for a number of strains including the type strain (Lindquist *et al.*, 2003).

Table 2. Cellular fatty acid compositions of strain Y9^T and the type strains of closely related species of the genus *Massilia*

Strains: 1, Y9^T; 2, *M. albidiflava* KCTC 12343^T; 3, *M. dura* KCTC 12342^T; 4, *M. lutea* KCTC 12345^T; 5, *M. niabensis* DSM 21312^T; 6, *M. niastensis* DSM 21313^T; 7, *M. timonae* DSM 16850^T; 8, *M. suwonensis* KACC 12635^T. Data in columns 2–4 were taken from Zhang *et al.* (2006) and data in columns 5–8 were taken from Weon *et al.* (2010). The reported data were obtained under the same or comparable conditions. Values are percentages of the total fatty acids; –, not present/not reported.

Fatty acid	1	2	3	4	5	6	7	8
C _{10:0} 3-OH	2.7	9.6	8.3	4.6	6.6	5.5	4.6	5.4
C _{12:0}	3.0	6.0	5.6	2.8	8.9	4.7	3.3	4.4
C _{12:0} 2-OH	–	0.7	–	–	–	2.4	2.2	2.4
C _{14:0}	6.9	2.8	3.2	1.9	–	–	–	1.2
C _{14:0} 2-OH	1.5	3.0	3.4	2.0	–	–	–	–
C _{16:0}	26.1	22.8	25.5	22.8	23.6	26.9	30.5	28.8
iso-C _{16:0}	1.6	–	–	–	–	–	–	–
C _{17:0} cyclo	–	–	–	–	–	3.4	3.7	2.9
iso-C _{17:1} ω9c	–	–	–	0.7	–	–	–	–
C _{18:1} ω7c	9.9	7.4	5.9	10.0	7.8	12.3	7.9	7.8
C _{18:0}	3.2	–	–	–	–	–	–	–
C _{20:0}	–	–	–	–	–	2.6	–	–
Summed feature 3*	35.8	46.4	46.7	54.7	46.0	38.1	47.0	45.8

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI Sherlock version 4.5 system. Summed feature 3 contained C_{16:1}ω7c and/or iso-C_{15:0} 2-OH. The database used was TSBA40.

1996). The predominant isoprenoid quinone in strain Y9^T was Q-8. The polar lipid profile of strain Y9^T contained major amounts of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol and moderate amounts of an unknown phospholipid and an unknown aminolipid (Supplementary Fig. S1, available in IJSEM Online). The DNA G+C content of strain Y9^T was 68.7 mol%. The fatty acid profile of strain Y9^T contained summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 35.8%) and C_{16:0} (26.1%) as major components and moderate amounts of C_{18:1}ω7c (9.9%), C_{12:0} (3.0%) and C_{10:0} 3-OH (2.7%), which were also found for recognized species of the genus *Massilia*. However, in comparison with recognized *Massilia* species, the fatty acid profile of strain Y9^T showed relatively large amounts of C_{14:0} (6.9%) and C_{18:0} (3.2%). Comparisons of the fatty acid profiles of the novel isolate and recognized members of the genus *Massilia* are given in Table 2. The major respiratory quinone, DNA G+C content and fatty acid profile of strain Y9^T were all consistent with the properties of the genus *Massilia*.

Extraction of genomic DNA and amplification of the 16S rRNA gene were performed as described by Rainey *et al.* (1996). The PCR product was sequenced directly by using the method of Lu *et al.* (2001). Sequence gel electrophoresis was performed and nucleotide sequences were obtained automatically by using an Applied Biosystems DNA sequencer (model 377) and software provided by the manufacturer. The resultant 16S rRNA gene sequence was compared with those available in GenBank, by using the BLAST program. Multiple alignment with sequences from closely related species was performed with the CLUSTAL X 1.8 program (Thompson *et al.*, 1997). Levels of 16S rRNA gene sequence similarity were

calculated by using the EzTaxon server (<http://www.eztaxon.org>; Chun *et al.*, 2007). Phylogenetic dendrograms were constructed by using the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Kluge & Farris, 1969) methods in MEGA, version 4.1 (Tamura *et al.*, 2007), with bootstrap values based on 1000 replications. Evolutionary distance matrices were generated following Kimura (1980). The almost-complete 16S rRNA gene sequence was obtained for strain Y9^T (1406 nt). Comparative 16S rRNA gene sequence analyses showed that strain Y9^T was affiliated phylogenetically with species of the genus *Massilia*. Strain Y9^T showed levels of 16S rRNA gene sequence similarity of 95.2–98.2% with respect to the type strains of recognized *Massilia* species, the highest levels being with *Massilia albidiflava* 45^T (98.2%), *Massilia lutea* 101^T (98.0%), *Massilia niastensis* 5516S-1^T (97.3%), *Massilia niabensis* 5420S-26^T (97.0%) and *Duganella violaceinigra* YIM 31327^T (97.0%). Other strains showed less than 97% 16S rRNA gene sequence similarity. Strain Y9^T showed relatively low sequence similarities (<95.6%) with respect to members of other genera of the family Oxalobacteraceae, except for *D. violaceinigra*. A phylogenetic tree based on the neighbour-joining method is shown in Fig. 1. Strain Y9^T fell within the evolutionary radius of the genus *Massilia*, clustering with *M. timonae*, the type species of the genus *Massilia*. The maximum-parsimony tree also supported the clustering of strain Y9^T and recognized *Massilia* species.

The major cellular fatty acids of strain Y9^T were summed feature 3 and C_{16:0}, identical to data for *M. timonae* DSM 16850^T. They also shared some common phenotypic characteristics (Table 1). Phylogenetically, strain Y9^T grouped with *Massilia* species and clustered with *M. timonae*

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain Y9^T and closely related species. Asterisks indicate that the corresponding branches were also recovered in the maximum-parsimony tree. Bootstrap values based on 1000 replications are listed as percentages; only values >50% are given. Bar, 0.005 substitutions per nucleotide position.

DSM 16850^T. Therefore, strain Y9^T should be assigned to the genus *Massilia*.

DNA–DNA hybridization experiments among strain Y9^T and its closest phylogenetic neighbours, *M. albidiflava* KCTC 12343^T, *M. lutea* KCTC 12345^T, *M. niastensis* DSM 21313^T, *M. niabensis* DSM 21312^T and *D. violaceinigra* KCTC 12193^T, were carried out applying the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992) under optimal hybridization conditions. Mean DNA–DNA relatedness between strain Y9^T and *M. albidiflava* KCTC 12343^T, *M. lutea* KCTC 12345^T, *M. niastensis* DSM 21313^T, *M. niabensis* DSM 21312^T and *D. violaceinigra* KCTC 12193^T was 37, 26, 21, 20 and 20%, respectively. These values were significantly lower than the threshold value of 70% that is commonly accepted for the definition of bacterial species (Wayne *et al.*, 1987).

Strain Y9^T showed a range of phenotypic characteristics that differentiated it from recognized species of the genus *Massilia* (Table 1). In addition, the results of our phylogenetic analysis (Fig. 1) and DNA–DNA relatedness experiments differentiate strain Y9^T from recognized *Massilia* species. It is apparent that strain Y9^T is not affiliated to any recognized species of the genus *Massilia*. Therefore, on the basis of the data presented above, strain Y9^T represents a novel species of the genus *Massilia*, for which the name *Massilia flava* sp. nov. is proposed.

Description of *Massilia flava* sp. nov.

Massilia flava (fla'va. L. fem. adj. *flava* yellow, pertaining to the yellow colony colour).

Cells are Gram-stain-negative, aerobic rods, 0.6–0.8 µm wide and 1.5–2.5 µm long. Colonies are circular to irregular, convex, yellow and 1–3 mm in diameter after 3 days cultivation at 28 °C on ISP2 medium. Growth occurs at 10–45 °C (optimal growth at 28–30 °C) and pH 6.5–8.5 (optimal growth at pH 7.0–7.5). Growth occurs with 0–1% NaCl (optimal growth without NaCl). Positive for catalase, oxidase and β-galactosidase (ONPG test), weakly positive for tryptophan deaminase and negative for urease, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase. Degrades starch and Tweens 20 and 80, but not casein or tyrosine. Nitrate is reduced. Neither indole nor hydrogen sulfide is produced. Methyl red reaction is negative. Milk coagulation and peptonization are positive. In API ZYM tests, positive for alkaline phosphatase, acid phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, naphthol-AS-BI-phosphohydrolase, β-galactosidase and α-glucosidase, but negative for esterase lipase (C8), lipase (C14), α-galactosidase, β-glucuronidase, β-glucosidase, N-acetylglucosaminidase, α-mannosidase and α-fucosidase. Assimilates L-arabinose, cellobiose, D-fucose, D-galactose, D-glucose, glycogen, lactose, maltose, L-rhamnose, sucrose, trehalose

and D-xylose. Weakly assimilates arbutin, D-fructose and D-mannose. Does not assimilate D-arabinose, D- or L-arabitol, L-fucose, N-acetyl-D-glucosamine, glycerol, *myo*-inositol, mannitol, melezitose, melibiose, raffinose, D-ribose, D-sorbitol, L-sorbose, D-tagatose, D-xylitol or L-xylose. The predominant ubiquinone is Q-8. The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The major fatty acids are summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), C_{16:0} and C_{18:1}ω7c. The G+C content of the DNA of the type strain is 68.7 mol%.

The type strain, Y9^T (=CGMCC 1.10685^T =KCTC 23585^T), was isolated from a soil sample collected from Ningxia Province, China. The species description is based on a single strain and hence serves as a description of the type strain.

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