

Massilia dura sp. nov., *Massilia albidiflava* sp. nov., *Massilia plicata* sp. nov. and *Massilia lutea* sp. nov., isolated from soils in China

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Four Gram-negative, motile, rod-shaped bacterial strains were isolated from soil samples collected from south-east China. A taxonomic study including phylogenetic analysis based on 16S rRNA gene sequences and phenotypic characteristics was performed. DNA G + C contents of the four strains were 63–66 mol%. Their predominant ubiquinone was Q-8. The fatty acid profiles contained C_{16:1ω7c} (36.9–54.7%) and C_{16:0} (22.8–25.5%) as the major components. Based on their phenotypic characteristics, phylogenetic position as determined by 16S rRNA gene sequence analysis and DNA–DNA hybridization results, the four isolates are considered to represent four novel species of the genus *Massilia*, for which the names *Massilia dura* sp. nov. (type strain 16^T = CCTCC AB 204070^T = KCTC 12342^T), *Massilia albidiflava* sp. nov. (type strain 45^T = CCTCC AB 204071^T = KCTC 12343^T), *Massilia plicata* sp. nov. (type strain 76^T = CCTCC AB 204072^T = KCTC 12344^T) and *Massilia lutea* sp. nov. (type strain 101^T = CCTCC AB 204073^T = KCTC 12345^T) are proposed.

The genus *Massilia* was first described by La Scola (1998) based on a single isolate from the blood of an immunocompromised patient with meningoencephalitis. Subsequently, the use of 16S rRNA gene sequence analysis led to the identification of a second isolate of *Massilia timonae* from a surgical wound infection in an immunocompetent 36-year-old male who had undergone orthopaedic surgery (Sintchenko *et al.*, 2000). More recently, Lindquist *et al.* (2003) presented taxonomic results for another four *Massilia*-like isolates (85A2206, 96A14209, 97A4424 and 99A9205) from different patients, including 16S rRNA gene sequence analysis, conventional biochemical test results,

morphological and flagellar characteristics and cellular fatty acid analysis. They provided an emended description of *M. timonae* as follows: 'Cells are Gram-negative medium straight rods. They are motile, predominantly by means of a single polar flagellum, lateral flagella may also occur. Tests for oxidase and catalase are positive.'

The present investigation was designed to establish the taxonomic position of four novel *Massilia*-like isolates, which formed a distinct clade with species of the genera *Massilia* and *Telluria* within the family *Oxalobacteraceae*. Genotypic and phenotypic data indicate that these strains should be recognized as representing four novel species of the genus *Massilia*.

Four strains designated 16^T, 45^T, 76^T and 101^T were isolated by using the dilution plating method from soil samples polluted with heavy metals from a farm situated in a suburb of Nanjing, Jiangsu Province, south-east China. The medium used for isolation was yeast extract/malt extract agar (4.0% yeast extract, 10.0% malt extract, 4.0% glucose, 2.0% agar) (ISP 2 medium; Shirling & Gottlieb, 1966); incubation was at 28 °C for 2 weeks. Biomass for molecular systematic and

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 16^T, 45^T, 76^T and 101^T are AY965998, AY965999, AY966000 and AY966001, respectively.

Tables detailing the cellular fatty acid profiles and levels of DNA–DNA relatedness among the four novel strains are available as supplementary material in IJSEM Online.

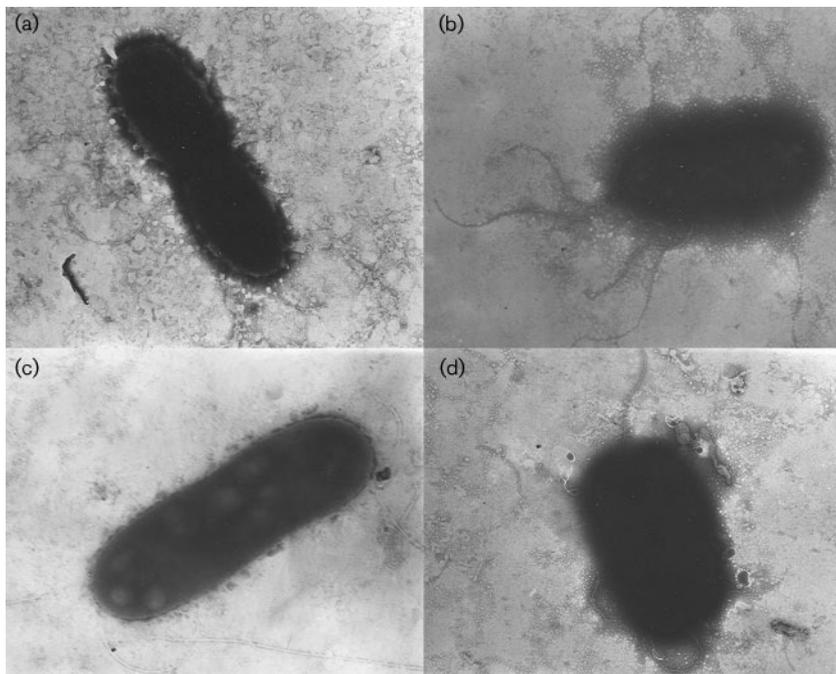


Fig. 1. Transmission electron micrographs of cells of strains 16^T (a), 45^T (b), 76^T (c) and 101^T (d) after 24 h growth on ISP 2 agar medium. Original magnification, $\times 20\,000$ (a), $\times 12\,000$ (b, d) and $\times 30\,000$ (c).

chemotaxonomic studies was obtained after incubation at 28 °C for 3 days in shake flasks of tryptone soy broth (TSB; Oxoid). Cellular morphological characteristics of the four new isolates were observed by light microscopy (model BH 2; Olympus) and by transmission electron microscopy (model H-800; Hitachi) after 24 h growth on ISP 2 medium. For transmission electron microscopy observation, cells were negatively stained with 1% (w/v) phosphotungstic acid after air drying. Motility of cells was studied on LB swarming agar (0.3%, w/v), and observation of flagella was performed using the method of Leifson (1960). Colony morphology was determined after 3 days growth at 28 °C on ISP 2 agar. Colour determination was made with colour chips from the ISCC-NBS Color Charts (Kelly, 1964).

Growth was tested at 4, 10, 28, 37, 40, 45 and 55 °C on ISP 2 medium. pH and NaCl tolerance experiments were performed as described by Xu *et al.* (2005). Other physiological and biochemical tests were carried out as described by Li *et al.* (2004).

Colonies of the four isolates shared several features such as whitish yellow to yellow colours, convex shape and plicate form on ISP 2 agar. Colonies of strains 76^T and 101^T reached a maximum of 2.0–3.0 mm in diameter after 3 days incubation at 28 °C, while those of strains 16^T and 45^T were about 1.0–1.5 mm in diameter. Cells of all four strains were Gram-negative, motile, non-spore-forming rods with one or more flagella, about 0.6–2.0 µm in width and 2.0–3.5 µm in length (Fig. 1). Detailed phenotypic characteristics and their variation among the four strains and reference strain *M. timonae* CIP 105350^T are given in Table 1 and in the species descriptions.

Ubiquinones were isolated using the methods of Minnikin *et al.* (1984) and separated by HPLC (Kroppenstedt, 1982). The predominant ubiquinone for the four strains was Q-8. Cellular fatty acid compositions were determined as described by Sasser (1990) using the Microbial Identification System (MIDI, Inc.). The major cellular fatty acids were C_{16:1}ω7c (36.9–54.7%) and C_{16:0} (22.8–25.5%); cellular fatty acid profiles for the four strains are given in Supplementary Table S1, which is available in IJSEM Online.

Extraction of genomic DNA and amplification of the 16S rRNA gene were performed as described by Xu *et al.* (2003). Phylogenetic analysis was performed using the software packages PHYLIP (Felsenstein, 1993) and MEGA version 2.1 (Kumar *et al.*, 2001) after multiple alignment of data by using CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to the Kimura two-parameter model; Kimura, 1980, 1983) and clustering were based on the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1000 resamplings (Felsenstein, 1985). Genomic DNA for determination of the base composition was prepared following the method of Marmur (1961). DNA G+C contents were determined using the thermal denaturation method of Marmur & Doty (1962). DNA–DNA hybridizations among the four isolates and their closest neighbour, *M. timonae* CIP 105350^T, were carried out applying the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992) under optimal hybridization conditions.

The almost complete 16S rRNA gene sequences for strains

Table 1. Differential phenotypic characteristics among strains 16^T, 45^T, 76^T and 101^T and their nearest phylogenetic neighbour, *M. timonae* CIP 105350^T

Data for *M. timonae* CIP 105350^T were taken from Lindquist *et al.* (2003). All strains show the following phenotypic characteristics. Cells are motile, non-spore-forming rods with flagella. Cellular fatty acids contain mainly C_{16:1}ω7c and C_{16:0}. Positive for catalase reaction and gelatin liquefaction, but negative for arginine dihydrolase, ornithine decarboxylase and indole production. Abbreviations: +, positive; –, negative; ND, no data.

Characteristic	16 ^T	45 ^T	76 ^T	101 ^T	<i>M. timonae</i> CIP 105350 ^T
Flagellation	Lateral	Lateral	Lateral	Lateral	Single or lateral
Nitrate reduction	+	+	+	–	–
Hydrolysis of starch	–	+	+	+	+
Enzyme activities:					
Lysine decarboxylase	–	+	–	–	–
β-Glucuronidase	+	+	–	+	ND
N-Acetyl-glucosaminidase	+	+	–	+	ND
L-Aspartic arylamidase	–	+	+	+	ND
Lipase	–	+	+	+	ND
Oxidase	+	+	–	+	+
Urease	+	+	+	–	–
Methyl red test	+	–	–	–	–
Voges–Proskauer test	–	–	+	–	ND
DNA G + C content (mol%)	65.9	65.3	65.1	63.3	62–67
Isolation source	Soil	Soil	Soil	Soil	Blood

16^T, 45^T, 76^T and 101^T were determined as consisting of 1478, 1470, 1471 and 1478 bp, respectively. These 16S rRNA gene sequences (corresponding to *Escherichia coli* positions 46–518) showed 97.1–99.5% similarity with each other. However, they shared relatively low 16S rRNA gene sequence similarity (<95%) with all recognized genera of the family *Oxalobacteraceae* except with the genus *Massilia* (96.5%). A neighbour-joining tree based on the 16S rRNA gene sequences of the four new isolates and related taxa is shown in Fig. 2. The four strains formed a distinct clade with the genus *Massilia* within the family *Oxalobacteraceae*.

According to the original description (La Scola *et al.*, 1998) and subsequently emended description (Lindquist *et al.*, 2003) of the genus *Massilia*, cells of the four novel strains have similar morphology to those of *Massilia* isolates: cells are non-spore-forming rods, motile by means of flagella. For all of these isolates, their major cellular fatty acids are C_{16:1}ω7c and C_{16:0}. They also share some other common phenotypic characteristics, as noted in Table 1. Phylogenetically, the four isolates were closest to *M. timonae* CIP 105350^T (96.5% 16S rRNA gene sequence similarity), and on this basis should be assigned to the genus *Massilia*.

Additionally, DNA–DNA hybridization among the four tested strains and the reference strain *M. timonae* CIP 105350^T (see Supplementary Table S2 in IJSEM Online) gave results much lower than 70%, the recommended threshold value for the delineation of genomic species (Wayne *et al.*, 1987). This provided decisive evidence that the four isolates represent members of different genomic species. The G + C contents of the genomic DNA from

strains 16^T, 45^T, 76^T and 101^T were 65.9, 65.3, 65.1 and 63.3 mol%, respectively.

Therefore, based on the phenotypic and genotypic data presented, we consider strains 16^T, 45^T, 76^T and 101^T to represent four novel species of the genus *Massilia*, *Massilia dura* sp. nov., *Massilia albidiflava* sp. nov., *Massilia plicata* sp. nov. and *Massilia lutea* sp. nov., respectively.

Description of *Massilia dura* sp. nov.

Massilia dura (du'ra. L. fem. adj. *dura* hard, referring to the nature of the colonies).

Colonies are 0.9–1.2 mm in diameter, circular, entire, convex, opaque, hard, compact and pale white to yellow on nutrient agar plates. Cells are 0.6–0.8 μm in width and 1.8–2.2 μm in length. Cells are motile, non-spore-forming, straight rods with one or more flagella, about 0.7–0.8 μm in width and 2.0–2.5 μm in length (Fig. 1a). Growth temperature and pH range for growth are 10–45 °C and pH 6.5–8.5, with optimum growth at 28–30 °C and pH 7.0–7.5. Cannot tolerate > 1% NaCl. Positive for oxidase, catalase, urease, α-galactosidase, α-glucosidase, α-maltosidase, β-glucosidase, β-glucuronidase, N-acetyl-glucosaminidase, β-galactosidase, nitrate reduction, casein and Tween 20 hydrolysis, gelatin liquefaction, NH₃ production and methyl red test. Negative for lysine decarboxylase, L-aspartic arylamidase, lipase, ornithine decarboxylase, arginine dihydrolase, Tween 80 and starch hydrolysis, melanin, indole and H₂S production, milk coagulation and peptonization. Utilizes glucose and sucrose as sole carbon sources, but not

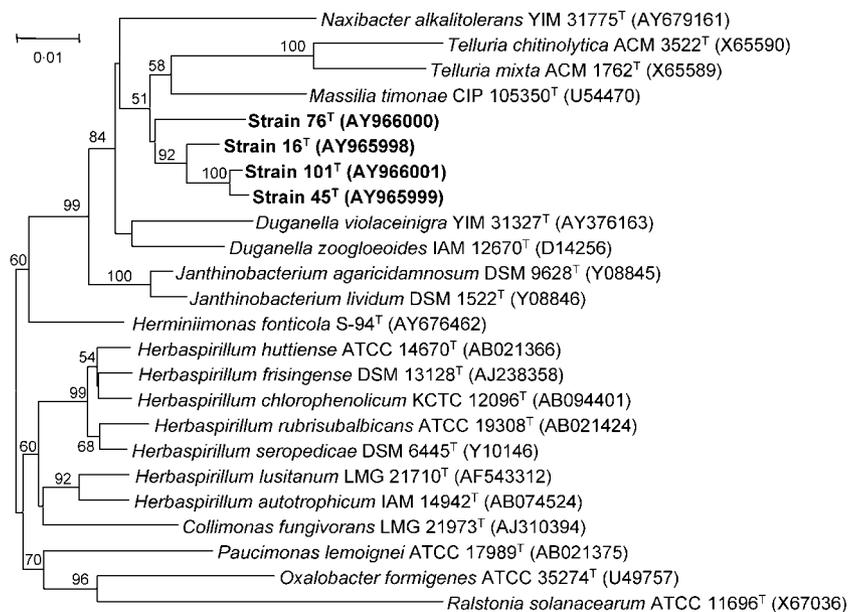


Fig. 2. Phylogenetic dendrogram obtained by neighbour-joining analysis based on 16S rRNA gene sequences, showing the position of strains 16^T, 45^T, 76^T and 101^T among their phylogenetic neighbours. Numbers on branch nodes are bootstrap values (1000 resamplings). Sequence accession numbers are given in parentheses. The sequence of *Ralstonia solanacearum* ATCC 11696^T was used as the root. Bar, 0.01 substitutions per nucleotide position.

malonate, maltose, trehalose, rhamnose, inositol, adonitol, palatinose, cellobiose, sorbitol, D-arabitol, L-arabinose, mannitol, phenol red, galacturonate or L-arabitol. Major cellular fatty acids are C_{16:1}ω7c and C_{16:0}. Q-8 is the predominant respiratory quinone. The G + C content of the genomic DNA is 65.9 mol%.

The type strain, strain 16^T (= CCTCC AB 204070^T = KCTC 12342^T), was isolated from heavy-metal-polluted farm soil, Nanjing, Jiangsu Province, China.

Description of *Massilia albidiflava* sp. nov.

Massilia albidiflava (al.bi.di.fl'a'va. L. adj. *albidus* whitish; L. adj. *flavus* yellow; N.L. fem. adj. *albidiflava* whitish yellow, referring to the colour of the colonies).

Colonies are 1.0–1.5 mm in diameter, circular, entire, convex, desiccated, opaque and pale white to yellow on nutrient agar plates. Cells are motile, non-spore-forming, short rods with peritrichous flagella, about 1.8–2.0 μm in width and 3.0–3.5 μm in length (Fig. 1b). Growth temperature and pH range for growth are 10–45 °C and pH 6.5–8.5, with optimum growth at 28–30 °C and pH 7.0–7.5. Cannot tolerate >1% NaCl. Positive for oxidase, catalase, α-galactosidase, α-glucosidase, α-maltosidase, β-glucosidase, β-galactosidase, nitrate reduction, urease, NH₃ production, gelatin liquefaction, starch, casein and Tween 20 hydrolysis. Negative for ornithine decarboxylase, arginine dihydrolase, indole, melanin and H₂S production, Voges–Proskauer and methyl red tests, milk coagulation and peptonization. Utilizes glucose and sucrose as sole carbon sources, but cannot utilize rhamnose, inositol, adonitol, palatinose, cellobiose, sorbitol, D-arabitol, L-arabinose, mannitol, phenol red, galacturonate or L-arabitol. Major cellular fatty acids are C_{16:1}ω7c and C_{16:0}. Q-8 is the predominant

respiratory quinone. G + C content of the genomic DNA is 65.3 mol%.

The type strain, strain 45^T (= CCTCC AB 204071^T = KCTC 12343^T), was isolated from heavy-metal-polluted farm soil, Nanjing, Jiangsu Province, China.

Description of *Massilia plicata* sp. nov.

Massilia plicata (pli.ca'ta. L. part. adj. *plicata* folded, coiled, referring to the nature of the colonies).

Colonies are 2.0–3.0 mm in diameter, circular, entire, convex, viscous, opaque and yellow to pale brown on nutrient agar plates. Cells are motile, non-spore-forming, straight rods with one or more flagella, about 0.6–0.7 μm in width and 2.0–2.5 μm in length (Fig. 1c). Soluble pigment is produced on ISP 2 and some other tested media. Cells are 0.6–0.7 μm in width and 1.8–2.2 μm in length. Growth temperature and pH range for growth are 10–45 °C and pH 6.5–8.5, with optimum growth at 28–30 °C and pH 7.0–7.5. Cannot tolerate >1% NaCl. Positive for catalase, α-galactosidase, α-glucosidase, α-maltosidase, β-glucosidase, β-galactosidase, L-aspartic arylamidase, lipase, urease, starch and casein, Tween 20 hydrolysis, gelatin liquefaction, Voges–Proskauer test, nitrate reduction and NH₃ production. Negative for oxidase, lysine decarboxylase, β-glucuronidase, N-acetyl-glucosaminidase, ornithine decarboxylase, arginine dihydrolase, methyl red test, indole, melanin and H₂S production, milk coagulation and peptonization. Can utilize malonate, glucose and sucrose as sole carbon sources, but cannot utilize maltose, trehalose, rhamnose, inositol, adonitol, palatinose, cellobiose, sorbitol, D-arabitol, L-arabinose, mannitol, phenol red, galacturonate or L-arabitol. Major cellular fatty acids are C_{16:1}ω7c and C_{16:0}. Q-8 is the predominant respiratory quinone. G + C content of the genomic DNA is 65.1 mol%.

The type strain, strain 76^T (= CCTCC AB 204072^T = KCTC 12344^T), was isolated from heavy-metal-polluted farm soil, Nanjing, Jiangsu Province, China.

Description of *Massilia lutea* sp. nov.

Massilia lutea (lu.te'a. L. fem. adj. *lutea* golden yellow, referring to the colony colour).

Colonies are 2.0–3.0 mm in diameter, circular, entire, convex, viscous, opaque and yellow on nutrient agar plates. Cells are motile, non-spore-forming, short rods with peritrichous flagella, about 1.8–2.0 µm in width and 3.0–3.5 µm in length (Fig. 1d). Growth temperature and pH range for growth are 10–45 °C and pH 6.5–8.5, with optimum growth at 28–30 °C and pH 7.0–7.5. Cannot tolerate > 1% NaCl. Positive for catalase, oxidase, α-galactosidase, α-glucosidase, α-maltosidase, β-glucosidase, β-galactosidase, NH₃ production and gelatin liquefaction. Negative for urease, ornithine decarboxylase, arginine dihydrolase, indole and H₂S production, nitrate reduction, Voges–Proskauer and methyl red tests, milk coagulation and peptonization. Can hydrolyse starch, casein and Tween 20, but not Tween 80 or cellulose. Utilizes glucose and sucrose as sole carbon sources, but not rhamnose, inositol, adonitol, palatinose, cellobiose, sorbitol, D-arabitol, L-arabinose, mannitol, phenol red, galacturonate or L-arabitol. Major cellular fatty acids are C_{16:1}ω7c and C_{16:0}. Q-8 is the predominant respiratory quinone. G + C content of the genomic DNA is 63.3 mol%.

The type strain, strain 101^T (= CCTCC AB 204073^T = KCTC 12345^T), was isolated from heavy-metal-polluted farm soil, Nanjing, Jiangsu Province, China.

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