Massilia dura sp. nov., Massilia albidiiflava 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 C16:1ω7c (36.9–54.7 %) and C16:0 (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 16T = CCTCC AB 204070T = KCTC 12342T), Massilia albidiiflava sp. nov. (type strain 45T = CCTCC AB 204071T = KCTC 12343T), Massilia plicata sp. nov. (type strain 76T = CCTCC AB 204072T = KCTC 12344T) and Massilia lutea sp. nov. (type strain 101T = CCTCC AB 204073T = KCTC 12345T) 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 16T, 45T, 76T and 101T 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
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 (0.3–3 %, 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. (2003). 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 76T and 101T reached a maximum of 2.0–3.0 mm in diameter after 3 days incubation at 28 °C, while those of strains 16T and 45T 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 105350T 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<sub>16:1ω7c</sub> (36.9–54.7 %) and C<sub>16:0</sub> (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 105350T, 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 16T, 45T, 76T and 101T and their nearest phylogenetic neighbour, *M. timonae* CIP 105350T

Data for *M. timonae* CIP 105350T 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<sub>16:1</sub>ω7c and C<sub>16:0</sub>. Positive for catalase reaction and gelatin liquefaction, but negative for arginine dihydrolase, ornithine decarboxylase and indole production. Abbreviations: +, positive; −, negative; ND, no data.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>16T</th>
<th>45T</th>
<th>76T</th>
<th>101T</th>
<th><em>M. timonae</em> CIP 105350T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flagellation</td>
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<td>Lateral</td>
<td>Lateral</td>
<td>Single or lateral</td>
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<td>−</td>
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<td>+</td>
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<td>Enzyme activities:</td>
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<td>−</td>
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</tr>
<tr>
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<td>−</td>
<td>+</td>
<td>−</td>
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<td>−</td>
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<td>DNA G+C content (mol%)</td>
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<td>65·3</td>
<td>65·1</td>
<td>63·3</td>
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</table>

16T, 45T, 76T and 101T 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<sub>16:1</sub>ω7c and C<sub>16:0</sub>. They also share some other common phenotypic characteristics, as noted in Table 1. Phylogenetically, the four isolates were closest to *M. timonae* CIP 105350T (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 105350T (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 16T, 45T, 76T and 101T were 65·9, 65·3, 65·1 and 63·3 mol%, respectively.

Therefore, based on the phenotypic and genotypic data presented, we consider strains 16T, 45T, 76T and 101T 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.


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 0·7–0·9 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, NH<sub>3</sub> 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 H<sub>2</sub>S production, milk coagulation and peptonization. Utilizes glucose and sucrose as sole carbon sources, but not...
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 C16:1ω7c and C16:0. Q-8 is the predominant respiratory quinone. The G+C content of the genomic DNA is 65.3 mol%.

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

Description of Massilia albidiflava sp. nov.

Massilia albidiflava (al.bi.di.flav.a. L. adj. albidus whitish; L. adj. flavus yellow; N.L. fem. adj. albidiflava whitish yellow, referring to the colour 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 peritrichous flagella, about 1.8–2.0 μm in width and 3.0–3.5 μm in length (Fig. 1c). 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, x-galactosidase, x-glucosidase, x-maltosidase, β-glucosidase, β-D-galactosidase, nitrate reduction, urease, NH3 production, gelatin liquefaction, starch, casein and Tween 20 hydrolysis. Negative for ornithine decarboxylase, arginine dehydroylase, indole, melain 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 C16:1ω7c and C16:0. Q-8 is the predominant respiratory quinone. The G+C content of the genomic DNA is 65.3 mol%.

The type strain, strain 45T (= CCTCC AB 204071T = KCTC 12343T), 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, desiccated, opaque and pale white to yellow 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, x-galactosidase, x-glucosidase, x-maltosidase, β-glucosidase, β-D-galactosidase, L-aspartic arylamidase, lipase, urease, starch and casein, Tween 20 hydrolysis, gelatin liquefaction, Voges–Proskauer test, nitrate reduction and NH3 production. Negative for oxidative, lysine decarboxylase, β-glucuronidase, N-acetyl-glucosaminidase, ornithine decarboxylase, arginine dehydroylase, methionyl red test, indole, melain 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 C16:1ω7c and C16:0. Q-8 is the predominant respiratory quinone. The G+C content of the genomic DNA is 65.3 mol%.
The type strain, strain 76\textsuperscript{T} (= CCTCC AB 204072\textsuperscript{T} = KCTC 12344\textsuperscript{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, x-glucosidase, x-glucosidase, α-maltosidase, β-glucosidase, β-galactosidase, NH\textsubscript{4} production and gelatin liquefaction. Negative for urease, ornithine decarboxylase, arginine dihydrolase, indole and H\textsubscript{2}S 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\textsubscript{16:1}ω7C and C\textsubscript{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\textsuperscript{T} (= CCTCC AB 204073\textsuperscript{T} = KCTC 12345\textsuperscript{T}), was isolated from heavy-metal-polluted farm soil, Nanjing, Jiangsu Province, China.

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**References**


