Massilia aurea sp. nov., isolated from drinking water

Virginia Gallego, Cristina Sánchez-Porro, Maria Teresa García and Antonio Ventosa

Correspondence
Antonio Ventosa
ventosa@us.es

Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain

A Gram-negative, motile, rod-shaped organism, strain AP13^T, able to produce yellow-pigmented colonies, was isolated from the drinking water distribution system of Seville (Spain) and was characterized by using a polyphasic taxonomic approach. In 16S rRNA gene sequence comparisons, strain AP13^T exhibited 96·9–95·6 % similarity with respect to the five recognized species of the genus *Massilia*. The DNA G+C content of strain AP13^T was 66·0 mol%, a value that supports the affiliation of strain AP13^T to the genus *Massilia*. DNA–DNA hybridization data and phenotypic properties confirmed that strain AP13^T represents a novel species of the genus *Massilia*, for which the name *Massilia aurea* sp. nov. is proposed. The type strain is AP13^T (=CECT 7142^T=CCM 7363^T=DSM 18055^T=JCM 13879^T).

The genus *Massilia* belongs to the family *Oxalobacteraceae* (*Betaproteobacteria*) and, to date, comprises five species, *Massilia timonae* (La Scola *et al.*, 1998), *Massilia dura*, *Massilia albidiflava*, *Massilia plicata* and *Massilia lutea* (Zhang *et al.*, 2006), with *M. timonae* as the type species. The genus *Massilia* was described by La Scola *et al.* (1998) on the basis of a single isolate from the blood of an immunocompromised patient. Moreover, strains related to *M. timonae* have been isolated from human patients (Lindquist *et al.*, 2003). However, the isolation from soil samples of phenanthrene-degrading (Bodour *et al.*, 2003), protease-producing (Wery *et al.*, 2003) and *N*-acyl homoserine lactone-producing (d'Angelo-Picard *et al.*, 2005) strains related to the genus *Massilia*, as well as the recently described four novel species of the genus *Massilia* isolated from different soil samples from south-east China (Zhang *et al.*, 2006), showed that species of this genus can be isolated from environmental samples.

To monitor the quality of the drinking water of Seville (Spain), we carried out several microbiological studies from April 2003 to January 2004. Samples (25 l) of drinking water were concentrated by using a tangential flow filtration system (Filtron; Pall), plated on plate count agar (PCA; Difco) and incubated at 28 °C for 7 days. Colonies with different morphologies were subsequently plated, using the same isolation medium, in order to obtain pure cultures. Strain AP13^T was isolated during the sampling campaign of April 2003 and was studied phylogenetically, phenotypically and genotypically. On the basis of the results of these studies, we propose that strain AP13^T represents a novel species of the genus *Massilia*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AP13^T is AM231588.

Chromosomal DNA was isolated and purified according to the method described by Marmur (1961). The 16S rRNA gene was amplified using the universal primers 16F27 and 16R1488, as described by Mellado *et al.* (1995). The almost-complete nucleotide sequence was determined by NBT-Newbiotechnic (Seville, Spain) using an automated DNA sequencer (model 3100; Applied Biosystems). A sequence analysis was subsequently conducted by using the ARB program package (Ludwig *et al.*, 2004). According to the recommendations of Ludwig *et al.* (1998), alternative treeing methods (maximum parsimony, distance matrix and maximum likelihood) were carried out. A comparison using 16S rRNA gene sequences from databases revealed that the sequence of strain AP13^T displays the highest levels of similarity with those from *Massilia* species. The sequence similarities with respect to *Massilia* species were $\leqslant$96·9 %, *M. timonae* being the most closely related species. The phylogenetic tree obtained with the maximum-parsimony method placed strain AP13^T within the branch constituted by *Massilia* species (Fig. 1). These results were consistent with those obtained with other algorithms. According to the phylogenetic data, the novel isolate belongs to the genus *Massilia*, but, as it shows relatively low similarity with other species in the genus, strain AP13^T could be considered a novel species (Stackebrandt & Goebel, 1994).

The G+C content of the genomic DNA was determined from the mid-point value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962), using the equation of Owen & Hill (1979), as previously described in detail by Ventosa *et al.* (1999). The DNA G+C content was found to be

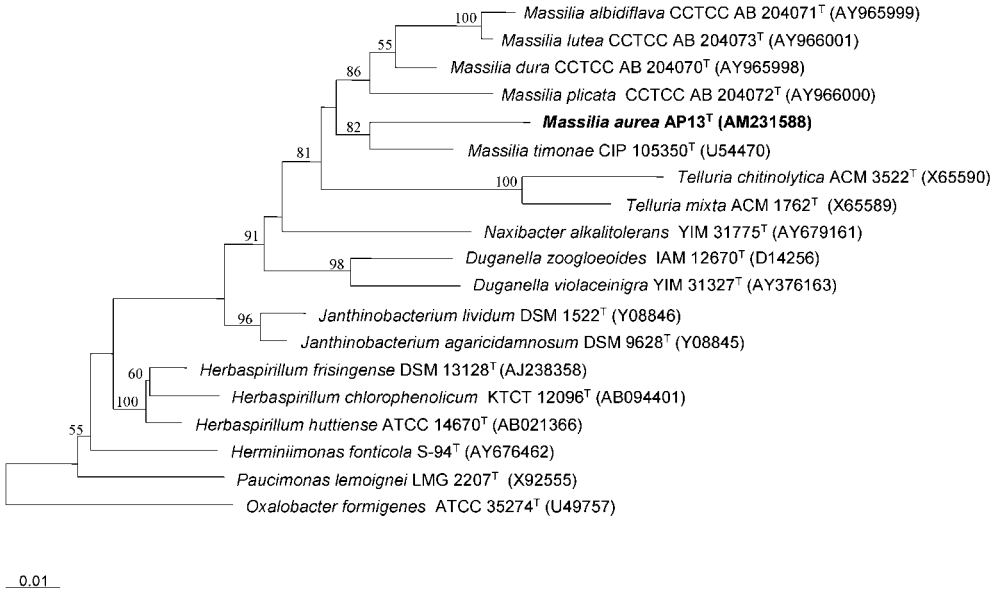

Fig. 1. Maximum-parsimony phylogenetic tree showing the relationships of strain AP13^T, species belonging to the genus *Massilia* and other related members of the *Betaproteobacteria*. Bootstrap values >50 % are indicated at the branch points. Bar, 1 % sequence divergence.

66·0 mol%, which is within the range for the genus *Massilia* (La Scola *et al.*, 1998; Zhang *et al.*, 2006). DNA–DNA hybridization studies were performed using the competition procedure of the membrane method (Johnson, 1994), which was described in detail by Mormile *et al.* (1999). The hybridization temperature was 59 °C, which is within the limit of validity for the filter method (De Ley & Tijtgat, 1970), and the degree of hybridization (%) was calculated according to Johnson (1994). The experiments were carried out in triplicate. The level of DNA–DNA hybridization between strain AP13^T and *M. timonae* DSM 16850^T was 13 %, confirming that strain AP13^T constitutes a novel species (Stackebrandt & Goebel, 1994).

The shape and motility of the bacterial cells were observed under a phase-contrast microscope (at ×1000) from a 24 h liquid culture (nutrient broth). Growth at different temperatures (4–40 °C), pH values (pH 4–9) and NaCl concentrations (0–5 % NaCl) was tested on PCA medium. The isolate was also tested for its ability to grow on nutrient agar medium, trypticase soy agar (TSA; Difco), R2A (Difco) and MacConkey agar (Scharlau). H_2S production was determined on Kligler iron agar (Difco). Oxidase activity was detected using a 1 % solution of tetramethyl-*p*-phenylenediamine (Difco) (Kovács, 1956). Catalase activity was tested by picking a young colony and smearing it in a drop of H_2O_2. The methyl red and Voges–Proskauer reactions were tested on Clark–Lubs' medium (Scharlau). Indole production was determined with Kovács' reagent on 1 % tryptone broth. A citrate test was performed on Simmons' citrate agar (Sigma). For the determination of acid production from different carbohydrates, a medium containing 0·5 % peptone, 0·5 % NaCl and 0·001 % phenol red was used (Cowan & Steel, 1974). The reduction of nitrate and nitrite was tested on nitrate broth containing 0·2 % KNO_3 (Skerman, 1967). Urease activity was studied in Christensen's medium (Christensen, 1946). The hydrolysis of gelatin, starch and DNA was tested on the corresponding agar media (Scharlau) (Cowan & Steel, 1974). Tween 80 hydrolysis was tested in PCA medium containing 1 % Tween 80 and 0·02 % $CaCl_2$ (Sierra, 1957). Casein hydrolysis was tested in PCA medium supplemented with 2 % skim milk (Difco) (Cowan & Steel, 1974).

Tests for carbon-source utilization, sugar fermentation and enzymes (qualitative) were carried out using API 20NE, API ID 32E and API ZYM kits (bioMérieux) inoculated according to the manufacturer's instructions and incubated at 28 °C. An API 50 CH strip was inoculated as described by Kersters *et al.* (1984). Antibiotic susceptibility was determined according to the conventional Kirby–Bauer method (Bauer *et al.*, 1966). The results of the phenotypic analysis are summarized in the species description. Several phenotypic characteristics that can be used to differentiate strain AP13^T from *Massilia* species are summarized in Table 1.

Fatty acid analysis and analysis of quinones were carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Cells were cultured on TSA medium for 24 h at pH 7·0 and 28 °C by using the methods described by Miller (1982) and Kämpfer & Kroppenstedt (1996). The predominant fatty acids of strain AP13^T were $C_{16:1}\omega7c$ and $C_{16:0}$. The fatty acid profile of strain AP13^T is very similar to

Table 1. Differential characteristics of strain AP13^T and species of the genus *Massilia*

Taxa: 1, strain AP13^T; 2, *M. timonae*; 3, *M. dura*; 4, *M. albidiflava*; 5, *M. plicata*; 6, *M. lutea*. Data for strain AP13^T and *M. timonae* DSM 16850^T are from this study. Data for *M. dura* CCTCC AB 204070^T, *M. albidiflava* CCTCC AB 204071^T, *M. plicata* CCTCC AB 204072^T and *M. lutea* CCTCC AB 204073^T are from Zhang *et al.* (2006). +, Positive; −, negative; W, weak; ND, not determined. All strains show the following phenotypic characteristics: cells are motile, non-spore-forming rods; colonies are yellow-pigmented; catalase-positive and β-galactosidase-positive; negative for indole and H_2S production.

Characteristic	1	2	3	4	5	6
Temp. range (°C) for growth	4–30	25–35	10–45	10–45	10–45	10–45
Optimum growth temp. (°C)	28	28	28–30	28–30	28–30	28–30
pH range for growth	4–9	ND	6·5–8·5	6·5–8·5	6·5–8·5	6·5–8·5
Optimum growth pH	7·0–8·0	ND	7·0–7·5	7·0–7·5	7·0–7·5	7·0–7·5
Oxidase	W	+	+	+	−	+
Nitrate reduction	−	−	+	+	+	−
Methyl red test	−	−	+	−	−	−
Voges–Proskauer test	−	−	−	−	+	−
Starch hydrolysis	+	+	−	+	+	+
Enzyme activities						
α-Chymotrypsin	−	+	ND	ND	ND	ND
α-Mannosidase	−	+	ND	ND	ND	ND
α-Fucosidase	−	+	ND	ND	ND	ND
α-Glucosidase	+	−	+	+	+	+
β-Glucosidase	−	+	+	+	+	+
α-Galactosidase	−	W	+	+	+	+
Lysine decarboxylase	−	−	−	+	−	−
β-Glucuronidase	−	−	+	+	−	+
N-Acetylglucosamidase	−	−	+	+	−	+
Lipase	−	−	−	+	+	+
Urease	−	−	+	+	+	−
Habitat	Drinking water	Blood	Soil	Soil	Soil	Soil
DNA G+C content (mol%)	66·0	62–67	65·9	65·3	65·1	63·3

those of recently described *Massilia* species, with $C_{16:0}$ and $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH as the predominant fatty acids (Table 2). Like members of the genus *Massilia*, strain AP13^T contains ubiquinone Q-8 as the major quinone.

On the basis of the phylogenetic, genotypic and phenotypic (and chemotaxonomic) data, we propose strain AP13^T as a novel species within the genus *Massilia* with the name *Massilia aurea* sp. nov.

Description of *Massilia aurea* sp. nov.

Massilia aurea (au′re.a. L. fem adj. *aurea* golden, referring to the yellowish pigment that the bacterium produces).

Cells are Gram-negative, straight rods 1·0 × 1·6–3·0 μm in size that occur singly or in pairs (on PCA medium at 28 °C after 48 h). Cells are motile, non-spore-forming and strictly aerobic. Colonies are circular, translucent, yellow-pigmented and 0·6–1·0 mm in diameter on PCA agar after 2 days incubation. Good growth occurs on TSA, R2A and nutrient agar medium. Does not grow on MacConkey agar. Cells have a tendency to form pellicles on the surface of static liquid cultures. Does not grow in the presence of 2 % NaCl. Growth occurs at 4–30 °C (optimum temperature, 28 °C) and at pH 4·0–9·0 (optimum pH, 6·0–7·0). Catalase-positive. Weakly oxidase-positive. Urease-negative. Negative for indole production, hydrogen sulfide production and nitrate reduction, and in the methyl red and Voges–Proskauer tests. Glucose is not fermented. Simmons' citrate test is positive. Starch, gelatin, casein and DNA are hydrolysed but Tween 80 is not. Acid is not produced oxidatively from D-galactose, D-mannose, D-glucose, D-fructose, D-maltose, glycerol, D-mannitol, D-trehalose, D-xylose or lactose. Alkaline and acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, β-galactosidase and α-glucosidase are present. Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, lipase (C14), cysteine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Utilizes D-xylose, D-glucose, D-fructose, D-mannose, L-rhamnose, *N*-acetylglucosamine, amygdalin, aesculin, D-cellobiose, D-maltose, starch, glycogen, gentiobiose, adipic acid, malic acid and trisodium citrate as sole carbon and energy sources, but not glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, L-sorbose, dulcitol, inositol,

Table 2. Cellular fatty acid content (%) of strain AP13^T and *Massilia* species

Taxa: 1, strain AP13^T; 2, *M. timonae*; 3, *M. dura*; 4, *M. albidiflava*; 5, *M. plicata*; 6, *M. lutea*. Data for *M. dura* CCTCC AB 204070^T, *M. albidiflava* CCTCC AB 204071^T, *M. plicata* CCTCC AB 204072^T and *M. lutea* CCTCC AB 204073^T are from Zhang *et al.* (2006).

Fatty acid	1	2	3	4	5	6
$C_{10:0}$	0·4	0·5	0·6	1·3	1·4	0·5
$C_{10:0}$ 3-OH	4·8	4·6	8·3	9·6	10·1	4·6
$C_{12:0}$	4·6	4·4	5·6	6·0	7·1	2·8
$C_{12:0}$ 2-OH	1·9	1·8	–	0·7	–	–
$C_{14:0}$	0·6	Trace	3·2	2·8	1·6	1·9
$C_{14:0}$ 2-OH	–	–	3·4	3·0	6·1	2·0
$C_{15:0}$	–	1·1	–	–	–	–
$C_{16:1}\omega7c$	48·3*	49·1	46·7	46·4	36·9	54·7
$C_{16:0}$	36·8	27·2	25·5	22·8	25·1	22·8
$C_{18:1}\omega7c$	2·5	11·2	5·9	7·4	11·7	10·0

*Fatty acid $C_{16:1}\omega7c$ is included in summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH).

D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, arbutin, salicin, D-lactose (bovine origin), D-melibiose, sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate, L-arabinose, capric acid or phenylacetic acid. Resistant to the following antibiotics: penicillin (10 U per disc), bacitracin (10 U per disc) and cephalothin (30 μg per disc). Sensitive to the following antibiotics (μg per disc): tetracycline (30), rifampicin (30), streptomycin (10), neomycin (10), erythromycin (15), kanamycin (30), vancomycin (30), nalidixic acid (30), novobiocin (30) and chloranphenicol (30). The predominant cellular fatty acids are $C_{10:0}$ (0·4 %), $C_{10:0}$ 3-OH (4·8 %), $C_{12:0}$ (4·6 %), $C_{12:0}$ 2-OH (1·9 %), $C_{14:0}$ (0·6 %), $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (48·3 %), $C_{16:0}$ (36·8 %) and $C_{18:1}\omega7c$ (2·5 %). The predominant ubiquinone is Q-8. The DNA G+C content of the type strain is 66·0 mol% (T_m).

The type strain, AP13^T (=CECT 7142^T=CCM 7363^T=DSM 18055^T=JCM 13879^T), was isolated from drinking water.

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