Massilia yuzhufengensis sp. nov., isolated from an ice core

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A Gram-negative, rod-shaped, aerobic, motile bacterium, strain Y1243-1^T, was isolated from an ice core drilled from Yuzhufeng Glacier, Tibetan Plateau, China. Cells had polar flagella. The novel strain shared 94.7–97.6 % 16S rRNA gene sequence similarity with the type strains of species of the genus *Massilia*. The novel isolate is thus classified in the genus *Massilia*. The major fatty acids of strain Y1243-1^T were summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) (43.98 %), $C_{16:0}$ (27.86 %), $C_{10:0}$ 3-OH (7.10 %), $C_{18:0}$ (6.95 %) and $C_{18:1}\omega7c$ (5.01 %). The predominant isoprenoid quinone was Q-8. The DNA G+C content of strain Y1243-1^T was 65.7 mol% (T_m). The major polar lipids were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. A number of phenotypic characteristics distinguished the novel isolate from the type strains of recognized *Massilia* species. Furthermore, in DNA–DNA hybridization tests, strain Y1243-1^T shared 45 % relatedness with its closest phylogenetic relative, *Massilia consociata* CCUG 58010^T. From the genotypic and phenotypic data, it is evident that strain Y1243-1^T represents a novel species of the genus *Massilia*, for which the name *Massilia yuzhufengensis* sp. nov. is proposed. The type strain is Y1243-1^T (=KACC 16569^T=CGMCC 1.12041^T).

The genus *Massilia* was first proposed by La Scola *et al.* (1998) for an isolate from the blood of an immunocompromised patient with a cerebrellar lesion. Members of the genus are characterized as aerobic, Gram-negative, motile, non-spore-forming rods, which contain several typical fatty acids, such as $C_{12:0}$, $C_{10:0}$ 3-OH, iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$, $C_{16:0}$ and $C_{18:1}\omega7c$. Q-8 is the predominant isoprenoid quinone. Phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol are the major polar lipids of these bacteria. The DNA G+C content ranges from 62.4 to 68.9 mol%. It was recently shown that four species of the genus *Naxibacter* were grouped together with the majority of species of the genus *Massilia* and no significant differences could be detected when comparing the chemotaxonomic makers described for species of the genera *Naxibacter* and *Massilia*. Therefore, Kämpfer *et al.* (2011) transferred all *Naxibacter* species to the genus *Massilia*. At the time of writing, a total of 18 *Massilia* species with validly published names have been described (La Scola *et al.*, 1998; Gallego *et al.*, 2006; Zhang *et al.*, 2006; Zul *et al.*, 2008; Weon *et al.*, 2008, 2009, 2010; Kämpfer *et al.*, 2011, 2012a; Wang *et al.*, 2012).

Ice core samples were drilled from Yuzhufeng Glacier on the Qinghai–Tibetan Plateau of China (94° 14.77′ E 35° 39.64′ N). Temperatures on the glacier are extremely low. Thawed water from sections of the ice core with depth was used for cultivation. After incubation at 4 °C for 30 days with R2A agar medium (Reasoner & Geldreich, 1985), several bacterial colonies were recovered.

The genomic DNA of these strains was extracted according to the methods of Marmur (1961) from cells grown on R2A agar for 2 days at 28 °C. Purity was assessed using a NanoDrop spectrophotometer (2000c; Thermo). The 16S rRNA gene was amplified with universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) (Embley, 1991). Nearly full-length 16S rRNA gene sequences were compared with those in GenBank using the BLAST program (NCBI) and the EzTaxon server 2.1 (Chun *et al.*, 2007) to determine their approximate phylogenetic affiliation. The 16S rRNA gene

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Y1243-1^T is JQ409016.

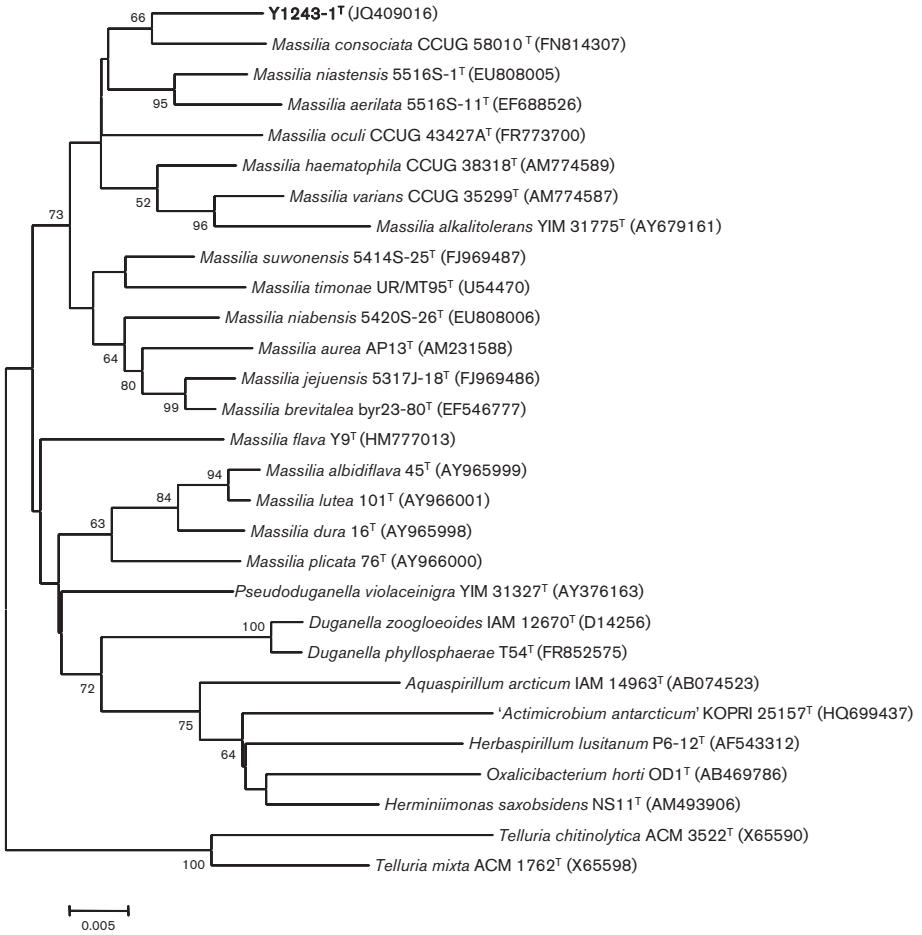

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain Y1243-1^T. Numbers at nodes are bootstrap percentages (based on 1000 replications); only values >50 % are shown. Bar, 0.005 accumulated changes per nucleotide.

sequences were then analysed with the software package MEGA 5.05 (Tamura *et al.*, 2011). A phylogenetic tree was constructed using the neighbour-joining and maximum-likelihood methods with bootstrap values based on 1000 replications (Fig. 1). One bacterial strain recovered from 119.6 m of the ice core, designated Y1243-1^T, was shown to be phylogenetically related to members of the genus *Massilia* (97.6–94.7 % 16S rRNA gene sequence similarity) with the closest relative being *Massilia consociata* CCUG 58010^T. We therefore considered that strain Y1243-1^T may represent a novel species of the genus *Massilia*.

It was recently shown that *Telluria* species fell together with *Massilia albidiflava*, *Massilia dura*, *Massilia lutea* and *Massilia plicata*, and *Duganella violaceinigra* was reclassified within a novel genus as *Pseudoduganella violaceinigra* (Kämpfer *et al.*, 2012b). The phylogenetic tree based on the neighbour-joining method in this study supports the reclassification of *D. violaceinigra*. *Telluria* species form the basal group in the tree. Furthermore, we found the topology of the maximum-likelihood tree and the neighbour-joining tree to be different for *Telluria* species (not shown). Therefore, further extensive taxonomic studies are required to determine whether to transfer members of the genus *Telluria* to the genus *Massilia*. Although the distinct phylogenetic positions and differences in chemotaxonomic markers such as polar lipids and fatty acids are usually used to distinguish bacteria at the genus level, assigning any novel isolate to *Massilia–Duganella–Telluria* species needs particular care because of the absence of

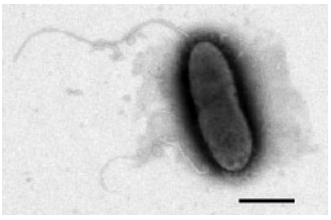

Fig. 2. Transmission electron micrograph of cells of strain Y1243-1^T after 24 h growth on R2A agar. Bar, 1 μm.

Table 1. Differential properties between strain Y1243-1^T and the type strains of recognized species of the genus *Massilia*

Strains: 1, Y1243-1^T; 2, *M. consociata* CCUG 58010^T; 3, *M. niabensis* KACC 12632^T; 4, *M. niastensis* KACC 12599^T; 5, *M. jejuensis* 5317J-18^T; 6, *M. brevitalea* DSM 18925^T; 7, *M. suwonensis* 5414S-25^T; 8, *M. varians* KACC 13770^T; 9, *M. haematophila* CCUG 38318^T; 10, *M. aurea* DSM 18055^T; 11, *M. aerilata* KACC 12505^T; 12, *M. alkalitolerans* KACC 12188^T; 13, *M. timonae* DSM 16850^T; 14, *M. flava* KCTC 23585^T; 15, *M. albidiflava* KCTC 12343^T; 16, *M. lutea* KCTC 12345^T; 17, *M. plicata* KCTC 12344^T; 18, *M. dura* KCTC 12342^T; 19, *Massilia oculi* CCUG 43427A^T. Data for taxa 3 and 6 are from Weon *et al.* (2009); for 9 and 12 from Weon *et al.* (2010); for 10, 11, 13 and 15–18 from Weon *et al.* (2008); for 14 from Wang *et al.* (2012); for 19 from Kämpfer *et al.* (2012a). Data for taxa 1, 2, 4, 5, 7 and 8 are from this study, except where indicated. +, Positive; (+), weakly positive; −, negative; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Catalase/oxidase	+/−	ND/−[a]	+/+	+/+[b]	ND/+[c]	(+)/−[d]	ND/+[c]	ND/+[e]	ND/+[e]	+/(+)[f]	+/+	ND/−[g]	+/+[h]	ND/+	+/+[i]	+/+[i]	+/−[i]	+/+[i]	ND/−
Temperature range (°C)	2–35	15–37[a]	5–35	5–35[b]	5–35[c]	4–30[d]	5–40[c]	15–37[e]	15–37[e]	4–30[f]	5–35	4–55[g]	25–35[j]	10–40	10–45[i]	10–45[i]	10–45[i]	10–45[i]	15–37
Isolation source	Ice core	Blood[a]	Air	Air[b]	Air[c]	Soil[d]	Air[c]	Eye[e]	Blood[e]	Water[f]	Air	Soil[g]	Blood[j]	Soil	Soil[i]	Soil[i]	Soil[i]	Soil[i]	Eye
Nitrate reduction	−	−	+	−	−	+[d]	−	−[c]	−	−	+	−[g]	−	+	+	−	−	−	−
Urease	−	−	−	−	−	−	−	−[c]	−	−	+	+[g]	−	−	−	−	−	−	−
Hydrolysis of:																			
Aesculin	+	+	−	+	+	+	−	(+)[c]	−	+	+	+[g]	+	ND	+	+	+	+	+
Gelatin	+	+	−	+	−	−	−	ND	ND	+	+	ND	+	(+)	+	+	+	+	ND
Assimilation of:																			
D-Glucose	−	+	−	+	−	+	−	−[c]	−	+	+	+	+	+	+	−	+	+	+
L-Arabinose	+	+	−	+	−	−	−	+[c]	−	−	+	+	+	+	+	+	+	+	+
Potassium gluconate	−	−	−	−	−	−	−	−[c]	−	−	−	−	+	ND	+	+	+	+	−
Adipic acid	−	−	−	−	−	+	+	−[c]	+	+	−	−	−	ND	+	+	−	−	−
Trisodium citrate	−	+	−	−	−	−	−	−[c]	−	+	−	+	+	ND	−	+	−	−	+
Enzyme activities																			
Esterase lipase (C8)	+	+	+	−	+	+	+	+	ND	+	+	ND	+	−	+	+	+	+	ND
Cystine arylamidase	−	+	−	+	+	−	(+)	+	ND	−	+	ND	+	+	−	−	−	−	ND
β-Glucuronidase	+	−	+	−	−	−	−	−	ND	−	−	ND	−	−	−	−	−	−	ND
α-Glucosidase	−	−	+	−	+	+	+	+	ND	(+)	+	ND	−	+	−	−	−	−	ND
β-Glucosidase	−	−	−	−	−	−	−	−	ND	−	−	ND	(+)	−	+	−	−	−	ND
DNA G+C content (mol%)	65.7	68.9	67.8	66.6[b]	66.1[c]	65.3[d]	67.8[c]	ND	ND	66.0[f]	68.9	62.4±0.3[g]	64.6[j]	68.7	65.3[i]	63.3[i]	65.1[i]	65.9[i]	ND

Data taken from: *a*, Kämpfer *et al.* (2011); *b*, Weon *et al.* (2009); *c*, Weon *et al.* (2010); *d*, Zul *et al.* (2008); *e*, Kämpfer *et al.* (2008); *f*, Gallego *et al.* (2006); *g*, Xu *et al.* (2005); *h*, Lindquist *et al.* (2003); *i*, Zhang *et al.* (2006); *j*, La Scola *et al.* (1998).

Table 2. Cellular fatty acid compositions (%) of strain Y1243-1^T and the type strains of recognized species of the genus *Massilia*

Strains: 1, Y1243-1^T; 2, *M. consociata* CCUG 58010^T; 3, *M. niabensis* KACC 12632^T; 4, *M. niastensis* KACC 12599^T; 5, *M. jejuensis* 5317J-18^T; 6, *M. brevitalea* DSM 18925^T; 7, *M. suwonensis* 5414S-25^T; 8, *M. varians* KACC 13770^T; 9, *M. haematophila* CCUG 38318^T; 10, *M. aurea* DSM 18055^T; 11, *M. aerilata* KACC 12505^T; 12, *M. alkalitolerans* KACC 12188^T; 13, *M. timonae* DSM 16850^T; 14, *M. flava* KCTC 23585^T; 15, *M. albidiflava* KCTC 12343^T; 16, *M. lutea* KCTC 12345^T; 17, *M. plicata* KCTC 12344^T; 18, *M. dura* KCTC 12342^T; 19, *Massilia oculi* CCUG 43427A^T. Data for taxa 6 from Weon *et al.* (2009); for 8 and 9 from Kämpfer *et al.* (2011); for 10 from Gallego *et al.* (2006); for 12 and 13 from Weon *et al.* (2010); for 14 from Wang *et al.* (2012); for 15–17 from Weon *et al.* (2008); for 18 from Zhang *et al.* (2006); for 19 from Kämpfer *et al.* (2012a). Data for taxa 1–5, 7 and 11 from this study. −, <1 % or not detected.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
$C_{10:0}$ 3-OH	7.1	5.35	4.32	4.01	6.02	4.9	5.39	3.3	3.0	4.8	4.49	3.2	4.6	2.7	7.0	5.7	10.1	5.5	9.6
$C_{12:0}$	4.47	5.41	6.85	3.92	4.95	5.0	4.05	3.7	3.3	4.6	3.69	3.3	3.3	3.0	5.3	4.0	7.1	3.9	7.7
$C_{12:0}$ 2-OH	1.89	1.92	−	2.01	1.77	2.0	1.97	2.0	1.5	1.9	−	2.2	2.2	−	−	−	−	−	4.3
$C_{14:0}$	1.73	−	−	1.07	−	−	−	−	−	0.6	1.91	−	−	6.9	2.6	1.2	1.6	1.1	1.0
$C_{14:0}$ 2-OH	−	−	−	−	−	−	−	−	−	−	2.68	−	−	1.5	2.4	2.9	6.1	2.6	−
$C_{15:0}$ 3-OH	1.01	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
$C_{16:0}$	27.86	20.17	19.74	26.72	23.67	23.0	26.38	28.5	26.2	36.8	31.01	26.6	30.5	26.1	23.4	26.6	25.1	27.5	20.9
$C_{17:0}$ cyclo	−	−	−	1.90	−	−	2.21	−	2.7	−	6.88	−	3.7	−	−	−	−	−	1.0
$C_{18:0}$	6.95	−	−	−	2.03	−	−	−	−		−	−	−	3.2	−	−	−	−	−
$C_{18:1}\omega7c$	5.01	14.35	11.93		12.76	9.0	8.88	8.1	8.7	2.5	6.25	6.6	7.9	9.9	7.4	7.8	11.7	7.03	6.0
Summed feature 3*	43.98	51.6	55.90	43.63	48.82	54.2	48.73	51.7	44.7	48.3	42.09	55.2	47.0	35.8	46.0	51.1	36.9	52.0	48.2

*Summed feature 3 included $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH.

clear phylogenetic boundaries and genus-specific chemotaxonomic markers.

To further determine the taxonomic position of the novel isolate, a series of phenotypic and genotypic approaches were employed. Morphology was examined by transmission electron microscopy (JEM-1230; JEOL) (Fig. 2). Phenotypic characteristics such as Gram staining, catalase activity, and hydrolysis of casein, hypoxanthine, Tween 80 and starch were performed using the methods of Smibert & Krieg (1994). The pH range for growth was determined in R2A broth at 30 °C. The pH (4–10) of the medium was adjusted with citrate phosphate buffer or Tris/HCl buffer (Breznak & Costilow, 1994). Growth in the absence of NaCl and in the presence of 1, 2, 3, 4 and 5 % (w/v) NaCl was also investigated in the same medium. Growth at various temperatures (0–40 °C) was measured. Other physiological and biochemical properties were further determined with API 20NE, API 20E and API ZYM strips according to the manufacturer's instructions (bioMérieux). Differences in physiological characterization between strain Y1243-1^T and the type strains of recognized *Massilia* species are given in Table 1. Of particular note, strain Y1243-1^T was able to grow at 2 °C, whereas other species of this genus cannot.

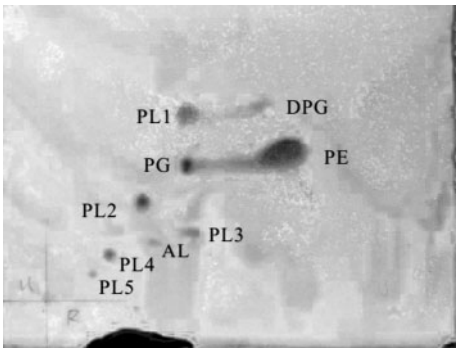

Fig. 3. Two-dimensional thin-layer chromatograph of polar lipid extracts from strain Y1243-1^T stained with molybdatophosphoric acid. PG, phosphatidylglycerol; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PL1–PL5, unknown phospholipids; AL, unknown aminolipid.

Fatty acid methyl esters were extracted and prepared using the standard protocol of the Microbial Identification System (MIDI, Version 6.0) with cells of strain Y1243-1^T and all strains under comparison harvested from R2A agar

after 48 h of growth at 28 °C. No significant differences in fatty acid profile were found between the novel isolate and closely related bacteria, but small quantitative differences were observed (Table 2). Strain Y1243-1^T had a relatively higher proportion of $C_{18:0}$ (6.95 %) compared with recognized species of the genus *Massilia*.

For isoprenoid quinone and polar lipid analyses, cells were harvested after 48 h of growth at 28 °C. Isoprenoid quinones were analysed as described by Hiraishi *et al.* (1998), using a Waters Acquity Ultra Performance LC (UPLC)-Q-TOF-MS spectrometer by electrospray ionization (Romano *et al.*, 2006). The predominant isoprenoid quinone of strain Y1243-1^T was Q-8. Polar lipids were extracted and analysed by two-dimensional TLC (Altenburger *et al.*, 1996; Tindall, 1990). The major polar lipids of strain Y1243-1^T were similar to those of species of the genus *Massilia* (Fig. 3) (Kämpfer *et al.*, 2008, 2011, 2012a; Weon *et al.*, 2010). Phospholipids PL1 and PL2 were also found in *Massilia oculi* (Kämpfer *et al.*, 2012a), *Massilia haematophila* and *Massilia varians* (Kämpfer *et al.*, 2008). One unknown aminolipid (AL), found in *M. consociata*, *M. haematophila* and *M. varians*, was also detected in strain Y1243-1^T. The genomic DNA G+C content of the novel strain was estimated from the midpoint value (T_m) of the thermal denaturation profile (Mandel *et al.*, 1970). The genomic DNA G+C content of strain Y1243-1^T was 65.7 mol%. The predominant isoprenoid quinones, major polar lipids and DNA G+C content of strain Y1243-1^T are consistent with the general characteristics of the genus *Massilia*.

The novel isolate shares a low 16S rRNA gene sequence similarity of 97.6 % with *M. consociata* CCUG 58010^T, and limited phenotypic variations can be observed between the two (Table 2). Therefore, we further compared the two bacteria at the genomic level by DNA–DNA hybridization experiments, which were carried out applying the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992). The level of DNA–DNA relatedness between strain Y1243-1^T and *M. consociata* CCUG 58010^T was 45 %.

Based on the genotypic and phenotypic data presented in this study, the psychrotolerant bacterium strain Y1243-1^T represents a novel species of the genus *Massilia*, for which the name *Massilia yuzhufengensis* sp. nov. is proposed.

Description of *Massilia yuzhufengensis* sp. nov.

Massilia yuzhufengensis (yu.zhu.feng.en′sis. N.L. fem. adj. *yuzhufengensis*, of Yuzhufeng Glacier, Tibetan Plateau, China, where the type strain was isolated).

Cells are aerobic, Gram-negative rods (0.7–1 μm wide and 2.3–2.7 μm long) and have polar flagella. Yellow, round, smooth, convex and opaque colonies are produced on R2A agar after incubation at 28 °C for 2–3 days. Grows at 2–35 °C (optimally at 25 °C) on R2A agar, at pH 5–8 (optimally at pH 7) and with 0–3 % NaCl (optimally at 2 % NaCl). Positive for catalase, but negative for oxidase, nitrate reduction and urease (API 20NE test strips). Degrades aesculin, gelatin and starch, but not casein or Tween 80. Assimilates L-arabinose, D-mannose, maltose and malic acid, but not D-glucose, D-mannitol, *N*-acetylglucosamine, potassium gluconate, capric acid, adipic acid, trisodium citrate or phenylacetic acid (API 20NE and API 20E test strips). In API ZYM tests, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β-glucuronidase, but negative for lipase (C14), cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The major fatty acids are summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) and $C_{16:0}$. The major respiratory quinone is Q-8 and major polar lipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol.

The type strain Y1243-1^T (=KACC 16569^T=CGMCC 1.12041^T) was isolated from a 119.6 m deep ice core section drilled from Yuzhufeng Glacier, Tibetan Plateau, China. The DNA G+C content of the type strain is 65.7 mol% (T_m).

Acknowledgements

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