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Massilia kyonggiensis sp. nov., Isolated from Forest Soil in Korea

Jaisoo Kim

Department of Life Science, College of Natural Sciences, Kyonggi University, Suwon 443-760, Republic of Korea

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A Gram-negative, short, rod-shaped bacterium, TSA1^T, was isolated from forest soil collected at Kyonggi University, South Korea. Assessment of 16S rRNA gene sequence similarity indicated that the strain is related to *Massilia niastensis* 5516S-1^T (98.3%), *M. haematophila* CCUG 38318^T (97.9%), *M. aerilata* 5516S-11^T (97.9%), *M. tieshanensis* TS3^T (97.6%), and *M. varians* CCUG 3529^T (97.1%). Colonies grown on Reasoner's 2A agar at 30°C for 2 days were transparent, white, round, smooth, and glossy. The cells grew at 10–42°C (optimum: 25–37°C) and pH 5–9 (optimum: 5–9) and in 0–2% NaCl (optimum: 0–1%). TSA1^T was able to grow on trypticase soy and nutrient agar, but not on Luria-Bertani or MacConkey agar. The strain was catalase- and oxidase-positive and able to degrade starch and casein, but not carboxymethyl cellulose. The predominant quinone of TSA1^T was Q-8, the major fatty acids were summed feature 3 and $C_{16:0}$, and the DNA G+C content was 66.7 mol%. Given these findings, we propose that this strain is a novel species of the genus *Massilia*. We suggest the name *Massilia kyonggiensis* sp. nov. (type strain, KACC 17471^T =KEMB 9005-031^T =JCM 19189^T).

Keywords: *Massilia kyonggiensis*, forest soil, taxonomy, soil bacteria

Introduction

Many kinds of bacteria live in the world, but we do not understand how they grow or how they affect our life. Soil contains almost all of the bacterial groups that have been identified through molecular analysis methods. However, a limited number of soil bacteria have been characterized because soil contains many uncultivable bacteria that cannot grow on laboratory media (Pham and Kim, 2012).

The genus *Massilia* was first proposed by La Scola *et al.* (1998), who described an isolate from the blood of an immunocompromised patient with cerebella lesions. Subsequently, many members of this genus have been isolated from various environments, including soil (Zhang *et al.*, 2006; Zul *et al.*, 2008; Wang *et al.*, 2012). Taxonomically, the genus belongs to the family Oxalobacteraceae, class Betaproteobacteria. The genus comprises aerobic, Gram-negative, motile, non-spore-forming, rod-shaped, or short rod-shaped bacteria. Summed feature 3 (comprising $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) and $C_{16:0}$ are the major fatty acids. Q-8 is the predominant isoprenoid quinone, and the DNA G+C content is relatively high (62–69 mol%). The putative new species described in this study originated from a soil sample collected in a forest at Kyonggi University, Suwon, Korea.

*For correspondence. E-mail: jkimtamu@kgu.ac.kr; Tel.: +82-31-249-9648; Fax: +82-31-253-1165

Materials and Methods

Sampling and cultivation

A soil sample from a forest at Kyonggi University, Suwon, Kyonggi-do, South Korea was collected and passed through a mesh sieve. Care was taken to avoid killing the microbes. The sampling site was located at 37° 17.990′ N and 127° 2.325′ E. Bacteria in the soil were isolated using a polycarbonate transwell plate as described previously (Pham and Kim, 2013). Briefly, 3 g of soil was placed in each well, and a transwell insert was placed in the soil. Tryptic soy broth (TSB; 3 ml) and a soil suspension (100 µl) as an inoculum were added to the insert, and the plates were incubated with shaking at 28°C for 2 weeks. The resulting cultures were serially diluted, and 100 µl of each diluted inoculum was spread on tryptic soy agar (TSA) plates. The plates were incubated at 28°C for 1 week under aerobic conditions until colonies appeared. In this study, *Massilia niastensis* KACC 12175^T, *Massilia haematophila* KACC 13771^T, *Massilia aerilata* KACC 12505^T, *Massilia tieshanensis* KACC 14940^T, and *Massilia varians* KACC 13770^T were used as reference species.

Cell morphology

The size of the cells after growth on Reasoner's 2A (R2A) agar plates for 2 days was determined by phase-contrast microscopy (BX50 microscope; Olympus, Japan) at 1000× magnification. Motility was assayed on TSA containing 0.4% agar. Gram staining was carried out as described by Doetsch (1981). The shape of the isolated colonies was examined after growth on R2A agar plates at 30°C for 2 days.

Physiological tests

The thermal tolerance and growth of the *Massilia* strains were tested at different temperatures (4, 10, 15, 20, 25, 30, 37, 45, 50, and 55°C) for 5 days. Salt tolerance was tested by incubating the cells for 5 days at 30°C in R2A supplemented with 0–10% NaCl (w/v). The effects of pH were tested from pH 4 to 12 (intervals of 0.5) using different buffers. Growth on various media was tested by incubating cells for 5 days at 30°C on nutrient agar, Luria-Bertani agar, R2A agar, and TSA. Growth at different O_2 levels was examined using the

traditional deep tube culture method with 0.7% agar medium (Johnson and Case, 2007). Oxidase activity was determined using 1% (v/v) *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride. Catalase activity was tested by bubble production in H_2O_2 (3%). Starch hydrolysis was assessed on 1% (w/v) soluble starch agar with I_2-KI solution, and casein degradation was tested on R2A agar using 1% (w/v) skim milk. Carbon-source utilization by TSA1^T and the reference strains was determined using the API 20NE, API ID32GN, and API ZYM systems from bioMérieux (France).

Table 1. Differential phenotypic characteristics of strain TSA1^T and the five closest species in genus *Massilia*
Strains: 1, strain TSA1^T; 2, *M. niastensis* 5516S-1^T; 3, *M. haematophila* CCUG 38318^T; 4, *M. aerilata* 5516S-11^T; 5, *M. tieshanensis* TS3^T; 6, *M. varians* CCUG 3529^T. The data for strain TSB1^T and its related taxa were obtained in this study. Some data (in parentheses) for other strains are taken from Du *et al.* (2012). +, positive; -, negative; w, weakly positive; ND, no data available.

Characteristics	*Massilia* strains					
	1	2	3	4	5	6
Nitrate reduction	+	-	+	-	-	-
Urease	-	-	-	+	-	-
Hydrolysis of:						
Esculin	+	-	-	+	+	+
Starch	+	(w)	(+)	(-)	(+)	(+)
Casein	+	(-)	(+)	(+)	(+)	(+)
Enzyme activities:						
Esterase (C4)	w	w	+	+	w	+
Esterase Lipase (C8)	-	w	w	+	w	w
Valine arylamidase	+	-	+	+	+	+
Cystine arylamidase	-	+	w	+	-	+
Trypsin	-	-	-	-	w	-
Naphthol-AS-BI-phosphohydrolase	+	+	+	+	+	w
Acid phosphatase	+	w	+	+	w	w
α-Galactosidase	+	w	-	-	-	-
β-Galactosidase	w	-	-	-	+	+
α-Glucosidase	+	-	+	+	-	+
β-Glucosidase	+	-	-	-	-	+
N-Acetyl-β-glucosaminidase	+	-	w	-	-	-
Assimilation of:						
(API 20NE)						
4-Nitrophenyl-β-D-galactopyranoside	+	+	-	w	-	+
D-Glucose	+	-	-	+	-	-
D-Mannose	+	+	+	+	+	-
Adipic acid	w	-	+	+	-	-
Phenylacetic acid	+	-	+	w	-	+
Potassium gluconate	w	-	-	-	w	-
(API ID 32GN)						
L-Rhamnose	+	+	-	+	+	+
Glycogen	+	-	+	+	+	+
L-Fucose	+	-	-	-	+	-
L-Serine	-	-	w	w	-	-
3-Hyroxybenzoic acid	-	+	+	-	-	-
Lactic acid	w	+	w	-	+	-
L-Alanine	-	-	-	w	w	+
Sodium acetate	-	+	+	-	-	+
Sodium malonate	-	-	w	-	-	-
Suberic acid	-	+	+	-	-	-
D-Saccharose	+	-	-	-	+	-
L-Rhamnose	+	+	-	+	+	+
(API 20NE+API ID 32GN)						
D-Glucose	+	-	-	+	w	-
Capric acid	-	+	+	-	-	-
Trisodium citrate	w	w	-	w	-	-
DNA G+C content (mol%)	66.7	(66.6)	ND	(68.9)	(65.9)	ND

Phylogenetic analysis

The primary structure of 16S rDNA gene was determined by directed sequencing of the 16S rRNA gene. Genomic DNA was extracted using an InstaGene Matrix kit (Bio-Rad, USA), and the 16S rRNA gene was amplified by PCR using the universal bacterial primer set 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′). The PCR product was purified with a multiscreen-filter plate (Millipore Corp., USA), and the sequencing reaction was prepared with the PRISM BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA). The reaction was incubated at 95°C for 5 min, cooled on ice for 5 min, and then analyzed with an ABI Prism 3730XL DNA Analyzer automated DNA sequencing system (Applied Biosystems). The nearly complete full sequence of the 16S rRNA gene was compiled using SeqMan software (DNASTAR Inc., USA). The phylogenetic neighbors were identified, and the pairwise 16S rRNA gene sequence similarities were calculated using the EzTaxon server (Kim *et al.*, 2012). The related 16S rRNA sequences (GenBank) were edited with the BioEdit program (Hall, 1999). Multiple alignments were completed with the CLUSTAL_X program (Thompson *et al.*, 1997). The phylogenetic tree was reconstructed using the neighbor-joining (Saitou and Nei, 1987), maximum likelihood, and maximum parsimony methods with the MEGA-5.03 program (Tamura *et al.*, 2011) and a bootstrap value of 1,000 replications (Felsenstein, 1985).

Chemotaxonomic and genotypic characteristics

Isoprenoid quinone was extracted with chloroform:methanol (2:1), filtered through Whatman paper (No. 2), evaporated under vacuum at 50°C, and extracted with *n*-hexane. The crude *n*-hexane-quinone mixture was purified with a Sep-Pak Vac silica cartridge (Watters, Ireland). The purified *n*-hexane-quinone was evaporated, eluted with hexane:diethyl ether (98:2) to produce menaquinone, and re-extracted with acetone before analysis with high-performance liquid chromatography (HPLC) (Collins and Jones, 1981). Cells grown for 3 days on TSA at 30°C were used for the fatty acid methyl ester analysis, in which the esters were saponified, methylated, and extracted according to the instructions of the Sherlock Microbial Identification System (TSBA6, Version 6.0; MIDI, Inc., USA) (Sasser, 1990). Polar lipids were extracted as instructed in a previously published procedure (Minnikin *et al.*, 1984), resolved by two-dimensional thin layer chromatography, and sprayed with the appropriate detection reagents (Minnikin *et al.*, 1984; Komagata and Suzuki, 1987).

The DNA G+C content of strain TSA1^T and the reference species was determined by reverse-phase HPLC as described by Mesbah *et al.* (1989). DNA from strain TSA1^T was labeled with photobiotin and used as the probe in hybridizations with the reference species. Fluorometry was used to determine the relatedness of the DNA-DNA hybridizations (Mehlen *et al.*, 2004).

Results and Discussion

Morphological and physiological characteristics

When assessed after 2 days' growth on R2A agar plates at 30°C, strain TSA1^T was short, rod-shaped, aerobic, Gram-negative, and motile with a cell length of 2–5 μm and a cell diameter of 0.5–1.0 μm. Colonies were white, brittle, circular,

Table 2. Cellular fatty acid composition of strain TSA1^T and the five closest species in genus *Massilia*

All data were obtained in this study and fatty acids comprising >1.0% of the total fatty acid content are listed. Strains: 1, strain TSA1^T; 2, *M. niastensis* 5516S-1^T; 3, *M. haematophila* CCUG 38318^T; 4, *M. aerilata* 5516S-11^T; 5, *M. tieshanensis* TS3^T; 6, *M. varians* CCUG 3529^T.

Fatty acid	1	2	3	4	5	6
Saturated						
$C_{10:0}$ 3OH	-	6.6	5.4	6.1	5.7	5.2
$C_{12:0}$	4.7	10.6	8.5	9.9	8.1	8.5
$C_{12:0}$ 2OH	-	2.1	1.3	-	3.3	1.2
$C_{14:0}$	3.5	-	-	2.8	-	-
$C_{14:0}$ 2OH	3.0	-	-	1.9	-	-
$C_{16:0}$	27.5	20.3	30.6	21.0	19.7	30.8
$C_{16:0}$ N alcohol	-	-	-	-	1.9	-
$C_{17:0}$ cyclo	2.8	-	-	-	-	-
$C_{18:0}$	-	-	-	-	1.8	-
Branched saturated						
Iso-$C_{10:0}$	-	-	-	-	1.2	-
Anteiso-$C_{15:0}$	-	-	-	-	1.9	
Anteiso-$C_{17:0}$	-	-	-	-	1.6	-
Anteiso-$C_{19:0}$	-	-	-	-	1.4	-
Unsaturated						
$C_{18:3}$ ω6c (6,9,12)	-	-	-	-	1.3	-
$C_{18:1}$ ω9c	-	-	-	-	3.3	
Summed features						
3 ($C_{16:1}$ ω7c/$C_{16:1}$ ω6c)	46.1	45.7	43.2	47.2	33.7	43.8
8 ($C_{18:1}$ ω7c/$C_{18:1}$ ω6c)	9.9	9.5	5.6	5.6	13.5	6.1

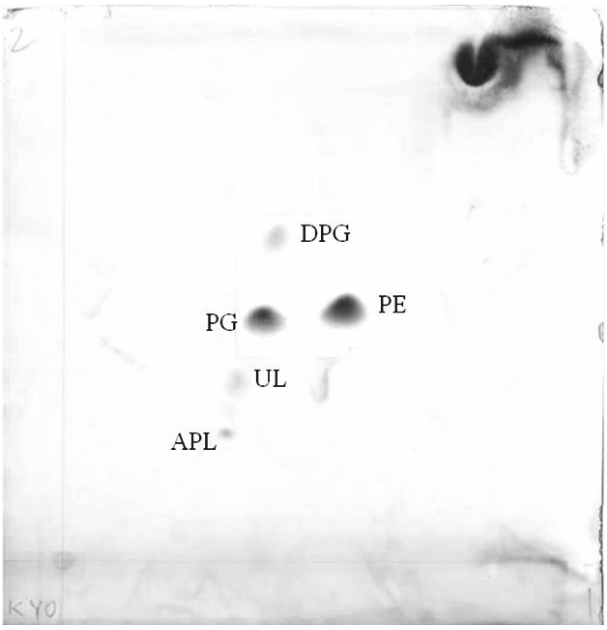

Fig. 1. Two-dimensional thin layer chromatography showing the polar lipid profile of strain TSB1^T. Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; APL, unknown aminophospholipid; UL, unknown polar lipid.

and convex, with entire margins and a diameter of 1.0–2.5 mm. Growth occurred across a wide range of temperatures (10–42°C) and pH (pH 5–9), with an optimum range of 25–37°C and pH 5–9. The salinity tolerance ranged from 0–2% NaCl with an optimal salinity of 0–1%. The cells were positive for oxidase and catalase activities, as well as for starch and casein degradation. The phenotypic features of TSA1^T and the reference species are shown in Table 1.

Chemotaxonomic characteristics

The predominant isoprenoid quinone found in strain TSA1^T was the ubiquinone Q-8, which is characteristic of the genus *Massilia* (Du *et al.*, 2012). For fatty acids comprising >1% of the total fatty acid content, the cellular fatty acid profile of strain TSA1^T consisted of summed feature 3 ($C_{16:1}$ ω7c/$C_{16:1}$ ω6c; 46.1%), $C_{16:0}$ (27.5%), summed feature 8 ($C_{18:1}$ ω7c/$C_{18:1}$ ω6c; 9.9%), $C_{12:0}$ (4.7%), $C_{14:0}$ (3.5%), $C_{14:0}$ 2OH (3.0%), and $C_{17:0}$ cyclo (2.8%). The fatty acid profiles of strain TSA1^T and the 5 reference species are shown in Table 2. The major two fatty acids in TSA1^T and the references were the same. The major polar lipid components of strain TSA1^T were phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), and the minor ones were diphosphatidylglycerol (DPG), an unknown polar lipid (UL), and an unknown aminophospholipid (APL) (Fig. 1).

DNA-DNA hybridization and DNA G+C content

The DNA G+C content of strain TSA1^T was 66.7 mol%, within the typical range observed in *Massilia* spp. (62–69 mol%) (Du *et al.*, 2012). Hybridization experiments between TSA1^T and the 5 reference species showed DNA similarity levels of 33.9% with *M. niastensis*, 19.3% with *M. haematophila*, 54.1% with *M. aerilata*, 50.0% with *M. tieshanensis*,

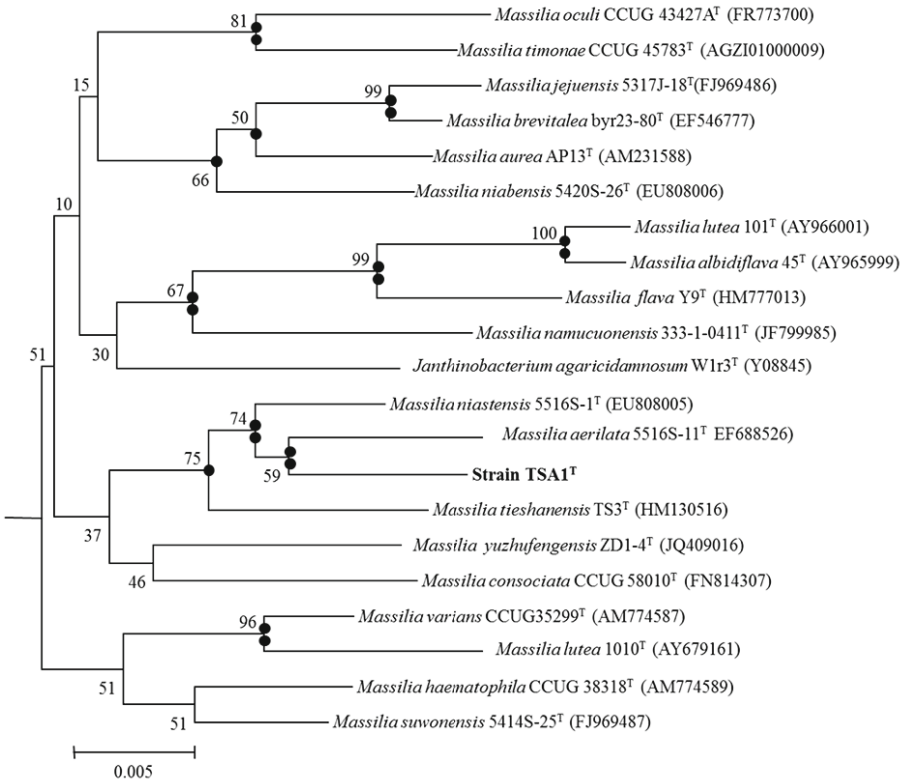

Fig. 2. Neighbor-joining phylogenetic tree of strain TSA1^T and related taxa based on the 16S rRNA gene sequence analysis. The numbers indicate bootstrap values from 1000 resamplings. Bar, 0.005 nucleotide substitutions per sequence position. One closed circle indicates a common node checked by the maximum-parsimony method or the maximum-likelihood method. Two closed circles indicate a common node checked by both methods.

and 43.0% with *M. varians*. These results demonstrate that strain TSA1^T differs from the closest taxa at the species level.

Phylogenetic analysis

The phylogenetic tree was constructed using the 16S rRNA gene sequences of strain TSA1^T, closely related species, and neighboring taxa (Fig. 2). Strain TSA1^T was located within the genus; it showed the highest similarity to *M. niastensis* (98.3%), *M. haematophila* (97.9%), *M. aerilata* (97.9%), *M. tieshanensis* (97.6%), and *M. varians* (97.1%). The 16S rRNA gene sequence for the strain was deposited in the EMBL database under the accession number KC574383.

Taxonomic conclusion

The phenotypic and chemotaxonomic properties, including 16S rRNA gene sequence and DNA-DNA hybridization similarities, indicate that strain TSA1^T represents a novel species, for which we propose the name *Massilia kyonggiensis* sp. nov.

Description of *Massilia kyonggiensis* sp. nov.

Massilia kyonggiensis (kyong.gi.en´sis. N.L. masc. *kyonggiensis*, referring to Kyonggi University where the soil sample was collected).

Cells are aerobic, short rod-shaped, Gram-negative, motile, 2–5 μm long, and 0.5–1.0 μm wide. Colonies on R2A at 30°C after 2 days are white, brittle, circular, and convex, with entire margins and a diameter of 1.0–2.5 mm. Growth occurs at 10–42°C (optimum: 25–37°C), at pH 5–9 (optimum: pH 5–9), and with 0–2% NaCl (w/v) (optimum: 0–1%). Cells are catalase and oxidase positive. Casein and starch are hydrolyzed. Enzyme activity is positive for leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, α-glucosidase, β-glucosidase, and *N*-acetyl-β-glucosaminidase; weakly positive for alkaline phosphatase, esterase (C4), and β-galactosidase; and negative for esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, β-glucuronidase, α-mannosidase, and α-fucosidase (API ZYM). Cells are positive for nitrate reduction, esculin hydrolysis, and β-galactosidase, but negative for indole production, glucose fermentation, arginine hydrolase, urease, and gelatin hydrolysis (API 20NE). The strain assimilates potassium nitrate, esculin, 4-nitrophenyl-β-D-galactopyranoside, D-glucose, L-arabinose, D-mannose, malic acid, D-maltose, phenylacetic acid, L-rhamnose, D-saccharose (sucrose), L-fucose, L-arabinose, and glycogen; weak for potassium gluconate, adipic acid, trisodium citrate, lactic acid, and 3-hydroxybutyric acid; negative for L-tryptophan, D-mannitol, *N*-acetyl-glucosamine, capric acid, D-ribose, inositol, itaconic acid, suberic acid, sodium malonate, sodium acetate, salicin, D-melibiose, D-sorbitol, propionic acid, valeric acid, L-alanine, potassium 5-ketogluconate, 3-hydroxybenzoic acid, L-serine, L-histidine, potassium 2-ketogluconate, 4-hydroxybenzoic acid, and L-proline (API 20NE and API ID 32GN). The predominant isoprenoid quinine is ubiquinone Q-8. The major cellular fatty acids (>10%) are summed feature 3 ($C_{16:1}$ ω7c/$C_{16:1}$ ω6c) and $C_{16:0}$. The predominant polar lipids are phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). The DNA G+C content is 66.7 mol% (HPLC). The type strain TSA1^T (=KACC 17471^T =KEMB 9005-031^T =JCM 19189^T) was isolated from the surface soil of a forest area at Kyonggi University in Suwon, South Korea.

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