Pseudomonas Tumuqiensis Sp. Nov., Isolated From Greenhouse Soil

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Abstract

A Gram-stain-negative, aerobic, rod-shaped and motile bacterium, named LAMW06^T, was isolated from greenhouse soil in Beijing, China. In the 16S rRNA gene sequence comparison, strain LAMW06^T had the highest similarity with *Pseudomonas cuatrocienegasensis* 1N^T was 97.4%. Phylogenetic analysis based on the 16S rRNA and three housekeeping gene sequences (*gyrB*, *rpoB* and *rpoD*) indicated that LAMW06^T represented a member of the genus *Pseudomonas*. The genome sequence size of the isolate was 5.5 Mb, with the DNA G+C content of 63.5 mol%. The average nucleotide identity and DNA–DNA hybridization values between strain LAMW06^T and closely related members of *Pseudomonas borbori* R-20821^T, *Pseudomonas taeanensis* MS-3^T and *Pseudomonas cuatrocienegasensis* 1N^T were 90.9%, 82.4%, 81.5 % and 43.0%, 25.9%, 24.6 % respectively. The major fatty acids contained summed feature 3 (C_{16:1} ω6c and/or C_{16:1} ω7c), C_{18:1} ω7c and C_{16:0}. The primary respiratory quinone was ubiquinone-9. The main polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, six aminophospholipids, six phospholipids, one aminolipid and one glycolipid. According to the genotypic, phylogenetic and chemotaxonomic data, strain LAMW06^T represents a novel species within the genus *Pseudomonas*, for which the name *Pseudomonas tumuqiensis* sp. nov. is proposed. The type strain is LAMW06^T (=GDMCC 1.2003^T =KCTC 72829^T).

Introduction

The first species of genus *Pseudomonas* was described by Migula (1894). At the time of writing, there are 341 species with validly published names on List of Prokaryotic names with Standing in Nomenclature (Parte et al. 2020). The characteristics of genus *Pseudomonas* are Gram-negative, rod-shaped, motile and catalase- and oxidase- positive (Anwar et al. 2017; Wang et al. 2020). The DNA G+C contents calculated from available genomes ranges from 58 to 69 mol% and the major respiratory quinone is ubiquinone-9 (Sun et al. 2018). Members of the genus *Pseudomonas* have been isolated from a wide variety of environments comprising soils (Zou et al. 2019), waters (Romanenko et al. 2008), plants (Timilsina et al. 2018) and animals (Lick et al. 2020). Here we isolated a novel strain designated as LAMW06^T from soil sample collected from a greenhouse. Based on the genotypic, phylogenetic, phenotypic and chemotaxonomic characterization, the isolate was represented a new taxon of the genus *Pseudomonas*.

Strain LAMW06^T was isolated from soil sample collected from a greenhouse in Beijing (39°96.73' N, 116°33.53' E), using an isolation medium of trypticase soy agar (TSA; Difco). The soil samples were diluted and cultured on the TSA medium after at 30 °C for 3 days, separated colonies were picked and serially streaked onto TSA plates incubating at 30 °C to obtain single colony. Strain LAMW06^T was obtained after several streakings and transferred onto TSA plates. The pure culture of strain LAMW06^T was preserved at -80 °C in tryptic soy broth (TSB; Difco) medium with 25% (v/v) glycerol. A biotechnology company where the strain LAMW06^T was researched and manufactured. The strains *Pseudomonas cuatrocienegasensis* DSM 23418^T (=1N^T) and *Pseudomonas borbori* DSM 17834^T (=R-20821^T) were

obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM); *Pseudomonas taeanensis* JCM 16046^T (=MS-3^T) was obtained from the Japan Collection of Microorganisms (JCM). All strains were chosen as reference strains in this study.

Materials And Methods

Bacterial strain and culture condition

Cells morphology were examined by using light microscope (Nikon 80i, Tokyo, Japan) and transmission electron microscope (Hitachi 7500, Tokyo, Japan) after incubation on TSA agar plates for 3 days at 30 °C (Ruan et al. 2014). The temperature range for growth was determined by incubating strain LAMW06^T at 10, 15, 20, 25, 30, 35, 37, 40 and 45 °C. The pH value range was determined in tryptic soy broth (TSB; Difco) with pH values of 4.0-11.0 at intervals of 0.5 pH units. The optimal concentration of NaCl for growth was investigated by using NaCl-free TSB medium (prepared according to the TSB formula without NaCl) with different NaCl concentrations (0, 0.5 and 1-12 % at 1.0 % increments, w/v). The pH of the media was adjusted by using the following buffer systems: MES (pH 4.0-6.0), MOPS (pH 7.0), Tricine (pH 8.0), TAPS (pH 9.0), CAPS (pH 10.0) and Na₂CO₃/ NaHCO₃ (pH 11.0) (Wang et al. 2017). Catalase and oxidase activities were detected by observing bubble production in 3 % (v/v) H₂O₂ solution and color variance of 1 % (w/v) tetramethyl-*p*-phenylenediamine, respectively. Cell motility was examined by using the hanging-drop technique. Anaerobic growth was tested on TSA plates at 30 °C for 10 days in GasPak EZ anaerobic container system (BD) added with sodium nitrite (10 mM) or sodium nitrate (20 mM) as potential electron acceptors (Skerman et al. 1967). Gram-stain reaction, H₂S production and methyl red test, hydrolysis of starch, gelatin and Tweens 20, 40, 60 and 80 were detected as described by Smibert and Krieg (1994). Additional physiological and biochemical features were determined by using API ZYM, 20NE and 50CH strips (bioMérieux) according to the manufacturers' instructions. Antibiotic susceptibility test was performed on TSA plates using antibiotic disks (Hangzhou Microbial Reagent) containing the following concentrations (µg per disk unless otherwise stated): tetracycline (30 µg), gentamicin (10 µg), erythromycin (15 µg), ampicillin (10 µg), sulfaisoxazole (300 µg), vancomycin (30 µg), clindamycin (2 µg) and amikacin (30 µg).

Phylogenetic analysis based on 16S rRNA gene sequences

The 16S rRNA gene of strain LAMW06^T was amplified by PCR using the bacterial universal primers 27F and 1492R (Weisburg et al. 1991) and compared with available sequences by using the EzBioCloud identify service (Yoon et al. 2017a). The genomic DNA was prepared using the method described by Sun et al. (2016). The purified PCR product was inserted into pGEM-T vector and sequenced by Shanghai Life Technologies Company. Multiple sequences were aligned by using clustal x program (Thompson et al. 1997). Phylogenetic relationships were analysed with neighbour-joining (NJ) (Saitou and Nei 1987), maximum-likelihood (ML) (Felsenstein 1981) and maximum-parsimony (MP) methods (Fitch 1971) by using mega 7.0 software (Kumar et al. 2016). According to the algorithm of the Kimura's two-parameter model, evolutionary distances were calculated using the NJ, ML and MP trees. The topologies of

phylogenetic trees were evaluated by bootstrap analysis based on 1000 replicates (Kimura 1980). Multilocus sequence analysis (MLSA) was performed based on the three housekeeping genes *gyrB*, *rpoB* and *rpoD* that gathered from the genome assembly (Table S1), following the method described by Anurat et al. (2016) based on the same taxonomic sampling.

Genomic analysis

The genomic DNA G+C content was calculated according to the draft genome sequence of strain LAMW06^T, which was performed on the Illumina MiSeq platform by Guangzhou Magigene Company. SOAPdenovo assembler software was applied to assemble the reads (Yoon et al. 2017b). Gene annotation was performed using NCBI Prokaryotic Genome Annotation Pipeline and Swiss-Prot database. The genes involved in metabolic pathways were analysed using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. Other genome sequences of closest phylogenetic relatives were obtained from the GenBank sequence database. The average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization (DDH) values were calculated according to the minimal standards proposed by Chun et al. (2018). The ANI value between two genomes was calculated using the OrthoANLu algorithm (<https://www.ezbiocloud.net/tools/ani>). The *in silico* DDH value between two strains was calculated by Genome-to-Genome Distance Calculator 2.1 (<http://ggdc.dsmz.de>).

Chemotaxonomic analyses

For fatty acids analyses, strain LAMW06^T, *P. cuatrocieneegasensis* DSM 23418^T, *P. taeanensis* JCM 16046^T and *P. borbori* DSM 17834^T were incubated on TSA medium after 2 days at 30 °C. According to the manufacturers' instructions, cellular fatty acids were analysed by the Sherlock Microbial Identification System with the standard MIS Library Generation Software (VERSION 6.0 and Date 4, Microbial ID) and a 6890N gas chromatograph (Agilent) (Sakamoto et al. 2002). Respiratory quinones of strain LAMW06^T were extracted from freeze-dried cells and analysed by using LC-MS (Minnikin et al. 1984). The polar lipid extracts were isolated by two-dimensional TLC using silica gel 60 F 254 aluminium-backed thin-layer plates (Merck) and identified by the method described by Xu et al. (2011). Molybdophosphoric acid was used for the detection of all lipids, ninhydrin reagent for aminolipids, molybdenum blue reagent for phospholipids and p-anisaldehyde reagent for glycolipids (Kates 1986).

Results

Morphological and physiological characteristics

Cells of strain LAMW06^T were Gram-stain negative, strictly aerobic, flagella, motile and rod-shaped (0.6-1.0 × 1.2-2.0 µm) (Fig. S1). Growth of strain LAMW06^T was observed at 15-37 °C (optimum: 30 °C), pH 5.0-10.0 (optimum: 8.0) and in the presence of 0-4% (w/v) NaCl (optimum: 1%). Positive for oxidase and catalase activities, negative for hydrolysis of starch, casein, chitin, Tweens 20, 40 and 60 as same as the related strains *P. cuatrocieneegasensis* DSM 23418^T, *P. borbori* DSM 17834^T and *P. taeanensis* JCM

16046^T. Negative for gelatin hydrolysis unlike *P. cuatrocienegasensis* DSM 23418^T. Esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase were positive in the API ZYM strip test. In the API 20NE strip test strain LAMW06^T showed positive results for nitrate reduction, and assimilation of glucose, maltose, gluconate, capric acid, malic acid and citrate. All results were negative in the API 50CH strip test (Table 1). Strain LAMW06^T was resistant to ampicillin, sulfaisoxazole and clindamycin, intermediately susceptible to gentamicin, and susceptible to tetracycline, erythromycin, vancomycin and amikacin. according to the antibiotic susceptibility tests.

Phylogenetic analysis

The 16S rRNA gene sequence (1418 bp) of strain LAMW06^T was obtained. Comparative analysis the degree of sequence similarity for strain LAMW06^T was found to be with *P. cuatrocienegasensis* 1N^T (97.4%), *P. borbori* R-20821^T (97.3%), *P. neuropathica* P155^T (97.0%), *P. taeanensis* MS-3^T (97.0%) and lower than 97.0% to other species. Phylogenetic analysis based on the NJ, ML and MP method revealed that strain LAMW06^T did not cluster with other type species (Fig. 1, S2 and S3). The phylogenetic trees analysis indicated that strain LAMW06^T belonged to the genus *Pseudomonas*. Alignment and comparison of the *gyrB*, *rpoB* and *rpoD* genes indicated that strain LAMW06^T and the type strain *Pseudomonas borbori* R-20821^T clustered on a branch (Fig 2).

Genomic analysis

The genome of strain LAMW06^T was 5.51 Mbp including 65 contigs with N50 as 351,006 coding sequences. The DNA G+C content of strain LAMW06^T was 63.5 mol%, which was consistent with the range of 58-69 mol% reported for the genus *Pseudomonas*. The ANI value between strain LAMW06^T and the relative reference species of genus *Pseudomonas* were less than 91.0%, for example, with *P. cuatrocienegasensis* 1N^T (accession number FOFP000000000 of the ncbi GenBank database), *P. borbori* R-20821^T (FOWX000000000), *P. neuropathica* P155^T (JACOPX000000000) and *P. taeanensis* MS-3^T (AWSQ000000000), and *P. peli* DSM 17833^T (JAAQXQ000000000) were 81.5%, 90.9%, 76.8% and 82.4%, respectively, which was significantly less than the threshold used for species recognition (94–96 %) (Meier-Kolthoff et al. 2013). The *in silico* DDH values between strain LAMW06^T and the relative reference species of genus *Pseudomonas* were less than 43.0%, for example, with *P. cuatrocienegasensis* 1N^T, *P. borbori* R-20821^T, *P. neuropathica* P155^T and *P. taeanensis* MS-3^T were 24.6%, 43.0%, 21.4% and 25.9% (Table S2), respectively, which was also lower than the threshold value recommended for the assignment of strains to the same genomic species (70%) (Goris et al. 2007).

Chemotaxonomic characteristics

The predominant fatty acids of strain LAMW06^T (>10%) were summed feature 3 (C_{16:1} ω6c and/or C_{16:1} ω7c) (38.2%), C_{18:1} ω7c (18.1%) and C_{16:0} (14.9%) consistent with strains *P. cuatrocienegasensis* DSM 23418^T, *P. borbori* DSM 17834^T and *P. taeanensis* JCM 16046^T. Strain LAM9210^T contained iso-

C_{12:0} (6.3%) was lower than that of strain *P. cuatrocienegasensis* DSM 23418^T shown in Table 2. The predominant menaquinone of strain LAMW06^T was ubiquinone-9 (Q-9) like other species of the genus *Pseudomonas*. Major polar lipids were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), six aminophospholipids (APL1-6), six phospholipids (PL1-6), one aminolipid (AL) and one glycolipid (GL) (Fig S4).

Therefore, based on the above phenotypic, phylogenetic analysis and genotypic data, it is proposed that strain LAMW06^T should be classified as representative of a novel species of the genus *Pseudomonas* with the name *Pseudomonas tumuqiensis* sp. nov.

Description of *Pseudomonas tumuqiensis* sp. nov.

Pseudomonas tumuqiensis (tu.mu.qi.en'sis. N.L. masc. adj. tumuqiensis arbitrary name formed from tumuqi biotechnology company, China, where taxonomic studies on this species were performed)

Cells are Gram-stain-negative, rod-shaped (0.6-1.0 × 1.2-2.0 μm), and motile by means of a single flagellum. Colonies of strain LAMW06^T are circular, smooth, light brown and with approximately 1.0-2.5 mm in diameter incubation on TSA after 3 days at 30 °C. Strain LAMW06^T can grow at 15-37 °C (optimum, 30 °C), pH 5.0-10.0 (optimum, 8.0) and 0-4% (w/v) NaCl (optimum, 1%). The positive results are catalase and oxidase, and negative for hydrolysis of starch, gelatin, casein, chitin, Tweens 20, 40, 60 and 80. In the API ZYM and 20NE strip test strain LAMW06^T show positive results for esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase, nitrate reduction, and assimilation of glucose, maltose, gluconate, capric acid, malic acid and citrate. Strain LAMW06^T is resistant to ampicillin, sulfaisoxazole and clindamycin. The major cellular fatty acids (>10%) are summed feature 8 (C_{18:1} ω7c and/or C_{18:1} ω6c), summed feature 3 (C_{16:1} ω6c and/or C_{16:1} ω7c) and C_{16:0}. The major menaquinone and polar lipids are ubiquinone-9 and diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, six aminophospholipids, one aminolipid, six phospholipids and one glycolipid, respectively. The genomic DNA G+C content is 63.5 mol%.

The type strain LAMW06^T (=GDMCC 1.2003^T =KCTC 72829^T), was isolated from greenhouse soil in Beijing, China.

Declarations

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Data availability The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LAMW06^T is MN503522. The GenBank accession number for the draft genome sequence of strain LAMW06^T is JAAOJA000000000.

Conflicts of interest The authors declare that there are no conflicts of interest.

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Figures

Figure 1

Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain LAMW06^T and its related taxa. The sequence of *Azotobacter chroococcum* LMG 8756^T was used as outgroup. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood method and maximum-parsimony method. Bootstrap

values (those above 50 %) are shown as percentages of 1000 replicates. Bar, 0.005 substitutions per nucleotide position.

Figure 2

Phylogenetic tree based on MLSA using the three housekeeping genes *gyrB*, *rpoB* and *rpoD* of type strains of species of the genus *Pseudomonas*, reconstructed by using the neighbour-joining method. Bootstrap

values were expressed based on 1000 replications; only values 50 % or above are shown at the nodes.
Bar, 0.02 nucleotide substitutions per 100 nucleotides.

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