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ORIGINAL PAPER

Paenibacillus vini sp. nov., isolated from alcohol fermentation pit mud in Sichuan Province, China

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Abstract A novel facultatively anaerobic bacterial strain, designated LAM0504^T, was isolated from a pit mud of Luzhou flavour liquor alcohol fermentation in Sichuan Province, China. Cells of strain LAM0504^T were observed to be Gram-stain negative, spore-forming, rod shaped and motile by means of peritrichous flagella. Strain LAM0504^T was found to be able to grow at 20–48 °C (optimum: 30 °C), pH 5.0–9.0 (optimum: 7.0) and 0–3 % NaCl (w/v) (optimum: 1.0 %). The 16S rRNA gene sequence similarity analysis showed that strain LAM0504^T was most closely related to *Paenibacillus konsisdensis* JCM 14798^T, *Fontibacillus phaseoli* LMG 27589^T and *Paenibacillus motobuensis* JCM 12774^T, with 97.0, 96.8 and 96.7 % sequence similarity, respectively. The DNA–DNA hybridization value between strain LAM0504^T and *P. konsisdensis* JCM 14798^T was 53.3 ± 1.2 %. The genomic DNA G+C content of strain LAM0504^T was 43.0 mol% as determined by the *Tm* method. The major fatty acids of strain LAM0504^T were identified as anteiso-$C_{15:0}$, $C_{16:0}$ and iso-$C_{15:0}$. The cell-wall peptidoglycan was found to contain *meso*-diaminopimelic acid. The predominant menaquinone was identified as MK-7. The major polar lipids were found to be diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, two unidentified phospholipids, two unidentified glycolipids and three unidentified lipids. On the basis of its physiological and phylogenetic characteristics, strain LAM0504^T is concluded to represent a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus vini* sp. nov. is proposed. The type strain is LAM0504^T (=ACCC 06420^T = JCM 19842^T).

Keywords *Paenibacillus vini* sp. nov. · Polyphasic taxonomy · 16S rRNA gene · Pit mud · Alcohol fermentation

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Introduction

The genus *Paenibacillus* belongs to the family *Paenibacillaceae* and was proposed by Ash et al. in 1993. At the time of writing, the genus *Paenibacillus* comprises 159 recognized species (http://www.bacterio.net/p/paenibacillus.html) and the type species is *Paenibacillus polymyxa*. *Paenibacillus* species are widely distributed throughout the biosphere such as blood (Ko et al. 2008), garbage (Iida et al. 2005), grassy sandbank (Traiwan et al. 2011) and hot spring (Zhou et al. 2012). Members of this genus have diverse physiological characteristics. Most of them are rod-shaped, facultatively anaerobic or strictly aerobic, endospore-forming and motile bacteria. Their optimal growth temperatures range from 28 to 37 °C. The optimal pH values for growth range from 6.0 to 9.0. Their DNA G+C content ranges from 39 to 54 mol%. The predominant menaquinone is MK-7 and the major fatty acids is anteiso-$C_{15:0}$ (Ash et al. 1993; Shida et al. 1997; Montes et al. 2004; Osman et al. 2006; Priest 2009).

While investigating the microbial community of an alcohol fermentation pit mud used for Luzhou flavour liquor production in Sichuan Province, China, a *Paenibacillus*-like strain, designated LAM0504^T, was isolated. By using a polyphasic taxonomic approach, we conclude that the new isolate represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus vini* sp. nov. is proposed.

Materials and methods

Isolation and culture of bacterial strains

Strain LAM0504^T was isolated from an alcohol fermentation pit mud for Luzhou flavour liquor production in Sichuan Province, China. For the isolation, 5 g of pit mud sample was added into a 150 ml Erlenmeyer flask with 50 ml sterile saline solution and incubated in the dark at 30 °C with a shaking speed of 150 rpm. After 24 h, the samples were diluted with sterilized water and spread onto TSA medium (BD/BBL 236950, Sparks, MD, USA) plates and incubated at 30 °C for 3 days. One of the isolates obtained, designated strain LAM0504^T, which was purified at least twice before preservation in 25 % (v/v) glycerol at −80 °C, was selected for further study.

Biomass for chemotaxonomic and molecular studies was obtained by cultivation in shaking flasks with TSB medium (BD/Difco 211825, Sparks, MD, USA) at 30 °C for 2 days. The recommended Minimal Standards for describing new taxa of aerobic, endospore-forming bacteria as described by Logan et al. (2009) were followed. The reference type strains *Paenibacillus konsisdensis* JCM 14798^T and *Paenibacillus motobuensis* JCM 12774^T were obtained from the Japan Collection of Microorganisms (JCM; Japan). *Fontibacillus phaseoli* LMG 27589^T was obtained from the Belgian Coordinated Collections of Microorganisms (BCCM/LMG; Belgian). The reference type strains were cultured under the same conditions as strain LAM0504^T for comparative analyses.

Morphological, physiological and biochemical characteristics

Cell morphological characteristics of an exponentially growing culture of strain LAM0504^T were examined by light microscope (Nikon 80i, Tokyo, Japan) and transmission electron microscope (Hitachi 7500, Tokyo, Japan). Gram-staining reaction and H_2S production tests were carried out according to the methods described by Smibert and Krieg (1994). Cell motility was tested in TSB medium with 0.4 % agar. Growth under anaerobic conditions was tested in TSB medium in Hungate tubes filled with oxygen-free N_2 at 30 °C for 3 days. The formation of endospores was tested on TSA agar supplemented with 5 mg L^{-1} $MnSO_4$ as described by Logan et al. (2009). Growth at temperatures of 4, 15, 20, 30, 37, 40, 45, 48, 50 and 55 °C was investigated in TSB medium. Growth at pH 4, 5, 6, 7, 8, 9, 10 and 11 was investigated in TSB medium. NaCl concentrations of 0, 1, 2, 3, 4 and 5 % (w/v) were produced in medium prepared according to the formula of TSB but varying the addition of NaCl. The medium was adjusted to the desired pH using the method described by Ruan et al. (2014). Catalase activity was determined by production of bubbles in 3 % (v/v) H_2O_2. Oxidase activity was detected by using tetramethyl-*p*-phenylenediamine. Methyl red and Voges–Proskauer tests were determined as described by Simbert and Krieg (1981). Hydrolysis of casein and starch were carried out on skimmed milk agar and starch agar, respectively. Egg yolk reaction was conducted on egg yolk agar. The utilisation of various substrates as the sole carbon source was

conducted using Biolog GN2 MicroPlates (Biolog, Hayward, CA, USA) according to the manufacturer's instruction. Other biochemical tests of strain LAM0504^T and the three closely related reference strains, including nitrate reduction, acid production and enzyme activity, were carried out with the API 20NE, API 50CH and API ZYM systems (bioMérieux, ÍLEtoile, France) according to the manufacturer's instructions.

Phylogenetic and genomic related analyses

Genomic DNA for PCR amplification was extracted and purified according to the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR with the universal bacterial primers 27F and 1492R (Weisburg et al. 1991) and purified using a PCR purification kit (TianGen, Beijing) according to the manufacturer's instructions. The purified PCR product was cloned into pMD19-T vector (TaKaRa) and sequenced by the Life Technologies Company (Shanghai, China). The 16S rRNA gene sequence similarities and multiple sequence alignment were analyzed using EzTaxon-e service (Kim et al. 2012) and CLUSTAL W software (Thompson et al. 1994). Phylogenetic trees were constructed using the MEGA 6 program package (Tamura et al. 2013) with the neighbour-joining method (Saitou and Nei 1987) and maximum-parsimony method (Fitch 1971), and evaluated by bootstrap analysis with 1000 replications as described by Felsenstein (1985).

The genomic DNA G+C content was determined by the thermal denaturation method (Marmur and Doty 1962) using a Beckman DU 800 spectrophotometer (Beckman Coulter, Brea, CA, USA). *Escherichia coli* K-12 was used as a reference strain. DNA–DNA hybridization between strain LAM0504^T and *P. konsisdensis* JCM 14798^T was performed according to the method described by De Ley et al. (1970) and Huss et al. (1983). The experiments were carried out in quadruplicate.

Chemotaxonomic characterization

For determination of cellular fatty acid composition, strain LAM0504^T, *P. konsisdensis* JCM 14798^T, *P. motobuensis* JCM 12774^T and *F. phaseoli* LMG 27589^T were incubated in TSB medium at 30 °C for 48 h. Cellular fatty acids of the four strains were analysed as described by Sakamoto et al. (2002). Their identification and quantification were carried out using the Sherlock Microbial Identification System with the standard MIS Library Generation Software (VERSION 6.0 and Date 4, Microbial ID Inc., Newark, DE, USA) and a 6890 N gas chromatograph (Agilent). The respiratory quinones were analyzed with reversed-phase HPLC as described by Komagata and Suzuki (1987). The polar lipids were extracted and separated on silica gel plates (10 × 10 cm, Merck 5554) (Kates 1986) and further analysed by using the methods described by Minnikin et al. (1984) and Xu et al. (2011). Sulfuric acid was used to reveal total polar lipids. Aminolipids were determined using ninhydrin reagent and phospholipids were identified by Zinzadze reagent. The data were interpreted as described by Fang et al. (2012). The cell wall peptidoglycan structure of strain LAM0504^T was tested by TLC (Komagata and Suzuki 1987) by using the methods described by Schleifer (1985).

Results and discussion

Morphological, physiological and biochemical characteristics

Cells of strain LAM0504^T were observed to be rod shaped, with a cell size of 0.6–1.0 μm in width and 1.0–3.0 μm in length and motile by peritrichous flagella (Fig. S1). The isolate was found to be Gram-stain negative, facultatively anaerobic and spore-forming (Fig S2). Colonies of strain LAM0504^T were observed to be milky, flat with regular edges after growth on TSA plates at 30 °C for 48 h. Growth was observed at 20–48 °C (optimum: 30 °C), 0–3 % (w/v) NaCl (optimum: 1.0 %) and pH 5.0–9.0 (optimum: 7.0). Strain LAM0504^T was found to be catalase positive, oxidase negative and to reduce nitrate to nitrite. Cells were found to be positive for the Voges–Proskauer test, negative for the methyl red test, egg yolk reaction and H_2S production. The hydrolysis of casein and starch were found to be positive. In the API 50CH system, strain LAM0504^T was observed to give positive reactions for L-arabinose, D-ribose, D-xylose, D-galactose, methyl β-D-xylopyranoside, D-glucose, methyl α-D-glucoside, *N*-acetylglucosamine, cellobiose, amygdalin, salicin, D-lactose, melibiose, sucrose, D-raffinose and glycogen tests; negative reactions for glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, D-fructose, L-rhamnose, dulcitol, inositol, D-mannitol and D-sorbitol tests; and a weak

Table 1 Comparison of the phenotypic, physiological and genotypic characteristics of strain LAM0504^T and its relatives

Characteristic	1	2	3	4
Optimum growth temperature (°C)	30	30	37	37
Catalase	+	+	+	−
Oxidase	−	+	+	− (w)
Nitrate reduction	+	+	−	+
Voges–Proskauer test	−	−	−	+
Hydrolysis of gelatin	+	−	+	+
Assimilation of (API 20NE)				
L-Arabinose	+	+	−	− (+)
D-Mannose	+	w	−	−
Potassium gluconate	−	−	+	+
Acid production from: (API 50CH)				
L-Arabinose	+	−	−	−
D-Ribose	+	+	+	−
D-Xylose	+	+	+	−
Methyl β-D-xylopyranoside	+	w	+	−
D-Fructose	−	+	−	−
D-Mannose	w	+	−	−
L-Rhamnose	−	+	+	−
Amygdalin	+	+	−	w
D-Turanose	−	+	+	−
D-Lyxose	−	−	−	+
Enzyme activities: (API ZYM)				
Alkaline phosphatase	−	−	−	+
Trypsin	−	−	+	−
α-Chymotrypsin	w	w	−	−
Acid phosphatase	−	w	−	+
α-Glucosidase	w	w	+	+
N-Acetyl-β-glucosaminidase	+	−	+	−
DNA G+C content (mol%)	43.0†	45.6†	51.3†	52.3†

Strains: 1, strain LAM0504^T; 2, *P. konsidensis* JCM 14798^T; 3, *P. motobuensis* JCM 12774^T; 4, *Fontibacillus phaseoli* LMG 27589^T

Data in the table are all from this study, except the data in parentheses which are taken from Flores-Félix et al. (2014)

Symbols: +, positive; −, negative; w, weakly positive

† Determined by *Tm* method

reaction with D-mannose. In the API ZYM system, activities of esterase (C4), esterase lipase (C8), leucine arylamidase, α-galactosidase, β-galactosidase, α-glucosidase and *N*-acetyl-β-glucosaminidase were determined to be positive; activities of α-chymotrypsin, naphthol-AS-BI-phosphohydrolase and β-glucosidase were weakly positive. In the API 20E system, the hydrolysis of *O*-nitrophenyl-β-D-galactopyranoside (ONPG) was found to be positive but the citrate utilization, gelatin and arginine hydrolysis tests were negative. In the API 20NE system, the hydrolysis of aesculin and urea, and the assimilation of D-glucose, L-arabinose, D-mannose, *N*-acetyl-glucosamine and D-maltose were found to be positive. The differences in the physiological and biochemical characteristics between strain LAM0504^T and its relatives are shown in Table 1.

Molecular results

Comparative sequence analysis based on the nearly complete 16S rRNA gene sequence of strain LAM0504^T (1459nt, GenBank accession number KJ005124) indicated that strain LAM0504^T was most closely related to *P. konsisdensis* JCM 14798^T, *F. phaseoli* LMG 27589^T and *P. motobuensis* JCM 12774^T, with 97.0, 96.8, 96.7 % sequence similarity, respectively. Although strain *F. phaseoli* LMG 27589^T shared a higher 16S rRNA gene sequence similarity than *P. motobuensis* JCM 12774^T, the phylogenetic analysis indicated that strain LAM0504^T and *F. phaseoli* LMG 27589^T were not in a close cluster and that strain LAM0504^T presents a closer relationship with *P. motobuensis* JCM 12774^T. Phylogenetic trees

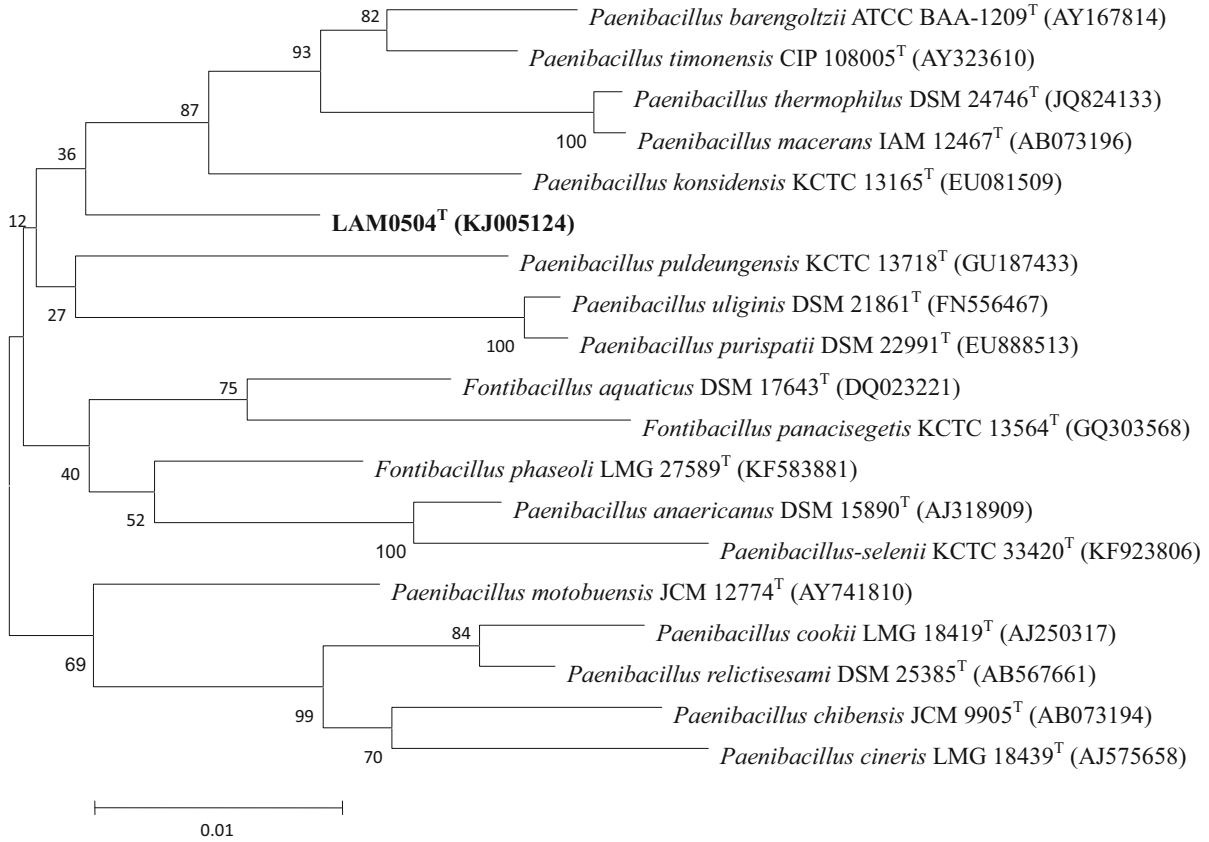

Fig. 1 Neighbour-joining phylogenetic tree based on a comparison of the 16S rRNA gene sequences of strain LAM0504^T and its closest relatives. Genbank accession numbers are given in *parentheses*. The *numbers* at the nodes indicate the percentages of bootstrap sampling derived from 1000 replications. Bar, 0.01 nucleotide substitution per nucleotide position

constructed with the neighbour-joining (Fig. 1) and maximum-parsimony (Fig. S3) methods showed that strain LAM0504^T shares a close relationship with the members of the genus *Paenibacillus*. The DNA–DNA hybridization value between strain LAM0504^T and *P. konsisdensis* JCM 14798^T was 53.3 ± 1.2 %.

The DNA G+C content of strain LAM0504^T was 43.0 mol% as determined by the *Tm* method, a value within the range of 39–59 mol% reported for the species of the genus *Paenibacillus*.

Chemotaxonomic characteristics

A comparison of the whole-cell fatty acid composition of strain LAM0504^T and the reference strains is shown in Table 2. The major fatty acids of strain LAM0504^T were identified as anteiso-$C_{15:0}$ (43.1 %), $C_{16:0}$ (15.9 %) and iso-$C_{15:0}$ (10.0 %). Minor differences existed in the proportions of the major fatty acids of strain LAM0504^T and the reference strains. The anteiso-$C_{15:0}$ content of strain LAM0504^T was notably higher than that in the reference strains (from 32.4 to 35.4 %), while the $C_{16:0}$ content (15.9 %) was in the range present in the reference strains (from 14.7 to 22.8 %). The diamino acid of the cell-wall peptidoglycan was determined to be *meso*-diaminopimelic. The predominant menaquinone was identified as MK-7. The main polar lipids of strain LAM0504^T were identified as diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, two unidentified phospholipids, two unidentified glycolipids and three unidentified lipids (Fig. S4). The main polar lipids of strain LAM0504^T were found to be similar to those of members of the genus *Paenibacillus*. The polar lipids of strain LAM0504^T show significant differences when compared with the type strain *F. phaseoli* LMG 27589^T in the absence of aminophosphoglycolipid, aminolipid and the presence of phosphatidylethanolamine.

Table 2 Major fatty acids of strain LAM0504^T and its relatives in the genus *Paenibacillus* and *Fontibacillus*

Fatty acids	1	2	3	4
Unbranched				
$C_{14:0}$	3.5	2.4	2.4	3.2
$C_{16:0}$	**15.9**	**22.8**	**14.7**	**22.3**
iso-unbranched				
iso-$C_{14:0}$	2.7	1.9	2.9	0.7
iso-$C_{15:0}$	**10.0**	8.4	**13.4**	5.1
iso-$C_{16:0}$	9.6	**11.4**	**13.2**	9.7
iso-$C_{17:0}$	4.9	6.9	7.8	6.8
Anteiso-unbranched				
Anteiso-$C_{15:0}$	**43.1**	**32.4**	**35.4**	**33.4**
Anteiso-$C_{17:0}$	6.1	8.4	7.8	**12.3**

Strains *1* strain LAM0504^T, *2 P. konsidensis* JCM 14798^T, *3 P. motobuensis* JCM 12774^T, *4 Fontibacillus phaseoli* LMG 27589^T

All data are from this study

Fatty acids present in amounts lower than 1 % in all strains are not shown and major components (higher than 10 %) in each strain are in bold

Taxonomic conclusion

The phenotypic properties (rod-shaped, facultatively anaerobic, endospore-forming and motile bacteria), major fatty acid (anteiso-$C_{15:0}$), respiratory quinone (MK-7), major polar lipids (diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine), cell-wall peptidoglycan diamino acid (*meso*-diaminopimelic) and G+C content (43.0 mol%) are consistent with the conclusion that strain LAM0504^T should be assigned to the genus *Paenibacillus*. However, the new isolated strain LAM504^T presents significant differences in its physiological and biochemical characteristics compared with the reference strains *P. konsisdensis* JCM 14798^T, *P. motobuensis* JCM 12774^T and *F. phaseoli* LMG 27589^T. Differences between strain LAM0504^T and its close relatives were observed in regards to oxidase activity, nitrate reduction, Voges–Proskauer test, acid production from carbohydrates (L-arabinose, D-ribose, D-xylose, methyl β-D-xylopyranoside, D-fructose, D-mannose, L-rhamnose, amygdalin, D-turanose and D-lyxose) and enzyme activities (alkaline phosphatase, trypsin, α-chymotrypsin, acid phosphatase, α-glucosidase and *N*-acetyl-β-glucosaminidase) as summarised in Table 1. The cellular fatty acid composition of strain LAM0504^T was mostly similar to that of the reference strains but notable differences in the proportions of some fatty acids were observed (Table 2). Differences in the polar lipid profile were also found compared to the reference strains (Fig. S4). The 16S rRNA gene sequence of strain LAM0504^T showed relatively low sequence similarities to the type strains *P. konsisdensis* JCM 14798^T, *F. phaseoli* LMG 27589^T and *P. motobuensis* JCM 12774^T, with 97.0, 96.8 and 96.7 % sequence similarity, respectively. The DNA–DNA hybridization value (53.3 ± 1.2 %) clearly differentiated strain LAM0504^T from *P. konsisdensis* JCM 14798^T. Based on the results of morphological, physiological, chemotaxonomic and phylogenetic characterisation, and combined with the DNA–DNA hybridization value (53.3 ± 1.2 %), strain LAM0504^T is considered to represent a novel species of the genus *Paenibacillus,* for which the name *Paenibacillus vini* sp. nov. is proposed.

Description of *Paenibacillus vini* sp. nov.

Paenibacillus vini (vi'ni. L. neut. gen. n. *vini* of wine, referring to the isolation of the type strain from pit mud of a Luzhou flavour liquor alcohol fermentationin Sichuan Province, China).

Cells are Gram-stain negative, facultatively anaerobic, spore-forming, catalase positive, oxidase negative and rod-shaped with a cell size of 0.6–1.0 μm in width and 1.0–3.0 μm in length. Endospores are spherical or ellipsoidal and lie at subterminal positions in slightly swollen sporangia. The temperature and pH ranges for growth are 20–48 °C (optimum: 30 °C) and pH 5.0–9.0 (optimum: 7.0). Does not require NaCl for growth but tolerates up to 3 % (w/v) NaCl (optimum: 1.0 %). Cells are positive for the Voges–Proskauer reaction but negative for methyl red reaction, H_2S and indole production. Nitrate is reduced to nitrite. The hydrolysis of ONPG, aesculin, casein, starch and urea is positive but that of the gelatin is negative. Positive for the utilisation of the following carbon sources: α-cyclodextrin, dextrin, glycogen, *N*-acetyl-D-glucosamine, L-arabinose, D-cellobiose, D-galactose, gentiobiose, α-D-glucose, α-D-lactose, maltose, D-mannose, D-melibiose, β-methyl-D-glucoside, D-raffinose, sucrose, D-trehalose, turanose, pyruvic acid methyl ester, α-ketobutyric acid, inosine, uridine and thymidine but negative for utilisation of Tween 40 and 80, L-rhamnose, D-sorbitol, citric acid, malonic acid,

propionic acid, ebacic acid, succinic acid, D-alanine, L-alanine, L-aspartic acid, L-glutamic acid, L-leucine and L-ornithine. The major fatty acids are anteiso-$C_{15:0}$, $C_{16:0}$ and iso-$C_{15:0}$. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The predominant menaquinone is MK-7. The main polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, two unidentified phospholipids, two unidentified glycolipids and three unidentified lipids. The DNA G+C content of the type strain is 43.0 mol% as determined by the *Tm* method.

The type strain is LAM0504^T (=ACCC 06420^T = JCM 19842^T), which was isolated from pit mud of a Luzhou flavour liquor alcohol fermentation in Sichuan Province, China.

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