Aestuariimicrobium ganziense sp. nov., a new Gram-positive bacterium isolated from soil in the Ganzi Tibetan Autonomous Prefecture, China

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Research Article

Keywords: Aestuariimicrobium ganziense sp. nov., Chemotaxonomy, 16S rRNA sequence analysis

Posted Date: February 11th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-215613/v1

Version of Record: A version of this preprint was published at Archives of Microbiology on March 12th, 2021. See the published version at https://doi.org/10.1007/s00203-021-02261-2.

Abstract

A novel Gram-stain positive, oval shaped and non-flagellated bacterium, designated YIM S02566^T, was isolated from alpine soil in Shadui Towns, Ganzi County, Ganzi Tibetan Autonomous Prefecture, Sichuan Province, PR China. Growth occurred at 23–35°C (optimum, 30°C) in the presence of 0.5-4 % (w/v) NaCl (optimum, 1%) and at pH 7.0–8.0 (optimum, pH 7.0). The phylogenetic analysis based on 16S rRNA gene sequence revealed that strain YIM S02566^T was most closely related to the genus *Aestuariimicrobium*, with *Aestuariimicrobium kwangyangense* R27^T and *Aestuariimicrobium soli* D6^T as its closest relative (sequence similarities were 96.3% and 95.4%, respectively). YIM S02566^T contained LL-diaminopimelic acid in the cell wall. MK-9(H4) was the predominant menaquinone. The major fatty acid patterns were anteiso-$C_{15:0}$ (60.0%). The major polar lipid was DPG. The genome size of strain YIM S02566^T was 3.1 Mb, comprising 3078 predicted genes with a DNA G + C content of 69.0 mol%. Based on these genotypic, chemotaxonomic and phenotypic evidences, strain YIM S02566^T was identified as a novel species in the genus *Aestuariimicrobium*, for which the name *Aestuariimicrobium ganziense* sp. nov. is proposed. The type strain is YIM S02566^T (= CGMCC 1.18751 ^{T} =KCTC 49477 ^{T}).

Introduction

The genus *Aestuariimicrobium*, first described by Jung et al. (2007), is a member of the family *Propionibacteriaceae* within the order *Propionibacteriales*, class *Actinomycetia* (Stackebrandt 2014; Stackebrandt et al. 1997; Stackebrandt et al. 2002a). Up to now, this genus comprises only two recognized species (http://www. bacterio. net/), *Aestuariimicrobium kwangyangense* (found in tidal flat sediment) and *Aestuariimicrobium soli* (found in farmland soil). In this study, a yellow bacterial strain YIM S02566^T was isolated from alpine soil in Shadui Towns, Ganzi County, Ganzi Tibetan Autonomous Prefecture, Sichuan Province, PR China, and was identified as the third strain of the genus *Aestuariimicrobium* (Chen et al. 2018) by means of a polyphasic taxonomic study. On the basis of the data obtained, we propose that isolate YIM S02566^T represents a novel species in the genus *Aestuariimicrobium*.

Methods And Materials

Bacterial isolation and cultivation

Strain YIM S02566^T was isolated from a soil sample in the Shadui Towns, Ganzi County, Ganzi Tibetan Autonomous Prefecture, Sichuan Province, PR China (31°49'24.19"N, 100°15'28.90"E). The altitude of the sample collection was 4643.88 m. 2 g of soil sample was serially diluted (10^{-1} dilution), and approximately 100 µl of the diluted culture suspension was spread on Luria-Bertani (LB) agar. The plates were then incubated at 30 °C for 10 days. Strain YIM S02566^T was one of the isolates that appeared on the LB plates under aerobic condition. Single colonies were purified by transferring them onto R2A

(Reasoner's 2A) agar plates with 1% NaCl. It was stored on R2A at 4 °C and as a glycerol suspension (20%, v/v) at -80 °C.

Phenotypic, physiological and biochemical characteristics

Cell morphology characteristics of strain YIM S02566^T was assessed by transmission electron microscopy (JEM-2100, JEOL) after 5 days of incubation on R2A with 1% NaCl at 30 °C. The Gram reaction was performed using the commercial kit, following the instruction of the manufacturer (Solarbio). The temperature range of growth (4, 10, 15, 18, 20, 23, 25, 28, 30, 33, 35, 37, 40 and 45 °C) and NaCl tolerance concentrations (0, 0.5, 1, 2, 3 and 4-12% at intervals of 2%, w/v) were measured by using R2A medium at 30 °C for 10 days. The pH tolerance (6, 7, 7.5, 8, 9, 10) for growth were determined on R2A liquid medium for 10 days at 30 °C using the buffer system described by Tang et al. (2010). Catalase activity was determined by production of bubbles after adding 3% H_2O_2 to the tested bacteria (Tarrand and Groschel 1982). Tests for hydrolysis of Tween 20, 40, 60 and 80 were determined by using traditional methods (Yang et al. 2020). Carbon source utilization tests, enzyme activity tests, acid production and additional physiological and biochemical tests were performed using Biolog GEN III microplates (Biolog), API ZYM, API 50 CH and API 20NE kits (bioMérieux) according to the manufacturer's instructions. Susceptibility to antibiotics was tested on R2A agar with 1% NaCl plates using discs containing the following concentrations of antibiotic (per disc): erythromycin (15 μg), cefoperazone (30 μg), tetracycline (30 μg), kanamycin (30 μg), ciprofloxacin (5 μg), furazolidone (300 μg), cefazolin (30 μg), rifampin (5 μg), polymyxin B (300 U), ampicillin (10 μg), penicillin (10 U), gentamicin (10 ug), chloramphenicol (30 ug), streptomycin (10 ug), bacitracin (0.04 U), cefoperazone (75 ug), clindamycin (2 ug), minomycin (30 ug), norfloxacin (10 ug), ofloxacin (5 ug), piperacillin (100 ug).

Phylogenetic analyses and genome sequencing

Genomic DNA for Polymerase Chain Reaction (PCR) amplification was extracted using the method described by Feng et al. (2020). The 16S rRNA gene was amplified by PCR with the universal primers PA (5'-CAG AGT TTG ATC CTG GCT-3') and PB (5'-AGG AGG TGA TCCAGC CGC A-3') (Yang et al. 2020). The amplicon was cloned into *PEASY*-Blunt (TRANSGEN Biotech) and then sequenced by the Tsingke Company (Beijing, PR China). Comparison of 16S rRNA with related strains was conducted by EzTaxon server (https ://www.ezbiocloud.net/) (Yoon et al. 2017). A total of 15 species form 13 genera were used in this study. *Terrabacter tumescens* was selected as the outgroup. All sequences used for phylogenetic analysis were obtained from GenBank, and accession numbers were listed in Fig.1. Multiple alignments with corresponding sequences of the most closely relatives were executed using the CLUSTAL X 1.8 program (Thompson et al. 1997). Phylogenetic analyses were performed by MEGA version 7.0 software (Kumar et al. 2016) using neighbor-joining (NJ) (Saitou and Nei 1987), Maximum Parsimony (MP) (Fitch 1971) and Maximum-Likelihood (ML) (Felsenstein 1981) methods, with bootstrap values based on 1000 replications (Felsenstein 1985).

The genome sequences of YIM S02566^T were determined using a PacBio + Illumina Hiseq at Shanghai Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China). The sequenced reads were assembled using SOAPdenovo software version 2.04 (https://soap.genom ics.org.cn/soap.enovo.html). The DNA G+C mol% value was obtained from the genomic sequences. A genome tree was constructed using RAxML (Stamatakis 2014), and fast bootstrapping (Stamatakis et al. 2008) was used to generate the support values in the tree.

Chemotaxonomic characteristics

The strain biomass for chemotaxonomic characterization was obtained from 5-days old cultures grown on medium R2A with 1% NaCl at 30 °C. The isomer type of the diaminopimelic acid of the cell wall was analyzed according to the method described by Lechevalier (Lechevalier and Lechevalier 1971). The respiratory quinones were isolated using the method of Collins et al. (1977), and analyzed by HPLC (Agilent Technologies 1260 Infinity) (Groth et al. 1996). Polar lipids profiles were analyzed as described by two-dimensional TLC (Minnikin et al. 1984; Toru et al. 1983), and the different spots were observed by spraying with the proper detection reagents (molybdophosphoric acid, molybdenum blue, ninhydrin, D reagent and α-naphthol). The cellular fatty acids were extracted and analyzed according to the standard $_{MIDI}$ protocol (Microbial Identification) and Sherlock Microbial Identification System (Sherlock version 6.1; midi database: TSBA6).

Results And Discussion

Morphological and physiological characteristics

The colonies of the strain YIM S02566^T was Gram positive, oval-shaped, non-flagellated, yellow and 0.5-1.0 μm in diameter after 5 days of incubation at 30 °C (Fig. S1). Other phenotypic and physiological characteristics of YIM S02566^T and its related members are given in Table 1, Table S1 and in the species description.

Phylogenetic analysis

The almost complete 16S rRNA gene sequence (1509 bp) of strain YIM S02566^T was generated, and displayed 96.3% 16S rRNA gene sequence identity with *Aestuariimicrobium kwangyangense* R27^T. The next highly related species was *Aestuariimicrobium soli* D6^T, with pairwise similarities of 95.4%. The NJ tree, MP tree and ML tree for the 16S rRNA shared the same topology and were presented in Fig. 1, Fig. S2 and Fig. S3, respectively. The phylogenetic tree indicated that strain YIM S02566^T clustered with *Aestuariimicrobium kwangyangense* R27^T and *Aestuariimicrobium soli* D6^T.

The draft genome sequence of strain YIM S02566^T consisted of 68 scaffolds with the N50 value of 449246, and contained 3078 coding sequences (CDSs), 3 complete rRNA genes, 44 tRNA genes. A phylogenetic tree based on genome sequences was reconstructed using RaxML to confirm the

relationships already displayed in the 16S rRNA gene based tree, with YIM S02566^T as a part of the genus *Aestuariimicrobium*(Fig. S4).

Chemotaxonomic characteristics

Strain YIM S02566^T contained LL-diaminopimelic acid as the diagnostic cell wall diamino acid. The predominant isoprenoid quinone was [MK-9(H_4)], which was the same as the members of the family *Propionibacteriaceae* (Bae et al. 2006; Chen et al. 2018; Collins et al. 2003; Jung et al. 2007; Nakamura et al. 1995; Stackebrandt et al. 2002a; Tamura et al. 1994; Yokota et al. 1994). The major polar lipid was diphosphatidylglycerol (DPG) (Fig. S5). The major fatty acids (>10%) of strain YIM S02566^T was anteiso-$C_{15:0}$ (60.0%). Compared with the data of other two reference strains, anteiso-$C_{15:0}$ was the major fatty acid of all strains (Table 2).

The combination of phenotypic (Table 1), phylogenetic (Fig. 1 and Fig. S4), and chemotaxonomic characteristics indicates that strain YIM S02566^T represents a novel species within the genus *Aestuariimicrobium*, for which the name *Aestuariimicrobium ganziense* is proposed.

Description of *Aestuariimicrobium ganziense* sp. nov.

Aestuariimicrobium ganziense (gan.zi.en'se. N.L. masc. adj. *ganziense* pertaining to Ganzi, China, from where the type strain was isolated).

Gram-stain positive, non-flagellated, oval shaped (approximately 0.5×0.7 μm) bacterium, and growth occurred at 23-35 °C (optimum, 30 °C) in the presence of 0.5-4 % (w/v) NaCl (optimum, 1%) and at pH 7.0-8.0 (optimum, pH 7.0). The gram staining reaction, catalase activity, hydrolysis of Tween 20, 40, 60 and 80 tests are positive. In API 20NE tests, positive for nitrate reduction, aesculin hydrolysis, protease hydrolysis, *β*-galactosidase activity, assimilation of L- arabinose, D-mannose, capric acid. In API ZYM tests, positive for esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphomonoesterase, naphthol-*AS-BI*-phosphoric acid, *α*-galactosidase, *β*-galactosidase, *α*-glucosidase, *β*-glucosidase, *N*-acetyl-*β*-glucosaminase, *α*-mannosidase, and *β*-fucosidase. In API 50CH tests, acid is produced from L-arabinose, D-xylose, L-xylose, D-galactose, D-glucose, fructose, D-mannose, L- sorbose, L-rhamnose, methyl-*ad*-glucopyranoside, amygdalin, arbulin, aesculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-melezitose, D-raffinose, amidon (starch), glycogen, xylitol, D-gentiobiose, D-turanose, D-lyxose, L-fucose. In the Biolog GEN III MicroPlate system, grows in the presence of dextrin, D-trehalose, gentiobiose, sucrose, stachyose, D-raffinose, *β*-methyl-D-glucoside, D-salicin, D-galactose, D-fucose, L-fucose, D-sorbitol, D-arabitol, myo-inositol, D-serine, gelatin, L-alanine, L-pyroglutamic acid, pectin, D-galacturonic acid, D-glucuronic acid, glucuronamide, *α*-keto-glutaric acid, tween 40, acetoacetic acid, propionic acid, acetic acid, formic acid. The strain is susceptible to penicillin (10 IU), chloramphenicol (30 μg), tetracycline (30 μg), ceftriaxone (30 μg), cefazolin (30 μg), ciprofloxacin (5 μg), ofloxacin (5 μg), erythromycin (15 μg), rifampin (5 μg), gentamicin (10 μg), minocycline (30 μg), but not toclindamycin (2 μg), kanamycin (30 μg), polymyxin B (300 IU), piperacillin

(100 μg), cefoperazone (75 μg), ampicillin (10 μg), bacitracin (0.04 IU), streptomycin (10 μg), norfloxacin (10 μg),furazolidone (300 μg). The major polar lipid is diphosphatidylglycerol (DPG). The predominant menaquinone is MK-9(H_4). The cell wall contains LL-diaminopimelic acid. The fatty acids are anteiso-$C_{15:0}$ (60.0%). The DNA G + C content of the type strain is 69.0 mol%.

The type strain YIM S02566^T (=CGMCC 1.18751 ^{T} =KCTC 49477 ^{T}) was isolated from a soil sample in the Shadui Towns, Ganzi County, Ganzi Tibetan Autonomous Prefecture, Sichuan Province, PR China (31°49'24.19"N, 100°15'28.90"E), the altitude of the sample collection is 4643.88 m. The GenBank accession numbers of the 16S rRNA gene and the genome sequence of YIM S02566^T are MW023203 and JACYIZ000000000, respectively.

Declarations

Acknowledgements

We thank Zhenyu Lv from the Kunming Institute of Botany, Chinese Academy of Sciences for the help in sample collection.

Funding

This work was supported by Major Science and Technology Projects of Yunnan Province (Digitalization, development and application of biotic resource 202002AA100007), National Natural Science Foundation of China (31760019) and Basic Research Project of Yunnan Province (202001BB050025).

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Tables

Table 1 Comparative characteristics of strain YIM S02566^T and type strains of closely related species

Characteristic	YIM S02566^T	*A. kwangyangense* R27^T	*A. soli* D6^T
Colony color	Yellow	Yellow	Yellow
Growth temperature			
Range	23-35 ℃	4-50℃	4-37℃
Optimum	30℃	30℃	30℃
Growth pH			
Range	7.0-8.0	7.5-8.5	6.5-8.5
Optimum	7.0	7.5-8.5	7.0
Growth NaCl (%, w/v)			
Range	0.5-4%	0-7%	0-7%
Optimum	1%	1%	0.5%
Gram reaction	+	+	+
Catalase activity	+	+	+
Oxidase activity	-	-	-
Urease activity	-	-	-
reactions for nitrate	+	+	+
Enzyme production (API ZYM)			
Cystine arylamidase	-	-	+
Naphthol-*AS-BI*-Phosphohydrolase	+	-	+
Esterase (C4)	+	+	-
Esterase lipase (C8)	+	+	-
Acid phosphatase	+	+	-
α-Galactosidase	+	+	-
β-Galactosidase	+	+	-
α-Glucosidase	+	+	-
β-Glucosidase	+	+	-
Hydrolysis of			
Tween 20, 40, 60, 80	+	+	+
Aesculin	+	+	+

Starch	-	-	-
Gelatin	+	+	+
Acid production (API 50CH)			
D-Xylose	+	+	-
L-Rhamnose	+	+	-
Sorbitol	-	+	+
myo-inositol	w	-	-
DNA G + C content (mol%)	69.0	68.8-69.2	69.2

Strains: YIM S02566^T; *A. kwangyangense* R27^T (data from Jung et al., 2007); *A. soli* D6^T (data from Chen et al., 2018).

+ positive, − negative, *w* weakly positive

Table 2 Cellular fatty acid profile (%) of strain YIM S02566^T and type strains of phylogenetically related species of the genus *Aestuariimicrobium*

Fatty acid	YIM S02566^T	*A. kwangyangense* R27^T	*A. soli* D6^T
iso-C14:0	7.0	1.5	2.9
iso-C15:0	0.4	**10.5**	**15.8**
iso-C16:0	-	1.6	1.0
anteiso-C15:0	**60.0**	**37.9**	**44.1**
Summed feature 2*	2.9	-	-
Summed feature 4*	-	**16.0**	**10.8**

Strains: YIM S02566^T; *A. kwangyangense* R27^T (data from Chen et al., 2018); *A. soli* D6^T (data from Chen et al., 2018).

*Summed feature 2 contains $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I

*Summed feature 4 contains iso-C17: 1 I and/or anteiso-C17: 1 B.

- not detected and not reported

Figures

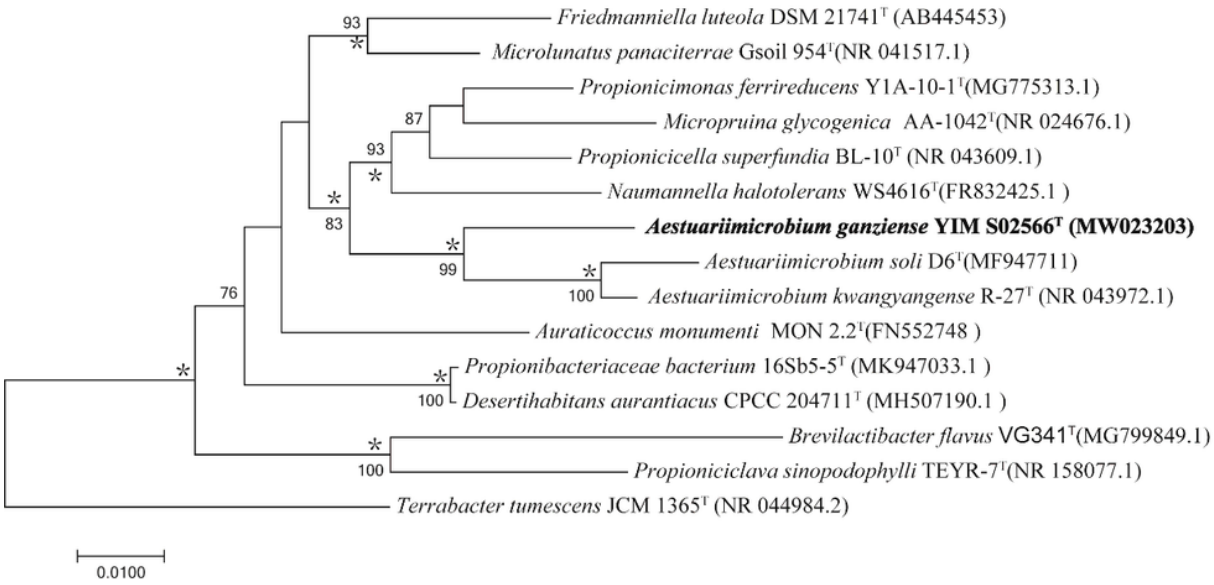

Figure 1

Neighbour-joining tree based on 16S rRNA gene sequences showing the relationships between strain YIM S02566T and some members of the family Propionibacteriaceae. Bootstrap values (> 50%) based on 1000 replicates were shown at the branch nodes. Asterisks (*) indicate that the corresponding branches were also recovered in trees generated with the maximum parsimony and maximum likelihood methods. Terrabacter tumescens JCM 1365T was used as an outgroup. Bar, 1% sequence divergence

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