Aeromicrobium camelliae sp. nov., isolated from Pu′er tea

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A novel Gram-reaction-positive, aerobic and non-spore-forming rod-shaped bacterial strain, YS17^T, was isolated from ripened Pu′er tea. Growth of the strain was observed at 15–50 °C (optimum 30–37 °C) and at pH 5.5–10.5 (optimum 6.0–9.5). Phylogenetic analysis of 16S rRNA gene sequences indicated that the strain represented a member of the genus *Aeromicrobium*. The strains most closely related to YS17^T were *Aeromicrobium erythreum* DSM 8599^T, *Aeromicrobium alkaliterrae* JCM 13518^T and *Aeromicrobium ginsengisoli* JCM 14732^T, with 16S rRNA gene sequence similarities of 96.8, 96.8 and 96.7 %, respectively. DNA–DNA hybridization of YS17^T with the type strains of the most closely related species, *A. erythreum* DSM 8599^T, *A. alkaliterrae* JCM 13518^T and *A. ginsengisoli* JCM 14732^T, yielded reassociation values of 10.9, 16.8 and 10.9 %, respectively. The diagnostic diamino acid of the cell wall peptidoglycan was LL-diaminopimelic acid. The predominant menaquinones were menaquinone MK-9(H_4) (76 %) and MK-8(H_4) (17 %). The major fatty acids were $C_{16:0}$, 10-methyl $C_{18:0}$ and $C_{18:1}\omega9c$. The DNA G+C content of YS17^T was 66 mol%. YS17^T could be differentiated from recognized species of the genus *Aeromicrobium* on the basis of phenotypic characteristics, chemotaxonomic differences, phylogenetic analysis and DNA–DNA hybridization data. On the basis of evidence from the polyphasic analyses performed as part of this study a novel species, *Aeromicrobium camelliae* sp. nov., is proposed, with strain YS17^T (=CGMCC 1.12942^T=JCM 30952^T) as the type strain.

The genus *Aeromicrobium* was first proposed by Miller *et al.* (1991) with *Aeromicrobium erythreum* as the type species, and the description was then emended by Yoon *et al.* (2005). At the time of writing, 11 species with validly published names have been identified, including *A. erythreum* (Miller *et al.*, 1991), *A. alkaliterrae* (Yoon *et al.*, 2005), *A. fastidiosum* (Tomohiko & Akira, 1994), *A. panaciterrae* (Cui *et al.*, 2007) and *A. ginsengisoli* (Kim *et al.*, 2008) that were isolated from soil; *A. marinum* (Bruns *et al.*, 2003), *A. tamlense* (Lee & Kim, 2007), *A. ponti* (Lee & Lee, 2008) and *A. halocynthiae* (Kim *et al.*, 2010) that were recovered from marine environment and *A. flavum* and *A. massiliense* isolated from the air and human stools, respectively (Tang *et al.*, 2008; Ramasamy *et al.*, 2012). Members of the genus *Aeromicrobium* are Gram-reaction-positive, non-endospore-forming rods or cocci and are characterized chemotaxonomically by having a tetrahydrogenated menaquinone with nine isoprene units [MK-9(H_4)] as the predominant respiratory quinone, and 10-methyl $C_{18:0}$, $C_{16:0}$ and both or either of $C_{18:1}\omega9c$ and $C_{16:0}$ 2-OH as the major cellar fatty acids (Yoon *et al.*, 2005).

During the course of our investigation on the community structure from Pu′er tea based on a culture-dependent method, a strain, YS17^T, was isolated from a ripened Pu′er tea in Yunnan province (China) and characterized using a polyphasic approach, including phylogenetic

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Aeromicrobium camelliae* YS17^T is KJ874351.

Two supplementary figures and two supplementary tables are available with the online Supplementary Material.

analyses based on 16S rRNA gene sequences, DNA–DNA relatedness and chemotaxonomic and phenotypic properties, in order to determine its precise taxonomic position. The results indicated that strain YS17^T represents a member of the genus *Aeromicrobium*, but it is clearly distinguished from the species of the genus *Aeromicrobium* with validly published names.

The Pu′er tea sample was suspended in sterile water and the supernatant was spread on Luria–Bertani (LB, laboratory-prepared) agar medium by serial dilution. After incubation at 30 °C for 2 weeks, strain YS17^T was recovered.

Genomic DNA preparation, 16S rRNA gene amplification and sequencing were carried out as described previously (Niu *et al.*, 2008). The partial 16S rRNA gene sequence (1512 nt) was obtained with primers 27F (5′-AGAGTTTGATCC/ATGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′). A similarity-based search was performed using the EzTaxon-e server (Kim *et al.*, 2012). The model of Jukes and Cantor was used to compute evolutionary distances, on the basis of which a phylogenetic tree was reconstructed using the neighbour-joining method with bootstrap analysis of 1000 replications in the MEGA6 software program (Tamura *et al.*, 2013). Another phylogenetic tree was built using the maximum-likelihood method in the MEGA6 software program, with bootstrap values based on 1000 replications. In the phylogenetic trees, strain YS17^T fell within the radiation of the cluster comprising species of the genus *Aeromicrobium* (Fig. 1; Fig. S1 available in the online Supplementary Material). All species of the genus *Aeromicrobium* were included in the phylogenetic tree. Sequence similarity calculations indicated that YS17^T shared relatively high similarity (96.0–96.8 %) with all other species of the genus *Aeromicrobium* depicted on the phylogenetic tree. Highest levels of 16S rRNA gene sequence similarity between YS17^T and the type strains of related species of the genus *Aeromicrobium* were with: *A. erythreum* (96.8 %), *A. alkaliterrae* (96.8 %) and *A. ginsengisoli* (96.7 %). Although the type strain of *A. erythreum* appeared to have the highest gene sequence similarity with strain YS17^T, it was located on another branch in the phylogenetic tree due to the higher levels of 16S rRNA gene sequence similarity with *A. fastidiosum* DSM 10552^T, 98.3 % (Fig. 1).

On the basis of 16S rRNA gene sequence similarity, DNA–DNA hybridization between strain YS17^T and the type strains of *A. erythreum*, *A. alkaliterrae* and *A. ginsengisoli* was assessed. DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) incorporating the modifications described by Huss *et al.* (1983), using a Model100 Bio UV/VIS spectrophotometer (Cary) equipped with a Peltier temperature-controlling programmer. YS17^T exhibited low levels of DNA–DNA relatedness to *A. erythreum* DSM 8599^T (10.9 %), *A. alkaliterrae* JCM 13518^T (16.8 %) and *A. ginsengisoli* JCM 14732^T (10.9 %). These values were well below the threshold value (70 %) recommended by Wayne *et al.* (1987) for the delineation of genomic species. The phylogenetic analysis results and DNA–DNA relatedness values described above indicated that YS17^T represents a novel species of the genus *Aeromicrobium*.

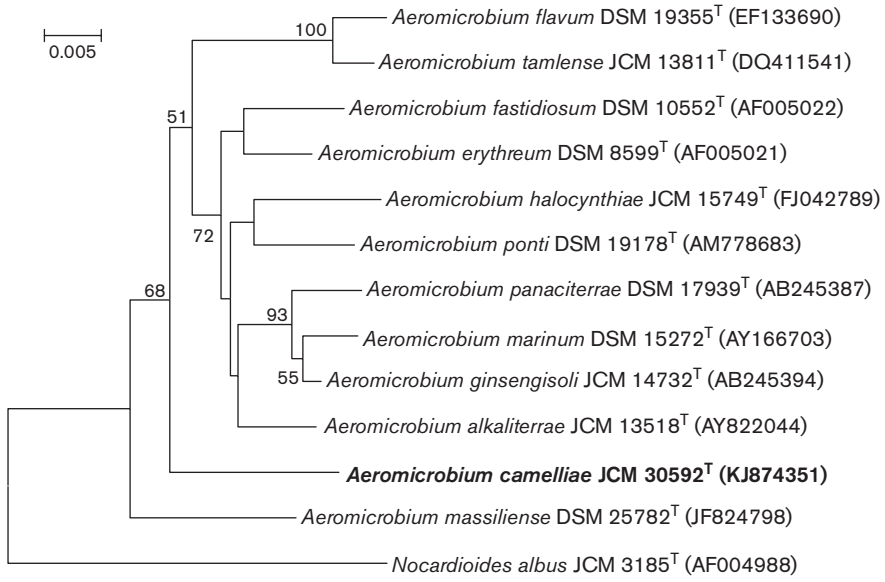

Fig. 1. Phylogenetic tree of *Aeromicrobium camelliae* sp. nov. YS17^T and related species based on 16S rRNA gene sequence similarity. The tree is rooted with *Nocardioides albus* JCM 3185^T and reconstructed using the neighbour-joining method. Bootstrap values (percentages of 1000 replications) >50 % are shown at nodes. Bar, 0.005 nt substitutions per site.

The tests for light and electron microscopy and analytical techniques were performed as described previously (Niu *et al.*, 2008). Gram staining was carried out according to the classical Gram-staining procedure described by Doetsch (1981) and the non-staining KOH method as described by Gregersen (1978). Motility was studied by puncture inoculation in semisolid agar medium. Oxidase activity was evaluated via oxidation of 1 % *p*-aminodimethylaniline oxalate and catalase activity was determined by measuring bubble production after application of a 3 % (v/v) hydrogen peroxide solution. Hydrolysis of aesculin, DNA, casein, starch, gelatin, pectin, xylan, CM-cellulose and Tweens 20, 60 and 80 was assessed according to the protocol of Dong & Cai (2001). Growth under anaerobic conditions was determined after incubation in an anaerobic chamber for about 2 weeks. Growth at various temperatures (4, 10, 13, 15, 20, 25, 28, 30, 35, 37, 40, 45, 50, 52 and 55 °C) was assessed on LB agar and growth at different pH values (pH 4.0–11.0 at intervals of 0.5 pH units) was assessed in LB broth. The different pH values were buffered with 200 mM Na_2HPO_4/100 mM citrate (for pH 4.0–6.0), 100 mM NaH_2PO_4/Na_2HPO_4 (for pH 6.5–8.0), 100 mM $NaHCO_3$/Na_2CO_3 (for pH 8.5–10.5) and 50 mM $NaHCO_3$/100 mM NaOH (for pH 11.0). Requirement for/tolerance of NaCl was tested in LB broth supplemented with 0–12 % (w/v) NaCl. Growth was also evaluated on trypticase soy agar (TSA) at 30 °C after 5 days of incubation. The ability to use different carbon sources was analysed in a basal medium containing different substrates as follows: sugars (0.5 %, final concentration), fatty acids (20 mM, final concentration) and amino acids (0.2 %, final concentration). The basal medium contained (per litre): 0.1 g $CaCl_2.2H_2O$, 0.5 g $MgSO_4.7H_2O$, 2.64 g $(NH_4)_2SO_4$, 2.38 g KH_2PO_4, 1.74 g K_2HPO_4, 9.0 g NaCl and 5 ml trace elements solution which contained (per litre): 0.4 g $ZnSO_4.7H_2O$, 0.2 g $MnSO_4.4H_2O$, 0.5 g H_3BO_3, 0.04 g $CuSO_4$, 0.2 g $Na_2MoO_4.2H_2O$, 0.1 g KI and 0.2 g $FeCl_3.6H_2O$. Other physiological and biochemical properties were obtained using the API ZYM and API 20E systems (bioMérieux) at 30 °C according to the instructions of the manufacturer. All tests were performed in duplicate.

Cells of strain YS17^T were Gram-reaction-positive, non-spore-forming, non-motile and rod-shaped, 0.3–0.5 μm × 0.9–1.6 μm in size (Fig. S2). Colonies on TSA were convex, circular, smooth, entire and yellow-pigmented, about 1.0 mm diameter after cultivation at 30 °C for 48 h.

Strain YS17^T grew strictly aerobically and growth occurred from 15 to 50 °C and at pH 5.5–10.5, with optimum growth at 30–37 °C and pH 6.0–9.5. The strain could grow in the presence of 0–11.0 % (w/v) NaCl. Additional physiological and biochemical characteristics of the strain are summarized in the species description, and a comparison of selected characteristics with related type strains is shown in Table 1 and Table S1.

For chemotaxonomic analyses, the biomass of strain YS17^T and the reference type strains were harvested from TSA plates incubated at 30 °C for 48 h. The diagnostic isomers of diaminopimelic acid in the cell wall were determined with established TLC procedures (Lechevalier & Lechevalier, 1980). Cellular fatty acid analyses were carried out by the Identification Service of the DSMZ (Braunschweig, Germany). Fatty acids were extracted using the method of Miller (1982), with the modifications of Kuykendall *et al.* (1988), and the profile of cellular fatty acids was analysed by GC using the Microbial Identification System (MIDI, Sherlock version 6.1; database, TSBA40; GC model 6890N, Agilent Technologies) using GC analysis according to the MIDI Microbial Identification System (e.g. Method: TSBA40). Respiratory lipoquinones were extracted from 100 mg of freeze-dried cells based on the two-stage method described by Tindall (1990a, b) and carried out by the Identification Service of the DSMZ. Respiratory lipoquinones were separated into their different classes (menaquinones, ubiquinones, etc.) by TLC on silica gel (Art. No. 805 023; Macherey–Nagel), using hexane/*tert*-butylmethylether (9 : 1, v/v) as solvent. UV-absorbing bands corresponding to the different quinone classes (e.g. menaquinones or ubiquinones) were removed from the plate and further analysed by HPLC. This step was carried out on an LDC Analytical (Thermo Separation Products) HPLC apparatus fitted with a reverse phase column (2 mm × 125 mm, 3 μm, RP18; Macherey–Nagel) using methanol/heptane (9 : 1, v/v) as the eluant. Respiratory lipoquinones were detected at 269 nm.

The major cellular fatty acids of strain YS17^T included $C_{16:0}$ (18.5 %), 10-methyl $C_{18:0}$ (30.2 %) and $C_{18:1}\omega9c$ (16.2 %). The four strains examined shared similar fatty acid profiles, with only minor differences in the respective proportions of some components (Table S2). YS17^T contained LL-diaminopimelic acid as the diagnostic diamino acid in the cell wall and the major isoprenoid quinones were MK-9(H_4) (76 %) and MK-8(H_4) (17 %), which were in accordance with the description of the genus *Aeromicrobium* (Yoon *et al.*, 2005).

The G+C content of the DNA was determined by the thermal denaturation method (Marmur & Doty, 1962) using a model 100 Bio UV/VIS spectrophotometer (Cary) equipped with a Peltier-thermostat-equipped 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian) with *Escherichia coli* K-12 as the reference. The G+C content of the genomic DNA of strain YS17^T was 66 mol%.

In conclusion, phylogenetic and chemotaxomic characteristics indicated that strain YS17^T represented a member of the genus *Aeromicrobium*. Furthermore, DNA–DNA relatedness between strain YS17^T and the type strains of recognized species of the genus *Aeromicrobium* was low. Physiological and biochemical traits distinguished strain YS17^T from other species of the genus *Aeromicrobium* (Table 1). On the basis of the results presented, a novel

Table 1. Characteristics differentiating *Aeromicrobium camelliae* YS17^T from its phylogenetic relatives

Strains: 1, YS17^T; 2, *A. erythreum* DSM 8599^T; 3, *A. alkaliterrae* JCM 13518^T; 4, *A. ginsengisoli* JCM 14732^T. All data are from this study. +, Positive; −, negative; w, weakly positive.

Characteristic	1	2	3	4
Isolated from	Pu′er tea	Tropical soil	Alkaline soil	Ginseng soil
Cell morphology	Rods	Irregular rods, cocci	Rods, cocci	Cocci
Temperature range (°C)	15–50	21–40	4–35	4–30
Optimum temperature (°C)	30–37	35	25	30
pH range	5.5–10.5	5.0–9.0	6.0–11.0	5.0–8.5
NaCl tolerance (%)	0–11.0	0–4.0	0–8.0	0–4.0
Oxidase	+	+	−	+
Catalase	+	+	+	−
Nitrate reduction	+	−	−	−
Voges–Proskauer reaction	+	+	−	−
API ZYM				
Esterase (C4)	+	w	+	+
Valine arylamidase	+	−	−	+
Cystine arylamidase	w	−	−	−
Trypsin	+	+	−	−
Naphthol-AS-BI-phosphohydrolase	+	w	w	+
Lipase (C14)	−	w	+	−
β-Glucosidase	−	+	−	−
Hydrolysis of:				
Aesculin	−	+	−	−
DNA	−	w	+	−
Gelatin	−	+	−	−
Utilization of:				
Maltose	w	−	+	+
D-Mannose	−	−	−	+
Rhamnose	−	−	−	+
Pyruvate	−	−	−	+
D-Xylose	−	−	−	+
L-Arabinose	−	+	−	+
Acetate	+	−	−	+
L-Aspartate	+	+	−	−
DNA G+C content (mol%)	66	73	68	70

species, *Aeromicrobium camelliae* sp. nov. with the type strain YS17^T, is proposed.

Description of *Aeromicrobium camelliae* sp. nov.

Aeromicrobium camelliae (ca.mel′li.ae: N.L. gen. n. *camelliae* of *Camellia*, referring to the isolation of the type strain from fermented green tea, *Camellia sinensis*).

Cells are Gram-reaction-positive, aerobic, non-motile and non-spore-forming rods, 0.3–0.5 μm in width and 0.9–1.6 μm in length. Colonies on TSA are convex, circular, smooth, entire and yellow-pigmented, about 1.0 mm diameter after cultivation at 30 °C for 48 h. Growth occurs between 15 and 50 °C (optimum 30–37 °C) and at pH 5.5–10.5 (optimum 6.0–9.5). Growth occurs at NaCl concentrations in the range of 0–11% (w/v). Oxidase- and catalase-positive. Nitrate is reduced to nitrogen gas. H_2S and indole are not produced. Voges–Proskauer reaction is positive. With API ZYM and API 20E systems, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and tryptophan deaminase are detected. Cystine arylamidase is weakly active. The following enzyme activities are not detected: lipase (C14), α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urase and gelatinase. Tween 60 and pectin are hydrolysed, but not casein, starch, aesculin, salicin, chitin, xylan, Tween 20, Tween 80, DNA, gelatin or CM-cellulose. The following carbon sources are utilized: D-galactose, maltose, D-fructose, sucrose, D-glucose, D-gluconate, trehalose, acetate, L-aspartate, L-alanine, glutamate and glycerol. D-Mannose, L-arabinose, xylose, rhamnose, salicin, cellobiose, L-phenylalanine, inositol, mannitol, pyruvate and

fumarate are not utilized as sole carbon and energy sources. The predominant fatty acids are $C_{16:0}$, 10-methyl $C_{18:0}$ and $C_{18:1}\omega 9c$. The major respiratory lipoquinones are MK-9(H_4) and MK-8(H_4). The cell wall peptidoglycan is based on LL-diaminopimelic acid.

The type strain, YS17^T (=CGMCC 1.12942^T=JCM 30952^T), was isolated from a ripened Pu′er tea sample obtained from a manufacturer in Yunnan province, China. The DNA G+C content of the type strain is 66 mol%.

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