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Lysobacter chengduensis sp. nov. Isolated from the Air of Captive *Ailuropoda melanoleuca* Enclosures in Chengdu, China

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Abstract A novel bacterial strain, designated as CF21^T, was isolated from the air of *Ailuropoda melanoleuca* enclosures in China. Cells were gram-negative, aerobic, non-motile, and rod shaped. Strain CF21^T grew at 10–40 °C (optimum 28–30 °C) and pH 6.0–9.0 (optimum pH 7.0–8.0) and in the presence of NaCl concentrations ranging from 0.0 % (w/v) to 2.0 % (optimum 0.0–1.0 %). 16SrRNA gene sequence analysis indicated that strain CF21^T belonged to genus *Lysobacter* within class *Gammaproteobacteria* and was most closely related to *Luteimonas dalianensi* OB44-3^T (95.8 % similarity), *Lysobacter ruishenii* CTN-1^T (95.1 %), *Lysobacter spongiicola* KMM329^T (94.8 %), and *Lysobacter daejeonensis* GH1-9^T (94.6 %). The genomic G+C DNA content was 68.72 mol%. Major cellular fatty acids of CF21^T were iso-$C_{16:0}$ (30.22 %), iso-$C_{15:0}$ (25.70 %), and the sum of 10-methyl $C_{16:0}$ and/or iso-$C_{17:1}\omega9c$ (21.94 %). The prominent isoprenoid quinone was ubiquinone 8 (Q-8). Primary polar lipids included diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and an unknown phospholipid. DNA sequence relatedness between strain CF21^T and *L. ruishenii* CTN-1^T was 56 %, which was clearly below the 70 % threshold for prokaryotic species delineation. These analyses indicated that CF21^T is a novel member of genus *Lysobacter*, for which the name *Lysobacter chengduensis* sp. nov. is proposed. The type strain is CF21^T (=CGMCC1.15145^T = DSM 100306^T).

Introduction

The genus *Lysobacter* was first proposed by Christensen and Cook [6] and was assigned to family *Lysobacteraceae*. Several years later, it was reclassified within the family *Xanthomonadaceae*, which belonged to class *Gammaproteobacteria* [23]. Species of genus *Lysobacter* are generally gram-negative, aerobic, and non-fruiting and lack flagella or gliding bacteria. Currently, the genus *Lysobacter* contains the following 29 species (http://www.bacterio.cict.fr/). These *Lysobacter* species are commonly found in diverse geographical and environmental habitats and have been isolated from soil, water, and sludge, especially agricultural soil [2, 17, 22, 25]. However, to the best of our knowledge, no representatives of genus *Lysobacter* have yet been acquired from the air. The typical characteristics of members of genus *Lysobacter* exhibit high genomic DNA G+C content (61.7–70.1 mol%), a predominance of iso-branched fatty acids, and ubiquinone 8 (Q-8) as the major respiratory quinine [11, 18, 31, 32]. Moreover, some members of this genus showed strong proteolytic abilities and could lyse many types of bacteria, fungi, yeasts, algae, and nematodes, suggesting great potential for use in the development of bio-control agents [14, 21].

Herein, we investigated the cultivable bacterial communities in the air of Giant Panda (*Ailuropoda melanoleuca*) enclosures at the Chengdu Giant Panda Breeding Research Base in Sichuan Province, located in south-

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western China. Based on differences in colony morphology and 16S rRNA sequence, the strain designated as CF21^T was further characterized using a polyphasic taxonomic approach. All of these tests were performed together with the most closely related type strains, *L. ruishenii* CTN-1^T and *L. daejeonensis* GH1-9^T, which were acquired from the Key Laboratory of Microbiological Engineering of Agriculture at Nanjing Agricultural University (Jiangsu, PR China). The data that we obtained indicated that strain CF21^T should be classified in genus *Lysobacter* as the type strain of a novel species.

Materials and Methods

Culture Conditions and Phenotypic Characteristics

Strain CF21^T was isolated on tryptic soy agar (TSA, Difco) from the cultivable bacterial community obtained from the air of an *Ailuropoda melanoleuca* enclosure. For further analysis of the morphological and physiological characteristics of the strain, CF21^T was cultivated on Luria–Bertani (LB) agar (Difco) medium at 30 °C. Cellular morphology was observed by light microscopy (Olympus; 61000) and transmission electron microscopy (H-600-A2; Hitachi) using cells from an exponentially growing culture. Gram staining was carried out using the non-staining method described by Buck [3]. Motility testing was performed using LB broth with 0.3 % agar. Growth at different temperatures (4, 10, 15, 20, 25, 28, 30, 37, 40, 45, and 50 °C) and at various pH ranges (2.0–10.0, at intervals of 1.0 pH unit) were monitored during a 7-day incubation period in LB broth. NaCl tolerance was tested in LN medium (LB without NaCl) broth supplemented with 0, 0.5, and 1–5 % (at intervals of 1 %; w/v) NaCl during a 7-day period of incubation. Growth under anaerobic conditions was investigated by incubation in an anaerobic chamber (Mitsubishi Gas Chemical) at 30 °C for 7 days on LB agar.

Biochemical Characteristics and Microbial Sensitivity Test

The physiological properties of strain CF21^T were determined using the BD Phoenix^{TM}-100 automated microbiology system (Becton–Dickinson, New Jersey), according to the manufacturer's instructions. The biological principles of the Phoenix automated microbiology and API systems are similar, including enzymatic hydrolysis of amide or glycosidic bonds, resistance to an antimicrobial agent or utilization of a carbon source, utilization of carbohydrates, hydrolysis of ornithine or urea, or hydrolysis of esculin. The tests included both traditional biochemical identification and a micro Kong integrated panel. Each negative identification (NID) panel contained 45 substrates plus two fluorescent positive control wells [4]. Sensitivity to various antibiotics was tested by spreading bacterial suspensions on LB plates and applying filter paper disks containing the following antibiotics (μg per disk): vancomycin (30), tetracycline (30), sulfamethoxazole (100), lincomycin (10), kanamycin (30), spectinomycin (100), furazolidone (100), erythromycin (15), streptomycin (10), trimesulf (23.75), rifampicin (5), ampicillin (10), ciprofloxacin (5), penicillin (10), gentamicin (10), florfenicol (30), cephalothin (30), doxycycline (30), amikacin (30), enrofloxacin (5), cephalothin (30), cefotaxime (30), and ceftazidime (30), which were all obtained from Hangzhou Microbial Reagent company. For these assays, the strain was incubated at 30 °C for 24 h.

16S rRNA Sequencing and Phylogenetic Analysis

The 16S rRNA gene was amplified by PCR using two universal primers, 27F and 1492R [12], and was cloned into the plasmid pMD-19T (Takara). The amplification products were sequenced by Tskingke Biotechnology. Similarity searches with the derived sequences were compared with available sequences found in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the EzTaxon Server (http://eztaxon-e.ezbiocloud.net/) [15]. Multiple alignments of strain CF21^T and the type strains for the published *Lysobacter* species were computed using the program CLUSTAL_X [28]. Phylogenetic analysis was performed using the PHYML online web server [13] and MEGA 6.0 offline software [27]. Phylogenetic trees were reconstructed using the 'neighbor-joining,' maximum-parsimony (Kluge and Farris [16]), and maximum-likelihood methods [9]. The robustness of the tree was viewed with MEGA 6.0 using a bootstrap analysis based on 1000 replicates [10].

Chemotaxonomic and Genomic Analyses

The DNA G+C content was determined by thermal denaturation according to the method of Mandel and Marmur [19], for which *Escherichia coli* K-12 was used as a standard. Genomic DNA from strain CF21^T was extracted and purified using standard procedures [24]. Polar lipid analysis of the strain was carried out using a two-dimensional TLC method, as described by Tindall [29]. Respiratory quinones were extracted and analyzed by HPLC, as described by Minnikin et al. [20] and Collins [7] (1985). For whole-cell fatty acid analysis, cell masses of strains CF21^T, *L. ruishenii* CTN-1^T, and *L. daejeonensis* GH1-9^T were harvested from LB plates after incubation at 30 °C for 2 days. Then, fatty acids were extracted, methylated, and

analyzed using a gas chromatograph, following the manufacturer's instructions for the Sherlock Microbial Identification System (MIDI Sherlock version 6.0, MIDI database TSBA6 6.00). All of the four tests mentioned above were carried out at the China General Microbiological Culture Collection Center (CGMCC). Additionally, DNA–DNA hybridization experiments were performed by fluorometric method with microplate wells to evaluate the DNA–DNA relatedness between strains CF21^T and *L. ruishenii* CTN-1^T on the basis of 16Sr RNA gene sequence similarities, according to the method developed by Ezaki et al. [8], at Guangdong Microbiology Culture Center (GIMCC).

Results

Morphological and Physiological Characteristics

Cells of strain CF21^T were gram-negative, aerobic, non-motile, and rod shaped with numerous fimbria. Colonies grown on LB agar were circular, smooth with entire edges, non-transparent, and light beige after 3 days of incubation at 30 °C. For cells grown in LN medium with 0–2 % (w/v) NaCl (optimum 0–1.0 %), no growth occurred in the presence of 3.0–5.0 % (w/v) NaCl. Strain CF21^T grew at 10–40 °C (optimum 28–30 °C) and at pH 6.0–9.0 (optimum pH 7.0–8.0). The physiological and biochemical characteristics of strain CF21^T that differentiated it from the reference strains are summarized in Table 1. Strain CF21^T exhibited characteristics that are consistent with those of genus *Lysobacter*, including the inability to assimilate 4MU-*N*-Acetyl-BD-Glucosaminide and so on. However, compared to the two reference strains, the strain was positive for acetate, D-mannitol, malonate, tiglic acid, and gamma-glutamyl-NA.

Phylogenetic Analysis

The 16S rRNA gene sequence of strain CF21^T consists of 1512 bp (GenBank accession number, KP756904) and has been shown to exhibit <95.8 % similarity to the type strain sequences for the genus *Lysobacter*. The highest sequence similarities were found with *Luteimonas dalianensi* OB44-3^T (invalid name) (95.8 %, similarity) and *L. ruishenii* CTN-1^T (95.1 %) [30], and the type strains of other species of the genus showed similarity values of <94.9 %, using EzTaxon server 2.1. A phylogenetic tree analysis showed that strain CF21^T formed a robust cluster with the type strain of *Luteimonas dalianensi* and was close to *L. ruishenii* and *L. daejeonensis*, forming a distinct phylogenetic lineage within genus *Lysobacter* (Fig. 1). The maximum-likelihood and maximum-parsimony trees also

Table 1 Differential physiological and biochemical characteristics of strain CF21^T and the related type strains of genus *Lysobacter*

Characteristic	1	2	3
Positive for all three strains			
Arginine-arginine-AMC	+	+	+
Glycine-proline-AMC	+	+	+
Lysine-alanine-AMC	+	+	+
Negative for all three strains			
4MU-*N*-acetyl-BD-glucosaminide	−	−	−
L-Pyroglutamic acid-AMC	−	−	−
Bis(PNP) phosphate	−	−	−
Beta-allose	−	−	−
Beta-gentiobiose	−	−	−
Dextrose	−	−	−
D-Fructose	−	−	−
D-Galactose	−	−	−
D-Gluconic acid	−	−	−
D-Melibiose	−	−	−
Sorbitol	−	−	−
Sucrose	−	−	−
Galacturonic acid	−	−	−
L-Arabinose	−	−	−
L-Rhamnose	−	−	−
Methyl-B-glucoside	−	−	−
Maltulose	−	−	−
N-acetyl-galactosamine	−	−	−
N-acetyl-glucosamine	−	−	−
Ornithine	−	−	−
Urea	−	−	−
Esculin	−	−	−
The difference among all three strains			
Glycine-AMC	+	−	+
Glutaryl-glycine-arginine-AMC	+	+	−
L-Arginine-AMC	−	−	+
L-Glutamic acid-AMC	+	+	−
L-Leucine-AMC	+	−	+
L-Phenylalanine-AMC	+	−	+
L-Proline-AMC	−	−	+
L-Tryptophan-AMC	+	−	+
Acetate	+	−	−
Adonitol	+	+	−
Citrate	+	+	−
Colistin	+	−	+
D-Mannitol	+	−	−
Alpha-ketoglutaric acid	+	+	−
Malonate	+	−	−
Polymyxin B	+	−	+
Tiglic acid	+	−	−
Gamma-glutamyl-NA	+	−	−
L-Proline-NA	−	−	+
PNP-BD-glucoside	−	−	+

Table 1 continued

Characteristic	1	2	3
Colony color on LB agar (3 days)	LB	PY	Y
DNA G+C content (mol%)	68.72	67.1[a]	61.7[b]

Strains: 1, CF21^T; 2, *L. ruishenii* CTN-1^T; and 3, *L. daejeonensis* GH1-9^T. Data are from this study unless otherwise stated; −, Negative; + positive

LB light beige, *PY* pale yellow, *Y* yellow

[a] Data taken from Wang et al. [30]

[b] Data taken from Weon et al. [31]

supported the phylogenetic position obtained with the neighbor-joining tree.

Chemotaxonomic and Genomic Characteristics

In Table 2, the cellular fatty acid profile of strain CF21^T is shown along with those of the reference strains under the same conditions. The major cellular fatty acids of strain CF21^T were iso-$C_{16:0}$ (30.22 %), iso-$C_{15:0}$ (25.70 %), and the sum of 9/10-methyl $C_{16:0}$ and/or iso-$C_{17:1}\omega9c$ (21.94 %), which correspond to those described for the recognized species of genus *Lysobacter* [5, 17]. Minor amounts of iso-$C_{11:0}$ (3.99 %), iso-$C_{11:0}$ 3-OH (5.51 %), iso-$C_{12:0}$ (1.16 %), iso-$C_{14:0}$ (2.83 %), iso-$C_{15:1}$ F(1.78 %), and iso-$C_{17:0}$ (3.29 %) were also detected. Compared to the two reference strains, CF21^T contained iso-$C_{12:0}$, iso-$C_{14:0}$, iso-$C_{15:1}$ F, and a relatively lower amount of iso-$C_{17:0}$, although the major fatty acids were similar to those of the reference strains. The predominant isoprenoid quinone detected in strain CF21^T was Q-8, which is a characteristic feature of members of genus *Lysobacter* [2]. The major polar lipids of strain CF21^T were phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and diphosphatidylglycerol (DPG), similar to other recognized species of genus *Lysobacter*, but an unknown phospholipid (UPL) was also detected (supplementary Fig. S1). The DNA G+C content was 68.72 mol%, and this value is within the expected range for genus *Lysobacter* [1, 6, 32]. The mean DNA–DNA relatedness between strain CF21^T and *L. ruishenii* CTN-1^T was 56 %, which is clearly far below the threshold of 70 % that is generally used for prokaryotic species delineation [26].

Conclusion

Based on the data that we obtained, strain CF21^T represents a novel species of genus *Lysobacter*, for which we propose the name *Lysobacter chengduensis* sp. nov.

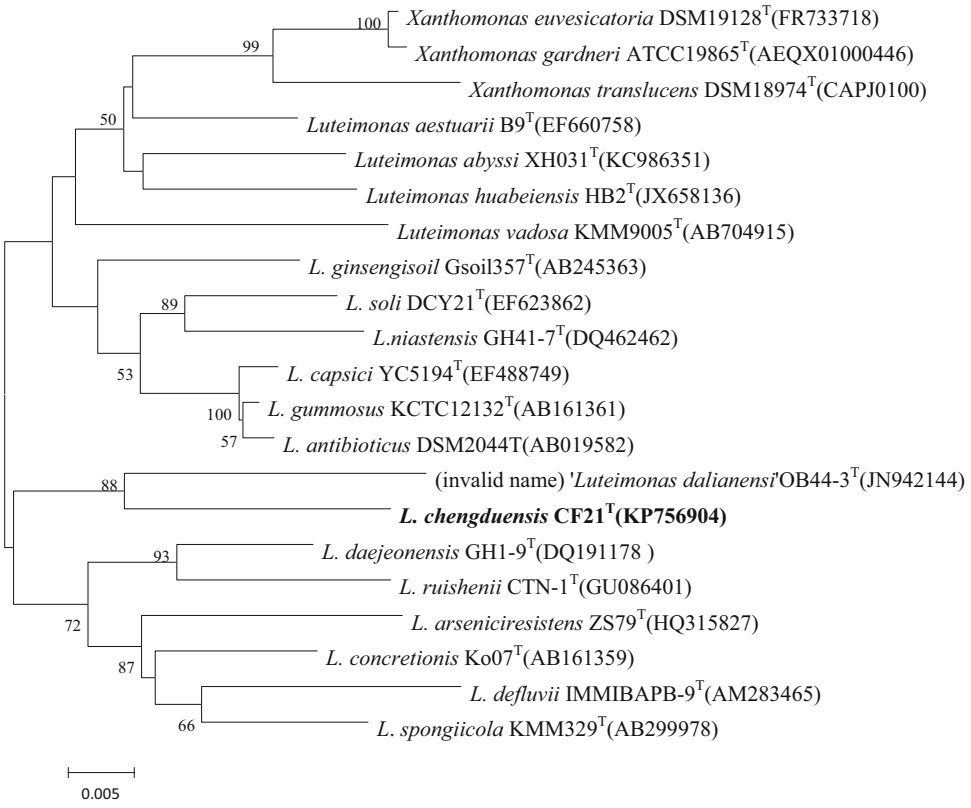

Fig. 1 A phylogenetic tree of strain CF21^T and the other type strains of recognized *Lysobacter* species constructed using the neighbor-joining method based on 16S rRNA gene sequences. GenBank accession numbers are indicated in *parentheses*. *Numbers* indicate the percentages of occurrence of branch points in 1000 bootstrapped trees. Each *bar* represents 0.5 % substitutions per nucleotide position

Table 2 Cellular fatty acid content of strain CF21^T and the related type strains of genus *Lysobacter*

Fatty acid	1	2	3
iso-$C_{11:0}$	3.99	10.88	5.17
iso-$C_{11:0}$ 3-OH	5.51	9.53	5.45
iso-$C_{12:0}$ 3-OH	–	–	1.08
iso-$C_{12:0}$	1.16	–	–
iso-$C_{13:0}$ 3-OH	–	–	2.77
iso-$C_{14:0}$	2.83	–	–
iso-$C_{15:0}$	25.70	28.49	9.20
anteiso-$C_{15:0}$	–	–	4.87
iso-$C_{15:1}$ F	1.78	–	–
iso-$C_{16:0}$	30.22	9.03	19.90
$C_{16:0}$	–	2.98	2.05
iso-$C_{17:0}$	3.29	14.71	13.45
anteiso-$C_{17:0}$	–	–	2.92
$C_{17:1}w6c$	–	–	1.60
$C_{17:0}$ cyclo	–	–	1.19
iso-$C_{17:0}$ 3-OH	–	–	1.01
iso-$C_{18:0}$	–	–	2.30
Summed feature 3[a]	–	3.25	4.95
Summed feature 9[a]	21.94	13.54	17.58

Strains: 1, *L. chengduensis* sp. nov. CF21^T; 2, *L.ruishenii* CTN-1^T; and 3, *L. daejeonensis* GH1-9^T. Data are from this study unless otherwise stated. Fatty acids that represent <1.0 % of the total in all strains are not shown; −, not detected/not reported or <1.0 %

[a] Summed features represent two or three fatty acids that could not be separated by the Microbial Identification System. Summed feature 3 and summed feature 9 represent $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$, or 10-methyl $C_{16:0}$ and/or iso-$C_{17:1}\omega9c$, respectively

Description of *Lysobacter chengduensis* sp. nov.

Lysobacter chengduensis (cheng.du.en′sis. N.L. masc. adj. *chengduensis* pertaining to Chengdu, in south-western China, where the type strain was isolated).

Cells are gram-staining negative, aerobic, non-motile, and rod shaped (0.60–0.65 μm wide and 0.90–0.95 μm long, with no flagellum). Colonies grown on LB agar were circular, smooth with entire edges, non-transparent, and light beige after 3 days of incubation at 30 °C. This strain can grow on LN medium with 0–2 % (w/v) NaCl, and no growth occurred in the presence of 3.0–5.0 % (w/v) NaCl. Growth occurred at 10–40 °C (optimum 28–30 °C) and at pH 6.0–9.0 (optimum pH 7.0–8.0). This bacterium was positive for arginine-arginine-AMC, glycine-proline-AMC, glycine-AMC, glutaryl-glycine-arginine-AMC, L-glutamic acid-AMC, L-leucine-AMC, L-phenylalanine-AMC, L-tryptophan-AMC, lysine-alanine-AMC, gamma-glutamyl-NA, adonitol, citrate, colistin, D-mannitol, alpha-ketoglutaric acid, malonate, and tiglic acid. Sensitivities to the following antibiotics were as follows (μg per disk, unless otherwise stated): vancomycin (30), tetracycline (30), kanamycin (30), spectinomycin (100), erythromycin (15), rifampicin (5), ciprofloxacin (5), florfenicol (30), doxycycline (30), amikacin (30), enrofloxacin (5), cephalothin (30), cefotaxime (30), and ceftazidime (30); this bacterium was not sensitive to sulfamethoxazole (100), lincomycin (10), furazolidone (100), streptomycin (10), trimesulf (23.75), ampicillin (10), penicillin (10), gentamicin (10), and cephalothin (30). The major cellular fatty acids were iso-$C_{16:0}$ (30.22 %), iso-$C_{15:0}$ (25.70 %), iso-$C_{17:1}$w9c (21.94 %), and the sum of 9(10-methyl $C_{16:0}$ and/or iso-$C_{17:1}\omega9c$) (21.94 %). The predominant isoprenoid quinone was Q-8. The DNA G+C content was 68.72 mol%. The primary polar lipids were phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), and an unknown phospholipid (UPL).

The type strain is CF21^T (=CGMCC 1.15145T = DSM 100306^T), which was isolated from the air of enclosures of *Ailuropoda melanoleuca* at the Chengdu Research Base of Giant Panda Breeding in south-west China.

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