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# *Changpingibacter yushuensis* gen. nov., sp. nov., isolated from fluvial sediment in Qinghai Tibet Plateau of China[§]

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**Two facultatively anaerobic, short rod-shaped, non-motile, Gram-stain-positive, unknown bacterial strains (JY-X040$^T$ and JY-X174) were isolated from fluvial sediments of Tongtian River in Yushu Tibetan Autonomous Prefecture, Qinghai province, China. Cells formed translucent, gray, round and convex colonies, with a diameter of less than 0.5 mm after 5 days of incubation at 30°C on brain heart infusion-5% sheep blood agar. The 16S rRNA gene sequence similarity between strain JY-X040$^T$ and *Fudania jinshanensis* 313$^T$ is 93.87%. In the four phylogenetic trees constructed based on the 16S rRNA gene and 423 core genes, the two isolates form an independent branch, phylogenetically closest to *F. jinshanensis* 313$^T$, but could not be classified as a member of the genus *Fudania* or any other genus of the family *Arcanobacteriaceae*. The DNA G + C content of strain JY-X040$^T$ was 57.8%. Calculation results of average nucleotide identity, digital DNA-DNA hybridization value and amino acid identity between strain JY-X040$^T$ and *F. jinshanensis* 313$^T$ are 69.9%, 22.9%, and 64.1%. The major cellular fatty acids were $C_{16:0}$ (23%) and $C_{18:1}\omega9c$ (22%). The cell-wall peptidoglycan type was A5$\alpha$ (L-Lys-L-Ala-L-Lys-D-Glu). The polar lipids comprised diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannoside and four unidentified components. The whole-cell sugars contained rhamnose and ribose. MK-10($H_4$) was the sole respiratory quinone. The minimum inhibitory concentration of streptomycin was 32 μg/ml. All physiological, biochemical, chemotaxonomic and genomic characteristics support that strains JY-X040$^T$ and JY-X174 represent members of a novel species in a new genus, *Changpingibacter yushuensis* gen. nov., sp. nov. The type strain is JY-X040$^T$ (GDMCC 1.1996$^T$ = KCTC 49514$^T$).**

***Keywords:*** *Changpingibacter yushuensis* gen. nov., sp. nov, Tongtian River, fluvial sediment, taxonomy, streptomycin resistance

## Introduction

The family *Actinomycetaceae* was firstly proposed by Buchanan in 1918 (approved Lists 1980) and emended a century later (Nouioui *et al.*, 2018). Recently, some of its members have been assigned into a new family, *Arcanobacteriaceae* (Oren and Garrity, 2020; Salam *et al.*, 2020). Most of the members in the family *Arcanobacteriaceae* come from infected humans, wild animals, and livestock, with a small number coming from animal faeces and sediment samples. Cells of the family are Gram-stain-positive, anaerobic or facultatively anaerobic, with shapes ranging from cocci, coccobacilli, rod to slightly curved rod, requiring an optimal growth temperature of 28–37°C (*i.e.*, mesophilic), with a G + C content between 50–66%, and $C_{16:0}$ and $C_{18:1}\omega9c$ as the main fatty acids. As of August 2021, the family *Arcanobacteriaceae* contains 6 valid genera (*Actinobaculum*, *Actinotignum*, *Arcanobacterium*, *Flaviflexus*, *Fudania*, and *Trueperella*) with *Arcanobacterium* as the type genus (Collins *et al.*, 1982; Oren and Garrity, 2020; Salam *et al.*, 2020) according to the List of Prokaryotic names with Standing in Nomenclature (LPSN) online database (https://www.bacterio.net). Some members of genera *Actinobaculum*, *Actinotignum*, and *Arcanobacterium* were thought to be directly related to certain human diseases. For example, *Actinotignum schaalii* had caused the most human infections among species of the genus *Actinotignum*, 172 cases (mainly urinary tract infections, though invasive infections had also been described) as recently reviewed (Lotte *et al.*, 2016); *Actinobaculum massiliense*, firstly isolated from the urine of an acute cystitis patient (Bakour *et al.*, 2016), was recently recovered from the vaginal exudates of a pelvic inflammatory disease

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woman (Galán-Relaño *et al.*, 2019); and *Arcanobacterium haemolyticum*, originally isolated from infections amongst American soldiers (Maclean *et al.*, 1946), was an understudied human pathogen that most commonly causes pharyngitis and wound infections in adolescents (Miller *et al.*, 1986; Mackenzie *et al.*, 1995; Linder, 1997). By contrast, members of the other half of this bacterial family (*Flaviflexus, Fudania*, and *Trueperella*) are not typical human pathogen (Yassin *et al.*, 2011; Du *et al.*, 2013; Jin *et al.*, 2014; Zhu *et al.*, 2019), but *Trueperella pyogenes* is an important opportunistic pathogen of livestock and carries genes mediating resistance to some antibiotics (Rzewuska *et al.*, 2019; Kwiecień *et al.*, 2020).

Using a polyphasic approach in this study, we determined the taxonomic position of two new environmental strains (JY-X040$^T$ and JY-X174) isolated from fluvial sediment samples collected from Yushu Autonomous Prefecture of Qinghai Province by culturomics (Fournier *et al.*, 2015), and propose that they represent a novel species in a new genus of the family *Arcanobacteriaceae*. Compared to their only nearest phylogenetic relative, *Fudania jinshanensis* 313$^T$, the genomes of strains JY-X040$^T$ and JY-X174 contain five times more accessory genes over unique genes contrary to the reverse trend in their relative, have more genes encoding glycoside hydrolases, possess a mutated *rpsL* gene conferring them streptomycin resistance.

## Materials and Methods

### Isolation and maintenance of novel bacterial strains

Strains JY-X040$^T$ and JY-X174 were isolated from fluvial sediments collected from Tongtian River (33°0′14″N/97°0′40″E, 3,595 m above sea level, frequented by animals and human) in Yushu Tibetan Autonomous Prefecture, Qinghai province, China. Samples below the water surface were collected with sterile sampling tubes, quickly transported and stored in a -80°C refrigerator, and sent to our laboratory in Beijing. Different sediment samples were subsequently thawed on ice, and then ground separately in sterile mortars into muddy suspension by adding cold (4°C) phosphate buffer solution (PBS) when needed. The mud suspension was serially diluted with PBS and plated onto peptone-yeast glucose (PYG) and brain heart infusion (BHI)-5% sheep blood plates. After culturing at 28°C for 4 days, single colonies were picked and purified by subculturing on BHI-5% sheep blood plates three times, then preserved at -80°C in BHI broth with 25% (v/v) glycerol.

### Genomic and phylogenetic analyses

The 16S rRNA gene sequences of strains JY-X040$^T$ and JY-X174 were amplified using universal bacterial primers (27F and 1492R) by PCR (Lane, 1991). Then, the TA cloning method was used to insert the fragment into Peasy-T3 Cloning Vector to amplify the gene in *Escherichia coli*. Finally, the nearly complete 16S rRNA sequences were obtained by Sanger sequencing. The newly generated 16S rRNA gene sequences (1,499 bp) were uploaded to the GenBank of NCBI database and compared with the database of EzTaxon server (www.ezbiocloud.net/identify) (Chun *et al.*, 2007).

The 16S rRNA gene sequences of strains JY-X040$^T$ (three copies) and JY-X174 (two copies) retrieved from their respective genomes (below) and those of all validly published species in the family *Arcanobacteriaceae* downloaded from NCBI were used to construct phylogenetic trees using MEGA version X (Kumar *et al.*, 2018). Three algorithms, neighbor-joining (NJ) (Saitou and Nei, 1987), maximum-likelihood (ML) (Guindon and Gascuel, 2003) and maximum-parsimony (MP) (Kolaczkowski and Thornton, 2004), were used to predict the topologies of phylogenetic trees. In the calculation process, gaps in all positions were eliminated by 1,000 repeated bootstrap analysis in pairs (Kimura, 1980).

Genomic DNA of strains JY-X040$^T$ and JY-X174 were purified by Genomic DNA Purification Kit (Cashion *et al.*, 1977). The genome of strain JY-X040$^T$ was sequenced on the Pacific Biosciences RS II with the single molecule real-time (SMRT) sequencing platform, and that of strain JY-X174 on the Illumina HiSeq TM2000 platform. Calculation results of the average nucleotide identity (ANI) using the orthoANI web software (Chun and Rainey, 2014), digital DNA-DNA hybridization (dDDH) value (Auch *et al.*, 2010) and average amino acid identity (AAI) using the AAI calculator from the Kostas lab (http://enve-omics.ce.gatech.edu/aai/) (Rodriguez-R and Konstantinidis, 2014) were compared between the two isolates and among those of the other related species in the family *Arcanobacteriaceae*.

In order to further verify the taxonomic status, we downloaded from NCBI the high-quality genomes of 13 type species validly published for the family *Arcanobacteriaceae*, with the genome of *Ruania albidiflava* CGMCC 4.3142$^T$ as an outgroup, and constructed a phylogenomic tree by using the FastTree software (Price *et al.*, 2009) and based on 423 core genes (Supplementary data Table S1) which were annotated in reference to *Actinotignum schaalii* (CP008802) and clustered with a 0.4 threshold by CD-HIT software (Fu *et al.*, 2012).

### Morphological, phenotypic and biochemical analyses

Cell morphology was observed on an optical microscope and transmission electron microscope. Semi-solid culture medium containing 0.3% (w/v) agar and hanging drop method were used to test cell motility and gliding motility, respectively. In order to select an optimal growth medium, strains JY-X040$^T$ and JY-X174 were incubated for 5 days by plating on BHI agar, BHI-5% sheep blood agar, PYG agar, tryptone soy agar (TSA), R2A agar, Luria-Bertani (LB) agar, MacConkey (MAC) agar, nutrient agar (NA) or marine broth (MB) agar. Air requirement for growth was tested under aerobic, anaerobic or aerobic plus 5% $CO_2$ conditions, respectively. Bacterial growth was sequentially tested at seven different temperatures (4, 16, 28, 30, 35, 37, and 42°C), with conditioned BHI broth containing one of the sixteen different salt concentrations (0.5%, 1–15% at one percent unit intervals) and eleven different initial pH values (pH 2–12, at one interval as adjusted with 1 M HCl or NaOH) (An *et al.*, 2006). For the following subsequent characterizations, strains JY-X040$^T$ and JY-X174 were aerobically cultured on BHI-5% sheep blood plates at 30°C, unless otherwise specified. Catalase activity was determined by mixing cell pellets with 3% (v/v) hydrogen peroxide solution. Oxidase test reagent was used to test the activity of

oxidase. Biochemical features were detected by API 50CH strips, API Coryne and API ZYM system as previously described (Zhu *et al.*, 2019).

### Chemotaxonomic analyses

Polar lipids were extracted (Ventosa *et al.*, 1993) and analyzed by two dimensional thin-layer chromatography (TLC) on silica gel60 thin layers (cut to 10 by 10 cm) by using chloroform-methanol-water (65:25:4, v/v) in the first dimension and chloroform-methanol-acetic acid-water (80:12:15:4, v/v) in the second dimension. Replicate of TLC plates was sprayed with ethanolic molybdatophosphoric acid, molybdenum blue, ninhydrin or α-naphthol to visualize the presence of total lipids, phospholipids, amino lipids and glycolipids (Wang *et al.*, 2018). Whole-cell sugars were analyzed and identified as described (Schleifer, 1985). Respiratory quinones were analyzed by reverse-phase high performance liquid chromatography (Collins and Jones, 1981). Cellular fatty acids of the isolates were extracted as described (Sasser, 1990). Peptidoglycan was isolated after trypsin digestion and mechanical breakage as described (Schumann, 2011).

### Gene function analysis

The circular genome map was constructed by the Circos (http://circos.ca/) (Wyatt *et al.*, 2013). Protein sequences were classified by using the cluster of orthologous groups (COG) of protein database (Galperin *et al.*, 2015; Huerta-Cepas *et al.*, 2019). The Comprehensive Antibiotic Resistance Database (CARD) was used to predict resistomes from protein or nucleotide data based on homology and SNP models (Alcock *et al.*, 2020). Carbohydrate-active enzymes were predicted using Carbohydrate-Active enzymes Database (CAZy) (Cantarel *et al.*, 2009). The function of target genes was annotated using KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway (Kanehisa *et al.*, 2016). After clustering by USEARCH 11 (Edgar, 2010), Bacterial Pan Genome Analysis pipeline was used to analyze the pan-genome orthologous groups (POGs) for the two isolates and 13 available species of the family *Arcanobacteriaceae* with 0.4 identity threshold of amino acid sequences.

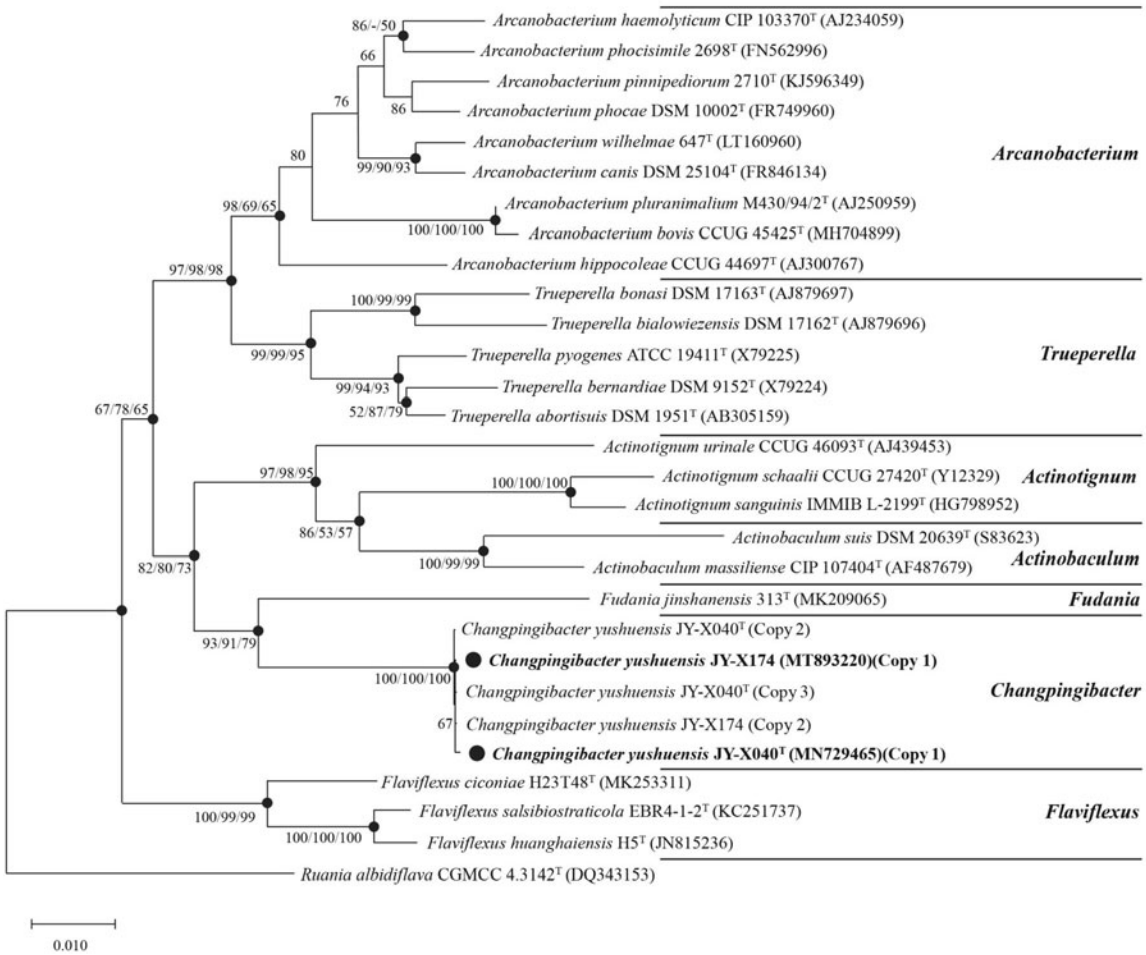


**Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing the phylogenetic position of strains JY-X040$^T$, JY-X174 and validly published species within the family *Arcanobacteriaceae*.** Multiple 16S rRNA gene sequences from strains JY-X040$^T$ (3 copies) and JY-X174 (2) were used for tree construction. Bootstrap values (≥ 50%) based on 1,000 replicates are shown at the branch nodes. Solid circles indicate the nodes were also supported by maximum-likelihood and maximum-parsimony analysis. Bar, 0.010 changes per nucleotide position. *Ruania albidiflava* CGMCC 4.3142$^T$ was used as an outgroup.

### Antimicrobial susceptibility test

The antimicrobial susceptibility of the strains was determined by broth microdilution method performed as outlined by the Clinical and Laboratory Standards Institute (CLSI, 2018, 2019). One hundred µl of serial twofold dilutions of antimicrobial agent in cation-adjusted Mueller-Hinton broth with 5% (v/v) of lysed horse blood was dispensed into each well of the 96-well microtiter plates. An equal amount (final total volume, 200 µl) of adjusted bacterial suspension ($5 \times 10^5$ CFU/ml) was added to designated wells (Galán-Relaño *et al.*, 2019). Microdilution plates were read after 96 h incubation at 30°C in aerobic environment or 37°C with 5% $CO_2$. *Escherichia coli* ATCC 25922 was included as quality control.

### Nucleotide sequence accession numbers

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains JY-X040$^T$ and JY-X174 are MN729465 and MT893220, respectively. The GenBank accession numbers, genome size (bp), gene number, N50 (bp), contig, rRNA, tRNA and G + C content (%) of strains JY-X040$^T$, JY-X174 and 14 available species of the family *Arcanobacteriaceae* used in this paper are all shown in Supplementary data Table S2.

## Results and Discussion

### Genomic characteristics and phylogenetic trees

By blasting against the database of EzTaxon server (www.ezbiocloud.net/identify) (Chun *et al.*, 2007), the near full-length 16S rRNA gene sequences (1,499 bp) of strains JY-X040$^T$ and JY-X174 had maximum similarity to *Arcanobacterium canis* DSM 25104$^T$ (94.7%), followed by *Arcanobacterium haemolyticum* CIP 103370$^T$ (94.1%), *Arcanobacterium wilhelmae* 647$^T$ (94.0%), and *F. jinshanensis* 313$^T$ (93.9%).

The topologies of the NJ, ML, and MP trees based on the 16S rRNA gene sequences (Fig. 1; Supplementary data Figs. S1 and S2) revealed that strains JY-X040$^T$ and JY-X174 form an independent branch closest to *F. jinshanensis* 313$^T$, but they are not clearly recognized as belonging to the genus *Fudania* or any other genus of the family *Arcanobacteriaceae*.

The genome size of strain JY-X040$^T$ is 2,661,583 bp (891.6× coverage; N50 = 2,661,635), containing 2,289 coding sequences (average length, 1,015 bp) as predicted by Prodigal software. The 2.6 Mb genome (102× coverage; N50 = 203,823) of strain JY-X174 was assembled from 31 contigs using VELVET (Zerbino and Birney, 2008). The DNA G + C contents of strains JY-X040$^T$ and JY-X174 are 57.8 and 57.9%, respectively.

When compared with closely related species of the family *Arcanobacteriaceae* (Supplementary data Tables S3 and S4), both isolates showed ANI and dDDH values lower than the recommended thresholds of 95–96% and 70% for bacterial species (Chun and Rainey, 2014), in contrast to the above-threshold value (98.5% and 88.5%, respectively) between strains JY-X040$^T$ and JY-X174, and their the AAI values lower than the recommended threshold value of 65% for bacterial genus (Konstantinidis *et al.*, 2017). The highest AAI values of strains JY-X040$^T$ and JY-X174 were 64.1% and 64.2% respectively, both with *Fudania jinshanensis* 313$^T$. Although one third of the POCP values (Supplementary data Table S5) were at borderline (slightly above) of the recommended threshold value of 50% for genus (Qin *et al.*, 2014), some recently proposed to raise the threshold to 65% for genus separation in the family *Geobacteraceae* (Xu *et al.*, 2020). If applying 65% as the genus threshold for the family *Arcanobacteriaceae*, all the POCP values would be within the new genus territory. These results indicated that strains JY-X040$^T$ and JY-X174 are different isolates of the same species, and the two isolates are different from other closely related species and genus, very likely representing a new species of a new genus.

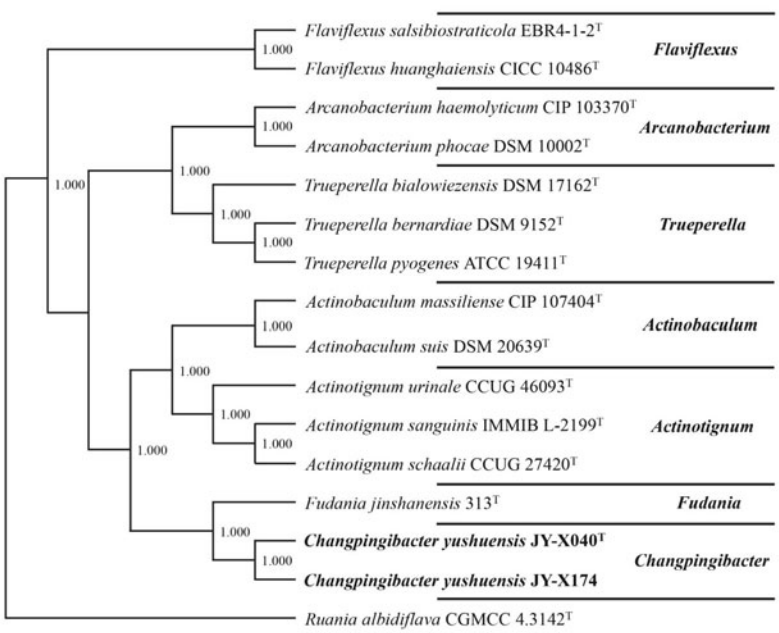


**Fig. 2. Neighbour-joining phylogenomic tree of strains JY-X040$^T$, JY-X174 and 13 validly published species within the family *Arcanobacteriaceae* based on 423 core genes from genome sequences.** Numbers on the tree indicate each split in the tree with support values from the Shimodaira-Hasegawa test calculated for 1,000 resamples. *Ruania albidiflava* CGMCC 4.3142$^T$ was used as an outgroup.

The topology of the phylogenomic tree based on 423 core genes also indicated that strains JY-X040$^T$ and JY-X174 form an independent branch, closest to *F. jinshanensis* 313$^T$ (Fig. 2) and next to genera *Actinotignum* and *Actinobaculum*, consistent to the aforementioned results based on 16S rRNA gene analysis.

## Morphological, phenotypic, and biochemical characteristics

Cells of strains JY-X040$^T$ and JY-X174 were Gram-stain-positive, non-motile (without observable gliding motility), catalase-positive, oxidase-negative and short rod-shaped, 0.3–0.6 μm wide and 0.7–1.3 μm long (Supplementary data Fig. S3). On BHI-5% sheep blood plate after 5 days, the colonies were translucent, gray, round and convex, with moist surface and clear boundaries, with a diameter of less than 0.5 mm. Strains JY-X040$^T$ and JY-X174 grew optimally under aerobic condition on BHI-5% sheep blood agar, with observable growth on other plates (BHI, PYG, TSA, R2A, LB, MAC, NA, and MB), and under anaerobic condition or with air plus 5% $CO_2$. Other requirements for growth included a temperature between 4–42°C (optimum, 28–30°C), in medium with pH 5.0–9.0 (optimum, 7.0), and 0.5–5% (w/v) of NaCl (optimum, 0.5%).

The following features were assessed using API 50CH strips, API Coryne and API ZYM system tests. Strains JY-X040$^T$ and JY-X174 produced acid from aesculin ferric citrate, D-fructose, D-glucose, D-lactose, D-xylose and potassium 5-ketogluconate (weak), but not from amygdalin, arbutin, D-

**Table 1. Differential characteristics of the proposed genus *Changpingibacter* gen. nov and its related genera of the family *Arcanobacteriaceae***
Genera: *Changpingibacter* gen. nov. (strains JY-X040$^T$ and JY-X174, data from this study); *Fudania* (*Fudania jinshanensis* 313$^T$, data from this study); *Actinotignum* (Hall *et al.*, 2003; Yassin *et al.*, 2015; Lotte *et al.*, 2016); *Actinobaculum* (Lawson *et al.*, 1997; Greub and Raoult, 2002; Cattoir, 2012; Yassin *et al.*, 2015); *Trueperella* (Lehnen *et al.*, 2006; Azuma *et al.*, 2009; Yassin *et al.*, 2011; Gilarranz *et al.*, 2016; Alssahen *et al.*, 2020); *Flaviflexus* (biochemical data of *Flaviflexus huanghaiensis* CICC 10486$^T$ and *Flaviflexus salsibiostraticola* JCM 19016$^T$ from this study) (Du *et al.*, 2013; Jin *et al.*, 2014; Lee *et al.*, 2020). +, Positive; −, negative; V, variable, W, weak; ND, no data available; V (w), type strain weak but another isolate negative; − (2/3) ND (1/3), among the three species in the genus, 2 negative while 1 no data available; DPG, diphosphatidylglycerol; GPL, unidentified glycophospholipid; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIDM, phosphatidylinositol dimannosides; PIM, phosphatidylinositol mannosides; PGL, phosphoglycolipid; PL, (unidentified) phospholipid; GL, unidentified glycolipid; L, unidentified lipid; MK-n(Hx) represents a menaquinone containing n isoprene units and partially hydrogenated (x: number of hydrogen atoms on the side chain).

| Characteristic | *Changpingibacter* | *Fudania* | *Actinotignum* | *Actinobaculum* | *Trueperella* | *Flaviflexus* |
|---|---|---|---|---|---|---|
| Source | River sediment | Tibetan antelope faeces | Patient blood/urine | Patient urine; cystitis/ pyelonephritis of pig | Patient pus/blood; balanoposthitis of European bison | Marine sediment; biofilm reactor; oriental stork faeces |
| Colony color | Translucent gray | White | Yellowish white; gray; white | Pale-gray; white | Translucent | Beige; yellow |
| Cell morphology | Short rod-shape | Straight to slightly curved rods | Straight to slightly curved rods | Cocci and rods | Cocci and rods | Cocci, coccobacilli and straight to curved rods |
| Optimal temperature | 28–30°C | 37°C | 37°C | 37°C | 37°C | 28–30°C |
| $O_2$ requirement | Facultatively anaerobic | Anaerobic/ facultatively anaerobic | Anaerobic/ facultatively anaerobic | Anaerobic/ facultatively anaerobic | Anaerobic/ facultatively anaerobic | Aerobic/ facultatively anaerobic |
| **Enzyme** | | | | | | |
| Catalase | + | − | − | − | V | V |
| Alkaline phosphatase | + | − | − | V | − | − (2/3) ND(1/3) |
| Esterase (C4) | V(w) | + | − | − | V | V |
| Esterase lipase (C8) | V(w) | + | − | − | V | V |
| α-Galactosidase | + | − | − | − | V | − (2/3) ND(1/3) |
| β-Galactosidase | + | − | − | − | V | V |
| Pyrazinamidase | + | − | − | − | V | ND |
| **Acid production from** | | | | | | |
| D-Ribose | − | + | + | + | V | + |
| D-Xylose | + | − | V | + | V | − |
| D-Galactose | − | + | − | − | ND | V |
| D-Maltose | − | + | V | + | V | V |
| D-Lactose | + | − | − | − | V | V |
| Starch | − | + | − | V | ND | + |
| Glycogen | − | + | − | V | V | V |
| **Respiratory quinone** | MK-10($H_4$) | − | − | − | MK-10($H_4$) | MK-9 series |
| **Major polar lipids** | DPG, GL2, PG, PI, PIM | DPG, GL2, PG, PI, PIM | DPG, PG, PI, PIDM | DPG, PG, PI, PIDM | DPG, PG, PI | PG, L, PL, GPL |
| **Whole-cell sugars** | Rhamnose, ribose | Glucose, rhamnose | Glucose, rhamnose, 6-deoxytalose | Glucose, rhamnose | Glucose, rhamnose | ND |
| **DNA G + C content (%)** | 57.8 | 60.6 | 50.0–61.1 | 55.0–60.2 | 56.0–66.0 | 59.5–65.6 |
| **Major fatty acids** | $C_{16:0}$ $C_{18:1}\omega9c$ $C_{18:0}$ | $C_{16:0}$ $C_{18:1}\omega9c$ $C_{14:0}$ | $C_{18:1}\omega9c$ $C_{16:0}$ | $C_{18:1}\omega9c$ $C_{16:0}$ | $C_{18:1}\omega9c$ $C_{16:0}$ | $C_{18:1}\omega9c$ $C_{16:0}$ |

adonitol, D-arabinose, D-cellobiose, D-galactose, D-gentiobiose, D-lyxose, D-maltose, D-mannose, D-melezitose, D-melibiose, D-raffinose, D-ribose, D-sucrose, D-trehalose, D-turanose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, dulcitol, erythritol, glycerol, glycogen, inositol, inulin, L-arabinose, L-rhamnose, L-sorbose, L-xylose, mannitol, methyl $\alpha$-D-glucopyranoside, methyl $\alpha$-D-mannopyranoside, methyl $\beta$-D-xylopyranoside, N-acetylglucosamine, potassium gluconate, potassium 2-ketogluconate, salicin, sorbitol, starch, or xylitol (API 50CH). The test results of acid phosphatase (weak), alkaline phosphatase, cystine arylamidase (weak), esculin, leucine arylamidase, malonate, naphthol-AS-BI-phosphohydrolase, pyrazinamidase, valine arylamidase (weak), $\alpha$-galactosidase, $\beta$-galactosidase, $\alpha$-glucosidase and $\beta$-glucosidase were positive, whereas the results for gelatin, lipase (C14), N-acetyl-$\beta$-glucosaminidase, pyrrolidonyl arylamidase, saccharose, trypsin, urease, $\alpha$-chymotrypsin, $\beta$-fucosidase, $\beta$-glucuronidase, or $\alpha$-mannosidase were negative. The results of esterase (C4) and esterase lipase (C8) were variable, the type strain was weakly positive and the other isolate negative (API Coryne and API ZYM).

### Chemotaxonomic characteristics

As summarized in Table 1, strains JY-X040$^T$ and JY-X174 had some chemotaxonomic features quite distinct from their related genera (*Fudania*, *Actinotignum*, *Actinobaculum*, *Trueperella*, *Flaviflexus*). The polar lipids of strain JY-X040$^T$ comprised four major known components (Supplementary data Fig. S4): diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylinositol mannoside (PIM), one major unidentified glycolipid-2 (GL2) and three minor components: unidentified glycolipid-1 (GL1), unidentified phospholipids 1-2 (PL1-2), contrasting to the existence of aminolipid and unidentified lipids and differential abundance of PIM and PI in *F. jinshanensis* 313$^T$ when analyzed in parallel (Supplementary data Fig. S4). By contrast, genera *Trueperella* and *Flaviflexus* lacked PIM (Yassin *et al.*, 2011; Du *et al.*, 2013) while *Actinotignum* and *Actinobaculum* contained phosphatidylinositol dimannosides and a choline-containing phosphoglycolipid (Yassin *et al.*, 2015). The whole-cell sugars of strain JY-X040$^T$ comprised rhamnose and ribose, different from that (rhamnose and glucose) of *Fudania*, *Actinobaculum*, *Trueperella*, *Flaviflexus* (Table 1). MK-10($H_4$) was the sole component in strain JY-X040$^T$ (Table 1), distinctively different from the principal MK-10($H_4$) in *Trueperella*, MK-9 series in *Flaviflexus*, and total absence of any respiratory quinone in *Fudania*, *Actinotignum* and *Actinobaculum* (Yassin *et al.*, 2011, 2015; Du *et al.*, 2013; Zhu *et al.*, 2019). Although sharing two major fatty acids ($C_{16:0}$ and $C_{18:1}\omega 9c$) with *F. jinshanensis* 313$^T$, the $C_{16:0}$ content in strains JY-X040$^T$ and JY-X174 was about one third less than that of *F. jinshanensis* 313$^T$ (Supplementary data Table S6). Moreover, $C_{18:0}$ was the third highest fatty acid in strains JY-X040$^T$ and JY-X174 instead of $C_{14:0}$ in *F. jinshanensis* 313$^T$. The amino acids found in the cell wall hydrolysate of strains JY-X040$^T$ and JY-X174 were alanine (Ala), glutamic acid (Glu) and lysine (Lys) in a molar ratio of Ala:Glu:Lys = 4.0:2.1:1, while this ratio was then unknown for *F. jinshanensis* 313$^T$; their cell-wall peptidoglycan type was A5$\alpha$ (L-Lys-L-Ala-L-Lys-D-Glu), same as *Flaviflexus* (Du *et al.*, 2013).

### Gene annotation and corresponding phenotypes

The number of genes assigned by the COG database to the genomes of strains JY-X040$^T$, JY-X174, and *F. jinshanensis* 313$^T$ are 2,054, 2,020, and 2,061, respectively. Among the 26 COG functional classes (Supplementary data Figs. S5 and S6) in the genomes of strains JY-X040$^T$, JY-X174 and *F. jinshanensis* 313$^T$ (histograms 1-3), five categories are the largest (Supplementary data Fig. S6): function unknown (317/323/346), replication, recombination and repair (222/182/155), amino acid transport and metabolism (187/178/170), carbohydrate transport and metabolism (178/169/164), and transcription (165/173/160). Comparing with the number of genes and the proportion of functional classification annotated by COG database, strains JY-X040$^T$, JY-X174 were more similar to *F. jinshanensis* 313$^T$ than to other members in the family *Arcanobacteriaceae*.

Drug resistance was predicted by the Comprehensive Antibiotic Resistance Database. One notable finding is that the *rpsL* gene (Supplementary data Fig. S7) in strains JY-X040$^T$, JY-X174, and *F. jinshanensis* 313$^T$ is almost identical to WP_003403453.1 which confers *Mycobacterium tuberculosis* resistance to streptomycin, suggesting the three strains could also be resistant to this antibiotic. Among the 17 amino acid sequence differences in the *rpsL*-encoded product (123 amino acids) between the four strains, 13 are identical among the strains JY-X040$^T$, JY-X174, and *F. jinshanensis* 313$^T$ (Supplementary data Fig. S7), another indication showing their close genetic relationship. In order to see whether these mutations change the susceptibility to streptomycin, minimum inhibitory concentrations (MIC) of streptomycin for strains JY-X040$^T$, JY-X174 and *F. jinshanensis* 313$^T$ were measured. The MIC of *F. jinshanensis* 313$^T$ and *Escherichia coli* ATCC 25922 (quality control strain) was 4 μg/ml, lower than that (32 μg/ml) of strains JY-X040$^T$ and JY-X174. Since the CLSI has no MIC breakpoint value for streptomycin, we borrowed the streptomycin MIC breakpoints (> 4 μg/ml) for *Trueperella pyogenes* (Dong *et al.*, 2017; Kwiecień *et al.*, 2020) as a reference, and concluded that strains JY-X040$^T$ and JY-X174 were resistant to streptomycin, in contrast to being sensitive for *F. jinshanensis* 313$^T$. It remains to be seen which (or more than one) of the four homologous amino acid changes in that narrow stretch (positions 14, 17, 19, and 21) caused the resistance. In another study (Bai *et al.*, 2016) we found about 4.6% of Shiga toxin-producing *Escherichia coli* isolated from the intestinal tracts of plateau pika collected from the same region (not exactly the same location) were also resistant to streptomycin. Since commercial antibiotics were virtually undetectable in soil, animal faeces and sediment in the core of the Tibetan plateau (Chen *et al.*, 2016), how the isolates in the two studies became resistant to streptomycin will be an interesting topic for future study.

CAZy analysis was performed to annotate the 45 and 28 genes encoding active carbohydrate enzymes in strains JY-X040$^T$ and *F. jinshanensis* 313$^T$, then the genes were classified and KEGG annotated. While the number and types of the other three enzyme genes (glycosyl transferases, glycoside hydrolases + carbohydrate-binding modules, and carbohydrate esterases) are almost comparable (the exact percentages differ though), the results (Supplementary data Table S7) showed that the number and types of glycoside hydro-

lase (GH) genes in strain JY-X040$^T$ were far more than *F. jinshanensis* 313$^T$ (24 genes/15 types *vs.* 10 genes/6 types). The CAZy annotation results are consistent to the biochemical results of the two type strains, for example, both were positive for *α*-glucosidase (GH13) and *β*-glucosidase (GH3) while having the respective GH; by contrast, strain JY-X040$^T$ has the corresponding GH and was positive for *α*-galactosidase (GH36) and *β*-galactosidase activity (GH35 and GH42) (Table 1) whereas *F. jinshanensis* 313$^T$ was negative for *α*- and *β*-galactosidase activity (Table 1), most probably due to lacking the corresponding GH (Supplementary data Table S7).

The KEGG annotation classified 1,309 (strain JY-X040$^T$) and 1,264 genes (*F. jinshanensis* 313$^T$) into various categories. Compared to *F. jinshanensis* 313$^T$, strain JY-X040$^T$ carries notably more pathway types and number of genes for metabolism, environmental information processing and cellular processes, especially having 15 types of genes that are lacking in *F. jinshanensis* 313$^T$ (Supplementary data Fig. S8), which might reflect their genetic potential to adapt to more diverse habitats (animal intestine *vs.* river sediment) and present other distinct phenotypes summarized in Table 1. In pathway modules, both strains contain a complete dTDP-L-rhamnose biosynthesis module (M00793) that might be related to the presence of rhamnose as the whole-cell sugar component (Table 1) (van der Beek *et al.*, 2019).

POGs analysis of *Arcanobacteriaceae* showed that the genomes of the two isolates and 14 other species of the family have 402 core genes (slightly less than used for genomic tree without *Flaviflexus ciconiae* H23T48$^T$), whereas strains JY-X040$^T$, JY-X174, and *F. jinshanensis* 313$^T$ have 1,526, 1,528, and 915 accessory genes and 303, 259, and 1,053 unique genes (Supplementary data Table S8), respectively. Most notably, *F. jinshanensis* 313$^T$ has the most unique genes compared to the other 15 genomes; or conversely, strains JY-X040$^T$ and JY-X174, similar to the three *Flaviflexus* species, have more accessory genes than *F. jinshanensis* 313$^T$ (Supplementary data Table S8).

## Taxonomic conclusion

The 16S rRNA phylogenetic tree (Fig. 1; Supplementary data Figs. S1 and S2) and genome-wide core gene phylogenomic tree (Fig. 2) indicated that strains JY-X040$^T$ and JY-X174 represent a new species of a new genus of the family *Arcanobacteriaceae*, closest to genus *Fudania*. The ANI and dDDH value (Supplementary data Table S3) ascertained that JY-X040$^T$ and JY-X174 were different strains of the same species, and easily distinguishable from the type strains of other neighboring species. The AAI values (Supplementary data Table S4) were lower than the recommended threshold value of 65% for bacterial genus demarcation, further supporting that the two isolates represent a new species of a new genus in the family *Arcanobacteriaceae*. Moreover, the two isolates were dramatically different from the genus *Fudania* and other related genera of the family *Arcanobacteriaceae* in eight major aspects: optimal growth temperature, requirement of $O_2$, activity of 5 enzymes (alkaline phosphatase, catalase, pyrazinamidase, *α*-galactosidase and *β*-galactosidase), acid production from 7 sugars (D-galactose, D-lactose, D-maltose, D-ribose, D-xylose, glycogen, and starch), respiratory quinone, polar lipid profile, whole-cell sugar composition, and DNA G + C content (Table 1).

Based on the phylogenetic, physiological, and chemotaxonomic characterizations, we propose that strains JY-X040$^T$ and JY-X174 represent a new species of a new genus of the family *Arcanobacteriaceae*, for which the name *Changpingibacter yushuensis* gen. nov., sp. nov. is proposed. The type strain is JY-X040$^T$ (GDMCC 1.1996$^T$ = KCTC 49514$^T$).

## Description of *Changpingibacter* gen. nov.

*Changpingibacter* (Chang.ping.i.bac'ter. N.L. masc. n. bacter, rod; N.L. masc. n. *Changpingibacter*, a rod-shaped bacterium isolated and characterized at Changping district of Beijing where our laboratory locates).

Gram-stain-positive, catalase-positive, oxidase-negative, nonmotile, facultatively anaerobic, short rod-shaped bacteria. Colonies on BHI-5% sheep blood plates after 5 days are translucent, gray, round and convex, with moist surface and complete boundaries, and a diameter of less than 0.5 mm. The predominant fatty acids are $C_{16:0}$, $C_{18:1}\omega9c$ and $C_{18:0}$. The type of murein is A5*α* (L-Lys-L-Ala-L-Lys-D-Glu). The whole-cell sugars consist of rhamnose and ribose. The only respiratory quinone is MK-10($H_4$). Major polar lipids include four known components (DPG, PG, PI, PIM) and one unknown GL2. The type species is *Changpingibacter yushuensis*.

## Description of *Changpingibacter yushuensis* sp. nov.

*Changpingibacter yushuensis* (yu.shu.en'sis. N.L. masc. adj. yushuensis named after Yushu Tibetan Autonomous Prefecture in Qinghai Province of China where the samples were collected from).

Displays the following characteristics in addition to those in the genus description. Cells are 0.3–0.6 μm wide and 0.7–1.3 μm long. Grow best aerobically, with moderate growth anaerobically or with air plus 5% $CO_2$. Grow at 4–42°C (optimum, 28–30°C) in medium of pH 5.0–9.0 (optimum, 7.0) and 0.5–5% concentration of NaCl (optimum, 0.5%). Produce acid from aesculin ferric citrate, D-fructose, D-glucose, D-lactose, D-xylose and potassium 5-ketogluconate (weak). Positive enzyme activity for acid phosphatase (weak), alkaline phosphatase, cystine arylamidase (weak), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, pyrazinamidase, valine arylamidase (weak), *α*-galactosidase, *β*-galactosidase, *α*-glucosidase and *β*-glucosidase, but variable for esterase (C4) and esterase lipase (C8). The polar lipids include five major components (DPG, PG, PI, PIM, and one unknown GL2) and three minor unknown components (GL1 and PL1-2).

The type strain, JY-X040$^T$ (GDMCC 1.1996$^T$ = KCTC 49514$^T$), isolated from fluvial sediment of Yushu Autonomous Prefecture in Qinghai Province of China, has a DNA G + C content of 57.8% and a genome size approximately 2.6 Mb. The accession numbers are MN729465 (16S rRNA gene sequence) and CP059492 (complete genome sequence). Another strain, JY-X174, similarly isolated, is also classified in the species, with accession numbers MT893220 (16S rRNA gene sequence) and JACEXJ000000000 (whole genome sequence).

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## Conflicts of Interest

The authors declare that there are no conflicts of interest.

## Ethical Statements

The ethical practice was approved by Ethical Committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention (# ICDC-2016004).

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