**Actinomyces gaoshouyii** sp. nov., isolated from plateau pika (Ochotona curzoniae)

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**Abstract**

Two strains (pika_113† and pika_114) of a previously undescribed Actinomyces-like bacterium were recovered from the intestinal contents of plateau pika (Ochotona curzoniae) on the Tibet-Qinghai Plateau, China. Results from biochemical characterization indicated that the two strains were phenotypically homogeneous and distinct from other previously described species of the genus *Actinomyces*. Based on the comparison of 16S rRNA gene sequences and genome analysis, the bacteria were determined to be a hitherto unknown subline within the genus *Actinomyces*, being most closely related to type strains of *Actinomyces denticolens* and *Actinomyces timonensis* with a respective 97.2 and 97.1 % similarity in their 16S rRNA gene sequences. Phylogenetic analyses confirmed that pika_113† was well separated from any other recognized species of the genus *Actinomyces* and within the cluster with *A. denticolens* and *A. timonensis*. The genome of strain pika_113† displayed less than 42 % relatedness in DNA–DNA hybridization with all the available genomes of existing species of the genus *Actinomyces* in the NCBI database. Collectively, based on the phenotypic characteristics and phylogenetic analyses results, we propose the novel isolates as representatives of *Actinomyces gaoshouyii* sp. nov. The type strain of *Actinomyces gaoshouyii* is pika_113† (†CGMCC 4.7372†=DSM 104049†), with a genomic DNA G+C content of 71 mol%.

The genus *Actinomyces* as presently defined encompasses heterogeneous, mostly Gram-staining-positive, non-spore-forming, non-motile, rod-shaped organisms which generally exhibit a high G+C content in their genomic DNA [1]. During the past few years, the taxonomy of genus *Actinomyces* has undergone much improvement, largely driven by 16S rRNA gene sequences and genome analyses. Several novel species of the genus *Actinomyces* from human clinical specimens and animal sources have been described in investigations cited herein, indicating their clinical importance [2–8]. Currently, the genus *Actinomyces* encompasses over 40 species. Despite this rapid increase in the number of recognized species of the genus *Actinomyces*, it is clear that much remains to be discovered, particularly from human and animal sources [9]. In this paper we report on the phenotypic and phylogenetic characterization of new isolates of a previously unknown *Actinomyces*-like bacterium isolated from plateau pika (*Ochotona curzoniae*), a wild mammal species. Based on the taxonomic results, a novel species of the genus *Actinomyces, Actinomyces gaoshouyii* sp. nov. is described.

With a preference to live at high elevations (mostly in the Tibetan plateau) and without a need to hibernate, the small, fast-reproducing, lagomorph animals named plateau pika are unique as a keystone mammal species in that area and have a high impact, both pro and con, on the local ecosystem by making burrows for other animals, creating microhabitats for plants and serving as prey for predators among other functions [10]. Human and animal guts are inhabited by millions of micro-organisms, which play fundamental roles in the host’s health and disease [11]. During an attempt to investigate the bacterial diversity of gut flora in wild animals, plateau pikas were captured in Yushu Tibetan autonomous prefecture and 200 samples from each animal sacrificed were collected from its colon and rectum. Fresh intestinal contents were aliquoted in 2 ml sterile tubes containing Luria–Bertani (LB) medium with addition of 20 % (v/v) glycerol. Upon inoculating one of the diluted samples

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain pika_113† is KY611802. The GenBank/EMBL/DDBJ accession numbers for the genome sequences of strain pika_113† and *Actinomyces denticolens* DSM 20671† are MVIW0000000 and MVIW0000000, respectively. One supplementary table and three supplementary figures are available with the online Supplementary Material.
on a Columbia-5% sheep blood agar (REF: 43041, bioMérieux) plate and incubation for 24 h in an anaerobic chamber filled with N₂/CO₂/H₂ (90:5:5), a greyish-white, opaque colony emerged. Pure colonies from subculturing that single colony on plates were stocked and designated as pika_113ᵀ. Strain pika_114 was similarly isolated from another pika sample.

Growth of the strains was tested repeatedly under different aeration conditions (anaerobically, microaerophilically and aerobically in the presence of 5 % CO₂), and they grew well, even under 10 % or higher oxygen pressure. Without any growth in air (in the absence of 5 % CO₂), pika_113ᵀ was facultatively anaerobic and grew well at 30°C. Pika_113ᵀ was cultured at 30°C in air plus 5 % CO₂ on Columbia-5% sheep blood agar plates for 24 h, and the morphological features and Gram staining were examined with a light microscope (Eclipse 80i, Nikon) or/and a transmission electron microscope (HT7700, Hitachi). Gram staining was tested with a Gram stain kit from bioMérieux according to the manufacturer’s instructions. Temperatures needed for bacterial growth (optical density at 600 nm; OD₆₀₀ value) were determined in brain heart infusion (BHI; REF: 241830, Becton, Dickinson and Company) broth containing 5 % (v/v) FBS at five different temperatures (20, 30, 37, 40 and 50°C). The pH and salt requirement for growth were determined in BHI broth under seven different pH (pH 4, 5, 6, 7, 8, 9 and 10) adjusted with HCl or NaOH (1 M) and with five different NaCl concentrations (0, 1, 2, 3 and 4 %, w/v), respectively. The optimum pH was determined by examining the growth at pH 4–10 using the following buffer systems: citric acid/sodium citrate (20 mM) for pH 4–5, HEPES (20 mM) for pH 6–8 and carbonate/bicarbonate buffer (20 mM) for pH 9–10. Cell motility was tested in soft agar plates as described previously [12]. All tests were performed in duplicate. Cells of the novel isolates were Gram-stain-positive, non-motile and straight to slightly curved rods, approximately 0.4–0.6×1.1–1.6 μm in diameter (Fig. S1, available in the online Supplementary Material). Optimal growth of pika_113ᵀ was achieved in BHI (pH 6–7) with 1 % (w/v) NaCl upon incubation at 30°C in the presence of 5 % CO₂. Unless stated otherwise, all the subsequent characterization tests for pika_113ᵀ were conducted under these optimized conditions.

The novel strains were characterized biochemically by using the API 50 CH strip, API ZYM system, API Rapid 32 Strept and API Coryne according to the manufacturer’s instructions (bioMérieux). The isolates could produce acid from glycerol, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, inositol, D-mannitol, methyl α-D-glucopyranoside (weakly for pika_113ᵀ), amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, gentiobiose, turanose, D-arabitol and potassium 5-ketogluconate (weakly). The strains could not produce acid from erythritol, D-arabinose, L-arabinose, D-xyllose, L-xyllose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, D-sorbitol, methyl α-D-mannopyranoside, N-acetylgucosamine, lactose, glyco-
gen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, potassium gluconate or potassium 2-ketogluconate (50 CH). Alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, α-galactosidase, β-galactosidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase, pyrazinamidase and alanyl-phenylalanine-proline arylamidase were produced by the isolates, but tests for esterase lipase (C8), lipase, trypsin, α-chymotrypsin, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, pyrrolidonyl arylamidase, arginine dihydrolase, pyroglytamic acid arylamidase, glycylic tryptophan arylamidase, urease and catalse were negative (API ZYM, API Rapid 32 Strept and API Coryne). The isolates reduced nitrates, and hydrolysed hippurate but not gelatin.

The biochemical characteristics of pika_113ᵀ are detailed in the species description and summarized in Table 1 in comparison with those of the most closely related type strains, both phylogenetically and phenotypically. As references, Actinomyces denticolens DSM 20671ᵀ and Actinomyces timoniensis DSM 23838ᵀ were also cultured and examined in parallel. Although similar in the majority of biochemical tests, no fermentation of lactose was the defining feature of pika_113ᵀ in differentiating it simultaneously from both of the closely related strains, A. denticolens DSM 20671ᵀ and A. timoniensis DSM 23838ᵀ. Its negativity in reacting with D-adonitol and potassium gluconate allowed the differentiation from A. denticolens DSM 20671ᵀ. It further was different from A. timoniensis DSM 23838ᵀ by many more tests, such as acid production from amygdalin, inulin, N-acetyl-glucosamine, D-lyxose and L-arabitol, and nitrate reduction (Table 1).

To study the chemotaxonomic features of strain pika_113ᵀ, its cellular fatty acid profile and polar lipid composition were compared with those of the reference strains (A.

### Table 1. Differential characteristics of strain pika_113ᵀ and its closest phylogenetic relatives in the genus Actinomyces

<table>
<thead>
<tr>
<th>Characteristic/test</th>
<th>Strain 1</th>
<th>Strain 2</th>
<th>Strain 3</th>
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<tbody>
<tr>
<td>Acid production (API 50 CH)</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>D-Adonitol</td>
<td>-</td>
<td>+</td>
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<td>Potassium gluconate</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>N-Acetylgulcosamine</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>D-Lyxose</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Arabitol</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Amygdalin</td>
<td>+</td>
<td>-</td>
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<td>Inulin</td>
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<td>Lactose</td>
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<td>Melezitose</td>
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<td>+</td>
<td>-</td>
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<tr>
<td>Reduction of nitrates</td>
<td>+</td>
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</table>
The cellular fatty acids of strain pika_113T and the reference strains were extracted according to the protocol of the Sherlock Microbial Identification System (MIDI) [13], and the cellular fatty acid profile and polar lipid compositions were further investigated as described previously [14, 15]. The major cellular fatty acids of strain pika_113T were mainly monounsaturated fatty acids and straight-chain saturated ones with C18:1ω9c (41.8 %) and C16:0 (39.0 %) predominant. The complete fatty acid profiles (Table 2) revealed that strain pika_113T shared two major cellular fatty acids (C19:1ω9c and C18:0) with A. denticolens DSM 20671T and A. timonensis DSM 23838T. Polar lipids were identified using two-dimensional TLC and staining with various specific reagents for detection of phosphate (molybdhenum blue spray reagent), free amino groups (ninhydrin), sugars (α-naphthol) and quaternary nitrogen compounds (Dragendorff), and the nonspecific spray reagent (phosphomolybdic acid hydrate). The major polar lipid detected in strain pika_113T was diphosphatidylglycerol. There were moderate amounts of phosphatidylinositol mannoside and phosphatidylinositol. In addition, an unidentified phosphoglycolipid, phosphatidylincholine, two unidentified aminolipids and two unidentified polar lipids were detected in trace amounts. The polar lipid profiles of strain pika_113T and its two reference strains were almost identical. In addition, trace amounts of phosphatidylglycerol (PG) and an unidentified glycolipid were detected in A. denticolens DSM 20671T and A. timonensis DSM 23838 (only PG) (Fig. S2). Thus, the profiling results of cellular fatty acids and polar lipids also supported the affiliation of the novel isolates with the genus Actinomyces.

For 16S rRNA gene sequencing and phylogenetic analyses, genomic DNA template was prepared from pika_113T using a Wizard Genomic DNA Purification kit (Promega) according to the manufacturer’s instructions. The almost-complete 16S rRNA genes of the two isolates were amplified by PCR with the universal forward primer 8F (5’-AGAGTTT-GATCCTGCGGTACG-3’) and reverse primer 1492R (5’-GTYTACCTTGGTACGACTT-3’), and sequenced directly [16]. The 16S rRNA gene sequence of strain pika_113T was submitted to the NCBI database with accession number KY611802. Upon comparison with all the 16S rRNA gene sequences from type strains of species of the genus Actinomyces retrieved from the NCBI database using the Basic Local Alignment Search Tool (BLAST), our novel isolates were most closely related to species of the genus Actinomyces with sequences highly similar to those of A. denticolens DSM 20671T (97.2 %) and A. timonensis DSM 23838T (97.1 %). In addition, the 16S rRNA sequence of pika_113T showed 94.6 % similarity with A. bovis ATCC 13683T, which is the type species of genus Actinomyces. The 16S rRNA gene phylogenetic trees were subsequently reconstructed with three different algorithms, the neighbour-joining algorithm (NJ) [17], the maximum-likelihood algorithm (ML) [18] and the maximum-parsimony (MP) algorithm [19] using MEGA 6 based on 1000 bootstrap replications. The evolutionary distances for the NJ and ML methods were computed using the Kimura 2-parameter method [20] and used to generate dendrograms with pairwise deletion gaps treatment. Partial deletion gaps treatment was also used with the MP algorithm with a 90 % site coverage cutoff value. The heuristic search methods of ML and MP were nearest-neighbour-interchange (NNI) and close-neighbour-interchange (CNI), respectively. Results from the reconstructed phylogenetic tree, including all the recognized species of the genus Actinomyces with Bifidobacterium bifidum as the outgroup, clearly placed strain pika_113T as a distinct cluster within the genus Actinomyces. It also revealed that strain pika_113T was most closely related to strains A. denticolens DSM 20671T and A. timonensis DSM 23838, with pika_113T forming a distinct lineage within the genus Actinomyces but not affiliating with any other recognized species (Fig. 1). Therefore, our phylogenetic analysis results demonstrate that the novel strain may very likely represent a novel species within the genus Actinomyces.

Chromosomal DNA from pika_113T was separately isolated with a Wizard Genomic DNA Purification kit (Promega) according to the manufacturer’s instructions, and the genome of strains pika_113T was sequenced with an Illumina Hiseq 2500 by a paired-end 125 bp sequencing.
strategy with the genomic DNA from *A. denticolens* DSM 20671\(^T\) and *A. timonensis* DSM 23838\(^T\) as calibration references in sequencing and sequence analysis. A total of 2.2 Mbp raw sequences, corresponding to a coverage depth of approximately 100\(\times\), were obtained for pika_113\(^T\) and assembled into 20 scaffolds using SOAP denovo (version

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**Fig. 1.** Phylogenetic tree showing the relationships of pika_113\(^T\) to other species of the genus *Actinomyces*. The tree was inferred from 16S rRNA gene sequences by the neighbour-joining (NJ), maximum-likelihood (ML) and maximum-parsimony (MP) methods. Numbers at nodes represent bootstrap values (NJ/ML/MP) as percentages of 1000 replicates. *B. bifidum* ATCC 29521\(^T\) is used as an outgroup. Names and accession numbers are given as cited in the GenBank database. Bar, 0.02 accumulated changes per nucleotide.
Our results revealed that pika_113 performed to assess the reliability of the phylogenetic tree. The joining tree reconstruction was calculated using MEA software (MEGA 6) with the Kimura model [21]. The approximate DNA G+C content of the draft genome sequence of strain pika_113T was 71.1 mol% (GenBank accession number of pika_113T: MVIV00000000). As the genome of A. denticolens DSM 20671T was not available when drafting the manuscript, we collected the type strain of A. denticolens, extracted its DNA and deposited the genome sequence to GenBank (accession number: EU621127). DNA–DNA hybridization (DDH) was conducted using the genome-to-genome distance calculator (GGDC) web software (http://ggdc.dsmz.de/), which improved DDH-prediction models and provided additional features such as confidence-interval estimation [22]. The DNA–DNA relatedness values between pika_113T and all the available genomes in the genus Actinomyces were below 70% (Table S1), the threshold for delineating bacterial species [23], further supporting our conclusion that strain pika_113T was phylogenetically different from any other existing species of the genus Actinomyces.

In order to further determine the phylogenetic position of strain pika_113T in the genus Actinomyces, five housekeeping genes, atpA (ATP synthase F1, EU620779), gyrA (DNA gyrase, EU621011), metG (methionyl-tRNA synthase, EU621127), pgI (glucose-6-phosphate isomerase, EU603149) and rpoB (DNA-directed RNA polymerase, EU621243) [24], were analysed for all the available genomes in the genus Actinomyces. Sequence alignment of the 30 genomes was done using the neighbour-joining method with the MEGA 6 software. The distance for neighbour-joining tree reconstruction was calculated using Kimura’s two-parameter model with pairwise deletion gaps treatment, and bootstrap analysis (1000 replications) was performed to assess the reliability of the phylogenetic tree [20]. Our results revealed that pika_113T was most closely related to A. denticolens and A. timonensis (Fig. S3), confirming the taxonomic classification of our strains.

It is apparent from both phenotypic and phylogenetic evidence that the isolates from plateau pika specimens represent a hitherto unknown species of the genus Actinomyces. We propose the name Actinomyces gaoshouyii sp. nov. for the novel bacterial species, with pika_113T as the type strain.

**DESCRIPTION OF ACTINOMYCES GAOSHOUYII SP. NOV.**

Actinomyces gaoshouyii (gao.shou.yii’i. N.L. gen. n. gaoshouyii in honour of professor Shouyi Gao, a well-known microbiologist, for his seminal contributions to the research of Vibrio cholerae).

Cells are Gram-staining-positive, facultatively anaerobic, straight to slightly curved rods, approximately 0.4–0.6 × 1.1–1.6 µm and catalase-negative. When grown on Columbia blood agar at 30 °C for 24 h, colonies are small (<1 mm in diameter), greyish white, opaque and shiny. Using API systems, acid is produced from glyceral, D-ribose, D-galactose, D-glucose, D-fructose, D-mannose, inositol, D-mannitol, methyl D-glucopyranoside, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, melibiose, sucrose, trehalose, inulin, raffinose, starch, gentiose, turanose, D-arabitol and potassium 5-ketogluconate, but not from erythritol, D-arabinose, L-arabinose, D-xyllose, L-xyllose, D-xylose, D-adonitol, methyl D-xyllopentoside, L-sorbose, L-rhamnose, dulcitol, D-sorbitol, methyl D-mannopyranoside, N-acetylglucosamine, lactose, glycochen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucitol, L-arabitol, potassium gluconate or potassium 2-ketoglucuronate. Alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, D-galactosidase, D-galactosidase, D-glucosidase, D-glucosidase, pyrazinamidase and alanly-phenylalanine-proline arylamidase are produced. Esterase lipase (C8), lipase, trypsin, a-chymotrypsin, β-glucuronidase, N-acetyl-β-glucosaminidase, D-mannosidase, D-fucosidase, pyrrolidinyl arylamidase, arginine dihydrolase, pyrogallaric acid arylamidase, glycol triptophan arylamidase and urease are negative. Nitrate reduction is positive. Hippurate is hydrolysed but not gelatin. The major fatty acids are C16:0, C18:1ω9c and C16:0.

Diphosphatidylglycerol is the major compound of the polar lipids.

The type strain, pika_113T (=CGMCC 4.7372T=DSM 104049T), was isolated from a plateau pika from Qinghai Tibet Plateau, China. The G+C content of the DNA of the type strain is 71 mol%.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


