Streptococcus marmotae sp. nov., isolated from the respiratory tract of Marmota himalayana

Lina Niu,1,2,3† Shan Lu,2† Shoukui Hu,2† Dong Jin,2† Xinhe Lai,4,5† Jing Yang,2 Cuixia Chen,2 Yi Wang,2 Yiting Wang,2 Xiangning Bai,2 Ruiting Lan,6 Gang Lv,3 Yingping Xie,1 Changyun Ye2 and Jianguo Xu2

1School of Life Science, Shanxi University, Taiyuan 030006, PR China
2State Key Laboratory for Infectious Disease Prevention and Control, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 100206, PR China
3School of Tropical and Laboratory Medicine, Hainan Medical University, Key Laboratory of Translation Medicine Tropical Diseases (Hainan Medical University), Ministry of Education, Haikou, PR China
4Institute of Translational Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, PR China
5Institute of Inflammation and Diseases, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, PR China
6School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, New South Wales, Australia

Five strains of a Gram-stain-positive, catalase-negative, α-haemolytic, coccus-shaped chain-forming organism were isolated separately from the lower respiratory tracts of five animals of Marmota himalayana in the endemic area of plague, the Qinghai-Tibet Plateau, China. Based on their morphological characteristics, biochemical features and molecular phylogenetic studies, the strains were placed as representing a new member of the genus Streptococcus. Comparative 16S rRNA gene sequence studies indicated that strain HTS5T shared 96.5, 96.2 and 96.0 % similarity with Streptococcus gallinaceus CCUG 42692T, Streptococcus parasanguinis ATCC 15912T and Streptococcus suis ATCC 43765T, respectively. Sequence analysis of its rpoB and sodA genes showed that strain HTS5T was most closely related to Streptococcus cuniculi CCUG 65085T with 9.2 and 10.9 % interspecies divergence, respectively. The whole genome phylogenetic tree based on 339 core genes of 65 Streptococcus genomes confirmed that HTS5T belongs to a distinct lineage that is well separated from recognized species of the genus Streptococcus. In silico DNA–DNA hybridization using 65 available genomes from GenBank showed that HTS5T displayed less than 70 % DNA–DNA relatedness with the other 65 species of the genus Streptococcus deposited in the GenBank database. The genome of strain HTS5T (2 322 791 bp) contained 2377 genes and had a G+C content of 41.6 mol%. Therefore, the five strains are considered to represent a novel species of the genus Streptococcus for which the name Streptococcus marmotae sp. nov. is proposed. The type strain is HTS5T (=DSM 101995T =CGMCC 1.15534T).

†These authors contributed equally to this work.

Abbreviation: DDH, DNA–DNA hybridization.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, rpoB and sodA gene sequences of S. marmotae strains HTS5T, HTS6, HTS7, HTS17 and HTS30 are KU992301, KU992311, KU992306; KU992302, KU992312, KU992307; KU992303, KU992313, KU992308; KU992304, KU992314, KU992309; and KU992305, KU992315, KU992310, respectively. The GenBank/EMBL/DDBJ accession number for the whole genome sequence of S. marmotae HTS5T is CP015196 (containing two plasmids: plasmid1=CP015197 and plasmid2=CP015198).

One supplementary figure and one supplementary table are available with the online Supplementary Material.
The genus *Streptococcus* consists of a large number of Gram-stain-positive, catalase-negative, coccus-shaped, chain-forming organisms. Due to the development of phenotypic and molecular biology identification techniques, the genus *Streptococcus* has undergone considerable expansion and revision with many novel species being described in recent years, especially from animal origins (Braden et al., 2015; Okamoto et al., 2015; Saito et al., 2016; Shinozaki-Kuwahara et al., 2014; Vela et al., 2015, 2016). In this study, we report on the phenotypic and phylogenetic characteristics of five *Streptococcus*-like strains isolated from tracheal samples of *Marmota himalayana* in the Qinghai-Tibet Plateau, China.

The Qinghai-Tibet plateau (altitude 3599.6 m, 33°21′ N 96°62′ E) is one of the remaining endemic plague foci in China and is under constant surveillance by the Chinese national plague surveillance programme due to its public health importance. Marmots (*Marmota himalayana*) are the primary animal reservoir of plague in the region. Monitoring plague outbreaks in animals is the key plague surveillance tool and involves capturing live healthy animals for isolation of *Yersinia pestis* (Stone, 2010). As an auxiliary programme to this surveillance, animals captured were also used for isolation of other potential human pathogens (Hu et al., 2015; Liu et al., 2015). Two novel bacterial species (*Helicobacter himalayensis* sp. nov. and *Escherichia marmota* sp. nov.) with pathogenic potential were isolated from samples of the gastrointestinal content of these marmots (Hu et al., 2015; Liu et al., 2015). We confirm here that the respiratory tract of these animals also carries bacteria with potential medical significance.

To test this hypothesis, respiratory tract tissues were collected from the marmots as previously described (Hu et al., 2015; Liu et al., 2015). Briefly, 30 mg of the mucosal tissue of the tracheal inner wall was collected by scraping with sterile scissors, mixed with 20% fetal bovine serum (Gibco) and ground together completely. The homogenates, 100 µl per plate, were spread on Columbia blood agar plates and incubated at 37 °C for 48 h under aerobic conditions. Ten respiratory tract tissue samples were plated on 20 Columbia blood agar plates. A single, circular, unpigmented, α-haemolytic colony of 0.5–1.0 mm in diameter emerged from one of the plates 24 h after aerobic incubation. The colony was purified by subculturing on Columbia blood agar plates, and pure colonies were collected and stored under the name HTS5T. Subsequently, four other strains (HTS6, HTS7, HTS17 and HTS30) were isolated from screening of an additional 40 respiratory tract tissue samples. As described below in detail, the five new strains were studied for (1) their colony morphology on plates and cell morphology under a light microscope; (2) their biological features (growth temperature, tolerance to salt, haemolysis, etc.); (3) their biochemical reactions as determined with the Rapid ID 32 Strep, API 50 CH and API ZYM systems; and (4) their phylogenetic relationships among each other and with other *Streptococcus* species by comparing their partial 16S rRNA gene sequences and segments of two housekeeping genes (*sodA* and *rpoB*). The genome of HTS5T was also sequenced and further whole genome phylogenetic analysis was performed by comparison of 339 core genes of 65 *Streptococcus* genomes; *in silico* DNA–DNA hybridization (DDH) analysis with the type strains of other species of the genus *Streptococcus* was also performed.

Morphological features were determined using cells cultured aerobically at 37 °C on Columbia blood agar for 24 h by means of light microscopy. Gram staining was performed using the classical Gram stain procedure (Austrian, 1960). Haemolytic activity was characterized by inoculating each strain on a Columbia agar plate containing 5.0% defibrinated sheep blood (bioMérieux) and incubating aerobically at 37 °C for 24 and 48 h (Facklam & Elliott, 1995). Determination of bacterial growth was performed in brain heart infusion broth (Difco) at pH 7.5 at 4, 15, 22, 30, 35, 37 or 42 °C (Facklam & Elliott, 1995). Salt tolerance was tested by the growth of the strains in the presence of 2.5, 3.5, 4.5 or 6.5% (w/v) NaCl and assessed as recommended by Facklam & Elliott (1995).

The commercial Slidex Strepto kit (bioMérieux) was used to determine whether the strains could be classified using the Lancefield antigen grouping; none of the strains of the proposed novel species reacted with any of the Lancefield group antisera. The five strains were further characterized biochemically using the Rapid ID 32 Strep, API 50 CH and API ZYM systems (bioMérieux) as per the manufacturer’s instructions. The results from the API 50 CH strips using CHB suspension medium, incubated at 37 °C, were recorded daily over 1 week (Table 1). The five strains showed the same biochemical characteristics as determined in the three identification systems used, producing acid from some carbohydrates (D-mannitol, inulin, melezitose, sorbitol, starch, trehalose and turanose) and in contrast to other related *Streptococcus* species (Notes of Table 1.)

The 16S rRNA gene sequences from these strains were obtained by cloning of PCR products amplified using the universal eubacterial primers 27F-1492R (5′-AGAGTTTGATCMTGCTCAG-3′ and 5′-GGYTACCTTGTTACGACTT-3′) from genomic DNA templates prepared using the Wizard Genomic DNA Purification kit (Promega) according to the manufacturer’s instructions. Phylogenetic analysis using 16S rRNA gene sequences demonstrated that strain HTS5T was 99.9%–100% similar to the other four strains but shared less than 97.0% similarity with the type strains of other species of the genus *Streptococcus* (Fig. S1, available in the online Supplementary Material). Strain HTS5T shared 96.5, 96.2 and 96.0% 16S rRNA gene sequence similarity with *S. gallinarum* CCUG 42692T, *S. parasanguinis* ATCC 15912T and *S. suis* ATCC43765T, respectively. This analysis demonstrated that the novel organism represents a novel species within the genus *Streptococcus*. Phylogenetic trees were reconstructed with three different algorithms, neighbour-joining (within the genus *Streptococcus*). Phylogenetic trees were reconstructed with three different algorithms, neighbour-joining (Figs 1 and S1), maximum-parsimony and maximum-likelihood, by using the software package MEGA version 6.0 (Tamura et al., 2013) and the program SeqTools (Rasmussen, 2002) to align the newly
determined 16S rRNA gene sequences of these five strains with sequences of the type strains of other Streptococcus species retrieved from GenBank. The genetic distance for the same species showed the same affinity to the suis group (Fig. S1). Bootstrap analysis (1000 replications) was performed to assess the reliability of the nodes. As shown in Fig. 1, which was reconstructed by using two representative strains from other Streptococcus species groups (S. pyogenes, S. mitis, S. salivaruis, S. bovis and S. mutans) and all strains of the suis group (Póntigo et al., 2015), it is clear that the newly isolated strains form a distinct lineage not affiliated with any known species of the genus Streptococcus but closely related to some described species (S. acidominimus, S. suis, S. gallinarus, S. entericus, S. minor, S. ovis and S. plurextorum), and thus falling into the suis 'species group' (Póntigo et al., 2015). Inclusion of more strains from other Streptococcus species showed the same affinity to the suis group (Fig. S1). Bootstrap analysis supported the branching of the proposed novel species from S. cuniculi CCUG 65085T and S. acidominimus CIP 82.4T with a bootstrap value of 72.0% (Fig. 1). Similar results were obtained with the other two algorithms.

Other than the 16S RNA gene, housekeeping genes such as sodA, rpoB, gyrB and groEL are also suitable and helpful for strain differentiation at the species level as demonstrated for several bacterial phyla (Saito et al., 2016; Shinozaki-

### Table 1. Characteristics useful in differentiating strain HTS5<sup>T</sup> from other closely related Streptococcus species

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<sup>*</sup>Lancefield serological group reaction uses group A-, B-, C-, D-, F- and G-specific streptococcal latex agglutinating antisera.
Kuwahara et al., 2014; Vela et al., 2015). The sodA and rpoB genes have specifically been used as useful phylogenetic tools for differentiating streptococci (Poyart et al., 1998; Drancourt et al., 2004; Vela et al., 2011). Therefore, we sequenced a segment of the sodA (450 bp) and rpoB (680 bp) genes from these strains by PCR sequencing using primer pairs d1 and d2 (Poyart et al., 1998) and StreptoR (Drancourt et al., 2004), respectively. The sodA and rpoB gene tree showed that strains HTS7, HTS17 and HT30 were phylogenetically closer to each other (98–100%) than to strain HTS5⁰ or HTS6 (Fig. 2). More importantly, the five strains were separated from the other suis group members and formed a distinct cluster by themselves but grouped together with S. cuniculi CCUG 65085⁰ with divergence values of 10.9–12.2% and 9.1–9.2% for the sodA and rpoB genes, respectively (Fig. 2). These results suggest strongly that these five newly isolated catalase-negative coccal strains from Marmota himalayana belong to a novel Streptococcus species.

The comprehensive database of full-length 16S rRNA gene sequences of bacteria and archaea, the Species Living Tree Project (LTP), is frequently used for delineating species in microbial taxonomy (www.arb-silva.de/living-tree) due to its regularly updated and corrected entries of best-quality sequences with a manually curated alignment for a single type strain (Yarza et al., 2008, 2014). As a principle, <98.7% similarity with a type strain but >94.5% of the genus threshold of a given 16S rRNA gene sequence suggests that a microbial strain be classified as a member of that genus (Yarza et al., 2008). The full-length 16S rRNA gene sequence of strain HTS5⁰ was extracted from the genome sequence obtained in this study (see below). In comparisons using the LTP database, levels of similarity between strain HTS5⁰ and other re-recognized Streptococcus species were below 98.7%, consistent with the findings based on its partial 16S rRNA gene sequence (Fig. 1). The data confirm that the new isolates represent a novel species.

The chromosomal DNA of HTS5⁰, the type strain of the proposed novel Streptococcus species, was isolated with a Wizard Genomic DNA Purification kit (Promega) and sequenced using an Illumina HiSeq2500 125 bp paired-end sequencer. A total of 10 684 674 reads were obtained, which gave a coverage depth of approximately 100×, and they were assembled into 122 contigs in 101 scaffolds using

![Fig. 1. Neighbour-joining phylogenetic tree inferred from a comparison of 16S rRNA gene sequences of strains HTS5⁰, HTS6, HTS7, HTS17 and HTS30 and closely related members of the main streptococcal ‘species groups’. The sequence of Enterococcus faecalis ATCC 19433⁰ was used as an outgroup. Bar, 0.01 substitutions per site. Bootstrap values ≥50 are indicated.](image-url)
Streptococcus marmotae sp. nov.

Description of Streptococcus marmotae sp. nov.

Streptococcus marmotae (mar.mo’tae, N.L. fem. n. marmotae referring to the isolation of the type strain from the marmot Marmota himalayana).

Cells are facultatively anaerobic, Gram-stain-positive, catalase-negative, non-motile and non-spore-forming cocci, 0.5–1.0 µm in diameter, and occur in short chains, pairs or groups. Colonies on Columbia blood agar are small, circular, non-pigmented, 0.5–1.0 mm in diameter and α-haemolytic after incubation at 37°C for 24 h. Growth is observed

Comparison of the genome of strain HTSST with 65 publically available Streptococcus genomes identified 339 core genes. The core genome phylogenetic tree showed unequivocally that strain HTSST grouped together with S. ovis and S. minor of the suis group (Fig. 3), which is in agreement with the 16S rRNA gene tree and the housekeeping gene tree, further supporting that strain HTSST represents a distinctive species.

Overall, the morphological features (Gram-stain-positive cocci in pairs or short chains), biochemical characteristics (as summarized above and listed in Table 1) and phylogenomic analysis results (DDH, phylogenetic trees built from 16S rRNA, two housekeeping and 339 core genes) showed that strains HTSST, HTS6, HTS7, HTS17 and HTS30 belonged to the suis group together with S. acidominimus, S. suis, S. gallinaceus, S. entericus, S. minor, S. ovis and S. plurextorum, and that these five novel strains represent a novel species, for which the name Streptococcus marmotae sp. nov. is proposed.

Fig. 2. Neighbour-joining phylogenetic tree inferred from a comparison of the sodA and rpoB gene sequences of strains HTSS, HTS6, HTS7, HTS17 and HTS30 and related members of the genus Streptococcus. Bar, 0.01 substitutions per site. Bootstrap values ≥50 are indicated.

SOAPdenovo (version 2.04). A complete genome was obtained using PacBio Single Molecule, Real-Time (SMRT) technology analysis 2.3.0 (Berlin et al., 2015). The genome contained one circular chromosome of 2 309 282 bp and two plasmids (of 3762 and 5997 bp, respectively). A total of 2377 genes were predicted by Glimmer (Guimaraes et al., 2011) and ten genomic islands were predicted using Island Viewer. The average gene length was 848 bp and the DNA G+C content was 41. mol%.

DDH has been used for delineating new bacterial species since the 1960s and a value of 70% was recommended as a cut-off for species demarcation by Wayne et al. (1987). With more genomes available, genome-sequence-based (in silico) DDH is replacing the conventional wet-lab DDH (Auch et al., 2010; Colston et al., 2014; Garrido-Sanz et al., 2016; Meier-Kolthoff et al., 2013). To further verify the taxonomic relationship of the proposed novel species with other Streptococcus species, an in silico DDH analysis was performed between the novel strains and the other Streptococcus species by using the DDH web-software at http://ggdc.dsmz.de (Auch et al., 2010). DNA–DNA relatedness values varied between 77.2 and 77.6% among the novel strains (HTSS, HTS6, HTS7, HTS17 and HTS30) and between 19.8 and 28.3% with other known species of the genus Streptococcus (Table S1), which firmly supports that strain HTSS and the other four marmot strains belong to the same species (>70%) and represent a novel Streptococcus species (<70.0%).

Comparison of the genome of strain HTSS with 65 publically available Streptococcus genomes identified 339 core genes. The core genome phylogenetic tree showed unequivocally that strain HTSS grouped together with S. ovis and S. minor of the suis group (Fig. 3), which is in agreement with the 16S rRNA gene tree and the housekeeping gene tree, further supporting that strain HTSS represents a distinctive species.

Overall, the morphological features (Gram-stain-positive cocci in pairs or short chains), biochemical characteristics (as summarized above and listed in Table 1) and phylogenomic analysis results (DDH, phylogenetic trees built from 16S rRNA, two housekeeping and 339 core genes) showed that strains HTSS, HTS6, HTS7, HTS17 and HTS30 belonged to the suis group together with S. acidominimus, S. suis, S. gallinaceus, S. entericus, S. minor, S. ovis and S. plurextorum, and that these five novel strains represent a novel species, for which the name Streptococcus marmotae sp. nov. is proposed.
Fig. 3. Rectangular cladogram of all 66 representative Streptococcus genomes, including strain HTS5\(^T\) and 65 streptococci from NCBI GenBank. The sequences of Lactococcus lactis subsp. lactis KF147 and Lactococcus lactis subsp. cremoris SK11 were used as an outgroup.
at 22, 30, 35, 37 and 42 °C but not at 4 or 15 °C; grows in broth containing not more than 2.5% (w/v) NaCl. With the API 50 CH and Rapid ID32 STREP kits, cells are able to produce acid from the following sugars: N-acetylglucosamine, ascorbin, amygdalin, arbutin, cellulose, erythritol, D-fructose, D-galactose, gentiobiose, D-glucose, glycerol, glycogen, 5-ketogluconate, lactose, maltose, D-mannose, melibiose, raffinose, salicin, sucrose and D-xylene, but not from D-adonitol, D-arabinose, L-arabinose, D-arabitol, L-arabitol, cyclodextrin, dulcitol, D-fucose, L-fucose, inositol, inulin, 2-ketogluconate, D-lxysose, D-mannitol, melezitose, methyl α-D-glucopyranoside, methyl α-D-mannoside, methyl β-D-xlyopyranoside, pullulan, L-rhamnose, D-ribose, sorbitol, L-sorbose, starch, tagatose, trehalose, turanose, xylitol or L-xylene. Enzyme activities are detected for α-chymotrypsin, esterase C4, β-glucuronidase, leucine arylamidase and naphitol-AS-BI-phophohydrolase. No activity is detected for α-fucosidase, glycyl-tryptophan arylamidase, lipase C14, cystine arylamidase, esterase lipase C8, phenylalanine-proline arylamidase, alkaline phosphatase, L-tryptophan arylamidase, pullulan, and α-D-mannosidase.

The type strain, HTS5^T (=DSM 101995^T=CGMCC 1.15534^T), was isolated from the respiratory tract of a wild marmot *Marmota himalayana* from Yushu, Tibetan autonomous Prefecture, Qinghai province, China. The DNA G+C content of the type strain is 41.6 mol%. An additional four strains (HTS6, HTS7, HTS17 and HTS30) of the same species have been isolated from other *M. himalayana* samples.

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**References**


