Streptococcus azizii sp. nov., isolated from naïve weanling mice

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Abstract

Three isolates of a previously reported novel catalase-negative, Gram-stain-positive, coccoid, alpha-haemolytic, Streptococcus species that were associated with meningoencephalitis in naïve weanling mice were further evaluated to confirm their taxonomic status and to determine additional phenotypic and molecular characteristics. Comparative 16S rRNA gene sequence analysis showed nearly identical intra-species sequence similarity (≥99.9%), and revealed the closest phylogenetically related species, Streptococcus acidominimus and Streptococcus cuniculi, with 97.0 and 97.5% sequence similarity, respectively. The rpoB, sodA and recN genes were identical for the three isolates and were 87.6, 85.7 and 82.5% similar to S. acidominimus and 89.7, 86.2 and 80.7% similar to S. cuniculi, respectively. In silico DNA–DNA hybridization analyses of mouse isolate 12-5202T against S. acidominimus CCUG 27296T and S. cuniculi CCUG 65085T produced estimated values of 26.4 and 25.7% relatedness, and the calculated average nucleotide identity values were 81.9 and 81.7, respectively. These data confirm the taxonomic status of 12-5202T as a distinct Streptococcus species, and we formally propose the type strain, Streptococcus azizii 12-5202T (=CCUG 69378T=DSM 103678T). The genome of Streptococcus azizii sp. nov. 12-5202 contains 2062 total genes with a size of 2.34 Mbp, and an average G+C content of 42.76 mol%.

Within the genus Streptococcus, there are currently over 100 valid named species and subspecies in the List of Prokaryotic Names with Standing in Nomenclature (LSPN) on the website at www.bacterio.net [1] as of 8 August, 2017. These species can be commensal, opportunistic pathogens, or pathogenic to humans and a variety of animal species. In 2015, Braden et al. published a detailed investigation into the potential pathogenesis of a novel alpha-haemolytic Streptococcus species that was isolated from symptomatic and asymptomatic dams and weanling C57BL/6NCrl mice in an experimental laboratory setting [2]. The bacterium was directly isolated from swabs of brain tissue during necropsy from mice that demonstrated increased mortality associated with running, abnormal gait, decreased activity and meningoencephalitis [2]. These authors speculated that the bacterium is an opportunistic pathogen that is likely transmitted from the dam’s oral cavity and/or urogenital tract during or after parturition and causes clinical disease due to a combination of stress to the animals during transport, declining maternal antibody and weanling immune incompetence [2]. This bacterium was given the name ‘Streptococcus azizii’; however, the species name was never formally proposed and validated [2]. Here we further analyse three of these formerly published isolates, and compare additional phenotypic data, 16S rRNA, rpoB, sodA and recN gene sequences, and whole-genome sequence analysis to the type strains of the two Streptococcus species, Streptococcus acidominimus and Streptococcus cuniculi, that have ≥97% 16S RNA gene sequence similarity to this novel bacterium. We formally propose the type strain, Streptococcus azizii, 12-5202T (=CCUG 69378T=DSM 103678T).

Strains 12-5202T (isolated from a brain swab of a 29-day-old male mouse found runted and hunched with neurologic signs), 12-5291 [isolated from an oral swab obtained from a timed-pregnant female (dam) mouse; litter found dead], and 13-1151-1 (isolated from a brain swab from a 1-month-old male mouse with symptoms of meningitis) were provided to the Centers for Disease Control and Prevention (CDC) Streptococcus laboratory by co-author Neil Lipman. S. acidominimus (CCUG 27296T) was originally isolated from a bovine vagina [3] and S. cuniculi (CCUG 65085T) was originally isolated from a healthy wild rabbit’s nasal sample [4]. Both isolates were obtained from the Culture Collection, University of Göteborg (CCUG) culture collection.

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Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization; GGDC, Genome-to-Genome Distance Calculator, The GenBank/EMBL/DDBJ accession numbers for the whole-genome sequences of S. azizii 12-5202T, 12-5291, 13-1151-1, and S. acidominimus CCUG 27296T and S. cuniculi 65085T are M8PR00000000, MSP000000000, M8PS000000000, MSJL0000000000 and MSJM0000000000, respectively. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, rpoB, sodA and recN genes are BVE84_10155, BVE84_07760, BVE84_10065 and BVE84_01660, respectively.

Two supplementary tables and three supplementary figures are available with the online version of this article.
The whole genomes of the three novel mouse strains (12-5202\textsuperscript{T}, 12-5291 and 13-1151-1), S. acidominimus CCUG 27296\textsuperscript{T} and S. cuniculi CCUG 65085\textsuperscript{T} were sequenced in the Biotechnology Core Facility at the CDC. DNA was extracted using the Zymo Fungal/Bacteria DNA MicroPrep kit (Zymo Research Corporation). Libraries were prepared using the NEBNext Ultra DNA library prep kit for Illumina (New England BioLabs), and paired-end reads (2 × 250 bp) were generated using the Illumina MiSeq reagent kit version 2 and the MiSeq instrument (Illumina). Reads were cleaned with BBduk version 36.71 to remove PhiX (NC_001422.1) with a 31 bp kmer query allowing for a single mismatch to occur. Adapters were clipped off reads, and a 20 bp sliding window requiring Phred 30 was applied with Trimmomatic version 0.36 [5]. All paired reads and reads at least 50 bp long that passed these filters were given to SPAdes version 3.9.0 with ‘-only-assembler’ and ‘-cov-cutoff auto’ arguments [6]. Completeness of each assembly was assessed with HMmer version 3.1b2 [7] and CheckM v1.0.7 [8]. From 226 reference Streptococcus genomes in NCBI’s GenBank, 553 single-copy marker genes arranged in 260 sets were queried against each assembly. All five assemblies were >99.5% complete and therefore designated as near-complete draft genomes.

To rapidly annotate each assembly, we used Prokka version 1.11 [9] and RNAmmer version 1.2 [10] along with the curated (setA) VFDB [11] as trusted proteins, which enabled extraction of full-length 16S rRNA, rpoB, sodA and recN virulence factor gene sequences from each isolate's genome. The novel isolates contained many (292–295 range) putative virulence factor encoding genes including machinery for Type IV pili, Type VII secretion system, alginate and capsule biosynthesis. PHASTER [12] was used to identify prophage in each genome and made use of the prophage database curated by its developers in January 2016. Both S. acidominimus and S. cuniculi lacked intact prophage, however all three isolates of the novel species harboured an intact 43–51 kb prophage most closely related to Streptococcus phage 2972 (NC_007019.1). Phylogenetic trees for 16S rRNA, rpoB, sodA, and recN gene sequences were each reconstructed with the JC69 substitution model. Sequences were aligned in MUSCLE version 3.8.31 [13]. RAxML version 8.2.9 [14] was used to generate 1000 maximum-likelihood (ML) trees, and 1000 bootstraps were projected onto the best ML tree. To calculate nucleotide similarity for 16S rRNA gene sequences, we applied the global Needleman–Wunsch aligner in the EMBOSS version 6.6.0.0 package suite [15] for all pairwise comparisons.

Our sequence comparison of the near full-length 16S rRNA gene sequence (1535 bp) of strain 12-5202\textsuperscript{T} to isolates 12-5291 and 13-1151-1 showed 99.9% intra-species similarity and revealed two phylogenetically closely related species, S. acidominimus CCUG 27296\textsuperscript{T} and S. cuniculi CCUG 65085\textsuperscript{T}, with 97.0 and 97.5% sequence similarity, respectively. In addition, the ML phylogenetic tree inferred from 16S rRNA gene sequence comparison, shown in Fig. 1, revealed that the novel bacterium 12-5202\textsuperscript{T} formed a distinct branch from the other Streptococcus species but clustered with S. acidominimus and S. cuniculi. Due to the 16S rRNA gene sequence similarity values being greater than the proposed cut-off similarity value of ≥97% for species demarcation [16–19], S. acidominimus and S. cuniculi were included in further comparative sequence and genome analyses.

Comparison of the rpoB, sodA and recN gene sequences has been used as a more discriminative tool to speciate or sub-speciate members of the genus Streptococcus [20–24]. The sequences of the rpoB (3564 bp) gene for the three isolates were identical, and were 87.6 and 89.7% similar to S. acidominimus and S. cuniculi, respectively. Analysis of the sodA gene (606 bp) showed 100% sequence similarity among the three isolates and 85.7 and 86.2% to S. acidominimus and S. cuniculi, respectively. The recN gene (1662 bp) analysis also showed 100% intra-species sequence similarity, and 82.5 and 80.7% similarity to S. acidominimus and S. cuniculi, respectively (Table S1, available in the online version of this article). These nucleotide differences in select phylomarker genes further establish the novel strains as distinct from its two closest species relatives. The ML phylogenetic trees inferred from rpoB, sodA and recN gene sequence comparison all showed a distinct branch for the novel strains (Figs S1, S2, and S3).

During these gene analyses, we noticed that there may be a discrepancy in the S. acidominimus type strains at different culture collections based on data in recent publications that are not consistent with our data. Streptococcus acidominimus is curated in several culture collections with their identifiers [ATCC 51725\textsuperscript{T}=CCUG 27296\textsuperscript{T}=CIP 82.4\textsuperscript{T}=DSM 20622\textsuperscript{T}=LMG 17755\textsuperscript{T}=NCIMB 702023\textsuperscript{T} (formerly NCDO 2025\textsuperscript{T}, NCFB 2025\textsuperscript{T})=NCTC 12957\textsuperscript{T}]. S. acidominimus strain NCDO 2025\textsuperscript{T} was confirmed to be a novel Streptococcus species based on 16S rRNA gene homology values, since it failed to cluster with other members of the streptococci [25]. Our 16S rRNA gene sequence data from CCUG 27296\textsuperscript{T} is consistent with the previously deposited 16S rRNA gene sequences in GenBank from S. acidominimus LMG 17755\textsuperscript{T} (JX98696), DSM 20622\textsuperscript{T} (DQ118676) and NCDO 2025\textsuperscript{T} (X58301) as well, when the 35N's were excluded from this sequence.

However, the sequence from S. acidominimus CCUG 27296\textsuperscript{T} was only 94.6% similar to the 16S rRNA gene sequence from CIP 82.4\textsuperscript{T} (https://research.pasteur.fr/en/team/biological-resources-center/). The rpoB, sodA and recN gene sequences from CCUG 27296\textsuperscript{T} were only 85.8, 80.0 and 67.3% similar to the CIP 82.4\textsuperscript{T} gene sequences deposited in GenBank: AF535181, Z95892 and EU917241, respectively. In addition, the biochemical results were different between CCUG 27296\textsuperscript{T} and CIP 82.4\textsuperscript{T}. Therefore, we conclude that CIP 82.4\textsuperscript{T} is not S. acidominimus. The novel

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strain, 12-5202\textsuperscript{T} 16S rRNA gene sequence showed only 95.6\% gene sequence similarity to the CIP 82.4\textsuperscript{T} sequence, so it is clearly not closely related to this strain. These data show that the gene sequences from the novel strain, 12-5202\textsuperscript{T}, are distinct from gene sequences from either type strain source of \textit{S. acidominimus}.

In an \textit{in silico} pairwise analysis for estimation of DNA–DNA hybridization (isDDH) using the Genome-to-Genome Distance Calculator (GGDC 2.0) [26], all three mouse isolates were predicted to give an isDDH relative binding ratio of \textgreater 70\% to one another indicating they belong to the same species (Table S2). The predicted isDDH values between 12-5202\textsuperscript{T} and \textit{S. acidominimus} CCUG 27296\textsuperscript{T} and \textit{S. cuniculi} CCUG 65085\textsuperscript{T} were 26.4 and 25.7, respectively, well below the established 70\% delineation value [16, 27] thus confirming it is a novel \textit{Streptococcus} species. The isDDH analysis comparing \textit{S. acidominimus} CCUG 27296\textsuperscript{T} to \textit{S. cuniculi} CCUG 65085\textsuperscript{T} showed an estimated value of 27.5\%, confirming their species status. The DNA G+C content of strain 12-5202\textsuperscript{T} was determined as 42.76 mol\% using the program biopython [28].

The average nucleotide identity (ANiB) values were calculated based on the assembled whole genomes [29, 30]. Two whole-genome sequences represent the same species if their ANiB value is \textgreater 95–96\% [29, 31]. Strain 12-5202\textsuperscript{T} exhibited \textgreater 99.9\% ANiB values to the other two mouse isolates and values of 81.9 and 81.7\% with the genomes of \textit{S. acidominimus}, CCUG 27296\textsuperscript{T} and \textit{S. cuniculi}, CCUG 65085\textsuperscript{T}, respectively, confirming that 12-5202\textsuperscript{T}, 12–5291 and 13–1151 belong together in a separate species (Table S2). The ANiB value for \textit{S. acidominimus} CCUG 27296\textsuperscript{T} compared to \textit{S. cuniculi} CCUG 65085\textsuperscript{T} was 83.3\% and confirms their taxonomic status as distinct species. In addition, the recently described OrthoANIu algorithm [32] was evaluated with these strains, and nearly identical ANI values (\pm 0.4\%) were obtained with values of 81.8 and 81.5\% when comparing strain 12-5202\textsuperscript{T} with the genomes of \textit{S. acidominimus} CCUG 27296\textsuperscript{T} and \textit{S. cuniculi} CCUG 65085\textsuperscript{T}, respectively (Table S2).

The three isolates (12-5202\textsuperscript{T}, 12-5291 and 13-1151-1), \textit{S. acidominimus} and \textit{S. cuniculi} were tested in duplicate with conventional biochemical tests [33, 34] and in triplicate with the rapid ID32 \textit{STREP} system (bioMérieux) and the Vitek 2 GP-ID System (bioMérieux) according to the manufacturer’s instructions. The broth microdilution method was used for susceptibility testing of the three novel reference isolates [35] using custom-made panels manufactured by Trek Diagnostic Systems.

Conventional biochemical testing showed nearly identical test results for the three isolates of the novel species, and the
phylogenetically closely related type strains, *S. acidominimus* and *S. cuniculi*. All three isolates of the novel species, and the type strains of *S. acidominimus* and *S. cuniculi*, tested weakly to strongly positive with both methods for pyrog glutamic acid arylamidase (PYR). This is a somewhat unusual finding, since most *Streptococcus* species test negative for PYR. In addition, the three isolates of the novel species, and the type strains of *S. acidominimus* and *S. cuniculi* tested positive for aesculin, hippurate and acid production from inulin, lactose, maltose, mannitol, raffinose, ribose, sucrose, trehalose, pullulan and variable with tagatose. These species also did not grow on bile-aesculin agar, aesculin agar or in tellurite or pyruvate media, failed to hydrolyse arginine or urea, were negative in the Voges–Proskauer test, and did not produce acid from arabinose, glycerol and sorbose. (Pullulan was negative in one of the three novel isolates and ribose tests negative with the ID32 Strep system.) The negative sorbitol test result obtained with the novel isolates in conventional and rapid ID32 testing systems can be used to differentiate it from *S. acidominimus* and *S. cuniculi* which tested positive (Table 1). Additional useful rapid ID32 system tests for differentiation of the novel strains from *S. cuniculi* were β-glucuronidase, which tests positive with the novel strain and negative with *S. cuniculi*, and α-galactosidase, which tests negative with the novel strain and positive with *S. cuniculi*. The rapid ID32 database gave an identification of ‘unacceptable profile’ which is the correct response, since this species is not in the database. The Vitek 2 GP-ID system yield variable results and were analysed in triplicate. Six of nine test runs had the correct result of ‘87% Probability Streptococcus suis’ and two runs had a misidentification ‘90% Probability Enterococcus cassilflavus’.

In contrast to previously published results for *S. acidominimus* CIP 82.4T [3], the results we obtained for *S. acidominimus* CCUG 27296T were somewhat different. In our study, both pullulan and mannitol were repeatedly positive in both the conventional and rapid ID32 Strep methods, whereas these tests were negative in that study.

Using the Interpretive Categories and MIC breakpoints listed for *Streptococcus* species, viridans group Clinical Laboratory Standards Institute, M100-S27 [35], all three novel isolates tested susceptible for penicillin, ampicillin, cefotaxime, ceftriaxone, meropenem, vancomycin, daptomycin, erythromycin, levofloxacin, chloramphenicol, clindamycin and linezolid, and were resistant to tetracycline. Using the Interpretive Categories and MIC breakpoints listed for *Streptococcus pneumoniae*, all three novel isolates tested susceptible to rifampin and cefuroxime, and two of the three strains showed an intermediate MIC to trimethoprim-sulfamethoxazole. In the previous study, using the Kirby–Bauer disc diffusion method, these isolates tested resistant to trimethoprim-sulfamethoxazole [2].

### DESCRIPTION OF STREPTOCOCCUS AZIZII SP. NOV.

(a.ziz’i. N.L. gen. n. azizii named in honor of Aziz Toma, a contemporary American microbiologist who provided many years of invaluable support to the Laboratory of Comparative Pathology at Memorial Sloan Kettering Cancer Center and Weill Cornell Medicine).

Colonies (diameter, 1 mm) are grey-white and convex, alpha-haemolytic when grown on TSA agar supplemented with 5% sheep blood and incubated for 24 h at 35°C in 5% CO2 atmosphere. Cells are Gram-stain-positive cocci and occur in chains, catalase-negative and non-motile, test optochin-resistant, vancomycin-sensitive and weak to strongly positive for pyrrolidonyl arylamidase and leucine aminopeptidase tests. Growth occurs weakly or not at all at 10 and 45°C in heart infusion broth. However, light to heavy growth is obtained on Columbia blood agar plates incubated at 45°C. Hippurate and aesculin are hydrolysed. Arginine, urea and starch are not hydrolysed and acetoin is not produced. No gas is

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<td>Raffinose</td>
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*+, Positive, –, negative, w+, weakly positive. The data in this table were generated in the CDC *Streptococcus* Laboratory.
produced in Mann–Rogosa–Sharp broth. The organism does not grow in broth containing 6.5% NaCl or in the presence of bile, pyruvate or tellurite. Acid is produced from inulin, lactose, maltose, mannitol, pullulan, raffinose, ribose, sucrose, trehalose and methyl β-D glucopyranoside. Melibiose and raffinose tests are variable. (Pullulan tests variable and ribose tests negative with the rapid ID32 Strep system.) Acid is not produced from arabinose, glycerol, sorbitol, sorbose, methyl α-glucopyranoside, xylose, D-arabitol, cyclodextrin, D-sorbitol, D-arabitol, glycollgen and melezitose. Enzyme activity is detected for β-glucosidase, β-galactosidase, β-glucuronidase, ala-phenylalanine-proline arylamidase, β-galactosidase, pyrogalluramic acid arylamidase, N-acetyl-β-glucosaminidase and urease, and weakly detected for β-manosidase. No enzyme activity was detected for arginine dihydrolase, α-galactosidase, alkaline phosphatase, N-acetyl-β-glucosaminidase and urase.

The type strain, 12-5202T (=CCUG 69378T=DSM 103678T), was isolated from brain tissue of a 29-day-old male mouse found runted, hunched and with neurologic signs. The CDC Streptococcus Laboratory designation for this strain is SS1966T. Strain 12-5291 was isolated from an oral swab of a 1-month-old male mouse with symptoms of meningitis. A 13-1151-1 was isolated from brain tissue retrieved from a 1-month-old male mouse with symptoms of meningitis. This source information was corrected in a recent (February 2017) erratum to [2]. The DNA G+C content of strain 12-5202T is 42.76 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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