Streptococcus Vaginalis Sp. Nov., a Novel Bacterial Species Isolated From Vaginal Swabs of a Pregnant Woman With Diabetes

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Research Article

Keywords: Streptococcus vaginalis, diabetes, pregnancy, vaginal environment

Posted Date: May 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-520083/v1

Version of Record: A version of this preprint was published at Archives of Microbiology on August 20th, 2021. See the published version at https://doi.org/10.1007/s00203-021-02532-y.

Abstract

Sequences targeted at the V3 and V4 16S rRNA hypervariable regions of a streptococcal strain (P1L01^T) isolated from vaginal swabs of a pregnant woman with diabetes were 100% similar to those of *Streptococcus anginosus* subsp. *whileyi*. However, phylogenetic analysis based on 16S rRNA full-gene sequencing (1562 bp) revealed highest sequence similarity to *Streptococcus periodonticum* (98.65%), followed by *Streptococcus anginosus* subsp. *whileyi* (98.65%), and *Streptococcus anginosus* subsp. *anginosus* (98.44%). Phylogenies of housekeeping genes *rpoB* and *groEL* were compared to improve classification, and the results showed a clear separation between strain P1L01^T and closely related *Streptococcus* type strains. The complete genome of strain P1L01^T consisted of 2,108,769 bp with a G+C content of 38.5 mol%. Average nucleotide identity values, based on genome sequencing, between strain P1L01^T and *Streptococcus periodonticum* KCOM 2412^T, *Streptococcus anginosus* subsp. *whileyi* CCUG 39159^T, and *Streptococcus anginosus* subsp. *anginosus* NCTC 10713^T were 95.48%, 94.33%, and 95.28%, respectively. The highest *in silico* DNA-DNA hybridization value with respect to the closest species was 66.2%, i.e., below the species cut-off of 70% hybridization. The main cellular fatty acids of strain P1L01^T were 16:0, 18:1ω7c, and 14:0. On the basis of phylogenetic, genotypic and phenotypic data, we propose to classify this isolate as representative of a novel species of the genus *Streptococcus*, *Streptococcus vaginalis* sp. nov., in reference to its isolation from vaginal swabs, with strain P1L01^T (=NBRC 114754^T = BCRC 81289^T) as the type strain.

Introduction

The genus *Streptococcus*, a heterogeneous group of Gram-positive bacteria, includes some of the most significant human pathogens – including *S. pyogenes*, *S. pneumoniae* (Megged 2020), *Streptococcus suis* (Yi et al. 2020), *S. iniae, S. parauberis* (Park et al. 2016), and *S. agalactiae* (Group B *Streptococcus*, GBS) (Opinion 2020). Of the 178 different species of species of the genus *Streptococcus* (https://lpsn.dsmz.de/), several are known to cause gynecological and obstetrics infections. Notably, GBS is the predominant infectious organism that causes bacteremia or sepsis in newborns and young infants (Opinion 2020). During labor and delivery, the vertical transmission of GBS from an infected mother to her fetus can also lead to pneumonia or meningitis (Scholl et al. 2016; Yu et al. 2011). For this reason, antibiotics are routinely given for preventing peripartum infections in women who test positive for GBS colonization in the anogenital region (Francois Watkins et al. 2019; Opinion 2020). However, only a limited number of studies have investigated the prevalence of other streptococcal species within the vaginal environment during pregnancy (Al Majid et al. 2020; Kolter and Henneke 2017; Rabe et al. 1988).

The *Streptococcus anginosus* group is a subgroup of viridans streptococci that comprises three closely related species: *S. intermedius*, *S. constellatus, and S. anginosus*, of which the last named is divided into two subspecies (*S. anginosus* subsp. *anginosus* and *S. anginosus* subsp. *whileyi*). In the current study, a streptococcal strain (termed P1L01^T) was isolated from vaginal swabs of a pregnant woman with type 2 diabetes at 35 weeks of gestation. 16S rRNA gene-based phylogenetic analyses were untaken, and species that showed > 98.5% similarity were selected for further characterization and comparison (Lim et al. 2019). Phylogenies of housekeeping genes *rpoB* (Glazunova et al. 2006; Saito et al. 2016) and *groEL* (Glazunova et al. 2006; Niu et al. 2017; Zbinden et al. 2012)were also analyzed to improve classification. While sequences targeted at the V3 and V4 16S rRNA hypervariable regions were 100% similar to those of *Streptococcus anginosus* subsp. *whileyi*, phylogenies of housekeeping genes *rpoB* and *groEL* revealed a clear separation between P1L01^T and related type strains of *Streptococcus*. Herein, we investigated the taxonomic status of strain P1L01^T and propose to classify this isolate as representative of a novel species of the genus *Streptococcus*, *Streptococcus vaginalis* sp. nov.

Materials And Methods

Sample isolation

As recommended by the National Health Bureau Care Program (Taiwan), vaginal swabs of a Taiwanese pregnant woman with type 2 diabetes were obtained at 35 weeks of gestation as part of screening for GBS colonization. Swab specimens were submitted for bacterial species identification. Under institutional review board approval (IRB number: 201701371A3), the patient gave written informed consent. Strain P1L01^T was isolated from cultures grown at 37°C in brain-heart infusion (BHI) medium under anaerobic conditions. As the patient was at high risk for preterm labor, steroids and antibiotics were administered at 37 weeks of gestation to accelerate lung maturation and prevent peripartum infections.

Genomic features and gene phylogeny

Genomic DNA was extracted from strain P1L01^T using the QIAGEN DNeasy Blood & Tissue kit (QIAGEN, Valencia, CA, USA). Phylogenies of housekeeping genes *rpoB* and *groEL* were compared to improve classification within the genus *Streptococcus* (Glazunova et al. 2006; Niu et al. 2017; Saito et al. 2016; Zbinden et al. 2012). Genomic sequences of the 16S rRNA, *rpoB*, and *groEL* genes were obtained from the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) using ARSA (accession number: NZ_CP034543, NZ_CP012805, and NZ_LR134283). Neighbour-joining phylogenetic analyses, based on 16S rRNA gene sequence comparison, were performed using the EzBioCloud database (http://www.ezbiocloud.net/identify). Sequence homology analyses for the *rpoB* and *groEL* genes were undertaken using the Genetyx-Win software (version 5.1; Genetyx Corporation, Tokyo, Japan).

The draft genome sequence of strain P1L01^T was obtained with an Illumina MiniSeq system (Illumina, San Diego, CA, USA) using the 600-cycle MiSeq Reagent Kit v3. *De novo* assembly of reads was performed using the SPAdes (version 3.10.1) (Bankevich et al. 2012). Gene annotation of the draft sequence was carried out using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html) (Klimke et al. 2009). Average nucleotide identity (ANI) values was calculated with Orthologous Average Nucleotide Identity tool (Lee et al. 2016). Digital DNA-DNA hybridization (dDDH) values were determined using Formula 2 of the Genome-to-Genome Distance (GGD) Calculator (Meier-Kolthoff et al. 2013).

Sequence alignment was carried out using the CLUSTAL W software (Thompson et al. 1997) and the MEGA X package (Kumar et al. 2018) was used for all analyses. Phylogenetic trees were constructed with different methods – including maximum-likelihood (ML) with the Jukes-Cantor model (Chor et al. 2006), minimum-evolution (ME) (Rzhetsky and Nei 1992) with the Tamura-Nei model (Tamura and Nei 1993) and neighbour-joining (NJ) with the Kimura's two-parameter model (Kimura 1980; Saitou and Nei 1987) – using 1000 bootstrap replications.

Phenotyping

Sheep blood (final concentration, 5% in BHI medium) was used for hemolytic activity assays. Fermentation capacity was examined using a commercial kit (API 50 CHL; bioMérieux, Lyon, France) according to the manufacturer's protocol. Halotolerance and ranges of permissive growth temperatures and pH were determined as described by Kozaki et al. (1992). The biochemical profiles of P1L01^T and closely related strains (*S. periodonticum*, *S. anginosus* subsp. *whileyi*, and *S. anginosus* subsp. *Anginosus*) were assessed using the API ZYM and API Rapid ID 32 Strep systems (bioMérieux). Whole-cell fatty acid methyl ester (FAME) profiling was performed using the Sherlock Microbial Identification system (MIDI). After incubation of bacterial cells on solid BHI medium for 48 h at 30°C under anaerobic conditions, FAME extraction and preparation were carried out as described by Sasser (1990).

Results

Gene phylogeny

The 16S rRNA gene sequences (1562 bp) of strain P1L01^T were compared with those stored in the EzBioCloud database. Three species – *Streptococcus periodonticum* JCM 33300^T (Japan Collection of Microorganisms, Ibaraki, Japan), *Streptococcus anginosus* subsp. *whileyi* DSM 25818^T (DSMZ GmbH, Braunschweig, Germany), and *Streptococcus anginosus* subsp. *anginosus* BCRC 14730^T (BCRC, Hsinchu, Taiwan) – showed > 98.0% similarity with strain P1L01^T on sequences targeted at the 16S rRNA region (Table 1). The highest sequence similarity was observed with *Streptococcus periodonticum* (98.65%), followed by *Streptococcus anginosus* subsp. *whileyi* (98.65 %), and *Streptococcus anginosus* subsp. *anginosus* (98.44%; Fig. 1). Similar findings were obtained using the NJ and ME algorithms (**Supplementary Figures S1 and S2**). The concatenated sequences (5190 bp) of two housekeeping genes (*rpoB*, 3567 bp; *groEL*, 1,623 bp) in strain P1L01^T had 93.64 – 95.07% identity with those of *S. periodonticum*, *S. anginosus* subsp. *whileyi* and *S. anginosus* subsp. *anginosus* (Table 1). Phylogenetic trees based on concatenated nucleotide alignments of housekeeping genes were constructed using the ML, NJ, and ME algorithms. The results showed a clear separation between P1L01^T and *Streptococcus* reference strains (Fig. 2 **and Supplementary Figures S3 and S4**).

Table 1
Genotypic characteristics of Streptococcus vaginalis sp. nov. P1L01T and the closely related species and subspecies

		Similarity of each gene with strain P1L01^T					P1L01^T		
Species	Strain No.	16S rDNA	*rpoB*	*groEL*	GenBank accession number of genome	GenBank assembly accession No.	G + C content (%)	ANI value (%)	GGD value (%)
S. periodonticum	KCOM 2412^T	98.65%	95.63%	93.83%	NZ_CP034543	GCA_003963555.1	38.95%	95.48%	66.20% [63.3–69.1%]
S. anginosus subsp. *whileyi*	CCUG 39159^T	98.65%	93.28%	94.45%	NZ_CP012805	GCA_000257765.1	38.50%	94.33%	56.50% [53.8–59.3%]
S. anginosus subsp. *whileyi*	MAS624	98.72%	93.27%	94.52%	NZ_AP013072	GCA_000478925.1	38.34%	94.25%	56.70% [53.9–59.4%]
S. anginosus subsp. *anginosus*	NCTC 10713^T	98.44%	95.10%	94.82%	NZ_LR134283	GCA_900636475.1	38.64%	95.28%	61.60% [58.7–64.4%]
S. anginosus subsp. *anginosus*	ATCC 33397^T	98.53%	95.04%	94.82%	NA	GCA_002088025.1	38.63%	95.1%	61.50% [58.6–64.3%]
S. anginosus	NCTC 11062	98.72%	94.09%	95.07%	NA	GCA_901542445.1	38.84%	94.05%	54.90% [52.1–57.6%]
S. anginosus	NCTC 11064	98.66%	96.02%	97.91%	NZ_LR594037	GCA_901542485.1	38.66%	95.82%	64.10% [61.2–66.9%]
S. intermedius	SK54^T	96.56%	95.04%	94.82%	NA	GCA_000258445.1	37.58%	86.96%	32.40% [30–34.9%]
S. intermedius	ATCC 27335^T	96.54%	91.67%	91.19%	NA	GCA_000413475.1	37.66%	87.04%	32.60% [30.2–35.1%]
S. constellatus subsp. *constellatus*	NCTC 11325^T	96.48%	92.09%	92.79%	NA	GCA_900459125.1	38.08%	88.78%	36.40% [34–39%]
S. constellatus subsp. *pharyngis*	CCUG 46377^T	96.39%	92.43%	92.67%	NA	GCA_000474135.1	37.94%	89.13%	36.80% [34.4–39.3%]

NA: Not available.

Genome features

The complete genome of the P1L01^T strain – which consisted of 2,108,769 bp with a G + C content of 38.5 mol% – contained 1,808 protein-coding genes and 53 predicted RNA genes. ANI values, based on genome sequencing, between strain P1L01^T and *Streptococcus periodonticum*, *Streptococcus anginosus* subsp. *whileyi*, and *Streptococcus anginosus* subsp. *anginosus* were 95.48%, 94.33%, and 95.28%, respectively. The dDDH value with respect to the closest species was 66.2% (Table 1).

Biochemical features

The biochemical characteristics of strain P1L01^T were determined in parallel to those of *Streptococcus periodonticum*, *Streptococcus anginosus* subsp. *whileyi*, and *Streptococcus anginosus* subsp. *anginosus* strains. Significant differences for strain P1L01^T were observed compared with other strains with respect to fermentation capacity and enzyme activities (Table 2). The major cellular fatty

acids of strain P1L01^T were $C_{16:0}$ (40.7%), $C_{18:1}$ ω7c (18.0%), and $C_{14:0}$ (14.7%) – with significant differences with the other three strains (**Supplementary Table 1**).

Table 2
Characteristics helpful in differentiating S. vaginalis sp. nov. P1L01T from closely related species of the genus Streptococcus. All data are from this study.

	Strains			
Characteristic	1	2	3	4
Hemolysis	γ	α	α	α
Acid production from (API 50 CHL)				
N-Acetyl glucosamine	+	–	+	+
Amygdaline	+	–	+	+
Melibiose	+	–	–	–
D-Raffinose	+	–	–	+
β-Gentiobiose	+	–	+	+
Enzyme activity (API ZYM)				
Alkaline phosphatase	1	3	2	4
Esterase (C4)	0	1	0	1
Cystine aminopeptidase	1	0	1	2
β-galactosidase	0	3	0	1
α-glucosidase	0	3	1	4
API Rapid ID 32 Strep				
α-galactosidase	+	–	–	–
APPA	–	+	+	+
Strains: 1, strain P1L01^T; 2, *S. periodonticum* JCM 33300^T; 3, *S. anginosus* subsp. *whileyi* DSM 25818^T; 4, *S. anginosus* subsp. *anginosus* BCRC 14730^T.				

Description of Streptococcus vaginalis **sp. nov.**

On the basis of phylogenetic, genotypic, and phenotypic data (Tables 1 and 2), we propose to classify strain P1L01^T as representative of a novel species of the genus *Streptococcus*. In reference to its isolation from vaginal swabs, the name *Streptococcus vaginalis* sp. nov. (vaginalis. L. gen. n. *vaginalis*, of the vagina) is suggested. Cells are Gram-stain-positive, catalase-negative, facultatively anaerobic, non-spore-forming cocci (diameter: 0.5 – 1.0 μm) that can be arranged in pairs, short chains, or small groups. The addition of 5% sheep blood to BHI agar showed γ-hemolytic activity (i.e., no hemolysis) after incubation at 37°C for 48. Growth was observed on BHI agar at 30 – 45°C under anaerobic conditions, but temperatures of 10°C and 50°C were not tolerated. Additionally, the new species tolerated 5.5% – but not 6.5% – NaCl. Growth was observed in a wide range of pH variations (4.5–8.5). Acid production was found from galactose, D-glucose, D-fructose, D-mannose, N-acetyl glucosamine, amygdalin, esculine, arbutine, salicine, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, D-raffinose, and β-gentiobiose. However, no production of acid was observed from glycerol, ribose, mannitol, sorbitol, erythritol, L-arabinose, D-arabinose, D-xylose, L-xylose, adonitol, β-methyl-xyloside, L-sorbose, rhamnose, dulcitol, α-methyl-D-mannoside, α-Methyl-D-glucoside, inuline, melezitose, glycogene, xylitol, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, D-tagatose, 2-keto-gluconate, gluconate, and 5-keto-gluconate. The major cellular fatty acids were 16:0, 18:1 ω7c, and 14:0. Strain P1L01^T (= NBRC 114754^T = BCRC 81289^T) was the type strain.

Discussion

Next-generation sequencing (NGS) is increasingly being applied for the analysis of complex microbial communities (Boers et al. 2019; Graspeuntner et al. 2018). Most published studies in the field have relied on sequences targeted at the V3 and V4 16S rRNA hypervariable regions (319F–806 R). During the analysis of vaginal swabs obtained from a Taiwanese pregnant woman with type 2 diabetes, we isolated a strain (P1L01^T) that could not be identified conclusively to the species level based on 16S V3–V4 rRNA gene sequence analysis. Highest sequence homology at these regions was obtained with *Streptococcus anginosus* subsp. *whileyi* and *Streptococcus anginosus* subsp. *anginosus* (100% and 99.78%, respectively). On analyzing sequences targeted at the V6 region (926F–1100R), strain P1L01^T was distinctly different from *S. anginosus* subsp. *whileyi* (similarity: 90.23%), *S. anginosus* subsp. *anginosus* (similarity: 90.23%), and *S. periodonticum* (similarity: 94.25%). The highest ANI and dDDH values among strain P1L01^T and the *S. periodonticum* type strain were 95.48 % and 66.2%, respectively. While dDDH values were lower than the commonly accepted cut-off (70%), the ANI value between strain P1L01^T and *S. periodonticum* KCOM 2412^T was of uncertain significance (Goris et al. 2007; Rossello-Mora and Amann 2015). Recent studies have suggested that bacterial isolates with ANI values < 95–96% or dDDH values < 70% with respect to type strains of their closest related species and genera should be classified in novel species Chun et al. 2018; Lim et al. 2019). Thus, a hypothesis was set forth that strain P1L01^T might represent a novel species of the genus *Streptococcus*.

Additional phenotyping of strain P1L01^T further supported this possibility. First, strain P1L01^T was γ-hemolytic, whereas the other three strains investigated in this study showed α-hemolytic activity. Second, biochemical analysis of strain P1L01^T demonstrated distinct characteristics compared to other strains in terms of the carbohydrate metabolism – with clear differences in acid production from melibiose, N-acetyl glucosamine, amygdaline, D-raffinose and β-gentiobiose. Additionally, strain P1L01^T can be distinguished from other strains through α-galactosidase and alanyl-phenylalanyl-proline arylamidase activities.

Based on phylogenetic, genotypic and phenotypic data, we therefore propose to classify this isolate as representative of a novel species of the genus *Streptococcus*, *Streptococcus vaginalis* sp. nov., in reference to its isolation from vaginal swabs. However, the question as to whether this novel species should be considered a pathogen or a cause of gynecological and/or obstetrics infections remains unanswered. While we found no clinical signs of infection in the patient from whom *Streptococcus vaginalis* sp. nov. was isolated, further research is needed to examine more rigorously its potential pathogenicity. Notably, bacteria of the *S. anginosus* group – which are closely related to the novel species of the genus *Streptococcus* identified in this study – have been shown to cause anogenital infections (mainly abscesses) and have been isolated in episiotomy site infections (Al Majid et al. 2020; Lampen and Bearman 2005; Rabe et al. 1988). The main virulence factors of the *S. anginosus* group include secreted toxins (exotoxins), hydrolytic enzymes, and capsular polysaccharides. To reveal the virulence potential of *Streptococcus vaginalis* sp. nov., further *in vitro* investigations would be required to evaluate its contribution to the cytotoxicity against human cell lines. Murine vaginal colonization models in preestrogenized mice may also be useful to investigate the mucosal biology of the novel species.

In conclusion, we have confidently recognized a novel species of the genus *Streptococcus*, termed *Streptococcus vaginalis* sp. nov., using phylogenetic, genotypic and phenotypic identification methods. The novel species was isolated from vaginal swabs of a pregnant woman with diabetes and was most closely related to *S. anginosus*. The role of *Streptococcus vaginalis* sp. nov. in the pathogenesis of gynecological and/or obstetrics infections is currently questionable. More studies are necessary to clarify whether the novel species should be considered a pathogen or a cause of human infection.

Declarations

Acknowledgements

We are grateful to Jung-Erh Yang and Hui-Jin Liao for their excellent technical assistance.

Ethical statement

All participants signed an informed consent to authorize the use of samples for research purposes. This study was approved by the Ethics Committee of Research of Chang Gung Memorial Hospital (IRB NO. 201701371A3).

Conflictsof interest: The authors declare that there are no conflicts of interest.

Funding information: This study was supported by Chang Gung Medical Foundation (CMRPG3H0711, CMRPG3J0771, CMRPG3J1731 and CMRPVVJ0031/2).

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Figures

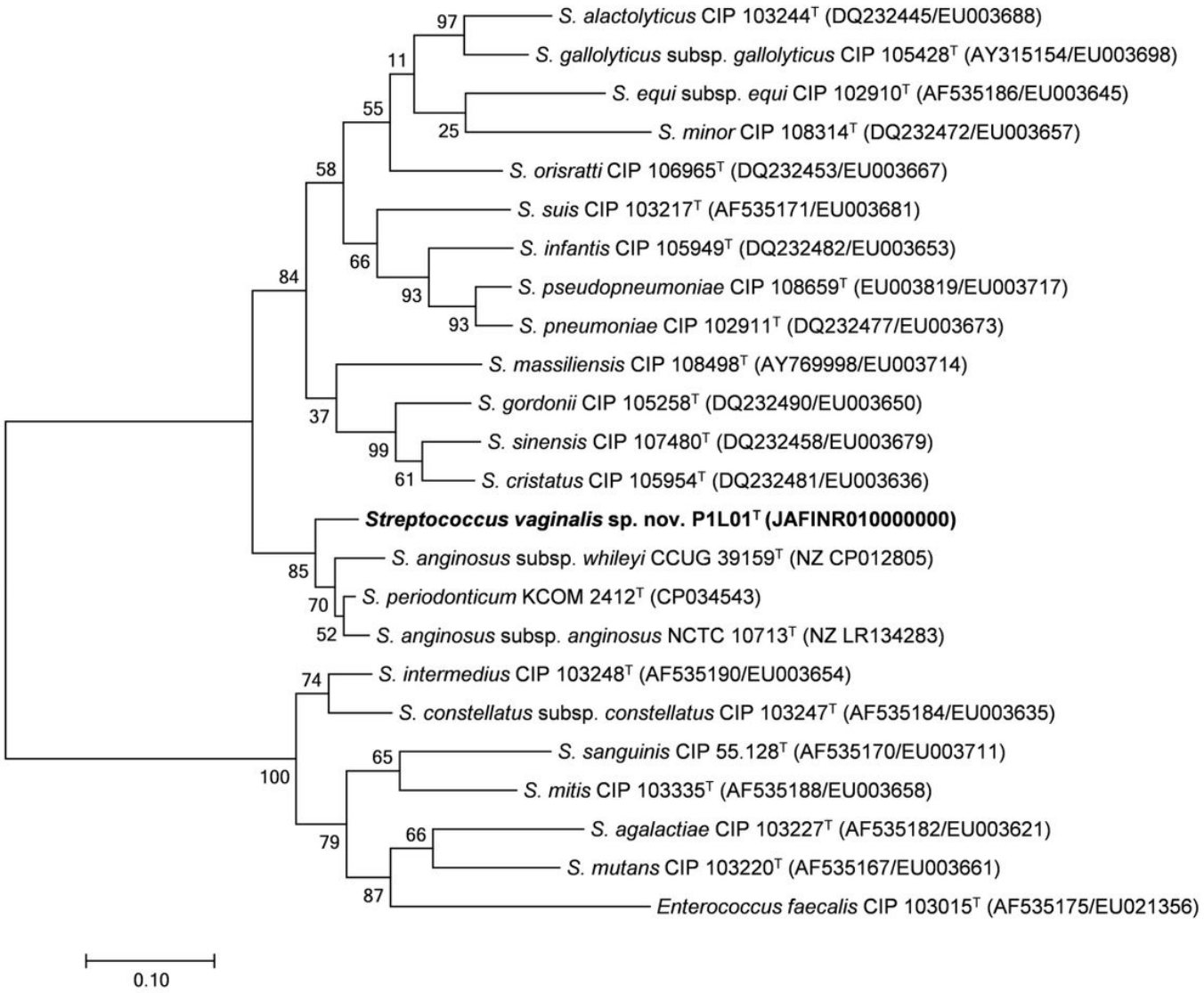

Figure 1

Phylogenetic tree analysis based on maximum-likelihood method with Kimura's two-parameter model of S. vaginalis sp. nov. (strain P1L01T) and closely related species of the genus Streptococcus according to 16S rRNA sequences. GenBank accession numbers are shown in parentheses. Enterococcus faecalis ATCC 19433T was used as an outgroup to root the trees. Numbers indicate bootstrap percentages (based on 1000 replications), and bootstrap values >70% are shown at branch points. The scale bar represents 0.002 substitutions per nucleotide position.

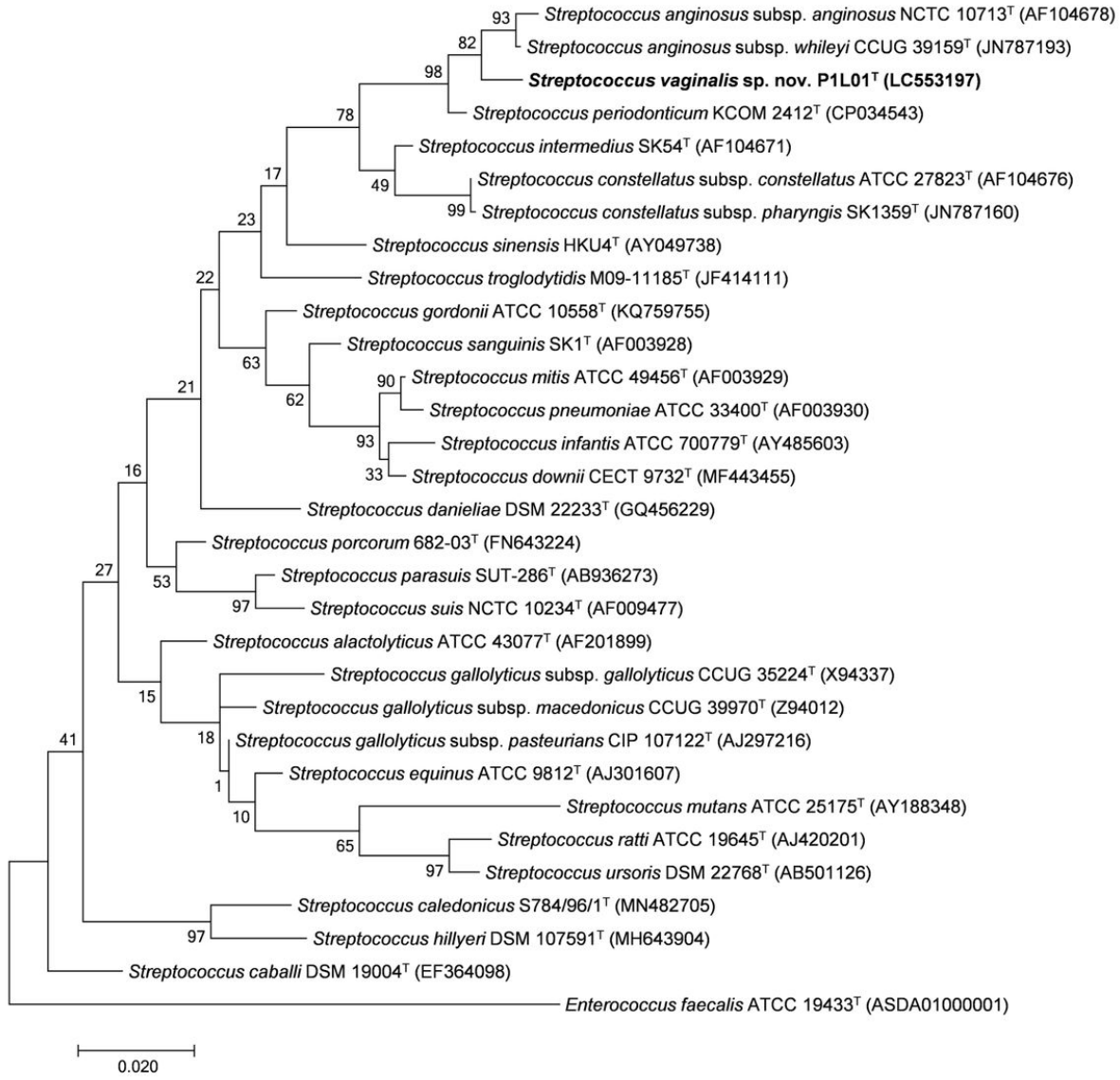

Figure 2

Phylogenetic tree analysis based on concatenated sequences of the two genes (rpoB and groEL) of S. vaginalis sp. nov. (strain P1L01T) and other species of the genus Streptococcus. The tree was reconstructed using the maximum-likelihood method with Kimura's two-parameter model; the target region used for comparison was 1430 bp in length. Enterococcus faecalis CIP 103015T was used as an outgroup to root the trees. Bootstrap values (based on 1000 replications) are shown at branch points. The bar indicates 10% estimated sequence divergence.

Supplementary Files

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- SupplementaryMaterials20210504.docx