Genomic characterization of a potentially novel *Streptococcus* species producing exopolysaccharide

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Abstract

Human oral streptococci, particularly the mitis group, often dwell in the upper respiratory tracts, oral mucosa, and tooth surfaces of healthy individuals. In this work, an α -hemolytic strain, designated LQJ-218, was isolated from the human oral cavity and evaluated for its ability to produce exopolysaccharides. Phylogenetic analysis based on 16S rRNA gene sequences showed that this strain is a potentially novel species belonging to the mitis group streptococci. Whole-genome sequence-based analysis indicated that the genome sequence of *Streptococcus* sp. LQJ-218 was 1,935,194 bp in length, with a mol% G + C content of 40.0, and contained 1897 coding DNA sequences and 91 RNA genes. Furthermore, four biosynthetic gene clusters relevant to exopolysaccharide production were identified in the genome. Both digital DNA–DNA hybridization (yielding a value of 56.60% between strain LQJ-218 and its nearest relative *S. mitis*) and average nucleotide identity analysis (revealing 91.29% identity of LQJ-218 with its nearest relative *S. mitis*) suggested that strain LQJ-218 should be classified as a novel *Streptococcus* species. This potentially novel strain may possess great potential for contributing to the development of new exopolysaccharides. The present study provides valuable genetic information that may be useful in comparative genomics and biotechnological applications.

Keywords Oral streptococci · Whole-genome sequencing · Exopolysaccharide · Novel species

Streptococci are assigned to the family *Streptococcaceae*, order *Lactobacillales*, class *Bacilli* within the phylum *Firmicutes* and are categorized as α -hemolytic, β -hemolytic or γ -hemolytic on the basis of their hemolysis on blood agar.

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A large number of streptococcal species are widespread in both human and animal oral cavities, and these oral bacteria have been phylogenetically distributed into four major clusters, namely, the anginosus, mitis, mutans, and salivarius groups, according to the analyses of the 16S rRNA gene as well as other housekeeping genes (Facklam 2002). Most of these strains are recognized as commensals that often inhabit the upper respiratory tracts, oral mucosa, and tooth surfaces, whereas a few are considered potential opportunistic pathogens. Several studies suggest that the mitis group streptococci (MGS) are closely associated with healthy individuals and may play a pivotal role in exacerbating influenza infection, particularly in immunocompromised individuals. Microbial exopolysaccharides (EPSs) have attracted increasing attention in the health and medical fields due to their unique physiological functions and biological activities, including antioxidant, antitumor, anticoagulant, antiviral, cholesterol-lowering, immunomodulatory, and hypoglycaemic activities (Nwodo et al. 2012; Yu et al. 2018; Zhou et al. 2019).

Although the mitis group currently comprises 19 known species and subspecies (Jensen et al. 2016), confirming

the accurate taxonomic position of newly isolated strains within this cluster remains a challenge. Clinical strains are easily misassigned owing to the limitations of conventional and commercial phenotypic test methods. In addition, the identification of MGS to the species level based on single-gene sequence analyses can also be unreliable, especially when many strains are used to calculate evolutionary distances. With the rapid development and increasing availability of next-generation sequencing (NGS) technology over the past decade, an increasing number of whole genomes from clinical MGS have been sequenced, assembled and annotated, producing adequate NGS data for comparative whole-genome analysis. Due to the increasing availability of NGS data, further exploration of the potential applications of EPS-producing strains is becoming feasible. Here, a potentially novel *Streptococcus* species is described that shows an ability to produce EPS. By studying its genetic characteristics, this strain has potential for application in identifying novel EPSs with unique properties. This novel strain, with its genome fully sequenced, seems valuable for obtaining specific information regarding the genomic basis of EPS production.

An oral specimen suspended in physiological saline solution and obtained from a human oral cavity was uniformly spread on Columbia agar plates containing 5% defibrinated sheep blood (bioMérieux) and incubated aerobically for 48 h at 37 °C. Single colonies with α -hemolytic activity visible on blood agar were selected and purified by transferring them onto fresh preparations of the same agar medium. One of the representative isolates, designated strain LQJ-218, was selected and maintained in a suspension of 20% (v/v) glycerol at –80 °C. This strain was subjected to phenotypic and molecular identification. The 16S rRNA gene of the strain was amplified using the universal bacterial primers 27F/1492R under standard cycling conditions (Gan et al.

2018). Next, the purified PCR product was ligated into the pUC19-T cloning vector and sequenced at Sangon Biotech. The almost-complete 16S rRNA gene sequence was compared to known sequences available from the EzBioCloud server (<https://www.ezbiocloud.net/>). Phylogenetic trees were constructed by MEGA 7.0 (Kumar et al. 2016) using neighbor-joining (Saitou et al. 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Fitch 1971) algorithms with bootstrap analysis of 1000 replicates.

The new isolate was phenotypically characterized as Gram-stain-positive, catalase-negative, chain-forming, and non-spore-forming cocci, 0.5–1.0 μ m in diameter. This strain was shown to utilize several carbohydrates, such as fructose, galactose, glucose, lactose, maltose, and sucrose. It shared the highest 16S rRNA gene sequence similarity with *Streptococcus mitis* NCTC 12261^T (99.39%), with lower similarity values (<98.98%) obtained for other recognized streptococcal species. Figure 1 shows that strain LQJ-218 formed a separate lineage from other MGS with 76% bootstrap support and was distantly related to those strains. Hence, this streptococcal strain can be initially classified as a potentially novel species belonging to the mitis group.

The EPS from *Streptococcus* sp. LQJ-218 was prepared according to a modified version of the method of Zhang et al. (2017). In brief, the activated cells were first grown in seed medium [containing (per liter) 5 g beef extract, 20 g glucose, 10 g peptone, 10 g yeast extract and 10 g NaCl, pH 7.5] at 37 °C for 18 h in a shaking incubator (150 rpm). Subsequently, the seed culture was inoculated into fermentation broth [containing (per liter) 10 g beef extract, 3 g peptone, 7 g yeast extract, and 5 g NaCl, pH 7.2] and cultured under the same conditions as above for 30 h. After centrifugation at 10,000 $\times$ g for 10 min, trichloroacetic acid was added to the supernatant to final concentration of 4% (w/v), and the mixture was continuously stirred at 4 °C overnight. Precipitated

Fig. 1 Neighbor-joining tree inferred from comparison of a 1443-bp portion of the 16S rRNA gene from the closely related *Streptococcus* species. GenBank accession numbers are given in parentheses after strain names. *Lactococcus lactis* subsp. *lactis* JCM 5805^T was used as the outgroup. Bootstrap values are based on 1000 replicates; values higher than 50% are shown. Filled circles indicate nodes also obtained in both maximum likelihood and maximum-parsimony trees. The scale bar indicates the substitution rate per nucleotide

proteins were eliminated by centrifugation at 12,000×g for 20 min at 4 °C, and the resulting solution was dialyzed against distilled water (Mw cutoff 8000–14,000 Da). A threefold volume of chilled absolute ethanol was added to the obtained filtrate, and the mixture was stored at 4 °C overnight to precipitate the EPS. The resulting residue was collected and lyophilized to gain the crude polysaccharide.

To analyze the different functional groups of the EPS extract, the absorption spectra of the freeze-dried product were recorded using an FTIR spectrometer (Nicolet iS10, USA) in the frequency range of 4000–500 cm⁻¹ following the KBr-disk method. As shown in Fig. 2, there were several characteristic absorption bands in the FTIR spectrum of the carbohydrates. The broad and intense peak observed at 3407.06 cm⁻¹ was ascribed to the stretch vibration of O–H. The signal observed at 2935.48 cm⁻¹ was attributed to the stretch vibration of C–H, and the band observed at 1650.11 cm⁻¹ was attributed to the bending vibration of O–H. The peak at 1546.43 cm⁻¹ was related to the vibration of C–O, whereas the absorption at 1381.03 cm⁻¹ was attributed to the stretching vibration of O=S=O in ester sulfate groups (Li and Shah 2014). The band at 1225.42 cm⁻¹ was associated with the stretch vibration of P=O, suggesting the existence of phosphate esters (Chen et al. 2013). The characteristic absorption appearing at 1058.80 cm⁻¹ corresponded to the stretch vibration of C–O–C linkages. The strong peaks in the regions of 916.28 and 814.08 cm⁻¹ indicated the presence of glycosidic linkage bonds between the glycosyl groups in the polysaccharides. Overall, the FTIR spectrum confirmed EPS production of the clinical isolate.

A 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay of the crude EPSs was conducted as described by Zeng et al. (2012), with minor modifications. Briefly, 2 ml of aqueous EPS of varying concentration (0, 2, 4, 6,

8, and 10 mg/ml) was mixed with 2 ml of DPPH ethanol solution (0.2 mM). The mixtures were vortexed intensely and maintained in a dark room for 30 min. After centrifugation of the supernatants at 10,000×g for 10 min, the absorbance was measured at 517 nm. The percent of DPPH radical scavenging ability was calculated according to the formula: scavenging ability (%) = [(A₀ – A₁)/A₀] × 100, where A₀ is the absorbance of the control, and A₁ is the absorbance of the solution containing a sample of given concentration. The results showed that the DPPH-scavenging ratio was in the range of 36.3–81.3% and that scavenging ability significantly increased with increasing sample concentration. Thus, the EPSs from *Streptococcus* sp. LQJ-218 can be considered as potential antioxidants, which attracted our interest.

To mine the genes involved in EPS biosynthesis, *Streptococcus* sp. LQJ-218 was subjected to whole-genome analysis. Genomic DNA from the strain was extracted using a Bacterial DNA Kit (Tiangen, China). The sequencing was performed by Personalbio (Shanghai Personal Biotechnology Co., Ltd, China) using the Illumina HiSeq platform, and it generated a total of 30,268,590 paired-end reads (2 × 151 bp). After quality filtering, the resulting 29,349,506 clean reads were assembled using SOAPdenovo version 2.04 (Li et al. 2010), which provided approximately 2171 × genome coverage. The assembly resulted in 32 contigs (N50, 126,357 bp; maximum length 236,439 bp) linked within 16 scaffolds (N50, 225,969 bp; maximum length 305,906 bp). GeneMarkS version 4.32 (<http://exon.gatech.edu/GeneMark/>) was applied to predict the coding DNA sequences (CDSs) in the whole genome. All the predicted CDSs were searched against the NCBI NR protein database and the Kyoto Encyclopedia of Genes and Genomes (KEGG), Swiss-Prot, Gene Ontology (GO) and Clusters of Orthologous Groups (COG) databases to assign functional annotation. In addition, clustered regularly interspaced short palindromic repeats (CRISPRs), rRNAs and tRNAs were identified using the CRISPRFinder server (<http://crispr.i2bc.paris-saclay.fr/Server/>), Barrnap version 0.4.2 and tRNAscan-SE version 1.3.1, respectively. Digital DNA–DNA hybridization (dDDH) and average nucleotide identity (ANI) analyses were conducted on the Genome-to-Genome Distance Calculator (GGDC) web server version 2.1 (<https://ggdc.dsmz.de/>) and Orthologous Average Nucleotide Identity Tool (OAT) software version 0.90 (<https://www.ezbiocloud.net/tools/orthoani>), respectively.

The genome size of the strain was estimated to be 1,935,194 bp, with a G + C content of 40.0 mol%, and 1897 CDSs, 2 rRNA (5S and 16S) genes, 38 tRNA genes, and 51 other ncRNA genes were located in the genome (Table 1). A total of 1660 protein-coding genes were categorized into 19 groups according to COG class description (Table 2). The CDSs were further annotated and divided into 76 and 42 functional classes through GO and KEGG analyses (Figs.

Fig. 2 FTIR spectrum for the EPS extracted from *Streptococcus* sp. LQJ-218

Table 1 Genomic features of *Streptococcus* sp. LQJ-218

Features	Value
Genome size (bp)	1,935,194
G + C content (%)	40.0
Scaffold number	16
Contig number	32
Scaffold N50 (bp)	225,969
Contig N50 (bp)	126,357
CDSs number	1897
Coding percentage (%)	87.4
rRNA genes (5S & 16S) (no.)	2
tRNA genes (no.)	38
Other ncRNA genes (no.)	51
CRISPRs (no.)	5
NR-annotated genes (no.)	1872
KEGG-annotated genes (no.)	1073
Swiss-Prot-annotated genes (no.)	1379
GO-annotated genes (no.)	1401

S1 and S2). The dDDH and ANI values between strain LQJ-218 and the closely related streptococci are presented in Table 3. According to the proposed standards (Chun et al. 2018), dDDH and ANI values between bacterial genomes of a new isolate and type species can be calculated to determine whether the strain belongs to a known species. In this study, the values of both dDDH and ANI were much lower than the thresholds currently accepted for delineating bacterial

species (< 70% for dDDH and < 95% for ANI; Wayne et al. 1987; Konstantinidis et al. 2005). These results indicated that the species status of strain LQJ-218 was distinct from those of closely related taxa. The genotypic data indicate that strain LQJ-218 should be assigned to a novel species within the genus *Streptococcus*.

Prediction of biosynthetic gene clusters (BGCs) for secondary metabolites was carried out using antiSMASH version 4.2.0 (Weber et al. 2015), and identification and classification of carbohydrate-active enzymes (CAZymes) were achieved using the dbCAN2 meta server through HMMER, DIAMOND, and Hotpep tools (Zhang et al. 2018). Eleven BGCs were inferred from the whole-genome sequence, including bacteriocin ($n = 2$), fatty acid ($n = 1$), putative ($n = 4$), and saccharide ($n = 4$) (Table 4). Notably, four EPS production gene clusters were found in the genome and displayed low similarities with those of other related strains, offering potential insight for the production of new EPSs at the whole-genome level. Furthermore, the CAZymes analysis revealed that glycoside hydrolase (GH) family was the major category (40 genes), followed by glycosyl transferase (GT; 24 genes), carbohydrate binding modules (CBM; 15 genes), and carbohydrate esterase (CE; 10 genes). The results presented herein will facilitate efforts to understand the underlying mechanism of EPS production from *Streptococcus* sp. LQJ-218. Microbial EPSs have been shown to possess different structures depending on the type of microorganism (Hong et al. 2017); due to its genetic characteristics, a potentially novel

Table 2 Number of genes associated with general COG functional categories

Code	Gene count	Percentage (%)	Description
C	46	2.42	Energy production and conversion
D	18	0.95	Cell cycle control, cell division, chromosome partitioning
E	133	7.01	Amino acid transport and metabolism
F	66	3.48	Nucleotide transport and metabolism
G	130	6.85	Carbohydrate transport and metabolism
H	40	2.11	Coenzyme transport and metabolism
I	33	1.74	Lipid transport and metabolism
J	152	8.01	Translation, ribosomal structure and biogenesis
K	113	5.96	Transcription
L	127	6.69	Replication, recombination and repair
M	89	4.69	Cell wall/membrane/envelope biogenesis
N	2	0.11	Cell motility
O	62	3.27	Posttranslational modification, protein turnover, chaperones
P	84	4.43	Inorganic ion transport and metabolism
Q	10	0.53	Secondary metabolites biosynthesis, transport and catabolism
S	405	21.35	Function unknown
T	56	2.95	Signal transduction mechanisms
U	25	1.32	Intracellular trafficking, secretion, and vesicular transport
V	69	3.64	Defense mechanisms
–	237	12.49	Not in eggNOG

Table 3 Digital DNA–DNA hybridization (dDDH) and average nucleotide identity (ANI) values for *Streptococcus* sp. LQJ-218 and closely related streptococci

Species	Accession no.	dDDH values (%)			ANI values (%)	
		Formula 1	Formula 2	Formula 3	OrthoANI	Original ANI
<i>S. mitis</i> B6	NC_013853	56.60	45.50	54.80	91.29	90.99
<i>S. pneumoniae</i> NCTC 7465 ^T	LN831051	54.00	42.60	51.80	90.58	90.12
<i>S. pseudopneumoniae</i> IS7493	NC_015875	52.00	43.40	50.30	91.03	90.56
<i>S. oralis</i> subsp. <i>tigurinus</i> osk_001	NZ_AP018338	51.70	31.00	45.80	85.87	85.51
<i>S. oralis</i> subsp. <i>oralis</i> ATCC 35037 ^T	NZ_GG749268	32.30	30.20	30.70	85.07	84.72
<i>S. infantis</i> ATCC 700779 ^T	NZ_GL732439	32.60	26.80	30.20	82.49	80.81
<i>S. peroris</i> ATCC 700780 ^T	NZ_GL732463	31.50	25.50	29.10	81.72	81.10
<i>S. cristatus</i> NCTC 12479 ^T	NZ_LS483383	17.00	26.40	17.10	76.15	75.50
<i>S. australis</i> ATCC 700641 ^T	NZ_GL636091	16.20	25.20	16.40	75.34	74.29
<i>S. parasanguinis</i> ATCC 15912 ^T	NC_015678	15.70	25.10	15.90	75.00	74.08
<i>S. gordonii</i> NCTC 7865 ^T	NZ_LS483341	15.70	24.10	15.90	74.94	74.14
<i>S. salivarius</i> subsp. <i>salivarius</i> NCTC 8618 ^T	NZ_CP009913	14.40	25.90	14.70	72.70	72.20
<i>S. constellatus</i> subsp. <i>pharyngis</i> C818	NC_022245	14.20	23.50	14.50	73.92	73.51
<i>S. marmotae</i> HTS5 ^T	NZ_CP015196	13.70	25.00	14.00	72.47	72.13
<i>S. sanguinis</i> SK1 ^T	NZ_GL878507	13.70	20.20	14.00	73.41	72.51
<i>S. vestibularis</i> ATCC 49124 ^T	NZ_GL831112	13.60	26.80	14.00	72.69	71.77

Table 4 Biosynthetic gene clusters for secondary metabolites identified in *Streptococcus* sp. LQJ-218 with antiSMASH 4.2.0

Cluster	Type	Start	Stop	Similarity to homologous gene clusters
1	Cf_saccharide	77,965	100,349	CMKL01000005_c1 (32% of genes show similarity)
2	Bacteriocin	117,108	135,156	CLOZ01000009_c1 (46% of genes show similarity)
3	Cf_saccharide	122,089	144,165	CGXP01000004_c1 (35% of genes show similarity)
4	Cf_putative	219,034	223,171	CJZN01000003_c1 (8% of genes show similarity)
5	Cf_putative	117,241	127,621	CRMK01000002_c1 (7% of genes show similarity)
6	Cf_fatty_acid	6952	32,246	CLAL01000013_c1 (40% of genes show similarity)
7	Cf_putative	58,097	64,315	CLNR01000002_c1 (28% of genes show similarity)
8	Cf_putative	63,178	67,918	AEVD01000017_c1 (12% of genes show similarity)
9	Bacteriocin	16,767	28,920	CKBD01000006_c1 (18% of genes show similarity)
10	Cf_saccharide	1	26,818	CMJA01000018_c1 (54% of genes show similarity)
11	Cf_saccharide	31,036	51,693	CFHA01000001_c1 (9% of genes show similarity)

species is likely to synthesize EPSs with unique structures. These findings will encourage further exploration of the fine structures, structural–functional relationships and bioactivities of polysaccharides produced by this novel strain.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Streptococcus* sp. LQJ-218 is MH636838. The Whole-Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession QQRU00000000. The version described in this paper is version QQRU01000000.

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Compliance with ethical standards

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

Ethical approval This work does not contain any studies with human participants or animals performed by any of the authors.

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