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Peptostreptococcus faecalis sp. nov., new bacterial species isolated from healthy indigenous congolese volunteer

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24 **ABSTRACT**

25 The Microbial Culturomics Project aiming to discover several bacterial species made it
26 possible to isolate the strain Marseille-P4308^T from a stool sample of a healthy indigenous
27 Congolese volunteer. Strain Marseille-P4308^T is a Gram-positive coccus shaped bacterium
28 that optimally grows at 37°C. The 16S rRNA gene sequence of the strain has a 96.2%
29 sequence similarity to *Peptostreptococcus anaerobius* strain NCTC 11460^T (GenBank
30 accession number: NR_042847.1). In addition, the average nucleotide identity of strain
31 Marseille-P4308^T with its closest related species was 71.1%, which was far below the
32 recommended threshold (>95-96%). The genome of the strain Marseille-P4308^T has a length
33 of 2.14 Mbp with G+C content of 30.4 mol%. Based on phenotypic, biochemical, genomic
34 and phylogenetic analysis, strain Marseille-P4308^T (= CSUR P4308 = CECT 9960) clearly
35 appears to be a new species for which the name *Peptostreptococcus faecalis* sp. nov., is
36 proposed.

37 INTRODUCTION

38 Different genera of anaerobic cocci bacteria are involved in a broad range of infections,
39 occurring in all parts of the human body [1]. In various environments, members of the
40 *Peptostreptococcaceae* family belonging to the order *Clostridiales*, phylum *Firmicutes* can be
41 found in the human body, manure, soil and sediment [2]. The closest phylogenetic neighbours
42 to this family belong to the genera *Alkaliphilus*, *Natronincola*, and *Tindallia* [2]. The species
43 of the genus *Peptostreptococcus* are commensal species that colonise almost every mucosal
44 human tissue, forming a part of the intestinal, urinary, vaginal, oral tract microbiotas and also
45 the skin. They are pathogenic under certain circumstances and can cause bacteraemia and
46 abscesses in different organs [3].

47 The genus *Peptostreptococcus* is a group of Gram-positive, mostly anaerobic cocci species
48 that are very diverse phenotypically and phylogenetically [4]. Cells measure between 0.3 and
49 2.0 μm and are arranged in chains, pairs, tetrads or masses. Some are aero-tolerant but do not
50 form spores. Their ability to use carbohydrates varies considerably. The main source of
51 energy would be the products of protein metabolism [1]. However, the pathogenic character
52 of some members of the genus *Peptostreptococcus* is complex to determine, because some
53 members are part of the normal microflora, while species such as *Peptostreptococcus*
54 *anaerobius* and *Peptostreptococcus stomatis* have been frequently identified from clinical
55 samples of diseased individuals [5–7]. Indeed, *Peptostreptococcus russellii* was isolated in
56 environment as a storage pit [8].

57 Numerous previously uncultured members of the human microbiome have been isolated by
58 the culturomics method [9]. Isolated species are then described by multiphasic approach
59 including a MALDI-TOF MS identification, 16S rRNA gene sequencing and the phenotypic
60 and biochemical analysis of the strain [10]. We report here a taxonogenomic description of

61 *Peptostreptococcus faecalis* sp. nov., strain Marseille-P4308^T isolated from human gut
62 microbiota.

63 MATERIAL AND METHODS

64 *Ethics and sample collection*

65 The stool sample was collected from a healthy indigenous Congolese volunteer living in Bene
66 Gain (1°59'24.7"S 15°52'18.1"E), in the Republic of the Congo, in August 2015
67 (supplementary Figure S1) [11]. Approval for this study was obtained from the Ministry for
68 Health of the Republic of Congo (000208/MSP/CAB.15 du Ministère de la Santé et de la
69 Population, 20 August 2015). The study was also approved by the ethics committee of the
70 Institut Fédératif de Recherche IFR48, Faculty of Medicine, Marseille, France, under
71 reference number 09-022. Prior to sampling, an informed consent form was obtained from
72 each individual. In the presence of representatives from a local health centre and village
73 elders, full information was given orally in the local language (Lingala) to ensure that the
74 project was fully understood, as the participants were illiterate.

75 *Strain isolation and growth conditions*

76 One gram of the stool sample was pre-incubated in a blood culture bottle containing rumen
77 and sheep blood, as described above for culturing human samples [12]. The culture medium
78 was serially diluted and inoculated on Columbia agar with 5% sheep blood (BioMérieux,
79 Marcy-L'Etoile, France). All pure colonies obtained by culture were identified by MALDI-
80 TOF MS [13]. If, despite the good quality of the bacterial spectrum, the bacteria could not be
81 identified, sequencing of the 16S rRNA gene of the bacterium was performed for
82 identification.

83 *16S rRNA sequencing and phylogenetic analysis*

84 The DNA of the bacterial strain was extracted using the EZ1 (Qiagen, Venlo, The
85 Netherlands) DNA tissue kit on the EZ1 (Qiagen) automate. The universal primers fD1 and

86 rP2 (Eurogentec, Angers, France) were used to amplify the 16S rRNA gene sequence.
87 Sequencing was performed using the BigDye® Terminator v1.1 Cycle Sequencing Kit and
88 ABI Prism 3130xl Genetic Analyzer capillary sequencer (Thermo Fisher, Saint-Aubin,
89 France), as previously described [14]. Using Codon Code Aligner software
90 (<http://www.codoncode.com>), the 16S rRNA nucleotide sequences were assembled and
91 corrected. Consensus sequence from 16S rRNA gene sequencing was compared by BLASTn
92 within the NCBI 16S rRNA database (<https://blast.ncbi.nlm.nih.gov/>). In order to create a
93 robust phylogenetic tree, the 16S rRNA sequences of species with a validly published name
94 were downloaded from the LPSN website (<https://lpsn.dsmz.de/>). Using MEGA X software
95 [15], the sequences were aligned and a phylogenetic tree was constructed with 1,000 bootstrap
96 replicates.

97 *Genome annotation and comparison*

98 Genomes of closely related species were downloaded from the GenBank database and
99 annotated with Prokka software v1.14.6 [16]. Coding sequences were predicted using
100 Prodigal software 2.6 [17], then the predicted bacterial protein sequences were searched
101 against the GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) database using BLASTp.
102 ARAGORN software 1.2 [18] was used to find tRNA and mRNA genes, whereas rRNA
103 genes were predicted with Barrnap software 0.4.

104 The genome of strain Marseille-P4308^T was compared to the genomes of the following
105 closely related species, including *Peptostreptococcus anaerobius* NCTC11460^T (GenBank
106 accession: UGTB000000000), *Peptostreptococcus russellii* RT-10B^T (JYGE000000000),
107 *Peptostreptococcus stomatis* DSM 17678^T (ADGQ000000000), *Peptostreptococcus canis*
108 DSM 27025^T (JABGBW000000000), *Asaccharospora irregularis* DSM 2635^T
109 (FQWX000000000), *Clostridioides difficile* ATCC 9689^T (AUOX000000000), *Clostridioides*
110 *mangenotii* LM2 (JIAA000000000) and *Clostridium hiranonis* DSM 13275^T (CP036523). The

111 DNA-DNA hybridisation (dDDH) was calculated to assess similarity between studied
112 genome sequences using the online server of Genome-to-Genome Distance Calculator
113 (GGDC 2.1) (<http://ggdc.dsmz.de/>), taking into account the method and formula two, as
114 suggested [19,20]. Furthermore, the genomic average nucleotide identity (ANI) was
115 determined using OrthoANI software v0.93.1 [21]. Clusters of Orthologous genes (COGs)
116 were detected by performing BLASTp of genomes against the COG database [22].

117 *Conditions of growth*

118 The strain was grown under different conditions. In terms of temperature, the growth of the
119 strain was evaluated at room temperature, 28°C, 37°C, 45°C and 55°C and incubated under
120 aerobic, anaerobic and microaerophilic conditions on Colombia agar enriched with 5% sheep
121 blood (BioMérieux), using the GENbag anaer and GENbag microaer systems (ThermoFisher
122 Scientific, Basingstoke), respectively. To determine the tolerated salt concentration and the
123 optimum pH for growth, the strain was cultured in different media with varied pH (6, 6.5, 7
124 and 8.5) and at different NaCl concentrations (5, 10, 50, 75 and 100 g/L NaCl) at 37°C under
125 anaerobic conditions.

126 *Phenotypic characteristics*

127 Motility test and Gram staining of the strain were verified using a DM1000 photomicroscope
128 (Leica Microsystems, Nanterre, France) under a 100× objective. In addition, a bacterial
129 suspension was fixed with a 2.5% glutaraldehyde solution at 0.1 mol/L with the aim of
130 observing the morphology of cells using a Hitachi TM4000 electron microscope (Hitachi
131 Group, Krefeld, Germany). Spore-forming was investigated by exposing a bacterial
132 suspension for 10 minutes under thermal shock at 80°C. Multiple biochemical criteria from
133 strain Marseille-P4308^T were revealed using API tests (50CH, ZYM and 20A; bioMérieux).
134 Oxidase and catalase reactions were determined using a BD BBL™ DrySlide (Becton
135 Dickinson, Le Pont-de-Claix, France).

136 *Antibiotic susceptibility*

137 The E test method was used to obtain an antimicrobial sensitivity profile and minimum
138 inhibitory concentration ($\mu\text{g/mL}$) of the strain Marseille-P4308^T [23]. Antimicrobial discs
139 placed on a blood agar Petri dish were amikacin, amoxicillin, benzylpenicillin, ceftazidime,
140 ceftriaxone, ciprofloxacin, clindamycin, daptomycin, erythromycin, imipenem, linezolid,
141 metronidazole, minocycline, rifampicin, teicoplanin, tigecycline,
142 trimethoprim/sulfamethoxazole, tobramycin and vancomycin.

143 **RESULTS**

144 *Identification and classification*

145 Analysis of the 16S rRNA gene sequence on NCBI Blast showed that the Marseille-P4308^T
146 strain has the highest similarity score of 96.2% to *Peptostreptococcus anaerobius* strain
147 NCTC 11460^T (GenBank accession number: NR_042847.1). This value obtained is below the
148 threshold value recommended for delimiting a new species of prokaryote [24]. Therefore, the
149 strain Marseille-P4308^T is presumed to be a potential new bacterial species belonging to the
150 genus *Peptostreptococcus* within the *Peptostreptococcaceae* family and phylum *Firmicutes*.
151 The phylogenetic tree constructed on the basis of the sequences of the 16S rRNA gene shows
152 the strain Marseille-P4308^T anchored in the *Peptostreptococcus* sp group with closely related
153 species (Figure 1). The position is confirmed with the phylogenomic tree basing on the
154 genomic sequences of the closely related species (Figure 2).

155 *Phenotypic characteristics*

156 Strain Marseille-P4308^T grows under anaerobic and micro-aerobic conditions between 25°C
157 and 42°C, with an optimal growth at 37°C. It is a Gram-positive coccus-shaped bacterium,
158 non-motile, non-spore-forming, oxidase-negative and catalase-negative. Using a Hitachi
159 electron microscope, the strain's morphology is highlighted; cells had a mean length of 0.84
160 μm and a mean diameter of 0.7 μm (supplementary Figure S2). Furthermore, this strain was

161 able to grow on media at a pH between 6 and 8.5 and tolerated NaCl concentration up to 50
162 g/L. Colonies were smooth, convex and regular in appearance, with a mean diameter of 0.5
163 mm on Columbia Agar with 5% Sheep Blood (bioMérieux).

164 Using API 50CH, the following carbohydrates are fermented: glycerol, erythritol, D-
165 arabinose, D-ribose, L-xylose, D-adonitol, methyl β -D-xylopyranoside, D-galactose, D-
166 fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α -D-
167 mannopyranoside, methyl α -D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin,
168 esculin ferric citrate, D-cellobiose, D-melibiose, D-saccharose, D-trehalose, inulin, D-
169 melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, D-lyxose, D-tagatose, L-
170 fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 5-ketogluconate. Negative
171 reactions were observed for D-glucose, D-lactose, D-maltose, D-mannitol, D-turanose, D-
172 xylose, salicin and sucrose. Indeed, the use of API ZYM strips revealed positive reactions for
173 esterase, leucine arylamidase, cystine arylamidase, α -glucosidase and α -galactosidase, while
174 the following tests, such as alkaline phosphatase, esterase lipase, lipase (C14), valine
175 arylamidase, trypsin, α -chymotrypsin, acid phosphatase, β -galactosidase, β -glucosidase, N-
176 acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase were negative. Finally, the use of
177 API 20A strips shows that strain Marseille-P4308^T was positive for gelatin and esculin ferric
178 citrate, while it was negative for tryptophan, urea, D-glucose, D-mannitol, D-lactose, D-
179 sucrose, D-maltose, salicin, and D-xylose. The comparison of the main phenotypic and
180 chemical differences between strain Marseille-P4308^T and its phylogenetically related species
181 is given in Table 1.

182 Antimicrobial susceptibility testing of strain Marseille-P4308^T showed the following minimal
183 inhibitory concentrations (in parenthesis) for the following antibiotics: ceftazidime (0.75
184 μ g/mL), trimethoprim (0.25 μ g/mL), daptomycin (0.016 μ g/mL), tobramycin (8 μ g/mL),
185 benzylpenicillin (5 μ g/mL), ciprofloxacin (1 μ g/mL), ceftriaxone (0.5 μ g/mL), linezolid

186 (0.094 µg/mL), ciprofloxacin (0.75 µg/mL), clindamycin (0.064 µg/mL) and amikacin (16
187 µg/mL).

188 *Genome properties and comparison*

189 Strain Marseille-P4308^T has a genome size of 2,145,294 bp, assembled into 29 contigs with
190 30.4 mol% G+C content. Its genome possesses 2,095 predicted genes, of which 2,031 are
191 protein-coding genes and 64 code for RNAs (9 rRNA genes, 53 tRNA genes and 2 tmRNA
192 gene). Of the 2,031 protein-coding genes, 1,442 were assigned to COG functional categories.
193 The genomic structure of the strain is represented by a circular map showing the coding
194 regions of the genome (supplementary Figure S3). The genome of strain Marseille-P4308^T
195 was compared with eight other genomes belonging to closely-related species in the
196 *Peptostreptococcaceae* family (Table 2). Among the *Peptostreptococcus* species, strain
197 Marseille-P4308^T has the second largest genome (2.14 Mbp), shorter only than the genome of
198 *Peptostreptococcus anaerobius* ATCC 27337^T (2.25 Mbp). The distribution of genes among
199 the different COG categories is almost the same proportion in all the genomes of the
200 compared *Peptostreptococcus* species (supplementary Figure S4).

201 The DDH values obtained after genomic analysis vary for strain Marseille-P4308^T from
202 18.9% with *Asaccharospora irregularis* DSM 2635^T to 23.7% with *Clostridioides mangenotii*
203 DSM 1289^T. Among species within *Peptostreptococcus* genus, DDH values ranged from
204 19.5%, between *P. faecalis* Marseille-P4308^T and *P. canis* CCUG 57081^T, to 27.9% between
205 *P. stomatis* W2278^T and *P. anaerobius* ATCC 27337^T (Table 3). These values are therefore
206 lower than the recommended threshold value of 70% for predicting a new prokaryote species
207 [19,24]. However, on this basis, strain Marseille-P4308^T is considered to be a new species in
208 the genus *Peptostreptococcus*. In addition, among the *Peptostreptococcus* species studied, the
209 lowest OrthoANI value was 71.16% (between *P. faecalis* Marseille-P4308^T and *P. anaerobius*
210 ATCC 27337^T or *P. stomatis* W2278^T) while 73.25% (between *P. canis* CCUG 57081^T and *P.*

211 *russellii* RT-10B^T) was the highest OrthoANI value obtained in this analysis (Table 3). The
212 mean nucleotide identity (ANI) analysis based on the genomes of species close to the
213 Marseille-P4308^T strain revealed genomic sequence similarities of less than 80%. Indeed, the
214 cut-off value of 95 ~ 96% was recommended to delineate the species barrier in
215 prokaryotes[25,26]. Therefore, our strain Marseille-P4308^T sharing with its all other related
216 species is considered as a new bacterial species.

217 **Conclusion**

218 Based on the results of phylogenetic, phenotypic and biochemical analyses, it appeared that
219 *Peptostreptococcus faecalis* sp. nov., strain Marseille-P4308^T, is formally considered as a new
220 species within the genus *Peptostreptococcus*. The type strain of this new species is Marseille-
221 P4803^T.

222 **Description of *Peptostreptococcus faecalis* sp. nov.**

223 *Peptostreptococcus faecalis* (fae.ca'lis. N.L. fem. adj. *faecalis* of faeces). Bacterial colonies
224 of strain Marseille-P4308^T are convex, smooth and regular with a mean diameter of 0.7 mm
225 on blood agar. Cells are non-motile and non-spore-forming. They are Gram-positive cocci
226 bacteria and are oxidase and catalase negative. Glycerol, erythritol, D-arabinose, potassium
227 gluconate, D-ribose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-
228 fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-
229 mannopyranoside, methyl α-D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin,
230 esculin ferric citrate, D-cellobiose, D-melibiose, D-saccharose, D-trehalose, inulin, D-
231 melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, D-lyxose, D-tagatose, L-
232 fucose, D-arabitol, L-arabitol, potassium 5-ketogluconate, esterase, leucine arylamidase,
233 cystine arylamidase, α-galactosidase, α-glucosidase and gelatin are fermented. The genome
234 size of strain Marseille-P4308^T is about 2.14 Mbp with a 30.4 mol% of G+C content. The 16S
235 rRNA and genome sequences are deposited in the GenBank database under Accession
236 numbers LT960583 and OERU00000000, respectively.

237 The type strain Marseille-P4308^T (=CSUR P4308 = CECT 9960) was isolated from stool
238 sample from a healthy indigenous Congolese volunteer.

239 **Author Contributions:**

240 Conceptualization and supervision, C.I.L., P.E.F., F.F., and D.R. Methodology, C.I.L., J.A.,
241 O.M., and F.F.; Validation, C.I.L. and F.F.; Formal analysis, F.M., C.I.L., R.Z., and G.M.N.;
242 Strain culture and collection, S.A.; Investigation, M.B. and F.M.; Writing-original draft
243 preparation, F.M., and R.Z.; Writing-review and editing, C.I.L.; P.E.F., J.A., O.M., F.F., and
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258

259 **Conflicts of interest:**

260 None to declare.

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375 **Table 1.** Differential characteristics of Marseille-P4308^T, *Peptostreptococcus stomatis* W2278^T [5], *Peptostreptococcus canis* CCUG 57081^T
 376 [27], and *Peptostreptococcus anaerobius* ATCC 27337^T [28].

Characteristics	Marseille-P4308 ^T	W2278 ^T	CCUG 57081 ^T	ATCC 27337 ^T
Oxygen requirement	Strictly anaerobic	Strictly anaerobic	Facultative anaerobic	Strictly anaerobic
Acid production from :				
D-Cellobiose	+	-	-	+
α-Galactosidase	+	+	-	+
D-Glucose	-	+	-	+
α-Glucosidase	+	+	NP	+
D-Lactose	-	-	-	+
D-Mannose	+	-	+	NP
D-Raffinose	+	-	-	NP
Sucrose	-	-	-	+
G+C content (mol%)	30.4	36	30.8	34-36
Habitat	Human	Human	Dog	Cat

377 NP, not performed.

378 **Table 2.** Comparison of the size, the content of G+C mol% and the number of proteins of the
 379 genome of *Peptostreptococcus faecalis* sp. nov., strain Marseille-P4308^T with the other
 380 genomes of related species.

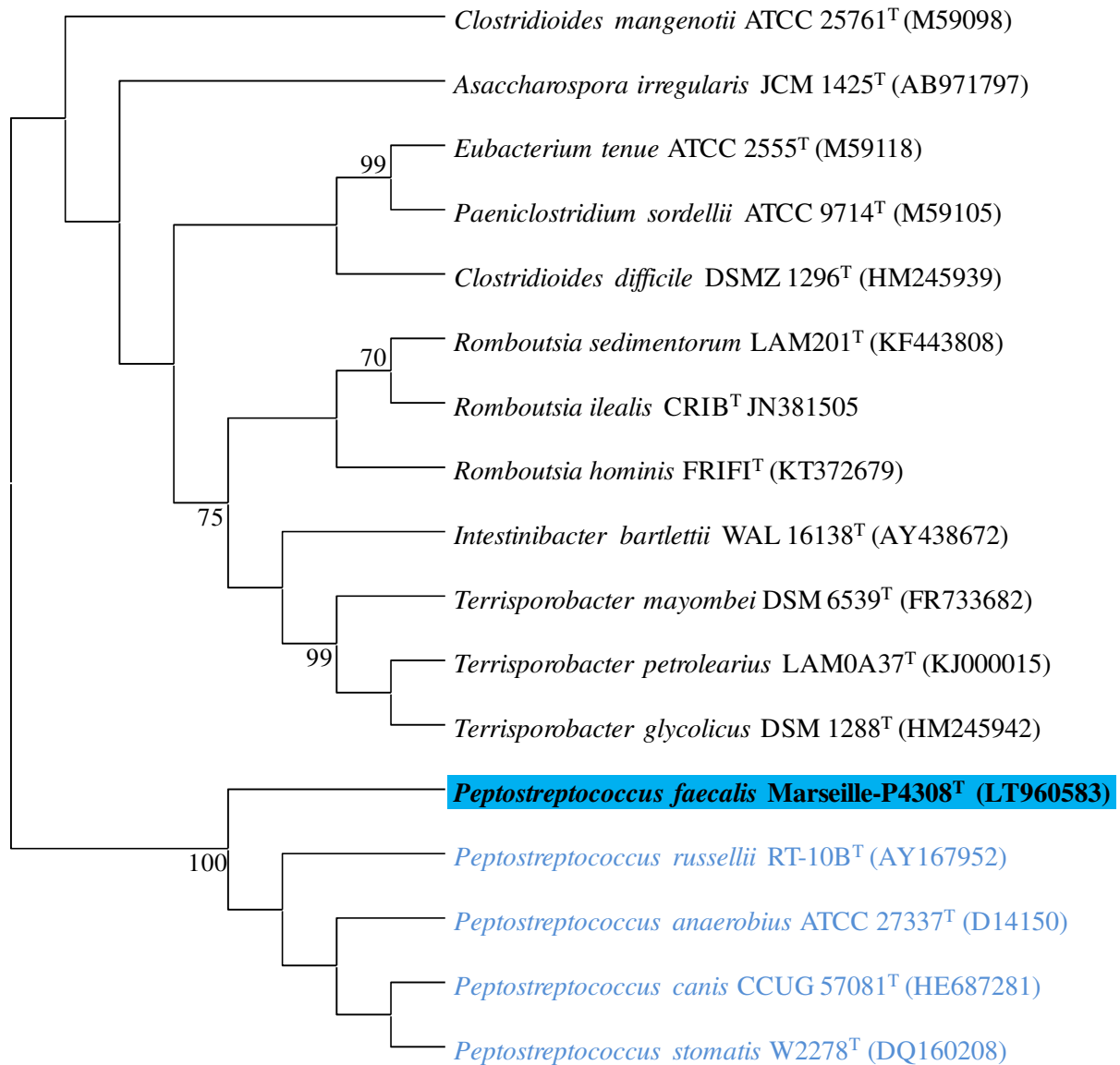
Species	Strain Number	Genbank accession	Size (bp)	G+C mol%	Nb of proteins
<i>Peptostreptococcus faecalis</i>	Marseille-P4308 ^T	OERU000000000	2,145,294	30.4	2,031
<i>Peptostreptococcus anaerobius</i>	ATCC 27337 ^T	UGTB000000000	2,256,756	35.7	2,070
<i>Peptostreptococcus canis</i>	CCUG 57081 ^T	JABGBW000000000	2,064,240	30.2	1,834
<i>Peptostreptococcus russellii</i>	RT-10B ^T	JYGE000000000	2,082,949	30.9	1,756
<i>Peptostreptococcus stomatis</i>	W2278 ^T	ADGQ000000000	1,988,044	36.6	1,799

381

382 **Table 3.** Average nucleotide identity (ANI) and dDDH values (%) obtained by pairwise comparison of the nine studied genomes.
 383 Compared genomes: 1, *Peptostreptococcus faecalis* Marseille-P4308^T; 2, *Peptostreptococcus anaerobius* ATCC 27337^T; 3, *Peptostreptococcus canis*
 384 CCUG 57081^T; 4, *Peptostreptococcus russellii* RT-10B^T; 5, *Peptostreptococcus stomatis* W2278^T; 6, *Asaccharospora irregularis* DSM 2635^T; 7,
 385 *Clostridioides difficile* DSM 1296^T; 8, *Clostridioides mangenotii* DSM 1289^T; 9, *Clostridium hiranonis* DSM 13275^T. OrthoANI values are
 386 shown on right bottom (in bold) and dDDH values calculated using GGDC formula 2 software (DDH estimates based on HSP identities / length)
 387 shown on upper left. The empty boxes between them are 100%.

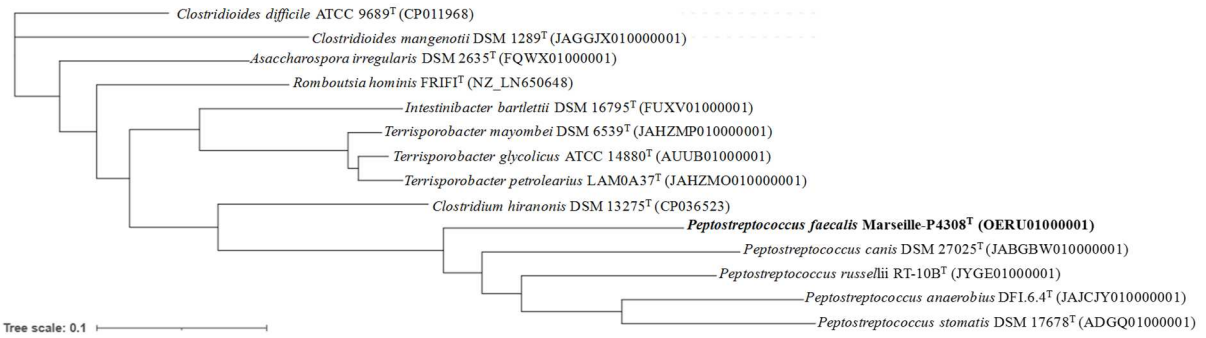
Strains	Marseille-P4308 ^T	ATCC 27337 ^T	CCUG 57081 ^T	RT-10B ^T	W2278 ^T	DSM 2635 ^T	DSM 1296 ^T	DSM 1289 ^T	DSM 13275 ^T
Marseille-P4308 ^T		22.6%	19.5%	21.2%	20.7%	18.9%	19.3%	23.7%	23.1%
ATCC 27337 ^T	71.16%		24.0%	22.5%	27.9%	20.4%	21.3%	28.3%	26.0%
CCUG 57081 ^T	71.17%	71.68%		21.3%	25.1%	18.9%	18.8%	25.8%	25.7%
RT-10B ^T	73.25%	72.26%	73.83%		23.3%	25.7%	19.4%	27.1%	22.6%
W2278 ^T	71.16%	73.83%	71.63%	72.29%		19.4%	21.2%	28.4%	27.8%
DSM 2635 ^T	69.14%	68.03%	68.85%	70.06%	67.83%		21.1%	20.3%	20.7%
DSM 1296 ^T	69.16%	68.00%	69.31%	69.40%	67.60%	75.83%		19.8%	21.6%
DSM 1289 ^T	68.82%	68.28%	68.58%	68.80%	67.50%	72.90%	73.53%		26.1%
DSM 13275 ^T	69.71%	69.54%	69.13%	69.66%	68.38%	72.03%	71.95%	71.65%	

388



389

390 **Figure 1.** Phylogenetic tree with the 16S rRNA gene sequences indicating the position of
 391 *Peptostreptococcus faecalis* sp. nov. Marseille-P4308^T among other closely related species.
 392 Sequences were aligned using MUSCLE, and phylogenetic inferences were obtained using
 393 the Neighbor-Joining method [29] within the MEGA X software [15]. Accession numbers are
 394 indicated in parenthesis.



395

396 **Figure 2.** Phylogenomic tree basing on genomic sequences of *Peptostreptococcus faecalis* sp.
 397 nov., Marseille-P4308^T and other its related species. Sequences were concatenated with union
 398 EMBOSS (<https://www.bioinformatics.nl/cgi-bin/emboss/union>) and aligned by Mugsy
 399 software [30]. The tree was built using iTOL an online bioinformatic tool [31].

400