Description of *Gemella massiliensis* sp. nov., a new bacterium isolated from the human gut.

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Short Report

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Abstract

Thanks to its ability to isolate previously uncultured bacterial species, culturomics has dynamized the study of the human microbiota. A new bacterial species, Gemella massiliensis Marseille-P3249 T , was isolated from a sputum sample of a healthy French man. Strain Marseille-P3249 T is a facultative anaerobe, catalase negative, Gram positive, coccus and unable to sporulate. The major fatty acids were C 16:0 (34%), C 18:1n9 (28%), C 18:0 (15%) and C 18:2n6 (13%). Its 16S rRNA sequence exhibits a 98.3% sequence similarity with Gemella bergeri strain 617-93, its phylogenetically closest species with standing in nomenclature. Its digital DNA-DNA hybridization (dDDH) and OrthoANI values with G. bergeri of only 59.7 ± 5.6% and 94.8%, respectively. These values are lower than the thresholds for species delineation (>70% and >95%, respectively). This strain grows optimally at 37°C and its genome is 1.80 Mbp long with a 30.5 mol% G+C content. Based on these results, we propose the creation of the new species Gemella massilienis sp. nov., strain Marseille-P3249 T (= CSUR P3249 = DSMZ 103940).

Introduction

The human microbiota has been strongly correlated to health and diseases (1) with numerous projects being launched to study its residing bacterial population (2, 3). Indeed, the metagenomics approach led to the production of a significant amount of data which helped the scientific community to better understand the residing population of different microbiota (4). However, several drawbacks can be faced when adopting this approach, such as the presence of unclassified genomic sequences, depth bias and inability to have biological material for further manipulation and studies (5). Thus, culturomics was developed in order to re-introduce the culture approach in the human microbiota description using a more sophisticated methodology (6). The latter relies on culturing samples with 18 different conditions assessing their bacterial content by direct seeding on solid media (7). Isolated colonies are efficiently identified using MALDI-TOF MS or 16S rRNA gene sequencing whenever MALDI-TOF MS fails (8, 9). Using this approach, a significant number of new bacterial species was isolated with some being correlated to previously detected operational taxonomic units (OTUs) (7, 10, 11). Recently, in our institute research was focused on the respiratory microbiota in order to assess its role in health or disease development (12). Accordingly, and as part of the project aiming to decipher the human microbiota, culturomics was applied to human sputum samples with the aim of profiling its bacterial content. Using this process, a new bacterial species, *Gemella massiliensis* strain Marseille-P3249^{T}, was isolated. Herein, we report the taxonogenomic description of this new species that was isolated from the sputum of a healthy French man (13).

Material And Methods

Growth conditions

A bacterial strain was isolated from a sputum sample from a healthy Frenchman by culturomics in order to explore the human microbiome. The study was approved by the ethics committee of the Institut

Federatif de Recherche IFR48 under the number 09–022 and then the patient the patient gave his formal agreement by signing the informed consent. Thus optimal growth conditions of strain Marseille-P3249 were evaluated using various culture conditions. Culture assays were done at 28, 37, 45 and 55°C under anaerobic (GENbag anaer, bioMérieux), microaerophilic (GENbag Microaer, bioMérieux) and aerobic conditions. Tolerance to acidity and halotolerance were evaluated independently with growth assays at pH 6, 6.5, 7 and 8.5 and by using 0, 5, 10, 50, 75 and 100 g/L NaCl concentrations, respectively.

Morphological, Biochemical And Antibiotic Susceptibility Analysis

The main biochemical features of strain Marseille-P3249 were tested using API strips (ZYM, 50CH and 20A (bioMérieux, France)). Motility and Gram stain were checked using a DM1000 photonic microscope (Leica Microsystems, Nanterre, France). Additionally, sporulation was evaluated after exposing a bacterial suspension to a 20 minutes heat shock at 80°C. Cell morphology images were obtained using a scanning electron (SEM) microscope (TM4000 Plus, Hitachi High-Technologies Corp., Tokyo, Japan).

Cellular fatty acid methyl ester (FAME) analyses were performed with GC/MS with 10 mg of bacterial biomass per tube. GC/MS and FAME analyses were performed as previously reported (14).

The minimal inhibitory concentrations (MIC) of strain Marseille-P3249 were evaluated using Etest (bioMérieux) for benzylpenicillin, amoxicillin, cefotaxime, ceftriaxone, imipenem, erythromycin, daptomycin, amikacin, rifampicin, minocycline, teicoplanin, vancomycin, metronidazole and colistin.

DNA Extraction And Genome Sequencing

A total of 82.1 ng/µL of genomic DNA (gDNA) were extracted from strain Marseille-P3249 as previously described (14). gDNA was sequenced using the MiSeq technology (Illumina Inc, San Diego, CA, USA) with the Mate-pair strategy and were run and barcoded with 11 additional projects using the Nextera Mate-Pair sample prep kit (Illumina) as formerly described (14). The DNA fragment size ranged from 1.5 kb up to 11kb with an optimal size of 6.29 kb. No size selection was done and 177.24 ng of tagmented fragments were circularized. The circularized DNAs were sheared mechanically to smaller fragments with an optimal size at 1393 bp on the Covaris device S2 in T6 tubes (Covaris, Woburn, MA, USA). Using a high sensitivity bioanalyzer LabChip (Agilent Technologies Inc, Santa Clara, CA, USA), the library profile was visualized with a final concentration of 15.59 nmol/l. The latter were normalized at 2nM and pooled with other samples, and finally diluted to 15pM. Automated cluster generation and sequencing run were performed in a single 2x251-bp run. Total information of 9.5 Gb was obtained from a 1050 K/mm2 cluster density with a cluster passing quality control filters of 92.5 % (18,644,000 passing filter paired-reads). Within this run, the index representation for strain Marseille-P3249^{T} was determined to 4.67%. The 870,362 paired reads were trimmed, assembled, annotated and analyzed as previously described (14).

Genome-to-Genome Distance Calculator (http://ggdc.dsmz.de) was used for digital DNA-DNA hybridization (dDDH) estimates with confidence intervals under recommended settings (Formula 2, BLASTP).

Phylogenetic Analysis

For phylogenetic analyses, 16S rRNA gene sequences of closely related species were recovered from the 16S RNA database of “The All-Species Living Tree” Project of Silva (LTPs121) (15). Muscle was used for sequence alignment and phylogenetic inferences were generated using the approximately-maximum-likelihood method within the FastTree software (16, 17).

Results And Discussion

Strain identification

MALDI-TOF MS failed to identify strain Marseille-P3249. Therefore, 16S rRNA gene sequencing was performed and using a blast comparison against the NCBI nucleotide database, strain Marseille-P3249 exhibited a 98.3% sequence similarity with *Gemella bergeri* strain 617 – 93, being the phylogenetically closest species with standing in nomenclature (Fig. 1) (18). Thus, and according to Kim *et al.*, this strain may be classified within a new bacterial species within the *Gemella* genus as it exhibits more than 1.35% sequence divergence with its phylogenetically closest species with a validly published name (19). A gel view performed to compare the mass spectra of strain Marseille-P3249 and its phylogenetically-close species confirmed the novelty of this strain with its unique peak profile (Fig. 2).

General Characteristics Of Strain Marseille-p3249

Cells from strain Marseille-P3249 were Gram-positive cocci. Colonies grew in both aerobic and anaerobic atmospheres at temperatures ranging between 25°C and 37°C in optimally at 37°C in aerobic conditions. This strain grew at a pH range between 6 and 8.5 and NaCl concentrations below 50 g/L. In aerobic conditions at 37°C strain Marseille-P3249 formed colonies after 24hrs on COS agar of 0.5 to 1.2 mm in diameter. Cells had an average diameter of 0.78 µm (Table 1, Fig. 3). Cells were not motile and non-spore forming.

Table 1

Differential characteristics of *Gemella massiliensis* strain Marseille-P3249^T (**GMA**), *Gemella assaccharolytica* EU427463 (**GAS**) (22), *Gemella cuniculi* AJ251987 (**GCU**) (23), *Gemella morbillorum* L14327 (**GMO**) (24), *Gemella bergeri* Y13365 (**GBE**) (18) and *Gemella sanguinis* Y13364 (**GSA**) (25).

Properties	**GMA**	**GAS**	**GCU**	**GMO**	**GBE**	**GSA**
Cell diameter (µm)	0.78	0.5	Na	0.3–0.8	Na	Na
Oxygen requirement	Fa	Fa	Fa	Fa	Fa	Fa
Gram stain	+	V	+	+	+	+
Endospore formation	-	-	Na	Na	-	-
Production of						
Alkaline phosphatase	-	-	+	Na	-	+
Catalase	-	-	-	-	-	-
Urease	-	-	-	Na	-	-
β-galactosidase	-	Na	Na	Na	-	-
N-acetyl-β-glucosamine	-	Na	-	Na	Na	-
L-Arabinose	-	-	-	Na	-	-
D-Ribose	-	-	-	Na	-	-
D-Mannose	-	-	Na	+	Na	Na
D-Mannitol	-	-	+	+	-	+
D-glucose	-	-	+	+	+	+
D-fructose	+	+	Na	-	-	Na
D-maltose	-	-	V	+	W	+
D-lactose	-	-	-	-	-	V
G + C content (mol%)	30.5	26.7	28.9	30.8	30.3	29.7
Habitat	Sputum sample	Clinical specimen	Abcess of a rabit	Clincal specimen	Clincal specimen	Clincal specimen

Fa = Facultative anaerobic; Na = data not available; V = Variable; W = weakly positive

Using an API 50CH strip (bioMérieux), this strain was able to metabolize D-fructose, amygdaline and L-sorbose. As for API ZIM (bioMérieux), positive reactions were observed for esterase (C4), esterase lipase (C8), leucine arylamidase, phosphatase acid and naphtol phosphohydrolase. Using API 20A (bioMérieux), the strain showed a positive reaction for esculin ferric citrate only. Negative reactions were obtained with alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

The major fatty acids were Hexadecanoic acid (34 %), 9-Octadecenoic acid (28 %), Octadecanoic acid (15 %) and 9,12-Octadecadienoic acid (13 %). A wide variety of other fatty acids were described with low abundances. Four of them, rarely detected as cellular fatty acids, were composed of longer aliphatic chains (C20 and C22) (Table 2).

Table 2
Cellular fatty acid composition (%).

Fatty acids	Name	Mean relative % (a)
16:00	Hexadecanoic acid	34.1 ± 0.3
18:1n9	9-Octadecenoic acid	27.6 ± 0.2
18:00	Octadecanoic acid	14.8 ± 0.1
18:2n6	9,12-Octadecadienoic acid	12.5 ± 0.4
18:1n7	11-Octadecenoic acid	2.3 ± 0.1
18:1n5	13-Octadecenoic acid	2.1 ± 0.1
14:00	Tetradecanoic acid	1.2 ± 0.1
17:00	Heptadecanoic acid	TR
15:00	Pentadecanoic acid	TR
15:0 anteiso	12-methyl-tetradecanoic acid	TR
16:1n7	9-Hexadecenoic acid	TR
(a) Mean peak area percentage; TR = trace amounts < 1 %		

Strain Marseille P3249 exhibited MICs (µg/mL) of 0.012, 0.016, 0.016, 0.016, 0.016, 0.19, > 6, 0.125, 0.03, 0.64, 0.032, 0.75, > 256 and > 256 for benzylpenicillin, amoxicillin, cefotaxime, ceftriaxone, imipenem, erythromycin, daptomycin, amikacin, rifampicin, minocycline, teicoplanin, vancomycin, metronidazole and colistin, respectively.

Genome Characteristics Of Strain Marseille-p3249

The genome was 1,804,813-bp long with a 30.5 mol% G + C content (Fig. 4). It is composed of 7 scaffolds (composed of 8 contigs). Of the 1,727 predicted genes, 1,677 were protein-coding genes and 50 were RNAs (5 genes were 5S rRNA, 2 genes were 16S rRNA, 2 genes were 23S rRNA, and 41 genes were tRNA genes). A total of 1 276 genes (76.09%) were assigned a putative function (by cogs or by NR blast). Twenty-six genes were classified as ORFans (1.55%). The remaining genes were annotated as hypothetical proteins (304 genes (18,13%)) (Table 3). The distribution of genes into COG functional categories is detailed in Table 4.

Table 3

Nucleotide gene content and gene count levels of strain Marseille-P3249^{T}

Infromation	Value	%
Size (bp)	1,804,813	100
Number of GC	551,183	30.54
Number total of genes	1,727	100
Number total of protein genes	1,677	97.10
Number total of RNA Genes	50	2.89
Number total of TRNA Genes	41	2.37
Number total of RNA (5S, 16S, 23S) Genes	9	0.52
Coding sequence size	1,547,868	85.76
Coding sequence gene protein size	1,535,274	85.06
Coding sequence tRNA gene size	3,178	0.17
Coding sequence (5S, 16S, 23S) gene size	9,416	0.52
Number of protein coding gene	1,677	100
Number of protein associated to cogs	1,136	67.74
Number of protein NOT associated to cogs	541	32.25
Number of protein associated to orfan	26	1.55
Number of gene associated to resistance genes	1	0.05
Number of genes associated to virulence	369	22.00

Table 4
Number of genes associated with the 25 general COG functional categories

Code	Value	% of total	Description
[J]	175	10.44	Translation
[A]	0	0.00	Rna processing and modification
[K]	75	4.47	Transcription
[L]	64	3.82	Replication, recombination and repair
[B]	0	0.00	Chromatin structure and dynamics
[D]	18	1.07	Cell cycle control, mitosis and meiosis
[Y]	0	0.00	Nuclear structure
[V]	43	2.56	Defense mechanisms
[T]	37	2.21	Signal transduction mechanisms
[M]	48	2.86	Cell wall/membrane biogenesis
[N]	9	0.54	Cell motility
[Z]	0	0.00	Cytoskeleton
[W]	2	0.12	Extracellular structures
[U]	20	1.19	Intracellular trafficking and secretion
[O]	46	2.74	Posttanslational modification, protein turnover,chaperones
[X]	63	3.76	Mobilome: prophages, transposons
[C]	63	3.76	Energy production and conversion
[G]	74	4.41	Carbohydrate transport and metabolism
[E]	100	5.96	Amino acid transport and metabolism
[F]	61	3.64	Nucleotide transport and metabolism
[H]	58	3.46	Coenzyme transport and metabolism
[I]	43	2.56	Lipid transport and metabolism
[P]	68	4.05	Inorganic ion transport and metabolism
[Q]	9	0.54	Secondary metabolites biosynthesis, transport and catabolism
[R]	96	5.72	General function prediction only
[S]	71	4.23	Function unknown
	541	32.26	Not in COGs

Genome Comparison

The draft genome sequence of strain Marseille-P3249^T was larger than those of *Gemella cuniculi* DSM 15828^T, *Gemella sanguinis* ATCC 700632^T and *Gemella haemolysans* ATCC 10379^T (1.86, 1.90 and 1.91 Mb respectively), but smaller than those of *Gemella asaccharolytica* WAL 1945J^T, *Gemella bergeri* ATCC 700627^T and *Gemella morbillorum* NCTC11323^T (1.28, 1.60 and 1.75 Mb respectively) (Table 5).

Table 5
Genome information of the species involved in the genomic comparative analyses.

Species	Size (Mb)	GC (%)	Gene Content
Gemella asaccharolytica WAL 1945J^T	1.28	26.6	1,251
Gemella bergeri ATCC 700627^T	1.60	30.3	1,524
Gemella morbillorum NCTC11323^T	1.75	30.7	1,622
Gemella massiliensis **Marseille-P3249^T**	**1.80**	**30.5**	**1,677**
Gemella cuniculi DSM 15828^T	1.86	28.9	1,687
Gemella haemolysans ATCC 10379^T	1.91	30.8	1,710
Gemella sanguinis ATCC 700632^T	1.90	29.6	1,861

Additionally, the G + C content of strain Marseille-P3249^T is smaller than those of *G. asaccharolytica* WAL 1945J^T, *G. cuniculi* DSM 15828^T, *G. sanguinis* ATCC 700632^T and *G. bergeri* ATCC 700627^T (26.6, 28.9, 29.6 and 30.3%, respectively), but larger than those of *G. morbillorum* NCTC11323^T and *G. haemolysans* ATCC 10379^T (30.7 and 30.8 %, respectively).

The gene content of strain Marseille-P3249^T was larger than those of *G. asaccharolytica* WAL 1945J^T, *G. bergeri* ATCC 700627^T and *G. morbillorum* NCTC11323^T (1,251, 1,524 and 1,622 respectively), but smaller than those of *G. cuniculi* DSM 15828^T, *G. haemolysans* ATCC 10379^T and *G. sanguinis* ATCC 700632^T (1,687, 1,710 and 1,861, respectively).

Strain Marseille-P3249 shared the highest number of orthologous proteins with *G. cuniculi* (1039). Furthermore, this bacterium shared 1031, 1032, 1054, and 778 orthologous proteins with *G. haemolysans, G. morbillorum, G. sanguinis* and *G. asaccharolytica*, respectively. Strain Marseille-P3249 exhibited OrthoANI values of 76.5, 76.8, 75.9, 75.4, 94.8 and 70.3% with *G. morbillorum*, *G. sanguinis, G. haemolysans* G. *cuniculi, G. bergeri* and *G. asaccharolytica*, respectively (Fig. 5).

Strain Marseille-P3249 exhibited dDDH values of 21.3 ± 4.7%, 22.6 ± 4.7%, 21.7 ± 4.7%, 22.1 ± 4.7%, 21.9 ± 4.7% and 59.7 ± 5.6% with *G. asaccharolytica*, *G. cuniculi, G. haemolysans, G. morbillorum, G. sanguini* and *G. bergeri* (Table 6). These results confirm the novelty of the isolated strain, since 70% is the recommended dDDH threshold to delimitate a new bacterial species (20, 21).

Table 6

Digital DNA-DNA hybridization values (%) obtained by strain Marseille-P3249^T with other closely-related species using the GGDC formula 2 software (dDDH estimates based on identities / HSP length).

	GMA	GAS	GCU	GHA	GMO	GSA	GBE
GMA	100%	21.30 ± 4.7%	22.60 ±4.7%	21.70 ±4.7%	22.10 ± 4.7%	21.90 ±4.7%	59.70 ±5.6%
GAS		100%	23.40 ±4.7%	23.20 ±4.7%	22.40 ± 4.7%	21.60 ±4.6%	21.00 ±4.7%
GCU			100%	21.80 ±4.7%	22.00 ± 4.7%	22.10 ±4.7%	22.70 ±4.7%
GHA				100%	22.90 ± 4.7%	23.50 ±4.8%	22.10 ± 4.7%
GMO					100%	23.00 ±4.8%	21.90 ±4.7%
GSA						100%	21.90 ±4.8%
GBE							100%

Abbreviations : GMA, *Gemella massiliensis* Marseille-P3249; **GAS**, *Gemella asaccharolytica* strain KA00071; **GCU**, *Gemella cuniculi* DSM 15828; **GHA**, *Gemella haemolysans* strain NCTC10459; **GMO**, *Gemella morbillorum* strain NCTC11323; **GSA**, *Gemella sanguinis* strain FDAARGOS 742; **GBE**, *Gemella bergeri* ATCC 700627.

Description of *Gemella massiliensis* sp. nov.

We propose strain Marseille-P3249 is the type strain of the new species *Gemella massiliensis* sp. nov. (mas.il.i.en'sis, L. gen. fem., adj., *massiliensis*, pertaining to Massilia, the Latin name of the city of Marseille, where this bacterium was discovered). Strain Marseille-P3249 is a facultative anaerobic bacterium but grows optimally at 37°C under aerobic conditions. Using a 50 CH strip, this strain exhibits positive reactions for D-fructose, amygdaline and L-sorbose. Positive reactions are also observed for esterase (C4), esterase lipase (C8), leucine arylamidase, phosphatase acid and naphtol phosphohydrolase using an API strip. In addition, using an API 20A (bioMérieux), positive reactions are observed for esculin ferric citrate only.

The major fatty acids are Hexadecanoic acid (34 %), 9-Octadecenoic acid (28 %), Octadecanoic acid (15 %) and 9,12-Octadecadienoic acid (13 %).

The genome is 1,804,813 bp long with 30.5 mol% G + C content. The 16S rRNA and whole genome sequences of *G. massiliensis* sp. nov., were deposited in EMBL-EBI under accession numbers LT628479 and FQLS00000000, respectively. The type strain Marseille-P3249^{T} (= CSUR P3249 = DSMZ 103940) was isolated from the sputum sample of a healthy French man.

Abbreviations

DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen

CSUR: Collection de Souches de l'Unité des Rickettsies

MALDI-TOF MS: Matrix-Assisted Laser Desorption Ionization Time-Of-Flight

MIC: Minimal Inhibitory Concentration

dDDH: DNA-DNA hybridization

COG: Clusters of Orthologous Groups

Declarations

The volunteer has given freely his authorization by signed and informed consent for advanced studies to be done on the collected sample. In addition, all the methods used in this study were performed in accordance with relevant guidelines and regulations conformed to Declaration of Helsinki.

Author Contributions: MDMF, MB and CIL drafted the manuscript and analyzed the data. MDMF, ZM, EK and ET performed the technical characterization on strain Marseille-P3249. PEF and DR conceived the study. CIL, GD, FF and PEF revised the manuscript and participated in its design and coordination. All authors read and approved the final manuscript.

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Conflicts of interest:

Prs Fournier and Raoult are co-founders of the Techno jouvence startup. The techno jouvence startup had not role in this study.

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Figures

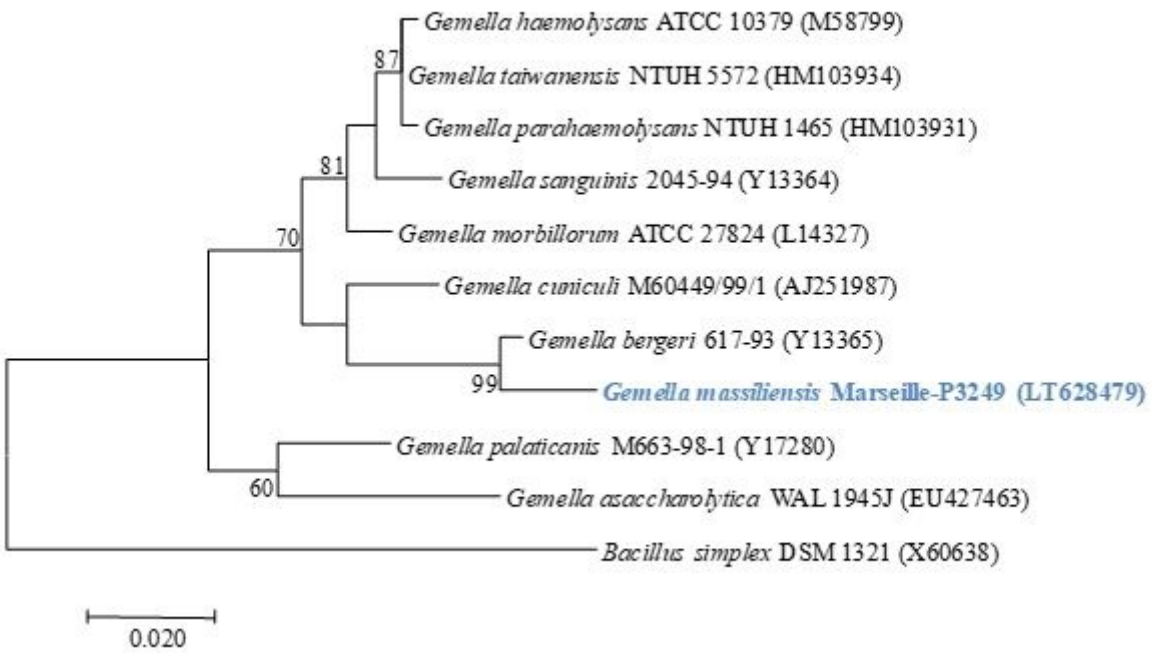

Figure 1

16S rRNA gene sequence phylogenetic analysis highlighting the position of strain Marseille-P3249 relative to other species. Sequence alignment and phylogenetic inferences were obtained using the maximum likelihood method within MEGA 7 software. The scale bar represents a 2% sequence divergence using 1000 replicates. GenBank accession numbers are indicated in parenthesis.

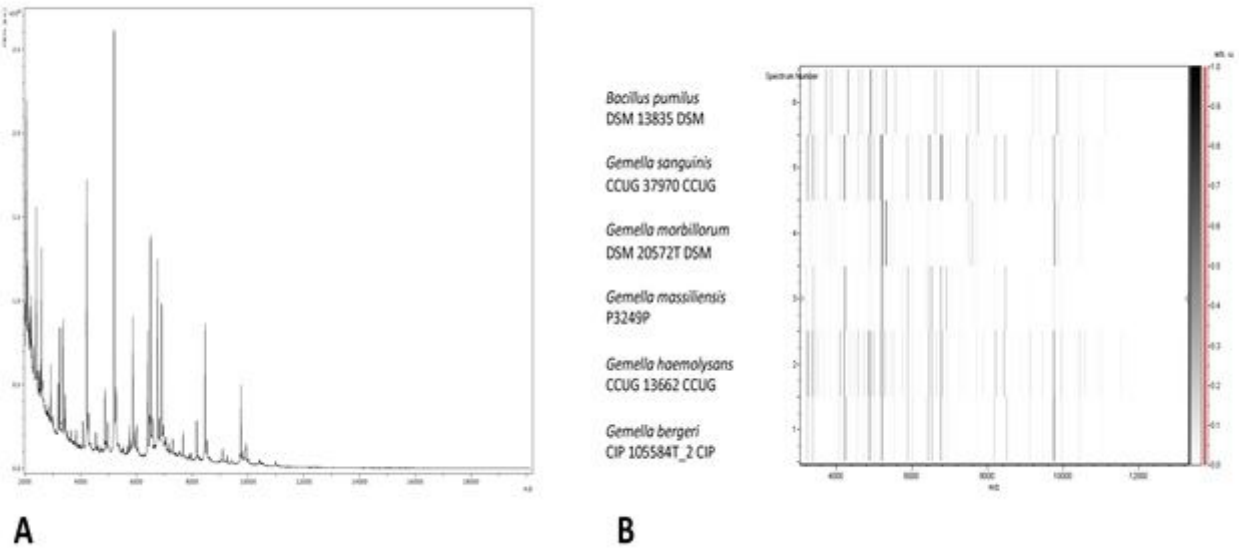

Figure 2

Reference mass spectrum representing G. massiliensis strain Marseille-P3249 (A). Gel view comparing the mass spectra of strain Marseille-P3249 to other species with the raw spectra on the left (B). The x-axis represents the m/z value. The left y-axis indicates the running spectrum number acquired from successive spectra loading. The intensity of the peaks is indicated with the different gray scale and the y axis indicates the relation between the peak color and its intensity.

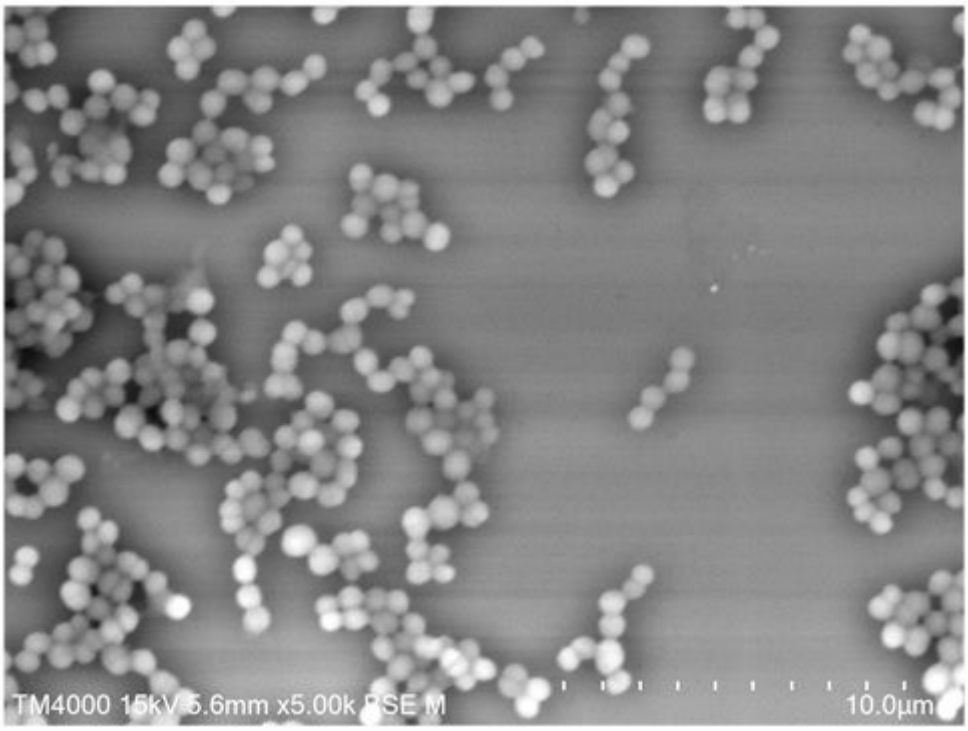

Figure 3

Electron micrographs of G. massiliensis strain Marseille-P3249.

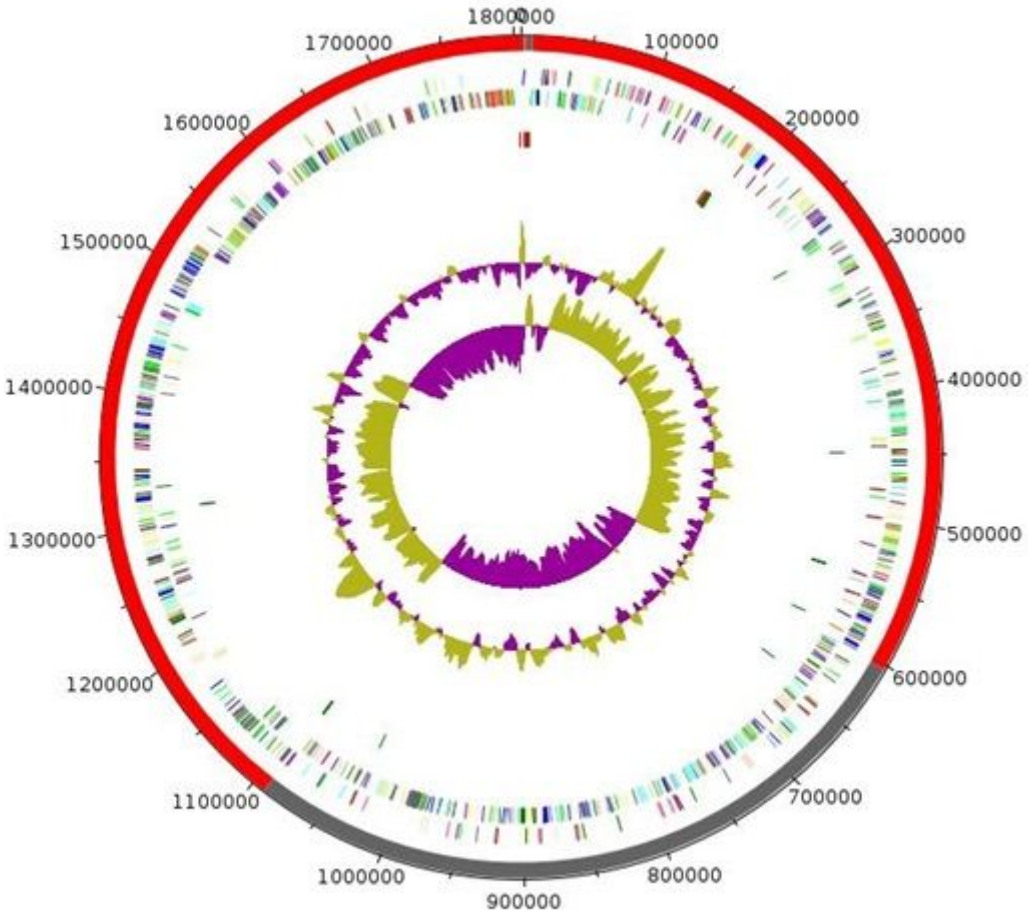

Figure 4

Graphical circular map of the chromosome. From outside to the center: Genes on the forward strand colored by COG categories (only genes assigned to COG), genes on the reverse strand colored by COG categories (only gene assigned to COG), RNA genes (tRNAs green, rRNAs red), GC content and GC skew.

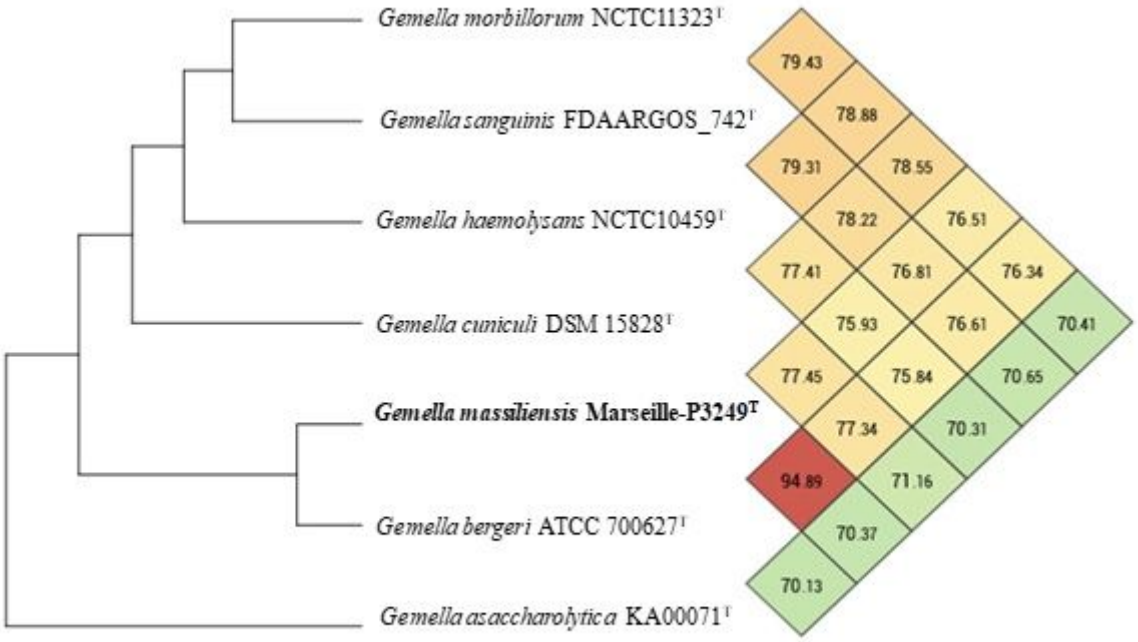

Figure 5

Heatmap generated with OrthoANI values calculated using the OAT software between Gemella massiliensis sp. nov., strain Marseille-P3249 and other closely related species with standing in nomenclature.