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Characterization and Whole Genome Sequencing of Two Novel *Anaerococcus* Isolated from Human Vagina: Description of *Anaerococcus genitaliorum* sp. nov., and *Anaerococcus* mediterraneensis sp. nov.

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Abstract

Two previously unidentified strictly anaerobic, Gram-positive coccoid bacteria, strains Marseille-P3625^T and Marseille-P2765^T, were isolated from vaginal samples of French women suffering from bacterial vaginosis (BV) and characterized using the taxono-genomics concept. Cells were negative for oxidase, catalase, urease, and indole production. The phylogenic and genotypic analyses, phenotypic characteristics, and genomic data presented in this review confirm that these two bacteria are distinct from previously known bacterial species with standing in nomenclature. Strains Marseille-P3625^T and Marseille-P2765^T exhibited 93.4% and 97.2% 16S rRNA gene sequence similarity with *Anaerococcus tetradius* strain F0127^T and *Anaerococcus lactolyticus* strain JCM 8140^T, the phylogenetically-closest species with validly published name, respectively. The major fatty acids of strains Marseille-P3625^T and Marseille-P2765^T were Hexadecanoic acid (45.0% and 42.0%, respectively) and 9-Octadecenoic acid (21.8% and 34.1%, respectively); their DNA G+C content are respectively, 31.3 and 34.6 mol%. We propose that strain Marseille-P3625^T (=CSUR P3625 = CECT 9562) and strain Marseille-P2765^T (=CSUR P2765 = DSM 103343), be the type strains of two new *Anaerococcus* species for which the names *Anaerococcus genitaliorum* sp. nov., and *Anaerococcus mediterraneensis* sp. nov., are proposed, respectively.

Keywords: *Anaerococcus genitaliorum; Anaerococcus mediterraneensis; Bacterial Vaginosis; Culturomics; Taxono-Genomics; Anaerobic Bacteria; News Species*

Abbreviations

BV: Bacterial Vaginosis; CSUR: Stands for 'Collection de Souches de l'Unite´ des Rickettsies'; DSMZ: Stands for 'Deutsche Sammlung von Mikroorganismen und Zellkulturen'; CECT: Colección Española de Cultivos Tipo; dDDH: Digital DNA-DNA Hybridization; MALDI-TOF MS: Matrix-Assisted Laser-Desorption/Ionization Time-of-Flight Mass Spectrometry; GGDC: Genome-to-Genome Distance Calculator; FAME:

Cellular Fatty Acid Methyl Ester; GC/MS: Gas Chromatography/Mass Spectrometry; MICs: Minimal Inhibitory Concentrations; gDNA: Genomic DNA; AAI: Average Amino Acid Identity

Introduction

The vaginal microbiota composition was investigated since the 1800s using both conventional culture and culture-independent methods including molecular techniques, with sequencing and phylogenetic analysis of the 16S rRNA gene [1,2]. The latter enhanced the understanding of the human vaginal microbiota, notably by enabling the detection of fastidious and uncultured bacteria such as those associated with bacterial vaginosis (BV) [3]. This disease was described as a dysbiosis characterized by a depletion of *Lactobacillus* species, first line of defense against genital infections, associated to an increase of Gram negative anaerobic bacteria [4-7].

The study of the vaginal microbiota diversity of health women and patient with bacterial vaginosis is part of the ongoing "microbial culturomics" study in our laboratory [8,9] that consists in optimizing culture conditions to explore in depth the human microbiota. Here, it enabled the isolation of two new Gram-positive-staining and strictly anaerobic bacteria in the vaginal discharge of a woman with bacterial vaginosis. Strains Marseille-P3625^T and Marseille-P2765^T were classified as belonging to the genus *Anaerococcus.*

The genus *Anaerococcus* belonging to the family *Peptoniphilaceae,* was first created by Ezaki., *et al*. in 2001 [10] after the division of the *Peptostreptococcus* genus into five genera*.* At the time of writing, the *Anaerococcus* genus contains 13 species with standing in nomenclature (www.bacterio.net) including *A. hydrogenalis, A. lactolyticus, A. octavius, A. prevotii, A. tetradius, A. vaginalis*, *A. murdochii, A. pacaensis, A. nagyae, A. degeneri, A. provencensis, A. rubeinfantis* and *A. senegalensis* (www.bacterio.net). All of these are strictly anaerobic Gram-positive, non-motile cocci [10]. Members of the genus are mostly isolated from the human vagina but have also been occasionally found in the nasal cavity or on the skin. Some were also isolated from various infectious sites [6,10-13].

Herein, we described the isolation process and taxono-genomic characterization [14] of strains Marseille-P3625^T and Marseille-P2765^T as type strains of two new *Anaerococcus* species for which the names *Anaerococcus genitaliorum* sp. nov. (=CSUR P3625 = CECT 9562), *Anaerococcus mediterraneensis* sp. nov. (=CSUR P2765 = DSM 103343), are proposed, respectively. Both strains were isolated from vaginal swabs of two patients with bacterial vaginosis.

Materials and Methods

Samples and ethics

Two bacterial strains, namely Marseille-P3625 and Marseille-P2765 were isolated in April 2016 from vaginal specimens of two French women, respectively, diagnosed with BV as previously described [15] at the Nord hospital in Marseille, France. The vaginal swabs were collected using a Sigma Transwab (Medical Wire, Corsham, United Kingdom). The patients had not received any antibiotic at the time of sampling. The patients gave the informed and signed consent and the study was authorized by the ethics committee of the Institut Federatif de Recherche IFR48 (Marseille, France) under agreement 09-022.

Bacterial strain isolation and identification by MALDI-TOF MS and 16S rRNA sequencing

After sampling, the vaginal swabs were initially preincubated at 37°C in an anaerobic blood culture bottle (Bactec Lytic/10 Anaerobic/F Culture Vials, Becton-Dickinson, Le Pont de Claix, Isère, France) enriched with 3 mL of sheep blood (bioMérieux, Marcy l'Etoile, France) and 4 mL filter-sterilized rumen fluid through a 0.2 µm pore filter (Thermo Fisher Scientific, Villebon-sur-Yvette, France). Various preincubation periods were tested (1, 3, 7, 10, 15, 21 and 30th days). Then 50 µL of the supernatant were inoculated on both Schaedler agar, Colistin-nalidixic acid (CNA) and Trypticase soy agar (BD Diagnostics, Le pont de Claix, France) and incubated for 4 days in anaerobic

atmosphere at 37°C. Isolated colonies were subcultured individually using the same conditions and each colony was subsequently identified by matrix-assisted laser-desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry with a Microflex spectrometer (Bruker Daltonics, Leipzig, Germany), as previously described [16,17]. The new protein spectra were compared with those of reference spectra in the Bruker database constantly updated with our own database [18]. If the score was greater than 1.9, the bacterium was identified at the genus (score between 2.0 and 2.299) and/or species levels (score from 2.3 to 3.0). When the score was lower than these thresholds, no identification was considered as reliable and the 16S rRNA gene was sequenced using the GeneAmp PCR System 2720 thermal cycler (Applied Bio systems, Bedford, MA, USA) and an ABI Prism 3130-XL capillary sequencer (Applied Biosciences, Saint Aubin, France), respectively, as previously described [19]. Finally, the resulting sequence was corrected using the Chromas Pro 1.34 software (Technelysium Pty. Ltd., Tewantin, Australia) and then compared to the NCBI nr database using the BLASTn algorithm (https://blast.ncbi.nlm.nih.gov/) for taxonomic assignment.

Phylogenetic analysis

The alignment of the 16S rRNA sequences of strains Marseille-P3625 and Marseille-P2765 with other species with validly published names and exhibiting the closest phylogenetic relationships was performed using CLUSTALW [20]. The phylogenetic trees were inferred by the Neighbor-joining method with the Kimura 2-parameter model within the MEGA software, version 6 [21]. If the 16S rRNA sequence similarity value was lower than 95% or 98.65% with the most closely related species with standing in nomenclature, as proposed by Stackebrandt and Ebers [22], the strain was proposed to belong to a new genus or species, respectively [23].

Phenotypic characteristics

For each isolate, cell morphology was observed using transmission electron microscopy after negative staining as previously described [24]. Electron microscopy analysis (TEM) was performed as described before [25]. Motility, oxidase, catalase, sporulation tests as well as Gram-stain were performed as previously reported [26]. To characterize the ideal growth conditions of each isolate, bacteria were cultivated on 5% sheep blood-enriched Columbia agar (bioMérieux) at various temperatures (25, 28, 37, 45, and 56°C) and atmospheres (aerobic conditions with or without 5% CO_2, anaerobic and microaerophilic). Several salinity conditions (NaCl concentrations of 0%, 5%, 10%, 15% and 20%) and pH values (5, 6, 6.5, 7 and 8.5) were also evaluated.

The biochemical properties of strains Marseille-P3625 and Marseille-P2765 were tested using API ZYM and API 20A strips (bioMérieux) according to the manufacturer's instructions. The strips were incubated in anaerobic conditions for 4 and 24 hours respectively.

Cellular fatty acid methyl ester (FAME) analysis was performed using Gas Chromatography/Mass Spectrometry (GC/MS). Both isolates were grown anaerobically on 5% sheep blood-enriched Columbia agar (bioMérieux) at 37°C. For each isolate, two samples were then prepared with approximately 14 - 26 mg of bacterial biomass per tube harvested from several culture plates. FAMEs were prepared as described by Sasser [27]. GC/MS analyses were carried out as described before [28].

Minimal inhibitory concentrations (MICs) of amoxicillin, benzylpenicillin, ceftriaxone, imipenem, ertapenem, erythromycin, Amikacin, ofloxacin, rifampicin, vancomycin and metronidazole were estimated for both isolates using E-test gradient strips (bioMérieux) according to the EUCAST recommendations [29,30].

DNA extraction, genome sequencing and analyses

After a pretreatment step by lysozyme incubation for 2 hours at 37°C, the genomic DNAs (gDNAs) of strains Marseille-P3625 and Marseille-P2765 were extracted using an EZ1 biorobot and the EZ1 DNA Tissue kit (Qiagen, Hilden, Germany). DNAs were quantified by a Qubit assay with the high sensitivity kit (Life Technologies, Carlsbad, CA, USA) at 71.2 and 69.6 ng/µl respectively. The gDNA of each strain was sequenced using a MiSeq sequencer with the mate pair strategy (Illumina Inc., San Diego, CA, USA) as previously described [24]. The high-quality paired-end reads obtained for each isolate were trimmed and then assembled using the Spades assembler program [31].

Prodigal was used for open reading frame (ORF) prediction [32] with default parameters. However, the predicted ORFs were excluded if they spanned a sequencing gap region. The predicted bacterial proteome was analyzed as previously reported [33]. The RNAmmer [34] and tRNAScanSE [35] programs were used to identify ribosomal RNAs and tRNAs, respectively. ORFans were identified when the BLASTP search was negative (E-value smaller than $1e^{-03}$ for ORFs with a sequence size larger than 80 aas or E-value smaller than $1e^{-05}$ for ORFs with sequence length smaller than 80 aas). Artemis [36] and DNA Plotter [37] were used for data management and for visualization of genomic features, respectively. Genomes from members of the *Anaerococcus* genus and closely related to both strains were used for the genomic comparison. Genomic informations from strains Marseille-P3625 and Marseille-P2765 and comparatively closest related species are presented in table 6. Annotation and comparison processes were performed using the multi-agent software system DAGOBAH [38], which includes Figenix [39] libraries that provide pipeline analysis. We estimated the degrees of genomic sequence similarity among compared genomes using the following tools. First, digital DNA-DNA hybridization (dDDH) analysis was performed using the GGDC web server, as previously reported [40]. Then, for genomes very divergent (average nucleotide identity (ANI) values between pairs of genomes below 75%), the average amino acid identity (AAI) was determined as recommended [41] available at (http://enve-omics.ce.gatech.edu/aai/index).

Results and Discussion

Identification and phylogenetic analysis of the new species

MS identification of the two isolates first cultivated on both Schaedler and Colistin-nalidixic acid (CNA) soy agar (strain Marseille-P3625) and on Schaedler soy agars (strain Marseille-P2765) in anaerobic atmosphere at 37°C and after 10 and 21 days of preincubation in a blood culture bottle enriched with rumen and sheep blood, respectively failed. This suggested that these isolates were not referenced in the database and may be unknown species. Their MALDI-TOF MS spectra were added to our database to improve its content. Pairwise similarity analysis of 16S rRNA gene sequences (deposited in EMBL-EBI under accession numbers LT900366 and LT598544 for strains Marseille-P3625 and Marseille-P2765, respectively) attested that strain Marseille-P3625 exhibited 92.6% sequence similarity with strain Marseille-P2765.

16S rDNA-based similarity analysis of strains Marseille-P3625 and Marseille-P2765 against GenBank yielded highest nucleotide sequence similarities of 93.4% with *Anaerococcus tetradius* strain F0127^{T} (GenBank accession no. GQ422749.1) and 97.2% sequence identity with *Anaerococcus lactolyticus* strain JCM 8140^{T} (NR_113565.1) respectively, the two phylogenetically closest species with a validly published name. As these similarity values were lower the 98.65% threshold established to delineate new species [22, 42], strains Marseille-P3625 and Marseille-P2765 were considered as putative new species within the genus *Anaerococcus*. The resulting Neighbor-joining tree highlighting the position of strains Marseille-P3625 and Marseille-P2765 relative to other closely related species with a validly published name is shown in figure 1.

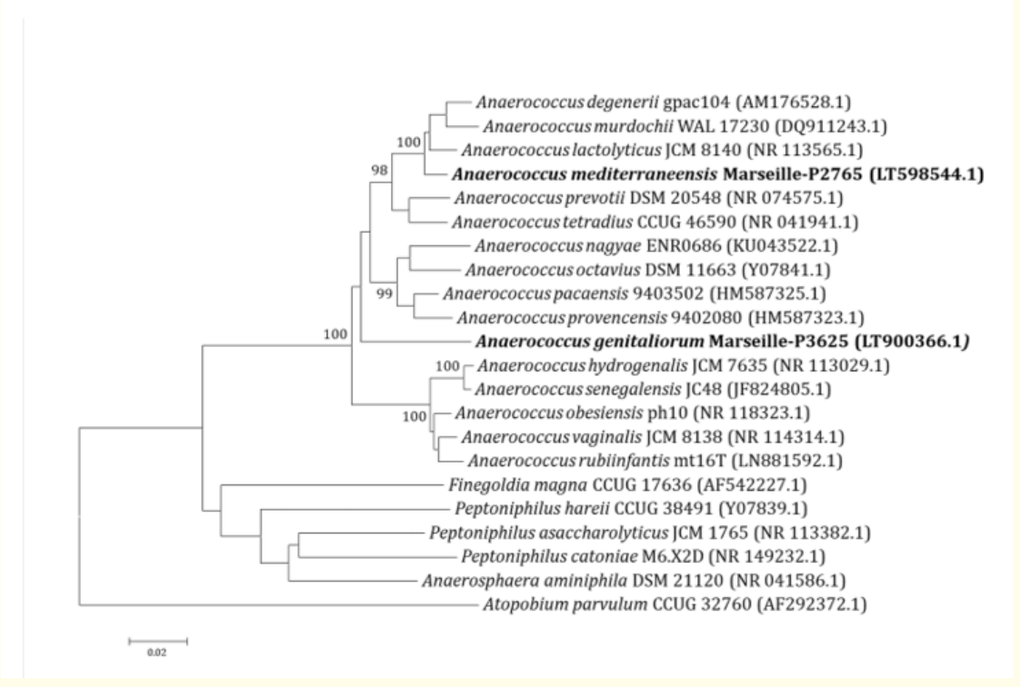

Figure 1: *Evolutionary relationships based on the 16S RNA gene sequence of Anaerococcus genitaliorum strain Marseille-P3625T and Anaerococcus mediterraneensis strain Marseille-P2765T and other closely related strains inferred under the Kimura 2-parameter model. GenBank accession numbers of each 16S rRNA are noted in parentheses. The numbers above the branches are support values when larger than 97% from neighbor-joining bootstrapping. Atopobium parvulum was used as outgroup. The scale bar represents a 2% nucleotide sequence divergence.*

Phenotypic characteristics

Cell from strains Marseille-P3625 and Marseille-P2765 were strictly anaerobic, Gram-stain-positive, non-motile cocci with a mean diameter of 1.1 and 1.0 µm, respectively. After 4 days of growth at 37°C in anaerobic atmosphere on blood-enriched Columbia agar (BD Diagnostics), colonies were white and circular, and had a mean diameter of 2 mm. They exhibited no catalase and oxidase activities. For both strains, growth occurred only in anaerobic atmosphere at temperatures ranging from 28 to 45°C, with optimal growth observed at 37°C in anaerobic atmosphere, with a pH ranging from 6.5 to 7.5 and NaCl concentrations lower than 5 g/L.

Using an API ZYM strip (bioMérieux), esterase (C4), alkaline phosphatase, acid phosphatase and naphtol-AS-BI-phosphohydrolase activities were positive for both strains. In addition, positive reaction of esterase lipase (C8) was observed for strain Marseille-P2765. In contrast, strain Marseille-P3625 had leucine arylamidase and valine arylamidase activities. No reaction was observed for lipase [14], trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, cystine arylamidase, α-mannosidase and α-fucosidase for both strains. Using an API 20A strip (bioMérieux), strain Marseille-P2765 produced acid from D-glucose, D-maltose and D-mannose, whereas strain Marseille-P3625 produced acid from D-glucose, D-maltose, D-trehalose and gelatin. Other fermentation tests including D-mannitol, D-lactose, D-saccharose, salicin, D-xylose, L-arabinose, glycerol, D-cellobiose, D-melezitose, D-raffinose, sorbitol and D-rhamnose were negative. In addition, indole production and urease activities were negative for both studied strains. However, esculin ferric citrate was hydrolyzed for strain Marseille-P3625 but not for strain Marseille-P2765. Strains Marseille-P3625 and Marseille-P2765 differed from other members of the *Anaerococcus* genus [10,43,44] in alkaline phosphatase activity (Table 1). The major cellular fatty acids found for these strains were Hexadecanoic acid (45.0% and 42.0% for strains Marseille-P3625 and Marseille-P2765, respectively) and 9-Octadecenoic acid (21.8% and 34.1% for strains Marseille-P3625 and Marseille-P2765, respectively). The most abundant fatty acids were saturated for both strains (Table 2). Cells from both strains were susceptible to amoxicillin, benzylpenicillin, ceftriaxone, imipenem, ertapenem, rifampicin, vancomycin and metronidazole, but resistant to erythromycin, amikacin and ofloxacin (Table 3).

Characteristic	1	2	3	4	5
Cell diameter (µm)	0.7 - 1.4	0.9 - 1.1	0.5 - 1.8	0.6 - 1.5	0.7 - 0.9
Oxygen requirement	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic
Gram stain	+	+	+	+	+
DNA G+C content (mol%)	31.3	34.6	32	33	31
Motility	-	-	-	-	-
Production of					
Alkaline phosphatase	+	+	-	-	-
Indole	-	-	-	-	-
Catalase	-	-	-	+	-
Urease	-	-	+	+/-	-
β-galactosidase	-	-	-	-	-
Acid form					
Mannose	-	+	+	+	+
Glucose	+	+	+	+/-	+
Lactose	-	-	-	-	-
Raffinose	-	-	-	+	-
Habitat	Vaginal discharges	Vaginal discharges	Human specimens	Human specimens	Human specimens

Table 1: *Differential phenotypic characteristics of Anaerococcus genitaliorum sp. nov, strain Marseille-P3625^T, Anaerococcus mediterraneensis sp. nov, strain Marseille-P2765^T and related species of the genus Anaerococcus. 1, Anaerococcus genitaliorum strain Marseille-P3625^T; 2, Anaerococcus mediterraneensis strain Marseille-P2765^T; 3, Anaerococcus tetradius strain ATCC 35098^T; 4, Anaerococcus prevotii strain DSM 20548^T; 5, Anaerococcus octavius strain NCTC 9810^T. +: Positive Reaction; -: Negative Reaction.*

Fatty acids	Name	Mean relative % (a)	
		Strain Marseille-P3625	Strain Marseille-P2765
16:00	Hexadecanoic acid	**45.0 ± 0.6**	**42.0 ± 1.6**
18:1n9	9-Octadecenoic acid	**21.8 ± 2.8**	**34.1 ± 1.0**
18:00	Octadecanoic acid	**10.9 ± 2.3**	4.2 ± 0.4
14:00	Tetradecanoic acid	8.3 ± 1.2	3.4 ± 0.6
18:2n6	9,12-Octadecadienoic acid	6.7 ± 1.5	**11.2 ± 0.9**
12:00	Dodecanoic acid	2.2 ± 0.7	1.4 ± 0.3
10:00	Decanoic acid	2.1 ± 1.0	2.3 ± 0.9
15:00	Pentadecanoic acid	1.9 ± 0.1	TR
17:00	Heptadecanoic acid	TR	-
15:0 anteiso	12-methyl-tetradecanoic acid	TR	-
16:1n7	9-Hexadecenoic acid	-	TR

Table 2: *Cellular fatty acid composition (%) of strain Marseille-P3625^T and strain Marseille-P2765^T.*

[a]: Mean peak area percentage; TR: Trace Amounts < 1 %, predominant products are shown in bold; –: Not Detected.

Antibiotics	Concentration (µg/ml)	*A. genitaliorum Strain Marseille-P3625*	*A. mediterraneensis Strain Marseille-P2765*
Amoxicillin	0.016-256	0.016	0.032
Benzylpenicillin	0.002-32	0.002	0.094
Ceftriaxone	0.002-32	0.064	0.064
Imipenem	0.002-32	0.002	0.004
Ertapenem	0.002-32	0.003	0.002
Erythromycin	0.016-256	2	1
Amikacin	0.016-256	> 256	> 256
Ofloxacin	0.002-32	> 256	> 256
Rifampicin	0.002-32	0.002	0.002
Vancomycin	0.016-256	0.094	0.094
Metronidazole	0.016-256	0.032	0.125

Table 3: *Minimal inhibitory concentrations (MIC **µg**/ml) of antibiotics for A. genitaliorum strain Marseille-P3625^T and A. mediterraneensis strain Marseille-P2765^T.*

Genome characteristics and comparison

Strains Marseille-P3625 and Marseille-P2765 exhibited genome sizes of 2,086,176 and 2,080,215-bp-long, respectively (Figure 2), with G+C content of 31.3 and 34.6 mol%, respectively. The genome characteristics of the two strains were detailed in table 4 and the repartition of their gene contents into the 25 general COG categories was represented in table 5 and figure 3. Comparison with the closest related species (Table 6) revealed that the two strains had genome sizes, G+C contents and total gene counts in the same range (Table 6). However, the distribution of genes into COG categories in all compared genomes was similar (Figure 3). In addition, we observed dDDH

values ranging from 19.3% ± 2.3% between *Anaerococcus lactolyticus* and *Anaerococcus prevotii* to 29.5% ± 2.5% between *Anaerococcus lactolyticus* and *Anaerococcus hydrogenalis* (Table 7). When comparing the two new strains to other *Anaerococcus* species, strains Marseille-P3625 and Marseille-P2765 exhibited dDDH values ranging from 20.2 ± 2.5% with *Anaerococcus prevotii* to 28.6 ± 2.5% with *Anaerococcus lactolyticus,* and 25.2 ± 2.4% with *Anaerococcus hydrogenalis,* respectively (Table 7). Furthermore, the AAI values ranged from 61.0% between strain Marseille-P3625 and *Anaerococcus hydrogenalis* to 78.9% *Anaerococcus tetradius* and *Anaerococcus prevotii.* Strain Marseille-P3625 shared AAI value with *Anaerococcus tetradius* (65.9%). Strain Marseille-P2765 shared AAI values ranging from 61.8% with *Anaerococcus hydrogenalis* to 75.8% with *Anaerococcus lactolyticus* (Table 7).

Attribute	*A. genitaliorum*		*A. mediterraneensis*	
	Value	% of total[a]	Value	% of total[a]
Size (bp)	2,086,176	100	2,080,215	100
G+C content (bp)	652,362	31.3	719,309	34.6
Coding region (bp)	1,871,084	89.7	1,849,157	88.9
Total genes	2,095	100	2,035	100
RNA genes	60	2.9	53	2.6
ncRNA genes	3	0.1	4	0.2
Protein-coding genes	1,966	93.8	1,930	94.8
Pseudogenes	66	3.2	48	2.4
Genes with function prediction	1,430	72.7	1,483	76.8
Genes assigned to COGs	1,340	68.2	1,356	70.3
Genes with peptide signals	172	8.7	158	8.2
Genes with transmembrane helices	452	23.0	501	26.0

Table 4: *Nucleotide content and gene count levels of the genomes.*

[a]: The total is based on either the size of the genome in base pairs or the total number of protein coding genes in the annotated genome.

Code	*A. genitaliorum*		*A. mediterraneensis*		Description
	Value	%value	Value	%value	
J	175	8,9	168	8,7	Translation
A	0	0,0	0	0,0	RNA processing and modification
K	96	4,9	98	5,1	Transcription
L	81	4,1	70	3,6	Replication, recombination and repair
B	0	0,0	0	0,0	Chromatin structure and dynamics
D	17	0,9	18	0,9	Cell cycle control, mitosis and meiosis
Y	0	0,0	0	0,0	Nuclear structure
V	69	3,5	79	4,1	Defense mechanisms
T	52	2,6	58	3,0	Signal transduction mechanisms
M	50	2,5	48	2,5	Cell wall/membrane biogenesis
N	9	0,5	8	0,4	Cell motility
Z	0	0,0	0	0,0	Cytoskeleton
W	4	0,2	3	0,2	Extracellular structures
U	22	1,1	18	0,9	Intracellular trafficking and secretion
O	64	3,3	65	3,4	Posttranslational modification, protein turnover, chaperones
X	14	0,7	14	0,7	Mobilome: prophages, transposons
C	66	3,4	84	4,4	Energy production and conversion
G	92	4,7	112	5,8	Carbohydrate transport and metabolism
E	101	5,1	84	4,4	Amino acid transport and metabolism
F	55	2,8	64	3,3	Nucleotide transport and metabolism
H	59	3,0	68	3,5	Coenzyme transport and metabolism
I	38	1,9	49	2,5	Lipid transport and metabolism
P	96	4,9	72	3,7	Inorganic ion transport and metabolism
Q	13	0,7	10	0,5	Secondary metabolites biosynthesis, transport and catabolism
R	101	5,1	98	5,1	General function prediction only
S	66	3,4	68	3,5	Function unknown
-	626	31,8	574	29,7	Not in COGs

Table 5: *Number of genes associated with the 25 general COG functional categories.*

Species	Strain	INSDC identifier	Size (Mb)	GC Percent	Gene Content	Number of contigs
Anaerococcus genitaliorum	Marseille-P3625	OCTP00000000	2.1	31.3	2,095	11
Anaerococcus mediterraneensis	Marseille-P2765	LT635772	2.1	34.6	2,035	1
Anaerococcus lactolyticus	ATCC 51172	ABYO00000000	2.2	34.9	2,241	298
Anaerococcus tetradius	ATCC 35098	ACGC00000000	2.2	34.1	2,081	143
Anaerococcus prevotii	DSM 20548	CP001708	1.9	36.1	1,782	1
Anaerococcus hydrogenalis	DSM 7454	ABXA00000000	1.9	29.6	1,884	52

Table 6: *Genome comparison of species closely related to strains Marseille-P3625^T and Marseille-P2765^T*

[a]: INSDC: International Nucleotide Sequence Database Collaboration.

Strains	1	2	3	4	5	6
dDDH						
1. Anaerococcus genitaliorum	100	26.7 ± 2.4	28.6 ± 2.5	28.1 ± 2.4	20.2 ± 2.3	24.3 ± 2.4
2. Anaerococcus mediterraneensis		100	22.3 ± 2.4	21.2 ± 2.4	20.2 ± 23	25.2 ± 2.4
3. Anaerococcus lactolyticus			100	25.3 ± 2.3	19.3 ± 2.3	29.5 ± 2.5
4. Anaerococcus tetradius				100	21.4 ± 2.3	25.8 ± 2.4
5. Anaerococcus prevotii					100	24.4 ± 2.4
6. Anaerococcus hydrogenalis						100
AAI						
1. Anaerococcus genitaliorum	100	65.4	65.5	65.9	64.2	61.0
2. Anaerococcus mediterraneensis		100	75.8	66.3	66.0	61.8
3. Anaerococcus lactolyticus			100	67.5	65.2	61.7
4. Anaerococcus tetradius				100	78.9	62.7
5. Anaerococcus prevotii					100	62.4
6. Anaerococcus hydrogenalis						100

Table 7: *Genomic similarity indices (dDDH and AAI) (%) between the genomes of Anaerococcus genitaliorum strain Marseille-P3526^T and Anaerococcus mediterraneensis strain Marseille-P2765^T and other members of the genus Anaerococcus.*

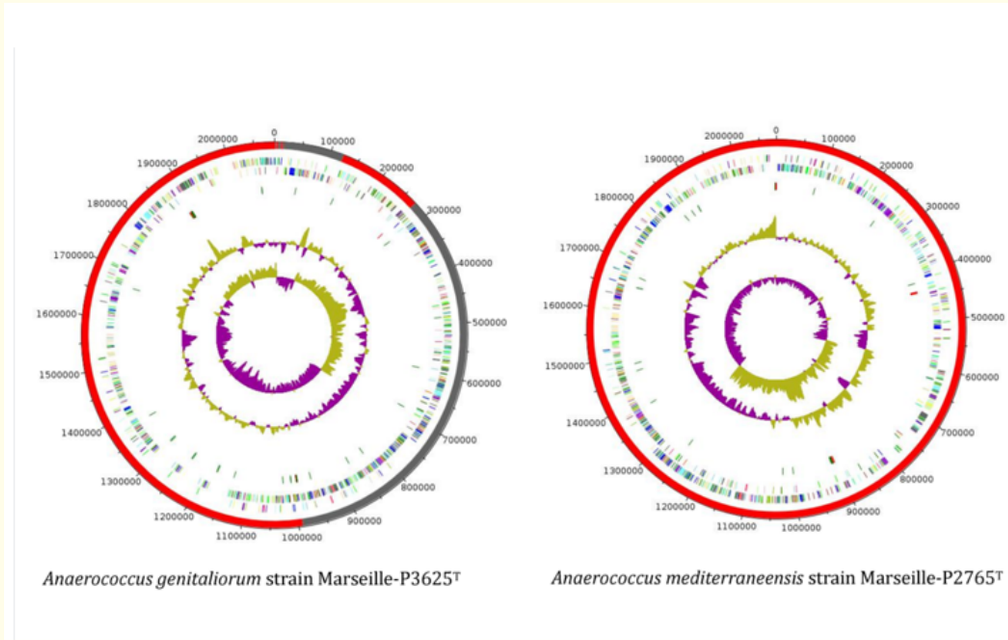

Figure 2: *Graphical circular map of the two genomes. From outside to the center: Contigs (red/gray), COG category of genes on the forward strand (three circles), genes on forward strand (blue circle), genes on the reverse strand (red circle), COG category on the reverse strand (three circles), GC content.*

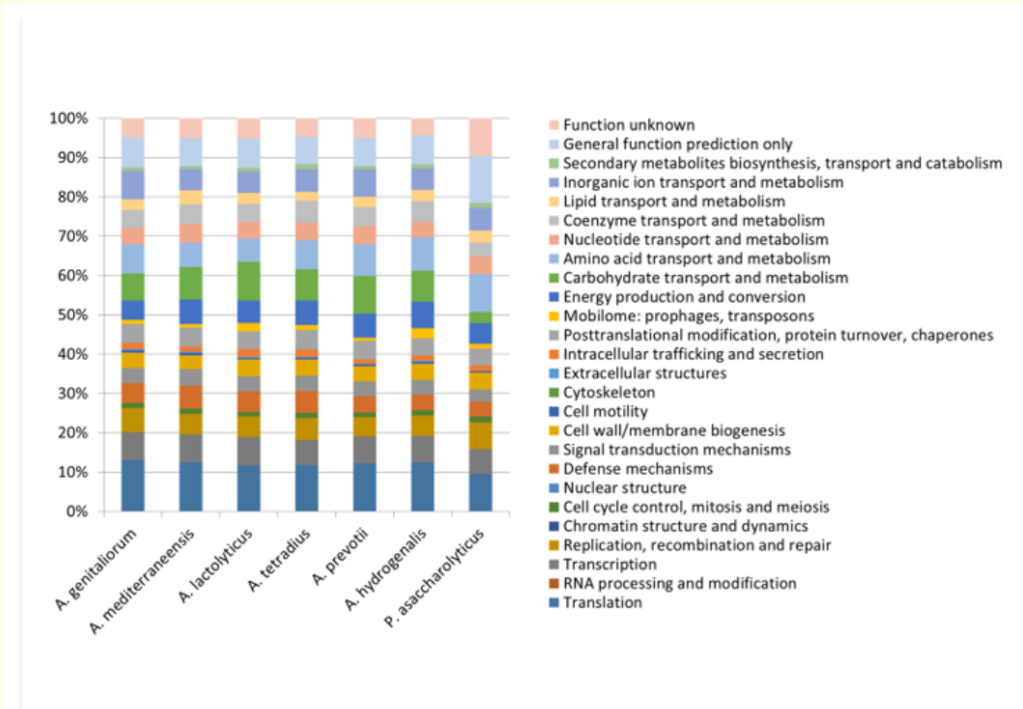

Figure 3: *Distribution of functional classes of predicted genes according to the clusters of orthologous groups of proteins of A. genitaliorum strain Marseille-P3625T and A. mediterraneensis strain Marseille-P2765T among other closely related species.*

The obtained dDDH and AAI values were lower than the 70% and 95-96% cutoff values for species delineation, respectively [40,41,45], supporting the status of new species of strains Marseille-P3625 and Marseille-P2765 within the *Anaerococcus* genus.

Conclusion

The strictly anaerobic strains Marseille-P3625 and Marseille-P2765 were isolated from vaginal samples of French women suffering from BV, as part of the investigation of the bacterial diversity of the vaginal microbiota in healthy individuals and patients suffering from BV. On the basis of the data from phenotypic, phylogenetic and genomic analyses, strain Marseille-P3625^T and strain Marseille-P2765^T were proposed to be the type strains of 2 new distinct species within the genus *Anaerococcus*, for which we propose the names *Anaerococcus genitaliorum sp. nov., and Anaerococcus mediterraneensis sp. nov.,* respectively.

Description of *Anaerococcus genitaliorum* sp. nov., and *Anaerococcus mediterraneensis* sp. nov.

Description of *Anaerococcus genitaliorum* sp. nov.

Anaerococcus genitaliorum (ge.ni.ta.lio'rum. L. fem. adj., genitaliorum referring to the human genital tract where strain Marseille-P3625^T was first isolated).

It is a strictly anaerobic Gram-stain-positive, non-motile coccus-shaped bacterium that grows from 28 to 45°C, at NaCl concentrations lower than 5 g/L and at pH values ranging from 6.5 to 7.5. Cells measure a mean diameter of 1.1 µm. Colonies are white and circular, with a mean diameter of 2 mm on blood-enriched Columbia agar in anaerobic atmosphere. Esculin ferric citrate is hydrolyzed but indole formation, catalase, oxidase and urease activities are not detected. Using an API ZYM strip (bioMérieux), esterase (C4), alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase and naphtol-AS-BI-phosphohydrolase activities are observed. No activity is for esterase lipase (C8), lipase (14), cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Using an API 20A strip (bioMérieux), strain Marseille-P3625^T produces acid from D-glucose, D-maltose, D-trehalose and gelatin but not from D-mannitol, D-lactose, D-saccharose, salicin, D-xylose, L-arabinose, glycerol, D-cellobiose, D-mannose, D-melezitose, D-raffinose, sorbitol and D-rhamnose. The most abundant fatty acids are Hexadecanoic acid and 9-Octadecenoic acid. Strain Marseille-P3625^T is susceptible to amoxicillin, benzylpenicillin, ceftriaxone, imipenem, ertapenem, rifampicin, vancomycin and metronidazole, but resistant to erythromycin, amikacin and ofloxacin.

The type strain Marseille-P3625^T (=CSUR P3625 = CECT 9562) was isolated from the vaginal sample of a French woman suffering from bacterial vaginosis. Its genome is 2,080,215-bp-long, with a 34.6 mol% G+C content. The 16S rRNA and whole-genome sequences are deposited in EMBL-EBI under accession numbers LT900366 and OCTP00000000, respectively.

Description of *Anaerococcus mediterraneensis* sp. nov.

Anaerococcus mediterraneensis (me.di.ter.ra.ne.en'sis, L. masc. adj., mediterraneensis, of Mediterraneum, the Latin name of the Mediterranean Sea by which Marseille, where was cultivated strain Marseille-P2765^T was isolated, is located).

The bacterium is strictly anaerobic Gram-stain-positive, non-motile and coccus-shaped. It grows from 28 to 45°C, at NaCl concentrations lower than 5g/L and at pH values ranging from 6.5 to 7.5. Cells measure a mean diameter of 1.0 µm. Colonies are white and circular, with a mean diameter of 2 mm on blood-enriched Columbia agar in anaerobic atmosphere. Esculin ferric citrate is not hydrolyzed. Indole formation, catalase, oxidase and urease activities are not detected. Using an API ZYM strip (bioMérieux), esterase (C4), esterase lipase (C8), alkaline phosphatase, acid phosphatase and naphtol-AS-BI-phosphohydrolase activities are observed. No activity is observed for lipase [14], leucine arylamidase, 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. Using an API 20A strip

(bioMérieux), strain Marseille-P2765^T produces acid from D-glucose, D-maltose and D-mannose but not from D-mannitol, D-lactose, D-saccharose, salicin, D-xylose, L-arabinose, glycerol, D-cellobiose, D-melezitose, D-raffinose, sorbitol, D-rhamnose, gelatin and D-trehalose. The most abundant fatty acids are Hexadecanoic acid and 9-Octadecenoic acid. Strain Marseille-P2765^T is susceptible to amoxicillin, benzylpenicillin, ceftriaxone, imipenem, ertapenem, rifampicin, vancomycin and metronidazole, but resistant to erythromycin, amikacin and ofloxacin.

The type strain Marseille-P2765^T (=CSUR P2765 = DSM 103343) was isolated from the vaginal sample of a French woman suffering from bacterial vaginosis. Its genome is 2,086,176-bp-long with 31.3 mol% G+C content. The 16S rRNA and whole-genome sequences are deposited in EMBL-EBI under accession numbers LT598544 and LT635772, respectively.

Conflict of Interest

The authors declare no competing interest in relation to this research.

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