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'*Massilibacteroides vaginae*' gen. nov., sp. nov., a new genus within the family of *Bacteroidaceae* isolated from a patient with vaginosis

J. Jneid¹, J. Y. Bou Khalil¹, S. Aherfi¹, C. Blanc-Tailleux¹, D. Raoult^{1,2}, B. La Scola^{1,2} and I. Pagnier¹

1) Aix Marseille Université, URMITE, UM63, CNRS 7278, IRD 798, INSERM 1095, Institut Hospitalo-Universitaire Méditerranée Infection, Marseille, France and 2) Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, Pôle des Maladies Infectieuses et Tropicales Clinique et Biologique, Fédération de Bactériologie-Hygiène-Virologie, Centre Hospitalo-Universitaire Timone, Assistance Publique—Hôpitaux de Marseille, Marseille, France

Abstract

'*Massilibacteroides vaginae*' sp. nov. strain MV12^T is the type strain of '*Massilibacteroides*' gen. nov., a new genus within the family of *Porphyromonadaceae*. This strain was isolated in Marseille from a patient with vaginosis. '*Massilibacteroides vaginae*' is a Gram-negative, aero-anaerobic bipolar bacillus. Here we describe the phenotypic characteristics and complete genome sequence of this bacterium. The EMBL accession number is FXDJ00000000 (FXDJ01000001–FXDJ01000009), and its genome consists of nine scaffolds. The strain was deposited in two culture collections under the numbers CSUR P1477 and DSM 100704.

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Corresponding author: I. Pagnier, Aix Marseille Université, URMITE, UM63, CNRS 7278, IRD 798, INSERM 1095, Institut Hospitalo-Universitaire Méditerranée Infection, 19-21 Bd Jean Moulin, 13005 Marseille, France

E-mail: isabelle.pagnier@univ-amu.fr

Introduction

Bacterial vaginosis presents a pressing health concern, as it regularly affects young women of childbearing age and is involved in cases of prematurity, with a prevalence of 10% to 30% in developed countries [1,2]. In eastern and southern Africa, the prevalence is even higher, at more than 50%, and is often associated as a risk factor for sexually transmitted diseases, such as herpes simplex virus type 2 and HIV [2].

In bacterial vaginosis, normal bacterial flora are modified. The main species present in a normal flora, *Lactobacillus* sp., are depleted and replaced by an abnormal population consisting of a higher diversity of bacterial species, including the anaerobic species *Gardnerella vaginae* and *Atopobium vaginae*, which are overrepresented compared to healthy patients [2].

Vaginal flora are now the subject of increasing interest, thanks to several advances in microbial investigation methods including molecular tools such as metagenomics, sequencing and phylogenetic analysis [3–5], as well as culture techniques like culturomics and bacterial identification using matrix-assisted desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) [6,7].

As a result of these advances, during the microbiologic exploration of vaginal samples from women with bacterial vaginosis in a postpregnancy study, we were able to isolate a new bacterial strain, which represents the first species of a new genus, named '*Massilibacteroides vaginae*,' strain MV12^T (= CSUR P1477 = DSM 100704).

Here we describe this new bacterial genus, including a summary classification, set of phenotypical characteristics and description of the complete genome sequencing and annotation. Those features support the circumscription of the genus and species '*Massilibacteroides vaginae*.'

Organism information

Classification and features

A vaginal specimen was collected during a prospective post-pregnancy study at the Conception University Hospital of Marseille (France). The vaginal swab was performed on 9 March 2013. The specimen was collected from a 27-year-old woman with bacterial vaginosis that had a molecular detection of 10¹⁰/mL *A. vaginae* [8]. After sample collection, the specimen was stored at –30°C. The '*Massilibacteroides vaginae*' MV12^T strain was isolated in October 2014 by anaerobic cultivation on 5% sheep's blood–enriched Columbia agar (bioMérieux, Marcy

l'Etoile, France) after incubation in an anaerobic blood culture bottle (Becton Dickinson [BD], San Diego, CA, USA), supplemented with sheep's blood and clarified sterile sheep rumen.

MALDI-TOF MS protein analysis was carried out using a Microflex spectrometer (Bruker Daltonics, Bremen, Germany) in order to identify the bacterium. Twenty separated colonies were deposited on a MTP 384 MALDI-TOF target plate (Bruker). Each spot was covered with 2 μ L of matrix solution (saturated solution of α -cyano-4-hydroxycinnamic acid) in 50% acetonitrile and 2.5% trifluoroacetic acid, then left to dry for 5 minutes at room temperature. The spectra were imported into the MALDI BioTyper software (version 2.0, Bruker) and analysed using standard pattern matching (with default parameter settings) against the main spectra of 6335 bacteria in the BioTyper database. A score made it possible to identify or not identify the tested species: a score of >2 with a validated species enabled identification at the species level; a score of >1.7 but <2 enabled identification at the genus level; and a score of <1.7 did not enable any identification. For strain MV12^T, the highest score reached was 1.34, which does not enable any identification. Therefore, this isolate could not be ranged within any known genus. The reference mass spectrum, generated by comparing 20 spectra obtained from 20 individual colonies of strain MV12^T, was integrated into our database (Fig. 1).

Because MALDI-TOF MS identification failed, we performed identification by amplifying and sequencing the 16S rRNA gene, as previously described [9]. The strain MV12^T exhibited a 93.5% 16S rRNA gene sequence similarity with *Parabacteroides goldsteinii*, the closest related species with a validly published name

[10]. This similarity percentage is lower than the threshold recommended to delineate a new genus without carrying out DNA-DNA hybridization [11,12]. The genus *Parabacteroides* belongs to the family of *Porphyromonadaceae* [13], which contains 18 different genera. Of them, the genus found to be the most closely related to '*Massilibacteroides vaginae*' strain MV12^T, according to the comparison of the 16S rRNA gene sequences, is first *Parabacteroides*, followed by *Tannerella*, *Porphyromonas* and *Dysgonomonas*. Phylogenetic analysis was performed, and in the inferred phylogenetic tree obtained by comparing the 16S rRNA gene sequence obtained for strain MV12^T with the closest related species, it forms a distinct lineage close to the genus *Parabacteroides* (Fig. 2). Sequences were aligned using CLUSTALW software, and the phylogenetic tree was built using MEGA software [14].

The strain was incubated at several growth temperatures (25, 28, 32, 35, 37, 40 and 50°C). The bacterium grew between 25 and 40°C, with optimal growth at 37°C. No growth was evident at 50°C. Morphologically, the colonies were light beige, slightly bright and nonhaemolytic, approximately 0.5 mm in diameter. On Gram staining they appeared as bipolar Gram-negative bacilli, nonmotile and unable to form spores (Fig. 3). The morphology of the bacteria was observed by electron microscopy and the following method: detection-coated grids were deposited on 40 μ L of bacterial suspension and incubated for 30 minutes at 37°C. The grids were then brought into contact for 1 second with a drop of ammonium molybdate 1%, dried on filter paper and observed with a Tecnai G20 Cryo electron microscope (FEI Company, Limeil-Brevannes, France)

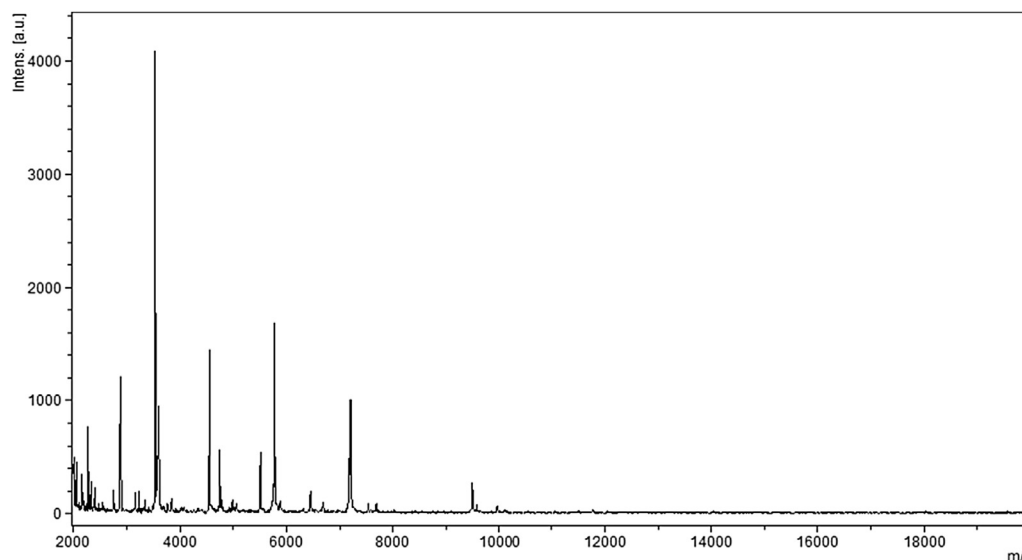


FIG. 1. Phylogenetic reconstruction based on 16S rRNA gene sequence, highlighting phylogenetic position of '*Massilibacteroides vaginae*' relative to other bacteria belonging to other genera in *Porphyromonadaceae* family. Sequences were aligned using Muscle [9] and phylogenetic tree was built using MEGA6 software [10] and maximum likelihood method.

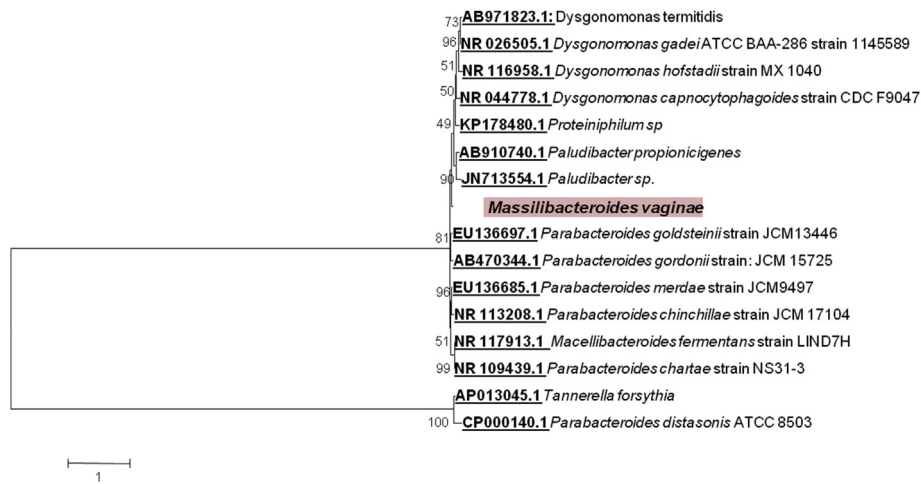


FIG. 2. Reference mass spectrum obtained by concatenation of 20 spectra generated from 20 colonies of ‘*Massilibacteroides vaginae*’ strain MV12^T.

at an operating voltage of 60 kV. Using the electron microscopy, we determined that the cells had a mean length of 2.06 μm (range 1.27–3.21 μm) and a width of 0.73 μm (range 0.62–0.81 μm) (Fig. 4).

The growth of the bacterium was tested in different atmospheric conditions using GENbag anaer and GENbag microaer systems (bioMérieux). The bacteria multiplied in aerobic and anaerobic atmospheres. It also multiplied in microaerophilic atmosphere supplemented with 5% CO₂. However, optimal growth was shown in anaerobic conditions. Colonies also grew on brain–heart infusion (BHI) agar and on BHI agar supplemented with 1% NaCl. This strain exhibited oxidase and catalase activities.

Using an API ZYM (bioMérieux), positive reactions were observed for phosphatase alkaline, leucine arylamidase, trypsin, phosphatase acid, naphthol phosphohydrolase, β-galactosidase, α-glucosidase, β-glucuronidase and *N*-acetyl β-glucosaminidase.

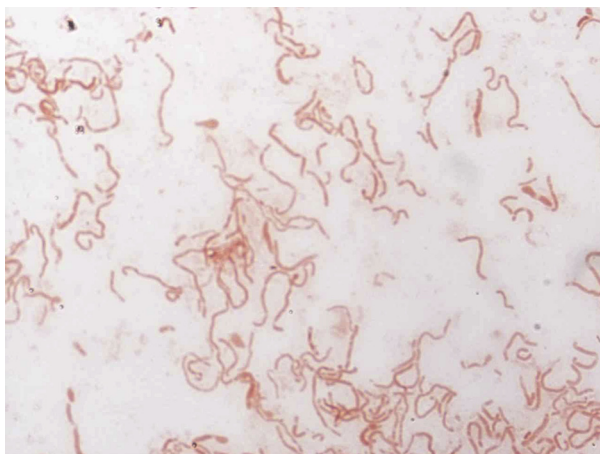


FIG. 3. Gram staining of ‘*Massilibacteroides vaginae*’ strain MV12^T.

Negative reactions were observed for esterase, esterase lipase, lipase, valine arylamidase, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase.

A rapid ID 32 A API strip (bioMérieux) was conducted, and positive reactions were obtained with arginine dihydrolase, β-galactosidase, β-galactosidase 6 phosphate, α-glucosidase, β-glucosidase, α-arabinosidase, *N*-acetyl-β-glucosaminidase, fermentation of mannose, fermentation of raffinose, glutamic decarboxylase acid, α-fucosidase, reduction of nitrates,

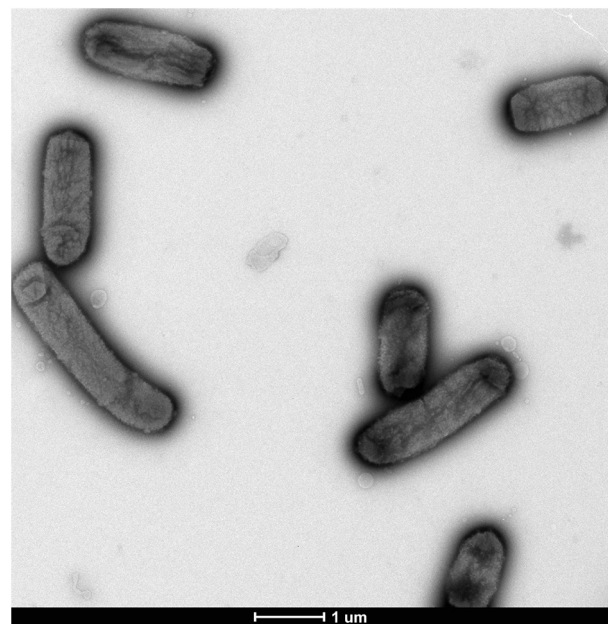


FIG. 4. Transmission electron microscopy of ‘*Massilibacteroides vaginae*’ strain MV12^T, obtained with Tecnai G20Cryo electron microscope, at operating voltage of 60 kV. Scale bar represents 1 μm.

phosphatase alkaline, arginine arylamidase, leucyl glycine arylamidase, leucine arylamidase, alanine arylamidase, glutamyl acid and glutamic arylamidase. The reaction was negative for the following enzymes: urease, α -galactosidase, β -glucuronidase, production of indole, proline arylamidase, phenylalanine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, glycine arylamidase, histidine arylamidase and serine arylamidase.

The susceptibility of *M. vaginae* strain MV12^T was tested *in vitro* using the Etest method. We observed susceptibility to benzylpenicillin (MIC = 0.094 μ g/mL), cefepim (MIC = 6 μ g/mL), imipenem (MIC = 0.5 μ g/mL), metronidazole (MIC = 0.094 μ g/mL), vancomycin (MIC = 3 μ g/mL) and amoxicillin/clavulanic acid (MIC = 125 μ g/mL).

Phenotypic features and comparison with those of the closest related genus are summarized in Table 1.

Genome sequencing information

Growth conditions and genomic DNA (gDNA) preparation

Strain MV12^T was grown under anaerobic conditions on 5% sheep's blood-enriched Columbia agar (bioMérieux) at 37°C. Colonies from five plates were collected and resuspended in 4 × 100 μ L of Tris-EDTA (TE) buffer. An aliquot of 200 μ L of this suspension was then diluted in 1 mL of TE buffer and

incubated with 2.5 μ g/ μ L of lysozyme at 37°C for 30 minutes, for lysis treatment. This was followed by overnight incubation with 20 μ g/ μ L proteinase K at 37°C. DNA was then extracted and purified with three successive phenol-chloroform extractions, then ethanol precipitation at -20°C overnight. After a final centrifugation, the DNA was resuspended in 160 μ L of TE buffer.

Genome sequencing and assembly

The gDNA of *M. vaginae* was sequenced on MiSeq Technology (Illumina, San Diego, CA, USA) using the mate-pair strategy. The gDNA was barcoded in order to be mixed with 11 other projects with the Nextera mate-pair strategy. The gDNA was quantified using a Qubit assay with a high-sensitivity kit (Life Technologies, Carlsbad, CA, USA) to 333.7 ng/ μ L. The mate pair library was prepared with 1.5 μ g of gDNA using the Nextera mate pair Illumina guide. The gDNA sample was simultaneously fragmented and tagged using a mate-pair junction adapter. The pattern of fragmentation was validated on an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) with a DNA 7500 labchip. The DNA fragments ranged in size from 1.5 to 11 kb, with an optimal size of 6.08 kb. No size selection was performed, and 716.8 ng of tagmented fragments were circularized. The circularized DNA was mechanically sheared to small fragments with an optimal size of 1049 bp on the Covaris device S2 in T6 tubes (Covaris, Woburn, MA, USA). The library profile was visualized using a High Sensitivity Bioanalyzer LabChip (Agilent Technologies), and the final concentration library was measured at 14.65 nmol/L. The libraries were normalized at 2 nM and pooled. After a denaturation step and dilution at 15 pM, the pool of libraries was loaded onto the reagent cartridge and then onto the instrument with the flow cell. Automated cluster generation and sequencing runs were performed in a single 39-hour run at a 2 × 251 bp read length. A total of 5.5 Gb of information was obtained from a 572 k/mm² cluster density with a cluster passing quality control filters of 96.3% (10 764 000 passed filter clusters). Within this run, the index representation for *M. vaginae* MV12^T was identified as 5.81%. The 625 022 paired reads were filtered according to the read qualities. These reads were trimmed then assembled.

Genome annotation

Open reading frames (ORFs) were predicted using Prodigal [15] with default parameters. Functional annotation was performed using comparative genomics by BLASTp search against the GenBank nonredundant (nr) and Clusters of Orthologous Groups (COGs) databases [16], with an *E*-value threshold of <1e-3 for amino acid sequences >80 aa and <1e-5 for sequences of <80 aa. The transfer RNAs were searched for using

TABLE 1. Phenotypic features differentiating *Maslibacteroides vaginae* strain MV12^T to closest related genera *Parabacteroides goldsteinii* strain DSM19448 and *Parabacteroides gordonii* strain DSM 23371

Property	<i>M. vaginae</i>	<i>P. goldsteinii</i>	<i>P. gordonii</i>
Cell width (μ m)	0.2–0.5	NA	0.5–1.7
Oxygen requirement	Aero-anaerobic	Anaerobic	Anaerobic
Gram stain	Negative	Negative	Negative
Optimal growth temperature	37°C	37°C	37°C
Habitat	Vagina	Specimens from humans	Blood culture
Use of:			
α -Glucosidase	+	+	+
α -Galactosidase	-	+	+
β -Galactosidase	+	+	+
<i>N</i> -acetyl- β -glucosaminidase	+	+	NA
Acid phosphatase	+	+	NA
Alkaline phosphatase	+	+	+
Leucine arylamidase	+	+	+
Arginine arylamidase	+	+	+
Leucyl glycine arylamidase	+	+	+
Phenylalanine arylamidase	-	+	-
Alanine arylamidase	+	+	+
Glycine arylamidase	-	+	-
Histidine arylamidase	-	+	-
Glutamyl glutamic acid arylamidase	+	+	-
Production of:			
Indole	-	-	-

+, positive result; -, negative result; NA, data not available.

tRNAScanSE tool [17], ribosomal RNAs by using RNAmmer [18] and BLASTn against GenBank. The ORFans were identified as sequences which were larger than 80 aa without a significant hit, which was defined as an *E* value of $>1e-03$ against the nr database. Artemis was used to visualize genomic features [19]. Pairwise genome comparisons were performed using Proteinortho software [20] to detect best BLASTp reciprocal hits between *M. vaginae* MV12^T and nine other bacteria (*Dysgonomonas capnocytophagoideis*, *Dysgonomonas mossii*, *Dysgonomonas gadei*, *Porphyromonas cangingivalis*, *Proteiniphilum acetatigenes*, *Tannerella forsythia*, *Bacteroides massiliensis*, *Parabacteroides gordonii* and *Parabacteroides distasonis*), using 1e-5, 30% and 70% as thresholds for the *E* value, identity amino acid percentage and coverage.

Genome properties

The genome of *M. vaginae* MV12^T is 3 710 320 bp long, composed of one chromosome and no plasmid, and harbouring

40.11% G+C content (Fig. 5). Of the 3062 predicted genes, 3001 were protein-coding genes and 61 were RNAs (10 rRNA and 51 tRNA genes). A total of 1969 genes (65.61%) were assigned a putative function. A total of 172 genes were identified as ORFans (5.73%). The remaining genes were annotated as hypothetical proteins (774 genes = 25.79%). The distribution of genes into COGs functional categories is provided in Table 2, and the properties of the genome are summarized in Table 3.

Insights from the genome sequence

The *M. vaginae* strain MV12^T genome (3.71 Mbp) is smaller than those of *D. mossii* (3.92 Mbp), *B. massiliensis* (4.51 Mbp), *D. gadei* (5.17 Mbp), *P. gordonii* (6.67 Mbp), *P. distasonis* (4.81 Mbp), *P. acetatigenes* (4.78 Mbp) and *D. capnocytophagoideis* (4.38 Mbp). It is larger than those of *T. forsythia* (3.41 Mbp) and *P. cangingivalis* (2.36 Mbp).

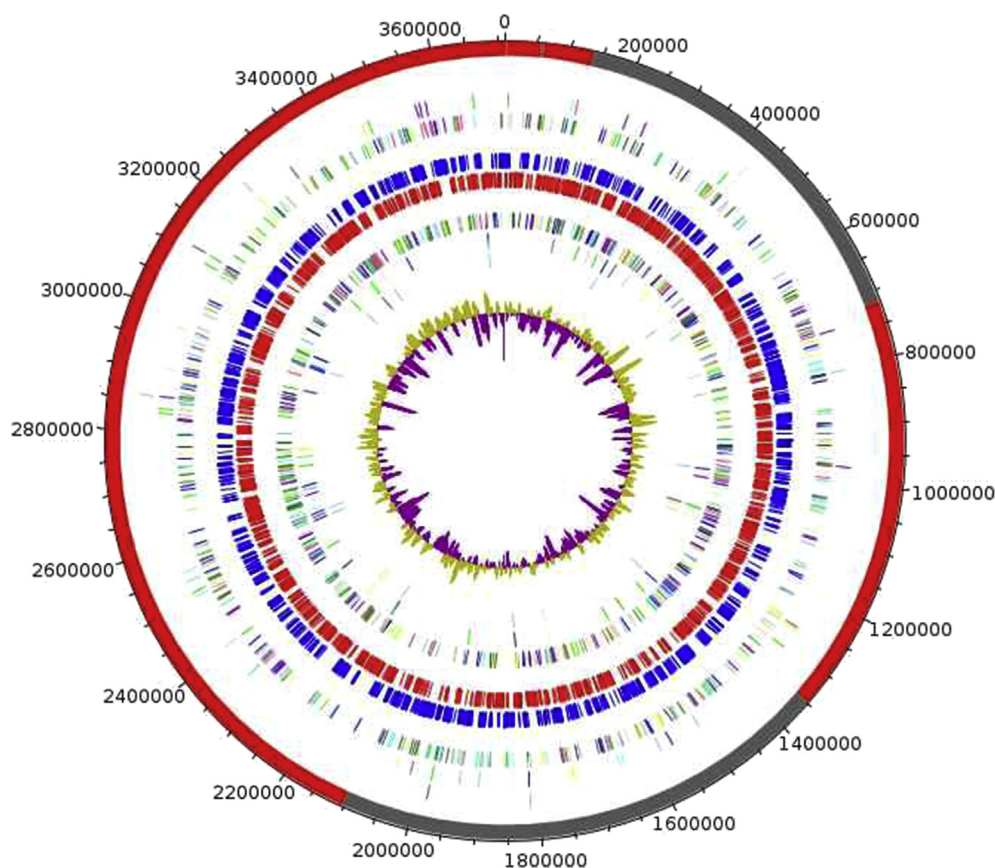


FIG. 5. Graphical circular map of '*Massilibacteroides vaginae*' genome. From outside to center: contigs (red/gray), COGs category of genes on forward strand (three circles), genes on forward strand (blue circle), genes on reverse strand (red circle), COGs category on reverse strand (three circles), GC content. COGs, Clusters of Orthologous Groups database.

TABLE 2. Number of genes associated with 25 general COGs functional categories

Code	Description	Value	Percentage
[J]	Translation	144.0	4.7
[A]	RNA processing and modification	0.0	0.0
[K]	Transcription	88.0	2.9
[L]	Replication, recombination and repair	119.0	3.9
[B]	Chromatin structure and dynamics	0.0	0.0
[D]	Cell cycle control, mitosis and meiosis	13.0	0.4
[Y]	Nuclear structure	0.0	0.0
[V]	Defense mechanisms	53.0	1.7
[T]	Signal transduction mechanisms	42.0	1.4
[M]	Cell wall/membrane biogenesis	119.0	3.9
[N]	Cell motility	0.0	0.0
[Z]	Cytoskeleton	0.0	0.0
[W]	Extracellular structures	0.0	0.0
[U]	Intracellular trafficking and secretion	15.0	0.5
[O]	Posttranslational modification, protein turnover, chaperones	55.0	1.8
[C]	Energy production and conversion	109.0	3.6
[G]	Carbohydrate transport and metabolism	139.0	4.5
[E]	Amino acid transport and metabolism	139.0	4.5
[F]	Nucleotide transport and metabolism	56.0	1.8
[H]	Coenzyme transport and metabolism	82.0	2.7
[I]	Lipid transport and metabolism	44.0	1.4
[P]	Inorganic ion transport and metabolism	68.0	2.2
[Q]	Secondary metabolites biosynthesis, transport and catabolism	18.0	0.6
[R]	General function prediction only	189.0	6.2
[S]	Function unknown	72.0	2.4

COGs, Clusters of Orthologous Groups database.

The G+C content of *M. vaginae* strain MV12^T (40.11%) is in the same range as those observed in the *Porphyromonadaceae* family, which vary between 37.47% for *D. mossii* and 47.60% for *P. cangingivalis*.

The *M. vaginae* strain MV12^T gene content is the same size as *T. forsythia* ATCC43037 (3001 Kb), larger than *P. cangingivalis* (2459 Kb) and smaller than the other bacteria of the family: *D. mossii* (3386 Kb), *B. massiliensis* (3935 Kb), *D. gadei* (4156 Kb), *P. gordonii* (5329 Kb), *P. distasonis* (3850 Kb), *P. acetatigenes* (3875 Kb) and *D. capnocytophagoides* (3578 Kb).

The comparison of *M. vaginae* strain MV12^T to the other related genera, regarding the number of orthologous proteins shared between genomes, is represented in Table 4.

TABLE 3. Nucleotide content and gene count level of genome

Attribute	Value	Percent
Genome size (bp)	3 710 320	100
DNA G+C content	1 488 269	40.1
No. of genes	3062	100
Protein-encoding genes	3001	98.0
RNA genes	61	2.0
DNA coding sequence size	3 361 204	90.6
Proteins associated to COGs	1324	44.1
ORFans	172	5.7
Hypothetical proteins	774	25.8
Paralogous genes (E value = 1e-25)	441	14.7
Proteins assigned with a predicted function	731	24.4
Protein with transmembrane helices	608	20.3
Genes with atypical GC and codon bias usage	19	0.6
Genes associated with antimicrobial resistance	0	0
Genes associated with bacteriocin	9	0.3
Genes associated with mobilome	1315	43.8
Genes associated with toxin/antitoxin	78	2.6

COGs, Clusters of Orthologous Groups database; ORF, open reading frame.

TABLE 4. Numbers of orthologous proteins shared between genomes

	Dysgonomonas mossii strain CCUG43457	Dysgonomonas capnocytophagoides	Dysgonomonas gadei	Porphyromonas cangingivalis	Proteiniphilum acetatigenes	Tannerella forsythia	Bacteroides massiliensis	Parabacteroides gordonii	Massilibacteroides vaginae	Parabacteroides distasonis
<i>D. capnocytophagoides</i>	—	—	—	—	—	—	—	—	—	—
<i>D. mossii</i> strain CCUG43457	1592	0.70	1702	715	1318	1023	1164	1372	1248	1284
<i>D. gadei</i>	—	0.74	1771	721	1320	1049	1178	1404	1267	1331
<i>P. cangingivalis</i>	0.74	—	—	720	1475	1112	1209	1530	1392	1404
<i>P. acetatigenes</i>	0.61	0.61	0.61	—	729	708	760	803	741	791
<i>T. forsythia</i>	0.65	0.65	0.65	0.61	—	1114	1182	1479	1316	1358
<i>B. massiliensis</i>	0.63	0.63	0.63	0.62	0.64	—	1081	1281	1197	1225
<i>P. gordonii</i>	0.65	0.65	0.65	0.61	0.64	0.64	—	1542	1227	1488
<i>M. vaginae</i>	0.65	0.65	0.65	0.62	0.65	0.66	0.67	—	1627	1910
<i>P. distasonis</i>	0.66	0.66	0.66	0.62	0.64	0.66	0.66	0.70	—	1538
	0.64	0.64	0.64	0.62	0.64	0.67	0.66	0.74	0.69	—

Conclusion

Regarding the analysis of the phenotypic, phylogenetic and genomic features of strain MV12^T, we propose the creation of the new genus '*Massilibacteroides*,' for which the type strain is '*Massilibacteroides vaginae*' strain MV12^T. This strain was isolated from the vaginal flora of a 27-year-old woman with bacterial vaginosis.

Description of *Massilibacteroides* gen. nov.

'*Massilibacteroides*' (Ma.ssi.li'bac.te.ro.i.des, from *Massilia*, the Roman name for Marseille, the city in which the strain was isolated, and *bacteroides*, as being phylogenetically close to the genus *bacteroides*) was chosen because the strain was isolated in Marseille, France. Gram-negative rods. Facultative anaerobic. Mesophilic. Nonmotile. Exhibits catalase and oxidase activity. Positive for phosphatase alkaline, leucine arylamidase, trypsin, phosphatase acid, naphthol phosphohydrolase, β -galactosidase, α -glucosidase, β -glucuronidase, *N*-acetyl β -glucosaminidase, arginine dihydrolase, β -galactosidase, β -galactosidase 6 phosphate, α -glucosidase, β -glucosidase, α -arabinosidase, *N*-acetyl- β -glucosaminidase, fermentation of mannose, fermentation of raffinose, glutamic decarboxylase acid, α -fucosidase, reduction of nitrates, phosphatase alkaline, arginine arylamidase, leucyl glycine arylamidase, leucine arylamidase, alanine arylamidase, glutamyl acid and glutamic arylamidase. Habitat: human vaginal flora. Type species: '*Massilibacteroides vaginae*.'

Description of '*Massilibacteroides vaginae*' sp. nov.

'*Massilibacteroides vaginae*' (va.gi'nae, L. n., from *vagina*, 'vagina'; L. gen. n. *vaginae*, 'of the vagina'). Gram-negative bacilli. Facultative anaerobic. Mesophilic. Nonmotile. Optimal growth at 37°C. Nonsporulating. Catalase and oxidase activity. Colonies are light beige, slightly bright, nonhaemolytic and approximately 0.5 mm in diameter. Cells are bacilli with a mean length of 2.06 μ m and a mean width of 0.73 μ m. Positive for phosphatase alkaline, leucine arylamidase, trypsin, phosphatase acid, naphthol phosphohydrolase, β -galactosidase, α -glucosidase, β -glucuronidase, *N*-acetyl β -glucosaminidase, arginine dihydrolase, β -galactosidase, β -galactosidase 6 phosphate, α -glucosidase, β -glucosidase, α -arabinosidase, *N*-acetyl- β -glucosaminidase, fermentation of mannose, fermentation of raffinose, glutamic decarboxylase acid, α -fucosidase, reduction of nitrates, phosphatase alkaline, arginine arylamidase, leucyl

glycine arylamidase, leucine arylamidase, alanine arylamidase, glutamyl acid and glutamic arylamidase. Strain MV12^T has susceptibility to penicillin G, cefotetan, imipenem, metronidazole, vancomycin and amoxicillin/clavulanic acid.

The 16S rRNA gene sequence was deposited in GenBank database under the accession number LN866992. The European Molecular Biology Laboratory (EMBL) accession number for the complete genome sequence is FXDJ00000000 (FXDJ01000001–FXDJ01000009), and it consists of nine scaffolds. The strain was deposited in two culture collections: the Collection de Souches de l'Unité des Rickettsies (CSUR) under number CSUR PI 477, and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) under number DSM 100704.

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Conflict of interest

None declared.

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