Two new bacteria isolated from vagina of a patient with vaginosis: *Atopobium massiliense* sp. nov. and *Butyricimonas vaginalis* sp. nov.

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Abstract

Two new bacterial strains, Marseille-P4126 (=CSURP4126) and Marseille-P4593 (=CSURP4593), were isolated from the vaginal sample of a French woman with vaginosis. These strains were identified and characterized using the taxonogenomics method. The findings from phylogenetic tree interpretation, phenotypic criteria and genomic analysis provided here distinctly display that *Atopobium massiliense* sp. nov. and *Butyricimonas vaginalis* sp. nov. are new members of the genus *Atopobium* and *Butyricimonas*, respectively.

Keywords: *Atopobium massiliense* sp. nov., bacteria, *Butyricimonas vaginalis*sp. nov., taxonogenomics, vaginosis
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Introduction

Imbalance of the vaginal flora can lead to vaginitis such as bacterial vaginosis in women of reproductive age. To better understand normal vaginal flora and dysbiosis, it is important to describe in detail the vaginal ecosystem. Under physiological conditions, *Lactobacillus* spp. dominance is observed, but a switch in vaginal microbiota composition towards anaerobic pathogenic bacteria, such as *Atopobium* sp. or *Gardnerella* sp., is characteristic of the bacterial vaginosis flora [1,2].

Our laboratory has developed a culturomics strategy combined with taxonogenomic analysis that has made it possible to isolate and describe many new bacterial species [3], many of which are found in the vagina [4–6].

The genus *Atopobium* was proposed in 1992 by Collins and Wallbanks, after comparative sequence analysis between some obligate anaerobic species [7]. The six species described for this genus have been isolated from human respiratory tract (*Atopobium parvulum*) [8], healthy vagina (*Atopobium vaginae*) [9], blood (*Atopobium deltae*) [10], perineal abscess infections (*Atopobium minutum*) and gingival crevices (*Atopobium rimae*) [8], as well as in healthy pharynx and oral and respiratory tract lesions of horses (*Atopobium fossor*) [11]. More recently, the *Butyricimonas* genus was proposed by Sakamoto et al. [12]. At the time of writing, this genus was composed of five species isolated from human (*Butyricimonas faecalis*, *Butyricimonas faecihominis* and *Butyricimonas paravirosa*) [13,14] or rat (*Butyricimonas synergistica* and *Butyricimonas virosa*) faeces [12].

Here, we report the description of two new species designated *Atopobium massiliense* sp. nov., strain Marseille-P4126, and *Butyricimonas vaginalis* sp. nov., strain Marseille-P4593, both isolated from a vaginal swab in the context of bacterial vaginosis.

Materials and methods

Strain isolation and identification

Two bacterial strains, Marseille-P4126 and Marseille-P4593, were isolated by culturomic strategy from a vaginal sample of a French woman with bacterial vaginosis. The woman gave informed consent and the study was authorized by the ethics committee of the Institut Federatif de Recherche IFR48

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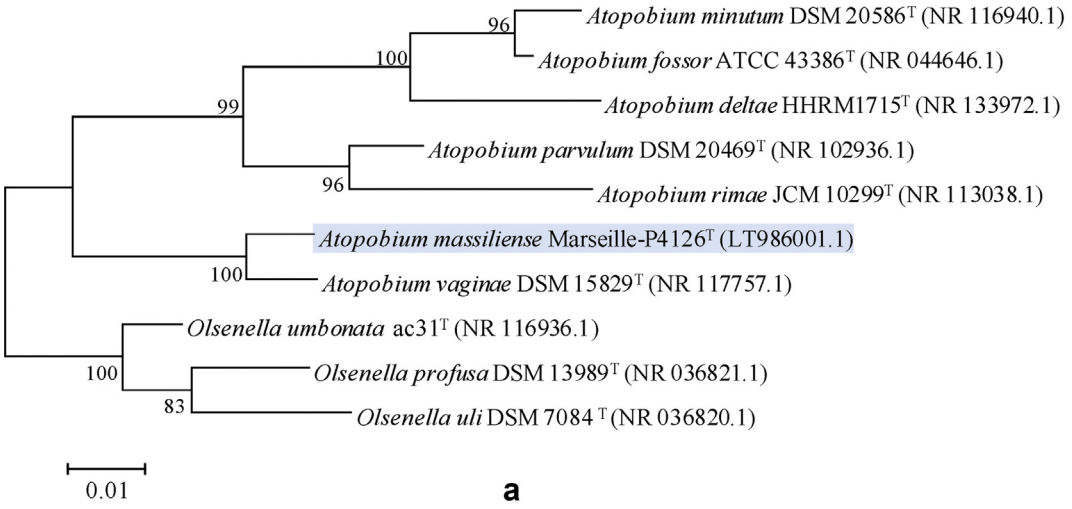
(agreement number: 09-022). The vaginal swab was treated as reported previously [5].

Bacterial identification was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany) and the spectra generated were analysed with BIOTYPER 3.0 software, using our local database, which is regularly incremented (https://www.mediterranee-infection.com/urms-data-base) [15]. As previously described, misidentification with MALDI-TOF MS led to 16S rRNA gene sequencing using fD1-rP2 primers (Eurogentec, Angers, France) and a 3500xLGenetic Analyzer capillary sequencer (Thermofisher, Saint- Aubin,



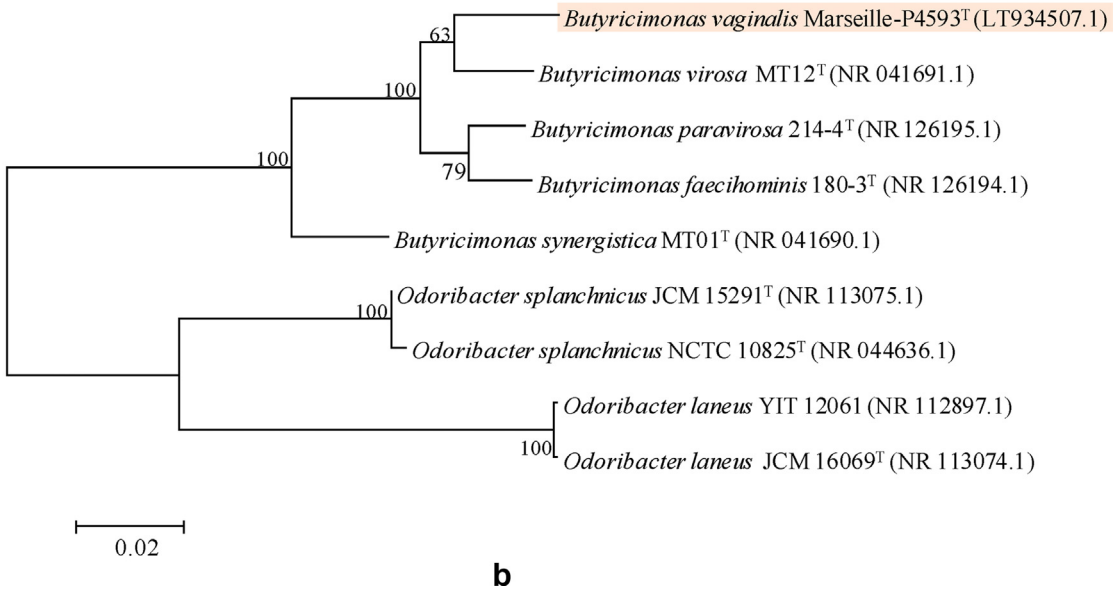


FIG. 1. Phylogenetic trees displaying the position of *Atopobium massiliense* strain Marseille-P4126^T (a) and *Butyricimonas vaginalis* strain Marseille-P4593^T (b) relative to their phylogenetically closest species. The respective GenBank accession numbers for 16S rRNA genes are indicated in parenthesis. Sequence alignment and phylogenetic inferences were obtained using the maximum likelihood method within MEGA 7 software. The numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 1000 times to generate a majority consensus tree.

TABLE 1. Different characteristics of 1, *Atopobium massiliense* sp. nov., strain Marseille-P4126; 2, *Atopobium rimae* strain DSM 7090 [7]; 3, *Atopobium parvulum* strain CCUG 32760 [7]; 4, *Atopobium minutum* strain DSM 20585 [7]; 5, *Atopobium vaginae* strain CCUG 38953^T [9]; 6, *Atopobium deltae* strain HHRM1715^T [10]

Characteristics	1	2	3	4	5	6
Cell diameter (µm)	0.4–0.88	NA	0.3–0.6	0.6–1	0.6–0.9	1
Oxygen requirement	OA	OA	OA	OA	FA	OA
Gram stain	+	+	+	+	+	+
Motility	—	—	—	—	—	—
Endospore formation	—	—	—	—	—	—
Alkaline phosphatase	+	NA	NA	NA	—	—
Leucine arylamidase	+	—	+	—	+	+
Acid phosphatase	+	+	+	—	+	NA
α-galactosidase	—	NA	NA	NA	—	—
β-galactosidase	—	—	+	—	—	—
N-acetyl-β-glucosaminidase	—	NA	NA	NA	—	—
α-fucosidase	—	NA	NA	NA	—	—
Catalase	—	—	—	—	NA	—
D-glucose	+	+	+	+	NA	NA
D-fructose	—	+	+	+	NA	NA
D-mannose	+	+	+	+	—	+
L-rhamnose	—	—	—	—	NA	NA
Inositol	—	—	—	—	NA	NA
D-mannitol	—	—	—	—	NA	NA
D-sorbitol	—	—	—	—	NA	NA
Amygdalin	—	—	—	—	NA	NA
Salicin	—	—	+	—	NA	NA
D-raffinose	+	—	—	—	—	NA
Glycogen	—	+	—	—	NA	NA
Source	Vagina	Gingival crevices	Respiratory tract	Perineal abscess	Vagina	Pathological blood

Abbreviations: **+**, positive reaction; **–**, negative reaction; FA, facultative anaerobe; NA, data not available; OA, obligate anaerobe.

France) [16]. All 16S rRNA nucleotide sequences were assembled and edited using CodonCode Aligner software (http://www.codoncode.com/) and the obtained sequence was matched against the NCBI database using the BLASTn algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Growth conditions and phenotypic characterization

In order to determine the best growth conditions of these two new strains, different temperatures and atmospheres (aerobic, anaerobic and microaerophilic) were tested as described by Diop et al. [17]. To assess bacterial biochemical characteristics, API ZYM and API 50 CH strips (bioMérieux, Marcy l'Étoile, France) were used, according to the manufacturer's recommendations. Gram-staining, catalase and oxidase activities, and sporulation were researched using standard procedures [18]. According to the Belkacemi's protocol, the morphological structure of these two new species was observed with a scanning electron microscope (Hitachi High-Technologies, Tokyo, Japan) [19].

Fatty acids analysis by gas chromatography/mass spectrometry

Approximately 50 mg of bacterial biomass collected from five culture plates for each strain allowed us to detect the amounts of fatty acids in these bacteria. Cellular fatty acid methyl esters were prepared as described by Sasser [20] and gas chromatography/mass spectrometry analyses were performed as described previously [21].

Genome sequencing and comparison

The genomic DNAs (gDNAs) of strains Marseille-P4126 and Marseille-P4593 were extracted with an EZ1 biorobot and the EZ1 DNA Tissue kit (Qiagen, Hilden, Germany), after being

TABLE 2. Different characteristics of 1, *Butyricimonas vaginalis* sp. nov., strain Marseille-P4593; 2, *Butyricimonas faecalis* strain H184^T [13]; 3, *Butyricimonas paravirosa* strain 214-4^T [14]; 4, *Butyricimonas virosa* strain DSM 23226^T [12]

Characteristics	1	2	3	4
Cell diameter (µm)	0.6–0.8	0.4–0.5	1	1–1.5
Oxygen requirement	—	—	—	—
Gram stain	—	–	–	–
Motility	–	–	–	–
Endospore formation	–	–	–	–
Alkaline phosphatase	—	—	+	+
Leucine arylamidase	—	—	—	—
α-galactosidase	—	—	—	—
β-galactosidase	—	+	+	+
β-glucuronidase	—	—	—	—
α-glucosidase	—	—	—	—
β-glucosidase	—	—	—	—
N-acetyl-β-glucosaminidase	+	+	+	+
α-fucosidase	—	+	+	—
Catalase	—	+	+	+
Glycerol	—	—	+	+
D-galactose	—	+	NA	NA
D-glucose	—	+	+	+
D-mannose	—	+	—	—
N-acetyl-glucosamine	—	+	NA	NA
Esculin ferric citrate	+	—	—	—
D-lactose	—	+	+	—
Potassium 5-ketogluconate	+	—	NA	NA
Source	Human vagina	Human faeces	Human faeces	Rat faeces

Abbreviations: **+**, positive reaction; **-**, negative reaction; NA, data not available.

TABLE 3. Cellular fatty acid compositions of strains Marseille-P4593 and Marseille-P4126 compared with their closely related species

Fatty acids	Names	Species				
***Atopobium* species**		**1A**	**2A**	**3A**	**4A**	**5A**
16:00	Hexadecanoic acid	60.2	33.3	0.8	1.1	34.8
18:1n9	9-Octadecenoic acid	24.0	27.7	38.2	32.5	25.4
18:2n6	9,12-Octadecadienoic acid	6.4	ND	ND	ND	ND
18:00	Octadecanoic acid	5.7	11.9	0.6	ND	17.5
16:1n7	9-Hexadecenoic acid	TR	ND	ND	ND	ND
15:00	Pentadecanoic acid	TR	ND	ND	ND	ND
17:1n7	10-Heptadecenoic acid	TR	ND	ND	ND	ND
***Butyricimonas* species**		**1B**	**2B**	**3B**	**4B**	**5B**
15:0 iso	13-methyl-tetradecanoic acid	75.4	64.6	57.6	61.8	68.6
16:00	Hexadecanoic acid	6.4	2.8	3.2	2.4	2.1
16:0 3-OH	3-hydroxy-Hexadecanoic acid	5.8	1.7	6.3	1.6	5.2
14:00	Tetradecanoic acid	5.2	TR	1.8	ND	1.3
17:0 3-OH iso	3-hydroxy-15-methyl-Hexadecanoic acid	1.7	5.3	10.6	14.9	10.4
18:1n9	9-Octadecenoic acid	TR	8.3	9.5	12.6	6.0
15:0 anteiso	12-methyl-tetradecanoic acid	TR	1.8	1.7	2.0	1.5
18:2n6	9,12-Octadecadienoic acid	TR	1.4	1.5	2.3	1.2
13:0 iso	11-methyl-Dodecanoic acid	TR	1.0	1.0	ND	TR

Abbreviations: 1A, *Atopobium massiliense* Marseille-P4126^T; 2A, *Atopobium deltae* HHRM 1715^T; 3A, *Atopobium parvulum* CCUG 32760^T; 4A, *Atopobium rimae* CCUG 31168^T; 5A, *Atopobium vaginae* CCUG 38953^T; 1B, *Butyricimonas vaginalis* Marseille-P4593; 2B, *Butyricimonas faecihominis* 180-3^T; 3B, *Butyricimonas paravirosa* 214-4^T; 4B, *Butyricimonas synergistica* JCM 15148^T; 5B, *Butyricimonas virosa* JCM 15149^T.

pretreated with lysozyme and incubated at 37°C for 2 hours. Sequencing of the gDNA from each strain was carried out with a MiSeq sequencer using the mate pair strategy (Illumina Inc., San Diego, CA, USA) as previously executed [4]. Then, assembly and annotation of the genomes of each strain were carried out using several softwares similar to those previously used [22].

Genomic comparison was performed by estimating the degrees of genomic sequence similarity among compared genomes using the different tools. The Genome-to-Genome Distance Calculator web server (available online at http://ggdc.dsmz.de) was used to calculate the DNA–DNA hybridization [23]. The average nucleotide identity analysis was assessed with the OAT software [24].

Results

Phylogenetic analysis

Analysis based on 16S rRNA gene sequences of Marseille-P4126 and Marseille-P4593 revealed nucleotide sequence similarities of 98.27% with *Atopobium vaginae* (accession number: NR_117757.1) and 96.34% with *Butyricimonas virosa* (accession number: NR_041691.1) as being, respectively, the phylogenetically closest species with a validly published name (Fig. 1). As these similarity percentages are far below the threshold value recommended (98.65%) by several authors [25] to delimit the species barrier between bacteria, strains Marseille-P4126 and Marseille-P4593 were both considered to be potentially new species belonging to the genera *Atopobium* and *Bytiricimonas*, respectively.

Strain phenotypic and biochemical characterization

Strain Marseille-P4126 is a Gram-positive, non-motile, rod-shaped bacterium measuring 0.4–0.9 µm in diameter. Its colonies appear white and small on blood agar after 48 hours of incubation. Strain Marseille-P4593 is a Gram-negative

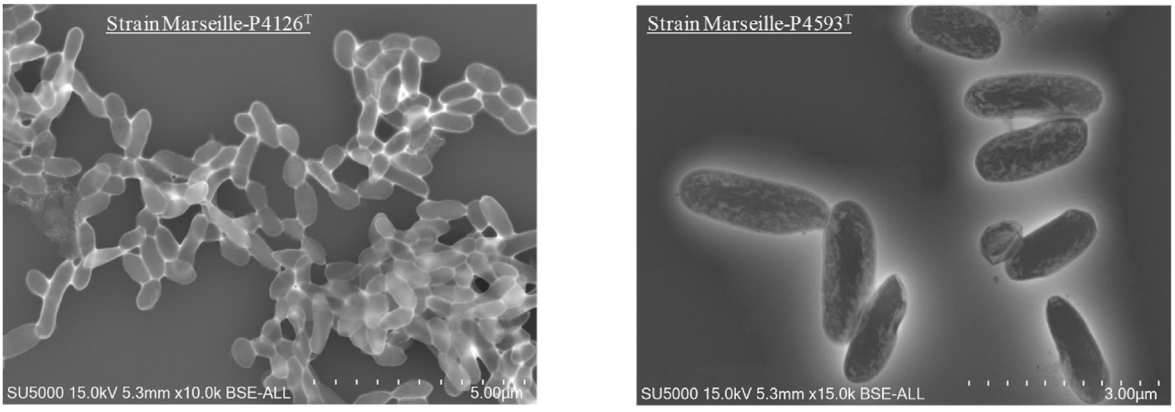


FIG. 2. Scanning electron micrograph of *Atopobium massiliense* strain Marseille-P4126^T and *Butyricimonas vaginalis* strain Marseille-P4593^T using the scanning electron microscope TM4000 from Hitachi. Scale bar and acquisition settings are presented on the micrographs.

TABLE 4. Genomic comparison of *Atopobium massiliense* strain Marseille-P4126 between its closely related species using genome-to-genome distance calculator and formula 2 (DNA–DNA hybridization estimates based on identities over high-scoring segment pair length)

% Similarity of *Atopobium* species	AFO	AMA	AMI	APA	ARI	AVA	ADE
AFO	100%	20.9 ± 4.7%	21.7 ± 4.7%	21.4 ± 4.7%	21.0 ± 4.6%	19.3 ± 4.5%	22.6 ± 4.7%
AMA		100%	22.2 ± 4.8%	23.6 ± 4.8%	26.4 ± 4.9%	25.8 ± 4.8%	26.4 ± 4.9%
AMI			100%	23.0 ± 4.8%	25.5 ± 4.8%	21.1 ± 4.9%	25.4 ± 4.8%
APA				100%	22.9 ± 4.7%	22.4 ± 4.8%	23.9 ± 4.8%
ARI					100%	23.3 ± 4.7%	21.5 ± 4.7%
AVA						100%	23.4 ± 4.7%
ADE							100%

Abbreviations: AFO, *Atopobium fossor* DSM 15642; AMA, *Atopobium massiliense* Marseille-P4126; AMI, *Atopobium minutum* strain DSM 20586; APA, *Atopobium parvulum* DNF00906; ARI, *Atopobium rimae* strain DSM 7090; AVA, *Atopobium vaginae* DSM 15829; ADE, *Atopobium deltae* strain DNF00019.

anaerobic, non-motile, rod-shaped bacillus with a mean diameter of 0.7 µm. Its colonies appear opalescent and beige with regular borders and a diameter of 2–3 mm. It should also be noted that both Marseille-P4126 and Marseille-P4593 strains have an optimal growth temperature of 37°C under anaerobic atmosphere and did not show catalase and oxidase activities. Using API ZYM strip, positive reactions were obtained for alkaline phosphatase, acid phosphatase, esterase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase with strain Marseille-P4126. Esterase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase were positive for strain Marseille-P4593. The API 50 CH strip revealed that strain Marseille-P4126 metabolized glycerol, glucose, mannose, *N*-acetyl-glucopyranoside and raffinose, whereas strain Marseille-P4593 presented hydrolysis of esculin ferric citrate and can assimilate potassium-5-ketogluconate. The phenotypic criteria of strains Marseille-P4126 and Marseille-P4593 are compared in Tables 1 and 2, respectively, with related type species within the genus *Atopobium* and *Butyricimonas*.

The most abundant fatty acid was hexadecanoic acid (60%), followed by 9-octadecenoic acid (24%) for Marseille-P4126 whereas it was 13-methyl-tetradecanoic acid (75%) for Marseille-P4593. Minor amounts of other fatty acids were also detected for both (Table 3). To reveal the shape of each bacterium, scanning electron microscopy was performed using the Hitachi TM4000 (Fig. 2).

Genome analysis and interpretation

The genomes of strains Marseille-P4126 and Marseille-P4593 were 1 548 103 bp and 4 929 720 bp long with 48.0 mol% and 43.6 mol% G + C content, respectively. The genomic assembly was performed into 16 contigs for Marseille-P4126 and into five scaffolds for Marseille-P4593; 1179 and 4049 protein-coding genes were counted within the genomes of Marseille-P4126 and Marseille-P4593, respectively.

Using DNA–DNA hybridization analysis, values ranged from 19.3% between *A. vaginae* and *A. fossor* to 25.5% between *A. massiliense* and *A. rimae* (Table 4). The DNA–DNA hybridization analysis of the *Butyricimonas* species used made it possible to obtain values ranging from 20.8% between *B. synergistica* and *B. virosa* to 65.4% between *B. faecalis* strain H184 and *B. vaginalis* strain Marseille-P4593 (Table 5). These values are lower than the 70% threshold used for the delineation of prokaryotic species, confirming that these two strains are new species [26,27].

To measure the overall similarity between genome sequences, an OrthoANI analysis (Fig. 3) was performed among closely related *Atopobium* species. The highest value of identity was 78.86%, shared between *A. fossor* and *A. minutum*, and the lowest value of similarity obtained was 66.78% between *A. rimae* and *A. vaginae* strain NCTC13935. In addition, analysis of the OrthoANI values obtained with the genomes of *Butyricimonas* species showed that *B. vaginalis* was <88% similar to the other species studied. Hence, we see that our Marseille-P4593 strain

TABLE 5. Genomic comparison of *Butyricimonas vaginalis* strain Marseille-P4593 between its closely related species using Genome-to-Genome Distance Calculator and formula 2 (DNA–DNA hybridization estimates based on identities over high-scoring segment pair length)

% Similarity of *Butyricimonas* species	BVA	BFA	BFH	BPA	BSY	BVI
BVA	100%	65.4 ± 2.7%	35.5 ± 5.0%	35.2 ± 5.0%	21.3 ± 4.7%	36.0 ± 4.9%
BFA		100%	35.9 ± 4.9%	35.3 ± 4.9%	21.3 ± 4.7%	36.0 ± 5.0%
BFH			100%	57.7 ± 5.5%	21.6 ± 4.7%	43.5 ± 5.1%
BPA				100%	21.0 ± 4.7%	44.6 ± 5.1%
BSY					100%	20.8 ± 4.7%
BVI						100%

Abbreviations: BVA, *Butyricimonas vaginalis* strain Marseille-P4593; BFA, *Butyricimonas faecalis* strain H184; BFH, *Butyricimonas faecihominis* strain 30A1; BPA, *Butyricimonas paravirosa* strain DSM 105722; BSY, *Butyricimonas synergistica* strain DSM 23225; BVI, *Butyricimonas virosa* strain DSM 23226.

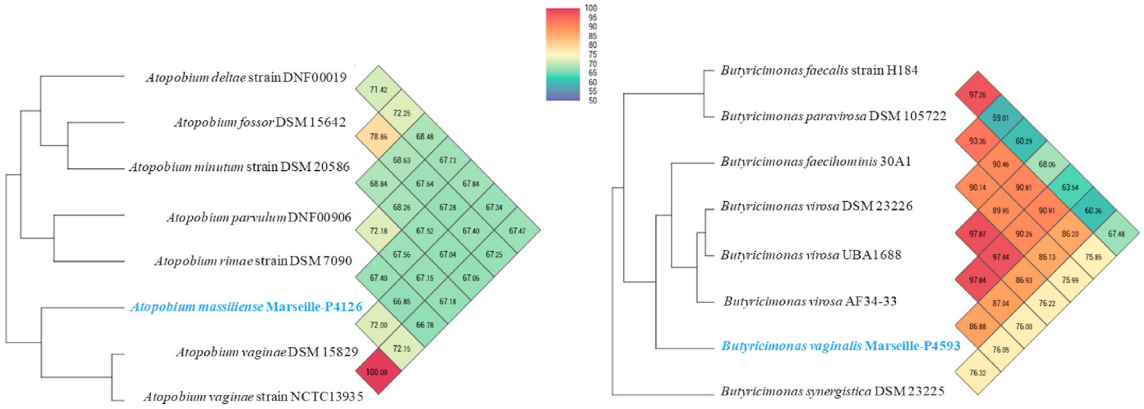


FIG. 3. Heatmap generated with OrthoANI values calculated using the OAT software for *Atopobium massiliense* sp. nov., strain Marseille-P4126 (left) and *Butyricimonas vaginalis* strain Marseille-P4593 (right) with their respective closely related species with standing in nomenclature.

shared 60.36% identity with *B. faecalis* strain H184 and 87.04% identity with *B. virosa* strain UBA1688. These values are below the recommended threshold value for being of the same species [28].

Conclusion

In short, the phenotypic, biochemical and genomic analyses carried out on the strains Marseille-P4126 and Marseille-P4593 were consistent in confirming the novelty of these species. In addition, the tests performed on these two strains showed that their sequence similarity of 16S rRNA, as well as their percentages of ANI, were below the recommended thresholds as 98.65% and <95%, respectively, to delimit the species barrier between bacteria. Hence, in view of all the evidence, we formally declare that *Atopobium massiliense* sp. nov. and *Butyricimonas vaginalis* sp. nov. are new bacterial species. The discovery of new anaerobic bacteria in women with bacterial vaginosis shows that further investigation is needed to better understand the vaginal microbiota. Members of the genus *Gardnella* and *Mobilincus* are not the only ones to cause dysbiosis. A study in our laboratory has shown that other microorganisms are involved in bacterial vaginosis; therefore, it is important to monitor and diagnose any imbalance in the vaginal flora as soon as possible.

Description of *Atopobium massiliense* sp. nov.

Atopobium massiliense sp. nov. (mas.si.li.en'se. L. fem. adj., from *massiliense* of Massilia, the Latin name of Marseille where the strain was first isolated). It is a Gram-positive anaerobic bacterium that appears as short rods, non-motile and non-spore forming. The bacterial cells have a size ranging from 0.4 to 0.88 µm. Small whitish colonies are observed on blood agar after 2 days of incubation in an anaerobic chamber at 37°C. $C_{16:0}$ (60%) and $C_{18:1n9}$ (24%) are the major cellular fatty acids found with this strain. Catalase and oxidase activities are negative. Alkaline phosphatase, acid phosphatase, leucine arylamidase, glycerol, glucose, mannose and raffinose are positive, whereas acid from rhamnose, inositol, mannitol, sorbitol, amygdalin, esculin ferric citrate, salicin, cellobiose, maltose, lactose, melibiose, sucrose and trehalose were not produced by strain Marseille-P4126.

The genome of strain Marseille-P4126 was 1.55 Mbp with 48.0 mol% of G + C content. The 16S rRNA and draft genome sequences are deposited in the GenBank database under Accession numbers LT986001 and OPYK00000000, respectively. The type strain of *Atopobium massiliense* sp. nov., strain Marseille-P4126 (=CSURP4126), was isolated from a vagina with bacterial vaginosis.

Description of *Butyricimonas vaginalis* sp. nov.

Butyricimonas vaginalis (va.gi.na'lis. N.L. masc. adj. *vaginalis* pertaining to vagina, of female genital organ from which the strain was isolated). This is a Gram-negative bacterium with short rod-shaped cells. They are non-motile and non-spore-forming. It doesn't present neither catalase nor oxidase activities. The type strain grows strictly under anaerobic conditions with an optimum temperature at 37°C. The main cellular fatty acid found is 13-methyl-tetradecanoic acid (75%). Positive reactions were observed for esterase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase. Acid is only produced from esculin ferric citrate and potassium-5-ketogluconate.

The genome of strain Marseille-P4593 was 4.93 Mbp with 43.6 mol% of G + C content. The 16S rRNA and genome sequences of strain Marseille-P4593 were deposited in GenBank database under accession numbers LT934507 and OIWZ00000000, respectively. The type strain Marseille-P4593 (=CSURP4593) was isolated from the vagina of a French woman with bacterial vaginosis.

Conflict of interest

The authors have stated that there are no conflicts of interest in relation to this article.

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