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

A. Bordigoni1,2, C. I. Lo1,2, E. Kuete Yimagou1,2, B. Nicaise1,3, K. Diop1,3, D. Raoult1,2, C. Desnues1,2 and F. Fenollar1,3

1) Aix Marseille Université, IRD, AP-HM, ME01, 2) IHU-Méditerranée Infection and 3) Aix Marseille Université, IRD, AP-HM, SSA, VITROME, Marseille, France

Abstract

Two new bacterial strains, Marseille-P4126 (=CSURP4126) and Marseille-P4593 (=CSURP4593), were isolated from the vaginal sample of a French woman with bacterial 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.

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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 rima*) [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...
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.mediterrane-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 3500xL Genetic Analyzer capillary sequencer (Thermofisher, Saint- Aubin, France).

**FIG. 1.** Phylogenetic trees displaying the position of *Atopobium massiliense* strain Marseille-P4126$^T$ (a) and *Butyricononas 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.

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According to the Belkacemi's protocol, the morphological characteristics of these two new species were 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 obtained sequence was matched against the NCBI database using the BLASTn algorithm [22].

**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 38953T [9]; 6, Atopobium deltae strain HHRM1715T [10]

| Characteristics                  | 1               | 2               | 3               | 4               | 5               | 6               |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cell diameter (µm)               | 0.4–0.88        | NA              | 0.3–0.6         | 0.6–1.0         | 0.6–0.9         | 1.0             |
| Oxygen requirement               | OA              | OA              | OA              | FA              | OA              | OA              |
| Motility                         | –               | –               | –               | –               | –               | –               |
| Endospore formation              | –               | –               | –               | –               | –               | –               |
| Alkaline phosphatase             | +               | NA              | NA              | NA              | NA              | NA              |
| Leucine arylamidase              | +               | +               | +               | +               | +               | +               |
| Acid phosphatase                 | +               | +               | +               | +               | +               | +               |
| β-galactosidase                  | NA              | NA              | NA              | NA              | NA              | NA              |
| N-acetyl-D-glucosaminidase       | NA              | NA              | NA              | NA              | NA              | NA              |
| α-fucosidase                     | –               | –               | –               | –               | –               | –               |
| Catalase                         | –               | –               | –               | –               | –               | –               |
| β-glucose                       | +               | +               | +               | NA              | NA              | NA              |
| α-fructose                      | –               | –               | –               | –               | –               | –               |
| β-mannose                       | +               | +               | +               | +               | +               | +               |
| D-mannose                       | –               | –               | –               | –               | –               | –               |
| Inositol                         | –               | –               | –               | –               | –               | –               |
| N-sorbitol                      | –               | –               | –               | –               | –               | –               |
| Amygdalin                        | –               | –               | –               | –               | –               | –               |
| Salicin                          | –               | –               | –               | –               | –               | –               |
| α-raffinose                     | +               | +               | +               | NA              | 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.

**TABLE 2.** Different characteristics of 1, Butyricimonas vaginalis sp. nov., strain Marseille-P4593; 2, Butyricimonas faecalis strain H184T [13]; 3, Butyricimonas paravirosa strain 214-4T [14]; 4, Butyricimonas virosa strain DSM 23226T [12]

| Characteristics                  | 1               | 2               | 3               | 4               |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|
| Cell diameter (µm)               | 0.6–0.8         | 0.4–0.5         | 1               | 1–1.5           |
| Oxygen requirement               | –               | –               | –               | –               |
| Motility                         | –               | –               | –               | –               |
| Endospore formation              | –               | –               | –               | –               |
| Alkaline phosphatase             | –               | –               | +               | +               |
| Leucine arylamidase              | –               | –               | +               | +               |
| N-acetyl-α-D-glucosaminidase     | –               | –               | –               | –               |
| α-glucosidase                    | –               | –               | –               | –               |
| β-glucosidase                    | –               | –               | –               | –               |
| a-glucosidase                    | –               | –               | –               | –               |
| N-acetyl-β-D-glucosaminidase     | +               | +               | +               | +               |
| α-fucosidase                     | –               | –               | –               | –               |
| Catalase                         | –               | –               | –               | –               |
| D-glucose                        | –               | –               | –               | –               |
| D-mannose                        | +               | +               | +               | +               |
| N-acetyl-D-fructose              | –               | –               | –               | –               |
| N-acetyl-D-glucosamine           | +               | +               | +               | +               |
| Esculin ferric citrate           | –               | –               | –               | –               |
| D-lactose                        | +               | +               | +               | +               |
| Potassium 5-ketoisocitrate       | +               | +               | +               | +               |

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

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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 *Butyricimonas*, 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

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

| Fatty acids          | Names                  | 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   | 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-Heptadecanoic acid  | TR      | ND      | ND      | ND      | ND      |
| **Butyricimonas species** |                      |         |         |         |         |         |
| 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-Octadecanoic acid    | TR      | 8.3     | 9.5     | 12.6    | 6.0     |
| 15:0 anteiso         | 12-methyl-tetradecanoic acid | 1.8    | 1.8     | 2.0     | 1.5     |         |
| 18:2n6               | 9,12-Octadecadienoic   | 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-P4126T; 2A, *Atopobium detace* HHRM 1715T; 3A, *Atopobium parvulum* CCUG 32760T; 4A, *Atopobium rini* CCUG 31168T; 5A, *Atopobium vaginae* CCUG 38953T; 1B, *Butyricimonas faecihominis* Marseille-P4593; 2B, *Butyricimonas paravirosa* 214-4T; 3B, *Butyricimonas paravirosa* 214-4T; 4B, *Butyricimonas synergistica* JCM 15148T; 5B, *Butyricimonas virosa* JCM 15149T.

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**FIG. 2.** Scanning electron micrograph of *Atopobium massiliense* strain Marseille-P4126T and *Butyricimonas vaginalis* strain Marseille-P4593T using the scanning electron microscope TM4000 from Hitachi. Scale bar and acquisition settings are presented on the micrographs.

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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% | 23.0 ± 4.8% | 23.8 ± 4.8% | 26.4 ± 4.9% | 25.8 ± 4.8% | 26.4 ± 4.9% |
| AMI                              | 100%| 22.4 ± 4.8% | 23.2 ± 4.8% | 25.5 ± 4.8% | 24.2 ± 4.8% | 23.9 ± 4.8% | 23.9 ± 4.8% |
| APA                              | 100%| 22.5 ± 4.7% | 23.3 ± 4.7% | 24.4 ± 4.7% | 23.4 ± 4.7% | 23.1 ± 4.7% | 23.4 ± 4.7% |
| ARI                              | 100%| 22.1 ± 4.7% | 21.0 ± 4.7% | 21.4 ± 4.7% | 22.6 ± 4.7% | 22.3 ± 4.7% | 22.6 ± 4.7% |
| AVA                              | 100%| 24.5 ± 4.8% | 24.4 ± 4.8% | 22.2 ± 4.8% | 23.3 ± 4.8% | 22.9 ± 4.8% | 23.8 ± 4.8% |
| ADE                              | 100%| 25.4 ± 4.8% | 23.8 ± 4.8% | 24.4 ± 4.8% | 24.7 ± 4.8% | 24.8 ± 4.8% | 24.8 ± 4.8% |

Abbreviations: AFO, Atopobium fossor DSM 15642; AMA, Atopobium massiliense Marseille-P4126; AMI, Atopobium minutum strain DSM 20586; APA, Atopobium parvulum DNFI00906; ARI, Atopobium rimae strain DSM 7096; AVA, Atopobium vaginae DSM 15829; ADE, Atopobium deotear DSM DNFI0019.

The genomes of strains Marseille-P4126 and Marseille-P4593 are compared in Tables 1 and 2, respectively, with phenotypic criteria of strains Marseille-P4126 and 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. vaginace 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. vaginale 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. vaginace strain NCTC13935. In addition, analysis of the OrthoANI values obtained with the genomes of Butyricimonas species showed that B. vaginale was <88% similar to the other species studied. Hence, we see that our Marseille-P4593 strain

**TABLE 5. Genomic comparison of Butyricimonas vaginale 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%                              | 65.4 ± 2.7% | 35.5 ± 5.0% | 35.2 ± 5.0% | 21.3 ± 4.7% | 36.0 ± 4.9% |
| BFH 100%                              | 35.5 ± 4.9% | 35.3 ± 4.9% | 21.3 ± 4.7% | 36.0 ± 5.0% |
| BPA 100%                              | 57.7 ± 5.5% | 21.6 ± 4.7% | 43.5 ± 5.1% |
| BSY 100%                              | 21.0 ± 4.7% | 44.6 ± 5.1% |
| BVI 100%                              | 20.8 ± 4.7% |

Abbreviations: BVA, Butyricimonas vaginale strain Marseille-P4593; BFA, Butyricimonas faecalis strain H184; BFH, Butyricimonas fibrisolvens strain 30A1; BPA, Butyricimonas parvalve strain DSM 105722; BSY, Butyricimonas synergistica strain DSM 23225; BVI, Butyricimonas virosa strain DSM 23226.

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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 *Butyrimonas 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. (ma.s.s.i.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-sporo-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. C16:0 (60%) and C18:1n9 (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 rafinose 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 Butyrimonas vaginalis** sp. nov.

*Butyrimonas vaginalis* (va.gi.nal.is. 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-sporo-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.

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