**Megasphaera vaginalis** sp. nov. and *Anaerococcus vaginimassiliensis* sp. nov., new bacteria isolated from vagina of French woman with bacterial vaginosis

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**Abstract**

Using the culturomics method, two strains were isolated, identified and characterized following the taxonogenomics concept. *Megasphaera vaginalis* sp. nov. strain Marseille-P4512 (= CSURP4512) and *Anaerococcus vaginimassiliensis* sp. nov. strain Marseille-P4857 (= CSURP4857) were isolated from the vagina of a French woman. The phylogenetic tree, phenotypic criteria and genomic analysis described here clearly show that these two bacteria are different from previously known bacterial species with standing in nomenclature and new members of *Firmicutes* phylum.

**Keywords:** *Anaerococcus vaginimassiliensis* sp. nov., Bacteria, Culturomics, *Megasphaera vaginalis* sp. nov., Taxonogenomics, Vagina

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**Introduction**

Healthy vaginal microbiota is a complex dynamic ecosystem, mainly dominated by *Lactobacillus* spp. and classified into five community state types (CST) depending on the following majority species: CST I (*Lactobacillus crispatus*), CST II (*Lactobacillus gasseri*), CST III (*Lactobacillus iners*) and CST V (*Lactobacillus jensenii*) [1,2]. These beneficial bacteria are the first line of defence against vaginal pathogens through competition and production of inhibitory compounds [3,4]. Bacterial vaginosis is a common infection due to an imbalance of the vaginal flora with an increase in CST IV, which is represented by anaerobic pathogenic bacteria, such as *Atopobium* sp., *Gardnerella* sp. and *Sneathia* sp.

The development of culturomics, combined with taxonogenomic analysis, has enabled the description of many previously unknown bacterial species [5,6]. Thanks to this strategy, our laboratory has characterized several new bacteria isolated from the vagina [7–9].

*Megasphaera* and *Anaerococcus* genera, respectively, belong to the *Veillonellaceae* and *Peptoniphilaceae* families within the *Firmicutes* phylum. At the time of writing and among validly published names, there are nine species described in *Megasphaera* and 13 species described in *Anaerococcus* [10]. Members of the *Megasphaera* genus, described in 1971 by Rogosa [11], can be found in human faecal flora [12,13], the mammalian digestive tract [14] and brewery samples [15]. Some *Anaerococcus* spp. were isolated from human clinical samples [16,17]. Among the 13 *Anaerococcus* species validly published, six were isolated from vaginal discharge or ovarian abscess samples: *Anaerococcus hydrogenalis*, *Anaerococcus lactolyticus*, *Anaerococcus vaginalis*, *Anaerococcus prevoti*, *Anaerococcus tetradius* and *Anaerococcus provencensis* [16,18].

We report here the description of two new designated species, *Megasphaera vaginalis* sp. nov. strain Marseille-P4857 and *Anaerococcus vaginimassiliensis* sp. nov. strain Marseille-P4512, belonging to the *Firmicutes* phylum.

**Material and methods**

**Strain isolation and identification**

As part of a culturomic study investigating the human microbiome, we isolated two bacterial strains from vaginal swabs of a
French woman with bacterial vaginosis. These were strains Marseille-P4857 and Marseille-P4512. The patient provided informed consent, and the study was authorized by the ethics committee of the Institut Federatif de Recherche IFR48 under the number 09-022. The vaginal swabs were directly seeded in Petri dishes containing 5% sheep blood agar (BioMérieux, Marcy l’Étoile, France) and incubated under anaerobic condition (Thermo Scientific, Dardilly, France) at 37°C after 3 days.
Identification was performed with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany) as previously reported [19]. The spectra generated were analysed by Biotyper 3.0 software, which is regularly incremented with the local URMS database (https://www.mediterranee-infection.com/urms-data-base). Misidentiﬁcation with MALDI-TOF MS led to ampliﬁcation of the 16S rRNA gene using the primer pair fD1 and rP2 (Eurogentec, Angers, France) and then sequencing using the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xLGenetic Analyzer capillary sequencer (Thermoﬁsher, Saint-Aubin, France), as previously reported [20]. All 16S rRNA nucleotide sequences were assembled and edited using CodonCode Aligner software (http://www.codoncode.com). Once a consensus sequence is obtained, it is submitted to the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide/) and a comparative analysis of nucleotides by BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blast&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) is performed. Hence, the sequences phylogenetically closest to the only typical species are recovered to build the phylogenetic trees.

**Phenotypic characterization**

Different growth conditions were tested for strains in aerobic, microaerophilic and anaerobic atmospheres (Thermo Scientiﬁc, Dardilly, France). The optimal temperature of growth was assessed (28°C, 37°C, 45°C and 55°C) on 5% sheep blood-enriched Columbia agar medium (BioMérieux). According to the manufacturer’s recommendations, API ZYM and API 50 CH strips (bioMérieux) were employed to assess the biochemical characteristics of each strain. Phenotypic tests, such as Gram-staining, catalase and oxidase were performed. Also, the spore-forming was searched for each strain as previously reported [21]. The morphological structure of these two isolates was highlighted with a scanning electron microscope (Hitachi High-Technologies, Tokyo, Japan) following the protocol described by Belkacemi et al. [22].

![FIG. 2. Scanning electron micrograph of Megasphaera vaginalis strain Marseille-P4857T and Anaerococcus vaginimassiliensis strain Marseille-P4512T using the scanning electron microscope TM4000 from Hitachi. Scale bar and acquisition settings are presented on the pictures.](image-url)

| TABLE 1. Different characteristics of Megasphaera species |
|----------------------------------------------------------|
| Properties | 1 | 2 | 3 | 4 |
| Cell diameter (μm) | 0.6–0.9 | 0.4–0.6 | 0.8 | 1.2–1.5 |
| Oxygen requirement | — | — | — | — |
| Gram stain | — | — | — | — |
| Motility | — | — | — | — |
| Endospore formation | — | — | — | — |
| α-glucosidase | — | NA | + | NA |
| Catalase | — | — | — | — |
| Oxidase | — | NA | — | — |
| Glyceraldehyde | + | — | W | — |
| Erythritol | — | — | NA | — |
| d-arabinose | + | — | W | NA |
| l-arabinose | + | — | — | — |
| d-ribose | + | — | — | — |
| d-xylene | + | — | — | — |
| d-glucose | — | — | — | — |
| d-fructose | + | — | — | — |
| l-rhamnose | — | — | NA | — |
| Dulcitol | — | — | NA | — |
| Inositol | + | — | — | — |
| d-mannitol | — | — | — | — |
| d-sorbitol | + | — | — | — |
| N-acetyl-glucosamine | + | — | — | NA |
| Esculin ferric citrate | — | — | — | — |
| Salicin | — | — | — | — |
| d-cellobiose | — | — | — | — |
| d-maltose | — | — | — | — |
| d-lactose | — | — | — | — |
| d-melibiose | — | — | — | — |
| d-trehalose | — | — | — | — |
| d-melezitose | — | — | — | — |
| d-raffinose | — | — | — | — |
| Glycogen | + | — | — | NA |
| Source | human vaginal swab | human stool | human stool | spoiled beer |

1, Megasphaera vaginalis sp. nov., strain Marseille-P4857; 2, Megasphaera micronuciformis strain AIP 412.00 [29]; 3, Megasphaera massiliensis strain NP3 [12]; 4, Megasphaera paucivorans strain DSM 16981 [11].

+, positive reaction; −, reaction; NA, not available data; w, weak reaction.
TABLE 2. Different characteristics of Anaerococcus species

| Properties                  | 1            | 2            | 3            |
|-----------------------------|--------------|--------------|--------------|
| Cell diameter (µm)          | 0.8–1.3      | 0.8–1.8      | 0.7–1.8      |
| Oxygen requirement          | +            | +            | +            |
| Motility                    | +            | +            | +            |
| Alkaline phosphatase        | +            | +            | +            |
| Leucine arylamidase         | +            | +            | +            |
| Acid phosphatase            | +            | NA           | NA           |
| β-galactosidase             | —            | —            | —            |
| β-galactosidase             | —            | —            | —            |
| β-glucosidase               | —            | —            | —            |
| β-glucuronidase             | —            | +            | +            |
| β-glucosidase               | —            | —            | —            |
| β-glucuronidase             | —            | —            | —            |
| Catalase                    | +            | D            | D            |
| Oxidase                     | —            | NA           | NA           |
| Glycerol                    | +            | NA           | —            |
| α-ribose                    | +            | +            | +            |
| Xylose                      | +            | —            | —            |
| α-glucose                   | +            | +            | D            |
| α-fructose                  | +            | +            | D            |
| α-maltose                   | +            | +            | D            |
| α-lactose                   | +            | D            | D            |
| Source                      | vaginal swab  | vaginal discharge  | vaginal discharge  |

1. Anaerococcus vaginnassiliensis strain Marseille-P4512; 2. Anaerococcus tetradius strain JCM 1964T; 3. Anaerococcus prevoti strain ATCC 9321T. +, positive reaction; —, reaction; NA, not available data; D, strain-dependent.

Genome characteristics

Genomic DNA extraction was performed with the EZ1 bio-robot using the EZ1 DNA tissue kit (Qiagen, Hilden, Germany), and sequencing was performed on the MiSeq instrument (Illumina Inc., San Diego, CA, USA) using the Nextera Mate Pair and Nextera XT Paired End (Illumina) sample preparation kit, as previously described [20]. The genomic assembly was carried out using the three following softwares: Velvet [23], Spades [24] and Soap Denovo [25]. MiSeq and Trimmomatic [26] softwares were used for trimmed or untrimmed sequences. To reduce assembly gaps, GapCloser software [27] was used. Best assembly was determined using different criteria, such as the number of scaffolds, N50 or number of N. Scaffolds were deleted when their nucleotide number was <800 bp and their depth value < 25% of the mean depths. Genome annotation of these two species was performed as described elsewhere [28]. In addition, the Genome-to-Genome Distance Calculator web server available online (http://ggdc.dsmz.de) made it possible to assess the similarity between the genomes being compared and to replace the DNA–DNA hybridization (DDH) with a digital DDH (dDDH) [29]. Average nucleotide identity analysis was also evaluated using the OAT software [30].

Results

Strain identification and phylogenetic analysis

Attempts to identify the strains cultivated on blood agar by mass spectrometry failed, indicating that these isolates were not known from the MALDI-TOF database. Therefore, their generated spectra were added to the local database. 16S rDNA-based similarity analysis of strain Marseille-P4857 and strain Marseille-P4512 against GenBank exhibited highest nucleotide sequence similarities of 95.12% with Megashaera micronuconiformis strain AIP 412.00 (accession number NR_025230.1) and 96.78% with Anaerococcus tetradius strain CCUG 46590 (accession number NR_041941.1), being respectively the two phylogenetically closest species. As these similarity values were below the 98.65% threshold recommended for the delimitation of new bacterial species [29,31], strain Marseille-P4857 and strain Marseille-P4512 were considered potentially new species within the phylum Firmicutes. The phylogenetic trees of Megashaera spp. (Fig. 1a) and Anaerococcus spp. (Fig. 1b) show their positions concerning their respective closely related species with a validly published name. In addition, the shape of each bacterium (shown in Fig. 2) was obtained from the Hitachi TM4000 instrument.

Biochemical properties of the strains

The two strains grow strictly under anaerobic conditions with an optimal temperature at 37°C. Strain Marseille-P4857 is a Gram-negative anaerobic coccus with a mean cell diameter of 0.70 µm. Colonies of strain Marseille-P4857 were white to yellow, shiny, opaque and convex with a diameter varying from 0.5 to 1 mm on blood agar after 3 days of incubation. It presents catalase-negative and oxidase-negative activities. Conversely, strain Marseille-P4512 is a Gram-positive anaerobic bacterium. Cells are cocccid with a mean diameter of 1.08 µm. They exhibit catalase-positive and oxidase-negative activities. Colonies of strain Marseille-P4512 are white with regular edges and a mean diameter of 2 mm.

Using the API ZYM strip, only acid phosphatase was positive for strain Marseille-P4857, while alkaline phosphatase, leucine arylamidase and acid phosphatase were also positive for strain Marseille-P4512. All remaining reactions were still negative with this API ZYM test. In addition, using the API 50 CH strip, Megashaera vaginalis strain Marseille-P4857 was positive for glycerol, erythritol, arabinose, ribose, xylose, D-fructose, inositol, sorbitol, methyl α-D-glucopyranoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin, D-maltose, D-lactose, D-melibiose, sucrose, inulin, D-melezitose, D-raffinose, glycogen, xylitol, gentiobiase, D-lyxose, D-tagalose, fucose, potassium gluconate and potassium 5-ketogluconate. For Anaerococcus vaginnassiliensis strain Marseille-P4512, glycerol, xylose, galactose, fructose, glucose, methyl-α-D-glucopyranoside, N-acetyl-glucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-trehalose, xylitol, gentiobiose, potassium 5-ketogluconate were positive. A large phenotypic comparison of Marseille-P4857 and Marseille-P4512 with closely related species is displayed in Tables 1 and 2. The
**TABLE 3. Genomic comparison of closely related species to *Megasphaera vaginalis* strain Marseille-P4857 and *Anaerococcus vaginimassiliensis* strain Marseille-P4512**

| Species                  | Size (Mb) | G + C mol% | Protein | rRNA | tRNA | Other RNA | Gene | Pseudogene |
|--------------------------|-----------|------------|---------|------|------|-----------|------|------------|
| *Megasphaera vaginalis*  | 2.21      | 50.2       | 2032    | 7    | 49   | 4         | 2137 | 45         |
| *Megasphaera cresviæ*  | 3.24      | 44.8       | 2933    | 17   | 55   | 4         | 3163 | 154        |
| *Megasphaera paucivorans* | 2.91     | 40.2       | 2598    | 14   | 51   | 4         | 2780 | 113        |
| *Megasphaera micronuiformis* | 1.77     | 45.4       | 1665    | 48   | 1746 | 29        |
| *Megasphaera elsdenii* | 2.50      | 52.8       | 2211    | 21   | 65   | 4         | 2378 | 75         |
| *Megasphaera stantoni* | 2.65      | 52.6       | 2397    | 18   | 57   | 4         | 2509 | 33         |
| *Megasphaera massiliensis* | 2.74     | 50.2       | 2388    | 3    | 56   | 4         | 2562 | 111        |
| *Megasphaera hexanica* | 2.88      | 49.0       | 2636    | 18   | 53   | 1         | 2750 | 42         |
| *Anaerococcus vaginimassiliensis* | 1.84     | 33.1       | 1722    | 13   | 48   | 3         | 1826 | 40         |
| *Anaerococcus vaginalis* | 1.89      | 29.0       | 1693    | 2    | 46   | 4         | 1793 | 48         |
| *Anaerococcus mediterraneensis* | 2.08    | 34.6       | 1936    | 9    | 44   | 4         | 2045 | 52         |
| *Anaerococcus tetradius* | 2.15      | 34.4       | 1895    | 5    | 45   | 4         | 2010 | 61         |
| *Anaerococcus prevotii* | 2.15      | 30.4       | 1978    | 2    | 40   | 4         | 2219 | 35         |
| *Anaerococcus micromedius* | 2.13     | 35.4       | 1953    | 14   | 49   | 4         | 2082 | 62         |
| *Anaerococcus senegalensis* | 1.80     | 28.6       | 1748    | 3    | 44   | 3         | 1735 | 77         |
| *Anaerococcus proveniens* | 2.27      | 33.7       | 2004    | 9    | 48   | 3         | 2146 | 82         |

**Table 4. Genomic comparison of *Megasphaera vaginalis* strain Marseille-P4857 and *Anaerococcus vaginimassiliensis* strain Marseille-P4512 between their closely related species using Genome-to-Genome Distance Calculator and formula 2 (dDDH estimates based on identities over HSP length)**

| % Similarity of *Megasphaera* species | MEL | MMI | MCE | MST | MHE | MPA | MMA | MVA |
|--------------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| MEL 100                              |     |     |     |     |     |     |     |     |
| MIM 26.0                              | 100 |     |     |     |     |     |     |     |
| MCE 19.2                              | 127 | 100 |     |     |     |     |     |     |
| MST 21.7                              | 197 | 100 | 100 |     |     |     |     |     |
| MHE 23.7                              | 212 | 100 | 100 | 100 |     |     |     |     |
| MPA 19.7                              | 232 | 100 | 100 | 100 | 100 |     |     |     |
| MMA 24.3                              | 234 | 100 | 100 | 100 | 100 | 100 |     |     |
| MVA 20.2                              | 235 | 100 | 100 | 100 | 100 | 100 | 100 |     |

| % Similarity of *Anaerococcus* species | AVG | APR | AYA | ATE | APA | ASE | AME | APV |
|---------------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| AVG 100                               | 21.4| 21.1| 21.9| 25.9| 21.7| 23.4| 22.6|     |
| APR 100                               | 21.6| 21.5| 20.5| 20.2| 21.2| 20.5|     |     |
| AYA 100                               | 28.8| 36.0| 29.4| 33.6| 26.2|     |     |     |
| ATE 100                               | 25.3| 24.6| 32.3| 21.2| 22.2|     |     |     |
| APA 100                               | 25.7| 22.1| 20.2| 17.7| 100 |     |     |     |
| ASE 100                               | 24.3| 21.6| 23.9|     |     |     |     |     |
| AME 100                               |     |     |     |     |     |     |     |     |
| APV 100                               |     |     |     |     |     |     |     |     |

**Genomic analysis**

The size of the genomes of strains Marseille-P4857 and Marseille-P4512 were 2,206,375 and 1,836,452 bp with 50.2 and 33.1 mol% G + C content, respectively. The genomic assembly was carried out into 17 contigs for Marseille-P4857 and into one scaffold for Marseille-P4512. Indeed, 2137 and 1826 were assigned as predicted genes for Marseille-P4857 and Marseille-P4512, respectively. In addition, 2032 and 1722 protein-coding genes and 56 and 61 RNA genes were found from the respective genomes of Marseille-P4857 and Marseille-P4512. The comparison of the genomes of *M. vaginalis* and *A. vaginimassiliensis* in terms of size and G + C content, as well as the number of genes compared with their phylogenetically closest species is presented in Table 3.

Using dDDH analysis, values ranged from 17.7% between *M. massiliensis* and *Megasphaera paucivorans* to 27.0% between *M. microurufornis* and *Megasphaera stantoni*. At the end of the dDDH analysis of *Anaerococcus* species used in this study, we obtained values ranging from 20.2% between *A. prevotii* ATCC 065-V-Col13 and *Megasphaera mediterraneensis* strain Marseille-P2765 to 33.6% between *A. vaginalis* ATCC 51170 and *M. mediterraneensis* strain Marseille-P2765. These values are lower than the 70% threshold used for the delineation of prokaryotic species, confirming that these three strains represent new species. The dDDH values obtained from genome analysis of the species studied here are shown in Table 4.

In addition, OrthoANI analysis among closely related species (Fig. 3) highlighted that *Megasphaera* species had a higher value of percentage of identity of 80.57% shared between *Megasphaera elsdenii* and *M. massiliensis*. The lowest value of similarity, 68.58%, was obtained between *M. elsdenii* and *M. paucivorans*. Hence, OrthoANI analysis for *ANAerococcus* species revealed that 71.78% was the highest value of similarity.
that the *M. vaginalis* Marseille-P4857 strain shared with *M. stantonii*. Analysis of *Anaerococcus* species revealed that OrthoANI values ranged from 92.09% of similarity with *A. prevotii* and *Anaerococcus marasmi* to 70.12% of similarity with *A. mediterraneensis* and *Anaerococcus senegalensis*. The highest percentage value obtained with strain Marseille-P4512 was 78.23% of similarity with *A. marasmi*.

**Conclusion**

Considering the phenotypic, biochemical and genomic analysis carried out on these bacteria, strains Marseille-P4857 and Marseille-P4512 are proposed as new species. In addition, the genomic evidence used in this study, such as the sequence similarity of the 16S rRNA gene below the threshold value of 98.65% or OrthoANI values < 95% allowed us to formally declare that *Megasphaera vaginalis* sp. nov. and *Anaerococcus vaginimassiliensis* sp. nov., are new species within the phylum *Firmicutes*.

**Description of *Megasphaera vaginalis* sp. nov**

*Megasphaera vaginalis* sp. nov. (va.gi.nal.is. L. n. fem. gen. vaginales from the vagina which is a female genital organ; vaginales referring to the vagina). This bacterium is Gram-negative, anaerobic and shell-shaped. Cells are 0.62–0.91 μm in diameter. Catalase and oxidase activities are negative. Acid phosphatase activity is present. Colonies are white, shiny and convex with a mean diameter of 0.5 mm on blood agar. The following tests were positive: glycerol, erythritol, arabinose, ribose, d-xylose, d-fructose, inositol, d-sorbitol, methyl α-d-glucopyranoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin, sucrose, inulin, d-maltose, d-lactose, d-melezitose, d-raffinose, glycogen, xylitol, gentiobiose, d-lyxose, d-tagatose, d-fucose, l-fucose, potassium gluconate and potassium 5-ketogluconate. C16:0 (22.0%), C16:1ω9 (14.8%), C12:0 (9.0%) and C14:0 3-OH (7.3%) were the major fatty acids found with *Megasphaera vaginalis* sp. nov. The genome of strain Marseille-P4857 was 2.20 Mbp with 50.2 mol% of G + C content. The 16S rRNA and draft genome sequences are deposited in the Genbank database under Accession numbers LT960586 and OEQB00000000, respectively. The type strain of *Megasphaera vaginalis* sp. nov., strain Marseille-P4857 was isolated from the vagina of a woman with bacterial vaginosis.

**Description of *Anaerococcus vaginimassiliensis* sp. nov**

*Anaerococcus vaginimassiliensis* sp. nov. (va.gi.ni.mas.si.leni.sis N.L. fem. adj. vaginimassiliensis: vagina refers to vagina and massiliensis to Massilia, the Latin name of Marseille where the type strain was isolated). Gram-staining is positive. It is a coccus-shaped bacterium with a diameter ranging from 0.8 to 1.2 μm.
Anaerococcus vaginimassiliensis sp. nov., is a strict anaerobic bacterium that grows preferentially at 37°C. It has catalase activity, but not oxidase. Colonies are white with regular boundaries and have a mean diameter of 2 mm. The A. vaginimassiliensis is able to ferment glycerol, xylose, D-galactose, D-glucose, D-fructose, methyl α-D-glucopyranoside, N-acetyl-D-glucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, trehalose, cellobiose, maltose, lactose, xyitol, gentiobiose and potassium 5-ketogluconate. Alkaline phosphatase, leucine arylamidase and acid phosphatase are positive. The major fatty acids were C₁₆:₀ (42%), C₁₈:₁ω₉ (25%) and C₁₈:ω₆ (19%). The genome size of A. vaginimassiliensis strain Marseille-P4512 is 1.83 Mbp with 33.1 mol% G + C content. The 16S rRNA and draft genome sequences of strain Marseille-P4512, available in GenBank database under accession numbers LT934505 and UZAS00000000, respectively. The type strain is Marseille-P4512T, which was isolated from the vagina of a woman with bacterial vaginosis.

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Conflict of interest

Authors declare that there are not conflict of interest.

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