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Characterization of a New *Ezakiella* Isolated from the Human Vagina: Genome Sequence and Description of *Ezakiella massiliensis* sp. nov.

Khoudia Diop¹ · Claudia Andrieu¹ · Caroline Michelle¹ · Nicholas Armstrong¹ · Fadi Bittar¹ · Florence Bretelle^{1,2} · Pierre-Edouard Fournier¹ · Didier Raoult^{1,3} · Florence Fenollar¹

Abstract

The study of the vaginal microbiota using the “culturomics concept” allowed us to isolate, from the vaginal swab of an asymptomatic 20-year-old woman who had sexual relations with another woman with bacterial vaginosis, an unknown Gram-positive anaerobic coccus-shaped bacterium that was designated strain Marseille-P2951^T and characterized using taxono-genomics. Strain Marseille-P2951^T is non-motile and non-spore forming and exhibits catalase and oxidase activities. Its 16S rRNA gene-based identification showed 98.5% identity with *Ezakiella peruensis*, the phylogenetically closest species. The major fatty acids are C18:1n9 (58%) and C16:0 (22%). With a 1,741,785 bp length, the G+C content of the genome is 36.69%. Of a total of 1657 genes, 1606 are protein-coding genes and 51 RNAs. Also, 1123 genes are assigned a putative function and 127 are ORFans. Phenotypic, phylogenetic, and genomics analyses revealed that strain Marseille-P2951^T (=CSUR P2951 =DSM 103122) is distinct and represents a new species of the genus *Ezakiella*, for which the name *Ezakiella massiliensis* sp. nov. is proposed.

Introduction

Gram-positive anaerobic cocci are mainly represented by the *Peptostreptococci*. These bacteria are part of the commensal flora of humans and animals, and are also often associated with a variety of human infections [21]. They represent approximately 24–31% of all anaerobic bacteria isolated in clinical samples and are the most frequent species

that belong to the genus *Peptostreptococcus* [20]. A taxonomic revision of this group has occasioned the division of *Peptostreptococcus* into 5 genera: *Anaerococcus*, *Finegoldia*, *Gallicola*, *Parvimonas*, and *Peptoniphilus* [8]. In 2015, a new genus of Gram-positive anaerobic cocci called *Ezakiella* was identified from a human fecal sample in a coastal traditional community in Peru [24, 25]. This genus has only one species: *Ezakiella peruensis* [16]. In a study characterizing the vaginal flora of women with and without bacterial vaginosis using the “culturomics concept,” based on the multiplication of culture conditions (media, temperature, pH, and atmosphere) combined with rapid bacterial identification by Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) [13, 14], we identified an unknown strain of *Ezakiella* designated Marseille-P2951^T which did not correspond to the other species of this genus.

We report the phenotypic, phylogenetic, and genomic characteristics of *Ezakiella massiliensis* sp. nov., strain Marseille-P2951^T (=CSUR [Collection de souches de l'Unité des Rickettsies] P2951 =DSM [Deutsche Sammlung von Mikroorganismen] 103122), isolated from the vaginal sample of a healthy woman who had sexual relations with another woman with bacterial vaginosis.

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Materials and Methods

Ethics and Sample Collection

In October 2015, a vaginal sample from an asymptomatic 20-year-old woman who had sexual relations with another woman with bacterial vaginosis was collected at the Hospital “La Timone” in Marseille (France). The diagnosis was conducted as previously reported [18]. At the time of sample collection, she was not being treated with antibiotics. She gave her written consent. This study was authorized by the local IFR48 ethics committee (Marseille, France) under agreement number 09-022. The sample was collected and transported using a Sigma Transwab (Medical Wire, Corsham, United Kingdom).

Strain Identification by MALDI-TOF MS and 16S rRNA Gene Sequencing

After sampling, the vaginal specimen was cultured using 1 of the 18 culturomics conditions previously described [14]. Isolated colonies were identified using MALDI-TOF, as previously described [4, 5, 28]. For unidentified colonies, the 16S rRNA gene was sequenced and the obtained sequence was matched against the NCBI database using the BLAST algorithm [7]. As suggested, if the 16S rRNA gene sequence similarity value was <98.7%, the strain was defined as a new species [12, 29].

Phylogenetic Tree

All species from the same order of the new species were retrieved and 16S sequences were downloaded from NCBI. Sequences were aligned using CLUSTALW, with default parameters and phylogenetic inferences obtained using the neighbor-joining method with 500 bootstrap replicates, using MEGA6 software.

Morphologic and Biochemical Characterization

To observe cell morphology, individual cells were visualized using a Tecnai G20 electron microscope [4]. Oxidase and catalase tests, Gram stain, motility, and sporulation were performed as previously conducted [23]. Biochemical tests were performed using API ZYM, API 20A, and API 50CH strips (bioMérieux, Marcy l’Etoile, France).

Growth Conditions

To determine optimal growth, the strain Marseille-P2951^T was cultivated on Columbia agar with 5% sheep blood (bioMérieux) and incubated at different temperatures in aerobic,

anaerobic, and microaerophilic atmospheres [4, 5]. The salinity and pH conditions were also tested [4, 5].

Cellular Fatty Acid Analysis

Cellular fatty acid methyl ester (FAME) analysis was performed by Gas Chromatography/Mass Spectrometry (GC/MS). Strain Marseille-P2951^T was grown on Columbia agar (bioMérieux). Then, two samples were prepared with approximately 9 mg of bacterial biomass per tube harvested from several culture plates. FAME was prepared as described by Sasser [27]. GC/MS analyses were carried out as described before [3]. Briefly, fatty acid methyl esters were separated using an Elite 5-MS column and monitored by mass spectrometry (Clarus 500—SQ 8S, Perkin Elmer, Courtaboeuf, France). A spectral database search was performed using MS Search 2.0 operated with the Standard Reference Database 1A (NIST, Gaithersburg, USA) and the FAMES mass spectral database (Wiley, Chichester, UK).

Antibiotic Susceptibility Testing

Amoxicillin, benzylpenicillin, ceftriaxone, imipenem, metronidazole, and vancomycin were used to test antibiotic susceptibility of strain Marseille-P2951^T. The minimal inhibitory concentrations (MICs) were then determined using E-test gradient strips (bioMérieux) [1, 17].

DNA Extraction, Genome Sequencing, and Assembly

After a pre-treatment of 2 h with lysozyme incubation at 37 °C, genomic DNA (gDNA) of strain Marseille-P2951^T was extracted on the EZ1 biorobot (Qiagen) with the EZ1 DNA tissue kit. The elution volume was 50 µL. gDNA was quantified by a Qubit assay with the high sensitivity kit (Life technologies, Carlsbad, CA, USA) at 74.2 ng/µL.

Then gDNA was sequenced on MiSeq Technology (Illumina Inc, San Diego, CA, USA) with the mate pair strategy. The gDNA was barcoded in order to be mixed with 11 other projects with the Nextera Mate Pair sample prep kit (Illumina) [4, 5]. Then, the genome sequencing and assembly were performed as previously described [4]. The reads were trimmed and assembled using the CLC genomics Workbench v4.7.2 software (CLC bio, Aarhus, Denmark).

Genome Annotation and Analysis

The Multi-Agent software system DAGOBAN [10] was used to perform annotation and comparison processes, which include Figenix [11] libraries that provide pipeline analysis. The genome of strain Marseille-P2951^T was compared to those of closest species. In order to evaluate the genomic similarity among all compared genomes, two parameters

were determined: dDDH, which exhibits a high correlation with DNA–DNA Hybridization (DDH) [19], and AGIOS (Average Genomic Identity of Orthologous gene Sequences) [26], which was designed to be independent from DDH.

Results

Strain Identification and Phylogenetic Analysis

The MALDI-TOF MS identification of our isolate failed (score <1.7). The 16S rRNA gene sequence of the strain showed 98.5% identity with *Ezakiella peruensis*, the phylogenetically closest species with a validly published name (Fig. 1). As this value was <98.7% recommended to delimit a species [12, 29], the strain Marseille-P2951^T was classified as a new species, named *Ezakiella massiliensis* sp. nov. The reference spectrum (Fig. 2a) was incremented in our database (<http://www.mediterranee-infection.com/article.php?leref=256&titre=urms-database>) and then compared to other closest species; their differences are shown in the gel view obtained (Fig. 2b).

Phenotypic and Biochemical Characteristics

Strain Marseille-P2951^T is a Gram-positive anaerobic coccus, 0.7 µm in diameter (Supplementary Figure S1A and B), non-motile, and non-spore forming. After 3 days of growth on blood agar (BD diagnostic), colonies were clear and gray, circular, and convex, with a diameter of approximately 0.8 mm. This strict anaerobe grows at temperatures between 28 and 37 °C, but optimal growth was observed at 37 °C after 48 h of incubation. Bacterial growth requires a NaCl concentration <5 g/L and a pH ranging from 6.5 to 8.5. Strain Marseille-P2951^T exhibited catalase and oxidase activities, whereas nitrate and nitrite were not reduced. The results of the API ZYM, 20A, and 50CH are summarized in Table 1 and were compared to those of the closest species [2, 8, 22, 25, 30]. The fatty acid profiles of strain Marseille-P2951^T and the closest species are recapped in Table 2. Strain Marseille-P2951^T was susceptible to amoxicillin (MIC 0.016 µg/mL), benzylpenicillin (MIC 0.003 µg/mL), ceftriaxone (MIC 0.016 µg/mL), imipenem (MIC 0.032 µg/mL), metronidazole (MIC 0.047 µg/mL), and vancomycin (MIC 0.19 µg/mL).

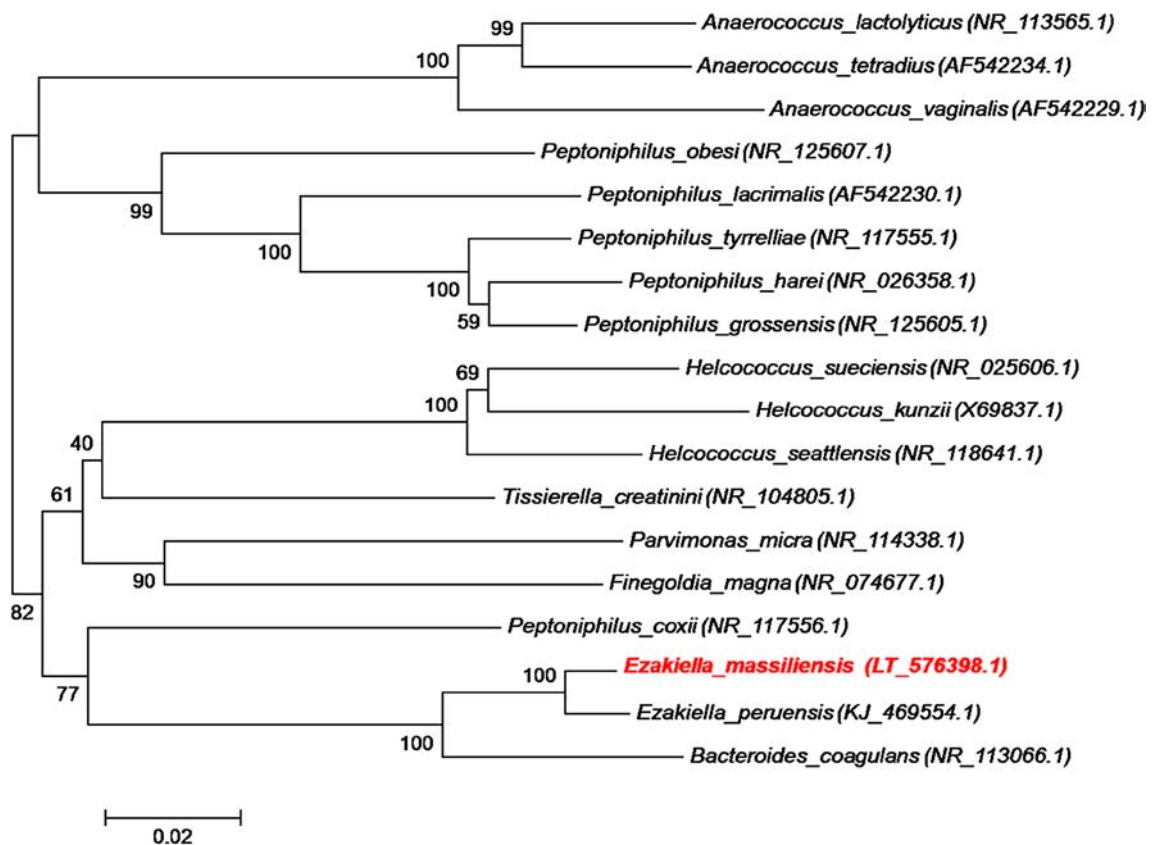


Fig. 1 Phylogenetic tree highlighting the position of *Ezakiella massiliensis* strain Marseille-P2951^T relative to other close strains. GenBank accession numbers for each 16S rRNA are noted in parenthesis. Sequences were aligned using Muscle v3.8.31 with default param-

eters and phylogenetic inferences were obtained using the neighbor-joining method with 500 bootstrap replicates, using MEGA6 software. The scale bar represents a 2% nucleotide sequence divergence

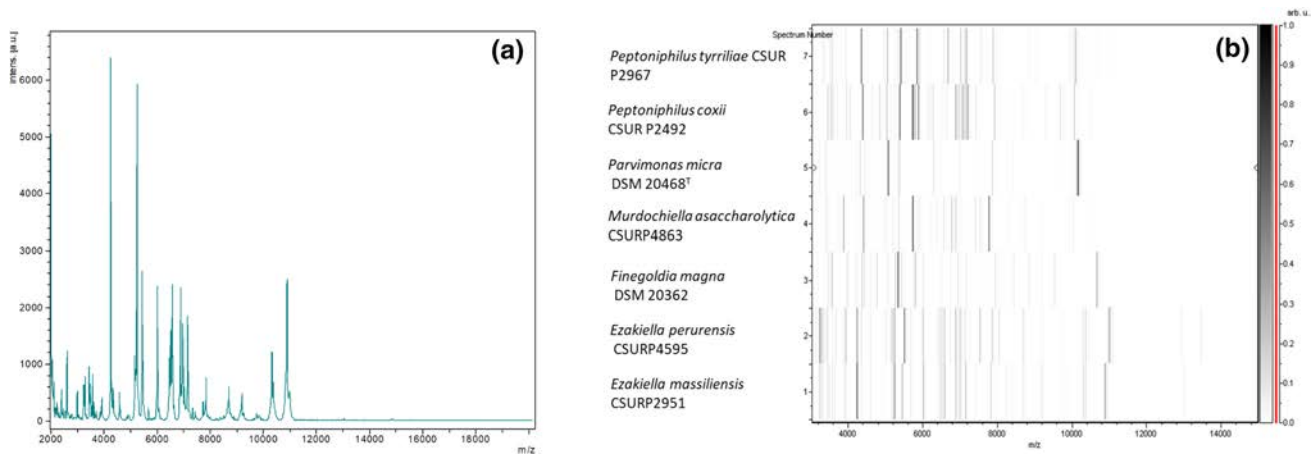


Fig. 2 **a** Reference mass spectrum from *Ezakiella massiliensis* strain Marseille-P2951^T spectra. **b** Gel view comparing the *Ezakiella massiliensis* strain Marseille-P2951^T to other species within the family. The gel view displays the raw spectra of loaded spectrum files arranged in a pseudo-gel-like look. The *x*-axis records the *m/z*

value. The left *y*-axis displays the running spectrum number originating from subsequent spectra loading. The peak intensity is expressed by a gray scale scheme code. The right *y*-axis indicates the relation between the color of a peak and its intensity, in arbitrary units. Displayed species are indicated on the left

Genome Properties

The draft genome of strain Marseille-P2951^T is 1,741,785 bp long with 36.69% of G+C content (Table 3; Fig. 3). It is composed of 1 scaffold (composed of 1 contig). Of the 1657 predicted genes, 1606 were protein-coding genes and 51 were RNAs (6 genes are rRNA, 45 genes are tRNA). A total of 1123 genes (69.93%) were assigned a putative function and 127 genes were identified as ORFans (7.91%). Tables 3 and 4 summarize the genome statistics and the distribution of genes into COG (Clusters of Orthologous Groups) functional categories, respectively.

Genomic Comparison

The comparison of the genome of strain Marseille-P2951^T with those of *Ezakiella peruensis* and other Gram-positive anaerobic cocci is detailed in supplementary Table S1. The distribution of genes into COG categories was similar among all compared genomes (Supplementary Figure S2). However, there were fewer genes of strain Marseille-P2951^T present in the COG categories C (Energy production and conversion) and G (Carbohydrate transport and metabolism) than other compared species. In addition, the AGIOS analysis revealed that strain Marseille-P2951^T shares 541 orthologous genes with *Helcococcus sueciensis* and 874 with *Ezakiella peruensis* (Supplementary Table S2). The analysis of the average percentage of nucleotide sequence identity ranged from 60.10 to 94.20% with *Peptoniphilus coxii* and *Ezakiella peruensis*, respectively (Supplementary Table S2). Moreover, the dDDH of strain Marseille-P2951^T and its closest species varied between 23.90 and 62 mol%

with *Helcococcus kunzii* and *Anaerococcus tetradius*, respectively (Supplementary Table S3).

Discussion

Only 20% of vaginal bacteria were identified by culture [15]. The understanding of vaginal flora was enhanced by the use of molecular techniques and culturomics. Molecular methods allowed the detection of uncultured and fastidious bacteria in the vagina, such as bacterial vaginosis-associated bacteria type 1 (BVAB1), BVAB2, and BVAB3 [9]. Also, culturomics concepts enabled the culture of new bacterial species, such as *Vaginella massiliensis* [4], *Olegusella massiliensis* [5], *Murdochiella vaginalis* [6], and *Ezakiella massiliensis*, reported in this paper.

Ezakiella massiliensis strain Marseille-P2951^T is a new member of the genus *Ezakiella*. The similarity of its 16S rRNA gene sequence less than the threshold delineated to distinguish a new species, the difference in the G+C content, the AGIOS values, and the GGDC values to 70% confirmed that strain Marseille-P2951^T is a distinct species than *Ezakiella peruensis*. The genus *Ezakiella* was created in 2015 [25] and contains Gram-positive anaerobic cocci that were detected in diverse human clinical samples. Bacteria of this group were also asaccharolytic. All these characteristics were observed in strain Marseille-P2951^T.

Table 1 Differential characteristics of *Ezakiella massiliensis* and phylogenetically related species: *Ezakiella peruensis* strain M6.X2^T, *Finegoldia magna* strain CCUG 17636^T, *Peptoniphilus asaccharolyticus* strain ATCC 14963, *Parvimonas micra* CCUG 46357^T, *Helcococcus kunzii* NCFB 2900^T, and *Anaerococcus prevotii* ATCC 9321^T [2, 8, 22, 25, 30]

Properties	<i>Ezakiella massiliensis</i>	<i>Ezakiella peruensis</i>	<i>Finegoldia magna</i>	<i>Peptoniphilus asaccharolyticus</i>	<i>Parvimonas micra</i>	<i>Helcococcus kunzii</i>	<i>Anaerococcus prevotii</i>
Cell diameter (µm)	0.7	<1	0.8–1.6	0.5–0.9	0.3–0.7	na	na
Oxygen requirement	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Facultatively anaerobic	Anaerobic
Gram stain	+	+	+	+	+	+	+
Spore formation	–	–	–	–	–	–	na
Motility	–	–	–	–	–	–	–
DNA G + C content (mol%)	36.69	38,4	na	31	28.65	30	33
Production of							
Alkaline phosphatase	+	+	variable	–	+	–	–
Indole	+	+	–	variable	–	–	–
Catalase	+	–	variable	+	variable	–	+
Nitrate reductase	–	–	–	–	–	–	–
Urease	–	–	–	–	–	–	+/-
β-galactosidase	–	–	–	–	–	–	–
N-acetyl-glucosamine	–	–	–	na	–	+	na
Acid from							
Mannose	–	–	–	–	–	–	+
Glucose	–	–	–	–	–	–	+/-
Lactose	–	–	–	–	–	+	–
Raffinose	–	–	–	–	–	–	+
Habitat	Vaginal discharge	Human feces	Human specimen	Human specimen	Human specimen	Human specimen	Human specimen

na no available data

+: positive reaction

–: negative reaction

Table 2 Cellular fatty acid profiles (%) of *Ezakiella massiliensis* strain Marseille-P2951^T (data from this study) compared with closest species [25]

Fatty acid	<i>Ezakiella massiliensis</i>	<i>Ezakiella peruensis</i>	<i>Finegoldia magna</i>	<i>Peptoniphilus asaccharolyticus</i>	<i>Parvimonas micra</i>	<i>Helcococcus kunzii</i>	<i>Anaerococcus prevotii</i>
C10:0	–	7.3	9.4	–	–	–	–
C12:0	–	–	4.8	12.0	–	–	–
C14:0	6.9	3.0	5.9	5.4	1.6	2.5	2.0
Iso-C _{15:0}	–	–	–	2.6	–	–	–
C16:0	21.8	18.3	17.6	14.4	13.4	30.0	17.1
C17:0	–	5.2	–	–	–	–	–
Anteiso-C _{17:0}	1.3	–	4.5	1.6	–	–	1.7
Iso-C17:1/C16:0 DMA	–	–	18.2	–	–	–	–
C18:0	5.0	–	–	9.4	6.8	16.0	11.5
C18:1 ω7c	–	4.2	–	–	–	–	–
C _{18:1} ω9c	58.1	39.8	3.6	20.2	15.5	19.3	19.3
C18:2ω6,9c/C18:0 ANTE	4.9	13.2	5.6	22.0	58.3	29.4	20.7
C _{18:1} ω9c DMA	–	–	11.1	6.6	–	–	–

Bold represents the majority fatty acid for this species; values <1% are not shown

Conclusion

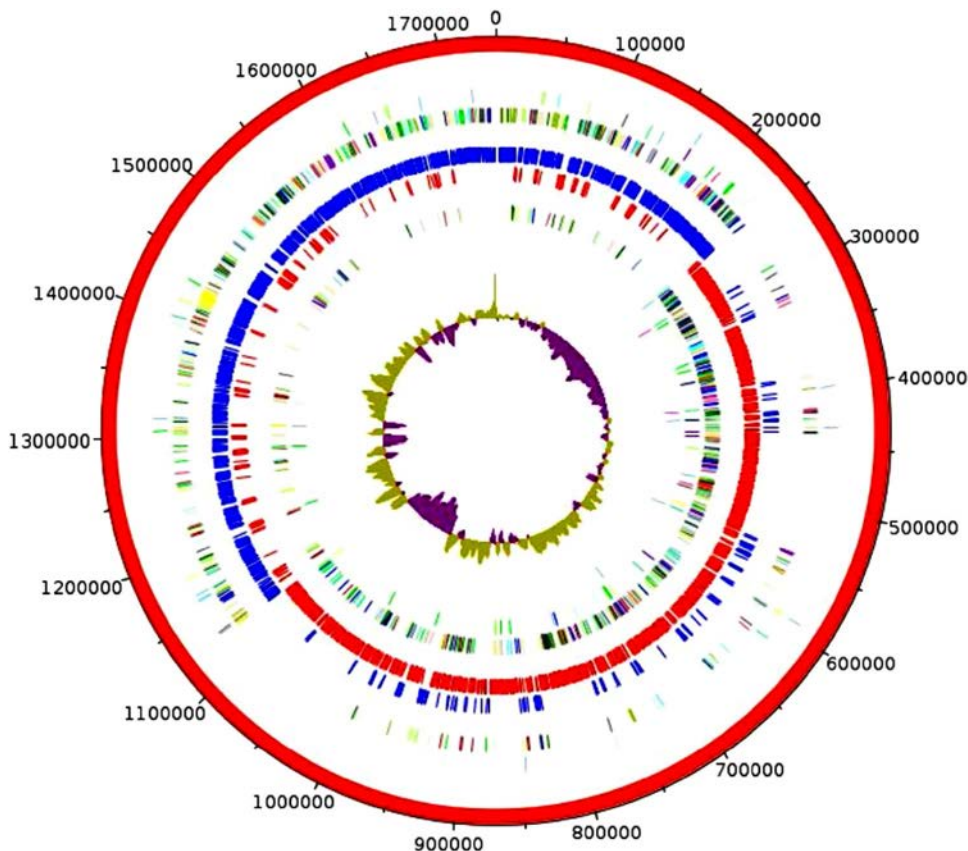
Phenotypic, phylogenetic, and genomic results confirmed that strain Marseille-P2951^T is distinct from its phenotypically closest species and can represent a new species, within the genus *Ezakiella*, for which we proposed the name

Table 3 Nucleotide content and gene count levels of the genome

Attribute	Genome (total)	
	Value	% of total ^a
Size (bp)	1,741,785	100
G+C content (bp)	639,071	36.69
Coding region (bp)	1,581,924	90.82
Total genes	1657	100
rRNA genes	6	0.36
Protein-coding genes	1606	100
Genes with function prediction	1123	69.93
Genes assigned to COGs	1029	64.07
Genes with peptide signals	210	13.08
Genes with transmembrane helices	408	25.40

^aThe total is based on either the size of the genome in base pairs or the total number of protein-coding genes in the annotated genome

Fig. 3 Graphical circular map of the genome. From outside to the center: Contigs (red/gray), COG category of genes on the forward strand (three circles), genes on forward strand (blue circle), genes on the reverse strand (red circle), COG category on the reverse strand (three circles), G+C content. (Color figure online)



Ezakiella massiliensis. The type strain Marseille-P2951^T was isolated from the vaginal sample of a healthy woman who had sexual relations with another woman with bacterial vaginosis.

Taxonomic and Nomenclature Proposal

Description of *Ezakiella massiliensis* sp. nov.

Ezakiella massiliensis (mas.si.li.en'sis, L. fem. adj. massiliensis, from *Massilia*, the Latin name of Marseille where the type strain was first isolated).

Ezakiella massiliensis is non-motile and non-sporulating. It is mesophilic, with optimal growth at 37 °C after 48 h. Colonies are clear and gray, circular, and convex, with 0.8 mm of diameter on blood agar. Obligate anaerobe, the cells are gram-positive and coccus-shaped, with a diameter of 0.7 µm, positive for indole, catalase, and oxidase, negative for nitrate reductase. Gelatin and starch were not hydrolyzed. Lipases, valine arylamidase, trypsin, α -chymotrypsin, phosphatase acid, α - and β -galactosidase, β -glucuronidase, α - and β -glucosidase, *N*-acetyl- β -glucosaminidase were negative, although alkaline phosphatase, leucine arylamidase, and naphthol-AS-BI-phosphohydrolase were positive.

Table 4 Number of genes associated with the 25 general COG functional categories

Code	Value	% of total	Description
[J]	171	10.647572	Translation
[A]	0	0	RNA processing and modification
[K]	61	3.7982564	Transcription
[L]	59	3.6737237	Replication, recombination, and repair
[B]	0	0	Chromatin structure and dynamics
[D]	21	1.3075966	Cell cycle control, mitosis and meiosis
[Y]	0	0	Nuclear structure
[V]	59	3.6737237	Defense mechanisms
[T]	36	2.241594	Signal transduction mechanisms
[M]	47	2.9265256	Cell wall/membrane biogenesis
[N]	5	0.3113325	Cell motility
[Z]	0	0	Cytoskeleton
[W]	2	0.124533	Extracellular structures
[U]	19	1.1830635	Intracellular trafficking and secretion
[O]	51	3.1755917	Posttranslational modification, protein turnover, chaperones
[X]	21	1.3075966	Mobilome: prophages, transposons
[C]	49	3.0510585	Energy production and conversion
[G]	36	2.241594	Carbohydrate transport and metabolism
[E]	100	6.2266498	Amino acid transport and metabolism
[F]	50	3.1133249	Nucleotide transport and metabolism
[H]	65	4.0473228	Coenzyme transport and metabolism
[I]	31	1.9302616	Lipid transport and metabolism
[P]	82	5.105853	Inorganic ion transport and metabolism
[Q]	13	0.8094645	Secondary metabolites' biosynthesis, transport, and catabolism
[R]	77	4.7945204	General function prediction only
[S]	61	3.7982564	Function unknown
–	577	35.92777	Not in COGs

It is asaccharolytic; acid is not produced from sugars. The major fatty acids are C18:1n9 (58%) and C16:0 (22%). Bacterial cells were susceptible to amoxicillin, ceftriaxone, benzylpenicillin, imipenem, metronidazole, and vancomycin. The genome is 1,741,785 bp long and the DNA G+C content is 36.69 mol%. The 16S rRNA and genome sequences are both deposited in EMBL/EBI under accession numbers LT576398 and LT635475, respectively. The respective DPD (Digital Protologue Database) TaxonNumber is TA00324. The type strain, strain Marseille-P2951^T (=CSUR P2951 =DSM 103122), was isolated from the vaginal sample of a healthy woman.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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