ORIGINAL ARTICLE

Libanicoccus massiliensis gen. nov., sp. nov., a new bacterium isolated from human stool

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Abstract

Strain Marseille-P3237 was isolated from a stool sample of a healthy 35-year-old Congolese pygmy female. This anaerobic, Gram-negative, non-spore-forming and non-motile coccus-shaped bacterium is a member of the order *Coriobacteriales*. It exhibits a 2 009 306-bp genome with a 65.46 mol% G+C content and is closely related to, but distinct from, members of the *Olsenella* genus. We propose the creation of the new genus *Libanicoccus* gen. nov. and of the new species *Libanicoccus massiliensis* sp. nov.

Keywords: Culturomics, Genome, *Libanicoccus massiliensis*, Pygmy, Taxonogenomics
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Introduction

The human gut contains 10^{11} to 10^{12} bacteria per gram of stool. This complex microflora is known for its microbial diversity and role in health and diseases [1]. Deciphering the gut microbiota has become a challenge in the twenty-first century [2] and has been attempted using different tools yielding increasingly complex results [3]. To date, more than 2000 different bacterial species belonging to the human gut microbiota have been reported [4]. In our laboratory, we have developed a new technique named culturomics to isolate previously uncultured human gut bacteria [5,6]. Basically, stool samples are cultured under various conditions and all isolated colonies are identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Among bacterial isolates that fail MALDI-TOF MS identification, those that show sufficient 16S rRNA gene sequence divergence with species with standing in nomenclature are further characterized using the taxonogenomics strategy, which combines phenotypic assays and genome sequencing and analysis [3,7]. In the present study, using the taxonogenomics approach, we describe the new genus *Libanicoccus* gen. nov. within the family *Coriobacteriaceae*. Strain Marseille-P3237^T (= CSUR P3237 = CCUG 71182) is the type strain of the new species *Libanicoccus massiliensis* gen. nov., sp. nov.

Material and methods

Ethics and sample collection

A stool sample from a healthy 35-year-old pygmy woman was collected in Congo and preserved at −80°C for further analysis at the URMITE Laboratory (Marseille, France). The sample donor gave a signed and informed consent. The study was approved by the ethics committee of the Institut Fédératif de Recherche IFR48 (Marseille, France) under number 09-022.

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Strain isolation

The stool sample was diluted in PBS (Life Technologies, Carlsbad, CA, USA) and pre-incubated for 3 days in a blood culture vial (BD BACTEC®, Plus Anaerobic/F Media, Le Pont de Claix, France) supplemented with 5 mL of sheep blood and 5 mL of filter-sterilized rumen at 37°C. Then, the culture suspension was inoculated on 5% sheep blood-enriched Columbia agar (bioMérieux, Marcy l'Etoile, France) and incubated at 37°C in anaerobic atmosphere.

MALDI-TOF MS and 16S rRNA gene sequencing identification

The individual identification of isolated colonies was first attempted using MALDI-TOF MS, as previously described [5,6]. The reference spectrum obtained for each colony was compared with the Bruker database using the MALDI Biotyper software version 3.0 (Bruker Daltonics, Bremen, Germany). Any score <1.9 was considered unreliable. In this case, colonies were subjected to 16S rRNA gene amplification and sequencing, using a GeneAmp 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3130xl Genetic Analyzer capillary sequencer (Applied Biosystems) as previously described [8]. Each 16S rRNA nucleotide sequence was compared with the nr database of the National Center for Biotechnology Information using the BLAST software (https://blast.ncbi.nlm.nih.gov/). We used the 16S rRNA sequence similarity thresholds of 95% and 98.65% proposed by Kim et al. to consider bacterial isolates as putatively belonging to a new genus or a new species without performing DNA–DNA hybridization [9]. Finally, to determine the phylogenetic position of strain Marseille-P3237 with regard to species with standing in nomenclature, its 16S rRNA gene sequence was compared with the 16S rRNA database of the 'All-Species Living Tree' project of Silva (LTPs121) [10]. Sequence alignment was obtained using Muscle [11] and phylogenetic relationships were inferred with the maximum-likelihood method within the FastTree software [12].

Growth conditions

Culture of strain Marseille-P3237 was attempted using several conditions to determine its optimal growth requirements. First, strain Marseille P3237 was inoculated on 5% sheep blood-enriched Columbia agar (bioMérieux) and incubated in aerobic, micro-aerophilic and anaerobic conditions at 28, 37, 45 and 55°C. The GENbag anaer and GENbag microaer systems (bioMérieux) were used to evaluate the bacterial growth in anaerobic and microaerophilic atmospheres, respectively. In addition, optimal halophily and pH were estimated using 0, 5, 15 and 45% NaCl concentrations and pH values of 6, 6.5, 7 and 7.5, respectively.

Morphological and biochemical assays

The API 20A, API ZYM and API 50CH strips (bioMérieux) were used to biochemically characterize strain Marseille-P3237. Sporulation ability was tested after exposing a bacterial suspension to a thermic shock at 80°C for 10 min. Motility was evaluated using a DM1000 photonic microscope (Leica Microsystems, Nanterre, France) with a 1000× magnification. Cell morphology was observed using electron microscopy and the following protocol. Bacteria were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for at least 1 h at 4°C. Then, a drop of cell suspension was deposited for approximately 5 min on glow-discharged formvar carbon film on 400-mesh nickel grids (FCF400-Ni, EMS). The grids were dried on blotting paper and cells were negatively stained for 10 seconds with a solution of 1% ammonium molybdate in filtered water at room temperature. Electron micrographs were acquired with a Tecnai G20 Cryo (FEI) transmission electron microscope operated at 200 keV.

Fatty acid methyl ester analysis

Cellular fatty acid methyl ester analysis was performed by gas chromatography/mass spectrometry. Two samples were prepared with approximately 21 mg of bacterial biomass per tube, harvested from several culture plates. FAME analysis was carried out as previously described [5].

Antibiotic susceptibility testing

The antibiotic susceptibility of strain Marseille-P3237 was assessed using the E-test method for the following molecules: benzylpenicillin, amoxicillin, cefotaxime, ceftriaxone, imipenem, amikacin, erythromycin, daptomycin, rifampicin, minocycline, teicoplanin, vancomycin, colistin and metronidazole (bioMérieux).

Genomic DNA extraction and genome sequencing

Genomic DNA (gDNA) of strain Marseille-P3237 was extracted as previously described [5]. A final concentration of 67.8 ng µL was measured with the Qubit assay and the high sensitivity kit (Life Technologies, Carlsbad, CA, USA). Afterwards, gDNA was sequenced on a MiSeq sequencer (Illumina, San Diego, CA, USA). Briefly, 1.5 µg of gDNA was used for mate-pair library preparation using the Nextera mate pair Illumina guide (Illumina). After tagmentation and fragmentation of the gDNA with a mate-pair junction adapter, the fragmentation pattern was confirmed using an Agilent 2100 BioAnalyzer (Agilent Technologies Inc, Santa Clara, CA, USA) with a DNA 7500 labchip. The DNA fragments ranged in size from 1.5 kb up to 11 kb with an optimal size at 5.282 kb. No size selection was performed and 191.8 ng of tagmented fragments were circularized. Mechanical shearing of the circularized DNA was