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Maryam Tidjani Alou, Saber Khelaifia, Caroline Michelle, Claudia Andrieu, Nicholas Armstrong, et al.. Anaerococcus rubiinfantis sp nov., isolated from the gut microbiota of a Senegalese infant with severe acute malnutrition. Anaerobe, 2016, 40, pp.85-94. 10.1016/j.anaerobe.2016.06.007 . hal-01455591

HAL Id: hal-01455591

<https://hal.science/hal-01455591>

Submitted on 26 Jul 2021

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Anaerococcus rubiinfantis sp. nov., isolated from the gut microbiota of a Senegalese infant with severe acute malnutrition

Maryam Tidjani Alou ^a, Saber Khelaifia ^a, Caroline Michelle ^a, Claudia Andrieu ^a,
Nicholas Armstrong ^a, Fadi Bittar ^a, Cheikh Sokhna ^b, Aldiouma Diallo ^b,
Pierre-Edouard Fournier ^a, Didier Raoult ^{a, b, c}, Matthieu Million ^{a, *}

^a Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes IRD 198, CNRS 7278, Aix-Marseille Université, Marseille, France

^b Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes IRD 198, CNRS 7278, Campus Commun UCAD-IRD of Hann, Dakar, Senegal

^c Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia

Anaerococcus rubiinfantis sp. nov. strain mt16^T is a new species within the genus *Anaerococcus*, which was isolated by the culturomics approach from the gut microbiota of an infant suffering from kwashiorkor. A phenotypic, biochemical and proteomic description of this strain is hereby presented alongside a complete annotation of its genome. This strictly anaerobic species forms Gram-positive non-sporeforming cocci. The major fatty acid was hexadecanoic acid. The phylogenetic analysis of strain mt16^T showed a 97.9% similarity level with *Anaerococcus vaginalis*, the closest validly published species. Its genome is 1,929,161 bp long with 29.5% G + C content and contains 1808 protein-coding genes and 56 RNA genes, among which are six rRNA genes. Genomic analysis identified 41/1864 coding genes as ORFans (2.2%) and at least 620/1808 (34.9%) orthologous proteins which are not shared with the closest phylogenetic species. We believe that the extension of the human anaerobic gut compendium by culturomics is one of the first steps that will improve the understanding of the links between the microbiome and health or disease.

1. Introduction

Severe acute malnutrition, as the leading cause of child mortality, is a major public health issue [1]. Recently, several studies have highlighted a dysbiosis of the gut microbiota of children affected with severe acute malnutrition [2,3]. In order to characterize the gut microbiota of severely undernourished children, we

used the “culturomics” approach, which consists in the multiplication of culture conditions with a variation of media, temperature, pH and atmosphere conditions to describe a bacterial ecosystem as exhaustively as possible [4]. A new member of the *Anaerococcus* genus was thus isolated. This genus was created by Ezaki in 2001 and contains eight validated species, the type species being *Anaerococcus prevotii* [5]. These Gram-positive, obligate anaerobic, and non-spore forming cocci were mostly isolated from human vaginal discharges and various purulent secretions [6].

The recent breakthrough in molecular techniques has allowed additional data such as the complete description of the genome, to be added to classical bacterial classification, which is based on phenotypic and genotypic characteristics and phylogeny based on the 16s rRNA gene [7–9]. Thanks to the decreasing cost of genome sequencing, a new concept of bacterial description [10], taxonomics, was created in our laboratory allying classical bacterial classification features to a proteomic description with the MALDI-TOF profile [11] and a description of the complete annotated genome of the described species [12].

Here follows the description of a new member of the

Abbreviations: AGIOS, average of genomic identity of orthologous gene sequences; bp, base pairs; COG, Clusters of Orthologous Groups; CSUR, Collection de souches de l'Unité des Rickettsies; dDDH: digital, DNA-DNA hybridization; DSM, Deutsche Sammlung von Mikroorganismen; EI, electron impact; FAME, fatty acid methyl ester; FTP, file transfer protocol; GC/MS, gaz chromatography/mass spectrometry; INSDC, International Nucleotide Sequence Database Collaboration; kb, kilobases; MALDI-TOF MS, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry; MIC, minimal inhibitory concentration; MS, mass spectrometry; NRPS, non-ribosomal peptide synthase; ORF, open reading frame; PKS, polyketide synthase; TE buffer, tris-EDTA buffer; SDS, sodium dodecyl sulfate; URMITE, Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes.

* Corresponding author.

E-mail address: matthieumillion@gmail.com (M. Million).

Anaerococcus genus isolated from the fecal sample of a Senegalese girl suffering from severe acute malnutrition (kwashiorkor), strain mt16^T (=CSUR P2032 = DSM 101186).

2. Materials and methods

2.1. Sample information

The fecal sample of a 7-month-old severely malnourished girl was collected with the consent of the child's parent. At the time of the collection, no antibiotic was administered to the patient and the sample was frozen at -80°C upon reception at the hospital La Timone in Marseille, France. Approval for this study was given by the Institut Fédératif de Recherche 48 (Faculty of Medicine, Marseille, France) under agreement number 09–022. The collected sample was cultivated using the eighteen conditions of standardized culturomics, as described previously [13].

2.2. Growth conditions

The ideal growth environment of strain mt16^T was determined by testing different temperatures (25, 28, 37, 45 and 56°C) under aerobic, anaerobic and microaerophilic growth. Aerobic growth was tested with or without 5% CO_2 . Anaerobic and microaerophilic atmospheres were created using respectively GENbag Anaer and GENbag microair systems (BioMerieux, Marcy l'Etoile, France).

2.3. Morphologic, biochemical and antibiotic susceptibility tests

Determination of phenotypic characteristics (oxidase, catalase, gram staining, motility, sporulation) was realized as carried out previously [14]. For electron microscopy with negative staining, a 40 μL bacterial suspension drop was deposited on formvar coated grids, and incubated at 37°C for 30 min. It was then followed by a 10 s incubation on ammonium molybdate 1%. The grids were dried on blotting paper and finally observed using a Tecnai G20 transmission electron microscope (FEI Company, Limeil-Brevannes, France).

Biochemical features were described using API 20A, ZYM and 50CH strips according to the manufacturer's instructions (BioMérieux). Since strain mt16 was isolated in anaerobic condition, the strips were all incubated in anaerobic atmosphere using GENbag Anaer (BioMerieux) for 4, 24 and 48 h for API ZYM, 20A and 50CH strips respectively. In order to test nitrate reduction, we also used an API 20NE strip in the same conditions as the API 20A strip.

Strain mt16 was grown on 5% sheep blood enriched Columbia agar (BioMerieux) for the fatty acids analysis which was carried out by GC/MS. For this purpose, two samples were prepared with approximately 15 mg of bacterial biomass each harvested from several culture plates. Cellular fatty acid methyl esters (FAME) were prepared as described by Sasser [15]. Briefly, GC/MS analyses were realized by using a Clarus 500 gas chromatograph equipped with a SQ8S MS detector (Perkin Elmer, Courtaboeuf, France). 2 μL of FAME extracts were volatilized at 250°C (split 20 mL/min) in a Focus liner with wool and separated on an Elite-5MS column (30 m, 0.25 mm i.d., 0.25 mm film thickness) using a linear temperature gradient ($70\text{--}290^{\circ}\text{C}$ at $6^{\circ}\text{C}/\text{min}$), allowing the detection of C4 to C24 fatty acid methyl esters. Helium flowing at 1.2 mL/min was used as carrier gas. The MS inlet line was set at 250°C and EI source at 200°C . Full scan monitoring was performed from 45 to 500 m/z . All data were collected and processed using Turbomass 6.1 (Perkin Elmer). FAMES were identified by a spectral database search using MS Search 2.0 operated with the Standard Reference Database 1A (National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA) and the FAMES mass spectral database (Wiley,

Chichester, UK). Retention time correlations with estimated nonpolar retention indexes from the NIST database were obtained using a 37-component FAME mix (Supelco; Sigma-Aldrich, Saint-Quentin Fallavier, France); FAME identifications were confirmed using this index. Our results were compared to that of *Anaerococcus* strains readily available in our in house collection, CSUR, namely *Anaerococcus vaginalis* CSUR P727, *Anaerococcus senegalensis* CSUR P156, *Anaerococcus murdochii* CSUR P1063, *Anaerococcus tetradius* CSUR P1064 and *Anaerococcus octavius* CSUR P179.

Antibiotic susceptibility was determined using the antibiotic gradient strip test method with Etest strips (BioMerieux) thus allowing to obtain the minimal inhibitory concentration (MIC) for each tested antibiotic. Inoculum suspension was prepared with a culture of strain mt16^T grown on sheep blood enriched Columbia agar (BioMerieux) suspended in a sterile saline solution (0.85% NaCl) to obtain a turbidity of 0.5 McFarland. The inoculum was then plated with cotton swabs on 5% horse blood and β -NAD enriched Mueller Hinton Agar (BioMerieux) as per EUCAST recommendations [16,17]. Etest strips (amoxicillin, amoxicillin/clavulanic acid, clindamycin, imipenem, metronidazole, penicillin G, vancomycin) were deposited. The plates were incubated for 48 h in an anaerobic atmosphere. Elliptical zones of inhibition are formed around the strip and the intersection with the strip indicated the MIC [17]. *Escherichia coli* strain DSM 1103 was used as a quality control strain. MICs were interpreted according to the EUCAST recommendations [18].

2.4. Strain identification by MALDI-TOF MS

Strain mt16 was identified using MALDI-TOF MS technology [11,19]. This proteomic analysis was conducted using a Microflex spectrometer (Bruker Daltonics, Leipzig, Germany) with a MTP 96 MALDI-TOF target plate (Bruker). The obtained spectra for a colony are imported into the MALDI BioTyper software (version 2.0, Bruker) and analyzed by standard pattern matching (with default parameter settings) against the 7567 references contained in our database (Bruker database incremented with our data). An identification score was obtained: a score ≥ 1.9 allowed an identification at the species level; a score between 1.9 and 1.7 allowed identification only at the genus level and finally a score < 1.7 gave no identification.

2.5. Strain identification by 16S rRNA sequencing

Due to an identification of < 1.7 for the MALDI-TOF MS analysis, the 16S rRNA gene was sequenced using fD1 and rP2 primers as previously described [20]. Stackebrandt and Ebers [21] determined a 98.65% similarity level threshold to define a new species without performing DNA-DNA hybridization.

2.6. Genomic DNA preparation

Strain mt16^T was cultured on 5% sheep blood-enriched Columbia agar (BioMerieux) at 37°C anaerobically. Bacteria grown on three Petri dishes were resuspended in 400 μL of TE buffer. Then, 200 μL of this suspension was diluted in 1 mL TE buffer for lysis treatment that included a 30-minute incubation with 2.5 $\mu\text{g}/\mu\text{L}$ lysozyme at 37°C , followed by an overnight incubation with 20 $\mu\text{g}/\mu\text{L}$ proteinase K at 37°C . Extracted DNA was then purified using 3 successive phenol-chloroform extractions and ethanol precipitations at -20°C overnight. After centrifugation, the DNA was resuspended in 160 μL TE buffer.

2.7. Genome sequencing and assembly

Genomic DNA (gDNA) of strain mt16 was sequenced on the 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). gDNA was quantified by a Qubit assay with the high sensitivity kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to 66.2 ng/ μ l. The mate pair library was prepared with 1 μ g of gDNA using the Nextera mate pair Illumina guide. The gDNA sample was simultaneously fragmented and tagged (tagmentation) with a mate pair junction adapter. The pattern of the fragmentation was validated on an Agilent 2100 BioAnalyzer (Agilent Technologies Inc, Santa Clara, CA, USA) with a DNA 7500 labchip. The DNA fragments ranged in size from 1 kb up to 11 kb with an optimal size at 3.927 kb. No size selection was performed and 505 ng of tagged fragments were circularized. The circularized DNA was mechanically sheared to small fragments with an optimal length at 597 bp on the Covaris device S2 in microtubes (Covaris, Woburn, MA, USA). The library profile was visualized on a High Sensitivity Bioanalyzer LabChip (Agilent Technologies Inc, Santa Clara, CA, USA) and the final concentration library was measured at 59.2 nmol/l. The libraries were normalized at 2 nM and pooled. After a denaturation step and dilution at 15 pM, the pool of libraries was loaded onto the reagent cartridge and then onto the instrument along with the flow cell. An automated cluster generation and sequencing run was performed in a single 39-h run in a 2×251 -bp.

2.8. Genome annotation and analysis

Open Reading Frames (ORFs) were predicted using Prodigal [22] with default parameters but the predicted ORFs were excluded if they were spanning a sequencing gap region (contain N). The predicted bacterial protein sequences were searched against the Clusters of Orthologous Groups (COG) using BLASTP (E-value $1e^{-03}$, coverage 0.7 and identity percent 30%). If no hit was found, it was searched against the NR database using BLASTP with E-value of $1e^{-03}$ coverage 0.7 and identity percent of 30%, and if the sequence length was smaller than 80 amino acids we used an E-value of $1e^{-05}$. The tRNAScanSE tool [23] was used to find tRNA genes, whereas ribosomal RNAs were found by using RNAmmer [24]. Lipoprotein signal peptides and the number of transmembrane helices were predicted using Phobius [25]. ORFans were identified if all the BLASTP performed did not give positive results (E-value smaller than $1e^{-03}$ for ORFs with sequence size larger than 80 aa or E-value smaller than $1e^{-05}$ for ORFs with sequence length smaller 80 aa).

Genomes were automatically retrieved from the 16S RNA tree using XEGEN software (Phylopattern) [26]. For each selected genome, the complete genome sequence, proteome genome sequence (all gene sequences encoding proteins in a genome) and Orfeome genome sequence (all gene sequences encoding orphan proteins in a genome) were retrieved from the FTP of NCBI. All proteomes were analyzed with proteinOrtho [27]. Then for each couple of genomes, a similarity score was computed. This score is the mean value of nucleotide similarity between all couples of orthologues between the two genomes studied (AGIOS) [28]. An annotation of the entire proteome was performed to define the distribution of functional classes of predicted genes according to the clusters of orthologous groups of proteins (using the same method as for the genome annotation). To evaluate the genomic similarity among studied *Anaerococcus* strains, we determined two parameters; dDDH, which exhibits a high correlation with DDH [29,30], and AGIOS [28], which was designed to be independent from DDH. dDDH was determined using the Genome-to-Genome

Distance Calculator (GGDC) server (<http://ggdc.dsmz.de/distcalc2.php>). Genomes were aligned by the GGDC 2.0 Blast + method and results were interpreted with the formula 2 as recommended [30]. Annotation and comparison processes were performed in the Multi-Agent software system DAGOBAN [31] that include Figenix [32] libraries to provide pipeline analysis.

3. Results

3.1. Phenotypic description

Growth was observed at 28, 37 and 45 °C in anaerobic atmosphere. Ideal growth was observed after 48 h at 37 °C in anaerobic atmosphere. No growth occurred in aerobic and microaerophilic conditions. Non-sporeforming and non-motile cells formed small and white colonies with a mean diameter of 2 mm. Gram-staining showed Gram-positive cocci (Fig. 1). Negative staining of strain mt16 observed in electron microscopy showed cocci arranged in pairs with a mean diameter 0.9 μ m (Fig. 2).

No oxidase activity was exhibited. Nevertheless, a positive catalase activity was observed for strain mt16. An API ZYM strip also showed positive activity for other enzymes: alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase and Naphthol-AS-BI-phosphohydrolase. No activity was found for the following enzymes: esterase (C4), lipase esterase (C8), lipase (C14), cysteine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Nitrate reductase and nitrite reductase activities were absent according to the API 20A strip. Using an API 20A strip, indole formation was positive and acid was produced from D-glucose, D-sucrose, D-maltose and D-mannose. No acid production was observed from D-lactose, D-salicine, D-xylose, L-arabinose, glycerol, D-cellobiose, D-melezitose, D-raffinose, D-sorbitol, L-rhamnose and D-trehalose. Urease, β -glucosidase and protease activities were absent. An API 50 CH also showed the metabolism of the following carbohydrates: tagatose and potassium-5-ketogluconate. The other carbohydrates sampled in the 50CH strip were not metabolized by strain mt16: glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl- β D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl- α D-mannopyranoside, methyl- α D-glucopyranoside, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate and potassium 2-ketogluconate.

The most abundant fatty acid was hexadecanoic acid (53%).

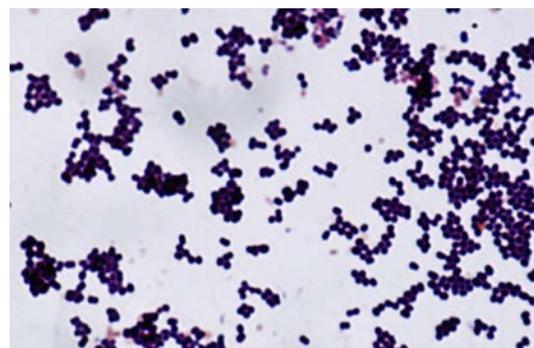


Fig. 1. Gram staining. Gram staining of *Anaerococcus rubiifantis* strain mt16^T.

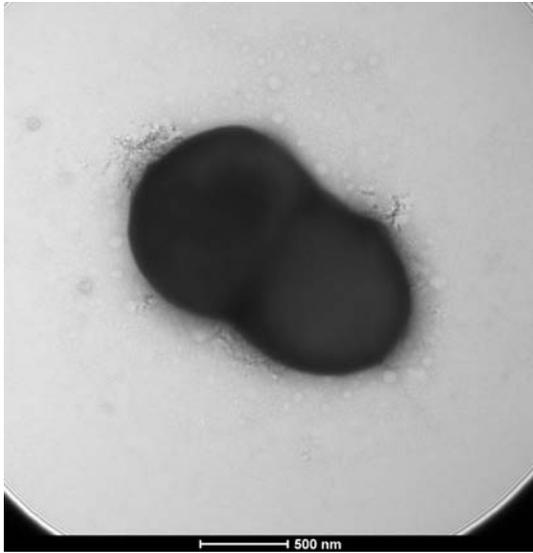


Fig. 2. Transmission electron microscopy. Transmission electron microscopy of *Anaerococcus rubiifantis* strain mt16^T using a Tecnai G20 transmission electron microscope (FEI Company). The scale bar represents 500 nm.

Unsaturated fatty acids 13-octadecenoic and 9,12-octadecadienoic were also measured with significant quantities. Minor amounts of other, mainly saturated, fatty acids, were also detected (Table 1). The comparison to other *Anaerococcus* species showed a relative similarity in the fatty acid profiles with 16:0 being the major fatty acid except in *A. murdochii* (major fatty acid: 18:1n9). All compared *Anaerococcus* species present core abundant fatty acids that are 16:0, 18:1n9, 18:2n6, 18:0 and 14:0 (Table 1).

Strain mt16 was susceptible to benzylpenicillin (MIC < 0.002 µg/mL), amoxicillin (MIC < 0.016 µg/mL), amoxicillin/clavulanic acid (MIC < 0.016 µg/mL), clindamycin (MIC < 0.023 µg/mL), imipenem (MIC < 0.002 µg/mL), metronidazole (MIC < 0.016 µg/mL) and vancomycin (MIC < 0.016 µg/mL). For *Escherichia coli* strain DSM 1103 which was used as a quality control strain, the following MICs (4, 4, 0.125 µg/mL) were obtained for amoxicillin, amoxicillin/clavulanic acid and imipenem respectively. For this quality control strain, no growth inhibition was observed with the following antibiotics: benzylpenicillin, clindamycin, metronidazole and

vancomycin.

3.2. Strain identification by MALDI-TOF MS

Strain mt16 was first isolated in April 2015 after a 10 days' anaerobic pre-incubation in liquid Colombia broth supplemented with 5% sheep blood at 37 °C. The liquid culture was then inoculated on 5% sheep blood Colombia agar (BioMerieux) and incubated in anaerobic condition at 37 °C. According to the MALDI-TOF analysis, strain mt16's highest match was *A. vaginalis* with a score of 1.575. This score under 1.7 did not allow the identification of strain mt16 at the species nor at the genus level and suggested that the closest species might be *A. vaginalis*. Strain mt16 was deposited on a MTP 96 target plate twelve times and the reference spectra obtained (Fig. 3) were incremented into our database. The spectrum of strain mt16 was also compared to other *Anaerococcus* species as represented in Fig. 4. The intensity of the peaks corresponding to strain mt16 was generally lower than that of compared *Anaerococcus* species, including its closest species, *A. vaginalis*.

3.3. Strain identification by 16S rRNA sequencing

In order to identify strain mt16, the 16S rRNA gene was sequenced and the obtained sequence (Accession number LN881592) showed a 97.9% similarity level with *A. vaginalis* strain CCUG 31349^T (Accession number AF542229.1), the phylogenetically closest validated species (Fig. 5). The general characteristics of strain mt16^T are presented in Table 2. According to the similarity level threshold defined by Stackebrandt and Ebers [20], we propose strain mt16^T as the type strain of a new species within the *Anaerococcus* genus for which we suggest the name *Anaerococcus rubiifantis* sp. nov.

3.4. Genome properties

Strain mt16^T's genome (Accession number FAVH00000000) is 1,929,161 bp with a 29.5% G + C content (Table 3, Fig. 6). At the final stage of assembly, it is composed of 3 scaffolds (composed of 5 contigs). There were 1864 predicted genes including 1808 protein-coding genes and 56 RNA genes. Among the RNA genes, there were 3 5S rRNA genes, 2 23S rRNA genes, a unique 16S rRNA gene and 50 tRNA genes. A putative function was assigned by COGS or NR blast to 1427 genes. 41 genes were identified as ORFans and the

Table 1
Cellular fatty acid profiles (%) of *Anaerococcus rubiifantis* strain mt16 compared with other *Anaerococcus* species.

| Fatty acids | Name | <i>A. rubiifantis</i> | <i>A. vaginalis</i> ^a | <i>A. senegalensis</i> ^a | <i>A. tetradius</i> ^a | <i>A. octavius</i> ^a | <i>A. murdochii</i> ^a |
|--------------|---------------------------------|-----------------------|----------------------------------|-------------------------------------|----------------------------------|---------------------------------|----------------------------------|
| 5:0 anteiso | 2-methyl-butanoic acid | TR | ND | ND | ND | ND | ND |
| 5:0 iso | 3-methyl-butanoic acid | ND | ND | ND | ND | 9.0 ± 1.6 | ND |
| 10:0 | Decanoic acid | ND | 1.2 ± 0.2 | TR | TR | ND | ND |
| 12:0 | Dodecanoic acid | TR | TR | TR | TR | TR | TR |
| 14:0 | Tetradecanoic acid | 2.1 ± 0.03 | 6.9 ± 0.2 | 4.4 ± 0.2 | 2.7 ± 0.1 | 4.1 ± 0.2 | 1.9 ± 0.0 |
| 15:0 | Pentadecanoic acid | TR | 1.0 ± 0.1 | TR | TR | TR | TR |
| 16:0 | Hexadecanoic acid | 52.7 ± 0.4 | 59.2 ± 1.1 | 54.6 ± 0.3 | 35.7 ± 1.2 | 49.6 ± 0.6 | 28.6 ± 0.2 |
| 16:1n7 | 9-Hexadecenoic acid | 1.1 ± 0.1 | TR | ND | TR | ND | ND |
| 17:0 | Heptadecanoic acid | TR | ND | TR | TR | ND | TR |
| 17:0 anteiso | 14-methyl-Hexadecanoic acid | ND | ND | ND | TR | ND | ND |
| 18:0 | Octadecanoic acid | 3.6 ± 0.3 | 5.7 ± 0.4 | 11.5 ± 1.4 | 16.7 ± 0.3 | 9.2 ± 0.3 | 13.4 ± 0.4 |
| 18:1n6 | 12-Octadecenoic acid | ND | ND | TR | 1.9 ± 0.2 | ND | 1.9 ± 0.1 |
| 18:1n7 | 11-Octadecenoic acid | ND | ND | ND | 1.0 ± 0.3 | ND | TR |
| 18:1n9 | 9-Octadecenoic acid | 28.2 ± 0.04 | 18.8 ± 0.8 | 17.6 ± 0.7 | 29.9 ± 0.5 | 23.5 ± 1.4 | 35.9 ± 0.3 |
| 18:2n6 | 9,12-Octadecadienoic acid | 10.1 ± 0.5 | 6.5 ± 0.3 | 9.2 ± 0.6 | 11.0 ± 0.2 | 3.8 ± 0.3 | 17.0 ± 0.7 |
| 20:4n6 | 5,8,11,14-Eicosatetraenoic acid | ND | ND | ND | ND | ND | TR |

These values represent the mean peak area percentage calculated from the analysis of FAMES in 3 sample preparations ± standard deviation (n = 3); TR = trace amounts < 1%. Bold represents the majority fatty acid for this species.

^a These strains are provided by the Collection des Souches de l'Unité des Rickettsies (CSUR).

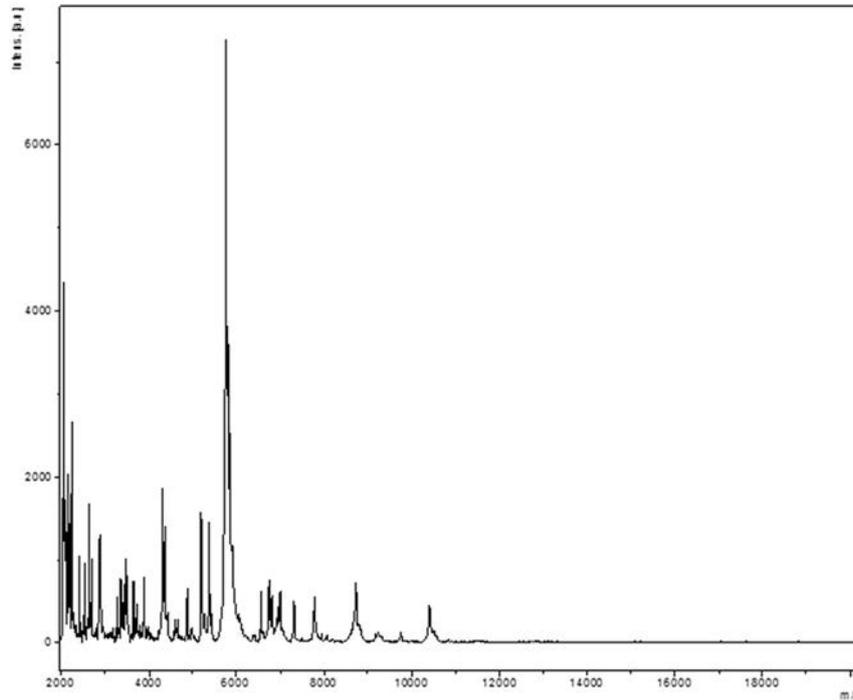


Fig. 3. Reference mass spectrum from *Anaerococcus rubiifantis* strain mt16^T. Spectra from 12 individual colonies were compared and a reference spectrum was generated.

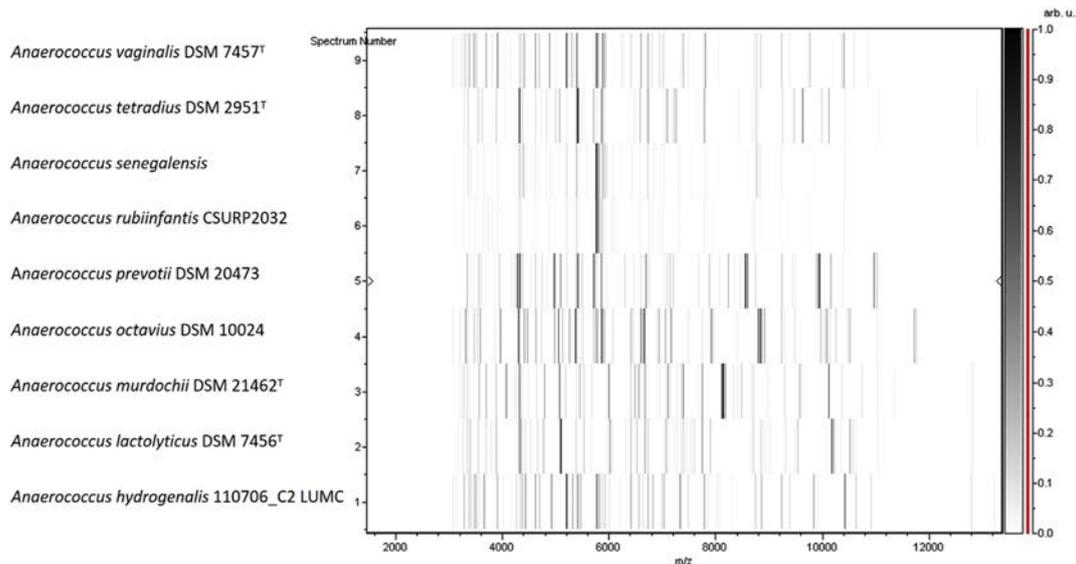


Fig. 4. MALDI-TOF MS comparative analysis of *Anaerococcus* species. Gel view comparing *Anaerococcus rubiifantis* strain mt16^T to other species within the *Anaerococcus* genus. The gel view displays the raw spectra of loaded spectrum files arranged in a pseudo-gel like look. The x-axis records the 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.

remaining 274 genes were identified as encoding hypothetical proteins. The gene distribution into COG functional categories is reported in Table 4.

3.5. Genome comparison

Strain mt16 genomic characteristics were compared to the characteristics of closely related species (Table 5).

The size of the draft genome sequence of strain mt16 (1.93 MB) is smaller than those of *A. prevotii* (2 MB), *A. tetradius* (2.10 MB) and

Anaerococcus lactolyticus (2.18 MB) but larger than the genomes of *Peptoniphilus harei* (1.84 MB), *Anaerococcus hydrogenalis* (1.89 MB) and *A. vaginalis* (1.87 MB). The G + C content of strain mt16 (29.45%) is smaller than most of the compared genomes: *P. harei* (34.44%), *A. tetradius* (34.15%), *A. hydrogenalis* (29.64%), *A. prevotii* (35.64%) and *A. lactolyticus* (34.94%). Only *A. vaginalis* G + C content (28.87%) is smaller than that of strain mt16.

The gene content of strain mt16 is larger than *P. harei*, *A. vaginalis* and *A. prevotii*'s gene contents (1,724, 1764 and 1806 respectively) but smaller than *A. tetradius*, *A. hydrogenalis* and

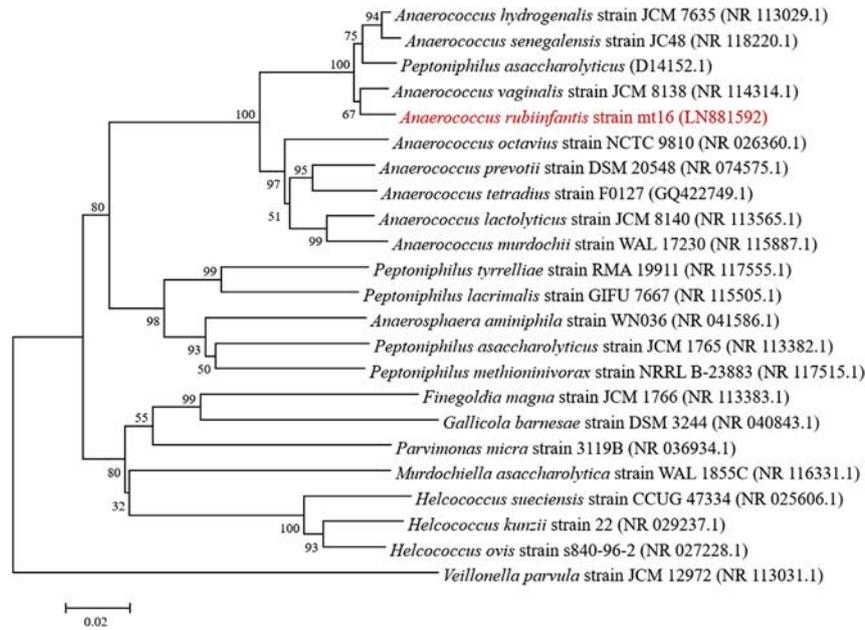


Fig. 5. Phylogenetic tree highlighting the position of *Anaerococcus rubiifantis* strain mt16^T relative to other close strains. The respective GenBank accession numbers for 16S rRNA genes are indicated in parenthesis. Sequences were aligned using CLUSTALW, and phylogenetic inferences were obtained using the maximum-likelihood method within the MEGA6 software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 1000 times to generate a majority consensus tree. *Parvimonas micra* strain 3119B was used as an outgroup. The scale bar represents a 1% nucleotide sequence divergence.

Table 2
Classification and general features of *Anaerococcus rubiifantis* strain mt16^T.

| Property | Term |
|------------------------|--|
| Current classification | Domain: <i>Bacteria</i> Phylum: <i>Firmicutes</i> Class: <i>Tissierellia</i> Order: <i>Tissierellales</i> Family: <i>Peptoniphilaceae</i> Genus: <i>Anaerococcus</i> Species: <i>Anaerococcus rubiifantis</i> Type strain: mt16 |
| Gram stain | Positive |
| Cell shape | Cocci |
| Motility | Non-motile |
| Sporulation | Non-sporulating |
| Temperature range | Mesophilic |
| Optimum temperature | 37 °C |

Table 3
Nucleotide content and gene count levels of the genome.

| Attribute | Genome (total) | |
|--|----------------|-------------------------|
| | Value | % of total ^a |
| Size (bp) | 1,929,161 | 100 |
| G + C content (%) | 568,166 | 29.45 |
| Coding region (bp) | 1,737,861 | 90.08 |
| Total genes (total ORFs) | 1864 | 100 |
| RNA genes | 56 | 3 |
| Protein-coding genes | 1808 | 97 |
| Genes with function prediction | 1427 | 78.9 |
| Genes assigned to COGs | 1243 | 68.75 |
| Genes with signal peptides | 163 | 9.01 |
| Genes with transmembrane helices | 412 | 22.78 |
| ORFan genes | 41 | 2.26 |
| Genes associated with PKS or NRPS ^b | 2 | 0.11 |
| N° of antibiotic resistance genes | 0 | 0 |

^a The 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.

^b PKS: Polyketide Synthase; NRPS: Non-Ribosomal Peptide Synthase.

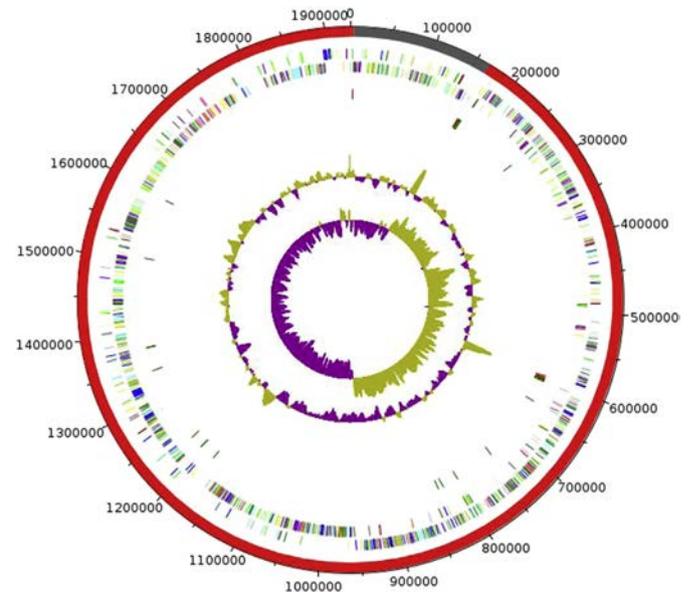


Fig. 6. Graphical circular map of the chromosome. From outside to the center: Contigs (red/grey), genes on the forward strand colored by COG categories (only gene assigned to COG), RNA genes (tRNAs green, rRNAs red), G + C content and G + C skew (purple: value below the mean, yellow: value above the mean). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

A. lactolyticus's gene contents (2,079, 2069 and 2253 respectively).

The distribution of genes into COG categories was similar in all compared genomes except for the carbohydrates metabolism and transport for *P. hareii*, for which this function is reduced (Fig. 7). Strain mt16 shared 1,003, 738, 1,061, 1,073, 1188 and 1091 orthologous genes with *A. lactolyticus*, *P. hareii*, *A. tetradius*, *A. hydrogenalis*, *A. vaginalis* and *A. prevotii* respectively (Table 6). Among species with standing in nomenclature, AGIOS values ranged from 58 to

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

| Code | Value | % of total ^a | Description |
|------|-------|-------------------------|--|
| [J] | 167 | 9.23 | Translation |
| [A] | 0 | 0 | Rna processing and modification |
| [K] | 90 | 4.97 | Transcription |
| [L] | 63 | 3.48 | Replication, recombination and repair |
| [B] | 0 | 0 | Chromatin structure and dynamics |
| [D] | 19 | 1.05 | Cell cycle control, mitosis and meiosis |
| [Y] | 0 | 0 | Nuclear structure |
| [V] | 43 | 2.37 | Defense mechanisms |
| [T] | 47 | 2.59 | Signal transduction mechanisms |
| [M] | 58 | 3.20 | Cell wall/membrane biogenesis |
| [N] | 5 | 0.27 | Cell motility |
| [Z] | 0 | 0 | Cytoskeleton |
| [W] | 3 | 0.16 | Extracellular structures |
| [U] | 16 | 0.88 | Intracellular trafficking and secretion |
| [O] | 57 | 3.15 | Posttranslational modification, protein turnover, chaperones |
| [X] | 20 | 1.10 | Mobilome: prophages, transposons |
| [C] | 88 | 4.86 | Energy production and conversion |
| [G] | 106 | 5.86 | Carbohydrate transport and metabolism |
| [E] | 121 | 6.69 | Amino acid transport and metabolism |
| [F] | 62 | 3.43 | Nucleotide transport and metabolism |
| [H] | 77 | 4.26 | Coenzyme transport and metabolism |
| [I] | 33 | 1.82 | Lipid transport and metabolism |
| [P] | 79 | 4.37 | Inorganic ion transport and metabolism |
| [Q] | 14 | 0.77 | Secondary metabolites biosynthesis, transport and catabolism |
| [R] | 121 | 6.69 | General function prediction only |
| [S] | 66 | 3.65 | Function unknown |
| - | 565 | 31.25 | Not in COGs |

^a The total is based on the total number of protein coding genes in the annotated genome.

Table 5
Genome comparison of closely related species to *Anaerococcus rubiinfantis* strain mt16^T.

| Name of organisms | INSDC identifier ^a | Size (Mb) | G + C (%) | Protein coding genes | Total genes (total ORFs) |
|---|-------------------------------|-----------|-----------|----------------------|--------------------------|
| <i>Anaerococcus rubiinfantis</i> Strain mt16 ^T | FAVH00000000 | 1.92 | 29.45 | 1808 | 1864 |
| <i>Anaerococcus hydrogenalis</i> DSM 7454 | ABXA00000000 | 1.89 | 29.64 | | 2069 |
| <i>Anaerococcus vaginalis</i> ATCC 51170 | ACXU00000000.1 | 1.87 | 28.87 | | 1764 |
| <i>Peptoniphilus harei</i> ACS-146-V-Sch2b | AENP00000000.1 | 1.84 | 34.44 | | 1724 |
| <i>Anaerococcus lactolyticus</i> ATCC 51172 | JRMW00000000.1 | 2.18 | 34.94 | | 2253 |
| <i>Anaerococcus prevotii</i> DSM 20548 | CP001708.1 | 2.00 | 35.64 | | 1806 |
| <i>Anaerococcus tetradius</i> ATCC 35098 | ACGC00000000.1 | 2.10 | 34.15 | | 2079 |

^a INSDC: International Nucleotide Sequence Database Collaboration.

79% among compared species except strain mt16. When comparing strain mt16 to other species, the AGIOS value ranged from 65% with *P. harei* to 88% with *A. vaginalis* (Table 6). dDDH values estimated between strain mt16^T and the compared genomes ranged from 18.8% to 34.7%. Well under 70%, these values confirm strain mt16^T as a new species [30].

3.6. Comparison with other *Anaerococcus* species

The characteristics of strain mt16 were compared to the characteristics of closely related *Anaerococcus* species. Differences were observed for several characteristics such as cell diameter, indole production, enzymes production, acid production from carbohydrates, G + C content, and habitat as reported in Table 7.

4. Discussion

Strain mt16^T was isolated from the stool sample of an 8-month

old infant afflicted with kwashiorkor. Phenotypic characterization, MALDI-TOF MS, 16S rRNA sequencing, and comparative genomic analyses among close phylogenetic relatives allowed identification of strain mt16^T as a new species within the genus *Anaerococcus*, with closest relative being *A. vaginalis*. However, several discrepancies with *A. vaginalis* including a 16S rRNA similarity, low dDDH and low AGIOS values confirmed it as a new species.

Typical features of the present strain included a susceptibility to all tested antibiotics consistent with the absence of known antibiotic resistance genes (Table 4). This high antibiotic susceptibility is clinically relevant and suggesting that gut *Anaerococcus* species could be altered by oral antibiotics. Further studies are needed to determine whether *Anaerococcus rubiinfantis*, found in this study in a severely malnourished child, is part of the Healthy Mature Anaerobic Gut Microbiota [3], is associated with malnutrition, or has no role on nutritional status. Moreover, future studies should elucidate if gut symbionts resistant to commonly used antibiotics are more likely to be pathogenic, such as *Clostridium difficile*

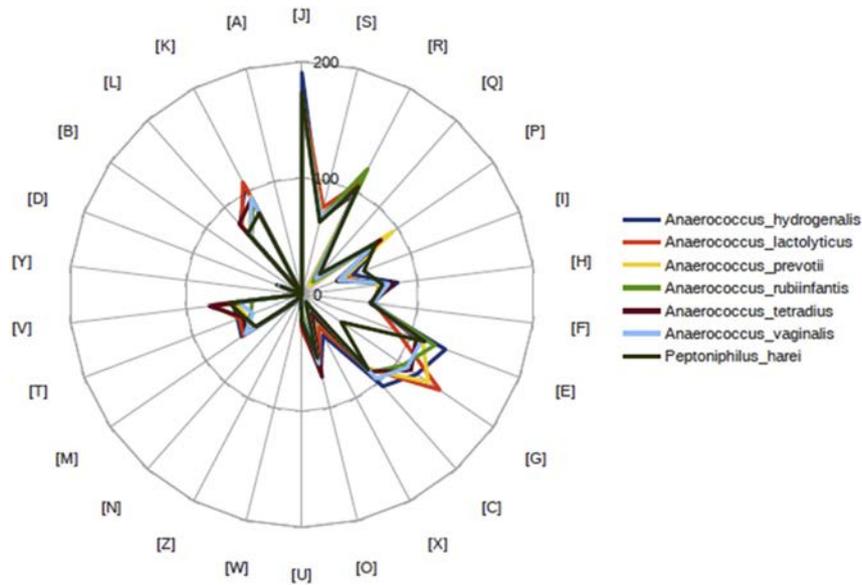


Fig. 7. Distribution of functional classes of predicted genes according to the clusters of orthologous groups of protein. For the codes of COG categories, see Table 4.

Table 6
The number of orthologous proteins shared between genomes (upper right)^a.

| | <i>Anaerococcus lactolyticus</i> | <i>Peptoniphilus harei</i> | <i>Anaerococcus tetradus</i> | <i>Anaerococcus hydrogenalis</i> | <i>Anaerococcus rubiinfantis</i> | <i>Anaerococcus vaginalis</i> | <i>Anaerococcus prevotii</i> |
|------------------------|----------------------------------|----------------------------|------------------------------|----------------------------------|----------------------------------|-------------------------------|------------------------------|
| <i>A. lactolyticus</i> | 2253 | 727 | 1145 | 938 | 1003 | 1047 | 1108 |
| <i>P. harei</i> | 0.65 | 1724 | 752 | 677 | 738 | 753 | 733 |
| <i>A. tetradus</i> | 0.73 | 0.65 | 2079 | 980 | 1061 | 1111 | 1193 |
| <i>A. hydrogenalis</i> | 0.6 | 0.58 | 0.60 | 2069 | 1073 | 1083 | 989 |
| <i>A. rubiinfantis</i> | 0.70 | 0.65 | 0.71 | 0.68 | 1808 | 1188 | 1091 |
| <i>A. vaginalis</i> | 0.70 | 0.66 | 0.71 | 0.67 | 0.88 | 1764 | 1082 |
| <i>A. prevotii</i> | 0.72 | 0.65 | 0.79 | 0.60 | 0.70 | 0.70 | 1806 |

Bold represents the total number of orthologous proteins for this species.

^a Average percentage similarity of nucleotides corresponding to orthologous proteins shared between genomes (lower left) and numbers of proteins per genome.

(antibiotic resistance by sporulation) and the aerotolerant *Klebsiella oxytoca* previously identified as a causative organism of antibiotic-associated hemorrhagic colitis [33].

A paradoxical feature observed in our strain and several other *Anaerococcus* members (*A. vaginalis*, *A. senegalensis*, *A. prevotii* and some strains of *A. tetradus* and *A. octavius* – Table 7) was the presence of a catalase enzyme [34]. In fact, a catalase activity has been found in several strictly anaerobic species such as some members of the genera *Bacteroides*, *Clostridium*, *Porphyromonas*, *Desulfovibrio* and even few archaeal species [34]. This suggest a different oxidative stress resistance level among anaerobic prokaryotes. Indeed, while *Methanobrevibacter smithii* and *Faecalibacterium prausnitzii* are prokaryotes among the most sensitive ones to oxygen (*F. prausnitzii* loses its viability within 2 min after exposure to ambient air [35]), we observed a trace but non-significant growth of *A. rubiinfantis* in microaerophilic conditions, as previously reported for *A. senegalensis* [12]. These results suggest that the aerobic/anaerobic bacterial culture dichotomy is no longer relevant [36] and that there is a continuum between species whose growth is not affected by a normal (ambient air) partial pressure of oxygen and/or positive redox potential, and species that only grow in total absence of oxygen and/or when redox potential is very low [37]. Moreover, tolerance to oxygen and to redox potential of the environment seems completely dependent on the presence of diverse antioxidants [36] and electron acceptors in the medium

[35]. This opens new perspectives for exploration in the field of the ‘anaerobic’ prokaryotes.

Finally, we confirmed the usefulness of genomic analysis as an updated tool to identify, classify and describe more accurately new bacterial strains as promoted by the taxonogenomics description concept [10]. Differences between strain mt16 and *A. vaginalis* (its closest neighbor) also included the production of alkaline phosphatase as well as the production of acids from the following carbohydrates: arabinose, mannitol and sucrose [38]. Strain mt16 was classified among the *Anaerococcus* genus that currently contains eight validated species [5] which are Gram-positive, strictly anaerobic, non-motile, and mostly non-sporeforming species [6,12]. The phylogenetic relationship between several members of the family *Peptoniphilaceae* to which the genus *Anaerococcus* belongs, displayed in Fig. 5, showed strain mt16 in the midst of the validated *Anaerococcus* species.

Given the phylogenetic analysis of the 16S rRNA gene sequence of strain mt16^T, its proteomic profile obtained with MALDI-TOF MS, its genomic description and annotation and dDDH values, we thus defined strain mt16^T as a new species called *Anaerococcus rubiinfantis* sp. nov. (=CSUR P2032 = DSM 101186).

Taxonomic and nomenclatural proposals Description of *Anaerococcus rubiinfantis* strain mt16^T sp. nov. (from ru.be.us (adj) infantis (L. gen. n.) meaning of a red infant, which is a reference to the hair discoloration observed in kwashiorkor patients).

Table 7

Differential characteristics of *Anaerococcus rubiinfantis* strain mt16^T, *Anaerococcus vaginalis* JCM 8138^T, *Anaerococcus hydrogenalis* JCM 7635^T, *Anaerococcus senegalensis* DSM 25366^T, *Anaerococcus prevotii* ATCC 9321^T, *Anaerococcus tetradius* JCM 1964^T, *Anaerococcus lactolyticus* JCM 8140^T, *Anaerococcus octavius* NCTC 9810^T, *Anaerococcus murdochii* ATCC-1385^T, *Bacillus sonorensis* DSM 13779^T [12,38–43].

| Properties | <i>Anaerococcus rubiinfantis</i> ^a | <i>Anaerococcus vaginalis</i> ^b | <i>Anaerococcus hydrogenalis</i> ^b | <i>Anaerococcus senegalensis</i> ^a | <i>Anaerococcus prevotii</i> ^b | <i>Anaerococcus tetradius</i> ^b | <i>Anaerococcus lactolyticus</i> ^b | <i>Anaerococcus octavius</i> ^b | <i>Anaerococcus murdochii</i> ^b |
|-------------------------------|---|--|---|---|---|--|---|---|--|
| Cell diameter (µm) | 0.9 | na | 0.7–1.8 | 0.87 | 0.7–1.8 | <1.8 | na | 0.7–0.9 | ≥0.7 |
| Indole | + | + | + | + | – | – | – | – | – |
| Major fatty acid ^a | 16:0 | 16:0 | na | 16:0 | na | 16:0 | na | 16:0 | 18:1n9 |
| G + C content (%) | 29.5 | 30–34 | 30–34 | 28.56 | 29–33 | 30–32 | 30–34 | 26–31 | na |
| Production of | | | | | | | | | |
| Alkaline phosphatase | + | – | – | + | – | – | – | – | + |
| Catalase | + | na | – | + | + | ± | na | ± | – |
| Nitrate reductase | – | na | – | – | – | – | na | – | – |
| Urease | – | – | ± | + | ± | + | + | – | – |
| β-galactosidase | – | – | – | – | – | – | + | – | + |
| Acid from | | | | | | | | | |
| L-Arabinose | + | – | na | – | – | – | – | – | na |
| Ribose | – | na | na | + | + | – | – | – | na |
| Mannose | + | ± | + | + | + | + | + | + | + |
| Mannitol | + | – | + | – | – | – | – | ± | na |
| Sucrose | + | – | – | + | – | + | – | – | na |
| D-glucose | + | + | + | + | ± | ± | + | + | + |
| D-fructose | – | na | – | + | w | + | na | – | na |
| D-maltose | + | na | – | – | – | – | na | – | na |
| D-lactose | – | – | + | – | – | – | + | – | – |
| Habitat | Human gut | Vaginal discharges | Vaginal discharges | Human gut | Clinical specimens | Vaginal discharges | Vaginal discharges | Nasal cavity | Clinical specimens |

na: non available data.

^a Testing was realized in house for these species or this property.

^b The test results reported in this table are provided by the literature except for the results for the major fatty acid.

The cells are Gram-positive, non-sporeforming, non-motile cocci with a diameter of 0.9 µm that form small white colonies on 5% sheep-blood enriched Colombia agar with a mean diameter of 2 mm. Strain mt16^T optimal growth is at 37° under anaerobic atmosphere. The major fatty acid was hexadecanoic acid. Catalase positive. Oxidase negative. Positive reactions were observed for alkaline phosphatase, leucine arylamidase, valine arylamidase, β-glucosidase, acid phosphatase and Naphthol-AS-BI-phosphohydrolase with an API ZYM strip. Neither urease nor nitrate reduction were observed. Indole was produced. Arabinose, glucose, sucrose, mannose, mannitol, tagatose, potassium-5-ketogluconate, N-acetyl-glucosamine, maltose, potassium gluconate, capric acid, adipic acid, trisodium citrate and phenylacetic acid were metabolized according to the API 50CH and API 20A strips. D-lactose, D-salicine, D-xylose, glycerol, D-cellobiose, D-melezitose, D-raffinose, D-sorbitol, L-rhamnose, D-trehalose, glycerol, erythritol, D-adonitol, methyl-β-D-xylopyranoside, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, amygdalin, arbutin, D-trehalose, inulin, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-lyxose, L-lyxose, D-arabitol, L-arabitol, D-fucose, L-fucose, potassium ketogluconate and potassium 2-gluconate were not metabolized. Strain mt16 was susceptible to amoxicillin, amoxicillin/clavulanic acid, benzylpenicillin, clindamycin, imipenem, metronidazole and vancomycin. The genome is 1,929,161 bp long with a 29.5% G + C content and accessible under FAVH00000000 in the GenBank collection. The 16S rRNA sequence is also accessible in the GenBank collection under number LN881592. The type strain mt16^T (= CSUR P2032 = DSM 101186) was isolated from the stool of a child living in Senegal and suffering from kwashiorkor.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors thank the Xegen Company (www.xegen.fr) for automating the genomic annotation process. This study was funded by the Méditerranée Infection Foundation.

We thank Karolina Griffiths and Magdalen Lardiere for English reviewing.

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