

Taxonogenomic description of four new *Clostridium* species isolated from human gut: ‘*Clostridium amazonitimonense*’, ‘*Clostridium merdae*’, ‘*Clostridium massilielmoense*’ and ‘*Clostridium nigeriense*’

M. T. Alou¹, S. Ndong¹, L. Frégère¹, N. Labas¹, C. Andrieu¹, M. Richez¹, C. Couderc¹, J.-P. Baudoin¹, J. Abrahão², S. Brah³, A. Diallo^{1,4}, C. Sokhna^{1,4}, N. Cassir¹, B. La Scola¹, F. Cadoret¹ and D. Raoult^{1,5}

1) Aix-Marseille Université, Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, UM63, CNRS 7278, IRD 198, INSERM 1095, Marseille, France, 2) Laboratório de Vírus, Departamento de Microbiologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, 3) Hopital National de Niamey, BP 247, Niamey, Niger, 4) Campus Commun UCAD-IRD of Hann, Route des pères Maristes, Hann Maristes, BP 1386, CP 18524, Dakar, Senegal and 5) Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia

Abstract

Culturomics investigates microbial diversity of the human microbiome by combining diversified culture conditions, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and 16S rRNA gene identification. The present study allowed identification of four putative new *Clostridium sensu stricto* species: ‘*Clostridium amazonitimonense*’ strain LF2^T, ‘*Clostridium massilielmoense*’ strain MT26^T, ‘*Clostridium nigeriense*’ strain Marseille-P2414^T and ‘*Clostridium merdae*’ strain Marseille-P2953^T, which we describe using the concept of taxonogenomics. We describe the main characteristics of each bacterium and present their complete genome sequence and annotation.

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Keywords: ‘*Clostridium amazonitimonense*’, ‘*Clostridium massilielmoense*’, ‘*Clostridium merdae*’, ‘*Clostridium nigeriense*’, culturomics, emerging bacteria, human microbiota, taxonogenomics

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Corresponding author: D. Raoult, Aix-Marseille Université, Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes (URMITE), CNRS 7278, IRD 198, INSERM 1095, UM63, Institut Hospitalo-Universitaire Méditerranée-Infection, Faculté de médecine, 27 Boulevard Jean Moulin, 13385, Marseille cedex 5, France.
E-mail: didier.raoult@gmail.com

Introduction

As showed by a remarkable number of studies, the human gut microbiota plays a tremendous role in human health and the homeostasis of the human body [1–3]. Conversely, an increasing number of links between the gut microbiota and several diseases has been uncovered; these diseases include nutritional disorders, metabolic diseases and pathologies of the

intestine [1,4–6]. Techniques used in exploratory studies have evolved alongside technology from culture-based techniques in early studies to today’s culture-independent techniques [7]. A turning point in the study of the gut microbiota is in progress, with a return to culture-based techniques. In fact, cultured strains are needed for *in vivo* and *in vitro* experiments to apply the extensive knowledge acquired thanks to metagenomic studies. Culturomics, a method invented in our laboratory, is a part of this turning point [8]. It consists of the multiplication of culture conditions with a variation of media and physico-chemical parameters such as atmosphere, temperature, pH and salinity in order to exhaustively explore a microbial ecosystem [9].

As part of a global exploratory study designed to improve the known and cultivated bacterial diversity of the human gut microbiota, several previously unknown species of *Clostridium* were isolated. Exploratory studies have shown that the healthy gut microbiota mostly consists of anaerobic bacteria, which represent 95% of the bacterial diversity [2]. Among these

anaerobic bacteria, the genus *Clostridium* is one of the most represented in the human gut, although taxonomic chaos is associated with this genus and its higher ranks in taxonomy, such as the family *Clostridiaceae* and the *Clostridiales* order [7]. This genus was created in 1880 by Prazmowski with the type species *Clostridium butyricum* [10,11]. In early taxonomy, which was based on phenotypic characteristics, all Gram-positive, anaerobic and spore-forming bacilli were attributed to the genus *Clostridium*. Consequently, with the advent of DNA sequencing and phylogenetic analysis, taxonomic chaos was uncovered in this genus, and several clusters were subsequently built, among which cluster I, *Clostridium sensu stricto*, was built around *C. butyricum* [11,12]. Among the 211 *Clostridium* species with standing in nomenclature, only a third are *Clostridium sensu stricto* species [10,12]. These mesophilic species are mostly strict anaerobes with Gram-positive staining and the ability to sporulate. Their metabolism is highly variable because they are able to metabolize various compounds such as carbohydrates, amino acids, alcohols, amino acids and purines. Acid and alcohol can be produced from carbohydrates or via protein metabolism [10,11].

Here we propose the creation of four putative new *Clostridium sensu stricto* species: '*Clostridium amazonitimonense*' strain LF2^T (= CSUR P1445 = DSM 28600), '*Clostridium massili-dielmoense*' strain MT26^T (= CSUR P2255 = CCUG 69893), '*Clostridium nigeriense*' strain Marseille-P2414^T (= CSUR P2414 = DSM 102218) and one new putative *Clostridium* cluster IV, '*Clostridium merdae*' strain Marseille-P2953^T (= CSUR P2953 = DSM 103669), which we describe using the concept of taxonogenomics [13]. This concept of new species description is based on the presentation of the phylogenetic, phenotypic and genomic features of the studied new species and their comparison to closely related species.

Materials and methods

Sample information

In order to explore the bacterial diversity of the human gut microbiota, stool samples were collected from several individuals: a healthy 8-month-old Senegalese boy, a healthy 44-month-old Senegalese girl, a healthy 37-year-old Amazonian woman from French Guyana and a healthy 29-year-old Senegalese woman. None of the donors was receiving antibiotic treatment at the time of sample collection. Oral consent was obtained from each adult. For the children, oral consent was provided by the parents. Samples were stored at -80°C upon arrival in the La Timone hospital in Marseille. This study received approval from the Institut Fédératif de

Recherche 48 (Faculty of Medicine, Marseille, France) under agreement 09-022.

Strain identification

Each stool sample was cultured using the 18 culture conditions of standardized culturomics [8,9]. For each condition, pre-incubation in the medium of an anaerobic blood culture bottle from BD BACTEC (Becton Dickinson [BD], San Diego, CA, USA) was performed during 30 days with seeding on 5% sheep's blood-enriched Colombia agar (bioMérieux, Marcy l'Étoile, France) every 3 days. Colonies were purified before identification using a Microflex spectrometer and a MTP 96 matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) target plate (Bruker Daltonics, Bremen, Germany) as described previously [14,15]. Each obtained spectrum was matched against the database from Bruker and from the La Timone hospital using MALDI Biotyper 3.0 software by standard pattern matching (with default parameter settings). Matching scores under 1.7 indicated the absence of a match in the database. For these unidentified colonies, the 16S rRNA gene was sequenced using fDI and rP2 primers as previously described [16]. Kim et al. [17] recommended the creation of a new species at the 16S similarity level threshold of 98.65% with the closest species with standing in nomenclature. The corresponding spectra are then incremented in the database and compared to the spectra of closely related species.

Growth conditions

For each species, optimal growth conditions were determined by testing various temperatures (25, 28, 37, 45 and 56°C), atmospheres (aerobic with or without 5% CO₂, microaerophilic and anaerobic atmospheres using the GENbag anaer and GENbag microaer systems, respectively; bioMérieux), pH values (6, 6.5, 7 and 8.5) and NaCl concentrations (0.5%, 1%, 5%, 7.5% and 10%).

Morphologic, biochemical and antibiotic susceptibility tests

The morphologic characteristics of each species, namely Gram staining, motility and spore formation, were determined as previously described [8]. To determine the size, and to confirm the shape of the cells as well as spore formation, the cells were negatively stained and observed with electron microscopy as follows: cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for at least 1 hour at 4°C. A drop of cell suspension was deposited for approximately 5 minutes on glow-discharged formvar carbon film on 400 mesh nickel grids (FCF400-Ni; Electron Microscopy Sciences (EMS), Hatfield, PA, USA). The grids were dried on blotting paper, and cells were

negatively stained for 10 seconds with 1% ammonium molybdate solution in filtered water at room temperature. Electron micrographs were acquired with a Tecnai G20 Cryo (FEI Company, Limeil-Brevannes, France) transmission electron microscope operated at 200 keV. Cells were then measured by ImageJ software (Image Processing and Analysis in Java; US National Institutes of Health, Bethesda, MD, USA; <http://imagej.nih.gov/>) [18,19].

Cellular fatty acid methyl ester (FAME) analysis was performed by gas chromatography/mass spectrometry (GC/MS). Two samples were prepared with approximately 23 mg of bacterial biomass per tube collected from several culture plates. Fatty acid methyl esters were prepared as described by Sasser [20]. GC/MS analyses were carried out as described previously [21]. Briefly, fatty acid methyl esters were separated using an Elite 5-MS column and monitored by mass spectrometry (Clarus 500–SQ 8 S; PerkinElmer, Courtaboeuf, France). A spectral database search was performed using MS Search 2.0 operated with the Standard Reference Database 1A (National Institute of Standards and Technology, Gaithersburg, MD, USA) and the FAME mass spectral database (Wiley, Chichester, UK).

The metabolic capacities of the strains were determined using API ZYM, 20A and 50CH strips according to the manufacturer's instructions. After inoculation of the strips, they were incubated in anaerobic atmosphere for 4, 24 and 48 hours. Other enzymatic features were tested separately, namely oxidase and catalase, which were tested as previously described [8].

The disc diffusion method was used to test antibiotic susceptibility according to European Committee on Antimicrobial Susceptibility Testing recommendations [22].

Genomic DNA preparation and genome sequencing

Bacterial cell lysis was achieved using pretreatments such as lysozyme incubation at 37°C for 2 hours, followed by proteinase K action. DNA was extracted by the EZ1 biorobot (Qiagen, Germantown, MD, USA) with the EZ1 DNA tissues kit. The elution volume was 50 µL. Genomic DNA (gDNA) was quantified by a Qubit assay with the high sensitivity kit (Life Technologies, Carlsbad, CA, USA) to 123.7 ng/µL.

gDNA was sequenced with a MiSeq sequencer (Illumina, San Diego, CA, USA) using the mate-pair strategy. The gDNA was barcoded in order to be mixed with 11 other projects using the Nextera Mate Pair sample prep kit (Illumina). The mate-pair library was prepared with 1.5 µg of gDNA using the Nextera mate-pair Illumina guide. The gDNA sample was simultaneously fragmented and tagged with a mate-pair junction adapter. The fragmentation pattern was validated on an Agilent 2100 Bio-Analyzer (Agilent Technologies, Santa Clara, CA, USA) with a

DNA 7500 labchip. The DNA fragments ranged in size from 1.5 to 11 kb, with an optimal size at 4.031 kb. No size selection was performed, and 385.5 ng of tagged fragments were circularized. The circularized DNA was mechanically sheared to small fragments with an optimal size of 1070 bp on the Covaris device S2 in T6 tubes (Covaris, Woburn, MA, USA). The library profile was visualized on a High Sensitivity Bioanalyzer LabChip (Agilent Technologies), and the final concentration library was measured at 2.40 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. The automated cluster generation and sequencing runs were performed in a single 2 × 301 bp run.

Genome annotation and analysis

Open reading frames (ORFs) were predicted using Prodigal [23] with default parameters, but the predicted ORFs were excluded if they spanned a sequencing gap region. The predicted bacterial protein sequences were searched against the GenBank [24] and Clusters of Orthologous Groups (COGs) databases using BLASTP (*E* value 1e-03, coverage 0.7, identity percentage of 30%). If no hit was found, it was searched against the NR database using BLASTP with an *E* value of 1e-03, a coverage of 0.7 and an identity percentage of 30%, and if the sequence length was smaller than 80 aa, we used an *E* value of 1e-05. The tRNAScanSE tool [25] was used to find tRNA genes, while ribosomal RNAs were found using RNAmmer [26]. Lipoprotein signal peptides and the number of transmembrane helices were predicted using Phobius [27]. Mobile genetic elements were predicted using PHAST [28] and RAST [29]. 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 than 80 aa). Such parameter thresholds have already been used in previous studies to define ORFans. Artemis [30] and DNA Plotter [31] were used for data management and the visualization of genomic features, respectively. The Mauve 2.3.1 alignment tool was used for multiple genomic sequence alignment [32].

Comparator species for genomic comparison were identified in the 16S RNA tree using PhyloPattern software [33]. For each selected genome, the complete genome sequence, proteome genome sequence and ORFeome genome sequence were retrieved from the National Center for Biotechnology Information FTP site. 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). Annotation and comparison processes were performed in the Multi-Agent software system DAGOBAN [34], which includes Figenix [35] libraries that provide pipeline analysis. To evaluate the genomic similarity between studied genomes, we determined two parameters: digital DNA-DNA hybridization (DDH), which exhibits a high correlation with DDH [36,37]; and average genomic identity of orthologous gene sequences (AGIOS) [38], which was designed to be independent from DDH. The AGIOS score is the mean value of nucleotide similarity between all couples of orthologous proteins between the two studied genomes [38,39].

Results

Strain identification and phylogenetic analyses

Isolation of strains Marseille-P2953 and LF2 occurred, respectively, after 7 days' and 30 days' preincubation in anaerobic blood culture bottles supplemented with rumen and sheep's blood, respectively. Strain Marseille-P2414 was first isolated after 15 days' preincubation in an aerobic blood culture bottle supplemented with rumen and sheep's blood. Finally, strain MT26 was first isolated after 30 days' preincubation in an anaerobic blood culture bottle supplemented with sheep's blood. Because the routine identification using MALDI-TOF MS failed, the 16S rRNA gene of each strain was sequenced. The 16S rRNA gene sequences of strains Marseille-P2953 (LT732642), LF2 (LK021125), Marseille-P2414 (LT161894) and MT26 (LN998063) revealed a 96.41%, 96.95%, 98.23% and 98.44% similarity level with, respectively, *Clostridium sporosphaeroides* strain DSM 1294, *Clostridium putrefaciens* strain DSM 1291, *Clostridium tertium* strain JCM 6289 and *Clostridium novyi* strain JCM 1406 (Fig. 1, Table 1). All these similarity levels were lower than the 98.65 cutoff defined to create a new species without performing DDH. We thus propose the creation of four new species within the genus *Clostridium* with the following names: '*Clostridium merdae*' (strain Marseille-P2953), '*Clostridium amazonitimonense*' (strain LF2), '*Clostridium nigeriense*' (strain Marseille-P2414) and '*Clostridium massilielmoense*' (strain MT26). The MALDI-TOF MS spectra of the four strains in this study were incremented in our database and compared to those of their closest relatives (Fig. 2).

Phenotypic description

Growth was observed between 28 and 45°C for all studied strains. While strain Marseille-P2414 grew in all tested atmospheres (namely aerobic, microaerophilic and anaerobic), growth was observed only in anaerobic atmosphere for strains Marseille-P2953, LF2 and MT26. All strains exhibited optimal

growth at 37°C in anaerobic atmosphere with an incubation period of 24 hours for strain Marseille-P2414 and 48 hours for strains Marseille-P2953, LF2 and MT26. None of the strains was tolerant to NaCl, only growing at a concentration of 0.5%. Conversely, all strains grew at all tested pH concentrations (6, 6.5, 7, 8.5).

The phenotypic characteristics of the four strains in this study and their closest relatives are summarized in Table 2. All strains were Gram positive and were spore-forming, rod-shaped cells (Fig. 3). Strains LF2 and Marseille-P2414 were motile, whereas strains Marseille-P2953 and MT26 were nonmotile. Major fatty acids are presented in Table 3. We observed that strains Marseille-P2414, LF2 and MT26 present hexadecanoic acid (16:0) as the major fatty acid composing their cell envelope, confirming these three strains belong to the same group. For strain Marseille-P2953, its major fatty acid was 9-octadecenoic acid (18:1n9), which is minor or absent in the other strains. Although phylogeny clearly classified Marseille-P2953 as a *Clostridium* species, these data confirmed other phenotypical observations, suggesting that this strain could belong to another *Clostridium* cluster.

Genome description and comparison

The genomic properties of all strains are presented in Table 4 and Fig. 4. Genome sizes of the strains ranged from 2.75 Mb for strain Marseille-P2953 to 3.8 Mb for strain Marseille-P2414, while the G+C content ranged from 28.2% for strain Marseille-P2414 to 45.4% for strain Marseille-P2953. The proportion of protein-coding genes was similar in all four genomes, and the distribution into COGs categories was also similar (Table 5). However, we observed that the genome of '*Clostridium merdae*' strain Marseille-P2953 presented a G+C% level significantly higher than the genomes of the other presented strains (Table 6). The G+C content of strain Marseille-P2953 sets it apart from the three other studied strains, as previously observed by its phenotypical features. Thus, the very large family of *Clostridiaceae* contains species with great phenotypical and genomic diversity, suggesting that this family, which was created before modern sequencing methods were in use, needs to be greatly reclassified. These observations demonstrate that phenotypical observations are no longer sufficient to classify all the newly discovered species. Whole genome sequencing must be used to characterize new bacterial species.

The DDH comparison presented in Table 7 demonstrates that the four studied new species are clearly distinct new species. Indeed, the DDH level between our four new species and the other *Clostridium* species comprises a range delimiting a distinct bacterial species.

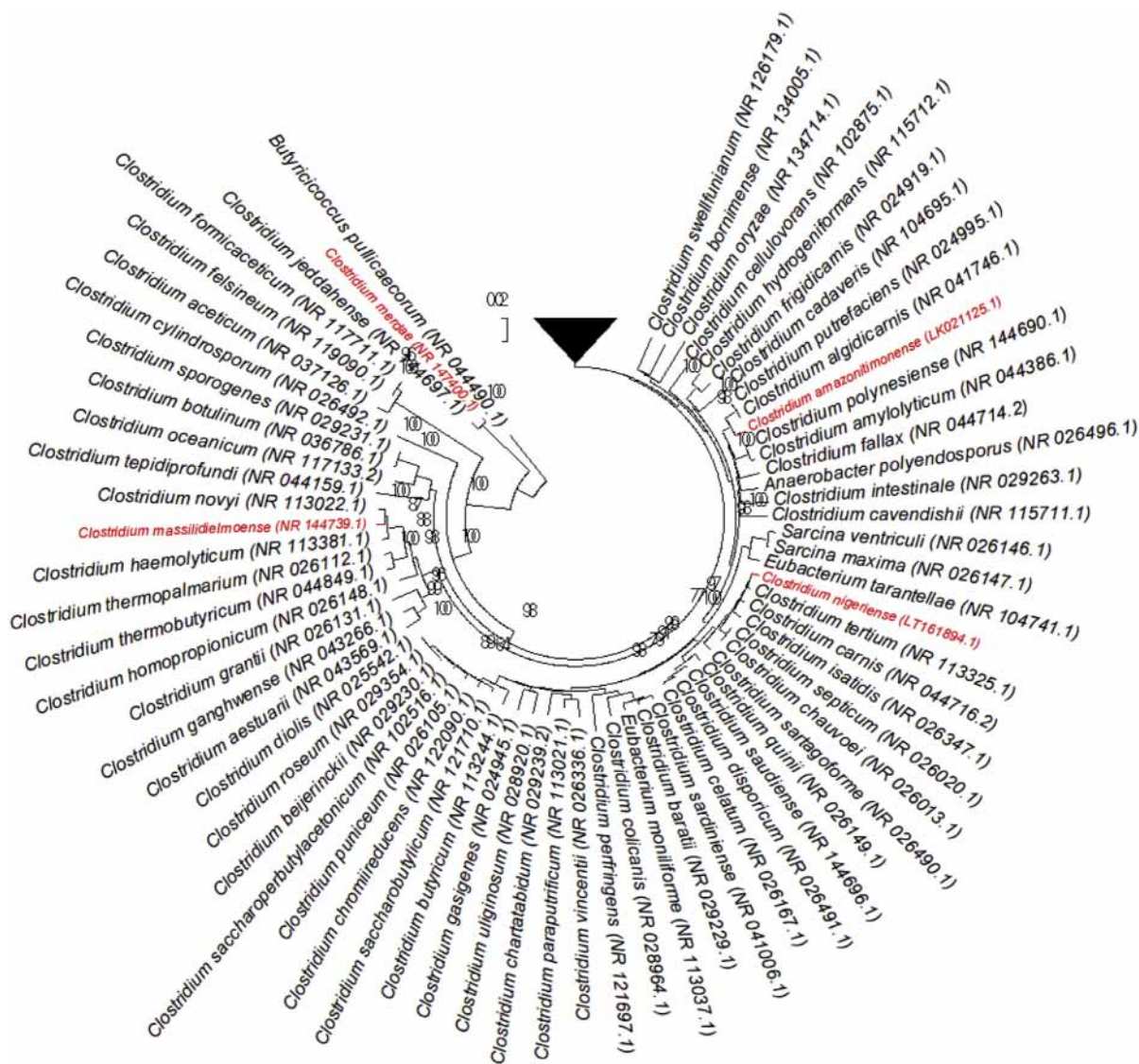


FIG. 1. Phylogenetic tree highlighting position of ‘*Clostridium amazonitimonense*,’ ‘*Clostridium merdae*,’ ‘*Clostridium massiliidemoense*’ and ‘*Clostridium nigeriense*’ relative to other type strains within *Clostridium* genus. Strains and their corresponding GenBank accession numbers for 16S rRNA genes are indicated in brackets. Sequences were aligned using Clustal W (<http://www.clustal.org/clustal2/>), and phylogenetic inferences were obtained using maximum-likelihood method within MEGA6 software (<http://www.megasoftware.net/mega.php>). Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 1000 times to generate majority consensus tree. *Butyrivibrio pullicaecorum* (NR_044490) was used as outgroup. Scale bar = 0.02% nucleotide sequence divergence.

TABLE I. Species information

Characteristic	LF2	MT26	Marseille-P2953	Marseille-P2414
Patient information	Healthy 37-year-old Amazonian woman	Healthy 8-month-old Senegalese boy	Healthy 29-year-old Senegalese woman	Healthy 44-month-old girl from Niger
Isolation conditions	30 days' anaerobic preincubation in blood culture bottle with rumen and sheep's blood	30 days' anaerobic preincubation in blood culture bottle with sheep's blood	7 days' anaerobic preincubation in blood culture bottle with rumen and sheep's blood	15 days' aerobic preincubation in blood culture bottle with rumen and sheep's blood
Optimal growth temperature	37°C	37°C	37°C	37°C
Atmosphere	Strict anaerobe	Strict anaerobe	Strict anaerobe	Facultative anaerobe
Similarity percentage with closest species	96.95% with <i>Clostridium putrefaciens</i> strain DSM 1291 ^T (AF127024)	98.44% with <i>Clostridium novyi</i> strain JCM 1406T (AB045606)	96.41% with <i>Clostridium sporosphaeroides</i> strain DSM 1294 ^T (X66002)	98.23% with <i>Clostridium tertium</i> strain JCM 6289 ^T (Y18174)

Strain Marseille-P2414 shared 1342, 1434, 1553, 1564, 1628 and 1664 orthologous proteins with *Clostridium perfringens*, *Clostridium baratii*, *Clostridium diolis*, *Clostridium beijerinckii*, *Clostridium paraputrificum* and *Clostridium sartagofforme*, respectively (Supplementary Table S1a). Strain MT26 shared 1111, 1114,

1151, 1165, 1253, 1254, 1261, 1294 and 1372 orthologous proteins with *Clostridium acetireducens*, *Clostridium acetobutylicum*, *Clostridium argentinense*, *Clostridium tetani*, *Clostridium carboxidivorans*, *Clostridium drakei*, *Clostridium homopropionicum*, *Clostridium novyi* and *Clostridium haemolyticum*, respectively

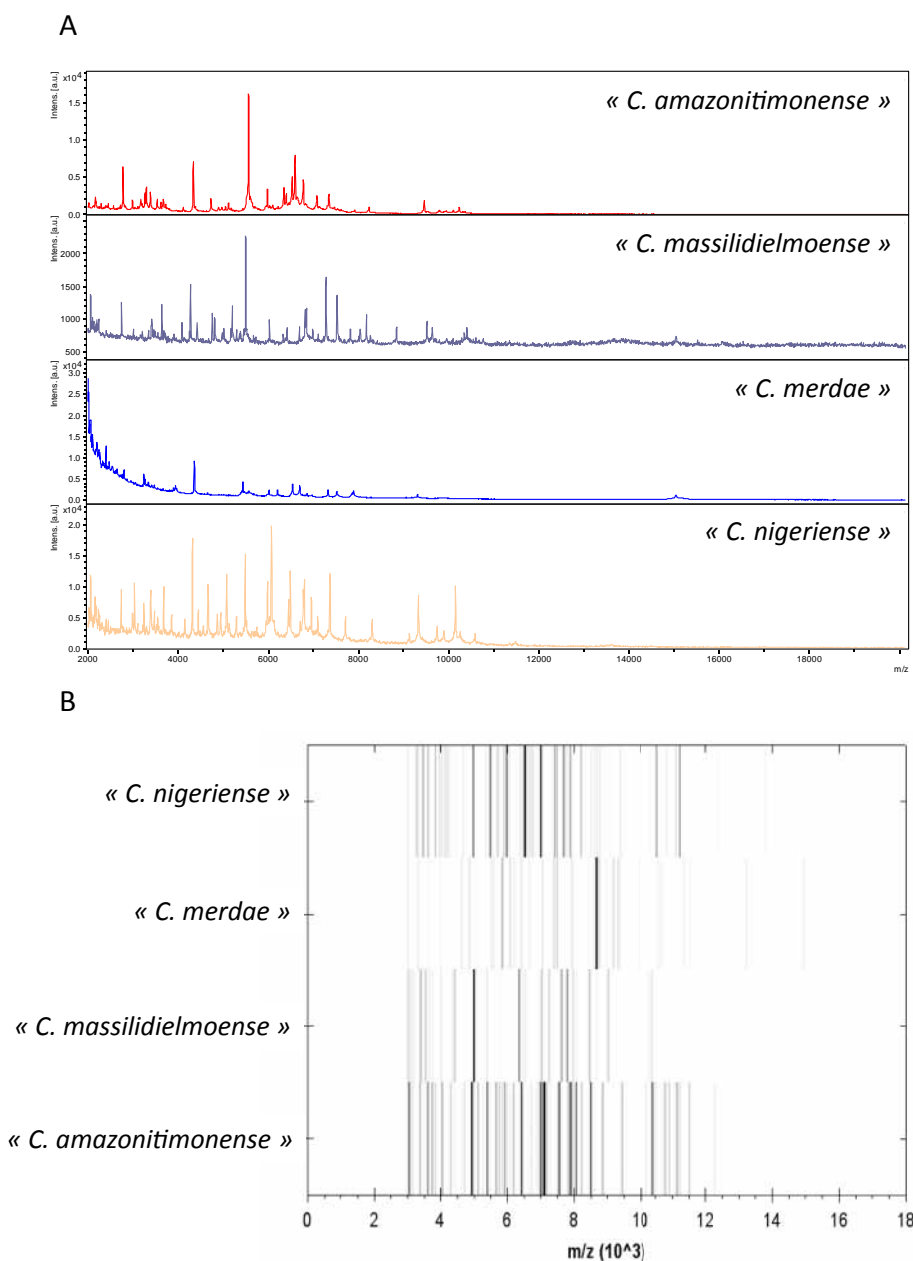


FIG. 2. Reference mass spectra from ‘*Clostridium amazonitimonense*,’ ‘*Clostridium merdae*,’ ‘*Clostridium massiliadielmoense*’ and ‘*Clostridium nigeriense*.’ Spectra from 12 individual colonies were compared and each reference spectrum generated (A). Gel view comparing ‘*Clostridium amazonitimonense*,’ ‘*Clostridium merdae*,’ ‘*Clostridium massiliadielmoense*’ and ‘*Clostridium nigeriense*’ to other species within genus *Clostridium*. Gel view displays raw spectra of loaded spectrum files arranged in pseudo-gellike look. x-axis records m/z value. Left y-axis displays running spectrum number originating from subsequent spectra loading. Peak intensity expressed by greyscale scheme code. Colour bar and right y-axis indicate relation between colour peaks, with peak intensity in arbitrary units. Displayed species are indicated at left (B).

TABLE 2. Differential characteristics of '*Clostridium amazonitimonense*,' '*Clostridium putrefaciens*,' '*Clostridium merdae*,' '*Clostridium sporosphaeroides*,' '*Clostridium nigeriense*,' '*Clostridium tertium*,' '*Clostridium massiliadielmoense*' and '*Clostridium novyi*' [1,2]

Characteristic	' <i>C. amazonitimonense</i> '	<i>C. putrefaciens</i>	' <i>C. merdae</i> '	<i>C. sporosphaeroides</i>	' <i>C. nigeriense</i> '	<i>C. tertium</i>	' <i>C. massiliadielmoense</i> '	<i>C. novyi</i>
Property								
Cell shape	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli	Bacilli
Cell size (µm)	2–2.5	NA	1.2–1.5	0.5–0.6	0.8–1.5	0.5–1.4	0.54	0.6–1.4
Gram stain	+	+	+	+	+	+	+	+
Oxygen tolerance	–	–	–	–	+	+	–	–
Salt tolerance	–	–	–	–	–	–	–	–
Motility	+	–	+	–	+	+	–	+
Endospore formation	+	+	+	+	+	+	+	+
Major cellular fatty acid	15:0 iso	NA	16:00	NA	16:00	NA	16:00	NA
Production of:								
Catalase	–	NA	–	NA	–	NA	–	NA
Oxidase	–	NA	–	NA	+	NA	–	NA
Alkaline phosphatase	+	NA	–	NA	+	NA	+	NA
Urease	–	NA	–	NA	–	NA	–	NA
β-Galactosidase	–	NA	–	NA	–	NA	–	NA
N-acetyl-glucosamine	+	NA	–	NA	+	NA	+	NA
Acid from:								
L-Arabinose	–	NA	–	NA	–	NA	–	NA
Ribose	+	NA	–	NA	+	NA	+	NA
Mannose	+	NA	–	NA	+	NA	+	NA
Mannitol	–	NA	–	NA	–	NA	–	NA
Sucrose	–	–	–	NA	+	NA	–	NA
D-Glucose	+	+	+	NA	+	NA	+	NA
D-Fructose	+	NA	–	NA	+	NA	+	NA
D-Maltose	–	–	+	NA	+	NA	+	NA
D-Lactose	–	–	–	NA	+	NA	–	NA
Habitat	Human gut	Hog muscle tissue	Human gut	Human gut	Human gut	Human gut	Human gut	Soil

+, positive result; –, negative result; NA, data not available.

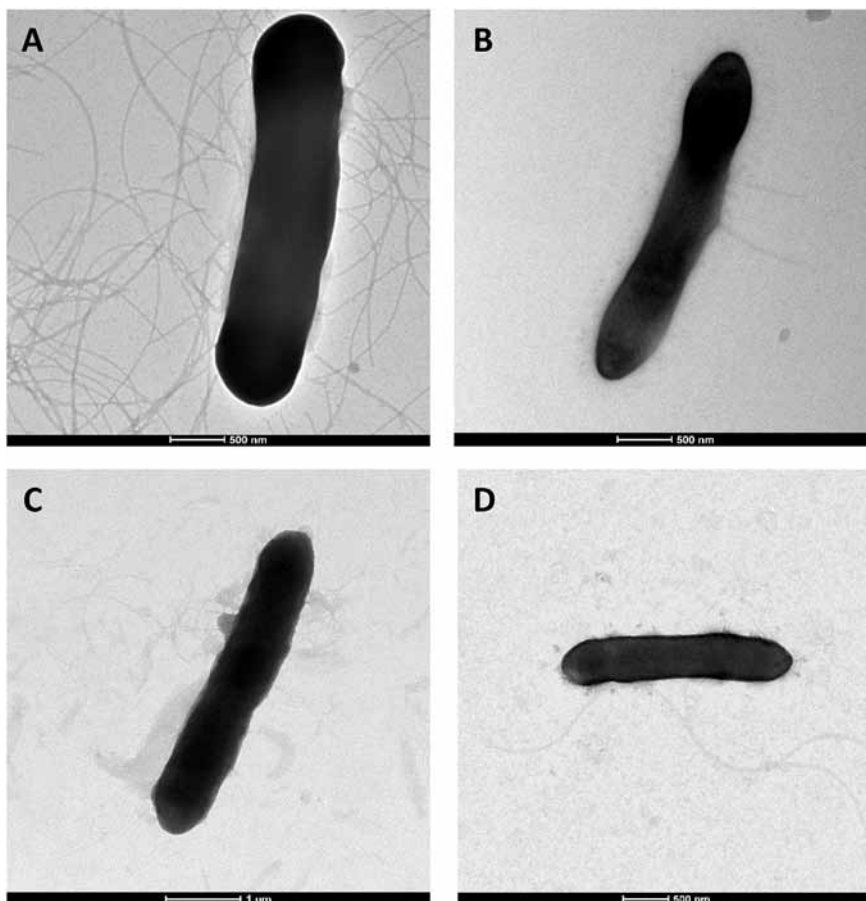
**FIG. 3.** Electron micrographs of '*Clostridium nigeriense*' (A), '*Clostridium amazonitimonense*' (B), '*Clostridium massiliadielmoense*' (C) and '*Clostridium merdae*' (D).

TABLE 3. Cellular fatty acid composition (%)

Fatty acid	Name	' <i>Clostridium nigeriense</i> '	' <i>Clostridium merdae</i> '	' <i>Clostridium massiliidiemoense</i> '	' <i>Clostridium amazonitimonense</i> '
16:00	Hexadecanoic acid	38.9 ± 0.8	38.5 ± 2.1	55.0 ± 2.5	15.3 ± 2.3
14:00	Tetradecanoic acid	ND	ND	15.7 ± 1.7	12.0 ± 0.6
18:00	Octadecanoic acid	6.7 ± 1.1	3.2 ± 0.6	3.6 ± 0.9	TR
13:00	Tridecanoic acid	ND	ND	3.0 ± 0.6	ND
12:00	Dodecanoic acid	1.8 ± 0.1	TR	1.5 ± 1.0	TR
04:00	Butanoic acid	ND	ND	ND	1.7 ± 1.0
15:00	Pentadecanoic acid	TR	ND	2.9 ± 0.6	ND
18:1n9	9-Octadecenoic acid	20.1 ± 0.4	35.4 ± 0.4	14.4 ± 1.0	2.3 ± 0.6
13:0 iso	11-methyl-Dodecanoic acid	ND	ND	ND	22.7 ± 1.8
5:0 iso	3-methyl-Butanoic acid	ND	ND	ND	11.2 ± 2.1
18:1n7	11-Octadecenoic acid	7.9 ± 0.2	ND	ND	TR
14:01	Tetradecanoic acid	6.6 ± 0.3	5.3 ± 1.4	ND	ND
18:2n6	9,12-Octadecadienoic acid	5.5 ± 0.4	15.2 ± 0.6	3.9 ± 1.3	TR
16:1n7	9-Hexadecenoic acid	5.3 ± 0.2	TR	ND	ND
16:1n9	7-Hexadecenoic acid	3.9 ± 0.2	ND	ND	ND
13:0 anteiso	10-methyl-Dodecanoic acid	ND	ND	ND	2.3 ± 0.3
15:0 anteiso	12-methyl-Tetradecanoic acid	TR	TR	ND	4.8 ± 0.5
15:0 iso	13-methyl-Tetradecanoic acid	TR	TR	ND	22.0 ± 1.5

ND, not detected; TR, traces.

(Supplementary Table S1b). Strain LF2 shared 774, 1005, 1017 and 1138 orthologous proteins with *Clostridium clariflavum*, *Clostridium perfringens*, *Clostridium cellulovorans* and *Clostridium beijerinckii*, respectively (Supplementary Table S1c).

Finally, strain Marseille-P2953 shared 735 orthologous proteins with *Ruminococcus albus* and *Clostridium thermocellum* as well as 740, 810, 833, 838, 867, 889 and 907 orthologous proteins with *Eubacterium siraeum*, *Clostridium leptum*, *Pseudoflavonifractor capillosus*, *Intestinimonas butyriciproducens*, *Flavonifractor plautii*, *Oscillibacter ruminantium* and *Oscillibacter valericigenes*, respectively (Supplementary Table S1d). Strain Marseille-P2953 shared fewer orthologous proteins than the other described genomes, as this strain belongs to *Clostridium* cluster IV and has been compared to more closely related

species with available genomes, which mostly belong to other genera, unlike the other described strains. AGIOS value ranges were similar among species with standing in nomenclature with compared species and with the described genomes, thus confirming that the described strains are new species.

Conclusion

Considering the 16S rRNA gene sequence similarity level with the closest species, all under the 98.65% cutoff to define a new species without performing DDH; considering the phenotypic characteristics distinguishing these strains from other known *Clostridium* species; and confirming that they belong to the

TABLE 4. Nucleotide content and gene count levels of genomes

Attribute	' <i>Clostridium amazonitimonense</i> '		' <i>Clostridium merdae</i> '		' <i>Clostridium nigeriense</i> '		' <i>Clostridium massiliidiemoense</i> '	
	No. of reads		No. of reads		No. of reads		No. of reads	
	5 235 372		1 841 718		1 832 750		3 097 182	
	4/15		4/4		4/19		9/9	
Scaffolds/contigs	Value	% of total ^a	Value	% of total ^a	Value	% of total ^a	Value	% of total ^a
Size (bp)	3 761 417	100	2 748 860	100	3 799 474	100	2 759 335	100
G+C content (%)	1 145 890	30.55	1 246 258	45.4	1 064 556	28.2	782 307	28.3
Coding region (bp)	3 129 293	83.2	2 373 508	86.3	3 322 705	87.4	2 453 243	88.9
Total genes	3553	100	2699	100	3659	100	2772	100
RNA genes	147	4.1	59	2.2	187	5.1	91	3.3
Protein-coding genes	3406	95.9	264	97.8	3472	94.9	2681	96.7
Genes with function prediction	2477	72.7	1966	74.4	245	70.5	1989	74.2
Genes assigned to COGs	2241	65.8	167	63.2	2149	61.9	1639	61.1
Genes with peptide signals	270	7.9	271	10.2	307	8.8	228	2.8
ORFans genes	189	5.5	117	4.4	119	3.4	40	1.5
Genes with transmembrane helices	849	24.9	569	21.5	935	26.9	639	23.8
Genes associated with PKS or NRPS	3	0.08	0	0	5	0.1	3	0.1
No. of antibiotic resistance genes	1	0.03	0	0	0	0	0	0
No. of genes associated with Pfam-A domains	NA	NA	2386	88	3182	86	246	88

COGs, Clusters of Orthologous Groups database; NRPS, nonribosomal peptide synthase; ORF, open reading frame; PKS, polyketide synthase.
^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.

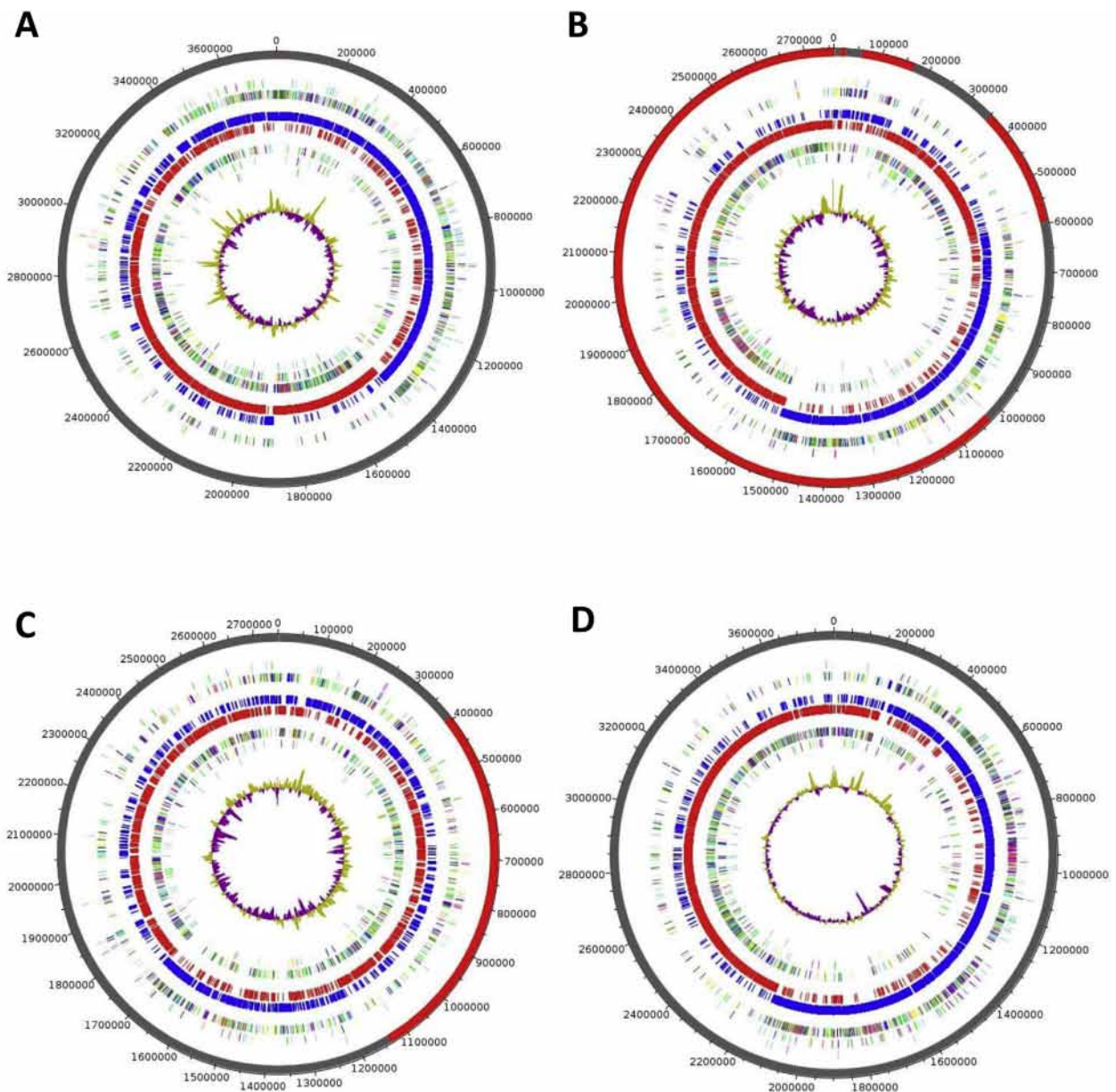


FIG. 4. Graphical circular map of genome of '*Clostridium nigeriense*' (A), '*Clostridium amazonitimonense*' (B), '*Clostridium massilieliemoense*' (C) and '*Clostridium merdae*' (D). From outside to center: Contigs (red/grey), COGs category of genes on forward strand (three circles), genes on forward strand (blue circle), genes on reverse strand (red circle), COGs category on reverse strand (three circles), G+C content. COGs, Clusters of Orthologous Groups database.

genus *Clostridium* and have the genomic characteristics of these strains, we propose the creation of four new *Clostridium* species: '*Clostridium nigeriense*,' '*Clostridium massilieliemoense*,' '*Clostridium amazonitimonense*' and '*Clostridium merdae*.'

Description of the new species '*Clostridium nigeriense*'
'*Clostridium nigeriense*' (ni.ge.rien'se, L. gen. adj., from *nigeriense*, 'of Niger,' referring to where the stool sample was collected)

was isolated from the stool sample of a healthy girl from Niger and was deposited in the DSMZ collection under accession number DSM 102218 and in the CSUR collection under accession number CSUR P2414.

'*Clostridium nigeriense*' is a Gram-positive bacillus that is motile, spore forming and facultative anaerobic. The test of catalase activity was negative but was positive for oxidase. '*Clostridium nigeriense*' forms translucent colonies with a mean

TABLE 5. Number of genes associated with 25 general COGs functional categories

Code	Description	' <i>Clostridium merdae</i> '		' <i>Clostridium nigeriense</i> '		' <i>Clostridium massiliadielmoense</i> '		' <i>Clostridium amazonitimonense</i> '	
		Value	% of total	Value	% of total	Value	% of total	Value	% of total ^a
[J]	Translation	189	7.16	217	6.25	192	7.16	181	5.31
[A]	RNA processing and modification	0	0	0	0	0	0	0	0
[K]	Transcription	171	6.48	191	5.50	125	4.66	238	6.99
[L]	Replication, recombination and repair	77	2.91	85	2.45	87	3.24	135	3.96
[B]	Chromatin structure and dynamics	0	0	0	0	0	0	1	0.03
[D]	Cell cycle control, mitosis and meiosis	31	1.17	40	1.15	39	1.45	24	0.70
[Y]	Nuclear structure	0	0	0	0	0	0	0	0
[V]	Defense mechanisms	53	2.01	75	2.16	58	2.16	105	3.08
[T]	Signal transduction mechanisms	97	3.67	117	3.37	94	3.50	135	3.96
[M]	Cell wall/membrane biogenesis	75	2.84	129	3.71	106	3.95	104	3.05
[N]	Cell motility	43	1.63	53	1.52	47	1.75	61	1.79
[Z]	Cytoskeleton	0	0	0	0	0	0	0	0
[W]	Extracellular structures	7	0.26	6	0.17	7	0.26	0	0
[U]	Intracellular trafficking and secretion	20	0.76	26	0.75	25	0.93	34	1
[O]	Posttranslational modification, protein turnover, chaperones	61	2.31	79	2.27	75	2.8	74	2.17
[X]	Mobilome: prophages, transposons	15	0.57	8	0.23	36	1.34	NA	NA
[C]	Energy production and conversion	106	4.01	139	4.00	93	3.47	137	4.02
[G]	Carbohydrate transport and metabolism	142	5.38	271	7.80	125	4.66	165	4.84
[E]	Amino acid transport and metabolism	212	8.03	195	5.61	150	5.59	219	6.43
[F]	Nucleotide transport and metabolism	58	2.19	85	2.45	68	2.53	72	2.11
[H]	Coenzyme transport and metabolism	90	3.41	104	2.99	111	4.14	88	2.58
[I]	Lipid transport and metabolism	69	2.61	84	2.42	64	2.39	62	1.82
[P]	Inorganic ion transport and metabolism	87	3.29	133	3.83	87	3.24	153	4.49
[Q]	Secondary metabolite biosynthesis, transport and catabolism	28	1.06	30	0.86	13	0.48	35	1.02
[R]	General function prediction only	158	5.98	202	5.82	151	5.63	320	9.39
[S]	Function unknown	86	3.26	133	3.83	91	3.39	190	5.58
—	Not in COGs	970	36.74	1.323	38.10	1.042	38.87	1.165	34.20

COGs, Clusters of Orthologous Groups database; NA, not applicable.

^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.

TABLE 6. Genome comparison of closely related species

Organism	INSDC	Size (Mb)	G+C (%)	Total genes
<i>Clostridium beijerinckii</i> ^a	CP000721	6.48	30	5.425
<i>Clostridium clariflavum</i>	CP003065	4.90	35.72	3.892
<i>Clostridium cellulovorans</i>	CP002160	56	311	4.524
<i>Clostridium perfringens</i> ^b	CP000246	35	28.38	2.876
' <i>Clostridium massiliadielmoense</i> '	FXL000000000	2.76	28.35	2.681
<i>Clostridium acetireducens</i>	LZFO000000000	2.42	26.75	2.238
<i>Clostridium acetobutylicum</i>	AE001437	43	30.93	3.848
<i>Clostridium argentinense</i>	AYSO000000000	4.74	28.6	4.279
<i>Clostridium carboxidivorans</i>	CP011803	5.75	29.93	4.973
<i>Clostridium drakei</i>	JIBU000000000	5.58	29	5.029
<i>Clostridium haemolyticum</i>	JENX000000000	2.61	27.43	2.201
<i>Clostridium homopropionicum</i>	LHUR000000000	3.65	31.09	3.5
<i>Clostridium novyi</i>	JENM000000000	2.46	28.58	1.833
<i>Clostridium tetani</i>	JRGJ000000000	2.79	28.57	2.516
' <i>Clostridium nigeriense</i> '	FLKB000000000	3.79	28.7	3.472
<i>Clostridium baratii</i>	JZTY000000000	37	27.95	2.839
<i>Clostridium diolis</i>	AQQG000000000	5.84	29.74	5.196
<i>Clostridium paraputrificum</i>	AUJC000000000	3.56	29.64	3.491
<i>Clostridium sartagoforme</i>	ASRV000000000	3.98	27.91	3.714
' <i>Clostridium merdae</i> '	FXLN000000000	2.75	45.34	2.64
<i>Clostridium leptum</i>	ABCB000000000	2.82	505	2.482
<i>Clostridium thermocellum</i>	CP000568	3.84	38.99	3.173
<i>Eubacterium siraeum</i>	ARNC000000000	2.62	44.85	2.308
<i>Flavonifractor plautii</i>	AGCK000000000	3.82	61.07	4.278
<i>Intestinimonas butyriciproducens</i>	CP011307	3.37	59.06	3.381
<i>Pseudoflavonifractor capillosus</i>	AAXG000000000	44	591	4.829
<i>Ruminococcus albus</i>	CP002403	4.33	43.62	3.991
' <i>Clostridium amazonitimonense</i> '	CCNN000000000	3.76	30.56	3.046

INSDC, International Nucleotide Sequence Database Collaboration.

^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.

diameter of 3 mm. Its major cellular fatty acid is hexadecanoic acid (16:0).

Its genome is 3 799 474 bp long with 28.27% G+C content. The 16S rRNA gene and genome sequences are available in the European Molecular Biology Laboratory–European

Bioinformatics Institute (EMBL-EBI) database under accession numbers LT161894 and FLKB000000000, respectively.

Strain Marseille-P2414^T is the type strain of the new species '*Clostridium nigeriense*'.

Description of the new species '*Clostridium amazonitimonense*'

'*Clostridium amazonitimonense*' (am.a.zo.ni.ti.mo.nen'se, N.L. neut. adj., from *amazoni* (a.ma.zo.ni, N.L. neut. adj.), referring to the Amazonian forest, the location where the stool sample was collected, and *timonense* (ti.mo.nen'se, N.L. neut. adj.), referring to the La Timone hospital, where the strain was isolated) was isolated from the stool sample of a healthy middle-aged woman from Amazonia and is deposited in the DSMZ collection under accession number DSM 28600 and in the CSUR collection under accession number CSUR PI445.

Strain LF2 is a Gram-positive bacillus that is motile, spore forming and a strict anaerobe. It is negative for catalase and oxidase activity. '*Clostridium amazonitimonense*' forms white colonies with a mean diameter of 5 mm.

The genome is 3 761 417 bp long with 30.56% G+C content. The 16S rRNA gene and genome sequences are available in the EMBL-EBI database under accession numbers LK021125 and CCNN000000000, respectively.

The type strain LF2^T is the type strain of the new species '*Clostridium amazonitimonense*'.

TABLE 7. Pairwise comparison of four studied species with nine other species using GGDC

	2	3	4	5	6	7	8	9	10	11	12	13	14
1	19.90 ± 2.2	18.10 ± 2.2	19.00 ± 2.2	19.40 ± 2.2	18.40 ± 2.1	22.60 ± 2.2	21.00 ± 2.2	20.30 ± 2.2	24.40 ± 2.3	39.90 ± 2.5	23.70 ± 2.3	23.20 ± 2.3	24.80 ± 2.3
2	100.00 ± 0.0	19.40 ± 2.2	24.80 ± 2.3	39.80 ± 2.5	19.70 ± 2.2	21.30 ± 2.3	19.50 ± 2.2	17.40 ± 2.1	26.70 ± 2.3	26.40 ± 2.4	26.00 ± 2.3	19.60 ± 2.2	25.70 ± 2.3
3	100.00 ± 0.0	100.00 ± 0.0	19.00 ± 2.2	18.10 ± 2.1	18.50 ± 2.1	21.30 ± 2.2	20.70 ± 2.2	20.00 ± 2.2	24.30 ± 2.3	26.70 ± 2.4	28.70 ± 2.3	20.10 ± 2.2	26.60 ± 2.4
4	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	24.70 ± 2.3	19.50 ± 2.2	21.30 ± 2.2	19.10 ± 2.2	18.30 ± 2.1	28.40 ± 2.4	26.10 ± 2.3	28.30 ± 2.3	20.00 ± 2.2	23.70 ± 2.3
5	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	18.30 ± 2.1	21.30 ± 2.3	19.40 ± 2.1	18.40 ± 2.2	23.30 ± 2.3	25.70 ± 2.3	26.70 ± 2.3	20.50 ± 2.2	26.80 ± 2.3
6	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	19.40 ± 2.1	18.00 ± 2.1	22.90 ± 2.3	25.80 ± 2.3	28.40 ± 2.4	21.20 ± 2.2	24.10 ± 2.3
7	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	23.60 ± 2.3	23.00 ± 2.3	24.70 ± 2.3	26.00 ± 2.3	24.90 ± 2.3	24.30 ± 2.4
8	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	20.20 ± 2.2	25.30 ± 2.3	24.70 ± 2.3	28.10 ± 2.4	25.10 ± 2.3	25.10 ± 2.4
9	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	25.10 ± 2.3	24.70 ± 2.3	28.10 ± 2.4	23.40 ± 2.3	25.40 ± 2.3
10	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	29.10 ± 2.3	26.80 ± 2.4	29.20 ± 2.4
11	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	27.30 ± 2.4	25.70 ± 2.3
12	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	23.80 ± 2.3
13	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0
14	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0	100.00 ± 0.0

DDH, DNA-DNA hybridization; GGDC, genome-to-genome distance; HSP, high-scoring pair. Data derived from formula 2 (DDH estimates based on identities/HSP length). Confidence intervals indicate inherent uncertainty in estimating DDH values from intergenomic distances based on models derived from empirical test data sets (which are always limited in size). These results are in accordance with 16S rRNA (Fig. 1) and phylogenetic analyses as well as GGDC results. 1, *Clostridium cellulovorans*; 2, *Clostridium massilielmoense*; 3, *Clostridium acetabulosum*; 4, *Clostridium haemolyticum*; 5, *Clostridium novyi*; 6, *Clostridium tetani*; 7, *Clostridium nigeriense*; 8, *Clostridium diolis*; 9, *Clostridium sartagoforme*; 10, *Clostridium merdae*; 11, *Clostridium leptum*; 12, *Clostridium thermocellum*; 13, *Clostridium amazonimonense*; 14, *Ruminococcus albus*.

Description of the new species '*Clostridium massilielmoense*'

'*Clostridium massilielmoense*' (mas.si.li.di.el.mo.en'se, L. neut. adj., a combination of *massili* (ma.ssi.li, N.L. neut. adj.), from Massilia, the Roman name of Marseille, where strain MT26 was isolated; and *dielmoense* (di.el.mo.en'se, N.L. neut. adj.), from Dielmo, the Senegalese village where the boy from whom the strain was cultivated lived) was isolated from the stool sample of a healthy 8-month-old Senegalese boy and is deposited in the CCUG collection under accession number CCUG 69893 and in the CSUR collection under accession number CSUR P2255.

Strain MT26 is a Gram-positive bacillus that is nonmotile, spore forming and a strict anaerobe. It shows positive catalase activity but no oxidase activity. '*Clostridium massilielmoense*' forms a translucent biofilm with cells of an average diameter of 0.54 µm.

The genome is 2 759 335 bp long with 28.35% G+C content. The 16S rRNA gene and genome sequences are available in the EMBL-EBI database under accession numbers LN998063 and FXLO00000000, respectively.

The type strain MT26^T is the type strain of the new species '*Clostridium massilielmoense*'.

Description of the new species '*Clostridium merdae*'

'*Clostridium merdae*' (mer'dae, L. gen. n., from *merdae*, 'of faeces,' referring to the fact that the strain was isolated from a stool sample) was isolated from the stool sample of a healthy 29-year-old Senegalese woman and is deposited in the DSMZ collection under accession number DSM 103669 and in the CSUR collection under accession number CSUR P2953.

Strain Marseille-P2953 is a Gram-positive bacillus that is motile, spore forming and a strict anaerobe. It does not show catalase or oxidase activity. '*Clostridium merdae*' forms small white colonies with a mean diameter of 1 mm.

The genome is 2 748 860 bp long with 45.34% G+C content. The 16S rRNA gene and genome sequences are available in the EMBL-EBI database under accession numbers LT732642 and FXLN00000000, respectively.

The type strain Marseille-P2953 is the type strain of the new species '*Clostridium merdae*'.

Conflict of interest

None declared.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.nmni.2017.11.003>.

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