Corynebacterium pacaense sp. nov., *Alistipes megaguti* sp. nov., *Alistipes provencensis* sp. nov., 3 new bacteria isolated from fresh human stool specimens

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Abstract

Here we describe the basic characteristics of *Corynebacterium pacaense* strain Marseille-P2417^T (= CSUR P2417), *Alistipes megaguti* strain Marseille-P5997^T (= CSUR P5997) and *Alistipes provencensis* strain Marseille-P2431^T (= CSUR P2431 = DSM 102308). The phenotypic criteria, the 16S ribosomal RNA sequencing and MALDI-TOF MS spectra analysis were used to identify and characterize these new bacteria species, which were isolated from fresh human stool specimens.
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Introduction

Culturomics is the concept of developing different culture conditions in order to enlarge our knowledge of the human microbiota through the discovery of previously uncultured bacteria [1–4]. However, after bacterial strain isolation, we used a taxonogenomics approach, including MALDI-TOF MS, phylogenetic analysis, main phenotypic description and genome sequencing, to describe the bacterium [5,6]. In this study, we describe three new species isolated for the first time from human stool samples.

Isolation and growth conditions

In 2016, strain Marseille-P2417^T was isolated from a fresh stool sample of a 24-year-old healthy woman. In 2017, strain Marseille-P5997^T was isolated from a fresh stool sample of a 25-year-old healthy woman. In 2015, strain Marseille-P2431^{T}was isolated from a 66-year-old man with hypertension and diabetes. Screening was performed by MALDI-TOF MS on a Microflex LT spectrometer (Bruker Daltonics, Bremen, Germany) as previously described [7]. The spectra generated from each strain (Fig. 1) were imported and analysed by Biotyper 3.0 software against the Bruker database, which is constantly updated with the Microbes Evolution Phylogeny and Infections (MEPHI) database.

The initial growth of strain Marseille-P2417^T was obtained after 24 hours of culture in Colombia agar enriched with 5% sheep's blood (bioMérieux, Marcy l'Etoile, France) in aerobic conditions at 37°C and pH 7.5. The initial growth of strains Marseille-P5997^T and Marseille-P2431^T was obtained after 48 hours of culture in a Colombia agar enriched with 5% sheep's blood (bioMérieux) in anaerobic conditions at 37°C and pH 7.5.

The study of these microbes (strains Marseille-P2417, Marseille-P5997 and Marseille-P2431) was validated by the ethics committee of IHU–Méditerranée Infection under numbers 2016-010, 2016-011 and 09-022 respectively.

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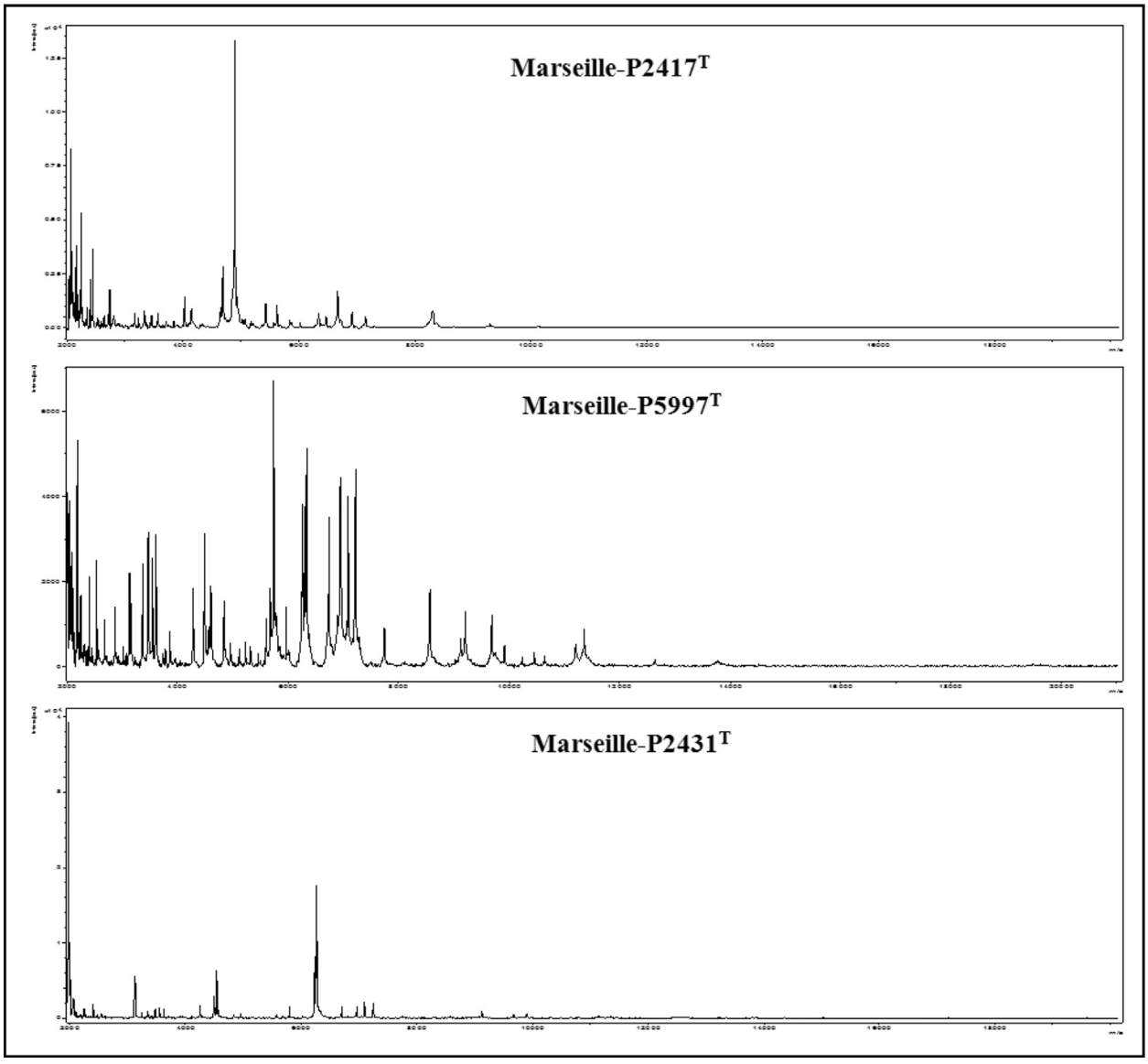

FIG. 1. MALDI-TOF MS reference mass spectrum. Spectra from 12 individual colonies were compared and reference spectrum generated.

Strain identification

The 16S ribosomal RNA (rRNA) gene was sequenced in order to classify these bacteria. Amplification was performed using the primers fD1, rP2 and rpoB (Eurogentec, Angers, France) and was sequenced using the Big Dye Terminator v1.1 Cycle Sequencing Kit and the sequencer (Thermo Fisher Scientific, Waltham, MA, USA), as previously described [8]. The 16S rRNA nucleotide sequences were assembled and corrected using CodonCode Aligner software (https://www.codoncode.com/). The 16S rRNA gene (accession no. LT223574) of strain Marseille-P2417^T exhibited a 97.7% sequence similarity with *Corynebacterium efficiens* strain YS-314^T (GenBank accession no. NR_102865), the phylogenetically closest species with standing in nomenclature (Fig. 2(A)). Those of strain Marseille-P5997^T (accession no. LS999984) and strain Marseille-P2431^T (accession no. LT223566) showed a sequence similarity of 96.9% and 98.5% with *Alistipes senegalensis* strain JC50^T (GenBank accession no. NR_118219) and *Alistipes timonensis* strain JC136^T (GenBank accession no. NR_125589) respectively; these were the phylogenetically closest species with standing in nomenclature (Fig. 2(B)). These values were lower than the 98.7% 16S rRNA gene sequence threshold recommended by Meier-Kolthoff et al. [9] to delineate a new bacterial species without performing DNA–DNA hybridization.

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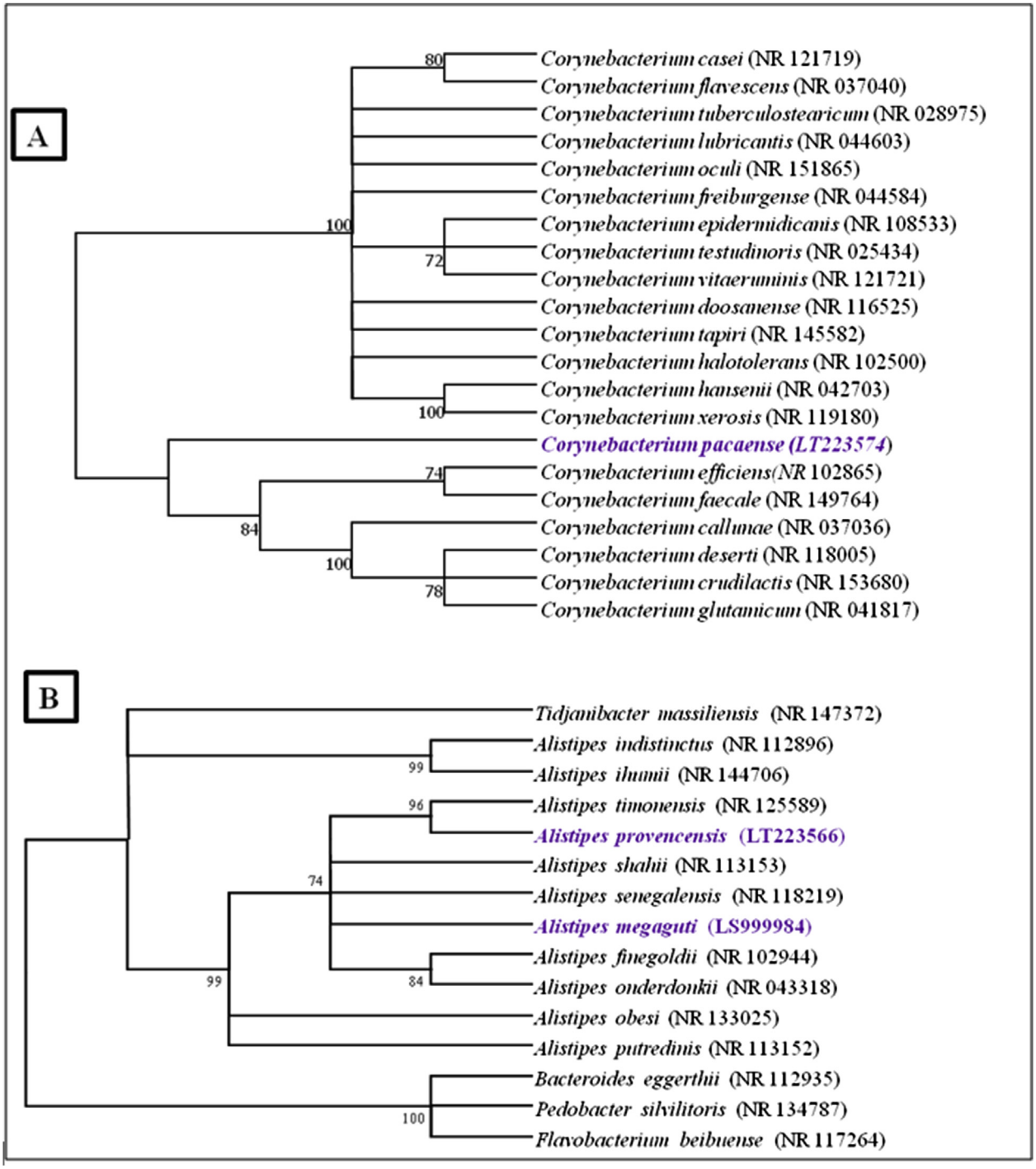

FIG. 2. Phylogenetic trees showing positions of *Corynebacterium pacaense* strain Marseille-P2417T (A), *Alistipes megaguti* strain Marseille-P5997T and *Alistipes provencensis* strain Marseille-P2431T (B) relative to other phylogenetically close neighbours. Respective GenBank accession numbers for 16S ribosomal RNA genes are indicated in parentheses. Sequences were aligned using Muscle v3.8.31 with default parameters, and phylogenetic inferences were obtained using maximum likelihood method within software. Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 1000 times to generate majority consensus tree. Only bootstrap values > 71% were retained. Scale bar indicates 2% nucleotide sequence divergence.

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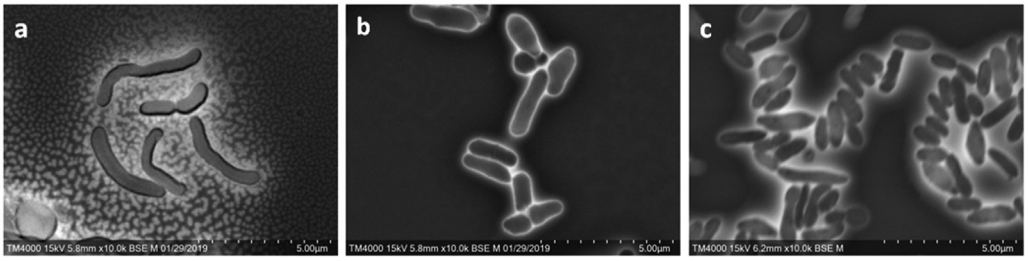

FIG. 3. Scanning electron micrograph of (a) *Corynebacterium pacaense*, (b) *Alistipes megaguti* and (c) *Alistipes provencensis* using TM4000Plus Tabletop microscope (Hitachi, Yokohama, Japan). Scale bar and acquisition settings are shown.

We consequently proposed to classify strains Marseille-P2417^T, Marseille-P5997^T and Marseille-P2431^T as new species within the genera *Corynebacterium* and *Alistipes* respectively.

TABLE 1. Biochemical tests of *Corynebacterium pacaense*, *Alistipes megaguti* and *Alistipes provencensis* (API 50 CH strips)

Test	Result		
	C. pacaense	*A. megaguti*	*A. provencensis*
Glycerol	−	+	−
Erythrol	−	−	−
D-Arabinose	−	−	w
L-Arabinose	−	+	−
D-Ribose	−	+	−
D-Xylose	−	+	+
L-Xylose	−	−	w
D-Adonitol	−	w	−
Methyl-βD-xylopyranoside	−	−	−
D-Galactose	−	+	−
D-Glucose	+	+	+
D-Fructose	+	+	−
D-Mannose	+	+	+
L-Sorbose	−	−	w
L-Rhammose	−	−	w
Dulcitol	−	w	−
Inositol	w	w	w
D-Mannitol	+	+	−
D-Sorbitol	−	−	−
Methyl-αD-mannopyranoside	−	−	w
Methyl-αD-glucopyranoside	−	−	−
N-Acetylglucosamine	−	+	−
Amygdalin	−	+	−
Arbutin	w	+	−
Esculin	+	+	+
Salicin	+	+	−
D-Cellobiose	−	+	−
D-Maltose	−	+	−
D-Lactose	−	+	+
D-Melibiose	−	+	−
D-Sucrose	+	−	−
D-Trehalose	w	+	−
Inulin	−	−	−
D-Melezitose	−	−	+
D-Raffinose	−	−	+
Starch	−	w	−
Glycogen	−	−	−
Xylitol	−	−	−
Gentiobiose	−	+	−
D-Turanose	−	−	−
D-Lyxose	−	w	−
D-Tagatose	−	+	−
D-Fucose	−	w	−
L-Fucose	−	w	−
D-Arabitol	−	−	−
L-Arabitol	−	−	w
Potassium gluconate	−	−	−
Potassium 2-ketogluconate	−	−	−
Potassium 5-ketogluconate	−	w	w

+, positive result; −, negative result; w, weakly positive result.

TABLE 2. Biochemical tests of *Corynebacterium pacaense*, *Alistipes megaguti* and *Alistipes provencensis* (API ZYM strips)

Test	Result		
	Corynebacterium pacaense	*Alistipes megaguti*	*Alistipes provencensis*
Alkaline phosphatase	−	−	−
Esterase (C4)	+	+	+
Esterase lipase (C8)	−	+	−
Lipase (C14)	−	+	+
Leucine arylamidase	+	−	−
Valine arylamidase	−	+	+
Cystine arylamidase	−	+	−
Trypsin	−	+	+
α-Chymotrypsin	−	+	−
Phosphatase acid	+	+	−
Naphthol-AS-BI-phosphohydrolase	+	+	+
α-Galactosidase	−	−	−
β-Galactosidase	−	+	+
β-Glucuronidase	−	+	+
α-Glucosidase	−	−	+
β-Glucosidase	+	−	+
N-Acetyl-β-glucosaminidase	−	−	−
α-Mannosidase	−	+	−
α-Fucosidase	−	+	−

+, positive result; −, negative result.

TABLE 3. Biochemical tests of *Corynebacterium pacaense* (API Coryne strips)

Test	Result
Potassium nitrate	−
Pyrazine carboxamide	−
Pyroglutamic acid−β-naphthylamide	−
2-Naphtyl-phosphate	−
Naphthol-ASBI-glucuronic acid	−
2-Naphtyl-βD-galactopyranoside	−
2-Naphtyl-αD-glucopyranoside	−
1-Naphtyl-N-acetyl-βD-glucosaminide	−
Esculin ferric citrate	+
Urea	−
Gelatin (bovine origin)	−
D-Glucose	+
D-Ribose	−
D-Xylose	−
D-Mannitol	+
D-Maltose	−
D-Lactose (bovine origin)	−
D-Sucrose	+
Glycogen	−

+, positive result; −, negative result.

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TABLE 4. Biochemical tests of *Alistipes megaguti* and *Alistipes provencensis* (API 20A strips)

Test	Result	
	Alistipes megaguti	Alistipes provencensis
L-Tryptophane	+	−
Urea	−	−
D-Glucose	+	+
D-Mannitol	+	−
D-Lactose	+	+
D-Saccharose	−	−
D-Maltose	+	+
Salicin	+	−
D-Xylose	+	+
L-Arabinose	+	−
Gelatin (bovine origin)	+	−
Esculin ferric citrate	+	+
Glycerol	−	−
D-Cellobiose	+	+
D-Mannose	+	+
D-Melezitose	−	+
D-Raffinose	−	+
D-Sorbitol	−	−
L-Rhamnose	+	−
D-Trehalose	+	−

+, positive result; −, negative result.

Phenotypic characteristics

Strain Marseille-P2417^T formed circular white colonies with a mean diameter of 1.15 mm. Bacterial cells of strain Marseille-P2417^T were Gram-positive and rod shaped, and they ranged in length from 1.29 to 1.5 µm and in width from 0.5 to µm (Fig. 3(a)). Strain Marseille-P2417^T showed catalase-positive and oxidase-negative activities. Colonies of the strain Marseille-P5997^T were 0.2 to 0.9 mm in diameter on blood-enriched Columbia agar. Bacterial cells of strain Marseille-P5997^T were Gram negative and rod shaped, and they ranged in length from 1.36 to 3.57 µm and in width from 0.45 to 0.6 µm (Fig. 3(b)). Strain Marseille-P5997^T showed catalase-negative and oxidase-negative activities. Colonies of the strain Marseille-P2431^T were 0.4 to 0.64 mm in diameter on blood-enriched Columbia agar. Bacterial cells were Gram negative and rod shaped, and they ranged in length from 0.9 to 1.43 µm and in width from 0.4 to 0.5 µm (Fig. 3(c)). Strain Marseille-P2431^T showed catalase-positive and oxidase-negative activities.

The main phenotypic properties of the three strains were studied by using API 50 CH strips (Table 1), API ZYM strips (Table 2), API Coryne strips (Table 3) and API 20A strips (Table 4). The main characteristics of strains Marseille-P2417^T, Marseille-P5997^T and Marseille-P2431^T are respectively summarized at the Digital Protologue website (http://imedea.uib-csic.es/dprotologue/) under the following numbers: TA00965, TA00955 and TA00966. A comparative study of the main biochemical and phenotypic features of the closest *Corynebacterium* species and *Alistipes* species is presented in Table 5.

Genome sequencing

Genomic DNA was extracted using the EZ1 biorobot (Qiagen, Courtaboeuf, France) with the EZ1 DNA tissue kit, then was sequenced using with the), as previously described [10]. The assembly was performed with a pipeline incorporating different

TABLE 5. Differential characteristics of *Corynebacterium pacaense* sp. nov. (1), *Corynebacterium efficiens* (2), *Corynebacterium humireducens* (3), *Alistipes megaguti* sp. nov. (4), *Alistipes provencensis* sp. nov. (5), *Alistipes ihumii* (6), *Alistipes senegalensis* (7) and *Alistipes timonensis* (8)

Property	1	2	3	4	5	6	7	8
Cell diameter (µm)	0.5–0.8	0.8–1.1	0.5–0.7	0.45–0.6	0.4–0.5	0.72	0.56	0.62
Oxygen requirement	Facultatively anaerobic	Facultatively anaerobic	Facultatively anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic
Gram stain	+	+	+	−	−	−	−	−
Motility	−	−	−	−	−	−	−	−
Endospore formation	−	−	−	−	−	−	−	−
Production of:								
Alkaline phosphatase	−	−	+	−	−	NA	NA	NA
Catalase	+	+	+	−	+	−	+	+
Oxidase	−	−	−	−	−	+	−	−
Urease	−	v	−	−	−	−	NA	NA
β-Galactosidase	−	−	−	−	−	−	w	+
N-Acetyl-glucosamine	−	−	−	−	−	+	NA	w
Acid from:								
L-Arabinose	−	−	−	+	−	NA	NA	NA
Ribose	−	+	+	+	−	NA	NA	NA
Mannose	+	+	+	+	+	+	+	−
Mannitol	+	−	−	+	−	NA	NA	NA
Sucrose	+	+	+	−	−	NA	NA	NA
D-Glucose	+	+	+	+	+	NA	NA	NA
D-Fructose	+	+	−	+	−	NA	NA	NA
D-Maltose	−	+	+	+	−	NA	NA	NA
D-Lactose	−	−	−	+	+	NA	NA	NA
G+C content (mol%)	63.7	59.0	59.0	58.6	58.3	57.9	58.4	58.8
Source	Human gut	Soil	Microbial fuel cell	Human gut	Human gut	Human gut	Human gut	Human gut

+, positive result; −, negative result; w, weakly positive result; NA, data not available.

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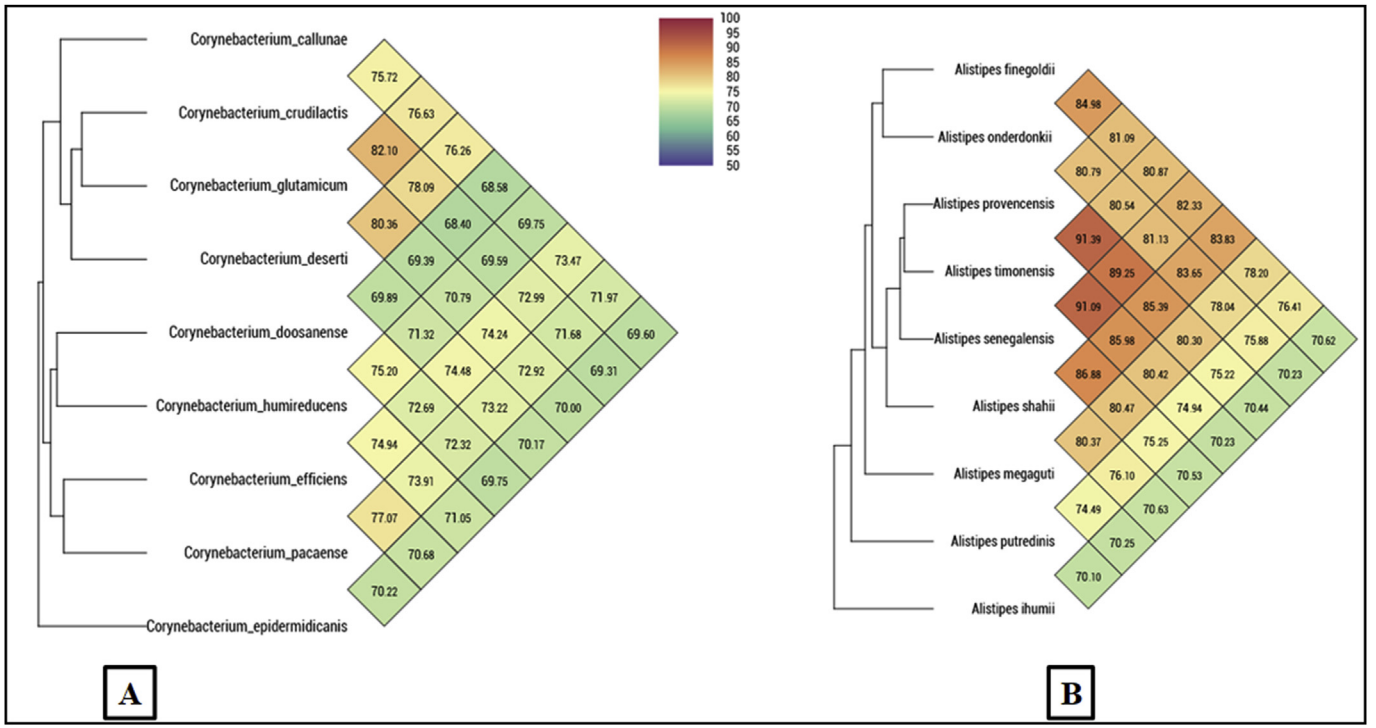

FIG. 4. Heat maps generated with OrthoANI values calculated using OAT software for *Corynebacterium pacaense* sp. nov. (A), *Alistipes megaguti* sp. nov., and *Alistipes provencensis* sp. nov. (B) with other closely related species with standing in nomenclature.

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softwares (Velvet [11], Spades [12] and Soap Denovo [13], and trimmed (MiSeq and Trimmomatic [14] softwares) or untrimmed data (only MiSeq software). GapCloser was used to reduce assembly gaps. Scaffolds <800 bp and scaffolds with a depth value lower than 25% of the mean depth was removed. The best assembly was selected by using different criteria (number of scaffolds, N50, number of N).

The genome of strain Marseille-P2417^T was 3 030 201 bp long (13 scaffolds, 16 N50) with a 63.75 mol% G+C content and contains 2820 predicted genes. The degree of genomic similarity of Marseille-P2417^T with closely related species was estimated using the OrthoANI software [15]. Values among closely related species (Fig. 4(A)) ranged from 68.40% between *Corynebacterium crudilactis* and *Corynebacterium doosanense* to 82.10% between *C. crudilactis* and *Corynebacterium gultamicum*. When the isolate was compared to these closely related species, values ranged from 70.22% with *Corynebacterium epidermidicanis* to 77.07% with *Corynebacterium efficiens*. Likewise, the genomes of strains Marseille-P5997^T and Marseille-P2431^T measured about 3 270 862 and 3 805 103 bp long respectively. They contain 2727 and 2973 predicted genes, with 58.6 and 58.3 mol% G+C content respectively. A genomic similarity analysis of *Alistipes* species was performed by OrthoANI software. Values among closely related species (Fig. 4(B)) ranged from 70.10% between *Alistipes putredinis* and *Alistipes ihumii* to 91.39% between *Alistipes provencensis* and *Alistipes timonensis*. When Marseille-P5997^T was compared to these closely related species, values ranged from 70.25% with *Alistipes ihumii* to 80.47% with *Alistipes senegalensis*. When Marseille-P2431^T was compared to these closely related species, values ranged from 70.44% with *Alistipes ihumii* to 91.39% with *Alistipes timonensis*.

Conclusion

Strains Marseille-P2417^T, Marseille-P5997^T and Marseille-P2431^T exhibited a 16S rRNA sequence similarity of <98.65% and an OrthoANI value < 95% with its phylogenetically closest species with standing in nomenclature, and we consequently propose them to be the type strains of the new *Corynebacterium pacaense* sp. nov., *Alistipes megaguti* sp. nov. and *Alistipes provencensis* sp. nov. respectively.

Description of *Corynebacterium pacaense* sp. nov.

Corynebacterium pacaense (pa.ca.en'se, L. adj. fem., 'PACA,' the name of the region Provence-Alpes-Côte d'Azur, France, where the strain was isolated). Cells were aerobic, Gram positive, rod shaped, nonmotile and non–spore forming, and catalase and oxidase. Cells had a length of 1.29 to 1.5 μm and a width of 0.5 to 0.8 μm. Colonies growing on 5% sheep's blood–enriched Columbia agar (bioMérieux) were circular and white after 72 hours of incubation in aerobic atmosphere at 37° C, and they showed a mean diameter of 1.15 mm. Positive reactions were observed for D-glucose, D-mannitol, D-sucrose, D-fructose, D-mannose, esculin, salicin, esterase (C4), leucine arylamidase, phosphatase acid, naphtalo-AS-BI-phosphohydrolase and β-glucosidase. Negative reactions were obtained with glycerol, D-maltose, D-ribose, D-xylose, glycogen, amygdalin, D-adonitol, D-arabinose, D-arabitol, D-cellobiose, D-fucose, D-galactose, D-lactose, D-lyxose, D-melezitose, D-melibiose, D-raffinose, D-sorbitol, D-tagatose, D-turanose, dulcitol, erythrol, gentiobiose, inulin, L-arabinose, L-arabitol, L-fucose, L-rhammose, L-sorbose, L-xylose, methyl-αD-glucopyranoside, methyl-αD-mannopyranoside, methyl-βD-xylopyranoside, *N*-acetylglucosamine, potassium 2-ketogluconate, potassium 5-ketogluconate, potassium gluconate, starch, xylitol, alkaline phosphatase, esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

The genome of strain Marseille-P2417^T was 3 030 201 bp long, and its G+C content was 63.75 mol%. The strain Marseille-P2417^T was isolated from a fresh stool sample from a 24-year-old healthy woman. It was deposited in the Collection de Souches de l'Unité des Rickettsies (CSUR) collection under accession number CSUR P2417. The 16S rRNA, *rpoB* genes and genome sequences are available in GenBank under accession numbers LT223574, LT223580 and FWCI00000000 respectively.

Description of *Alistipes megaguti* sp. nov.

Alistipes megaguti (mega.gu'ti, N.L. masc. adj. *megaguti,* 'mega-gut,' the name of a project aiming to individually cultivate all species from the digestive tract of healthy humans). Cells were anaerobic, Gram-negative, nonmotile and asporogenous rods. Catalase and oxidase activities were negative. Bacterial cells had a length of 1.36 to 3.57 μm and a width of 0.4 to 0.6 μm. Colonies of the strain Marseille-P2431^T were 0.2 to 0.9 mm in diameter on blood-enriched Columbia agar. Growth occurred at 37°C under anaerobic conditions. Positive reactions were observed for esterase (C4), esterase lipase (C8), cystine arylamidase, trypsin, α-chymotrypsin, phosphatase acid, naphtalo-AS-BI-phosphohydrolase, β-galactosodase, α-mannosidase, L-arabinose, D-xylose, glucose, fructose, mannose, D-mannitol, esculin, D-maltose, D-lactose, D-tagatose and α-fucosidase.

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Negative reactions were observed for alkaline phosphatase, leucine arylamidase, α-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β glucosaminidase, D-arabinose, L-xylose, methyl β-D-xylopyranoside, L-rhammose, D-sorbitol, methyl α-D-mannopyranoside, sucrose, glycogen and potassium 2-ketogluconate. The most abundant fatty acid by far was hexadecanoic acid (53%), followed by 13-methyltetradecanoic acid (16%) and 9-octadecenoic acid (8%).

The genome of strain Marseille-P5997^T was 3 270 862 bp long, and its G+C content was 58.6 mol%. Strain Marseille-P5997^T was isolated from a fresh stool sample from a 25-year-old healthy woman and was deposited in the CSUR collection under accession number CSUR P5997. The 16S rRNA and genome sequences are available in GenBank under accession numbers LS999984 and LR027382 respectively.

Description of *Alistipes provencensis* sp. nov.

Alistipes provencensis (pro.ven.cen'sis, N.L. adj. neut., from Provence, the region in France, where the strain was isolated). Cells were anaerobic, Gram-negative, nonmotile, asporogenous rods that were catalase positive and oxidase negative. Cells had a length of 0.9 to 1.43 μm and a width of 0.4 to 0.5 μm. Colonies of the strain Marseille-P2431^T were 0.4 to 0.64 mm in diameter on blood-enriched Columbia agar. Growth occurred at 37°C under anaerobic conditions. Positive reactions were observed for esterase (C4), lipase (C14), β-galactosodase, β-glucuronidase, α-glucosidase, β-glucosidase, D-xylose, D-glucose, D-mannose, esculin, D-lactose, D-melezitose and D-raffinose. Negative reactions were observed with alkaline phosphatase, esterase lipase (C8), α-chymotrypsin, phosphatase acid, α-galactosidase, *N*-acetyl-β-glucosaminidase, glycerol, erythrol, L-arabinose, D-ribose, D-galactose, D-fructose, dulcitol, D-mannitol, amygdalin, arbutin, D-cellobiose, D-maltose, D-sucrose, D-trehalose, inulin, starch, glycogen, D-fucose and potassium 2-ketogluconate.

The genome of strain Marseille-P2431^T was 3 805 103 bp long, and its G+C content was 58.3 mol%. Strain Marseille-P2431^T was isolated from a 66-year-old man with diabetes and hypertension, and was deposited in the CSUR collection under accession number CSUR P2431. The 16S rRNA and genome sequences are available in GenBank under accession numbers LT223566 and FKYL00000000 respectively.

Nucleotide sequence accession number

The 16S rRNA gene, the *rpoB* gene and genome sequences of strain Marseille-P2417^T were deposited in GenBank under accession numbers LT223574, LT223580 and FWCI00000000 respectively. The 16S rRNA gene and genome sequences of strain Marseille-P5997^T and strain Marseille-P2431^T were deposited in GenBank under accession numbers LS999984 and LT223566 respectively, and NR027382 and FKYL00000000 respectively.

Deposit in culture collection

Strain Marseille-P2417^T, strain Marseille-P5997^T and strain Marseille-P2431^T were respectively deposited in the CSUR collection under the following numbers: CSUR 2417^T, CSUR P5997^T and CSUR 2431^T = DSM 102308.

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Conflict of Interest

None declared.

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