

Corynebacterium bouchesdurhonense sp. nov., and *Corynebacterium provencense* sp. nov., two new species isolated from obese patients

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Abstract

Corynebacterium bouchesdurhonense sp. nov. strain Marseille-P2067^T (= CSURP2067; = DSM100846) and *Corynebacterium provencense* sp. nov. strain Marseille-P2161^T (= CSURP2161; = DSM101074) are two new species from the order *Corynebacteriales* that were isolated from obese French individuals.

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Introduction

It is important to understand the implications of bacterial diversity in normal physiological functions and for disease [1]. To explore the diversity of human intestinal bacteria, the culturomic approach, based on diversified culture conditions, was designed to isolate species that had never been cultivated before, and also to complete the metagenomics of 16S rRNAs [2–4]. In addition, a new taxonomic method called taxonogenomics has been developed that provides descriptions associating the analysis of complete sequences of the genome and the phenotypic characteristics of novel bacterial species [5]. Based on this new approach, we report here a brief description of two new species of *Corynebacterium*, both isolated for the first time in humans.

Isolation and growth conditions

In 2015, the strain Marseille-P2067^T and the strain Marseille-P2161^T were isolated from stool samples from obese French individuals. The strains of these bacteria have not been identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The screening was carried out on a Microflex LT spectrometer (Bruker Daltonics, Bremen, Germany), as previously reported [6,7]. Spectra obtained (Fig. 1) were imported and analysed using the BIOTYPER 3.0 software against the Bruker database, which is regularly updated from the MEPHI database <http://www.mediterranee-infection.com/article.php?larub=280&titre=umrs-database> [1]. The strains Marseille-P2067^T and Marseille-P2161^T were obtained by culture on Columbia agar supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France) incubated for 48 hours at 37°C, under microaerophilic conditions using CampyGen (Thermo Scientific, Villebon-sur-Yvette, France) [8].

Phenotypic characteristics

Colonies of the strain Marseille-P2067^T were white, circular, non-haemolytic and opaque on Columbia agar enriched with

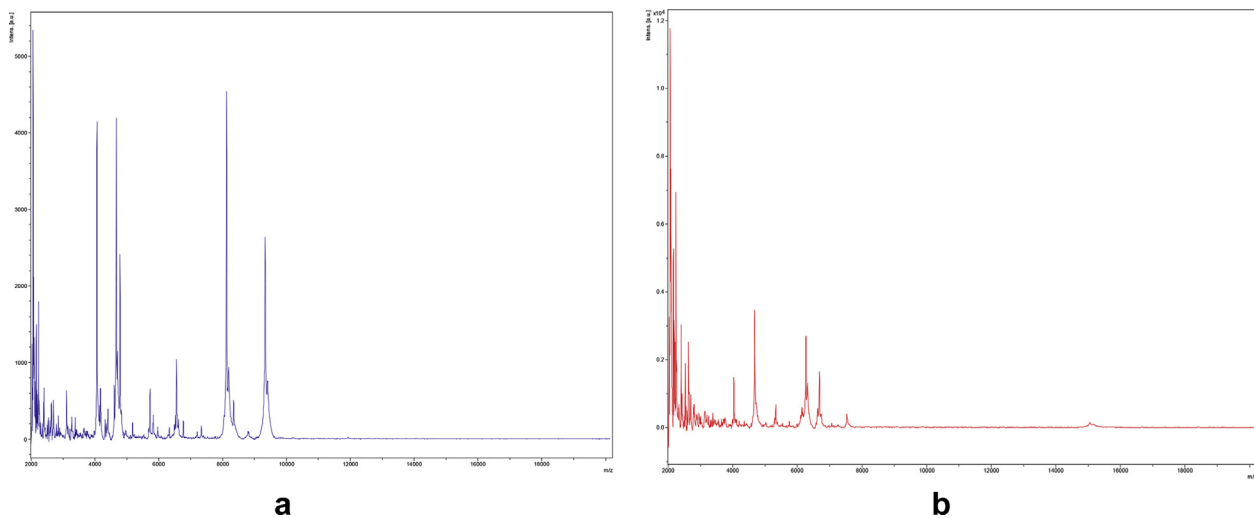


FIG. 1. MALDI-TOF MS reference spectra of *Corynebacterium bouchesdurhonense* sp. nov. (a) and *Corynebacterium provencense* sp. nov. (b). Each reference spectrum was generated by comparison of spectra from 12 individual colonies.

5% sheep blood (bioMérieux) at 37°C under aerobic conditions, and measured 1 mm in diameter after 48 hours of incubation. Bacteria were Gram-positive and rod-shaped. Cells were non-motile, spore-forming and facultatively anaerobic. On electron microscopy, cells had mean length and diameter of 0.8 and 1.8–5.0 µm, respectively (Fig. 2). Strain Marseille-P2067^T exhibited positive oxidase and catalase activities.

Strain Marseille-P2161^T colonies vary between 1 and 1.5 mm in diameter, and appear circular, milky white, non-haemolytic and opaque after 48 hours of incubation on

Columbia agar enriched with 5% sheep blood (bioMérieux) under aerobic conditions. Cells were rod-shaped with a diameter ranging from 1.2 to 1.7 µm and a length of 0.6 µm (Fig. 2). Strain Marseille-P2161^T was Gram-positive, motile, facultatively anaerobic and spore-forming. Strain Marseille-P2161^T exhibited catalase activity but not oxidase activity. API ZYM and API 20NE tests were performed at 37°C under aerobic conditions (Table 1). Table 2 compares the main biochemical characteristics of the closest *Corynebacterium* species with standing in nomenclature.

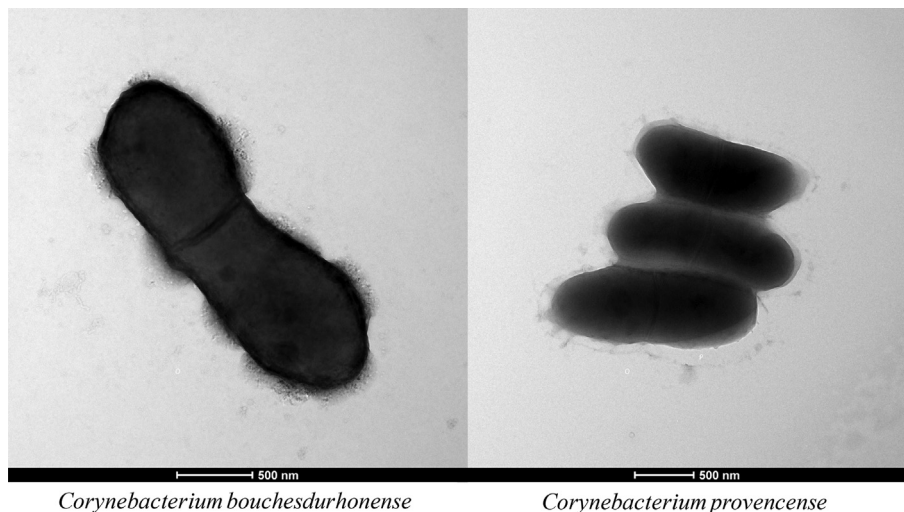


FIG. 2. Scanning electron microscopy (SEM) of stained *Corynebacterium bouchesdurhonense* sp. nov. and *Corynebacterium provencense* sp. nov. A colony was collected from agar and immersed into a 2.5% glutaraldehyde fixative solution. Then a drop of the suspension was directly deposited on a poly-L-lysine-coated microscope slide for 5 minutes and treated with 1% phosphotungstic acid aqueous solution (pH 2.0) for 2 minutes to increase the SEM image contrast. The slide was gently washed in water, air-dried and examined in a tabletop SEM (Hitachi TM4000), approximately 60 cm in height and 33 cm in width, to evaluate bacteria structure. Scales are shown on figures.

TABLE 1. Phenotypic characterization of *Corynebacterium bouchesdurhonense* sp. nov. and *Corynebacterium provencense* sp. nov. based on analytical profile index (API) tests

Tests	Characteristics	Results	
		<i>C. bouchesdurhonense</i> sp. nov	<i>C. provencense</i> sp. nov
API ZYM	Alkaline phosphatase	+	-
	Esterase (C4)	-	+
	Esterase lipase (C8)	-	+
	Lipase (C14)	-	+
	Leucine arylamidase	+	-
	Valine arylamidase	-	+
	Cystine arylamidase	-	+
	Trypsin	-	-
	α-chymotrypsin	-	-
	Acid phosphatase	+	-
	Naphthalo-AS-BI-phosphohydrolase	+	+
	α-galactosidase	-	-
	β-galactosidase	-	-
	β-glucuronidase	-	-
	α-glucosidase	-	+
	β-glucosidase	-	+
	N-acetyl-β-glucosaminidase	+	-
	α-mannosidase	-	-
	α-fucosidase	-	-
	API 20 NE	Nitrates to nitrites	+
Indole		-	-
Glucose fermentation		-	-
Arginine dihydrolase		-	+
Urease		-	+
β-glucosidase		+	-
Protease		-	-
β-galactosidase		-	-
Glucose assimilation		-	-
Arabinose		-	-
Mannose		-	-
Mannitol		-	-
N-acetyl-glucosamine		-	-
Maltose		-	-
Potassium gluconate		-	-
Capric acid		-	-
Adipic acid		-	-
Malate	-	-	
Trisodium citrate	-	-	
Phenylacetic acid	-	-	

Strains identification

For each strain, the 16S rRNA gene was amplified using the primer pair fD1 and rP2 (Eurogentec, Angers, France) and sequenced using the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xLGenetic Analyzer capillary sequencer (ThermoFisher, Saint-Aubin, France), as previously described [9]. All 16S rRNA nucleotide sequences were assembled and corrected using CODONCODE ALIGNER software (<http://www.codoncode.com>).

Strain Marseille-P2067^T exhibited a 97.3% 16S rRNA similarity with *Corynebacterium tuscaniense* strain ISS-5309 (NR_043093), the phylogenetically closest species with standing in nomenclature. Strain Marseille-P2161^T showed 98.5% sequence similarities to *Corynebacterium variabile* strain DSM 20132 (NR_025314) (Fig. 3). We consequently proposed to classify strain Marseille-P2067^T and strain Marseille-P2161^T as new species within the genus *Corynebacterium* in the phylum *Actinobacteria*.

Genome sequencing

Genomic DNA was extracted using the EZ1 biorobot with the EZ1 DNA tissue kit (Qiagen, Hilden, Germany) and then sequenced on a MiSeq sequencer (Illumina Inc., San Diego, CA, USA) with the Nextera Mate Pair sample prep kit and Nextera XT Paired End (Illumina), as previously described [10]. Assembly was performed using a pipeline containing several

TABLE 2. Differential phenotypic characteristics of *Corynebacterium bouchesdurhonense* sp. nov. (1), *Corynebacterium provencense* sp. nov. (2), *Corynebacterium urinapleomorphum* (3), *Corynebacterium phoceense* (4), *Corynebacterium aurimucosum* (5), and *Corynebacterium appendicis* (6)

Property	1	2	3	4	5	6
Cell diameter (µm)	1.8–5.0	1.2–1.7	0.2	0.5	0.5	0.3
Oxygen requirement	+	+	+	+	+	-
Gram stain	+	+	+	+	+	+
Salt requirement	-	-	-	-	-	-
Motility	-	+	-	-	-	-
Endospore formation	+	+	-	+	-	-
Alkaline phosphatase	+	-	+	+	+	+
Catalase	+	+	+	+	+	+
Oxidase	+	-	-	-	NA	NA
Nitrate reductase	+	-	-	+	-	-
Urease	-	+	+	-	-	+
β-galactosidase	-	-	-	-	-	-
N-acetyl-glucosamine	+	-	-	-	-	-
Arabinose	-	-	-	NA	+	-
Lipase (C8)	-	+	+	+	-	-
β-glucosidase	-	+	-	+	-	-
Mannose	-	-	-	+	-	+
Mannitol	-	-	-	-	-	-
Indole	-	-	-	NA	-	-
D-glucose	-	-	-	+	+	+
D-maltose	-	-	-	+	+	+
Source	Human stool	Human stool	Human urine	Human urine	Human sample	Human gut

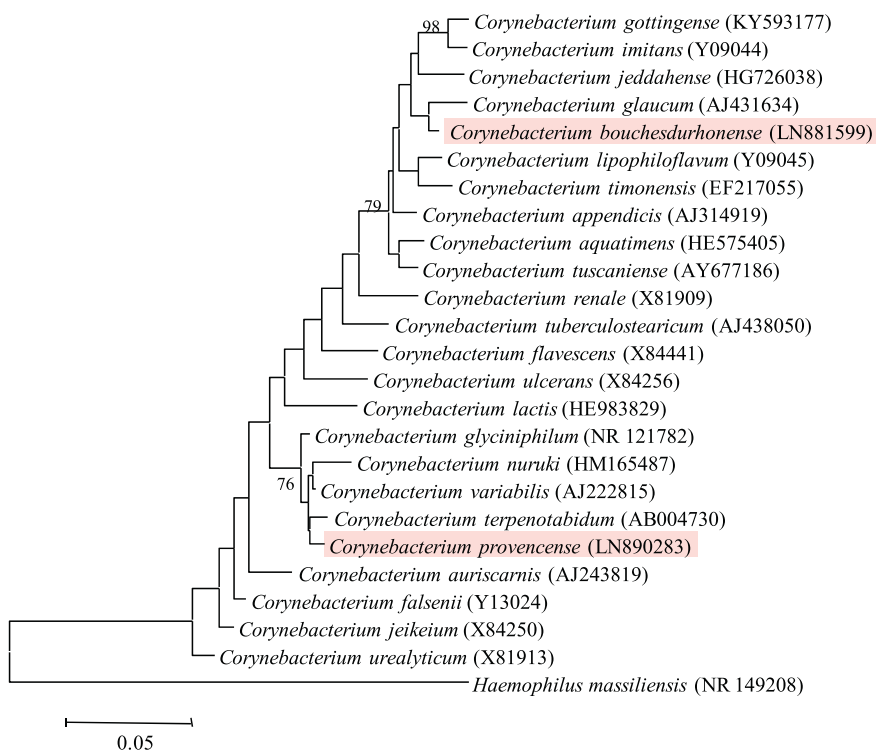


FIG. 3. Phylogenetic tree highlighting the position of *Corynebacterium bouchesdurhonense* sp. nov. and *Corynebacterium provencense* sp. nov. relative to the most closely related type strains within the genus *Corynebacterium*. GenBank accession numbers of 16S rRNA are indicated in parentheses. Sequences were aligned using MUSCLE with default parameters, phylogenetic inference was obtained using the maximum likelihood method and MEGA 7 software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 1000 times to generate a majority consensus tree. The scale bar indicates a 5% nucleotide sequence divergence. *Haemophilus massiliensis* was used as an outgroup.

softwares (VELVET [11], SPADES [12] and SOAP DENOVO [13]), and trimmed (MiSEQ and TRIMMOMATIC [14] softwares) or untrimmed (only MiSEQ software) data. GAPCLOSER was used to reduce assembly gaps. Scaffolds <800 bp and scaffolds with a depth value < 25% of the mean depth were removed. The best assembly was selected by using different criteria (number of scaffolds, N50, number of N). Strains Marseille-P2067^T and

Marseille-P2161^T have genome sizes of 2.26 Mb and 3.03 Mb, respectively, with a 68% and 66.85% G + C content, respectively. The degree of genomic identity of these strains with closely related species was calculated using ORTHOANI software [15]. For the strain Marseille-P2067^T, OrthoANI values among closely related species (Fig. 4) ranged from 68.34%, between *Corynebacterium appendicis* and *Corynebacterium ulcerans*, to

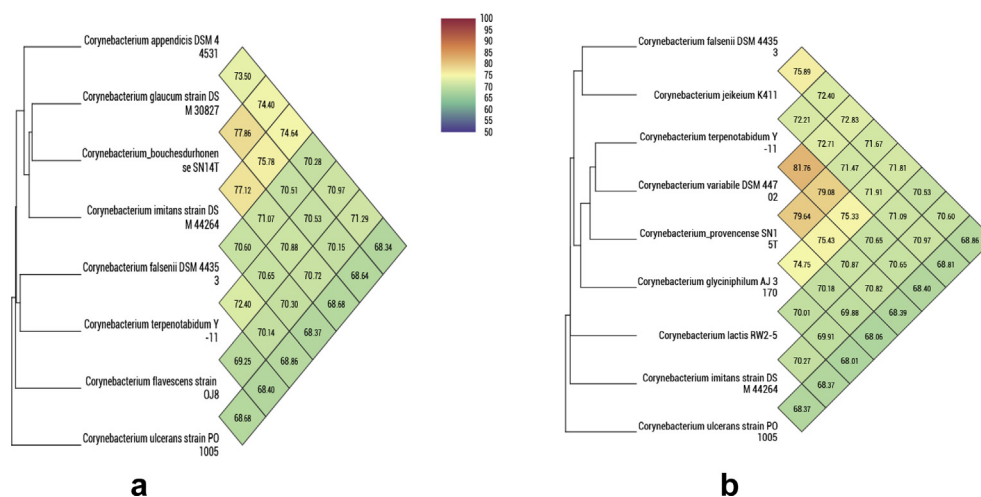


FIG. 4. Heatmaps generated with ORTHOANI values calculated using the OAT software for *Corynebacterium bouchesdurhonense* sp. nov. (a) and *Corynebacterium provencense* sp. nov. (b) with other closely related species with standing in nomenclature.

77.86%, between *Corynebacterium bouchesdurhonense* and *Corynebacterium glaucum*. When *Corynebacterium bouchesdurhonense* was compared with these closely related species, values ranged from 68.68% with *Corynebacterium terpenotabidum* to 77.86% with *Corynebacterium glaucum*. For the strain Marseille-P2161^T, ORTHOANI values among closely related species (Fig. 4) ranged from 68.01%, between *Corynebacterium glyciniphilum* and *Corynebacterium ulcerans*, to 81.76%, between *Corynebacterium terpenotabidum* and *Corynebacterium variabile*. When *Corynebacterium bouchesdurhonense* was compared with these closely related species, values ranged from 68.06% with *Corynebacterium ulcerans* to 77.86% with *Corynebacterium variabile*.

Conclusion

On the basis of unique phenotypic features, including MALDI-TOF spectrum, a 16S rRNA sequence divergence >1.3% and an ORTHOANI value < 95% with the phylogenetically closest species with standing in nomenclature, we formally proposed strain Marseille-P2067^T and strain Marseille-P2161^T as the type strains of *Corynebacterium bouchesdurhonense* sp. nov. and *Corynebacterium provencense* sp. nov. respectively, which are new species within the genus *Corynebacterium*.

Description of *Corynebacterium bouchesdurhonense* strain Marseille-P2067^T sp. nov.

Corynebacterium bouchesdurhonense (bou.ches.du.rhon.en.se, M. L. adj. Bouches-du-Rhône is a department of the Provence-Alpes-Côte d'Azur region (France), where strain Marseille-P2067^T was isolated). The strain grows at temperatures ranging between 25°C and 45°C in aerobic conditions (at an optimum temperature of 37°C). The potential pathogenicity of the type strain Marseille-P2067^T (= CSURP2067; = DSM100846) is unknown, but it was isolated from the stool of an obese patient consulting at our hospital. This strain Marseille-P2067^T has a genome size of 2.26 Mb and exhibited a G + C content of 68%.

Description of *Corynebacterium provencense* strain Marseille-P2161^T sp. nov.

Corynebacterium provencense (pro.ven.cen.se, M. L. adj. Provence is an old province in the southeast of France, where

strain Marseille-P2161^T was isolated). The strain grows at temperatures ranging between 25°C and 45°C in aerobic conditions (at an optimum temperature of 37°C). The potential pathogenicity of the type strain Marseille-P2161^T (= CSURP2161; = DSM101074) is unknown, but it was isolated from the stool of an obese individual consulting at our hospital. Besides, *C. provencense* was associated recently with otitis in a cat [16]. Strain Marseille-P2161^T has a genome size of 3.03 Mb and exhibited a G + C content of 66.85%.

Nucleotide sequence accession number

The 16S rRNA gene and genome sequences were deposited in GenBank under Accession numbers LN881599 and FJVG00000000, respectively, for strain Marseille-P2067^T, and LN890283 and FIZC00000000, respectively, for strain Marseille-P2161^T.

Deposit in culture collections

All these strains were deposited in two different strain collections under following numbers: strain Marseille-P2067^T (= CSURP2067; = DSM100846) and strain Marseille-P2161^T (= CSURP2161; = DSM101074).

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Conflicts of interest

None to declare.

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Ethics and consent

The study was approved by the ethics committee of the Institut Federatif de Recherche 48 under reference 2016-010. The patients gave and approved and signed consent for participating in this study.

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