Corynebacterium neomassiliense sp. nov., a new bacterium isolated in a stool sample from a healthy male pygmy

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Abstract

An obligate aerobic, Gram-positive, non-sporulating, rod-shaped bacterium designated Marseille P3888^T was isolated from the stool sample of a healthy male pygmy. We described its main characteristics, and sequenced and annotated its genome. The 16S rRNA analysis revealed 98.10% sequence similarity with Corynebacterium terpenotabidum, the phylogenetically closest species with standing in nomenclature. The genome had a size of 3142051 bp with a guanine + cytosine content of 66.83%. We proposed the creation of the new *Corynebacterium neomassiliense* sp. nov. strain Marseille-P3888^T.

Keywords: Bacteria, *Corynebacterium neomassiliense*, culturomics, genome, species, sp. nov., taxono-genomics
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Introduction

Corynebacterium is a large genus of bacteria in which some species are of medical, veterinary or biotechnological interest [1]. To explore the bacterial diversity of the human gut, the culturomics approach, based on various culture conditions, was chosen to isolate species that had never been cultivated before and also to complete metagenomics targeting the 16SrRNA gene [2–4]. The combination of culturomics with taxono-genomics, which is a new method based on genomic, proteomic and phenotypic analysis, is an efficient strategy to provide a complete description of the bacterial species isolated [3–5]. By adopting this new approach, we propose here a brief description of a new species within the family *Corynebacteriaceae*, isolated from a human stool sample.

Isolation and growth conditions

In June 2017, we isolated from a stool sample of a 49-year-old healthy male pygmy an unidentified bacterial strain. Screening was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a Microflex LT spectrometer (Bruker Daltonics, Bremen, Germany) as previously described [6]. The obtained spectra (Fig. 1) were imported into MALDI Biotyper 3.0 software (Bruker Daltonics) and analysed against the main spectra of the bacteria included in two databases (Bruker and the constantly updated MEPHI databases; https://www.mediterranee-infection.com/acces-ressources/base-de-donnees/urms-data-base/). The study was validated by the ethics committee of Institut Federatif de Recherche 48 under number 09-022. Strain Marseille-P3888^T was first isolated in aerobic conditions after incubation in a culture bottle (bioMerieux, Marcy l'Étoile, France) supplemented with 5 mL sheep blood at 37°C.

Phenotypic characteristics

Characteristics of the strain were obtained as previously described [7]. Colonies were white and punctiform. Bacterial

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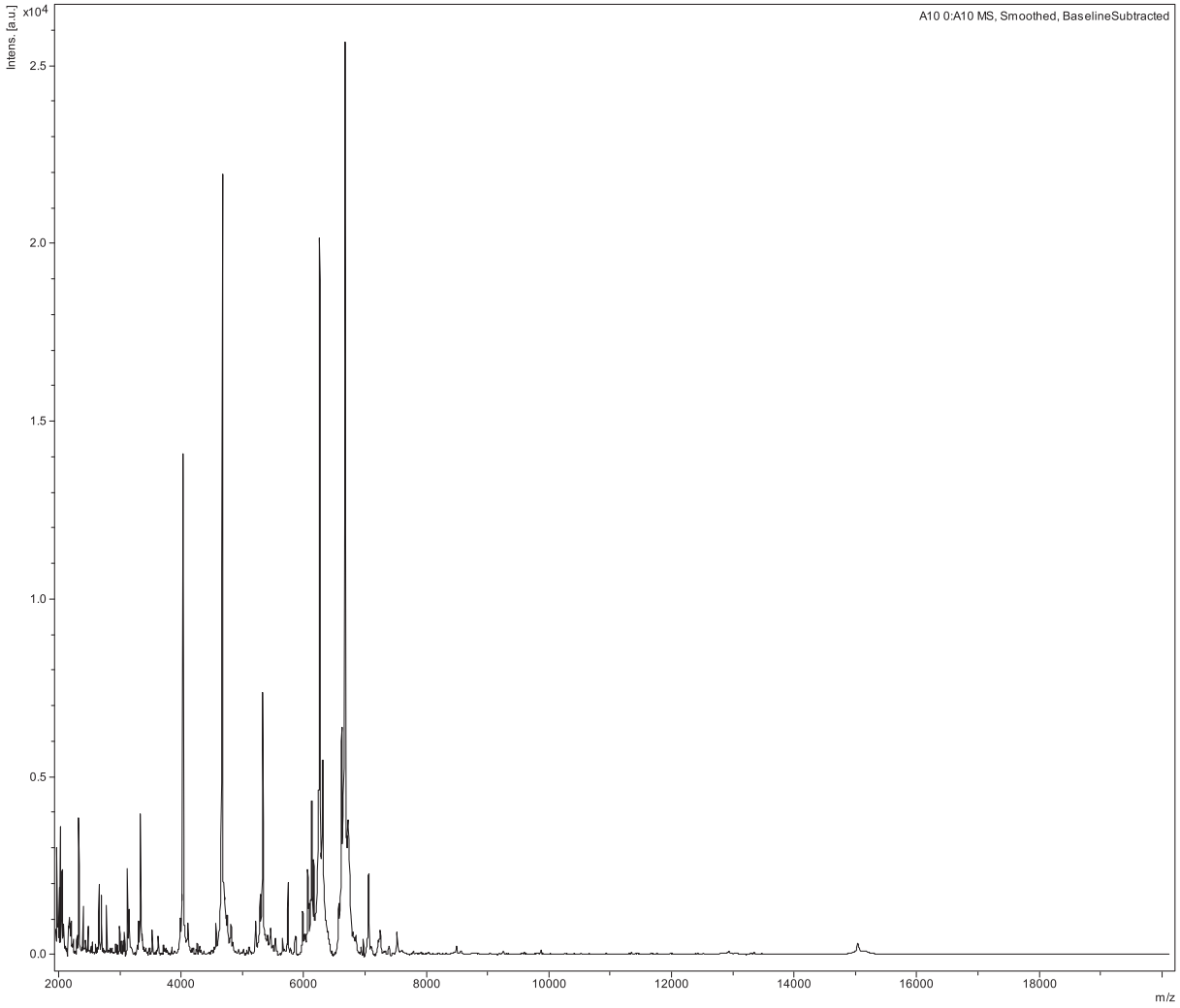

FIG. 1. MALDI-TOF MS reference mass spectrum; spectra from 12 individual colonies were compared and a reference spectrum was generated.

cells were Gram-positive. For scanning electronic microscopy, a colony was collected from agar and immersed into a 2.5% glutaraldehyde fixative solution. The slide was gently washed in water; air-dried and examined using a TM4000 microscope (approximately 60 cm in height and 33 cm in width) to evaluate bacterial structure. Cells appeared to be rod-shaped, with mean length and diameter of 3 µm and 0.5 µm, respectively (Fig. 2).

Cellular fatty acid methyl ester analysis was performed by gas chromatography/mass spectrometry. Two samples were prepared with approximately 85 mg of bacterial biomass per tube harvested from several culture plates. Fatty acid methyl esters were prepared as described by Sasser (2006) [8]. Gas chromatography/mass spectrometry analyses were carried out as described before [9]. Briefly, fatty acid methyl esters were separated using an Elite 5-MS column and monitored by mass spectrometry (Clarus 500—SQ 8 S; Perkin Elmer, Courtaboeuf, France). A spectral database search was performed using MS SEARCH 2.0 operated with the Standard Reference Database 1A (NIST, Gaithersburg, MD, USA) and the fatty acid methyl ester mass spectral database (Wiley, Chichester, UK). The major fatty acids by far were hexadecenoic acid (61%) and 9-octadecenoic acid (32%). No branched structures were described (Table 1).

The sporulation test (10 min at 80°C) was negative. Different growth temperatures (20, 28, 32, 37, 45 and 56°C), pHs (5, 6, 7, 7.5, 8 and 8.5), NaCl content (5, 10 and 15 g) and atmospheres (aerobic, anaerobic, microaerophilic (CampyGEN, Oxoid, Basingstoke, UK)) were tested on 5% sheep-blood-enriched Columbia Agar. Strain Marseille-P3888^T was a very-easy-to-cultivate bacterium and grew in all these conditions except at 56°C. API ZYM, API Coryne and API 50CH strips (BioMerieux) were used to evaluate the biochemical properties of the strain according to the manufacturer's instructions. Marseille-P3888^T showed activity for the esterase C4, esterase-

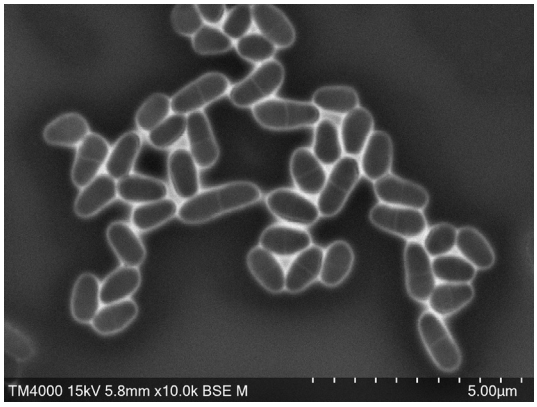

FIG. 2. Scanning electron microscopy of *Corynebacterium neomassiliense* gen. nov., sp. strain Marseille-P3888^T using a tabletop microscope TM 4000 plus (Hitachi, Tokyo, Japan). Scale bar represents 5 µm.

TABLE 1. Fatty acid profiles (%) of *Corynebacterium neomassiliense* strain Marseille-P3888^T

Fatty acids	Name	Mean relative % [a]
16:00	Hexadecanoic acid	61.4 ± 1.4
18:1n9	9-Octadecenoic acid	32.4 ± 1.4
18:00	Octadecanoic acid	3.7 ± 0.2
14:00	Tetradecanoic acid	1.2 ± 0.1
18:2n6	9,12-Octadecadienoic acid	TR
16:1n9	7-Hexadecenoic acid	TR

[a]Mean peak area percentage. TR, trace amounts <1%.

TABLE 2. Phenotypic characterization of *Corynebacterium neomassiliense* gen. nov. sp. nov., based on Analytical Profile Index 20A and ZYM (API) tests

	Characteristics	Results
API 20A	Indol formation	–
	Urease	+
	Acidification – glucose	–
	Acidification – mannitol	–
	Acidification – lactose	–
	Acidification – saccharose	–
	Acidification – maltose	–
	Acidification – salicin	–
	Acidification – xylose	–
	Gelatin hydrolysis	–
	Acidification – xylose	–
	Acidification– cellobiose	–
	Acidification – mannose	–
	Acidification – melezitose	–
	Acidification– raffinose	–
	Acidification – sorbitol	–
	Acidification – rhamnose	–
	Acidification– trehalose	+
API ZYM	Alkaline phosphatase	–
	Esterase (C4)	+
	Esterase lipase (C8)	+
	Lipase (C14)	+
	Leucine arylamidase	–
	Valine arylamidase	–
	Cystine arylamidase	–
	Trypsin	–
	α-chymotrypsin	–
	Alkaline phosphatase	–
	Acid phosphatase	–
	Naphthol-AS-BI-phosphohydrolase	+
	α-galactosidase	–
	β-galactosidase	–
	β-glucuronidase	–
	α-glucosidase	+
	β-glucosidase	+
	N-acetyl-β-glucosaminidase	–
	α-mannosidase	–
	α-fruconidase	–

lipase C8, esterase-lipase C14, acid-phosphatase, naphthol-AS-BI-phosphohydrolase, -glucosidase, -glucosidase, urease and a carbohydrate metabolism positive for D-fructose and D-trehalose. All the other reactions tested were negative. (Detailed results are given in Tables 2 and 3). Marseille-P3888^T showed catalase-positive and oxidase-negative activities. Characteristics of the strain are summarized in Table 4. A comparative study of the differential characteristics of strain Marseille-P3888^T with other closely related species is shown in Table 5.

Strain identification

The 16S rRNA gene was sequenced to classify this bacterium. Amplification was performed using the primer pair fD1 and rP2 (Eurogentec, Angers, France) and sequencing used the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xL sequencer (Thermofisher, Saint-Aubin, France), as previously described [10]. The 16S rRNA nucleotide sequences were assembled and corrected using CodonCode Aligner software (http://www.codoncode.com). We have placed the 16S rRNA of C. neomassiliense in GenBank (LT984641.1). Strain Marseille-P3888^T exhibited a 98.10% sequence identity with Corynebacterium terpenotabidum strain Y-11 (GenBank accession number NR_121699.2), the phylogenetically closest species with standing in nomenclature (Fig. 3a). The *RpoB* gene was shown to be more discriminant for *Corynebacterium* species [11], so we analysed the phylogenetic position of *C. neomassiliense* (Fig. 3b). We consequently classified this strain as a member of a new species within the genus *Corynebacterium*, family *Corynebacteriaceae*, phylum *Actinobacteria*.

DNA extraction

Genomic DNA of *C. neomassiliense* was extracted in two steps. First, a mechanical treatment was performed using acid-washed glass beads (G4649-500g Sigma) using a FastPrep-24™ 5G Grinder (mpBio, Illkirch-Graffenstaden, France) at maximum speed (6.5) for 90 s. After 2 hours of incubation at 37°C with lysozyme, DNA was extracted on the EZ1 biorobot (Qiagen, Hilden, Germany) with an EZ1 DNA tissues kit. The genomic DNA was suspended in an elution volume of 50 µL and quantified using a Qubit assay with the high sensitivity kit (Life Technologies, Carlsbad, CA, USA) to 37 ng/µL.

TABLE 3. Study of carbohydrate metabolism of strain Marseille-P3888^T using API 50 CH strips

Test	Characteristics	Results	Characteristics	Results
50 CH	Glycerol	–	Esculin ferric citrate	–
	Erythritol	–	Salicin	–
	D-arabinose	–	D-cellobiose	–
	L-arabinose	–	D-maltose	–
	D-ribose	–	D-lactose	–
	D-xylose	–	D-melibiose	–
	L-xylose	–	D-saccharose	–
	D-adonitol	–	D-trehalose	+
	Methyl βD-xylopyranoside	–	Inulin	–
	D-galactose	–	D-melezitose	–
	D-glucose	–	D-raffinose	–
	D-fructose	+	Amidon	–
	D-mannose	–	Glycogen	–
	L-sorbose	–	Xylitol	–
	L-rhamnose	–	Gentiobiose	–
	Dulcitol	–	D-turanose	–
	Inositol	–	D-xylose	–
	D-mannitol	–	D-tagalose	–
	D-sorbitol	–	D-fucose	–
	Methyl αD-mannopyranoside	–	L-fucose	–
	Methyl αD-glucopyranoside	–	D-arabitol	–
	N-acetyl-glucosamine	–	L-arabitol	–
	Amygdalin	–	Potassium gluconate	–
	Arbutin	–	Potassium 2-ketogluconate	–
			Potassium 5-ketogluconate	–

Genome sequencing

Genomic DNA was sequenced on MiSeq Technology (Illumina Inc., San Diego, CA, USA) with the paired end strategy and was barcoded to be mixed with 16 other projects for the Nextera XT DNA sample prep kit (Illumina). To prepare the paired end library, dilution was performed to obtain 1 ng of each genome as input. The 'tagmentation' step fragmented and tagged the DNA. Then limited cycle PCR amplification (12 cycles) completed the tag adapters and introduced dual-index barcodes. The library profile was validated on an Agilent 2100 BioAnalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) with a DNA High-sensitivity labchip and the fragment size was estimated to be 1.5 kb. After purification on AMPure XP beads (Beckman Coulter Inc., Fullerton, CA, USA), the libraries were normalized on specific beads according to the Nextera XT protocol (Illumina). Normalized libraries were pooled for sequencing on the MiSeq. Automated cluster generation and paired end sequencing with dual index reads were performed in a single 39-hour run in 2 × 250 bp. Total information of 7.5 Gb was obtained from an 802 000/mm^2 cluster density with a cluster passing quality-control filters of 96.4%. Within this run, the index representation for *C. neomassiliense* was determined as 3.91%. The 564 703 paired end reads were trimmed using TRIMMOMATIC software [12]. The genome was assembled using SPADES software [13], then GAPCLOSER [14] was used to reduce gaps.

TABLE 4. Description of *Corynebacterium neomassiliense* according to the digitalized protologue ex: TA00521 on the www.imedea.uib.es/dprotologue website

Taxonumber	TA00888
Type of description	New description
Species name	*Neomassiliensis*
Genus name	*Corynebacterium*
Specific epithet	*Neomassiliensis*
Species status	sp. nov.
Species etymology	*Corynebacterium neomassiliensis* (Gr. n. korune, a club;L. neut. n. bacterium, a rod, bacterium and (Neo adj. new' mas.si.li.en'sis L masn. adj. massiliensis, of Massilia, the ancient Roman name for Marseille, where the strain was isolated).
Authors	Manon Boxberger, Issam Hasni, Melhem Bilen, Didier Raoult and Bernard La Scola
Designation of the type strain	Marseille-P3888
Strain collection number	CSURP3888 = CCUG72352
16S rRNA gene accession number	LT984641.1
Genome accession number	UZAZ01000001
Genome status	Draft
Genome size	3142051 pb
GC%	66.83%
Country of origin	France, Marseille
Date of isolation	01/06/2017
Source of isolation	Human gut
Gram strain	Positive
Cell shape	Rod
Cell size	Mean diameter 0.5 µm and length 3 µm
Motility	Non-motile
Sporulation	No
Colony morphology	White, smooth
Temperature range	22–45°C
Lowest temperature for growth	22–45°C
Temperature optimum	37°C
Highest pH for growth	5
Lowest pH for growth	8.5
pH optimum	6
Lowest NaCl concentration for growth	5
Highest NaCl concentration for growth	15
Salinity optimum	5
Relationship to O_2	Facultative anaerobe
O_2 for strain testing	Anaerobiosis, microaerophilic, Aerobiosis
Oxidase	Negative
Catalase	Positive
Major fatty acids	Hexadecanoic acid (61.4%)

The total length of the *C. neomassiliense* genome was 3.1 megabases encompassing 18 scaffolds with a guanine-cytosine (GC) content of 66.83%. The gene prediction analysis reported 2768 predicted genes, including 2693 coding DNA sequences and 75 RNA sequences (11 rRNAs, 61 tRNAs and 3 ncRNAs).

The degree of genomic similarity of strain Marseille-P3888^T with closely related species was estimated using the ORTHOANI software [15]. Values among closely related species (Fig. 4) ranged from 66.51% between *Corynebacterium accolans* and *Corynebacterium variabile* to 79.03% between *Corynebacterium nuruki* S6-4 and *C. terpenotabidum* Y-11. When the isolate was compared to these closely related species, values ranged from 67.26% with *C. variabile* DSM 20132 to 78.86% with *C. terpenotabidum* Y-11.

TABLE 5. Comparison of phenotypic criteria between *Corynebacterium neomassiliense* and the phylogenetically closest bacterial species

	Corynebacterium neomassiliense	*Corynebacterium terpenotabidum*	*Corynebacterium nuruki*	*Corynebacterium glyciniphilum*	*Corynebacterium urealyticum*
Cell diameter (μm)	0.5–1	0.5–0.7 × 1.0–1.5	NA	0.3–0.4 × 1	0.5–1
Oxygen requirement	Aerobe	Aerobe	Aerobe	Aerobe	Aerobe
Gram stain	Gram-positive	Gram-positive	Gram-positive	Gram-positive	Gram-positive
Salt requirement	–	–	–	NA	NA
Motility	–	–	–	–	–
Endospore formation	–	–	NA	–	–
Alkaline phosphatase	+	NA	+	+	NA
Catalase	+	+	+	+	+
Oxidase	–	–	+	NA	–
Nitrate reductase	–	–	–	–	–
Urease	+	+	+	+	+
β-galactosidase	–	–	–	–	NA
N-acetyl-glucosamine	–	NA	–	+	NA
D-arabinose	–	NA	NA	+	NA
L-arabinose	–	NA	NA	+	–
Esterase lipase (C8)	+	NA	NA	–	NA
Mannose	–	+	+	+	NA
Mannitol	–	–	+	–	–
D-sorbitol	–	–	+	–	–
D-glucose	–	+	+	+	–
D-fructose	+	+	–	+	NA
D-maltose	–	–	NA	–	–
Source	Human faeces	Soil	Alcohol fermentation starter	Putrefied banana	Human urine and skin

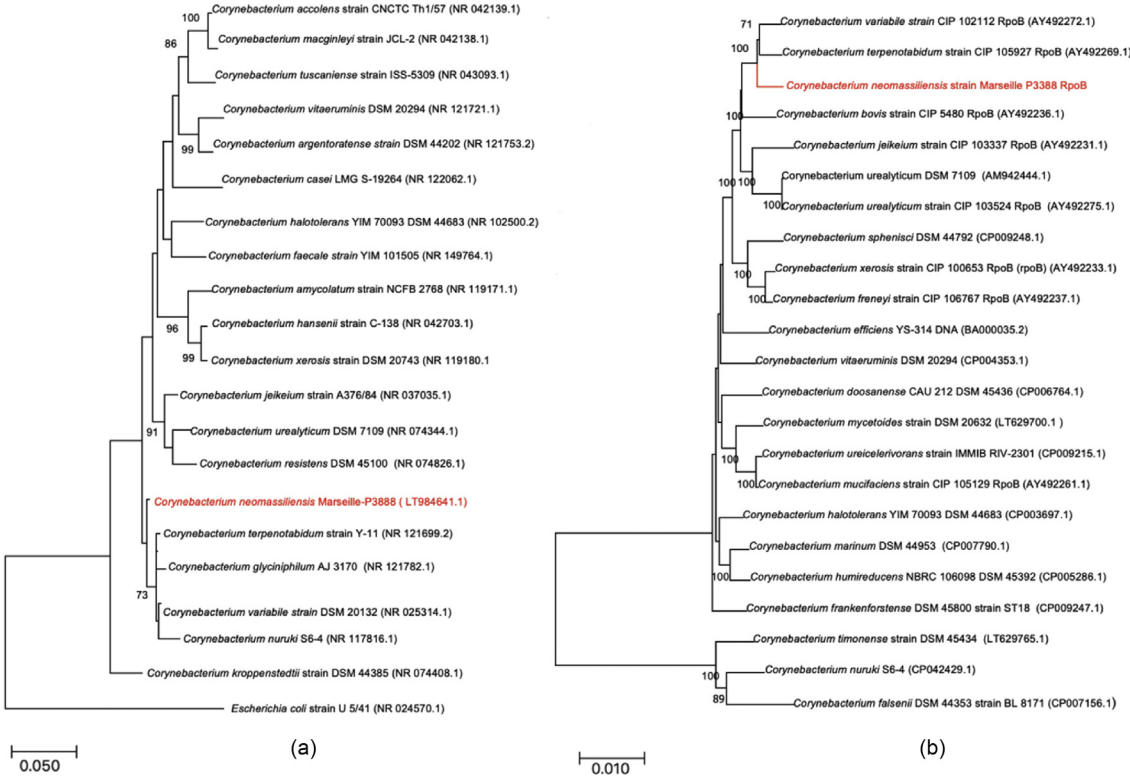

FIG. 3. Phylogenetic tree showing the position of *Corynebacterium neomassiliense* strain Marseille-P3888T relative to other phylogenetically closest neighbours. The respective GenBank accession numbers for 16S rRNA genes (a) and RpoB (b) are indicated in parenthesis. Sequences were aligned using Muscle v3.8.31 with default parameters and phylogenetic inferences were obtained using the maximum likelihood method within the software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 1000 times to generate a majority consensus tree. Only bootstrap values >70% were retained.

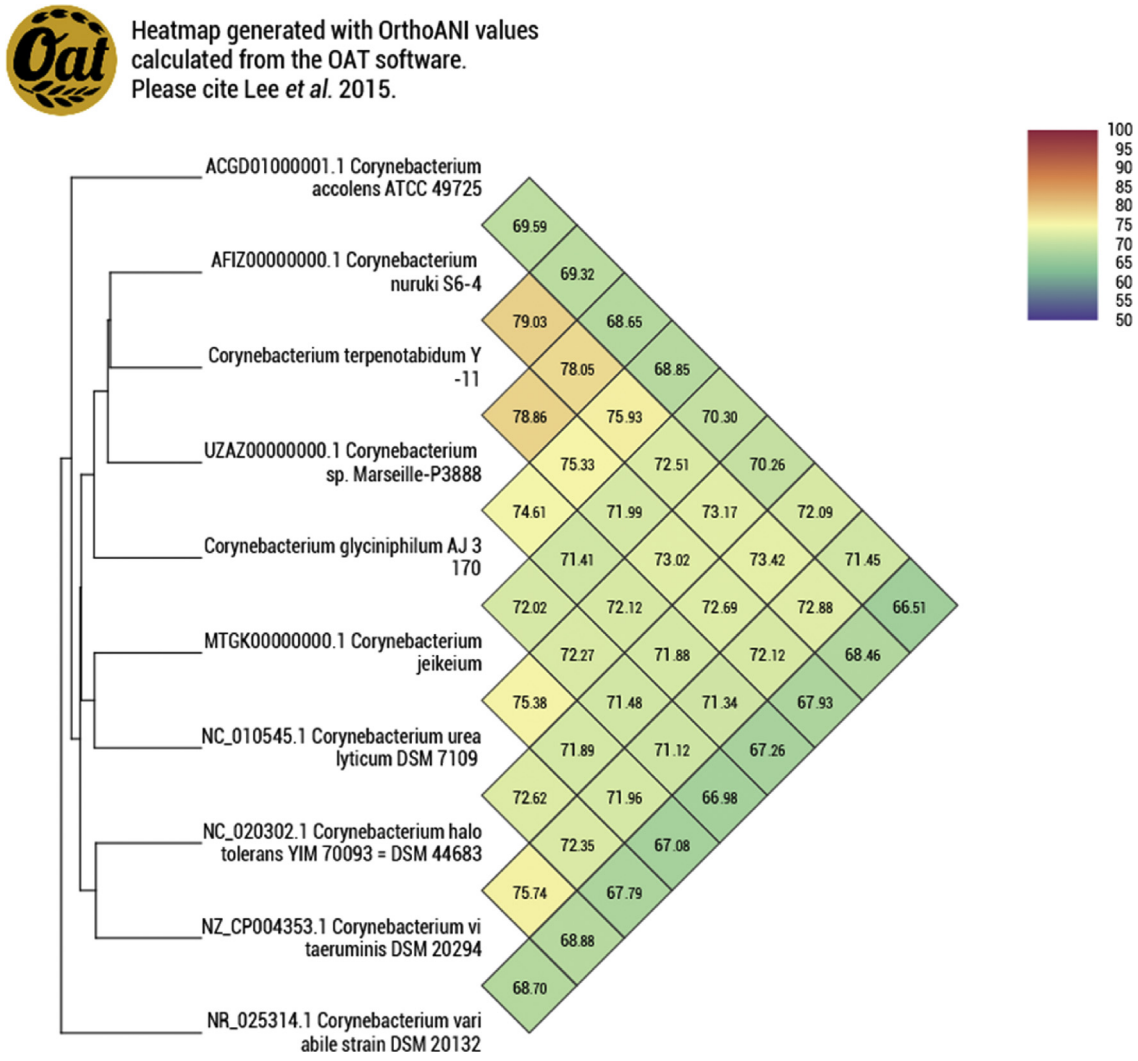

FIG. 4. Heatmap generated with OrthoANI values calculated using the OAT software between *Corynebacterium neomassiliense* strain Marseille-P3888^T and other closely related species with standing in nomenclature.

Conclusion

Strain Marseille-P3888^T, exhibiting a 16S rRNA sequence divergence >98.10% with its phylogenetically closest species with standing in nomenclature, is consequently proposed as the type strain of the new genus *Corynebacterium neomassiliense* sp. nov. (mas.si.li.en'se, L. neut. adj., massiliense for Massilia, the Latin name of Marseille, where the strain was first isolated).

Nucleotide sequence accession number

The 16S rRNA gene and genome sequences were deposited in GenBank under accession numbers LT984641.1 and UZAZ01000001, respectively.

Deposit in culture collections

Strain Marseille-P3888^T was deposited in two different strain collections (numbers = CSURP3888; = CCUG72352).

Conflict of interest

None to declare.

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