NEW SPECIES

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 P3888T 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-P3888T.

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 16S rRNA 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-P3888T 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
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-P3888T 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-P3888T showed activity for the esterase C4, esterase-
Strain identification

The 16S rRNA gene was sequenced to classify this bacterium. Amplification was performed using the primer pair 51F1 and rP2 (Eurogentec, Angers, France) and sequencing used the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xL sequencer (Thermo Scientific, 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. neo-massiliense 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. neo-massiliense (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. neo-massiliense 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 1.** Fatty acid profiles (%) of Corynebacterium neo-massiliense 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-Octadecadecanoic acid| TR                |
| 16:1n9               | 7-Hexadecenoic acid       | TR                |

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

**TABLE 2.** Phenotypic characterization of Corynebacterium neo-massiliense 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 – salcin           | –       |
| Acidification – xylene           | –       |
| Gelatin hydrolysis               | –       |
| Acidification – xylase           | –       |
| Acidification – cellobiose       | –       |
| Acidification – mannose          | –       |
| Acidification – melatose         | –       |
| Acidification – raffinose        | –       |
| Acidification – sorbitol         | –       |
| Acidification – rhamnose         | –       |
| Acidification – trehalose        | +       |
| Alkaline phosphatase             | –       |
| Esterase (C4)                    | +       |
| Esterase lipase (C8)             | +       |
| Lipase (C14)                     | +       |
| Leucine arylamidase              | –       |
| Valine arylamidase               | –       |
| Cysteine 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-D-glucosaminidase     | –       |
| α-Mannosidase                    | –       |
| α-Fructosidase                   | –       |

| **API ZYM**                      |         |
| Alkaline phosphatase             | –       |
| Esterase (C4)                    | +       |
| Esterase lipase (C8)             | +       |
| Lipase (C14)                     | +       |
| Leucine arylamidase              | –       |
| Valine arylamidase               | –       |
| Cysteine 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-D-glucosaminidase     | –       |
| α-Mannosidase                    | –       |
| α-Fructosidase                   | –       |
TABLE 3. Study of carbohydrate metabolism of strain Marseille-P3888 using API 50 CH strips

| Test    | Characteristics | Results       | Characteristics | Results |
|---------|-----------------|---------------|-----------------|---------|
| 50 CH   | Glycerol        | Esculin ferric citrate | –              | –       |
|         | Erythritol      | Salicin       | –              | –       |
| C. arabinose | –             | O-cellobiose  | –              | –       |
| C. arabinose | –             | O-maltose     | –              | –       |
| C. ribose  | –              | O-lactose     | –              | –       |
| C. xylose  | –              | O-melibiose   | –              | –       |
| C. xylitol | –              | O-saccharose  | –              | –       |
| C. adonitol | –             | O-trehalose   | –              | –       |
| Methyl β-D-xylopyranoside | –          | Inulin         | –              | –       |
| C. galactose | –             | O-melezitose  | –              | –       |
| C. glucose  | –              | O-caffeine    | –              | –       |
| C. fructose | +              | Amidon        | –              | –       |
| C. mannose | –              | Glycogen      | –              | –       |
| C. sorbose | –              | Xylitol       | –              | –       |
| C. rhamnose | –             | Gentiobiase   | –              | –       |
| Dulcitol  | –              | O-turanose    | –              | –       |
| Inositol  | –              | O-xylene      | –              | –       |
| C. mannositol | –           | O-taglucose   | –              | –       |
| C. arabinol | –             | O-fucose      | –              | –       |
| Methyl β-D-mannopyranoside | –       | O-arabinol    | –              | –       |
| Methyl β-D-glucopyranoside | –       | O-arabinol    | –              | –       |
| N-acetylglucosamine | –        | O-arabinol    | –              | –       |
| Amygdalin  | –              | Potassium     | –              | –       |
| Arbutin   | –              | Glucuronate   | –              | –       |
|          |                | 2-ketogluconate| Potassium       | –       |
|          |                | 5-ketogluconate| –              | –       |

Genomic 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/mm² 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.

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 with closely related species was estimated using the OrTHOANI software [15]. Values among closely related species (Fig. 4) ranged from 66.51% 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

| Phenotypic Criteria                      | *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                                 |                                  |                                  |                        | NA                            | NA                          |
| Endospore formation                      |                                  |                                  |                        | NA                            | NA                          |
| Alkaline phosphatase                     | +                                | +                               | +                      | +                             | +                           |
| Catalase                                 |                                  |                                  |                        | NA                            | NA                          |
| Oxidase                                  |                                  |                                  |                        | NA                            | NA                          |
| Nitratreductase                          |                                  |                                  |                        | NA                            | NA                          |
| Urease                                   | +                                | +                               | +                      | +                             | +                           |
| *β*-galactosidase                        |                                  |                                  |                        | NA                            | NA                          |
| N-acetylglucosamine                      |                                  |                                  |                        | NA                            | NA                          |
| *α*-arabinose                            |                                  |                                  |                        | NA                            | NA                          |
| *γ*-arabinose                            |                                  |                                  |                        | NA                            | NA                          |
| Esterase lipase (C8)                     |                                  |                                  |                        | NA                            | NA                          |
| Mannose                                  |                                  |                                  |                        | NA                            | NA                          |
| Mannitol                                 |                                  |                                  |                        | NA                            | NA                          |
| *α*-sorbitol                             |                                  |                                  |                        | NA                            | NA                          |
| *α*-glucose                              |                                  |                                  |                        | NA                            | NA                          |
| *α*-fructose                             |                                  |                                  |                        | NA                            | NA                          |
| *α*-maltose                              |                                  |                                  |                        | NA                            | 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.
Conclusion

Strain Marseille-P3888<sup>T</sup>, 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.sil.i.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<sup>T</sup> was deposited in two different strain collections (numbers = CSURP3888; = CCUG72352).

Conflict of interest

None to declare.

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