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Description of *Gracilibacillus phocaeensis* sp. nov., a new halophilic bacterium isolated from Senegalian human stool

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Abstract

Using the taxonogenomics method, we describe *Gracilibacillus phocaeensis* strain Marseille-P3801, a new species previously isolated from a salty stool of a 20-year-old man from N'Diop, Senegal. It is a Gram-positive, aerobic and motile bacillus. The major fatty acids are $C_{15:0\text{-anteiso}}$ (59%), $C_{16:0}$ (16%) and $C_{17:0\text{-anteiso}}$ (11%). Strain Marseille-P3801 exhibits a 98.45% sequence similarity with *Gracilibacillus thailandensis* strain TP2-8, the phylogenetically closest species. Its genome is 4.66 Mb with 39.6 mol% G + C content.

Keywords: Culturomics, genome, *Gracilibacillus phocaeensis* sp. nov., gut, taxonogenomics
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Introduction

The genus *Gracilibacillus* has been described as moderately halophilic, motile endospore-forming bacteria [1]. Moderately halophilic bacteria have been found in a variety of fermented foods; indeed, one of the most important food preservation methods in history has been the use of salt [2]. Salt is the main source of sodium in our diet. Some gut bacteria, such as *Lactobacillus*, are highly sensitive to salt [3]. However, it has been demonstrated to favour the emergence and growth of other, mainly halophilic, bacteria, including *Gracilibacillus* [4]. Halophilic and halotolerant bacteria are most commonly isolated from the human gut microbiota [5,6].

The culturomics approach, which is based on the multiplication of culture conditions (variation of media, temperature and atmosphere) with a more rapid bacterial identification by matrix-assisted desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) [7] was used to explore the human gut halophilic microbiota. Our culturomic approach included the use of high-salt-containing culture media, which allowed us to isolate a new moderately halophilic bacterial strain, Marseille-P3801T, which belongs to the genus *Gracilibacillus* [8]. The genus *Gracilibacillus* currently compromises 13 species with valid standing in nomenclature [9]. *Gracilibacillus* species were isolated from diverse salty environmental samples, including seawater, salty lakes [4,10,11], soil [12,13], food [1,14,15] and gut microbiota [6].

Various parameters, including phenotypic and genotypic characteristics, such as DNA-DNA hybridization, are used to define a new species, but they have certain limitations [16]. In our study, we sought to get around these limitations by using a taxonogenomic approach that includes phenotypic characteristics, proteomic information obtained by MALDI-TOF MS and analysis of the complete genome sequence. From this information, we were able to construct a complete description of a new halophilic species, *G. phocaeensis,* with type strain Marseille-P3801T (= CSUR P3801).

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Materials and methods

Bacterial strains and growth conditions

Strain Marseille-P3801T was isolated from stool of a 20-year-old man from N'Diop, Senegal. The study was approved by the ethics committee of the Institut Hospitalo-Universitaire Méditerranée Infection (approval 2016-011), and the patient provided written informed consent.

The percentage of salt in the stool sample was determined using a salinity refractometer (Thermo Fisher Scientific, Villebon-sur-Yvette, France) by diluting 1 g of NaCl in 10 mL of distilled water and centrifuging it for 10 minutes at 5000*g*. For a second run, 100 µL of supernatant was deposited in the refractometer; the results were in a straight line, displayed on screen as per-mille values, then reported in percentage of NaCl. To culture the bacteria from stool samples, we used an aerobic blood culture bottle (Becton Dickinson, Le Pont-de-Claix, France) containing a halophilic medium prepared in a modified Columbia broth (Sigma-Aldrich, Saint-Quentin-Fallavier, France), by adding (per litre): 1% (w/v) $MgSO_4$, 0.1% (w/v) $MgCl_2$, 0.4% (w/v) KCl, 0.1% (w/v) $CaCl_2$, 0.05% (w/v) $NaHCO_3$, 0.2% (w/v) of glucose, 0.5% (w/v) of yeast extract (Becton Dickinson) and 10–15% (w/v) NaCl according to the required salinity, with pH adjusted to 7.5. This was incubated for 3 days in an aerobic atmosphere at 37°C [8]. All strains were first isolated in a halophilic culture medium with 15% (w/v) NaCl.

The initial growth of colonies on agar was obtained after 24 hours' incubation at 37°C under aerobic conditions. The oxygen requirement was evaluated by incubating strain Marseille-P3801T under aerobic, microaerophilic and anaerobic conditions using AnaeroGen (Atmosphere Generation Systems, Dardilly, France) at 37°C. The isolated colonies were identified using MALDI-TOF MS as previously described [17]. For the unidentified colonies, the 16S ribosomal RNA (rRNA) gene was sequenced, and the obtained sequence was matched against the National Center for Biotechnology Information (NCBI) database using the BLAST algorithm [18].

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene sequence of the strain was determined for subsequent phylogenetic analysis. The genomic DNA of the strain was amplified by PCR using the primer pair fD1 and rP2 (Eurogentec, Angers, France) [19] and sequenced with the MiSeq Technology (Illumina, San Diego, CA, USA) as previously described [20]. The 16S rRNA nucleotide sequences were assembled and corrected using CodonCode Aligner software (https://www.codoncode.com/). A BLAST (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi) search was further performed against the GenBank nucleotide collection. If the 16S rRNA sequence similarity value was lower than 98.65% with the most closely related species with standing in nomenclature, as proposed by Stackebrandt [21], the strain was proposed as belonging to a new species [22].

Phenotypic and biochemical characteristics

The morphology of strain Marseille-P3801T was revealed by negative staining observed with a Hitachi SU5000 scanning electron microscope (Hitachi Group, Krefeld, Germany) and Gram staining observed on a Leica DM2500 photonic microscope (Leica Microsystems, Nanterre, France) with a 100× oil-immersion objective. Sporulation, motility catalase and oxidase were tested, as previously reported [23,24]. To determine the optimal growth conditions, strain Marseille-P3801 was cultivated in Müller-Hinton agar (Sigma-Aldrich) by varying the NaCl concentrations (from 5% to 20% (w/v)) as well as the pH (5, 5.5, 6, 6.5, 7, 7.5 and 8). It was also seeded on 5% sheep's blood–enriched Columbia agar (bioMérieux, Marcy l'Etoile, France) and incubated under different temperatures (25, 28, 37, 45 and 55°C). API 50 CH, ZYM and 20 NE test strips (bioMérieux) were used according to the manufacturer's instructions to study the carbohydrate metabolism, enzyme activity and biochemical characteristics of strain Marseille-P3801.

Cellular fatty acid methyl ester (FAME) analysis was performed by gas chromatography/mass spectrometry (GC/MS). Two samples were prepared with approximately 90 mg of bacterial biomass per tube, collected from several culture plates. FAMEs were prepared as described by Sasser [25]. GC/MS analyses were carried out as previously described [26]. Briefly, FAMEs were separated using an Elite 5-MS column and monitored by MS (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 (NIST), Gaithersburg, MD, USA) and the FAME MS database (Wiley, Chichester, UK).

Extraction, sequencing and assembly of genome

Genomic DNA of *Gracilibacillus phocaeensis* was extracted in two steps; first a mechanical treatment was performed by washing with glass beads and acid (G4649-500g; Sigma-Aldrich) using a FastPrep BIO 101 instrument (Qbiogene, Strasbourg, France) at maximum speed (6.5 m/s) for 90 seconds. After 30 minutes' incubation of the lysozyme at 37°C, the DNA was extracted by the EZ1 biorobot (Qiagen, Germantown, MD, USA) with the EZ1 DNA tissue kit. The elution volume was 50 µL. Genomic DNA was evaluated with a Qubit test (Life Technologies, Carlsbad, CA, USA). The mate pair library was prepared with 1.5 µg of genomic DNA using the Nextera mate pair Illumina

guide. The DNA sample was simultaneously fragmented and tagged with a mate pair junction adapter. The pattern of fragmentation was validated on an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) with a DNA LabChip 7500 kit. The DNA fragments' sizes ranged from 1.5 to 11 kb, with an optimal size of 8.10 kb. No size selection was performed, and 600 ng of tagmented fragments were circularized. The circularized DNA was mechanically sheared into small fragments with an optimal of 1086 bp with a Covaris S2 device in T6 tubes (Covaris, Woburn, MA, USA). The library profile was visualized on a High Sensitivity Bioanalyzer LabChip kit (Agilent), and the final concentration library was measured at 31.31 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. Automated cluster generation and sequencing run were performed in a single 39-hour run with a 2 × 251 bp read length. Total information of 8.2 Gb was obtained from a 932K/mm^2 cluster density, with a cluster passing quality control filters of 91%. Within this run, the index representation for *Gracilibacillus phocaeensis* was determined at 13.20%. The 2<thinsp>141<thinsp>870 paired-end reads were filtered according to the read qualities.

The assembly was performed with a pipeline incorporating different software, including Velvet [27], Spades [28] and SOAPdenovo2 [29] on trimmed (Trimmomatic) [30] or raw data. To reduce assembly gap, GapCloser software was used. Scaffolds less than 800 bp and scaffolds with a depth value of <25% of the mean depth were discarded. The best assembly was selected using several different criteria (number of scaffolds, N50, number of N).

Genome annotation and comparisons

Open reading frames were predicted using the Prodigal tool (http://prodigal.ornl.gov) with default parameters. Transfer RNAs (tRNAs) and rRNAs were detected using tRNAscan-SE v.1.2129 and RNAmmer v.1.230 respectively [31,32]. The protein sequence annotation was performed on the NCBI GenBank nonredundant protein sequence database (nr) using BLAST protein with an *E* value of 1e-03 as the significance thresholds [33]. We then obtained the functional classification of gene families (Clusters of Orthologous Groups (COGs) database ID and letters) by using eggNOG against the COGs database [34]. The genome of *Gracilibacillus phocaeensis* strain Marseille-P3801T (EMBL EBI accession no. UZBG00000000) was compared with that of *Gracilibacillus boraciitolerans* strain JCM 21714 (BAVS00000000), *Gracilibacillus massiliensis* strain Awa-1T (CZRP00000000), *Gracilibacillus lacisalsi* DSM 19029 (ARIY00000000), *Gracilibacillus ureilyticus* CGMCC (FOGL00000000), *Gracilibacillus dipsosauri* (QGTD00000000) and *Halobacillus karajensis* DSM 14948 (FNWW00000000) using OrthoANI software [35].

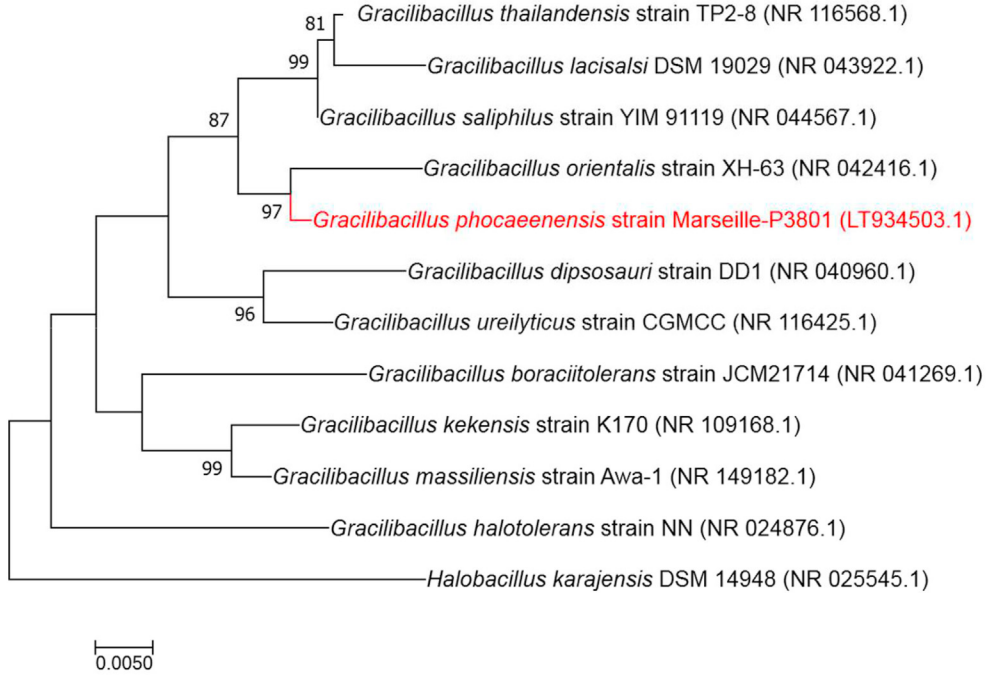

FIG. 1. Phylogenetic tree highlighting phylogenetic position of *Gracilibacillus phocaeensis* strain Marseille-P3801T relative to other phylogenetically close members of family *Bacillaceae*. Sequences were aligned using Clustal W; phylogenetic inferences were obtained using maximum likelihood method within MEGA 7 software. Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 500 times to generate majority consensus tree.

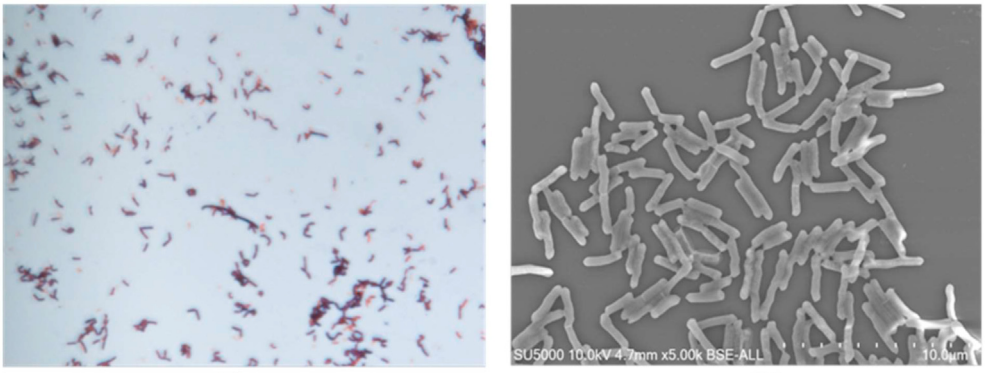

FIG. 2. (a) Gram staining of *Gracilibacillus phocaeensis* strain Marseille-P3801T. (b) Morphologic structure of *G. phocaeensis* strain Marseille-P3801T obtained with Hitachi SU5000 (Hitachi Group, Krefeld, Germany) scanning electron microscope. Scale bar and acquisition settings are shown.

Results

Strain identification and phylogenetic analysis

Strain Marseille-P3801T formed yellow colonies after 1 day's culture on agar with horse's blood, ranging from 2% to 20% (w/v) NaCl (optimum at 7.5% (w/v)) at 37°C. The spectrum resulting from the eight pure colonies of strain Marseille-P3801 deposited on MALDI-TOF MS target plate did not allow the identification of this bacterium because there was no spectrum match with those in the Bruker database (Supplementary Fig. S1). Using the 16S rRNA sequence of *G. phocaeensis* (LT934503.1), phylogenetic analysis revealed that strain Marseille-P3801 exhibited a sequence similarity of 98.39% with *Gracilibacillus thailandensis* strain TP2-8 (GenBank accession no. NR_116568.1), the phylogenetically closest species with standing in nomenclature (Fig. 1). Therefore, we classified this strain as a member of a new species within the genus *Gracilibacillus*, family *Bacillaceae*, and *Firmicutes*. This value was lower than the 98.7% 16S rRNA gene sequence threshold advised by Meier-Kolthoff *et al.* [36] to delineate a new species without carrying out DNA-DNA hybridization. Classification and general features of

TABLE 1. **Differential characteristics of 1, *Gracilibacillus phocaeensis* strain Marseille-P3801 compared with other close bacteria of the genus *Gracilibacillus:* 2, *G. timonensis* strain Marseille-P2481 [6]; 3, *G. massiliensis* strain Marseille-P1441 [15]; 4, *G. alcaliphilus* strain SG103 [38]; 5, *G. saliphilus* strain YIM 91119 [39]; 6, *G. orientalis* strain XH-63 [40]; 7, *G. halophilus* strain YIM-C55.5 [13]**

Property	1	2	3	4	5	6	7
Cell diameter (μm)	0.3–0.6	0.5–0.8	0.3–1.8	0.5–0.7	0.7–0.9	0.7–0.9	0.3–0.5
Pigmentation	Yellow	Creamy orange	White	Creamy white	Creamy white	Creamy	White
Oxygen requirement	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
Gram stain	+	+	+	+	+	+	+
Salt requirement	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+
Sporulation	+	+	—	+	+	+	+
Indole	—	—	—	—	—	—	—
Production of:							
Alkaline phosphate	—	—	—	—	+	NA	+
Catalase	+	+	+	+	+	+	+
Oxidase	+	—	—	—	+	—	+
Nitrate reductase	—	—	—	+	+	—	+
Urease	+	+	+	+	+	—	—
β-Galactosidase	+	+	—	NA	+	NA	+
α-Galactosidase	—	—	+	—	—	NA	—
N-Acetyl-glucosamine	—	—	—	+	+	NA	—
Acid from:							
L-Arabinose	—	—	—	+	+	+	—
D-Mannose	—	—	—	—	+	—	—
D-Mannitol	—	—	—	+	+	+	+
D-Glucose	—	—	—	+	+	+	+
D-Fructose	—	—	—	+	+	+	+
D-Maltose	—	—	—	+	+	+	—
D-Lactose	—	—	—	+	+	+	—
DNA G + C content (mol%)	39.6	39.8	36.05	41.3	40.1	37.1	42.3
Habitat	Human gut	Human gut	Cooking salt	Fermentation liquor	Salt lake	Salt lake	Salt soil

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

TABLE 2. Phenotypic characterization of *Gracilibacillus phocaeensis* sp. nov. strain Marseille-P3801, based on analytical profile index (API) tests

Characteristic	Result
API ZYM	
Alkaline phosphatase	—
Esterase (C4)	+
Esterase lipase (C8)	+
Lipase (C14)	+
Leucine arylamidase	+
Valine arylamidase	—
Cystine arylamidase	—
Trypsin	—
α-Chymotrypsin	—
Acid phosphatase	+
Naphthol-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	—
API 50 CH	
Glycerol	—
Erythritol	—
D-Arabinose	—
L-Arabinose	—
D-Ribose	—
D-Xylose	—
L-Xylose	—
D-Adonitol	—
Methyl-βD-xylopyranoside	—
D-Galactose	—
D-Glucose	—
D-Fructose	—
D-Mannose	—
L-Sorbose	—
L-Rhamnose	—
Dulcitol	—
Inositol	—
D-Mannitol	—
D-Sorbitol	—
Methyl-αD-mannopyranoside	—
Methyl-αD-glucopyranoside	—
N-Acetyl-glucosamine	—
Amygdalin	—
Arbutin	—
Esculin ferric citrate	+
Salicin	—
D-Cellobiose	—
D-Maltose	—
D-Lactose	—
D-Melibiose	—
Sucrose	—
D-Trehalose	—
Inulin	—
D-Melezitose	—
D-Raffinose	—
Starch	—
Glycogen	—
Xylitol	—
Gentiobiose	—
D-Turanose	—
D-Lyxose	—
D-Tagatose	—
D-Fucose	—
L-Fucose	—
D-Arabitol	—
L-Arabitol	—
Potassium gluconate	—
Potassium 2-ketogluconate	—
Potassium 5-ketogluconate	—

+, positive result; −, negative result.

strain Marseille-P3801 are summarized in Supplementary Table S1.

Physiologic and biochemical characteristics

G. phocaeensis sp. nov. strain Marseille-P3801T (= CSUR P3801) is Gram positive. Colonies are yellow and circular, with a mean diameter of 2 mm after 2 to 3 days' growth on 5% sheep's blood–enriched Columbia agar medium (bioMérieux). Bacterial cells of strain Marseille-P3801 were motile, rod shaped and polymorphic (Fig. 2).

The major fatty acids were saturated structures, mainly branched ones: 12-methyl-tetradecanoic acid (59%), hexadecanoic acid (16%) and 14-methyl-hexadecanoic acid (11%) (Supplementary Table S2). Other saturated and branched fatty acids were also described. 7-Hexadecenoic acid was the only unsaturated structure detected. Catalase and oxidase were positive. Using API ZYM strips, positive reactions were detected for lipases (C4, C8 and C14), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase; however, no reaction was observed for alkaline phosphatase, valine, cysteine arylamidase, α-chymotrypsin, α-galactosidase, trypsin, β-glucuronidase, α-mannosidase and α-fucosidase. The API 20 NE strip indicated positive reactions of fermentation of glucose, urease activity and metabolism of L-arginine and esculin. In contrast, negative reactions were observed for nitrate and indole production as well as metabolism of D-glucose, L-arabinose, D-mannose, D-maltose, D-mannitol, *N*-acetyl-glucosamine, potassium gluconate, capric acid, malic acid and phenylacetic acid. Using the API 50 CH strip, strain Marseille-P3801T exhibited esculin hydrolysis and negative reactions for D-galactose, D-lactose, D-maltose, D-ribose, D-saccharose, D-lyxose, D-mannose, L-sorbose, D-tagatose, D-turanose, D-xylose, L-xylose, D-arabinose, L-arabinose, D-sorbitol, D-cellobiose, D-melezitose, D-melibiose, D-trehalose, D-raffinose, D-arabitol, L-arabitol, D-glucose, D-fructose, D-fucose, L-rhamnose, D-adonitol, D-mannitol, L-fucose, amygdalin, arbutin, erythritol, dulcitol, gentiobiose, glycerol, glycogen,

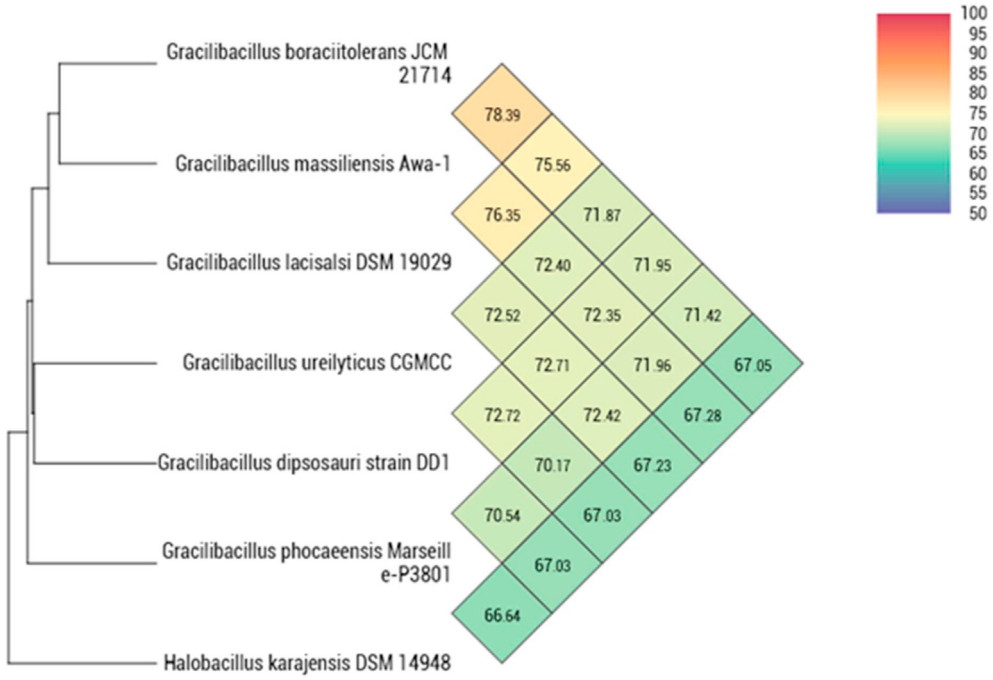

FIG. 3. Heat map generated with OrthoANI values calculated by OAT software between *Gracilibacillus phocaeensis* sp. nov. strain Marseille-P3801 and other closely related species with standing in nomenclature.

inositol, inulin, salicin, starch, xylitol, αD-glucopyranoside, methyl-βD-xylopyranoside, methyl-αD-mannopyranoside, potassium gluconate and *N*-acetylglucosamine.

The differential characteristics of *Gracilibacillus phocaeensis* with respect to other bacteria related to the genus *Gracilibacillus* are outlined in Table 1. Phenotypic characterization of *Gracilibacillus phocaeensis* sp. nov. based on the analytical profile index (API) tests we performed is summarized in Table 2.

Genome properties

The genome length of *Gracilibacillus phocaeensis* strain Marseille-P3801T is 4.66 Mb encompassing 12 scaffolds (11 contigs). The G + C content is 39.6 mol%. Among the 4390 predicted genes, 4255 were protein-coding genes, 67 were RNAs (11 rRNA, 52 tRNAs, four noncoding RNAs) and 68 were pseudogenes. The BLASTp annotation of *G. phocaeensis* strain Marseille-P3801 assigned a putative function to 3606 genes, and 649 genes were annotated as being hypothetical proteins. For further

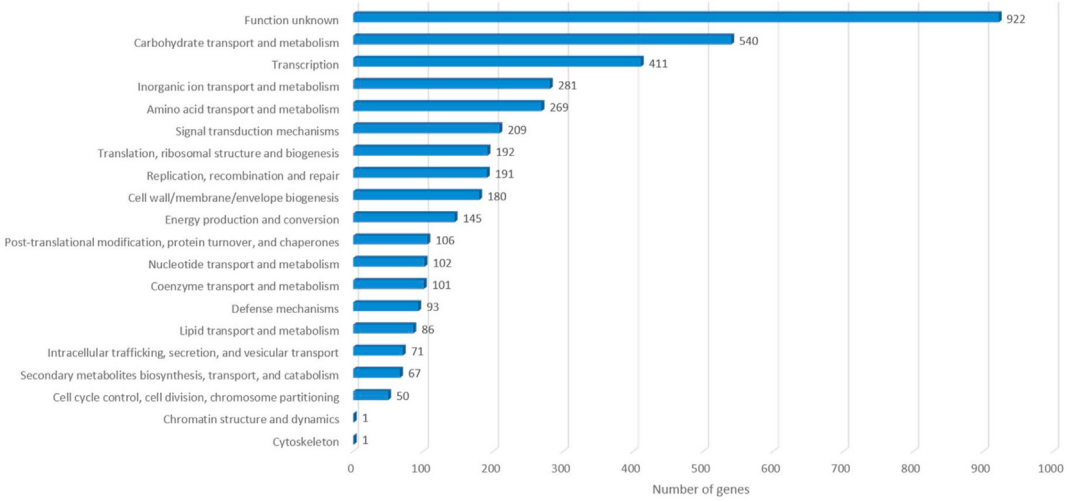

FIG. 4. Distribution of functional classes of predicted genes according to Clusters of Orthologous Groups (COGs) database of proteins of *Gracilibacillus phocaeensis* strain Marseille-P3801.

insight into gene functions, we compared the *G. phocaeensis* protein sequences with sequences in the COGs database. Of the 4390 protein-encoding genes we found, 3774 were assigned to a COGs function (86%) distributed among 20 COGs categories (Supplementary Fig. S2).

Genome comparison

To explore the genomic similarity of *G. phocaeensis* with closely related bacteria, we performed analysis using OrthoANI. Among closely related species, we found OrthoANI values ranging from 66.64% between *Gracilibacillus phocaeensis* strain Marseille-P3801 and *Halobacillus karajensis* DSM 14948, to 78.39% between *Gracilibacillus boraciitolerans* strain JCM 21714 and *Gracilibacillus massiliensis* strain Awa-1. When *Gracilibacillus phocaeensis* strain Marseille-P3801 was compared with these closely related species, we found values ranging from 66.64% with *Halobacillus karajensis* DSM 14948, to 72.42% with *Gracilibacillus lacisalsi* strain DSM 19029 (Fig. 3). Fig. 4 provides representations of the genome of strain Marseille-P3801 and its genes organized into functional categories.

Description of *Gracilibacillus phocaeensis*

Gracilibacillus phocaeensis (pho.ca.een'sis, N.L. masc. adj., from *phocaeensis*, related to the Phocaeans, the founders of Marseille, France, where the type strain was isolated and characterized, like many other species). It is a Gram-positive, motile and aerobic bacterium. The colonies are yellow and circular, with a mean diameter of 2 mm. Bacterial cells were rod shaped and polymorphic. Strain Marseille-P3801 grows at an optimal temperature of 37°C, pH 7, with 7.5% (w/v) NaCl. It is catalase and oxidase positive. Positive reactions were observed for esterase (C4), esterase lipase (C8), acid phosphatase, naphthol-AS-BI-phosphohydrolase, leucine arylamidase, β-galactosidase, α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase. *G. phocaeensis* strain Marseille-P3801T was isolated from a stool sample from a 20-year-old man from N'Diop, Senegal. This strain exhibited a G + C content of 39.6 mol%. Its 16S rRNA sequence was deposited in GenBank under accession number LT934503, and the whole genome shotgun sequence was deposited in GenBank under accession number UZBG00000000.

Discussion and conclusion

The concept of microbial culturomics, which is based on varying the physicochemical parameters of culture conditions, permits us to explore the microbial diversity of different ecosystems, such as gut microbiota [7]. Microbial culturomics provides culture conditions that simulate, reproduce or mimic all the selective constraints that have shaped the natural microbiota for millions of years. Many new bacterial species have been discovered, particularly those belonging to the *Bacillales* order, which is one of the most represented bacterial orders [37]. To explore the halophilic microbiota of the human gut, the use of culture media with a high salt content allowed us to isolate a new moderately halophilic bacterial strain, Marseille-P3801T, which belongs to the genus *Gracilibacillus* [8]. To our knowledge, this is the second *Gracilibacillus* species described to be isolated from the human gut. On the basis of its phenotypic, phylogenetic and genomic characteristics, this strain is proposed to represent a novel species in the genus *Gracilibacillus*, for which we propose the name *Gracilibacillus phocaeensis* sp. nov., with Marseille-P3801T as the type strain.

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Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nmni.2020.100799.

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