Noncontiguous finished genome sequences and descriptions of *'Paenibacillus bouchesdurhonensis,' 'Paenibacillus rubinfantis,' 'Paenibacillus senegalimassiliensis'* and *'Paenibacillus tuaregi'* identified by culturomics

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Abstract

Microbial culturomics represents a completely new approach to investigate microbial diversity by using different optimized culture conditions, mass spectrometry, genome sequencing and annotation and phenotypic description that allow for an extensive characterization of new species and the study of the human microbiome. Here we present four new species within the genus *Paenibacillus*: *'Paenibacillus bouchesdurhonensis'* strain Marseille-P3071^T, *'Paenibacillus rubinfantis'* strain MT18^T, *'Paenibacillus senegalimassiliensis'* strain SIT18^T and *'Paenibacillus tuaregi'* strain Marseille-P2472^T, which are all facultatively aerobic and Gram-positive bacilli.

Keywords: Culturomics, human gut microbiota, new species, *"Paenibacillus bouchesdurhonensis"*, *"Paenibacillus rubinfantis"*, *"Paenibacillus senegalimassiliensis"*, *"Paenibacillus tuaregi"*
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Paenibacillus is a genus of facultatively anaerobic and endospore-forming bacteria, originally included within the genus *Bacillus* and then reclassified as a separate genus in 1993 by Ash *et al.* [1]. This novel classification is based on 16S rRNA gene sequence data and the fact that this group was distinct from other groups which they defined within the *Bacillus* genus. The Latin word *paene* means 'almost,' so the 'paenibacilli' are literally 'almost bacilli.' *Paenibacillus* species have been detected and isolated in a variety of environments, such as soil, water, rhizosphere, vegetable matter and forage or insect larvae, as well as in clinical samples [2–5]. Interest in *Paenibacillus* spp. has been detected and rapidly growing because many bacteria belonging to this genus have been shown to be important for agriculture (e.g. *Paenibacillus polymyxa*) and could have industrial (e.g. *Paenibacillus amylolyticus*) and medical (e.g. *Paenibacillus peoriate*) applications [6–8]. Various *Paenibacillus* spp. also produce antimicrobial substances that affect a wide spectrum of microorganisms [9–11].

Since the creation of the *Paenibacillus* genus in 1993, the genus description was emended by Shida *et al.* in 1997 [12]. To date, the genus comprises 183 species with validly published names with standing in nomenclature (Fig. 1). The development of PCR techniques at the end of the 1980s and now faster genome-sequencing methods have resulted in a significant increase in the number of *Paenibacillus* species identified. In this study, a new approach—microbial culturomics, including genome sequencing, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and main phenotypic characteristics [13–18]—enabled us to identify and describe four new *Paenibacillus* species.

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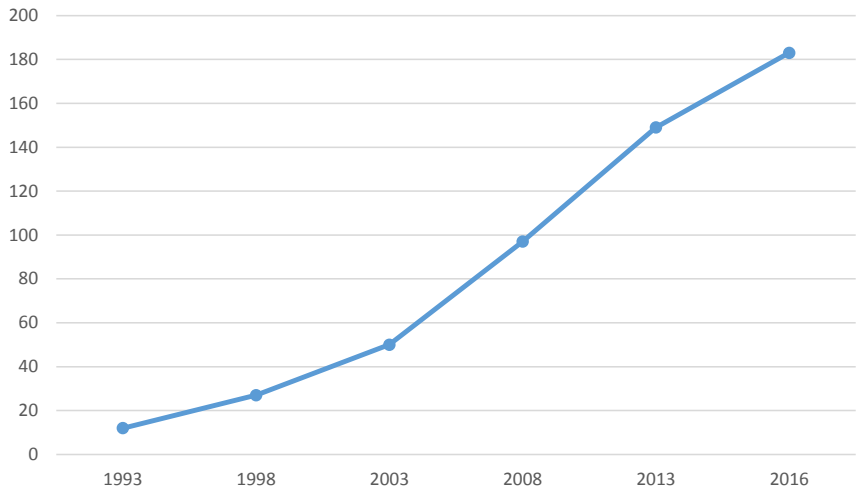

FIG. 1. Identification of *Paenibacillus* new species since recommendation and reclassification of *Paenibacillus* gen. nov. as separate genus by Ash *et al.* in 1993 [1].

'Paenibacillus bouchesdurhonensis' strain Marseille-P3071^T, *'Paenibacillus rubinfantis'* strain MT18^T, *'Paenibacillus senegalimassiliensis'* strain SIT18^T and *'Paenibacillus tuaregi'* strain Marseille-P2472^T are type strains of the corresponding species. They are all Gram-positive bacilli and facultatively anaerobic. Strain Marseille-P3071^T was isolated from a 3.3-month-old Senegalese girl with severe acute malnutrition (marasmus form). She was 70 cm tall and weighted 7 kg with the following anthropometric criteria: weight-for-height z score −1.75 and weight-for-age z score 1.28. Strain MT18^T was isolated from a Nigerian child with severe acute malnutrition (kwashiorkor form) with the presence of oedema. Meanwhile, strain SIT18^T was isolated from a 13-month-old healthy boy in Senegal, and strain Marseille-P2472^T was isolated from a healthy Nigerian girl who was 72 cm tall and weighted 8 kg. The patients' parents provided signed informed consent, and the study was validated by the ethics committee of the Institut Fédératif de Recherche IFR48 under number 09-022 (Table 1). These isolations were part of the culturomics study aimed at exploring microbial diversity using multiple culture conditions [14,15].

For the description of these four new species, we here use a new concept of bacterial description based on proteomics analysis with the MALDI-TOF MS profile [15] combined with phenotypic and genomic descriptions. We thus present a summary classification, as well as the main features and complete genomic sequencing and annotation of *'Paenibacillus bouchesdurhonensis'* strain Marseille-P3071^T (= CSUR P3071 = DSM 103972), *'Paenibacillus rubinfantis'* strain MT18^T (= CSUR P2076 = DSM 101191), *'Paenibacillus senegalimassiliensis'* strain SIT18^T (= CSUR P2144 = CCUG 69869) and *'Paenibacillus tuaregi'* strain Marseille-P2472^T (= CSUR P2472 = DSM 102801).

Material and methods

Strain identification and phylogenetic analysis

The analysed samples were collected and stored at −80°C. Eighteen standard culture conditions were tested on the samples under variable conditions in a dilution series ranging from 1/10 to 1/10^{10} to isolate these strains, as previously described [13]. The samples' origin and conditions of isolation are summarized in Table 1. Blood culture bottles were monitored 1 month after inoculation.

According to the manufacturer's recommendations, identification of isolated colonies was performed using a Microflex LT

TABLE 1. Sample information for *Paenibacillus* species

Characteristic	*'P. bouchesdurhonensis'*	*'P. rubinfantis'*	*'P. senegalimassiliensis'*	*'P. tuaregi'*
Strain	Marseille-P3071^T	MT18^T	SIT18^T	Marseille-P2472^T
Sample origin	Human stool	Human stool	Human stool	Human stool
Patient information	Marasmus, Senegalese girl	Kwashiorkor, Nigerian child	Healthy Senegalese boy	Healthy Nigerian girl
Authorization/consent	No. 09-022 (IFR 48, Marseille)	No. 09-022 (IFR 48, Marseille)	No. 09-022 (IFR 48, Marseille)	No. 09-022 (IFR 48, Marseille)
Storage	−80°C	−80°C	−80°C	−80°C
Isolation conditions	Haemoculture + rumen; day 10 aerobic 37°C	Haemoculture after thermic shock at 80°C during 20 minutes; day 20 aerobic 37°C	Haemoculture + sheep's blood + rumen; day 7 aerobic 37°C	Liquid marine medium; day 15 aerobic 37°C

spectrometer (Bruker Daltonics, Bremen, Germany) and a MSP 96 MALDI-TOF MS target plate (Bruker Daltonics), as previously described [15]. The obtained spectra were imported into MALDI Biotyper 3.0 software (Bruker) and were compared with the computer databases at the Bruker base and the base-specific laboratory at the hospital of La Timone, Marseille, France. We previously updated our database with the spectra of the new bacterial species cultured during our previous study. The resulting score allowed us to identify (or not) the tested species: samples were labeled as correctly identified at the species level with a score of $\geq$2; they permitted identification at the genus level with a score of $\geq$1.7 but <2; and samples provided no identification with a score of <1.7. No significant score was obtained for our strains, thus suggesting that our isolated species were not members of a known species.

Consequently, identification of these strains was realized by 16S rRNA gene amplification and sequencing. For nucleotide sequence analyses, DNA was previously extracted by EZ1 DNA Tissue Kit using BioRobot EZ1 Advanced XL (Qiagen, Courtaboeuf, France). The 16S rRNAgene was amplified by PCR by using universal primers pair fD1 and rP2 (Eurogentec, Angers, France). Sequencing was then realized by using the Big Dye Terminator v1.1 Cycle Sequencing Kit and ABI Prism 3130xl Genetic Analyzer capillary sequencer (Applied Biosystems, Foster City, CA, USA) [16]. The obtained sequences were assembled and corrected by CodonCode Aligner software (http://www.codoncode.com) and were compared with the sequences available in the GenBank database by BLASTn (http://blast.ncbi.nlm.nih.gov.gate1.inist.fr/Blast.cgi). A similarity threshold of <98.7% allowed identification at the species level (new species), whereas a threshold of <95% allowed identification at the genus level (new genus) [19]. All species from the same family of the new species were automatically retrieved by using a custom Python script, which was able to download 16S rRNA gene sequences from the National Center for Biotechnology Information (NCBI), then separate 16S rRNA gene sequences in two groups: group A, containing the sequences of strains from the same genus, and group B, containing the rest. It

TABLE 2. Comparison of phenotypic characteristics among *Paenibacillus* strains

Property	*'P. bouchesdurhonensis'*	*P. lentus*	*'P. rubinfantis'*	*P. barengoltzii*	*'P. senegalimassiliensis'*	*P. sanguinis*	*'P. tuaregi'*	*P. telluris*
Strain	Marseille-P3071^T	GMG12401	MT18^T	SAFN 016	SIT18^T	2301083	Marseille-P2472^T	PS38
Temperature	37°C	35–41°C	37°C	37°C	37°C	30–37°C	37°C	37°C
Atmosphere	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
pH range	6–8.5	6.0–10.0	6–8.5	4.5–9.0	6–8.5	NA	6–8.5	5.0–10.0
Colony aspect	Circular, smooth, crateriform, grey and intact edges	Occur singly or in pairs	Circular, smooth, convex, grey and intact edges	Flat, smooth, circular, entire and brownish yellow	Circular, smooth, flat, grey and intact edges	Translucent, shiny, grey	Irregular form, smooth and grey	Yellow, low convex with irregular edges, translucent and glossy
Cell shape	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped
Cell size (µm)	2.5–3.0 0.5–0.7	1.7–2.3 0.8–0.9	2.0–2.5 0.5–0.7	3.0–5.0 0.5–0.8	1.5–2.0 0.3–0.5	2.0–4.0 0.5	3.5–4.5 0.6–0.8	4.0–5.0 0.7–1.0
Gram stain	Negative	Positive	Negative	Positive	Variable	Positive	Variable	Positive
Salt tolerance (g L^{-1})	0–50	NA	0–50	NA	0–50	NA	0–50	NA
Motility	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
Endospore formation	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Major cellular fatty acid	15:0 anteiso	15:0 anteiso	15:0 anteiso	NA	15:0 anteiso	15:0 anteiso	15:0 anteiso	15:0 anteiso
Production of:								
Alkaline phosphatase	−	NA	−	NA	−	NA	+	+
Catalase	+	+	+	+	−	+	+	+
Oxidase	−	−	−	+	−	−	−	+
Nitrate reductase	−	+	−	+	−	+	−	+
Urease	−	NA	−	NA	−	NA	−	NA
β-Galactosidase	+	NA	+	+	+	NA	+	+
N-Acetyl-glucosamine	−	NA	−	NA	−	−	−	+
Acid from:								
L-Arabinose	+	NA	+	+	+	−	+	+
Ribose	NA	NA	NA	+	NA	NA	NA	NA
Mannose	−	NA	−	+	−	+	−	+
Mannitol	−	+	−	+	−	+	−	+
D-Saccharose	+	NA	+	+	+	NA	+	NA
D-Glucose	+	+	+	−	+	+	+	+
D-Fructose	+	NA	+	+	+	NA	+	NA
D-Maltose	+	NA	+	+	+	NA	+	+
D-Lactose	+	NA	+	+	+	NA	+	NA
Habitat	Human gut	Soil mixture	Human gut	Clean room floors	Human gut	Human blood	Human gut	Soil

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

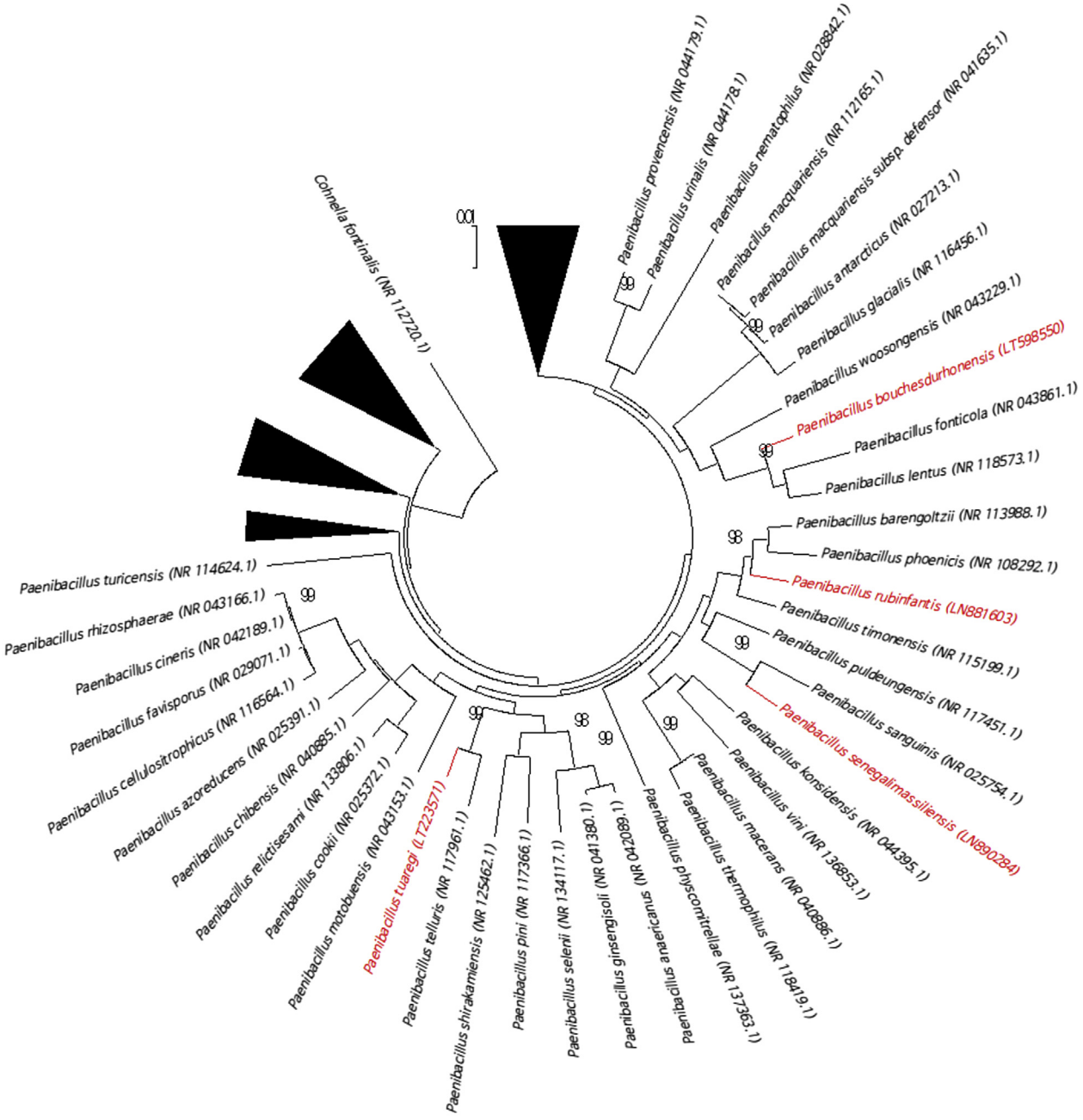

FIG. 2. Phylogenetic tree highlighting position of *'Paenibacillus bouchesdurhonensis'* strain Marseille-P3071^T, *'Paenibacillus rubinfantis'* strain MT18^T, *'Paenibacillus senegalimassiliensis'* strain SIT18^T and *'Paenibacillus tuaregi'* strain Marseille-P2472^T relative to other type strains within *Paenibacillus* genus. Strains and their corresponding GenBank accession numbers for 16S rRNA gene sequences are indicated in brackets. Sequences were aligned using Clustal W (http://www.clustal.org/clustal2/), and phylogenetic inferences were obtained using maximum-likelihood method within MEGA 6 (http://www.megasoftware.net/mega.php). Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 1000 times to generate majority consensus tree. *Cohnella fontinalis* (NR_112720) and *Saccharibacillus kuerlensis* (NR_044389) were used as outgroup. Scale bar = 1% nucleotide sequence divergence.

finally only kept the 48 closest strains from group A and the closest three from group B. Some of the closest species were then selected for each of the four studied strains.

All the spectra have been integrated to the URMITE database (http://www.mediterranee-infection.com/article.php?laref=256&titre=urms-database). The comparison of their proteomic profiles was made between our strains and their closest species.

Phenotypic features

As previously described [17], phenotypic characteristics such as Gram staining, motility, sporulation, and catalase and oxidase activities were tested with these four species. We also determined the ideal growth conditions of our strains by testing five different growth temperature conditions (20, 25, 30, 37 and 45° C) in an aerobic atmosphere with or without 5% CO_2, and under anaerobic and microaerophilic conditions using the GENbag anaer and GENbag microaer systems, respectively (bioMérieux, Marcy l'Etoile, France). To characterize their phenotypic features and observe cell morphology, negative staining was performed.

Biochemical analysis of strains Marseille-P3071^T, MT18^T, SIT18^T and Marseille-P2472^T was carried out using API 50CH, API 20A and API ZYM strips according to the manufacturer's instructions (bioMérieux). Table 2 compare data of our four new species to published data of closely related species: *Paenibacillus lentus* strain GMG 12401 [20], *Paenibacillus telluris* strain P538 [21], *Paenibacillus barengoltzii* strain SAFN 016 [22] and *Paenibacillus sanguinis* strain 2301083 [23].

Fatty acid methyl esters were prepared as described by Sasser [24]. Gas chromatography/mass spectrometry analyses were carried out as previously described [25]. 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 FAMEs mass spectral database (Wiley, Chichester, UK).

Antibiotic susceptibility was tested using the disk diffusion method [26] according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2015 recommendations.

Genome description and comparison

Genomic DNA (gDNA) of all four *Paenibacillus* strains was extracted on the EZ1 biorobot (Qiagen) with EZ1 DNA tissues kit after a 2-hour lysozyme incubation at 37°C. The elution volume was 50 µL. Then gDNA was quantified by a Qubit assay with a high sensitivity kit (Life Technologies, Carlsbad, CA, USA).

Sequencing of gDNA was carried out by the MiSeq Technology (Illumina, San Diego, CA, USA) with the mate-pair strategy. The gDNA was barcoded in order to be mixed with 11 other projects with the Nextera Mate Pair sample prep kit (Illumina). The Nextera mate-pair Illumina guide was used to prepare the mate-pair library. The gDNA sample was simultaneously fragmented and tagged with a mate-pair junction adapter. The pattern of the fragmentation was validated on an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) with a DNA 7500 labchip. The library profile was visualized on a High Sensitivity Bioanalyzer LabChip (Agilent Technologies), and the final concentration library was measured.

Open reading frames (ORFs) were predicted using Prodigal with default parameters (excluding the spanned sequencing gap region) [27]. The predicted bacterial protein sequences were searched against the GenBank [28] and the Clusters of Orthologous Groups database (COGs) databases using BLASTP (*E* value 1e-03, coverage 0.7 and identity percent 30%). On the contrary, if no hit was found, it was searched against the NR database using BLASTP with an *E* value of 1e-03 with a sequence size larger than 80 aa or an *E* value of 1e-05 if the sequence length was smaller than 80 aa; coverage of 0.7 and identity percentage was 30%. The RNAmmer tool [29] was used to find ribosomal RNAs, while tRNA genes were found using tRNAScanSE [30]. Predicting of the lipoprotein signal peptides and the number of transmembrane helices was carried out using Phobius (a combined transmembrane topology and signal peptide predictor) [31]. In addition, the mobile genetic elements were predicted using genome annotation technologies, PHAST (PHAge Search Tool) and RAST (Rapid Annotation using Subsystem Technology) [32,33]. ORFans were identified if the results of all BLASTP performed were positive (E value smaller than 1e-03 or E value smaller than 1e-05 if the sequence length was smaller than 80 aa). Artemis and DNA Plotter were used to generate images of circular and linear DNA maps to display the data management and the visualization of genomic features [34,35]. The Mauve alignment tool (version 2.3.1) was used for multiple genomic sequence alignment [36]. Closest species were identified in the 16S RNA gene sequence tree using Phylopattern software [37] for genomic comparison. At that point, the complete genome sequence, proteome genome sequence and Orfeome genome sequence of each selected genome were retrieved from the NCBI FTP site. Then an annotation of the entire proteome was performed to determine the distribution of functional classes of predicted genes according to the COGs of proteins (same method as for the genome annotation). Annotation and comparison processes were performed by the Multi-Agent software system DAGOBAH including Figenix libraries to provide pipeline analysis [38,39]. Finally, to evaluate the genomic similarity between

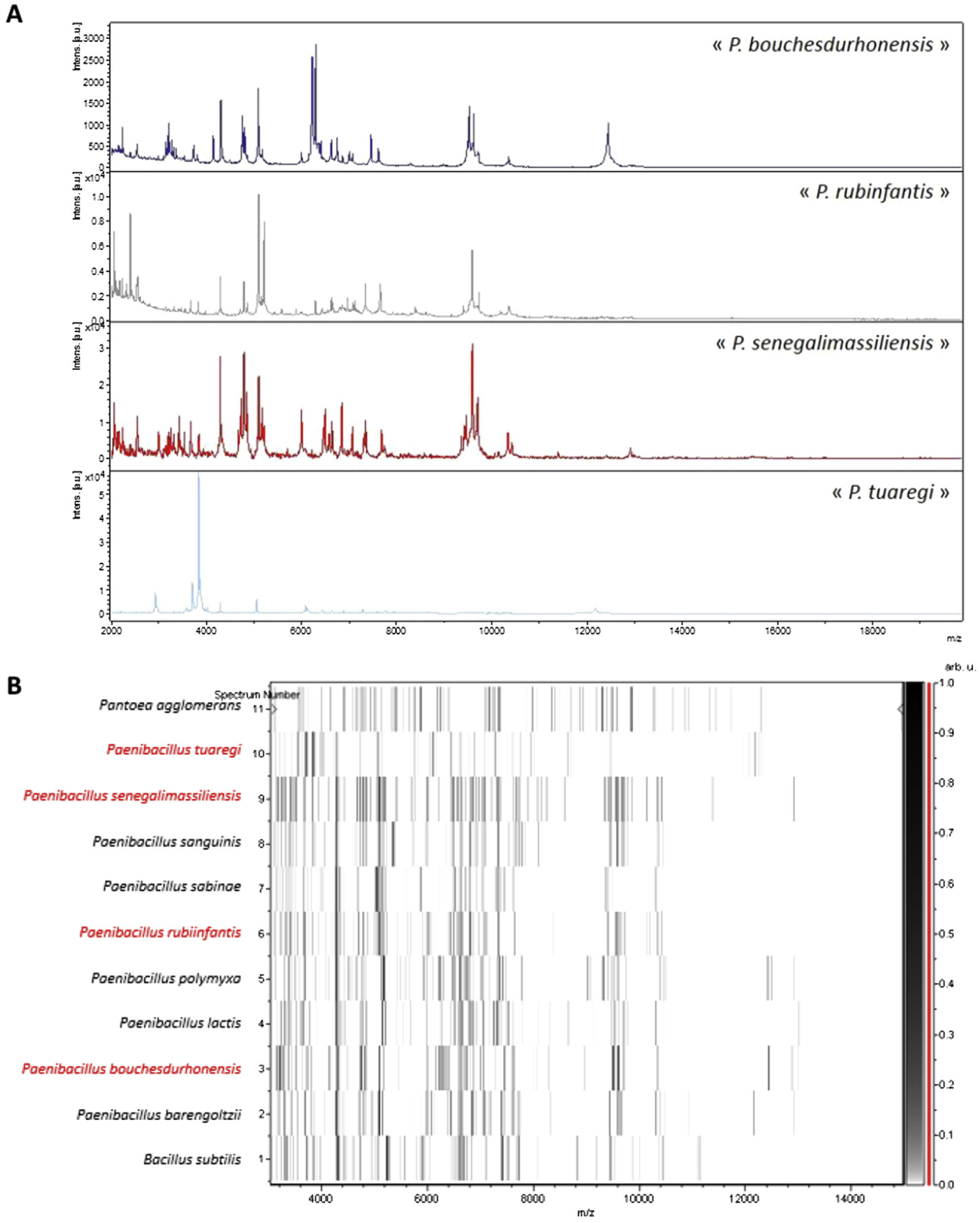

FIG. 3. Reference mass spectra from *'Paenibacillus bouchesdurhonensis'* strain Marseille-P3071^T, *'Paenibacillus rubinfantis'* strain MT18^T, *'Paenibacillus senegalimassiliensis'* strain SIT18^T and *'Paenibacillus tuaregi'* strain Marseille-P2472^T. Spectra from 11 individual colonies were compared. Each reference spectrum generated is displayed (A), and gel views (B) compare *'Paenibacillus bouchesdurhonensis'* strain Marseille-P3071^T, *'Paenibacillus rubinfantis'* strain

studied genomes, two main parameters, digital DNA-DNA hybridization (dDDH)—which exhibits a high correlation with DDH—and average genomic identity of orthologous gene sequences (AGIOS), were determined [19,40]. The AGIOS score was defined as the mean value of nucleotide similarity between all couples of orthologous proteins between the two studied genomes [41].

Results

Strain identification and phylogenetic analysis

The phylogenetic tree of our strains is shown in Fig. 2. Strain Marseille-P3071^T (accession no. LT598550) revealed a 97.75% sequence similarity with the 16S rRNA gene sequence of *Paenibacillus lentus* strain CMG1240^T (accession no. KC800716), the closest species with a validly published name. We therefore suggest that our strain is a representative strain of a new species within the genus *Paenibacillus* for which we suggest the name *'Paenibacillus bouchesdurhonensis'* strain Marseille-P3071^T (= CSUR P3071 = DSM 103972). Strain MT18^T (accession no. LN881603) revealed a 98.65% sequence similarity with the 16S rRNA of *Paenibacillus barengoltzii* strain SAFN-016^T (accession no. AY167814), the closest species with a validly published name. We therefore suggest that our strain is a representative strain of a new species within the genus *Paenibacillus* for which we suggest the name *'Paenibacillus rubinfantis'* strain MT18^T (= CSUR P2076 = DSM 101191). Strain SIT18^T (accession no. LN890284) revealed a 97.63% sequence similarity with the 16S rRNA gene sequence of *Paenibacillus* sanguinis strain 2301083^T (accession no. AY323609), the closest species with a validly published name. We therefore suggest that our strain is a representative strain of a new species within the genus *Paenibacillus* for which we suggest the name *'Paenibacillus senegalimassiliensis'* strain SIT18^T (= CSUR P2144 = CCUG 69869). Strain Marseille-P2472^T (accession no. LT223571) revealed a 96.9% sequence similarity with the 16S rRNA gene sequence of *Paenibacillus telluris* strain PS38^T (accession no. HQ257247), the closest species with a validly published name. We therefore suggest that our strain is a representative strain of a new species within the genus *Paenibacillus* for which we suggest the name *'Paenibacillus tuaregi'* strain Marseille-P2472^T (= CSUR P2472 = DSM 102801).

The analysis of the gel view (Fig. 3) shows that all the profiles of our studied strains have similar general characteristics with the other *Paenibacillus* species used for the comparison. Furthermore, the outsider species *Bacillus subtilis* and *Pantoea agglomerans* profiles show several unique differences.

Phenotypic features

The main phenotypic results of each studied strain are listed in Table 2. All the observations were permitted by Gram staining and electronic microscopy (Fig. 4), which revealed that our four new species had similar morphology. All four bacterial species were rod shaped, aerobic or facultatively anaerobic, and could form endospores with Gram-positive results.

All the results of biochemical analysis of the four strains Marseille-P3071^T, MT18^T, SIT18^T and Marseille-P2472^T, carried out using API 50CH, API 20A, API ZYM strips (bioMérieux), are detailed in Supplementary Tables S2–S4.

The cellular fatty acid composition of our strains is listed in Table 3. We observed that the majority cellular fatty acid of the all the presented *Paenibacillus* strains is 12-methyl-tetradecanoic acid, as for the other paenibacilli strains (Table 2). All these observations, along with the fatty acid results, support the notion that these four new species are all members of the *Paenibacillus* genus.

Additionally, the antibiotic susceptibility of the four *Paenibacillus* sp. strains was tested; the results are presented in Table 4. These results were interpreted by critical diameters (mm) of disk antibiotic diffusion, which are described in Table 4. All four strains were sensitive to β-lactams, aminoglycosides, glycopeptides, tetracyclines, lincosamides and trimethoprim/sulfamethoxazole.

Genome description and comparison

The properties and statistics of the genomes are summarized in Table 5, and the distribution of predicted genes of our strains according to COGs categories is shown in Table 6. For all the 25 general COGs functional categories, values of our four new *Paenibacillus* species are in the same range. Genomic characteristics of our strains were compared to those of closely related species with an available genome (Table 7). With the genome size ranging from 5 to 5.8 Mbp, they all had the same GC percentage of approximately 50% (±3%), as does the characterized genome of other known *Paenibacillus* species [42]. These COGs categories (only gene assigned to COGs), RNA genes (tRNAs green, rRNAs red), GC content and GC skew are illustrated in the graphical circular map of the genome (Supplementary Fig. S1).

MT18^T, *'Paenibacillus senegalimassiliensis'* strain SIT18^T and *'Paenibacillus tuaregi'* strain Marseille-P2472^T to other species within genus *Paenibacillus* was realized. *Bacillus subtilis* and *Pantoea agglomerans* are used as outgroup. Gel view displays raw spectra of loaded spectrum files arranged in pseudo-gel-like look. *x*-axis records *m/z* value. Left *y*-axis displays running spectrum number originating from subsequent spectra loading. Peak intensity is expressed by greyscale scheme code. Colour bar and right *y*-axis indicate relation between colour peaks, with peak intensity in arbitrary units. Displayed species are indicated at left.

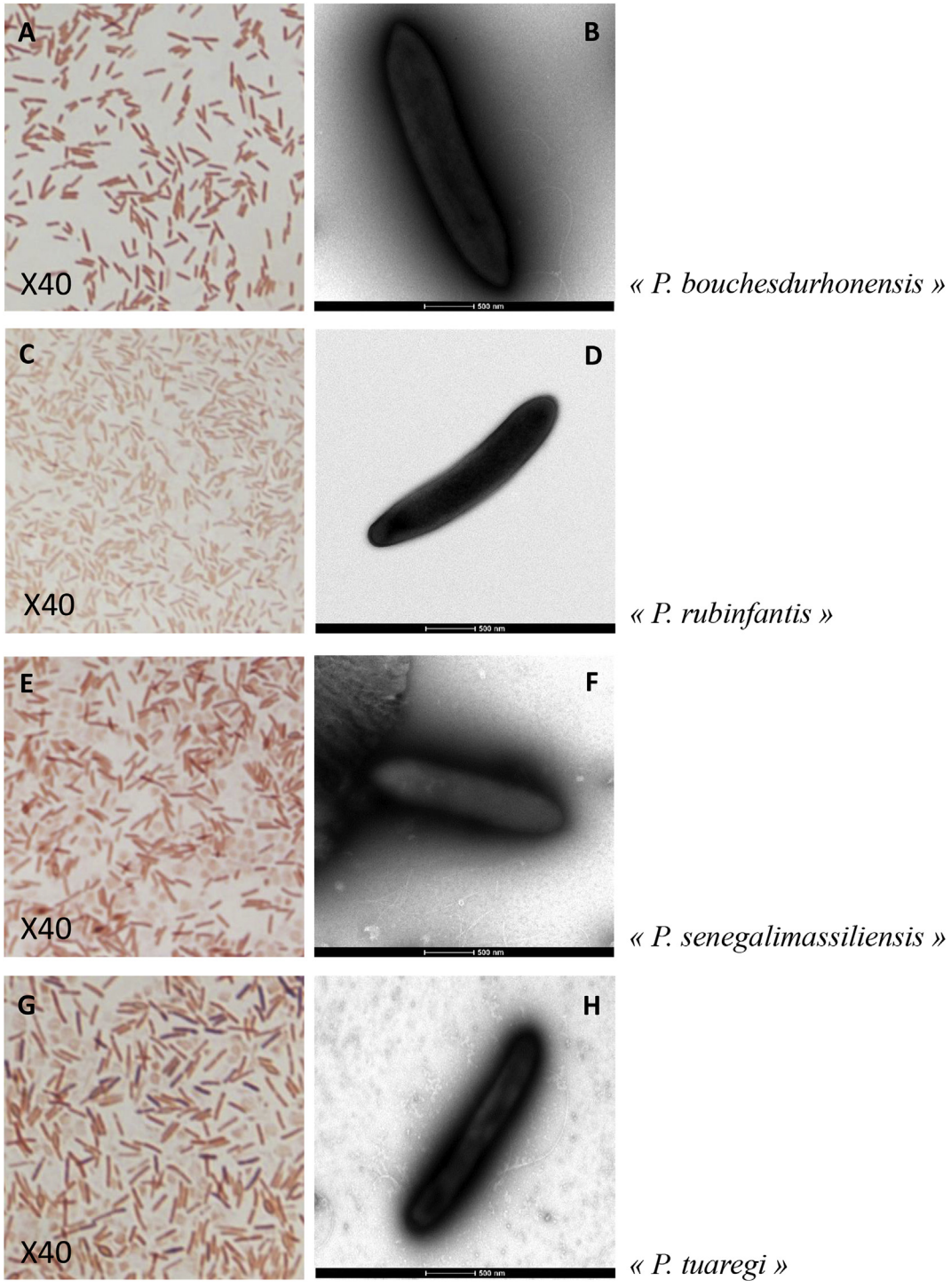

FIG. 4. Gram staining and electron micrographs, respectively, of *'Paenibacillus bouchesdurhonensis'* strain Marseille-P3071^T (A, B), *'Paenibacillus rubinfantis'* strain MT18^T (C, D), *'Paenibacillus senegalimassiliensis'* strain SIT18^T (E, F) and *'Paenibacillus tuaregi'* strain Marseille-P2472^T (G,H).

Furthermore, dDDH values (Table 8) are inferior to 70% and confirm that all the studied species are distinct species [40,41]. These results are supported by their AGIOS values (Supplementary Table S1), which demonstrated that comparison between our strains and other species within the *Paenibacillus* genus resulted in a similar range of values compared to the comparison between the same species except our strains. This confirmed their new species status.

TABLE 3. Cellular fatty acid composition (%) for *Paenibacillus* strains[a]

Fatty acid	IUPAC name	*'P. bouchesdurhonensis'*	*'P. rubinfantis'*	*'P. senegalimassiliensis'*	*'P. tuaregi'*
14:0	Tetradecanoic acid	1.7 ± 0.2	TR	1.3 ± 0.3	TR
14:0 iso	12-methyl-Tridecanoic acid	2.3 ± 0.3	1.9 ± 0.1	10.4 ± 0.5	2.0 ± 0.1
15:0	Pentadecanoic acid	1.3 ± 0.2	2.3 ± 0.2	TR	TR
15:0 iso	13-methyl-tetradecanoic acid	16.1 ± 0.9	10.3 ± 0.1	3.1 ± 0.3	6.5 ± 0.2
15:0 ante iso	12-methyl-tetradecanoic acid	50.6 ± 1.0	51.2 ± 2.4	52.6 ± 1.2	48.6 ± 2.4
16:0	Hexadecanoic acid	11.8 ± 0.4	12.6 ± 0.3	13.3 ± 0.6	8.4 ± 0.6
16:0 iso	14-methyl-Pentadecanoic acid	6.2 ± 0.6	11.4 ± 0.5	15.9 ± 0.1	20.2 ± 0.5
16:1n9	7-Hexadecenoic acid	2.8 ± 0.3	No	No	No
17:0 iso	15-methyl-Hexadecanoic acid	2.5 ± 0.2	2.8 ± 0.5	TR	3.1 ± 0.4
17:0 anteiso	14-methyl-Hexadecanoic acid	2.5 ± 0.1	5.8 ± 0.8	TR	9.5 ± 0.8
18:1n9	9-Octadecenoic acid	1.1 ± 0.2	No	TR	No
18:2n6	9,12-Octadecadienoic acid	TR	No	1.3 ± 0.1	No

IUPAC, International Union of Pure and Applied Chemistry; TR, trace amounts <1%.
[a]Mean peak area percentage ± standard deviation.

TABLE 4. Results of antibiotic resistance tests (critical diameters (mm)) for *Paenibacillus* strains

Characteristic	Concentration (µg/mL)	*'P. bouchesdurhonensis'*	*'P. rubinfantis'*	*'P. senegalimassiliensis'*	*'P. tuaregi'*
Penicillin G	10	30.7 (S)	30.2 (S)	45 (S)	28 (I)
Amoxicillin/clavulanic acid	30	42.8 (S)	34.6 (S)	36.5 (S)	27.6 (S)
Colistin	50	18.2 (S)	17 (S)	14.6 (R)	0 (R)
GEN500 gentamicin	500	37 (S)	40.7 (S)	41.1 (S)	34.8 (S)
FOX30 cefoxitin	30	12.8 (R)	0 (R)	33.2 (S)	10.7 (R)
AMX25 amoxicillin	25	40 (S)	40.7 (S)	40.4 (S)	35.9 (S)
E15 erythromycin	15	40.9 (S)	28.9 (S)	43.2 (S)	20.4 (I)
DO30 doxycycline	30	46.7 (S)	41.5 (S)	46.7 (S)	25 (S)
RA30 rifampicin	30	47.2 (S)	24.6 (S)	48 (S)	20.7 (S)
VA30 vancomycin	30	29.8 (S)	30.2 (S)	33.2 (S)	23 (S)
SXT25 trimethoprim/sulfamethoxazole	25	26.9 (S)	28.7 (S)	37.2 (S)	18.7 (S)
DA15 clindamycin	15	26.1 (S)	0 (R)	42.6 (S)	18.7 (S)
MET4 metronidazole	4	0 (R)	0 (R)	0 (R)	0 (R)
TOB10 tobramycin	10	16.9 (I)	23 (S)	18.3 (S)	0 (R)
IPM10 imipenem	10	29.4 (S)	35 (S)	35.7 (S)	33.2 (S)

I, intermediate; R, resistant; S, sensitive.

TABLE 5. Nucleotide content and gene counts levels of genome for *Paenibacillus* strains

Attribute	*'P. bouchesdurhonensis'*		*'P. rubinfantis'*		*'P. senegalimassiliensis'*		*'P. tuaregi'*	
	8;33		20;25		15;17		4;24	
Scaffolds/contigs	Value	% of total[a]	Value	% of total[a]	Value	% of total[a]	Value	% of total[a]
Size (bp)	5 823 754	100	5 370 420	100	5 059 702	100	5 668 612	100
G+C content (%)	2 759 740	47.45	28 47 155	53.0	2 488 213	49.2	2 783 054	49.1
Coding region (bp)	5 050 020	86.7	4 689 593	87.3	4 509 792	89.1	4 872 040	85.9
Total genes	5213	100	4983	100	4736	100	5169	100
Protein-coding genes	5113	98.1	4901	98.4	4655	98.3	5061	97.9
RNA genes	100	1.9	82	1.6	81	1.7	108	2.1
Proteins with function prediction	3507	68.6	3523	71.9	3194	68.6	3574	70.6
Proteins assigned to COGs	2198	43.0	3025	61.7	2828	60.8	3040	60.1
Proteins with peptide signals	670	13.1	688	14.0	638	13.7	703	13.9
No. of protein associated to ORFan	237	4.6	122	2.5	164	3.5	216	4.3
Genes with transmembrane helices	1215	23.8	12.1	24.5	1126	24.2	1255	24.8
Genes associated with PKS or NRPS	12	0.2	8	0.2	13	0.3	24	0.5
No. of antibiotic resistance genes	0	0	3	0.1	0	0	0	0
No. of genes associated with Pfam-A domains	4590	88	4522	90	4240	89	4624	89

COGs, Clusters of Orthologous Groups database; NRPS, nonribosomal peptide synthase; PKS, polyketide synthase.
[a]Total is based on either size of genome in base pairs or total number of protein-coding genes in annotated genome.

Conclusion

In this study, we used a new concept based on genome sequence, MALDI-TOF MS identification and main phenotypic characteristics, to describe four new species of the *Paenibacillus* genus which have been isolated from diverse stool clinical samples but possess similar morphologic properties. Thus, their cellular fatty acid composition supports the idea that these four strains belong to the same genus. Their 16S rRNA gene

TABLE 6. No. of genes associated with 25 general COGs functional categories for *Paenibacillus* strains

Code	Description	*'P. bouchesdurhonensis'*		*'P. rubinfantis'*		*'P. senegalimassiliensis'*		*'P. tuaregi'*	
		Value	% of total[a]	Value	% of total[a]	Value	% of total[a]	Value	% of total[a]
J	Translation	231	4.52	237	4.83	234	5.03	259	5.11
A	RNA processing and modification	0	0	0	0	0	0	0	0
K	Transcription	281	5.50	286	5.83	260	5.59	338	6.67
L	Replication, recombination and repair	115	2.25	120	2.45	105	2.26	110	2.17
B	Chromatin structure and dynamics	1	0.02	1	0.02	1	0.02	1	0.02
D	Cell cycle control, mitosis and meiosis	47	0.92	53	1.08	50	1.07	48	0.94
Y	Nuclear structure	0	0	0	0	0	0	0	0
V	Defense mechanisms	115	2.25	109	2.22	93	2.00	103	2.03
T	Signal transduction mechanisms	151	2.95	172	3.51	147	3.16	194	3.83
M	Cell wall/membrane biogenesis	159	3.11	153	3.12	143	3.07	147	2.9
N	Cell motility	52	1.02	62	1.26	56	1.20	53	1.04
Z	Cytoskeleton	2	0.04	3	0.06	2	0.04	2	0.04
W	Extracellular structures	3	0.06	6	0.12	13	0.28	2	0.04
U	Intracellular trafficking and secretion	33	0.65	35	0.71	34	0.73	29	0.57
O	Posttranslational modification, protein turnover, chaperones	113	2.21	121	2.46	113	2.43	129	2.54

COGs, Clusters of Orthologous Groups database.
[a]Total is based on either size of genome in base pairs or total number of protein-coding genes in annotated genome.

sequencing compared to other strains of *Paenibacillus* genus indicated that *'Paenibacillus bouchesdurhonensis'* strain Marseille-P3071^T, *'Paenibacillus rubinfantis'* strain MT18^T, *'Paenibacillus senegalimassiliensis'* strain SIT18^T and *'Paenibacillus tuaregi'* strain Marseille-P2472^T are all members of the *Paenibacillus* genus.

Description of *'Paenibacillus bouchesdurhonensis'* sp. nov.

Cells of the strain Marseille-P3071^T are Gram-negative bacilli and are rod shaped, with a length varying from 2.5 to 3 µm and a width from 0.5 to 0.7 µm. This strain exhibits catalase activity but no oxidase activity. *'Paenibacillus bouchesdurhonensis'* is motile and endospore forming. Colonies are circular, smooth, crateriform, grey and have intact edges, with a diameter of 1 to 3 mm. Optimum growth occurs at 37°C in an aerobic atmosphere on Colombia agar enriched with 5% of rumen after a 24-hour growth.

Strain Marseille-P3071^T is susceptible to penicillin (10 µg/mL), colistin (50 µg/mL), gentamycin (500 µg/mL), erythromycin (15 µg/mL), doxycycline (30 µg/mL), rifampicin (30 µg/mL), trimethoprim/sulfamethoxazole (25 µg/mL), clindamycin (15 µg/mL), amoxicillin (25 µg/mL), amoxicillin/clavulanate (30 µg/mL), clindamycin (15 µg/mL), imipenem (10 µg/mL) and vancomycin (30 µg/mL). The major fatty acid is 12-methyl-tetradecanoic acid.

TABLE 7. Genome comparison of closely related species for *Paenibacillus* strains

Organism	Strain	INSDC	Size (Mb)	G+C%	Total genes
P. terrae	AM141	CP003107	6.08	46.77	5525
P. jamilae	CECT 5266	LDRX00000000	5.59	45.58	4772
P. pabuli	HSCC 492T NRRL NRS-924T	BCNM00000000	7.33	46.52	6524
P. massiliensis	2301065	ASSE00000000	6.39	48.50	5496
P. fonticola	ZL	ARMT00000000	6.31	47.68	5639
P. panacisoli	Gsoil 1411	AUFO00000000	6.33	48.27	5596
'P. bouchesdurhonensis'	Marseille-P3071T	FTLT00000000	5.82	47.45	5113
P. peoriae	KCTC 3763	CP011512	5.77	46.44	5122
P. sabinae	T27	CP004078	5.27	52.64	4788
P. polymyxa	IAM 13419	CP002213	6.02	45.58	5283
P. macerans	IAM 12467	JMQA00000000	7.34	52.57	6561
'P. rubinfantis'	MT18	FAUQ00000000	5.37	53.02	4901
P. forsythiae	T98	ASSC00000000	5.08	52.94	5313
P. sanguinis	2301083	ARGO00000000	4.80	49.31	4403
P. wynnii	LMG 22176T	JQCR00000000	5.99	44.87	4891
P. borealis	FSL	CP009285	8.16	51.39	6213
P. stellifer	IS1	CP009286	5.66	53.54	4464
P. alvei	DSM 29	AMBZ00000000	6.83	45.90	6605
'P. senegalimassiliensis'	SIT18T	FAUP00000000	5.06	49.18	4655
P. elgii	SD17	LQRA00000000	7.96	52.56	7597
P. terrigena	A35	ARGP00000000	6.36	46.08	5817
P. pini	JCM 16418 S22	BAVZ00000000	4.96	42.02	5131
P. dendritiformis	CIP 105967T	AHKH00000000	6.37	54.12	5660
P. popilliae	ATCC 14706	BALG00000000	3.83	51.02	3855
P. taiwanensis	BCRC 17411	AULE00000000	5.24	44.83	4641
'P. tuaregi'	Marseille-P2472T	FLKE00000000	5.66	49.13	5061
P. assamensis	GPTSA 11	AULU00000000	2.02	43.28	4422

INSDC, International Nucleotide Sequence Database Collaboration.

TABLE 8. Digital DNA-DNA hybridization values for *Paenibacillus* strains

	1	2	3	4	5	6	7	8	9	10	11	12
1	100% ± 00	22.30% ± 2.4	24.50% ± 2.4	19.60% ± 2.3	20.10% ± 2.3	19.10% ± 2.25	21.90% ± 2.35	21.00% ± 2.35	18.80% ± 2.3	20.20% ± 2.35	19.20% ± 2.25	19.20% ± 2.3
2		100% ± 00	20.00% ± 2.3	19.60% ± 2.3	19.60% ± 2.3	20.70% ± 2.35	21.30% ± 2.35	21.20% ± 2.35	20.00% ± 2.3	19.40% ± 2.25	19.90% ± 2.3	21.80% ± 2.35
3			100% ± 00	26.10% ± 2.45	26.30% ± 2.4	19.00% ± 2.3	24.90% ± 2.4	25.30% ± 2.4	18.70% ± 2.3	19.40% ± 2.3	21.80% ± 2.35	20.30% ± 2.3
4				100% ± 00	40.10% ± 2.5	22.50% ± 2.35	28.90% ± 2.45	41.30% ± 2.5	23.40% ± 2.35	21.70% ± 2.35	34.80% ± 2.5	24.10% ± 2.4
5					100% ± 00	23.20% ± 2.35	30.10% ± 2.45	40.90% ± 2.5	22.90% ± 2.35	22.20% ± 2.35	41.80% ± 2.45	25.60% ± 2.4
6						100% ± 00	22.60% ± 2.4	24.60% ± 2.4	19.70% ± 2.3	19.00% ± 2.25	19.90% ± 2.3	20.20% ± 2.3
7							100% ± 00	30.00% ± 2.45	23.70% ± 2.4	22.30% ± 2.35	22.10% ± 2.4	26.00% ± 2.45
8								100% ± 00	22.20% ± 2.35	26.10% ± 2.45	22.40% ± 2.35	28.40% ± 2.45
9									100% ± 00	18.70% ± 2.25	20.20% ± 2.3	19.80% ± 2.3
10										100% ± 00	20.30% ± 2.3	22.90% ± 2.35
11											100% ± 00	21.80% ± 2.35
12												100% ± 00

1, *P. fonticola*; 2, *P. panacisoli*; 3, *P. bouchesdurhonensis*; 4, *P. peoriae*; 5, *P. polymyxa*; 6, *P. rubinfantis*; 7, *P. stellifer*; 8, *P. alvei*; 9, *P. senegalimassiliensis*; 10, *P. pini*; 11, *P. dendritiformis*; 12, *P. tuaregi*.

The genome of strain Marseille-P3071^T is 5 823 754 bp long with 47.45% G+C content. The 16S rRNA gene and genome sequences are available in the European Molecular Biology Laboratory/European Bioinformatics Institute (EMBL/EBI) database under accession numbers LT598550 and FTLT00000000, respectively. The type strain Marseille-P3071^T (= CSUR P3071 = DSM 103972) was isolated from the stool sample of a Senegalese girl with severe acute malnutrition (marasmus form). Its habitat is the human gut. Strain Marseille-P3071^T is the type strain of the new species *'Paenibacillus bouchesdurhonensis'* (bou.ches.du.rho.nen'sis, NL. adj. masc., to refer to Bouches-du-Rhône, the name of the French department where the strain was isolated).

Description of *'Paenibacillus rubinfantis'* sp. nov.

Cells of the strain MT18^T are Gram-negative bacilli and are rod shaped, with a length varying from 2 to 2.5 µm and a width from 0.5 to 0.7 µm. This strain exhibits catalase activity but no oxidase activity. *'Paenibacillus rubinfantis'* is motile and endospore forming. Colonies are circular, smooth, convex, grey and have intact edges with a diameter of 1 to 3 mm. This strain was isolated after 20 days in a blood culture bottle after thermic shock at 80°C during 20 minutes. Optimum growth occurs at 37°C in an aerobic atmosphere on Colombia agar after a 24-hour growth.

Strain MT18^T is susceptible to penicillin (10 µg/mL), colistin (50 µg/mL), gentamycin (500 µg/mL), erythromycin (15 µg/mL), doxycycline (30 µg/mL), rifampicin (30 µg/mL), trimethoprim/sulfamethoxazole (25 µg/mL), tobramycin (10 µg/mL), amoxicillin (25 µg/mL), amoxicillin/clavulanate (30 µg/mL), clindamycin (15 µg/mL), imipenem (10 µg/mL) and vancomycin (30 µg/mL). The major fatty acid is 12-methyl-tetradecanoic acid.

The genome of strain MT18^T is 5 370 472 bp long with 53% G+C content. The 16S rRNA gene and genome sequences are available in the EMBL/EBI database under accession numbers LN881603 and FAUQ00000000, respectively. The type strain MT18^T (= CSUR P2076 = DSM 101191) was isolated from the stool sample of a Nigerian child with severe acute malnutrition (kwashiorkor form). Its habitat is the human gut.

Strain MT18^T is the type strain of the new species *'Paenibacillus rubinfantis'* (*rubinfantis* is composed of 'ru.bi' (L. adj. neut.), for *rubeus* (adj.), meaning 'red,' and 'in.fan.tis' (L. adj. neut.), meaning 'infant,' which is a reference to the hair discolouration observed in children with kwashiorkor).

Description of *'Paenibacillus senegalimassiliensis'* sp. nov.

Cells of the strain SIT18^T are Gram-variable bacilli and are rod shaped with a length varying from 1.5 to 2 µm and a width from 0.3 to 0.5 µm. This strain exhibits neither catalase nor oxidase activities. *'Paenibacillus senegalimassiliensis'* is motile and endospore forming. Colonies are circular, smooth, flat, grey and have intact edges with a diameter of 1 to 3 mm. Strain SIT18^T was isolated after 7 days in a blood culture bottle + sheep blood + rumen, under aerobic conditions at 37°C.

Strain SIT18^T is susceptible to penicillin (10 µg/mL), clindamycin (15 µg/mL), metronidazol (4 µg/mL), cefoxitin (30 µg/mL), erythromycin (15 µg/mL), doxycycline (30 µg/mL), rifampicin (30 µg/mL), trimethoprim/sulfamethoxazole (25 µg/mL), tobramycin (10 µg/mL), amoxicillin (25 µg/mL), amoxicillin/clavulanate (30 µg/mL), clindamycin (15 µg/mL), imipenem (10 µg/mL) and vancomycin (30 µg/mL). The major fatty acid is 12-methyl-tetradecanoic acid.

The genome of strain SIT18^T is 5 059 702 bp long with 49.20% G+C content. The 16S rRNA and genome sequences are available in the EMBL/EBI database under accession numbers LN890284 and FAUP00000000, respectively. The type strain SIT18^T (= CSUR P2144 = CCUG 69869) was isolated from the stool sample of a healthy Senegalese boy. It habitat is the human gut.

Strain SIT18^T is the type strain of the new species *'Paenibacillus senegalimassiliensis'* (*'senegalimassiliensis'* is composed of 'se.ne.ga.li' (L. masc. adj.), referring to the Republic of Senegal, the West African country where the stool sample was retrieved, and

'ma.si.li.en'sis' (L., masc. adj.), referring to Massilia, the Roman name of Marseille, where the strain SIT18^T was isolated).

Description of *'Paenibacillus tuaregi'* sp. nov.

Cells of the strain Marseille-P2472^T are Gram-variable bacilli and are rod shaped with a length varying from 3.5 to 4.5 μm and a width from 0.6 to 0.8 μm. This strain exhibits catalase activity but no oxidase activity. *'Paenibacillus tuaregi'* is motile and endospore forming. Colonies are irregular, smooth and grey with a diameter of 1 to 4 mm. Optimum growth occurs at 37°C in an aerobic atmosphere on Colombia agar enriched with liquid marine medium after a 24-hour growth.

Strain Marseille-P2472^T was susceptible to gentamycin (500 μg/mL), doxycycline (30 μg/mL), rifampicin (30 μg/mL), trimethoprim/sulfamethoxazole (25 μg/mL), clindamycin (15 μg/mL), amoxicillin (25 μg/mL), amoxicillin/clavulanate (30 μg/mL), clindamycin (15 μg/mL), imipenem (10 μg/mL) and vancomycin (30 μg/mL). The major fatty acid is 12-methyl-tetradecanoic acid.

The genome of strain Marseille-P2472^T is 5 668 612 bp long with 49.10% G+C content. The 16S rRNA and genome sequences are available in the EMBL/EBI database under accession numbers LT223571 and FLKE00000000, respectively. The type strain Marseille-P2472^T (= CSUR P2472 = DSM 102801) was isolated from the stool sample of a healthy Nigerian girl. Its habitat is the human gut.

Strain Marseille-P2472^T is the type strain of the new species *'Paenibacillus tuaregi'* (*'tuaregi'* from 'tua.re.gi,' L. masc. adj., *tuaregi* from 'Touareg,' the people of the sample donor from which the strain was isolated).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.nmni.2017.07.004.

Conflict of interest

None declared.

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