Neobacillus massiliamazoniensis sp. nov., a new bacterial species isolated from stool sample of an inhabitant of the Amazon region

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Abstract

Using a culturomics approach, a strain was isolated, identified and characterised following the taxonogenomics concept. *Neobacillus massiliamazoniensis* sp. nov., strain LF1^T (=CSURP1359) was isolated from human stool. The 16S rRNA gene sequence analysis of strain LF1^T (accession number: LK021124) exhibits 98.32% similarity levels with *Neobacillus bataviensis* strain IDA1115 (accession number: NR_036766.1), the phylogenetically closest related species with standing in nomenclature. The draft genome size of strain LF1^T (accession number: CVRB00000000) is 4.6 Mbp with a G+C content of 34.1 mol%. Analysis of phylogenic tree, genomic analysis and phenotypic criteria described here sufficiently prove that this bacterium is different from previously known bacterial species with standing in nomenclature and represents a new *Neobacillus* species belonging to *Firmicutes* phylum.
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Introduction

Bacillus species represent most of the bacteria present in the environment, particularly in soil, food and water [1–3]. This genus was created by Ferdinand Julius Chon in 1872 [4] and includes today 95 species. Recently, this genus has been reclassified through comparative phylogenomic and genomic analyses. This has revealed the existence of six new clades divided into six new genera, namely *Peribacillus* gen. nov., *Cytobacillus* gen. nov., *Mesobacillus* gen. nov., *Neobacillus* gen. nov., *Metabacillus* gen. nov. and *Alkalihalobacillus* gen. nov. [5].

The use of culturomics and metagenomics methods has led to a better understanding of the microbial diversity of the human microbiota [6]. Thus, the revolutionary techniques, including the sequencing of the 16S rRNA gene and bacterial genomes and the MALDI-TOF mass spectrometry, have led to the discovery of several new and as yet unknown bacteria. Here, we report the full description of the *Neobacillus massiliamazoniensis* sp. nov. strain LF1^T, isolated from a human stool sample, using a combination of phenotypic and genotypic characters as per the taxonogenomic strategy [7,8].

Materials and methods

Isolation and identification

In 2017, as part of a study of gut microbiota, the strain LF1^T was first isolated from a stool sample from human using culturomics approach. It was cultivated on under blood culture flask enriched with 5% rumen fluid sterilised by filtration at 0.2 μm then inoculated on 5% sheep blood Columbia agar (bioMérieux, Marcy L'Etoile, France) after two days of incubation at 37°C.

Bacterial identification was carried out using MALDI-TOF mass spectrometry (Bruker, Daltonics, Bremen, Germany) and the reference spectrum generated was added in the local database (https://www.mediterranee-infection.com/urms-database). After several unsuccessful identification attempts with MALDI-TOF instrument, the 16S rRNA gene was amplified

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using universal primers pairs fD1 and rP2 (Eurogentec, Angers, France) and sequenced with the Big Dye® Terminator v1.1 cycle sequencing kit and the 3500xL capillary sequencer Genetic Analyser (Thermo Fisher, Saint-Aubin, France), as previously described [9]. The nucleotide sequences obtained were assembled and edited with the Codoncode Aligner software (http://www.codoncode.com). The consensual corrected sequence was used as a reference sequence and compared against the 16S gene sequences of the type strains existing in the NCBI database to assess the phylogenetically closest species. For this purpose, a Blastn method was used since the NCBI database, to compare the 16S rRNA gene sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Growth conditions and phenotypic characterisation

To determine the best growing condition for this new species, different approaches were used. Therefore, the strain LF1^T was inoculated on Columbia agar with 5% sheep blood (bioMérieux, Marcy l'Etoile, France) and incubated at different temperatures (28, 37, 42 and 52°C) and atmospheres (aerobic, anaerobic and microaerophilic) according to Diop et al., 2018 [10]. The growth at different pH (6.5 to 8.5) and salt concentrations (50 to 150 g/L) is also tested in parallel. API ZYM and API 50 CH strips (bioMérieux) were used to evaluate the biochemical characteristics of the bacterium, following the manufacturer's recommendations. Gram stain, catalase and oxidase tests and sporulation ability were done using standard procedures [11].

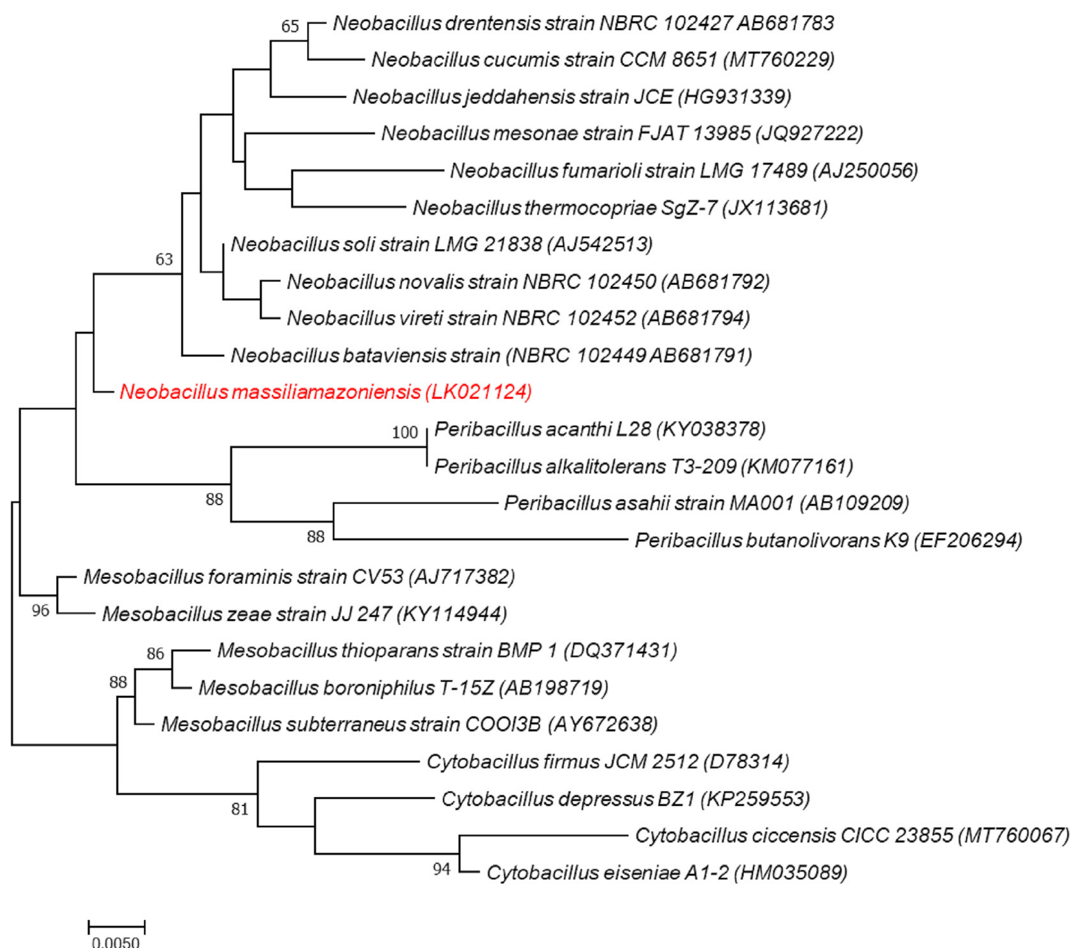

FIG. 1. Phylogenetic tree displaying the position of *Neobacillus massiliamazoniensis* strain LF1^T compared with its closest phylogenetically species. The respective GenBank accession numbers for 16S rRNA genes are indicated in parenthesis. Sequence alignment and phylogenetic inferences were obtained using the maximum likelihood method within MEGA 7 software. The numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 1000 times to generate a majority consensus tree. Only percentage values higher than 70% are shown in the figure with a 5% scale.

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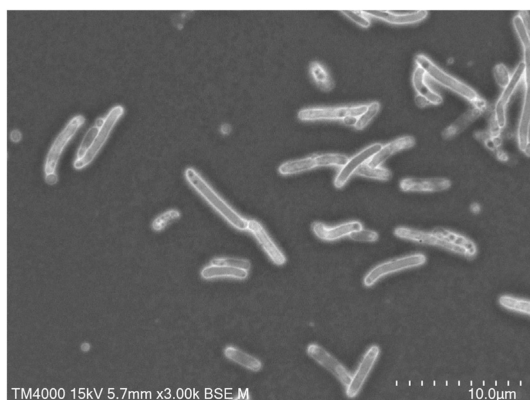

FIG. 2. Scanning electron micrograph of *Neobacillus massiliamazoniensis* LF1^T using the Scanning Electron Microscope TM4000 from Hitachi. Scale bar and acquisition settings are presented on the picture.

The morphological structure of the bacterium was observed with a scanning electron microscope (Hitachi Group, Tokyo, Japan) as per the protocol described by Belkacemi et al. [12].

Genome extraction and sequencing

Genomic DNA was extracted using the EZ1 biorobot (Qiagen, Courtaboeuf, France) with the EZ1 DNA tissue kit preceded by a pre-treatment with lysozyme and incubation at 37°C for 2 hours. Sequencing of the gDNA was performed using MiSeq technology using the Nextra Mate Pair sample preparation kit and the Nextera XT Paired (Illumina) tip, as described previously [13]. The assembly was performed using a pipeline incorporating different softwares (Velvet [14], Soap Denovo [15] and Spades [16]). Data from Illumina MiSeq were trimmed using Trimmomatic software or untrimmed using MiSeq software only [17]. GapCloser was used to reduce assembly deviations. Scaffolds <800 base pairs (bp) and scaffolds with a depth value less than 25% of the average depth were removed. The best assembly was selected using different criteria (number of scaffolds, N50, number of N). The degree of genomic similarity of this strain with closely related species was estimated using OrthoANI software [18]. Then, an online server named Genome-to-Genome Distance Calculator (http://ggdc.dsmz.de) [19] was used to calculate the DNA-DNA hybridisation (DDH) values shared between strain LF1^T and its closest related species.

TABLE 1. Differential characteristics of 1, *Neobacillus massiliamazoniensis* sp. nov., strain LF1^T compared with 2, *Neobacillus bataviensis* strain IDA 1115, 3, *Neobacillus novalis* strain and 4, *Neobacillus jeddahensis* strain CSURP732

Property	1	2	3	4
Cell diameter (μm)	0.5-1.0	0.7-1.2	0.4-0.6	0.7-0.9
Oxygen requirement	facultative anaerobic	Aerobic	Aerobic	Aerobic
Gram stain	+	+	+	+
Motility	—	NF	+	—
Endospore formation	—	+	+	+
Production of:				
Alkaline phosphatase	+	NF	—	—
Acid phosphatase	+	NF	NF	+
Catalase	—	—	—	—
Oxidase	+	NF	—	+
β-Galactosidase	—	w	—	—
α-Glucosidase	+	NF	NF	—
Esterase	—	NF	NF	+
Esterase lipase	—	NF	NF	+
Naphthol-AS-BI-phosphohydrolase	—	NF		+
Nitrate reductase	—	+	—	+
N-acetyl-glucosamine	—	w	+	+
Urease	—	—	—	—
N-acetyl-β-glucosaminidase	—	NF	NF	—
Utilisation of:				
Potassium 5-ketogluconate	—	—	—	—
D-Xylose	—	—		+
D-Fructose	w	+	+	+
D-Glucose	+	+	+	+
Maltose	—	NF	+	+
D-Mannose	+	+	+	+
Habitat	Human stool	Agricultural area	Agricultural area	Human skin

+, positive result; −, negative result; NF, data not found; w, weak reaction.

TABLE 2. Cellular fatty acid profiles (%) of *Neobacillus massiliamazoniensis* strain LF1^T compared with closest species

Fatty acid	1	2	3	4
$C_{15:0\ iso}$	55.9 ± 1.2	36.9 ± 4.3	32.2 ± 4.2	47.1 ± 3.5
$C_{14:00}$	1.3 ± 0.1	1.5 ± 0.6	1.4 ± 1.3	1.6 ± 0.2
$C_{14:0\ iso}$	8.1 ± 0.9	6.9 ± 1.1	8.7 ± 2.5	3.1 ± 1.2
$C_{16:00}$	3.9 ± 0.7	7.7 ± 3.2	3.4 ± 1.6	3.8 ± 1.0
$C_{16:0\ iso}$	2.1 ± 0.5	2.4 ± 0.7	2.1 ± 1.0	2.5 ± 1.8

1, *Neobacillus massiliamazoniensis*; **2,** *Neobacillus bataviensis*; **3,** *Neobacillus drentensis*; **4,** *Neobacillus vireti*.

Results

Phylogenetic analysis

Because there is no spectrum in the MALDI-TOF database that corresponds to the strain LF1^T, the latter cannot be identified correctly. Therefore, a similarity analysis based on 16S rDNA of the strain LF1^T showed that *N. massiliamazoniensis* strain LF1^T had 98.32% sequence identity with *Neobacillus bataviensis* strain IDA1115 (Accession number: NR_036766.1). This value is lower than the recommended threshold value (<98.65%) to delineate new bacterial species [20,21]. Given this result, we therefore propose to classify strain LF1^T as a new species within the genus *Neobacillus* which belongs to the family *Bacillaceae* and the phylum *Firmicutes* [5]. The performed phylogenetic tree (Fig. 1) shows the position of *N. massiliamazoniensis* sp. nov. strain LF1^T among closely related species with a validly published name. The shape of the bacterium (Fig. 2) was observed using the Hitachi TM4000 instrument (Hitachi Group, Tokyo, Japan).

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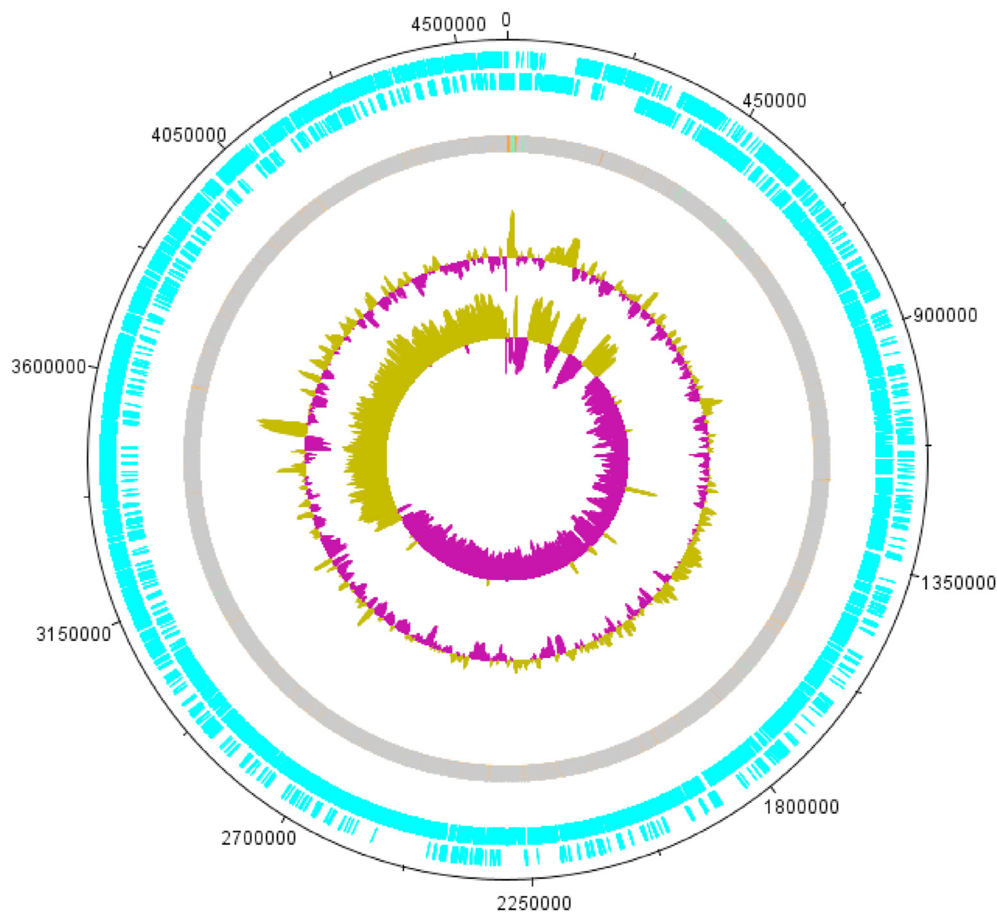

FIG. 3. Graphical circular map of the *Neobacillus massiliamazoniensis* sp. nov., strain Maresille-P1359. The fourth circle shows the G+C mol% content plot. The inner-most circle shows GC skew, with purple indicating negative values and olive indicating positive values.

Phenotypic and biochemical features

N. massiliamazoniensis sp. nov. strain LF1^T is able to grow both aerobically and anaerobically but better aerobically with an optimal growth temperature of 37°C. It is a Gram-positive rod-shaped facultative anaerobic bacterium with an average cell diameter of 1μm. This strain does not grow in salted media but it could grow with pH ranging from 6 to 7.5. Indeed, the optimum growth was observed at pH = 7. It has catalase-negative and oxidase-positive activities. Using the API ZYM strip, only reactions with alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, α-galactosidase, α-glucosidase, β-glucosidase were positive. Other tests with esterase (C4), esterase lipase (C8), lipase (C14), trypsin, acid phosphatase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase were negative. In addition, using a 50 CH strip, strain LF1^T was positive for D-glucose, N-acetyl-glucosamine, glycogen, starch and D-mannose. A weak reaction was observed with D-fructose. However, tests including glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl

TABLE 3. Comparison of genome size and GC content of the new *Neobacillus massiliamazoniensis* strain LF1^T with its closely related species belonging to the genus *Neobacillus*

Species (strain)	Size (pb)	GC (mol%)	Total genes	Genome accession numbers
***Neobacillus massiliamazoniensis* (LF1)**	**4,588,940**	**34.1**	**4491**	CVRB00000000
Neobacillus bataviensis (IDA 1115)	5,371,144	39.6	5277	NZ_AJLS00000000.1
Neobacillus cucumis (DSM 101566)	5,707,899	38.6	5580	NZ_PGVE00000000.1
Neobacillus drentensis (DSM 15600)	5,305,306	38.9	5229	BCUX00000000
Neobacillus soli (DSM 15604)	5,579,901	39.7	5486	BCVI00000000
Neobacillus jeddahensis (DSM 28281)	4,762,944	39.4	4684	NZ_CCAS000000000.1
Neobacillus fumarioli (DSM 18237)	3,294,206	40.4	3355	BCUZ00000000

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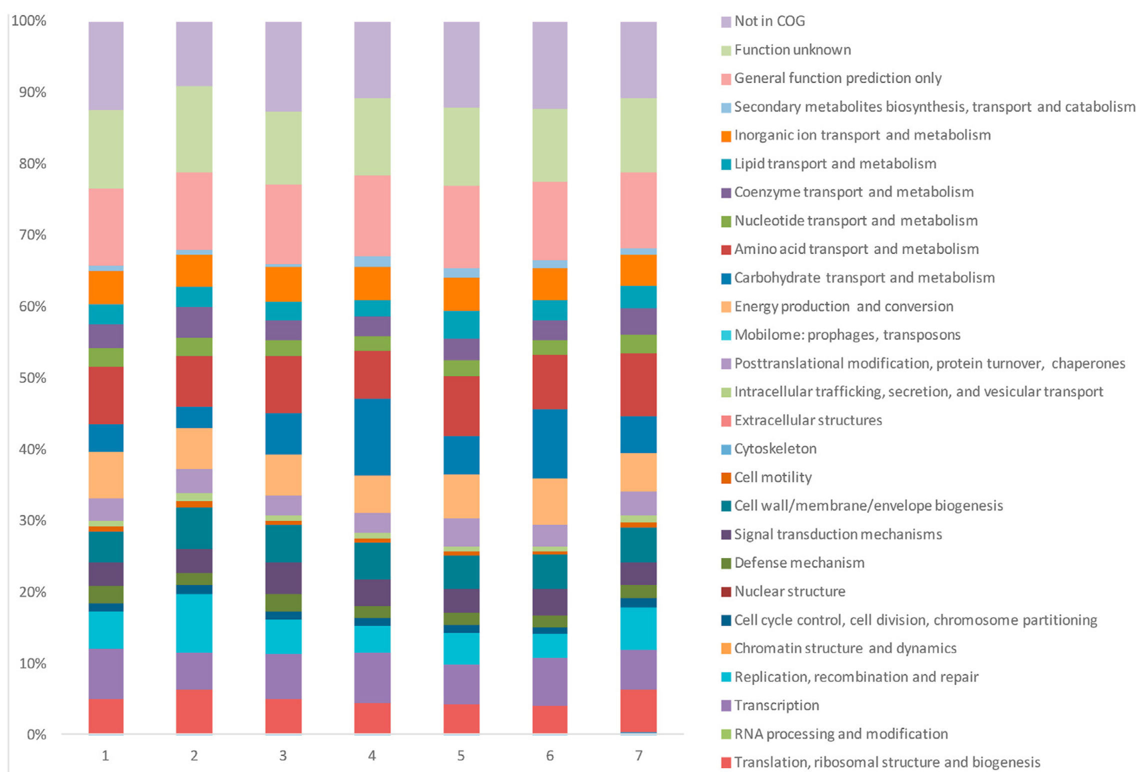

FIG. 4. Distribution of functional classes of predicted genes in accordance with the COG of proteins of strain LF1^T among related other species: **1**, *Neobacillus massiliamazoniensis* LF1^T; **2**, *Neobacillus jeddahensis*; **3**, *Neobacillus drentensis*; **4**, *Neobacillus cucumis*; **5**, *Neobacillus fumarioli*; **6**, *Neobacillus soli*; **7**, *Nebacillus bataviensis*.

βD-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl αD-mannopyranoside, methyl αD-glucopyranoside, amygdalin, arbutin, salicin, D-cellobiose, D-lactose, D-melibiose, sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagalose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate and potassium 5-ketogluconate were negative. Using the API NE system, only the arginine dihydrolase test was positive, but the other strips were negative. Phenotypic comparison of strain LF1^T with closely related species showed that *N. massiliamazoniensis* differs from other compared with *Neobacillus* species by alkaline phosphatase positive and N-acetyl glucosamine and maltose negative (Table 1). The major fatty acids were 13-methyl-tetradecanoic acid (56%) and 12-methyl-tetradecanoic acid (17%). Comparison of fatty acid composition of *N. massiliamazoniensis* to other *Neobacillus* species is shown in Table 2.

Genomic properties and comparison

Our new species has a genome size of 4,588,940 bp and a G+C percentage of 34.1 mol%. Its genomic assembly was performed in 120 contigs with 4491 genes, of which 4391 are coding

TABLE 4. Genomic comparison of *Neobacillus massiliamazoniensis* strain LF1^T between their closely related species using GGDC and formula 2 (dDDH estimates based on identities over HSP length)

	N.ma	N.fu	N.jd	N. bt	N.cu	N.de	N.so
N.ma	100%	22.70 ± 4.7%	21.80 ± 4.7%	20.3 ± 2.3%	21.60 ± 4.7%	27.20 ± 4.9%	27.20 ± 4.8%
N.fu		100%	18.70 ± 4.6%	18.9 ± 2.3%	18.70 ± 4.6%	20.30 ± 4.6%	20.20 ± 4.6%
N.jd			100%	21.8 ± 2.4%	21.00 ± 4.7%	20.80 ± 4.7%	21.20 ± 4.7%
N.bt				100%	21.1 ± 2.3%	21.50 ± 2.3%	23.3 ± 2.4%
N.cu					100%	20.70 ± 4.7%	20.70 ± 4.7%
N.de						100%	23.40 ± 3.2%
N.so							100%

Abbreviations: N.ma, *Neobacillus massiliamazoniensis* LF1 (Genome accession number: CVRB00000000); **N.fu**, *Neobacillus fumarioli* DSM 18237 (BCUZ00000000); **N.jd**, *Neobacillus jeddahensis* DSM 28281 (CCAS000000000); **N.ba**, *Neobacillus bataviensis* IDA 1115 (AJLS00000000); **N.cu**, *Neobacillus cucumis* DSM 101566 (PGVE00000000); **N.de**, *Neobacillus drentensis* DSM 15600 (BCUX00000000); **N.so**, *Neobacillus soli* DSM 15604 (BCVI00000000); GGDC, Genome-to-Genome Distance Calculator.

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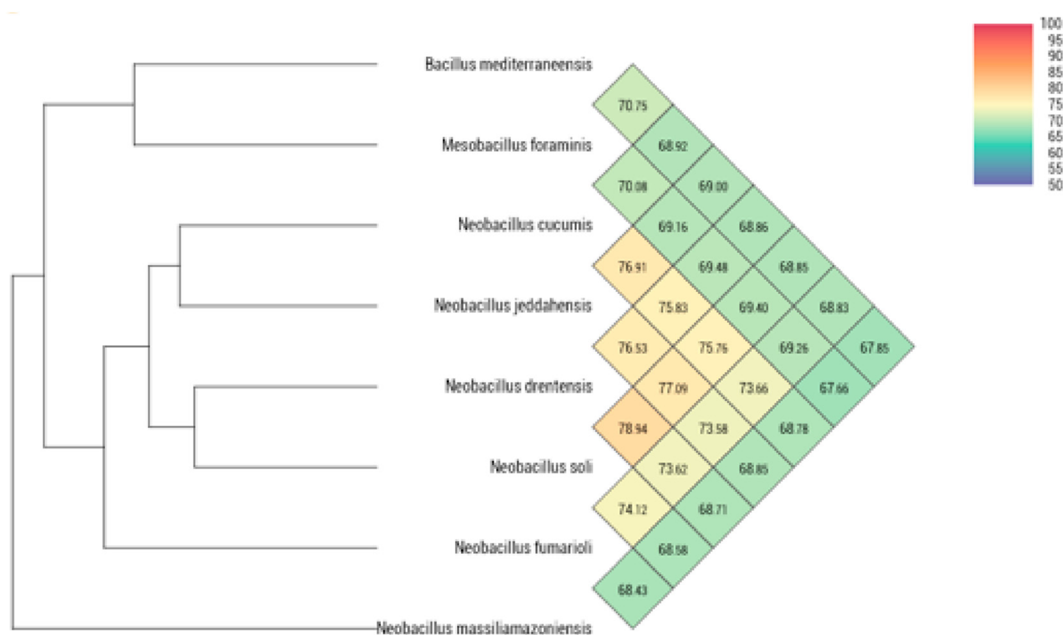

FIG. 5. Heatmap generated with OrthoANI values calculated using the OAT software for *Neobacillus massiliamazoniensis* sp. nov., strain LF1^T with their respective closely related species with standing in nomenclature.

(Fig. 3). The total number of proteins is equal to 4195 and that of rRNA and tRNA are 8 and 91, respectively. A brief statistical comparison of our *Neobacillus* genome with other related in terms of size, G+C content and number of genes, is presented in Table 3. Similarly, the distribution of genes in the 25 general COG categories has been illustrated in Fig. 4. DDH analysis shows values ranging from 18.7% between *N. fumarioli* and *N. jeddahensis* and *N. cucumis* to 27.2% between *N. massiliamazoniensis* and *N. drentensis*. The analysis of DDH values shared between strain LF1^T and other studied strains, showed low percentages of similarity ranging from 20.3% with *N. bataviensis* and 27.2% with *N. drentensis* (Table 4). These values are below the 70% threshold used for the delimitation of prokaryotic species, confirming that our strain represents a new species [21]. Furthermore, OrthoANI analysis with closely related species (Fig. 5) showed that *N. massiliamazoniensis* had a higher similarity value with *N. soli* (68.85%) and a lower value with *M. foraminis* (67.6%).

Conclusion

Based on phenotypic characteristics, but also on the phylogenetic and genomic analyses such as 16S rRNA sequence similarity of less than 98.65%, DDH value less than 70% and OrthoANI value of less than 95% [22], *N. massiliamazoniensis* strain LF1^T is declared as new species in the genus *Neobacillus*.

Description of *Neobacillus massiliamazoniensis* sp. nov.

Neobacillus massiliamazoniensis (mas.si.li.a.ma.zo.ni.e′n.sis. N. L. gen. neutr. n. *massiliamazoniensis*, a combination of Massilia, the Latin name of Marseille where strain LF1^T was isolated and described for the first time and Amazonia, the origin of the patient who provided the stool sample). The colonies of the strain appear beige and circular on blood agar plate, with a diameter of 1 mm. Bacterial cells are immobile and free of spores. They are Gram-positive bacilli and show positive oxidase and negative catalase activities. The strain grows both under aerobic and anaerobic conditions at temperatures ranging from 28°C to 37°C. The optimum grow occurs under aerobic condition at 37°C. *N. massiliamazoniensis* is able to ferment D-glucose, D-mannose, N-acetyl-glucosamine, D-maltose, starch and glycogen. Alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, α-galactosidase, α-glucosidase and β-glucosidase are present. The major fatty acids are 13-methyl-tetradecanoic acid (56%) and 12-methyl-tetradecanoic acid (17%). The genome size of strain LF1^T is approximately 4.6 Mbp with a G+C content of 34.1 mol%. The 16S rRNA gene sequence and the whole genome sequence of *N. massiliamazoniensis* have

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been deposited in GenBank under accession numbers LK021124 and CVRB00000000, respectively. Strain LF1^T is the type strain of *N. massiliamazoniensis* which was isolated from human feaces.

Transparency declaration

None to declare.

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