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 LF1T (=CSURP1359) was isolated from human stool. The 16S rRNA gene sequence analysis of strain LF1T (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 LF1T (accession number: CVRB00000000) is 4.6 Mbp with a G+C content of 34.1 mol%. Analysis of phylogenetic 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 LF1T, 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 LF1T 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/hrms-database). After several unsuccessful identification attempts with MALDI-TOF instrument, the 16S rRNA gene was amplified...
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 LF1T 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 LF1T 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.
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 Nextera 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 LF1T and its closest related species.

### Results

**Phylogenetic analysis**

Because there is no spectrum in the MALDI-TOF database that corresponds to the strain LF1T, the latter cannot be identified correctly. Therefore, a similarity analysis based on 16S rDNA of the strain LF1T showed that *N. massiliamazoniensis* strain LF1T 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 LF1T 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 LF1T 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).

| Fatty acid | 1  | 2  | 3  | 4  |
|------------|----|----|----|----|
| C15:0 iso | 55.9 ± 1.2 | 36.9 ± 4.3 | 32.2 ± 4.2 | 47.1 ± 3.5 |
| C16:00    | 1.3 ± 0.1  | 1.5 ± 0.6  | 1.4 ± 1.3  | 1.6 ± 0.2  |
| C16:0 iso | 8.1 ± 0.9  | 6.9 ± 1.1  | 8.7 ± 2.5  | 3.1 ± 1.2  |
| C18:0     | 3.9 ± 0.7  | 7.7 ± 3.2  | 3.4 ± 1.6  | 3.8 ± 1.0  |
| C18:1 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*.
Phenotypic and biochemical features

*N. massiliamazoniensis* sp. nov. strain LF1<sup>T</sup> 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 1 μm. 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<sup>T</sup> 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-xylene, L-xylene, D-adonitol, methyl

### TABLE 3. Comparison of genome size and GC content of the new *Neobacillus massiliamazoniensis* strain LF1<sup>T</sup> 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        | CV9800000000                     |
| *Neobacillus batavensis* (IDA 1115) | 5,371,144 | 39.6      | 5277        | NZ_A5000000000.1                  |
| *Neobacillus cucumis* (DSM 101566)   | 5,707,899 | 38.6      | 5580        | NZ_A5000000000.1                  |
| *Neobacillus drentensis* (DSM 15600)  | 5,305,306 | 38.9      | 5229        | BCUX00000000                      |
| *Neobacillus sali* (DSM 15604)       | 5,579,901 | 39.7      | 5486        | BCU000000000                      |
| *Neobacillus jeddahensis* (DSM 28281) | 4,762,944 | 39.4      | 4684        | NZ_CCAS000000000.1                |
| *Neobacillus fumarioli* (DSM 18237)  | 3,294,206 | 40.4      | 3335        | BCU200000000                      |

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β-D-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-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<sup>T</sup> with closely related species showed that <i>N. massiliamazoniensis</i> differs from other compared with <i>Neobacillus</i> 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 <i>N. massiliamazoniensis</i> to other <i>Neobacillus</i> 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<sup>T</sup> 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 ± 2.3% | 20.30 ± 4.6% | 20.20 ± 4.6% | 20.70 ± 4.7% |
| N.jd | 100% | 21.8 ± 2.4% | 21.00 ± 4.7% | 20.80 ± 2.4% | 21.20 ± 2.4% | 23.3 ± 2.4% | 23.3 ± 2.4% |
| N.bt | 100% | 21.1 ± 2.3% | 21.1 ± 2.3% | 21.50 ± 2.3% | 20.70 ± 4.7% | 20.70 ± 4.7% | 23.40 ± 3.2% |
| N.cu | 100% | 20.70 ± 4.7% | 20.70 ± 4.7% | 20.70 ± 4.7% | 20.70 ± 4.7% | 100% | 100% |
| N.de | 100% | 23.40 ± 3.2% | 23.40 ± 3.2% | 23.40 ± 3.2% | 23.40 ± 3.2% | 100% | 100% |
| N.so | 100% | 27.20 ± 4.8% | 27.20 ± 4.8% | 27.20 ± 4.8% | 27.20 ± 4.8% | 27.20 ± 4.8% | 100% |

**Abbreviations:** N.ma, <i>Neobacillus massiliamazoniensis</i> LF1 (Genome accession number: CVRB00000000); N.fu, <i>Neobacillus fumarioli</i> DSM 18237 (BCUZ00000000); N.jd, <i>Neobacillus jeddahensis</i> DSM 28281 (CCAS00000000000); N.bt, <i>Neobacillus bataviensis</i> IDA 1115 (AJLS00000000); N.cu, <i>Neobacillus cucumis</i> DSM 101566 (PGVE00000000); N.de, <i>Neobacillus drentensis</i> DSM 15600 (BCUX00000000); N.so, <i>Neobacillus soli</i> DSM 15604 (BCVI00000000); GGDC, Genome-to-Genome Distance Calculator.

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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 LF1T 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 LF1T 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 LF1T 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 LF1T 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
been deposited in GenBank under accession numbers LK021124 and CVRB00000000, respectively. Strain LF1T is the type strain of *N. massiliamazoniensis* which was isolated from human feaces.

Transparency declaration

None to declare.

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References

[1] Schoeni JL, Wong ACL. Bacillus cereus food poisoning and its toxins. J Food Prot 2005;68:636–48. https://doi.org/10.4315/0362-028x-68.3.636.

[2] Pal D, Mathan Kumar R, Kaur N, Kumar N, Kaur G, Singh NK, et al. Bacillus maritimus sp. nov., a novel member of the genus Bacillus isolated from marine sediment. Int J Syst Evol Microbiol 2017;67:60–6. https://doi.org/10.1099/ijsem.0.01569.

[3] Saxena AK, Kumar M, Chakdar H, Anuroopa N, Bagyaraj DJ. Bacillus species in soil as a natural resource for plant health and nutrition. J Appl Microbiol 2020;128:1583–94. https://doi.org/10.1111/jam.14506.

[4] Sneath PHA, MCGOWAN V, Skerman VBD. Approved lists of bac-

[5] Sneath PHA, MCGOWAN V, Skerman VBD. Approved lists of bacterial names. Int J Syst Evol Microbiol 1980;30:225–420. https://doi.org/10.1099/001623.

[6] Patel S, Gupta RS. A phylogenomic and comparative genomic frame-

[7] Lopez DR, Birney E. Veloce: algorithms for de novo short read as-

[8] Lee I, Ouk Kim Y, Park S-C, Chun J. OrthoANI: an improved algorithm for calculating average nucleotide identity. Microbiol Today 2006;8:6.

[9] Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for high quality short-read de novo assembler. GigaScience 2013;2:18. https://doi.org/10.1186/2047-217X-1-18.

[10] Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 2012;4135–77. https://doi.org/10.1093/cmb.2012.0021.

[11] Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for high quality short-read de novo assembler. GigaScience 2013;2:18. https://doi.org/10.1186/2047-217X-1-18.

[12] Lee I, Ouk Kim Y, Park S-C, Chun J. OrthoANI: an improved algorithm for calculating average nucleotide identity. Int J Syst Evol Microbiol 2014;64:384–91. https://doi.org/10.1128/ijsem.0.03709-1.

[13] Loy R, Liu B, Xie Y, Li Z, Huang W, Yuan J, et al. SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. GigAscience 2012;1:18. https://doi.org/10.1186/2047-217X-1-18.

[14] McCarthy BJ, Bolton ET. An approach to the measurement of genetic relatedness among organisms. Proc Natl Acad Sci U S A 1963;50:37.

[15] GigaScience 2012;1:18. https://doi.org/10.1186/2047-217X-1-18.

[16] Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for high quality short-read de novo assembler. GigaScience 2013;2:18. https://doi.org/10.1186/2047-217X-1-18.

[17] Lee I, Ouk Kim Y, Park S-C, Chun J. OrthoANI: an improved algorithm for calculating average nucleotide identity. Int J Syst Evol Microbiol 2014;64:384–91. https://doi.org/10.1128/ijsem.0.03709-1.

[18] Loy R, Liu B, Xie Y, Li Z, Huang W, Yuan J, et al. SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. GigAscience 2012;1:18. https://doi.org/10.1186/2047-217X-1-18.

[19] Meier-Kolthoff JP, Göker M, Spröer C, Klenk H-P. When should a new genus be recognized? An overview on Bacillus. Int J Syst Evol Microbiol 2013;63:290–313. https://doi.org/10.1099/ijsem.0.05162.

[20] Stackebrandt E, Ebers J. Taxonomic parameters revisited: tarnished gold standards. Microbiol Today 2006;8:6.

[21] McCarthy BJ, Bolton ET. An approach to the measurement of genetic relatedness among organisms. Proc Natl Acad Sci U S A 1963;50:156–64.

[22] Meier-Kolthoff JP, Göker M, Spröer C, Klenk H-P. When should a new genus be recognized? An overview on Bacillus. Int J Syst Evol Microbiol 2013;63:290–313. https://doi.org/10.1099/ijsem.0.05162.