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

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Running Title: *Neobacillus massiliamazoniensis* sp. nov.

Keywords: *Neobacillus massiliamazoniensis*; genome; culturomics; taxono-genomics; gut microbiota.

1 **Abstract:**

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

13 **Introduction:**

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

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

28 **Materials and Methods**

29 *Isolation and identification*

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

35 Bacterial identification was carried out using MALDI-TOF mass spectrometry (Bruker,
36 Daltonics, Bremen, Germany) and the reference spectrum generated was added in the local

37 database(<https://www.mediterranee-infection.com/urms-data-base>).After several unsuccessful
38 identification attempts with MALDI-TOF instrument, the 16S rRNA gene was amplified
39 using universal primers pairs fD1 and rP2 (Eurogentec, Angers, France) and sequenced with
40 the Big Dye® Terminator v1.1 cycle sequencing kit and the 3500xL capillary sequencer
41 Genetic Analyzer (Thermofisher, Saint-Aubin, France), as previously described [9]. The
42 nucleotide sequences obtained were assembled and edited with the Codoncode Aligner
43 software (<http://www.codoncode.com>). The consensual corrected sequence was used as a
44 reference sequence and compared against the 16S gene sequences of the type strains existing
45 in the NCBI database to assess the phylogenetically closest species. For this purpose, a Blastn
46 method was used since the NCBI database, to compare the 16S rRNA gene sequences
47 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

48 *Growth conditions and phenotypic characterization*

49 In order to determine the best growing condition for this new species, different approaches
50 were used. Therefore, the strain LF1^T was inoculated on Columbia agar with 5% sheep blood
51 (bioMérieux, Marcy l'Etoile, France) and incubated at different temperatures (28, 37, 42 and
52 52°C) and atmospheres (aerobic, anaerobic and microaerophilic) according to Diop *et al.*
53 2018[10]. The growth at different pH (6.5 to 8.5) and salt concentrations (50 to 150 g/L) is
54 also tested in parallel. API ZYM and API 50 CH strips (bioMérieux) were used to evaluate
55 the biochemical characteristics of the bacterium, following the manufacturer's
56 recommendations. Gram stain, catalase and oxidase tests and sporulation ability were done
57 using standard procedures [11]. The morphological structure of the bacterium was observed
58 with a scanning electron microscope (Hitachi Group, Tokyo, Japan) according the protocol
59 described by Belkacemi *et al.*[12].

60 *Genome extraction and sequencing*

61 Genomic DNA was extracted using the EZ1 biorobot (Qiagen, Courtaboeuf, France) with the
62 EZ1 DNA tissue kit preceded by a pre-treatment with lysozyme and incubation at 37°C for 2
63 hours. Sequencing of the gDNA was performed using MiSeq technology using the Nextra
64 Mate Pair sample preparation kit and the Nextera XT Paired (Illumina) tip, as described above
65 [13]. The assembly was performed using a pipeline incorporating different softwares (Velvet
66 [14], Soap Denovo [15] and Spades [16]). Data from Illumina MiSeq were trimmed using
67 Trimmomatic software or untrimmed using MiSeq software only [17]. GapCloser was used to
68 reduce assembly deviations. Scaffolds < 800 base pairs (bp) and scaffolds with a depth value
69 less than 25% of the average depth were removed. The best assembly was selected using
70 different criteria (number of scaffolds, N50, number of N). The degree of genomic similarity
71 of this strain with closely related species was estimated using OrthoANI software [18]. Then,
72 an online server named Genome-to-Genome Distance Calculator (GGDC)
73 (<http://ggdc.dsmz.de>) [19] was used to calculate the DNA-DNA hybridization (DDH) values
74 shared between strain LF1^T and its closest related species.

75 **Results**

76 *Phylogenetic analysis*

77 Since there is no spectrum in the MALDI-TOF database that corresponds to the strain LF1^T,
78 the latter cannot be identified correctly. Therefore, a similarity analysis based on 16S rDNA
79 of the strain LF1^T showed that *Neobacillus massiliamazoniensis* strain LF1^T had 98.32%
80 sequence identity with *Neobacillus bataviensis* strain IDA1115 (Accession number:
81 [NR_036766.1](#)). This value is lower than the recommended threshold value (<98.65%) to
82 delineate new bacterial species [20,21]. Given this result, we therefore propose to classify
83 strain LF1^T as a new species within the genus *Neobacillus* which belong to the family
84 *Bacillaceae* and the phylum *Firmicutes* [5]. The performed phylogenetic tree (Figure 1)

85 shows the position of *N. massiliamazoniensis* sp. nov. strain LF1^T among closely related
86 species with a validly published name. The shape of the bacterium (Figure 2) was observed
87 using the Hitachi TM4000 instrument (Hitachi Group, Tokyo, Japan).

88 *Phenotypic and biochemical features*

89 *N. massiliamazoniensis* sp. nov., strain LF1^T is able to grow both aerobically and
90 anaerobically but better aerobically with an optimal growth temperature of 37°C. It is a Gram-
91 positive rod-shaped **facultative anaerobic bacterium** with an average cell diameter of 1µm.
92 **This strain does not grow in salted media but it could grow with pH ranging from 6 to 7.5.**
93 **Indeed, the optimum growth was observed at pH = 7.** It has catalase-negative and oxidase-
94 positive activities. Using the API ZYM strip, only reactions with alkaline phosphatase,
95 leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, α-
96 galactosidase, α-glucosidase, β-glucosidase were positive. Other tests with esterase (C4),
97 esterase lipase (C8), lipase (C14), trypsin, acid phosphatase, β-galactosidase, β-glucuronidase,
98 N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase were negative. In addition, using
99 a 50 CH strip, strain LF1^T was positive for D-glucose, N-acetyl-glucosamine, glycogen,
100 starch and D-mannose. A weak reaction was observed with D-fructose. However, tests
101 including glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-
102 adonitol, methyl βD-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol,
103 D-mannitol, D-sorbitol, methyl αD-mannopyranoside, methyl αD-glucopyranoside,
104 amygdalin, arbutin, salicin, D-cellobiose, D-lactose, D-melibiose, sucrose, D-trehalose, inulin,
105 D-melezitose, D-raffinose, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagalose, D-fucose,
106 L-fucose, D-arabitol, L-arabitol, potassium 2-ketogluconate and potassium 5-ketogluconate
107 were negative. Using the API NE system, only the arginine dihydrolase test was positive, but
108 the other strips were negative. Phenotypic comparison of strain LF1^T with closely related
109 species showed that *N. massiliamazoniensis* differs from other compared *Neobacillus* species

110 by alkaline phosphatase positive and N-acetyl glucosamine and maltose negative (Table 1).
111 The major fatty acids were 13-methyl-tetradecanoic acid (56%) and 12-methyl-tetradecanoic
112 acid (17%). Comparison of fatty acid composition of *N. massiliamazoniensis* to other
113 *Neobacillus* species is shown in Table 2.

114 *Genomic properties and comparison*

115 Our new species has a genome size of 4,588,940 bp and a G+C percentage of 34.1 mol%. Its
116 genomic assembly was performed in 120 contigs with 4,491 genes, of which 4,391 are coding
117 (Figure 3). The total number of proteins is equal to 4,195 and that of rRNA and tRNA are 8
118 and 91, respectively. A brief statistical comparison of our *Neobacillus* genome with other
119 related in terms of size, G+C content and number of genes, is presented in Table 3. Similarly,
120 the distribution of genes in the 25 general COG categories has been illustrated in Figure 4.
121 DDH analysis shows values ranging from 18.7% between *N. fumarioli* and *N. jeddahensis* and
122 *N. cucumis* to 27.2% between *N. massiliamazoniensis* and *N. drentensis*. The analysis of DDH
123 values shared between strain LF1^T and other studied strains, showed low percentages of
124 similarity ranging from 20.3% with *N. bataviensis* and 27.2% with *N. drentensis* (Table 4).
125 These values are below the 70% threshold used for the delimitation of prokaryotic species,
126 confirming that our strain represents a new species [21]. Furthermore, OrthoANI analysis
127 with closely related species (Figure 5) showed that *N. massiliamazoniensis* had a higher
128 similarity value with *N. soli* (68.85%) and a lower value with *M. foraminis* (67.6%).

129

130 **Conclusion**

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

135 **Description of *Neobacillus massiliamazoniensis* sp. nov.**

136 *Neobacillus massiliamazoniensis* (mas.si.li.a.ma.zo.ni.e'n.sis. N. L. gen. neutr. n.
137 *massiliamazoniensis*, a combination of Massilia, the Latin name of Marseille where strain
138 LF1^T was isolated and described for the first time and Amazonia, the origin of the patient who
139 provided the stool sample). The colonies of the strain appear beige and circular on blood agar
140 plate, with a diameter of 1 mm. Bacterial cells are immobile and free of spores. They are
141 Gram-positive bacilli and show positive oxidase and negative catalase activities. The strain
142 grows both under aerobic and anaerobic conditions at temperatures ranging from 28°C to
143 37°C. The optimum grow occurs under aerobic condition at 37°C. *N. massiliamazoniensis* is
144 able to ferment D-glucose, D-mannose, N-acetyl-glucosamine, D-maltose, starch and
145 glycogen. Alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine
146 arylamidase, acid phosphatase, α -galactosidase, α -glucosidase, β -glucosidase are present. The
147 major fatty acids are 13-methyl-tetradecanoic acid (56%) and 12-methyl-tetradecanoic acid
148 (17%). The genome size of strain LF1^T is approximately 4.6 Mbp with a G+C content of 34.1
149 mol%. The 16S rRNA gene sequence and the whole genome sequence of *Neobacillus*
150 *massiliamazoniensis* have been deposited in GenBank under accession numbers LK021124
151 and CVRB00000000, respectively. Strain LF1^T is the type strain of *N. massiliamazoniensis*
152 which was isolated from human feces.

153 **Conflict of interest:**

154 None to declare

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157 submitting the genomic sequence to GenBank and Nicholas Armstrong for performing fatty
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232 **Table 1:** Differential characteristics of **1**, *Neobacillus massiliamazoniensis* sp. nov., strain
 233 LF1^T compared with **2**, *Neobacillus bataviensis* strain IDA 1115, **3**, *Neobacillus novalis* strain
 234 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	-
Utilization of:				
Potassium 5-ketogluconate	-	-	-	-
D-Xylose	-	-		+
D-Fructose	w	+	+	+
D-Glucose	+	+	+	+
Maltose	-	NF	+	+
D-Mannose	+	+	+	+
Habitat	Human Stool	Agricultural area	Agricultural area	Human skin

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

236 **Table 2:** Cellular fatty acid profiles (%) of *Neobacillus massiliamazoniensis* strain LF1^T
237 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

238 **1:** *Neobacillus massiliamazoniensis*; **2:** *Neobacillus bataviensis*; **3:** *Neobacillus drenensis* and
239 **4:** *Neobacillus vireti*.

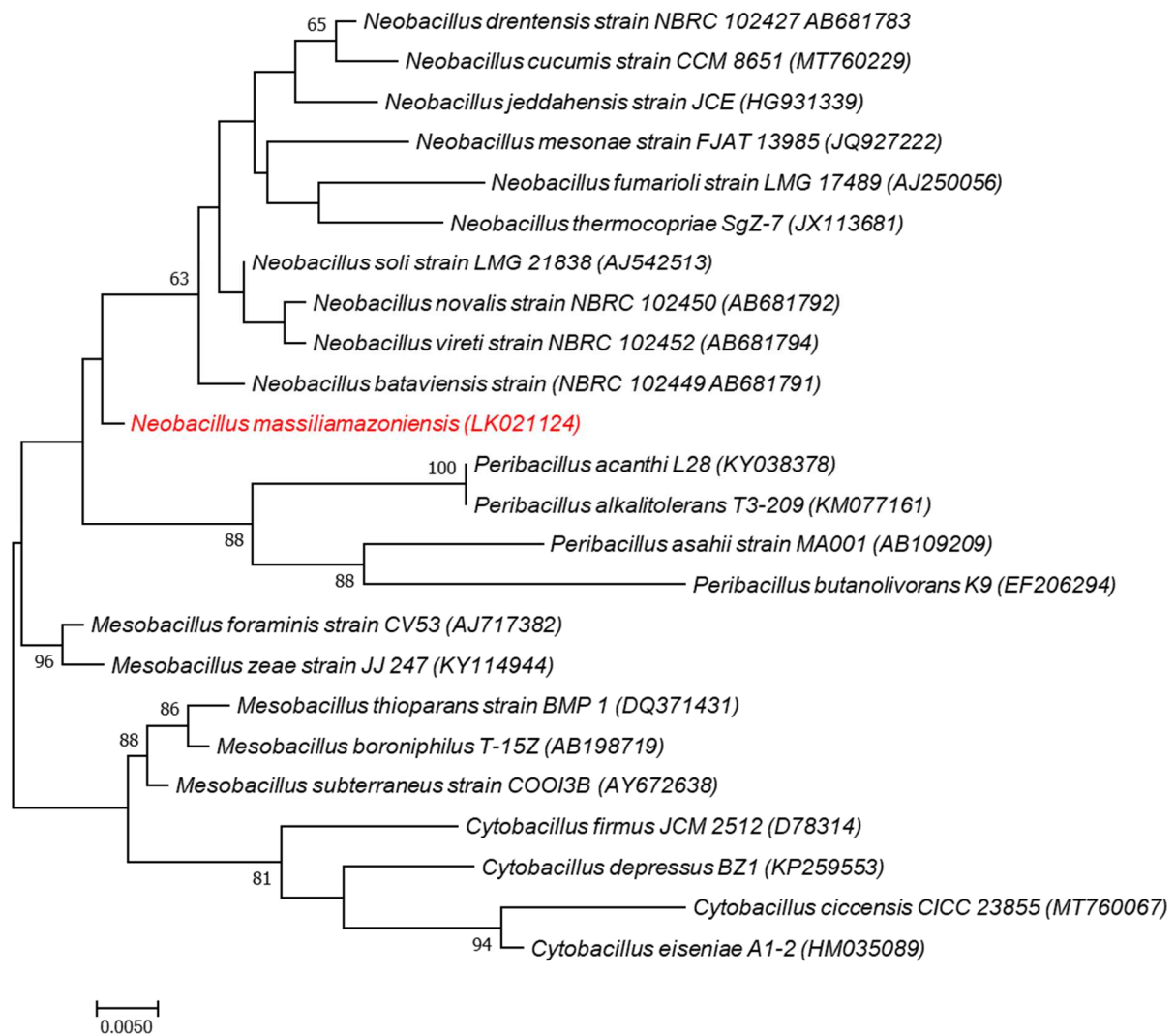
240 **Table 3:** Comparison of genome size and GC content of the new *Neobacillus*
 241 *massiliamazoniensis* strain LF1^T with its closely related species belonging to the genus
 242 *Neobacillus*.

Species (strain)	Size(pb)	GC(mol%)	Total genes	Genome accession numbers
<i>Neobacillus massiliamazoniensis</i> (LF1)	4,588,940	34.1	4,491	CVRB000000000
<i>Neobacillus bataviensis</i> (IDA 1115)	5,371,144	39.6	5,277	NZ_AJLS000000000.1
<i>Neobacillus cucumis</i> (DSM 101566)	5,707,899	38.6	5,580	NZ_PGVE000000000.1
<i>Neobacillus drentensis</i> (DSM 15600)	5,305,306	38.9	5,229	BCUX000000000
<i>Neobacillus soli</i> (DSM 15604)	5,579,901	39.7	5,486	BCVI000000000
<i>Neobacillus jeddahensis</i> (DSM 28281)	4,762,944	39.4	4,684	NZ_CCAS000000000.1
<i>Neobacillus fumarioli</i> (DSM 18237)	3,294,206	40.4	3,355	BCUZ000000000

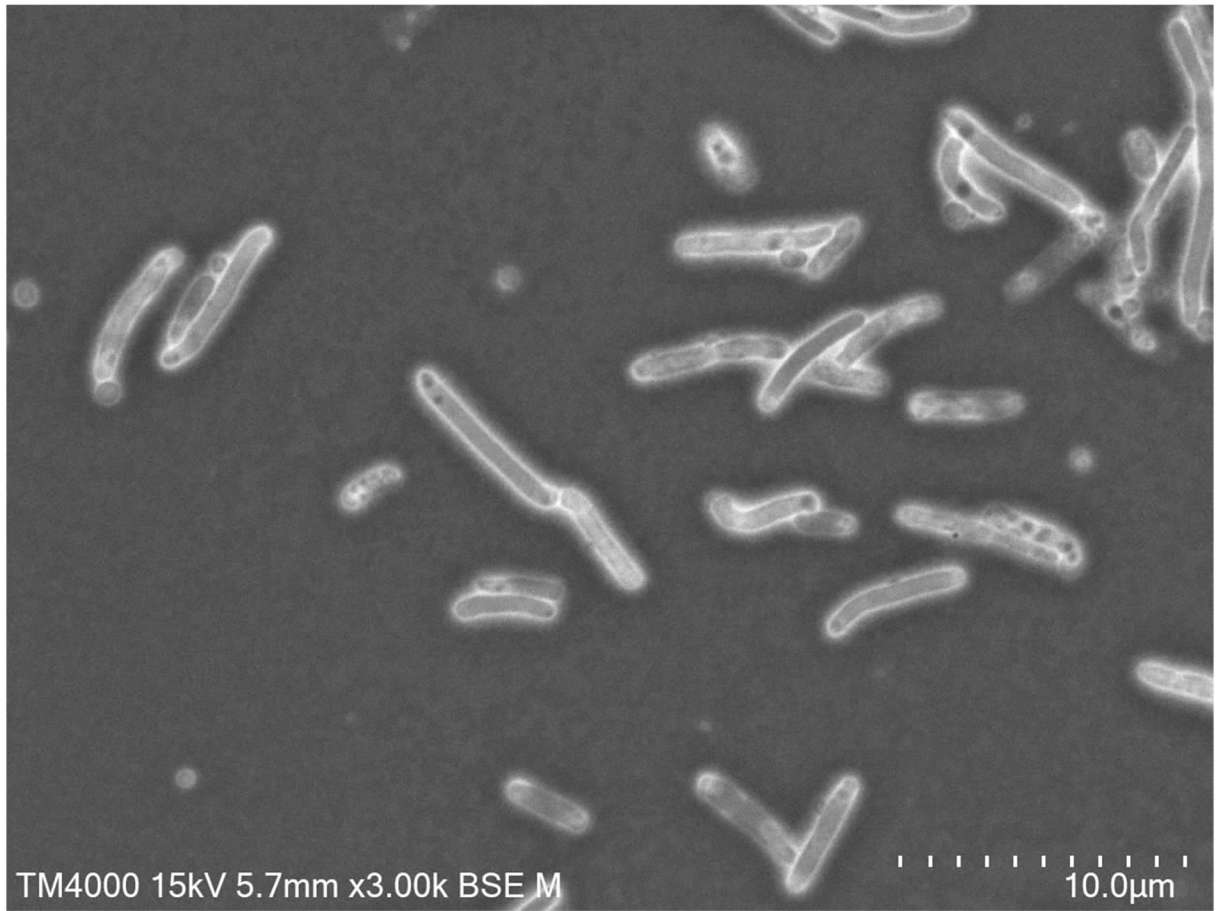
243 **Table 4:** Genomic comparison of *Neobacillus massiliamazoniensis* strain LF1^T between their
 244 closely related species using GGDC and formula 2 (dDDH estimates based on identities over
 245 HSP length).

	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%

246 **Abbreviations:** **N.ma**, *Neobacillus massiliamazoniensis* LF1 (Genome accession number :[CVRB000000000](#)); **N.fu**,
 247 *Neobacillus fumarioli* [DSM 18237 \(BCUZ000000000\)](#); **N.jd**, *Neobacillus jeddahensis* [DSM 28281 \(CCAS000000000\)](#);
 248 **N.ba**, *Neobacillus bataviensis* [IDA 1115 \(AJLS000000000\)](#); **N.cu**, *Neobacillus cucumis* [DSM 101566 \(PGVE000000000\)](#);
 249 **N.de**, *Neobacillus drementensis* [DSM 15600 \(BCUX000000000\)](#); **N.so**, *Neobacillus soli* [DSM 15604 \(BCVI000000000\)](#).

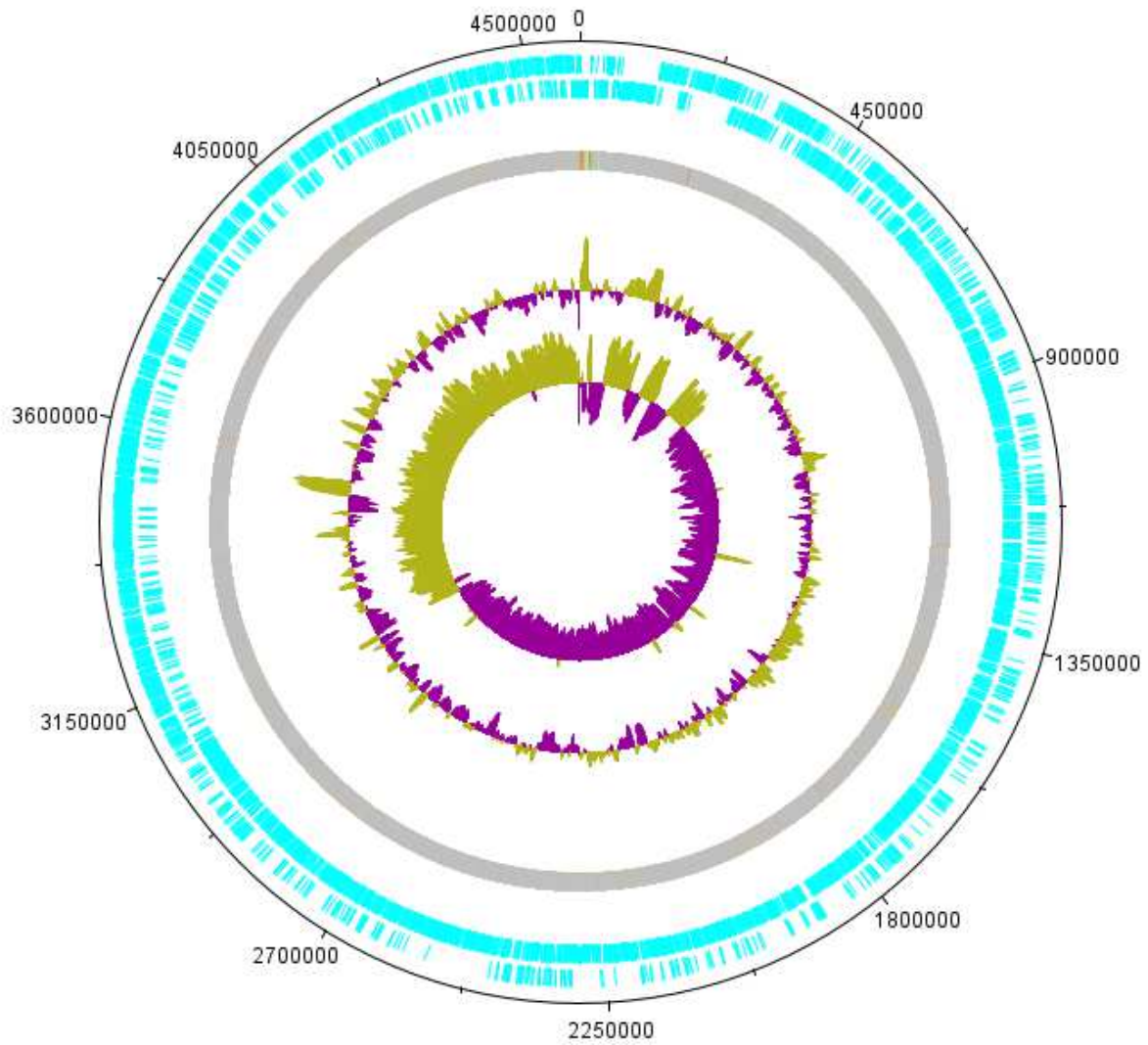


250
 251 **Figure 1:** Phylogenetic tree displaying the position of *Neobacillus massiliamazoniensis* strain
 252 LF1^T compared to its closest phylogenetic species. The respective GenBank accession
 253 numbers for 16S rRNA genes are indicated in parenthesis. Sequence alignment and
 254 phylogenetic inferences were obtained using the maximum likelihood method within MEGA
 255 7 software. The numbers at the nodes are percentages of bootstrap values obtained by
 256 repeating the analysis 1000 times to generate a majority consensus tree. Only percentage
 257 values higher than 70% are shown in the figure with a 5% scale.



258

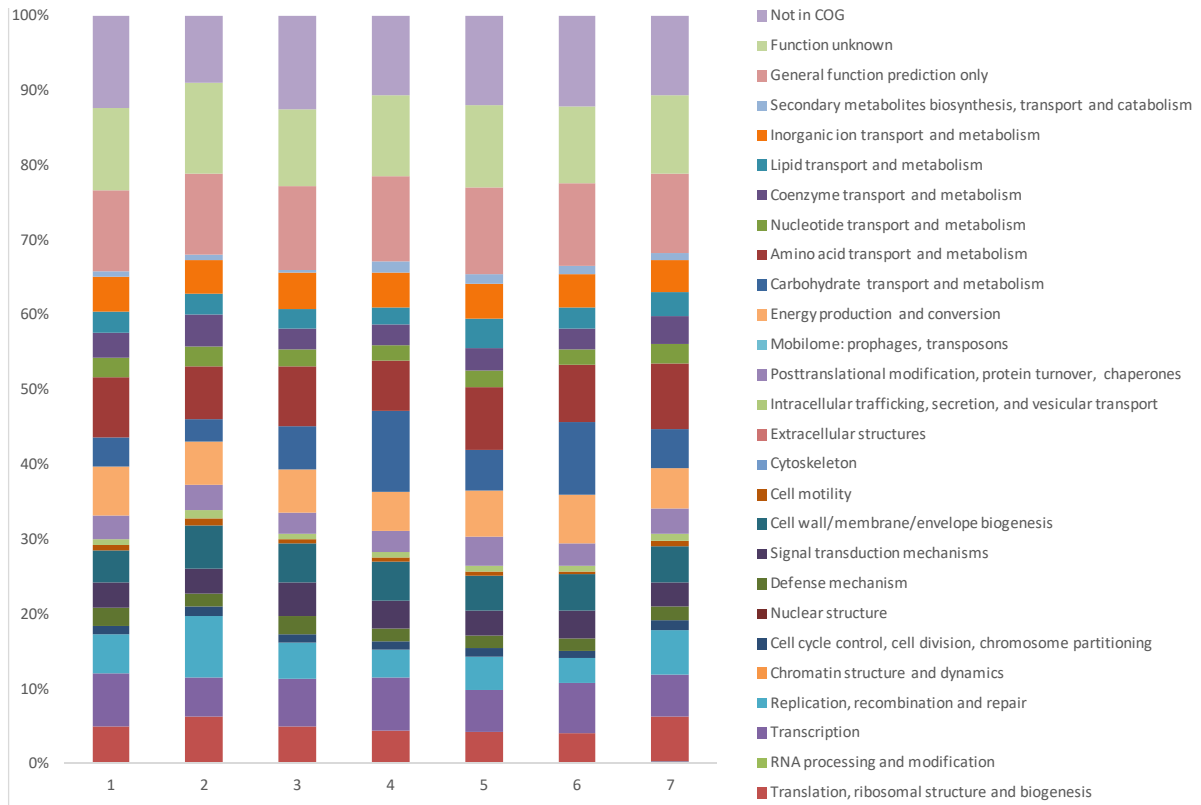
259 **Figure 2:** Scanning electron micrograph of *Neobacillus massiliamazoniensis* LF1^T using the
260 Scanning Electron Microscope TM4000 from Hitachi. Scale bar and acquisition settings are
261 presented on the picture.



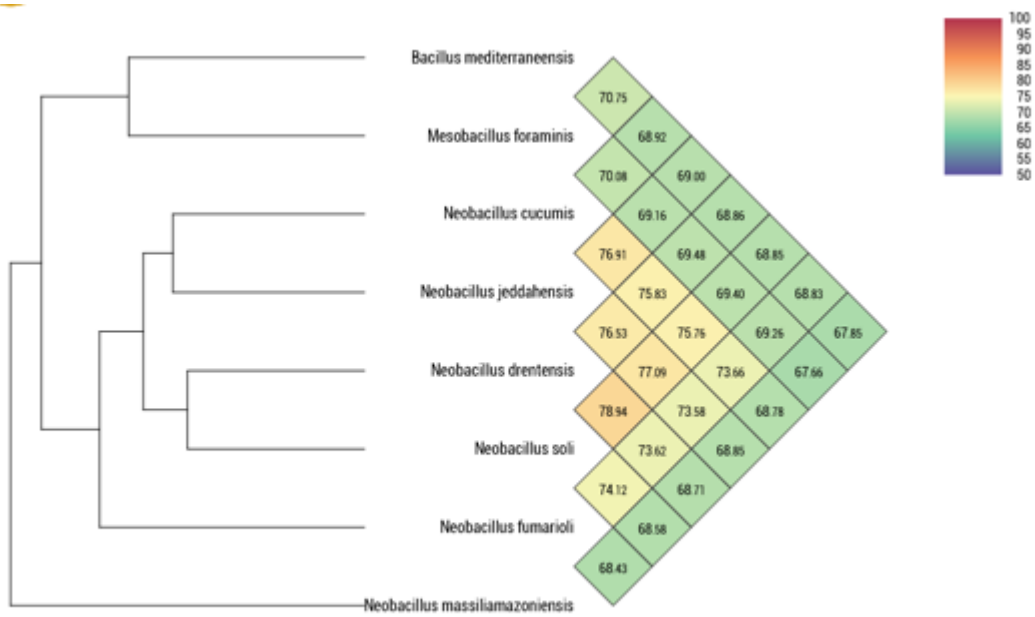
262

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

266



267 **Figure 4:** Distribution of functional classes of predicted genes according to the COG of
268 proteins of strain LF1^T among related other species: **1**, *Neobacillus massiliamazoniensis*
269 LF1^T; **2**, *Neobacillus jeddahensis*; **3**, *Neobacillus drementensis*; **4**, *Neobacillus cucumis*; **5**,
270 *Neobacillus fumarioli*; **6**, *Neobacillus soli*; **7**, *Neobacillus bataviensis*;



271
 272 **Figure 5:** Heatmap generated with OrthoANI values calculated using the OAT software for
 273 *Neobacillus massiliamazoniensis* sp. nov., strain LF1^T with their respective closely related
 274 species with standing in nomenclature.