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**Taxonogenomics description of *Bacillus marasmi* sp. nov., a new species isolated from the stool sample.**

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**Running title:** *Bacillus marasmi* sp. nov.

**Keywords:** *Bacillus marasmi* sp. nov.; Taxonogenomics; Human stool; Marasmus; Child.

1 **Abstract:**

2 Using culturomics method, two strains were isolated, identified and characterized following  
3 the taxono-genomics concept. *Bacillus marasmi* sp. nov. strain Marseille-P3556  
4 (=CSURP3556) is isolated from 13-month-old girl living in **Nigerien**. The phylogenic tree,  
5 phenotypic criteria and genomic analysis described here clearly show that this bacterium is  
6 different from previously known bacterial species with standing in nomenclature and new  
7 members of *Bacillus* genus.

8

## 9 **Introduction**

10 In 1872, Ferdinand Julius Cohn described a new genus belonging to the family *Bacillaceae*  
11 and named *Bacillus* [1]. Currently hundreds of new *Bacillus* species have been described with  
12 validly published names [2]. Most often members of the genus *Bacillus* are bacteria that live  
13 in various environments such as soil, food, fresh water and the sea [3-6]. *Bacillus species*  
14 *have varied lifestyles and can be saprophytes [7] or plant endophytes [8-9]*. In addition, there  
15 are two species which constitute a burden for public health: one is associated with food  
16 poisoning (*Bacillus cereus*) [10] and the other is responsible for anthrax (*Bacillus anthracis*)  
17 [11].

18 An understanding of the origin of disease or the human health status depends in part on the  
19 study of bacteria involved in normal physiological functions [12]. Thus, the exploration of the  
20 human intestinal microbiota has been at the focus of scientific studies in recent years. The  
21 culturomics method was initiated in our laboratory to isolate bacteria under different culture  
22 conditions [13-14]. This method is associated with identification by MALDI-TOF MS and  
23 systematic sequencing of the 16S rRNA gene; which made it possible to better understand the  
24 microbial diversity of the human intestine [15-16]. New bacterial species have been isolated  
25 and described using these methods which combine phenotypic, morphological, biochemical  
26 and genotypic characteristics [17-18].

27 Herein, we report the details of the isolation and taxono-genomics characterization of strain  
28 Marseille-P3556<sup>T</sup>, as type strain of *Bacillus marasmi* sp. nov., *for which its creation was*  
29 *previously announced [19]*.

## 30 **Materials and methods**

31 *Strains isolation and identification*

32 The human microbiome study was an opportunity for us to isolate bacterial strain from stool  
33 sample from a Nigerien child with severe acute malnutrition. The patient's guardian approved  
34 informed consent and then the study was validated by the ethics committee of the Institut  
35 Federatif de Recherche IFR48 under number 09-022. The shipments of the sample as well as  
36 its isolation were done as previously described [20]. Then, strain Marseille-P3556 was seeded  
37 in petri dishes containing 5% sheep blood agar (bioMérieux, Marcy l'Etoile, France) and  
38 incubated under anaerobic condition (Thermo Scientific, Dardilly, France) at 37°C.  
39 **Identification by MALDI-TOF mass spectrometry has failed with this strain despite numerous**  
40 **attempts because this reference spectrum is unknown in the MADI-TOF database.** The  
41 Microflex LT spectrometer (Bruker, Daltonics, Bremen, Germany) was used during the  
42 MALDI-TOF identification as previously described [21]. The obtained spectra were analyzed  
43 with Biotyper 3.0 software and added in the local URMS database  
44 (<https://www.mediterranee-infection.com/urms-data-base>). The 16S rRNA gene was  
45 amplified using the primer pair fD1 and rP2 (Eurogentec, Angers, France) and then sequenced  
46 using the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xLGenetic Analyzer  
47 capillary sequencer (ThermoFisher, Saint-Aubin, France), as previously described [22]. The  
48 nucleotide sequences of the 16S rRNA gene were analyzed by CodonCode Aligner software  
49 (<http://www.codoncode.com>). An obtained consensus sequence is compared to the NCBI  
50 nucleotide database (<https://www.ncbi.nlm.nih.gov/nucleotide/>).

#### 51 *Phenotypic and biochemical characterization*

52 Aerobic, microaerophilic and anaerobic atmospheres (Thermo Scientific, Dardilly, France)  
53 were tested to assess the different growth conditions of this strain. Varied temperatures were  
54 tested (28, 37, 45 and 55 °C) to determine the optimal temperature of strain Marseille-P3556  
55 on 5% sheep blood-enriched Columbia agar medium (bioMérieux, Marcy l'Etoile, France).  
56 API ZYM and API 50 CH strips (bioMérieux) were used to establish the biochemical

57 characteristics of the strain in accordance with the manufacturer's recommendations. Further  
58 phenotypic tests, such as Gram-staining, catalase, oxidase and spore-forming were performed  
59 as previously reported [23]. The morphological details were highlighted with a scanning  
60 electron microscope (Hitachi High-Technologies, Tokyo, Japan) as previously described [24].  
61 The fatty acid methyl ester (FAME) analysis was performed by GC/MS. Samples were  
62 prepared with approximately 10 to 20 mg of bacterial biomass per tube harvested from several  
63 culture plates. FAMEs were prepared as described by Sasser [25] followed by GC/MS  
64 analyses [26]. Briefly, FAMEs were separated using an Elite 5-MS column and monitored  
65 using mass spectrometry (Clarus 500 - SQ 8 S, Perkin Elmer, Courtaboeuf, France). Spectral  
66 database search was performed using MS Search 2.0 operated with the Standard Reference  
67 Database1A (NIST, Gaithersburg, USA) and the FAMEs mass spectral database (Wiley,  
68 Chichester, UK).

### 69 *Genome characteristics*

70 Extraction of the genomic DNA (gDNA) was carried out on EZ1 biorobot using the EZ1  
71 DNA tissue kit (Qiagen, Hilden, Germany). Then, it's sequenced on the MiSeq instrument  
72 (Illumina Inc, San Diego, CA, USA) using the Nextera Mate Pair and Nextera XT Paired End  
73 (Illumina) sample preparation kit, as previously reported [17]. The genomic assembly was  
74 performed with three softwares such as Velvet [27], Spades [28] and Soap Denovo [29].  
75 Sequences were trimmed or untrimmed using MiSeq and Trimmomatic [30] softwares. Best  
76 assembly was done using different criteria as previously described [20]. Annotation of  
77 Genome of Marseille-P3556 was performed as reported elsewhere [20]. The Genome-to-  
78 Genome Distance Calculator (GGDC) web server available online (<http://ggdc.dsmz.de>) was  
79 used to calculate similarity between the related closed genomes. Thus the DNA–DNA  
80 hybridization (DDH) was also determined [31]. Average nucleotide identity analysis was  
81 assessed using the OAT software [32].

## 82 **Results**

### 83 *Strain identification and phylogenetic analysis*

84 Colonies of strain Marseille-P3556 was not identified by mass spectrometry. **In other words**  
85 **the spectral reference of the strain is absent in the MALDI-TOF database.** Therefore, its  
86 generated reference spectrum was incremented in our local database (**Figure 1**). A similarity  
87 analysis of the 16S rDNA gene of strain Marseille-P3556 revealed a nucleotide sequence  
88 identity of 97.29% with *Bacillus subterraneus* strain COOI3B (accession number  
89 NR\_104749.1), the phylogenetically closest species. This similarity value was lower than the  
90 recommended cut-off value (98.65%) to delimit the species barrier in bacteria [33]. Therefore,  
91 strain Marseille-P3556 was potentially new species within the family *Bacillaceae*. The 16S  
92 rRNA-based phylogenetic tree of *Bacillus* species (Figure 2) highlighted the position of strain  
93 Marseille-P3556 among its closely related species with a validly published name. In addition,  
94 the shape of bacterial cell was visualized using the Hitachi SU5000 instrument (Figure 3).

### 95 *Biochemical and phenotypic properties of the strain*

96 Strain Marseille-P3556 grows under aerobic conditions with an optimal temperature at 37°C.  
97 It is Gram-positive aerobic rod-shaped bacterium with a mean cell diameter of 0.8 µm.  
98 Colonies of the strain Marseille-P3556 were flat, smooth, small, circular and pale grey with a  
99 mean diameter of 0.5 to 2 mm. It presents catalase-negative and oxidase-negative activities.

100 Using the API ZYM strip, **esterase lipase (C 8)** and naphthol-AS-BI-phosphohydrolase were  
101 positive for strain Marseille-P3556, while alkaline phosphatase, valine arylamidase, trypsin, α  
102 -chymotrypsin, acid phosphatase, **lipase (C14)**, **esterase (C4)** β-galactosidase, β-  
103 glucuronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase  
104 were negative. In addition, using 50 CH strip, *Bacillus marasmi* strain Marseille-P3556 was  
105 positive for glycerol, ribose, D-turanose, adonitol, galactose, glucose, mannose, D-fructose,

106 inositol, sorbitol, methyl  $\alpha$ D-glucopyranoside, N-acetyl-glucosamine, amygdalin, arbutin,  
107 salicin, D-maltose, D-lactose, D-melibiose, starch, sucrose, inulin, D-melezitose, D-raffinose,  
108 glycogen, xylitol, gentiobiose, D-cellobiose, D-trehalose, D-lyxose, and potassium 5-  
109 ketogluconate. But negative reactions were observed for erythritol, arabinose, xylose, D-  
110 tagalose, fucose and potassium 2-ketogluconate. Phenotypic criteria of strain Marseille-P3556  
111 were compared with closely related species in Table 1. The major fatty acids found for  
112 Marseille-P3556 were C<sub>16:0</sub> (31.4%), C<sub>14:0iso</sub> (20%) and C<sub>16:1n7</sub> (14.8%). Minor amounts of  
113 saturated fatty acids were also found with strain Marseille-P3556.

#### 114 *Genomic analysis*

115 The size of the genome of strains Marseille-P3556 was 4,638,282 bp long with 38.2 mol%  
116 G+C content (Figure 4). The genomic assembly was carried out into 47 contigs and into 46  
117 scaffolds. Strain Marseille-P3556 has 4,586 assigned as predicted genes. Furthermore, 4,415  
118 protein-coding genes and 116 RNAs genes (28 rRNAs, 83 tRNAs and 5 ncRNAs) were  
119 detected inside genome of Marseille-P3556. In Table 2, the composition of the genome of *B.*  
120 *marasmi* is contrasted against that of the genomes of phylogenetically close species.

121 DDH analysis showed values ranged from 19.1% between *B. drentensis* and *B. subterraneus*  
122 to 31.8% between *B. circulans* and *B. subterraneus*. Obtained values are below to the 70%  
123 recommended threshold to delineate new prokaryotic species, thus confirming that this strain  
124 is new species. The DDH values comparison of species in the study here is detailed in Table  
125 3. Moreover, OrthoANI analysis among closely related species (Figure 5) showed that  
126 *Bacillus* species had higher value of percentage of identity of 77.22% shared between *B.*  
127 *drentensis* and *B. bataviensis*. On the other hand, 67.53% was lowest value of similarity  
128 obtained between *B. subterraneus* and *B. circulans*. These OrthoANI values are lower than  
129 the recommended threshold (<95%), which suggest strain Marseille-P3556 is new member of  
130 *Bacillus* genus.

131 **Conclusion**

132 Considering the phenotypic tests, biochemical criteria and genomic analysis performed on  
133 strain Marseille-P3556, we proposed it as new bacterial species. Therefore, in this study, the  
134 genomic findings obtained such as the sequence identity of the 16S rRNA gene below the  
135 threshold value of 98.65%, the OrthoANI values (<95%) and DDH averages allowed us to  
136 formally report that *Bacillus marasmi* sp. nov., is new species within the family *Bacillaceae*  
137 in the phylum *Firmicutes*.

138 **Description of *Bacillus marasmi* sp. nov.**

139 *Bacillus marasmi* sp. nov. (ma.ras.mi, L. adj. fem, 'marasmus,' the disease meaning a form of  
140 severe malnutrition in children). It is Gram-positive aerobic bacterium and rod-shaped. Cells  
141 have diameter varying between 0.6 to 1 µm. Catalase and oxidase activities are negative.  
142 Colonies are small, circular and pale grey with a mean diameter of 1.25 mm on blood agar.  
143 The strain Marseille-P3556 was positive for glycerol, ribose, D-turanose, adonitol, galactose,  
144 glucose, mannose, D-fructose, inositol, sorbitol, methyl αD-glucopyranoside, N-acetyl-  
145 glucosamine, amygdalin, arbutin, salicin, D-maltose, D-lactose, D-melibiose, starch, sucrose,  
146 inulin, D-melezitose, D-raffinose, glycogen, xylitol, gentiobiose, D-lyxose, and potassium 5-  
147 ketogluconate. C<sub>16:0</sub> (31.4%), C<sub>14:0iso</sub> (20%) and C<sub>16:1n7</sub> (14.8%). are the major fatty acids  
148 detected in cells of *Bacillus marasmi* sp. nov. The genome of strain Marseille-P3556 was 4.64  
149 Mbp with 38.2 mol% of G+C content. The 16S rRNA and draft genome sequences are  
150 deposited in the Genbank database under Accession numbers LT671590 and  
151 CABHPS0000000000, respectively. The type strain of *Bacillus marasmi* sp. nov., strain  
152 Marseille-P3556 was isolated from a Nigerian child with clinical aspects of marasmus.

153 **Conflict of interest:**

154 Authors declare that there are not conflicts of interest.

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283 P. *Bacillus novalis* sp. nov., *Bacillus vireti* sp. nov., *Bacillus soli* sp. nov., *Bacillus*  
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285 Int J Syst Evol Microbiol. 2004 Jan;54(Pt 1):47-57. doi: 10.1099/ijss.0.02723-0.

286 **Table 1:** Different characteristics of **1**, *Bacillus marasmi* sp. nov., strain Marseille-P3556; **2**, *Bacillus dakarensis* strain Marseille-P3515 [18]; **3**,  
 287 *Bacillus massiliogabonensis* strain Marseille P2639 [18]; **4**, *Bacillus subterraneus* strain DSM 13966 [35]; **5**, *Bacillus drentensis* strain DSM  
 288 15600 [36]; **6**, *Bacillus bataviensis* strain DSM 15601 [36].

<b>Properties</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>
Cell diameter (µm)	0.6-1	0.5-1	0.7-1	0.5–0.8	0.6-1.2	0.7-1.2
Oxygen requirement	Aerobic	Aerobic	Aerobic	FA	FA	FA
Gram stain	+	+	–	–	+	+
Motility		+	–	+	+	+
Endospore formation	+	+	+	–	+	+
<b>Production of:</b>						
Alkaline phosphatase	–	–	+	na	na	na
Catalase	–	+	+	+	na	na
Oxidase	–	+	–	–	na	na
β-Galactosidase	–	–	–	–	+	+
α-Glucosidase	–	–	–	+	na	na
Naphthol-AS-BI-phosphohydrolase	+	–	–	na	na	na
N-acetyl-β-glucosaminidase	+	–	–	–	+	+
<b>Utilization of:</b>						
Potassium 5-ketogluconate	+	–	–	na	–	–
D-Xylose	–	+	–	+	w	–
D-Fructose	+	–	–	+	+	+
D-Glucose	+	–	–	+	+	+
D-Mannose	+	–	–	–	w	+
G+C content (mol%)	38.2	38.6	37.9	43.1	39.4	39.6
Habitat	Human stool	Human stool	Stool sample	Thermal waters	Soil	Soil

289 FA, facultative anaerobic; +, positive reaction; -, reaction; na, not available data.

290

291 **Table 2:** Genome comparison of closely related species to *B. marasmi* strain Marseille-P3556<sup>T</sup>

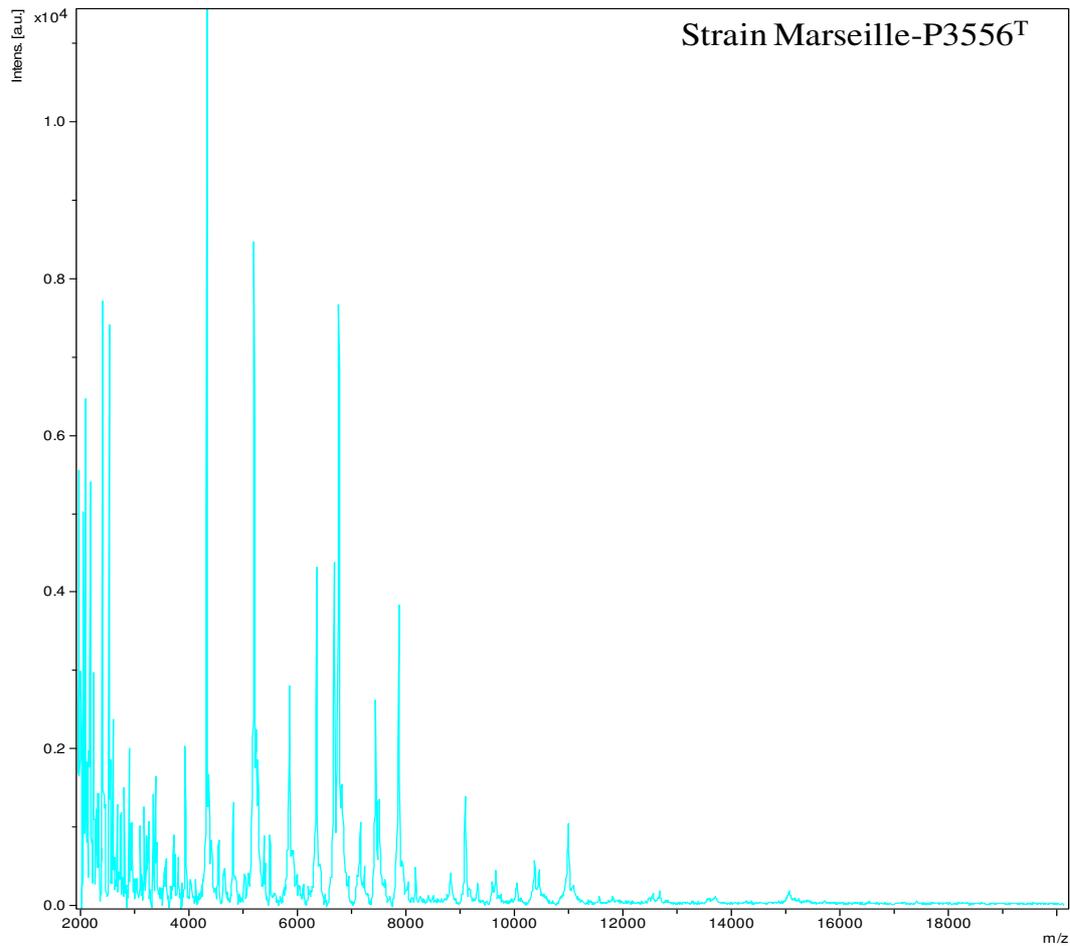
Species	Size (Mb)	G+C (mol%)	Proteins	RNAs	Genes	Pseudogenes
<i>Bacillus marasmi</i>	4.64	38.2	4,415	116	4,585	54
<i>Bacillus subterraneus</i>	4.57	43.9	4,526	93	4,691	72
<i>Bacillus circulans</i>	5.10	35.6	4,836	79	5,008	93
<i>Bacillus massiliogabonensis</i>	5.23	38.1	4,917	193	5,153	48
<i>Bacillus drementensis</i>	5.31	38.9	4,946	185	5,213	82
<i>Bacillus bataviensis</i>	5.37	39.6	5,126	36	5,277	115
<i>Bacillus mediterraneensis</i>	3.34	42.3	3,253	108	3,467	106
<i>Bacillus niacini</i>	6.18	38.2	5,793	46	5,920	81

292

293 **Table 3:** Genomic comparison of *Bacillus marasmi* strain Marseille-P3556 between its closely related species using GGDC and formula 2 (dDDH  
294 estimates based on identities over HSP length).

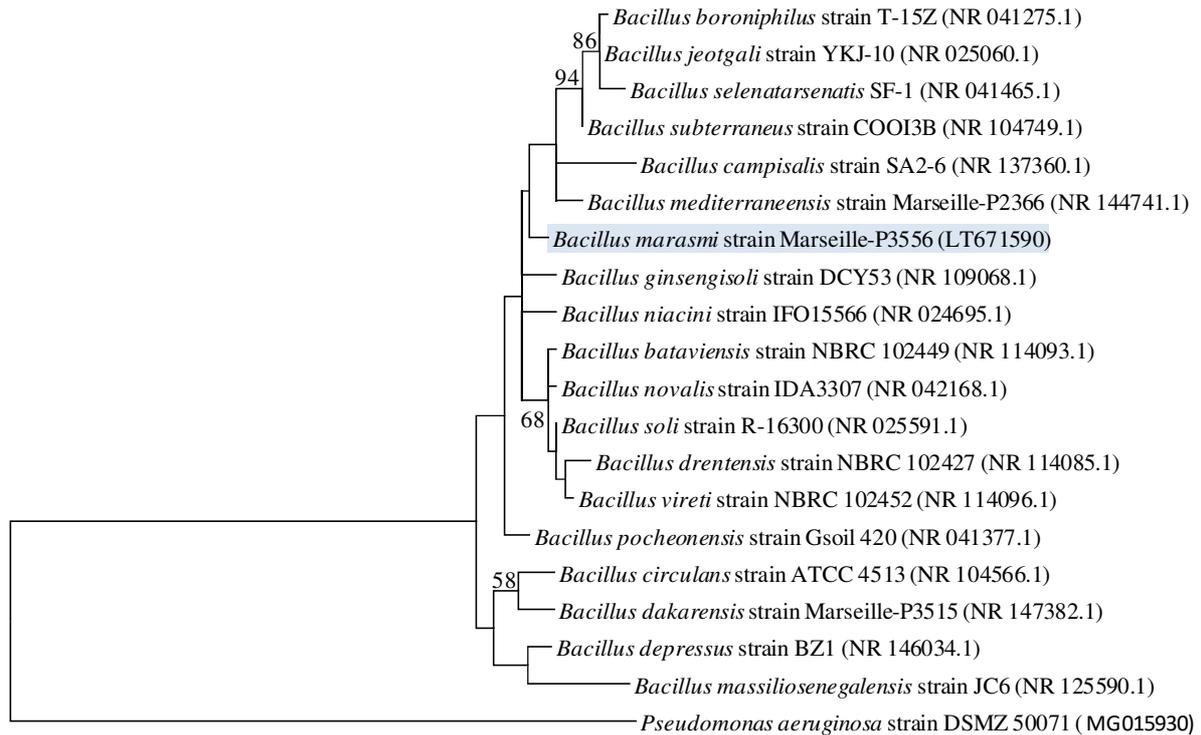
	BMA	BDA	BMG	BCI	BSU	BDR	BBA	BME
BMA	100%	21.90 ±4.7%	22.60 ±4.8%	28.80 ±4.9%	22.40 ±4.7%	20.60 ±4.7%	20.60 ±4.7%	21.40 ±4.6%
BDA		100%	24.30 ±4.8%	28.50 ±4.9%	21.60 ±4.7%	21.30 ±4.7%	22.40 ±4.7%	23.00 ±4.7%
BMG			100%	27.40 ±4.8%	26.20 ±4.9%	23.80 ±4.8%	23.10 ±4.7%	27.10 ±4.8%
BCI				100%	31.80 ±4.9%	30.60 ±4.9%	25.40 ±4.8%	28.60 ±4.9%
BSU					100%	19.10 ±4.5%	19.60 ±4.6%	19.70 ±4.6%
BDR						100%	21.00 ±4.7%	20.50 ±4.6%
BBA							100%	21.60 ±4.7%
BME								100%

295 **BMA**, *Bacillus marasmi* Marseille-P3556 (CABHPS000000000); **BDA**, *Bacillus dakarensis* Marseille-P3515 (FTOZ000000000); **BMG**, *Bacillus*  
296 *massiliogabonensis* Marseille-P2639 (FZRJ000000000); **BCI**, *Bacillus circulans* NBRC 13626 (NZ\_CP026033.1); **BSU**, *Bacillus subterraneus* DSM 13966  
297 (RSFW000000000); **BDR**, *Bacillus drementensis* NBRC 102427 (BCUX000000000); **BBA**, *Bacillus bataviensis* LMG 21833 (AJLS000000000); **BME**, *Bacillus*  
298 *mediterraneensis* Marseille-P2366 (FOJL000000000).

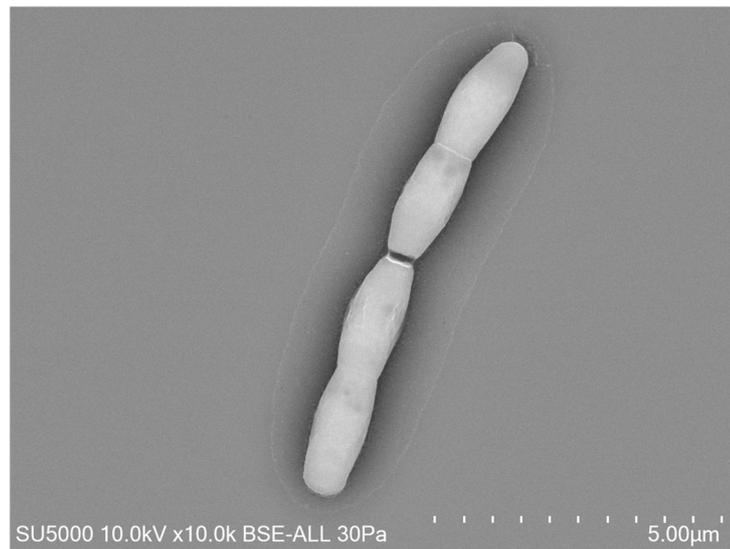


300

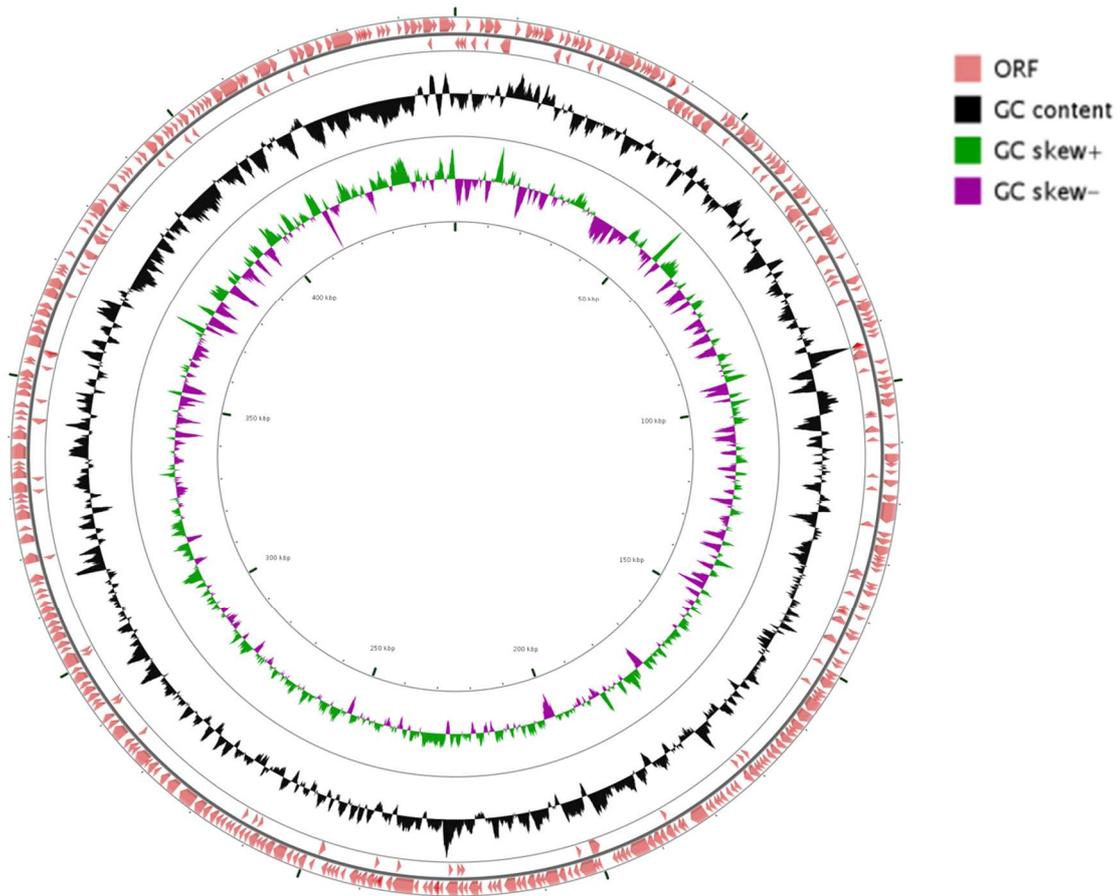
301 **Figure 1:** Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry  
302 (MALDI-TOF MS) reference spectrum of *Bacillus marasmi* sp. nov. The reference spectrum  
303 was generated by comparison of spectra from 12 individual colonies of strain Marseille-  
304 P3556.



305  
 306 **Figure 2:** The 16S rRNA phylogenetic tree displaying the position of *Bacillus marasmi* strain  
 307 Marseille-P3556<sup>T</sup> relative to its closest phylogenetically species. The respective GenBank  
 308 accession numbers for 16S rRNA genes are indicated in parenthesis. Sequence alignment and  
 309 phylogenetic inferences were obtained using the maximum likelihood method within MEGA  
 310 7 software. The numbers at the nodes are percentages of bootstrap values obtained by  
 311 repeating the analysis 1000 times to generate a majority consensus tree.



313 **Figure 3:** Scanning electron micrograph of *Bacillus marasmi* strain Marseille-P3556T using  
314 the Scanning Electron Microscope SU5000 from Hitachi. Scale bar and acquisition settings  
315 are presented on the pictures.



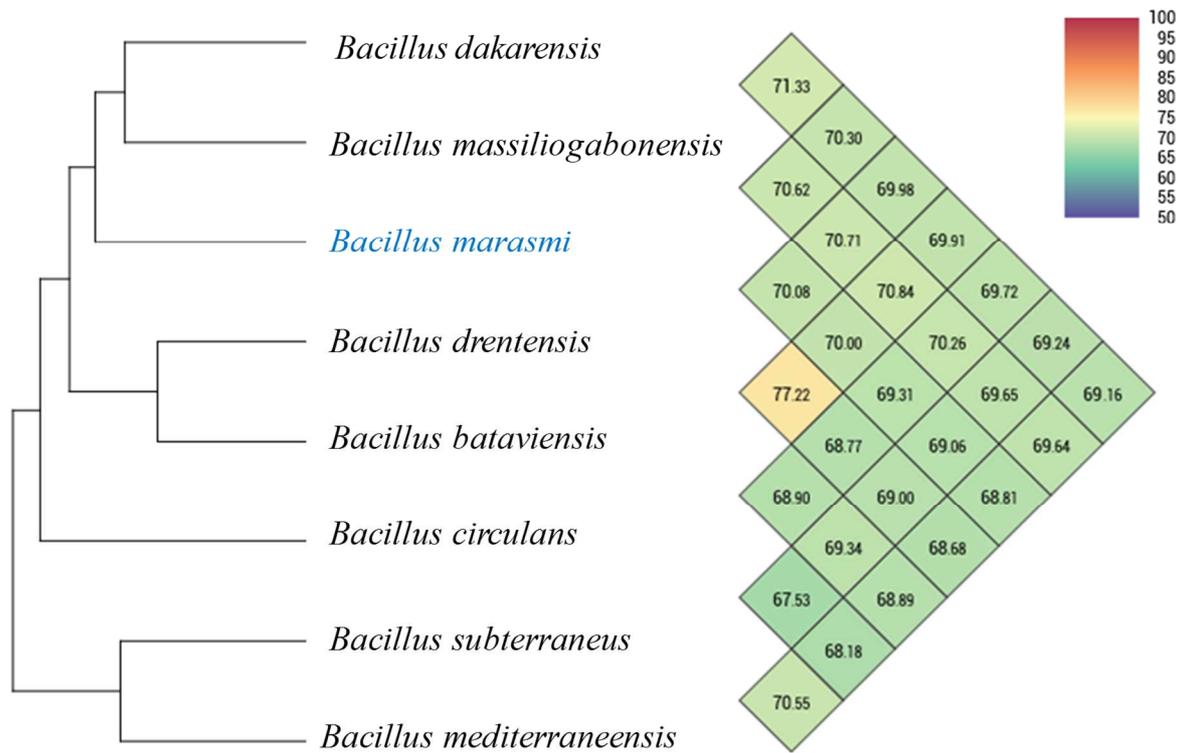
316

317 **Figure 4:** Circular map of the genome *Bacillus marasmi* strain Marseille-P3556 (4,638,282 bp).

318 CGView Server [34] was used to perform this genome cartography. From outside to the center: region

319 coding genes and RNA genes from the forward and reverse strands, respectively, GC content (black)

320 and GC skew (green/mauve).



322

323 **Figure 5:** Heatmap generated with OrthoANI values calculated using the OAT software for  
 324 *Bacillus marasmi* sp. nov., strain Marseille-P3556 with its respective closely related species  
 325 with standing in nomenclature.