

***Virgibacillus douboii* sp. nov., an halophilic bacterium isolated from healthy children stool in Mali.**

Salimata KONATE^{1,2,4}, Aminata CAMARA^{1,2,4}, Cheikh Ibrahima LO^{2,3}, Maryam TIDJANI ALOU^{1,2}, Amadou TOGO⁴, Safiatou NIARE⁴, Nicholas ARMSTRONG^{1,2}, Abdoulaye DJIMDÉ⁴, Mahamadou Ali THERA⁴, Florence FENOLLAR^{2,3}, Didier RAOULT^{1,2} and Matthieu MILLION^{1,2*}

¹ Aix Marseille Univ, IRD, AP-HM, MEPHI, Marseille, France

² IHU-Méditerranée Infection, Marseille, France

³ Aix Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France

⁴ Université des Sciences, des Techniques et des Technologies de Bamako.

* **Corresponding author** : Matthieu MILLION, Institut Hospitalo-Universitaire Méditerranée-Infection, 19-21 Boulevard Jean Moulin, 13385, Marseille cedex 05, France ; tel +33 413 732 401 ; fax : +33 413 732 402 ; e-mail: matthieumillion@gmail.com.

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Abstract

A moderate halophilic and strictly aerobic bacterium was isolated from a human stool as part of a study on the diagnosis of childhood malnutrition in Mali. Strain Marseille-Q1616^T is a Gram stain-positive, rod-shaped, catalase positive and oxidase negative bacterium. It has a genome size of 3.91 Mbp with 39.79 % G+C content which contains 3,954 protein-coding genes including genes encoding Fosfomycin resistance and *Listeria monocytogenes*, 16 rRNA genes and 64 tRNA. Strain Marseille-Q1616^T exhibited a 96.3% 16S rRNA gene sequence similarity and shared an OrthoANI value of 70.64% (the highest observed) with *Virgibacillus kekensis*, the phylogenetically closest validly published species. Based on phenotypic and phylogenetic evidence and genomic average nucleotide identity values, we suggest the creation of a new species within the *Virgibacillus* genus, named *Virgibacillus dumboii* sp. nov., type strain Marseille-Q1616^T (= CSURQ1616).

1 **Introduction**

2 The instrumental role of the gut microbiota in severe acute malnutrition has been confirmed
3 by recent studies that highlight the loss of methanogenic archaea [1], the proliferation of
4 *Proteobacteria* and *Bacteroidetes* [2] associated to a depletion of antioxidants in the gut
5 environment [3]. To explore the gut microbiota of severely malnourished children, we
6 applied the culturomics approach which consists of combining multiple culture conditions to
7 sequencing (whole genome or 16S rRNA gene) and bacterial identification using Matrix
8 Assisted Laser Desorption Ionization -Time of Flight Mass Spectrometry (MALDI-TOF MS)
9 [4,5]. In this context, we isolated the strain Marseille-Q1616^T, a species belonging to the
10 *Virgibacillus* genus.

11 The genus *Virgibacillus*, belonging to the *Firmicutes* phylum, was first described by
12 Heyndrickx *et al.* in 1998 to accommodate *Bacillus pantothenicus* [6] and transfer *Bacillus*
13 *halodenitrificans* [7]. This genus includes 36 species validly published including 3 synonyms
14 [8]. Members of the *Virgibacillus* genus are moderate or slightly halophilic and mostly
15 isolated from the environment or fermented sea food [9]. In this study, phenotypic,
16 phylogenetic and genomics characteristics were provided to describe *Virgibacillus doumboii*
17 sp. nov., strain Marseille-Q1616^T, a moderate halophilic bacterium isolated from a healthy
18 child under five years of age living in Mali.

19 **Material and methods**

20 *Stool sample collection*

21 A stool sample was collected from a healthy boy under the age of five living in Mali. This
22 collection was carried out as part of a study on the diagnosis of severe acute malnutrition. The
23 child's legal guardian gave his written and informed consent. The Malian ethics committee
24 approved the study under number N°2014/46/CE/FMPOS on May 22, 2014.

25 *Stool salinity assessment*

26 The salinity of the stool was measured by a salinity refractometer (Thermo Scientific,
27 Villebon-sur-Yvette, France). One gram of stool was diluted in 10 mL of distilled water and
28 centrifuged for 10 minutes at 5000 rpm. 100 μ L of supernatant are measured using the
29 refractometer according to the manufacturer's instructions [10].

30 *Strain isolation and growth conditions*

31 The stool sample was inoculated into an aerobic blood culture bottle (Becton Dickinson, Le
32 Pont-de-Claix, France) containing a halophilic medium prepared using a Columbia broth basis
33 (Sigma-Aldrich, Saint-Quentin-Fallavier, France) to which were added per liter: (1% (w / v)
34 of MgSO₄, 0.1% (w / v) of MgCl₂, 0.4% (w / v) of KCl, 0.1% (w / v) of CaCl₂, 0.05% (w /
35 v) of NaHCO₃, 0, 2% (w / v) glucose, 0.5% (w / v) of yeast extract (Becton Dickinson) and
36 10% (w / v) of NaCl depending on the salinity required. After a two-day incubation at 37°C in
37 an aerobic atmosphere, 50 μ L of culture were inoculated on a Chapman medium (Malt Agar
38 Salt medium) then incubated again for 48 hours. The capacity of the strain Marseille-Q1616^T
39 to grow at different temperatures (25°C, 28°C, 37°C, 42°C and 56°C) and varied pH (5 to 10)
40 was tested. Growth of this strain was assessed concomitantly in different atmospheres namely
41 anaerobic, aerobic and microaerophilic. Cellular fatty acid methyl ester (FAME) analysis was
42 performed by GC/MS method as described before[11].

43 *Strain identification*

44 Strain Marseille-Q1616^T was first tentatively using Matrix assisted Laser Desorption
45 Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) which is used for routine
46 identification of bacteria and fungi in microbiology laboratories [12]). After an identification
47 attempt using MALDI-TOF MS (score <1.7), the 16S rRNA gene of the Marseille-Q1616^T
48 strain was sequenced. Thus, the primer pair fD1 and rP2 (Eurogentec, Angers, France) and

49 the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xLGenetic Analyzer capillary
50 sequencer (ThermoFisher, Saint-Aubin, France) were used to sequence this gene as previously
51 reported (12). Codon Code Aligner software (<http://www.codoncode.com>) was subsequently
52 used for assembly and correct sequences.

53 *Genome sequencing, annotation and comparison*

54 To extract genomic DNA, the EZ1 biorobot was used with the EZ1 DNA tissue kit (Qiagen,
55 Hilden, Germany). Then, the MiSeq sequencer (Illumina Inc, San Diego, CA, USA) with the
56 Nextera Mate Pair and Nextera XT Paired End (Illumina) sample preparation kit, allowed the
57 sequencing of the complete genome of strain Marseille-Q1616^T, as previously described [13].
58 This genome was assembled using a pipeline consisting of several softwares as mentioned in
59 a previous study [14]. **The presence of resistance-encoding genes as well as that of virulence**
60 **factors was assessed using ABRICATE with the virulence and resistance database (VFDB,**
61 **ResFinder, Megares).** Genome analyses of this strain comparing it to the genomes of the
62 closest species were performed by calculating digital DNA-DNA hybridization (dDDH) and
63 studying genomic similarity with the OrthoANI software [15].

64 **Results**

65 *Phenotypic and biochemical description*

66 The strain Marseille-Q1616^T is a Gram-stain positive, motile and asporogenous bacterium.
67 This strain was catalase positive but oxidase negative. Bacterial cells are elongated rod-
68 shaped with a length ranging from 1.3 to 2.7 µm and a width ranging from 0.3 to 0.7 µm
69 (Figure 1). It was able to ferment glucose and D-turanose, hydrolyze esculin and also
70 exhibited esterase and lipase activities (Table 1). Strain Marseille-Q1616^T was not able to
71 grow in microaerophilic and anaerobic atmospheres but was able to withstand temperatures
72 ranging from 28 to 42°C with a pH ranging from 6.0 to 9.5 in an aerobic atmosphere. In fact,
73 analysis of cellular fatty acids revealed that the main fatty acids identified were 12-methyl

74 tetradecanoic acid (49.0%), 14-methyl pentadecanoic (23%) and 12-methyl tridecanoic acid
75 (10%) (Table 2). Using the E-test strips, the minimal inhibitory concentration was assessed
76 for strain Marseille-Q1616^T: ceftazidime (0.075 µg/ml), ciprofloxacin (0.047µg/ml),
77 oxacilline (0.094µg/ml), vancomycin (0.38µg/ml), imipenem (0.064µg/ml), clindamycin
78 (0.023µg/ml), doxycycline (4.000 µg/ml), erythromycin (0.5 µg/ml), rifampicin (0.004µg/ml),
79 daptomycin (0.047µg/ml), benzylpenicillin (0.004µg/ml) and linezolid (0.125µg/ml).
80 However, the strain shows a MIC >256 µg/ml for trimethoprim sulfamethoxazole association
81 and amikacin.

82 Strain Marseille-Q1616^T exhibits a 96.36% 16S rRNA similarity with *Virgibacillus kekensis*
83 strain YIM kkn16 (AY121439), the phylogenetically closest species with a valid name
84 (Figure 2). Since this value is below the recommended threshold to define new species
85 [16,17], strain Marseille-Q1616^T is consequently proposed as a new species within the
86 *Virgibacillus* genus.

87 *Genomic properties and comparison*

88 The genome of strain Marseille-Q1616^T has a length of 3.91 Mbp with 39.79 mol% G+C
89 content. It contains 3,954 protein-coding genes, 16 rRNA genes and 64 tRNA genes. When
90 compared with others **shared** genomes, it was smaller than those of *Virgibacillus massiliensis*
91 Vm-5 (4.35 Mbp), but larger than those of *Virgibacillus panthotenticus* DSM 26T and
92 *Virgibacillus senegalensis* SK-1 (4.75 and 3.92 Mbp respectively). Overall, the distribution of
93 genes into Clusters of Orthologous Groups (COGs) of strain Marseille-Q1616^T is similar to
94 that of *Virgibacillus halodenitrificans* strain Marseille P736. **Using ABRICATE with the**
95 **virulence and resistance database (VFDB, ResFinder, Megares), three genes were detected as**
96 **resistance genes (fosB (fosfomycine resistance gene)) and virulence factors encoding genes**
97 **(clpP (ATP-dependent Clp protease proteolytic subunit [ClpP (VF0074)] [*Listeria***
98 ***monocytogenes* EGD-e]) and clpC (endopeptidase Clp ATP-binding chain C [ClpC**

99 (VF0072)] [*Listeria monocytogenes* EGD-e])). However; no heavy metal genes were found in
100 the genome of the strain Marseille-Q1616^T.

101 Genome-to-Genome Distance Calculator (GGDC) online calculator (<http://ggdc.dsmz.de/>)
102 with formula 2 was used to compute dDDH between the genome of strain Marseille-Q1616^T
103 and those of phylogenetically shared species. *Virgibacillus doumboii* sp. nov., strain
104 Marseille-Q1616^T shared a similarity of 25.1, 20.4, 18.2, 22.0 and 19.5% with *Virgibacillus*
105 *dokdonensis* strain DSW-10, *Virgibacillus halodenitrificans* strain 1806, *Virgibacillus*
106 *panthotenticus* strain DSM 26, *Virgibacillus proomii* strain V-P, *Virgibacillus picturae* strain
107 LMG 19492, respectively (Table 3). These values, all below the recommended DDH
108 threshold value (<70%), suggest that *V. doumboii* strain Marseille-Q1616^T is different from
109 the compared species.

110 In addition, the degree of genomic similarity of strain Marseille-Q1616^T with related species
111 was estimated using the Orthologous Average Nucleotide Identity Tool (OAT) software [15].
112 Furthermore, genomic analyzes showed OrthoANI values ranging from 69.11% between *V.*
113 *doumboii* and *V. panthotenticus* to 80.63%, between *V. panthotenticus* and *V. dokdonensis*.
114 Additionally, 70.64% was the highest OrthoANI value shared by *V. doumboii* and *V.*
115 *halodenitrificans* (Figure 3). Given that all the estimates of genomic similarity were below the
116 95% threshold delimiting new species, we confirm that *V. doumboii*, is a new species with the
117 *Virgibacillus* genus.

118 **Conclusion**

119 Phenotypic, biochemical, phylogenetic and genomic evidence have shown that strain
120 Marseille-Q1616^T is different from its phylogenetic relatives and thus represents a new
121 species, within the *Virgibacillus* genus, for which we suggest the name *Virgibacillus*
122 *doumboii*. The type strain Marseille-Q1616^T was isolated from the stool of a healthy Malian
123 child.

124 **Description of *Virgibacillus doumboii* sp. nov.**

125 *Virgibacillus doumboii* (*do.um.bo'i.i* N.L. masc. adj. *doumboii* of Prof. Ogobara Doumbo, an
126 eminent biologist who recently passed away, to whom we pay tribute for his numerous
127 scientific works and the initiation of the project as a part of which strain Marseille-Q1616^T
128 was isolated). Cells are Gram positive rod-shaped bacteria with a mean length of 1.9µm and a
129 width of 0.5µm. They are motile and non-spore-forming. It presents positive catalase and
130 negative oxidase activities. The type strain grows strictly under aerobic conditions with an
131 optimum temperature at 37°C. The main cellular fatty acids found are 12-methyl-
132 tetradecanoic acid (49.0 %), hexadecanoic acid (23.3 %) and the 14-methyl-hexadecanoic
133 acid (9.4 %). Positive reactions were observed for esterase (C4), esterase Lipase (C8), esculin
134 ferric citrate, D-turanose, arginine dihydrolase, urease, β-glucosidase and protease. The strain
135 Marseille-Q1616^T ferments the glucose and reduces nitrates to nitrites. Its genome was 3.91
136 Mbp with 39.79 mol% of G+C content. The 16S rRNA and genome sequences of strain
137 Marseille-Q1616^T were deposited in GenBank database under accession numbers LR746133
138 and CADCWQ010000001 respectively. The type strain Marseille-Q1616^T (= CSUR Q1616)
139 was isolated from the stool of a healthy Malian child under five years of age.

140 **Conflicts of interest**

141 None to declare.

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214 **Table 1.** Differential characteristics of **1**, *Virgibacillus doumboii* strain Marseille Q1616^T; **2**,
 215 *Virgibacillus senegalensis* strain SK-1; **3**, *Virgibacillus massiliensis* strain Vm-5; **4**,
 216 *Virgibacillus siamensis* strain JCM 15395; **5**, *Virgibacillus panthotenticus* strain LMG 7129.

Characteristics	1	2	3	4	5
Cell diameter (µm)	0.3-0.7	0.6-0.9	0.5-0.8	0.5-0.7	NF
Gram stain	+	+	+	+	+
Motility	+	+	+	+	+
Endospore formation	-	+	+	+	+
Oxygen Requirement	Aerobic	Aerobic	Aerobic	FA	FA
Salt tolerance (%)	5-15	0.5-10	5-20	1-20	5-15
Catalase	+	-	+	+	+
Oxydase	-	-	+	+	NF
Alkaline phosphatase	-	-	-	NF	NF
Acid phosphatase	-	-	-	NF	NF
N-acetyl-β-glucosaminidase	-	-	-	NF	+
Glycerol	-	-	-	NF	+
L-xylose	-	-	-	+	NF
D-galactose	-	-	-	NF	+
D-glucose	-	-	+	+	+
D-fructose	-	-	+	NF	+
Esculin ferric citrate	+	-	-	-	+
Salicin	-	-	-	NF	+
D-cellobiose	-	-	-	+	NF
D-maltose	-	-	-	NF	+
D-lactose	-	-	-	-	-
Starch	-	-	-	+	+
Arabinose	-	-	-	-	+
Trisodium citrate	-	-	-	NF	+
G+C (mol%)	39.7	42.9	36.8	38.0	36.9
Habitat	Human	Human	Human	Fish	Soil

217 FA, facultative anaerobic; +, positive reaction; -, reaction; **NF**, Not Available data.

218 **Table 2.** Cellular fatty acid composition (%) of **1**, *Virgibacillus doumboii* sp. nov. strain
 219 Marseille-Q1616^T; **2**, *Virgibacillus siamensis* strain JCM 15395 and **3**, *Virgibacillus*
 220 *panthotenticus* strain LMG 7129.

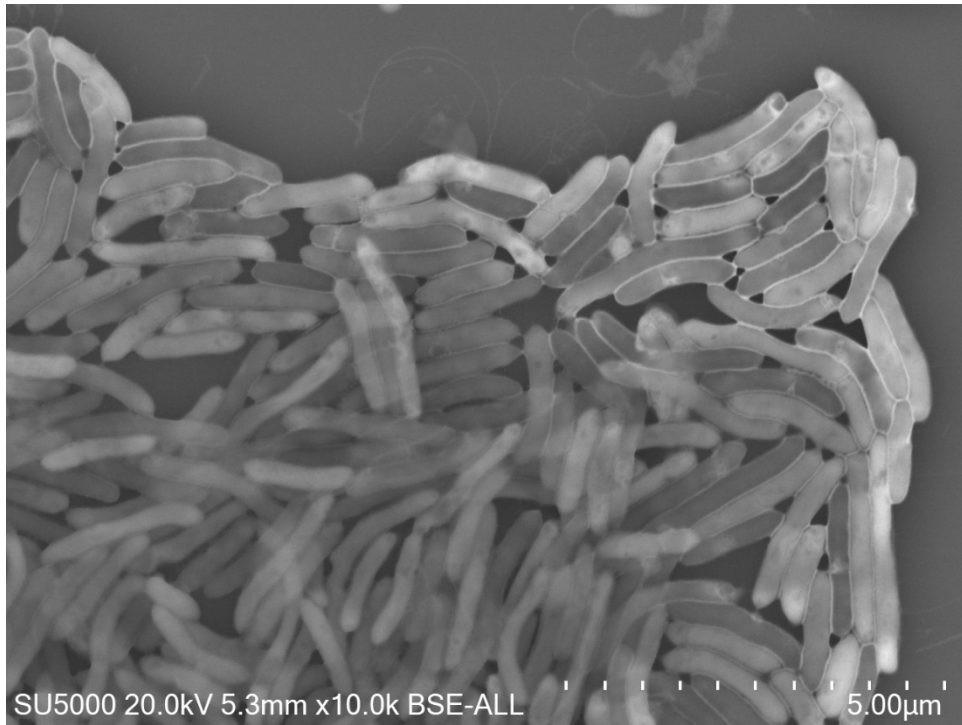
Fatty acids	Name	1	2	3
C _{15:0} anteiso	12-methyl-tetradecanoic acid	49.0	55.8	47.4
C _{5:0} iso	3-methyl-Butanoic acid	2.0	NA	NA
C _{17:0} anteiso	14-methyl-Hexadecanoic acid	9.4	17.7	13.5
C _{15:0} iso	13-methyl-tetradecanoic acid	4.2	11.3	15.8
C _{14:0} iso	12-methyl-Tridecanoic acid	9.8	3.9	4.3
C _{16:00}	Hexadecanoic acid	23.3	1.5	5.2
C _{17:0} iso	15-methyl-Hexadecanoic acid	TR	1.5	2.8
C _{14:00}	Tetradecanoic acid	NA	0.2	1.2

221 TR, trace amounts <1%; -, not detected; NA, not applicable

222 **Table 3.** Genomic comparison of *Virgibacillus doumboii* strain Marseille-Q1616^T with its closely
 223 related species using GGDC and formula 2 (dDDH estimates based on identities over HSP length).

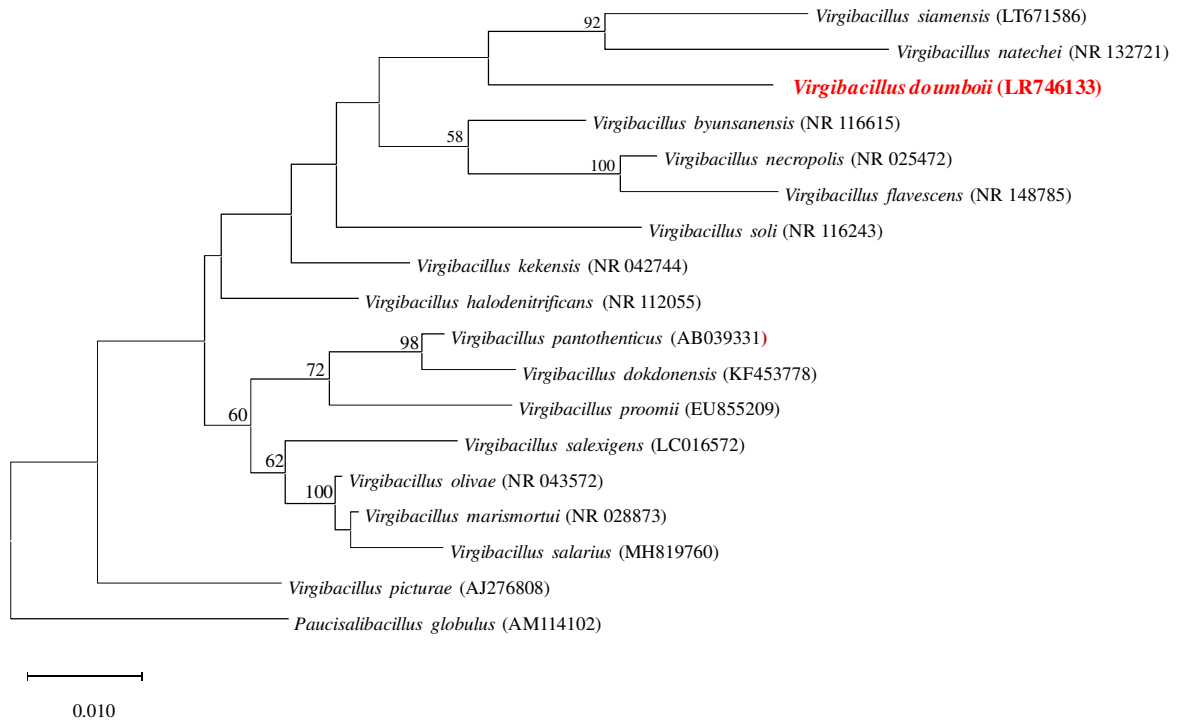
	Vdou	Vdok	Vhal	Vpan	Vpro	Vpic
Vdou	100%					
Vdok	25.1±4.5%	100%				
Vhal	20.4±4.6%	23.3±4.8%	100%			
Vpan	18.2 ±4.5%	25.5±4.8%	17.7 ±4.4%	100%		
Vpro	22.0 ±4.7%	22.1±4.7%	23.8±4.7%	25.3±4.8%	100%	
Vpic	19.5 ±4.6%	23.5±4.8%	20.8 ±4.7%	19.9±4.6%	18.7±4.5%	100%

224 **Vdou**, *Virgibacillus doumboii* ; **Vdok**, *Virgibacillus dokdonensis* ; **Vhal**, *Virgibacillus*
 225 *halodenitrificans* ; **Vpan**, *Virgibacillus pantothenticus* ; **Vpro**, *Virgibacillus promii* ; **Vpic**,
 226 *Virgibacillus picturae*.



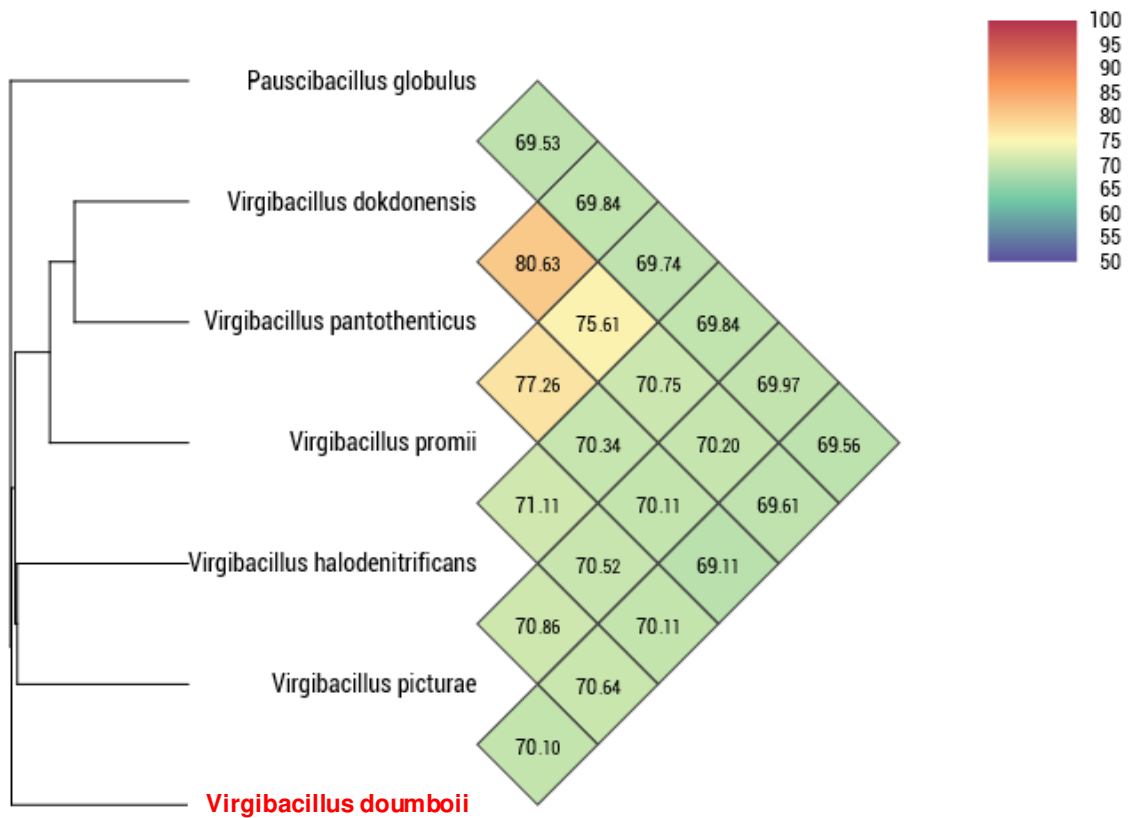
227

228 **Figure 1:** Morphological structure of *Virgibacillus doumboii* sp. nov., strain Marseille-
229 Q1616^T, obtained by scanning electron microscopy using Hitachi SU5000 instrument. Scale
230 and parameters are shown on the figure.



231

232 **Figure 2:** Phylogenetic tree highlighting the position of *Virgibacillus douboii* sp. nov.,
 233 strain Marseille-Q1616^T. The GenBank accession numbers for 16S rRNA genes are indicated
 234 in parenthesis. Sequences were aligned using MUSCLE v7.0.26 with default parameters and
 235 phylogenetic inferences were obtained using the maximum likelihood method with MEGA 7
 236 software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the
 237 analysis 1000 times to generate a majority consensus tree. Only bootstrap values > 50% are
 238 shown and the minimum value is 58%. The scale bar indicates a 1% nucleotide sequence
 239 divergence.



240

241 **Figure 3:** Heatmap generated with OrthoANI values calculated using the OAT software
 242 between *Virgibacillus douboii* sp. nov., strain Marseille-Q1616^T and other closely related
 243 species with standing in nomenclature.