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

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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 doumboii* sp. nov., type strain Marseille-Q1616^T (= CSURQ1616).

1 Introduction

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

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

19 Material and methods

20 Stool sample collection

A stool sample was collected from a healthy boy under the age of five living in Mali. This collection was carried out as part of a study on the diagnosis of severe acute malnutrition. The child's legal guardian gave his written and informed consent. The Malian ethics committee approved the study under number N°2014/46/CE/FMPOS on May 22, 2014. The salinity of the stool was measured by a salinity refractometer (Thermo Scientific, Villebon-sur-Yvette, France). One gram of stool was diluted in 10 mL of distilled water and centrifuged for 10 minutes at 5000 rpm. 100 μ L of supernatant are measured using the refractometer according to the manufacturer's instructions [10].

30 Strain isolation and growth conditions

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

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

- the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xLGenetic Analyzer capillary 49
- sequencer (Thermofisher, Saint-Aubin, France) were used to sequence this gene as previously 50
- reported (12). Codon Code Aligner software (http://www.codoncode.com) was subsequently 51
- used for assembly and correct sequences. 52
- 53 Genome sequencing, annotation and comparison
- To extract genomic DNA, the EZ1 biorobot was used with the EZ1 DNA tissue kit (Oiagen, 54
- Nextera Mate Pair and Nextera XT Paired End (Illumina) sample preparation kit, allowed the 56

Hilden, Germany). Then, the MiSeq sequencer (Illumina Inc, San Diego, CA, USA) with the

- sequencing of the complete genome of strain Marseille-Q1616^T, as previously described [13].
- 57
- 58 This genome was assembled using a pipeline consisting of several softwares as mentioned in
- a previous study [14]. The presence of resistance-encoding genes as well as that of virulence 59
- factors was assessed using ABRICATE with the virulence and resistance database (VFDB, 60
- 61 ResFinder, Megares). Genome analyses of this strain comparing it to the genomes of the
- closest species were performed by calculating digital DNA-DNA hybridization (dDDH) and 62
- studying genomic similarity with the OrthoANI software [15]. 63
- **Results** 64

55

Phenotypic and biochemical description 65

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

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

Strain Marseille-Q1616^T exhibits a 96.36% 16S rRNA similarity with *Virgibacillus kekensis*strain YIM kkny16 (AY121439), the phylogenetically closest species with a valid name
(Figure 2). Since this value is below the recommended threshold to define new species
[16,17], strain Marseille-Q1616^T is consequently proposed as a new species within the *Virgibacillus* genus.

87 Genomic properties and comparison

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

- with formula 2 was used to compute dDDH between the genome of strain Marseille-Q1616^T
- and those of phylogenetically shared species. *Virgibacillus doumboii* sp. nov., strain
- 104 Marseille-Q1616^T shard 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
- threshold value (<70%), suggest that *V. doumboii* strain Marseille-Q1616^T is different from
- 109 the compared species.
- In addition, the degree of genomic similarity of strain Marseille-Q1616^T with related species 110 111 was estimated using the Orthologous Average Nucleotide Identity Tool (OAT) software [15]. Furthermore, genomic analyzes showed OrthoANI values ranging from 69.11% between V. 112 doumboii and V. panthotenticus to 80.63%, between V. panthotenticus and V. dokdonensis. 113 Additionally, 70.64% was the highest OrthoANI value shared by V. doumboii and V. 114 115 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 Virgibacillus genus. 117

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.

Virgibacillus doumboii (do.um.bo'i.i N.L. masc. adj. doumboii of Prof. Ogobara Doumbo, an 125 eminent biologist who recently passed away, to whom we pay tribute for his numerous 126 scientific works and the initiation of the project as a part of which strain Marseille-O1616^T 127 was isolated). Cells are Gram positive rod-shaped bacteria with a mean length of 1.9µm and a 128 129 width of 0.5µm. They are motile and non-spore-forming. It presents positive catalase and negative oxidase activities. The type strain grows strictly under aerobic conditions with an 130 optimum temperature at 37°C. The main cellular fatty acids found are 12-methyl-131 tetradecanoic acid (49.0 %), hexadecanoic acid (23.3 %) and the 14-methyl-hexadecanoic 132 acid (9.4 %). Positive reactions were observed for esterase (C4), esterase Lipase (C8), esculin 133 ferric citrate, D-turanose, arginine dihydrolase, urease, β -glucosidase and protease. The strain 134 Marseille-Q1616^T ferments the glucose and reduces nitrates to nitrites. Its genome was 3.91 135 Mbp with 39.79 mol% of G+C content. The 16S rRNA and genome sequences of strain 136 Marseille-Q1616^T were deposited in GenBank database under accession numbers LR746133 137 and CADCWQ010000001 respectively. The type strain Marseille-Q1616^T (= CSUR Q1616) 138 was isolated from the stool of a healthy Malian child under five years of age. 139

140 **Conflicts of interest**

141 None to declare.

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Table 1. Differential characteristics of **1**, *Virgibacillus doumboii* strain Marseille Q1616^T; **2**, *Virgibacillus senegalensis* strain SK-1; **3**, *Virgibacillus massiliensis* strain Vm-5; **4**, 214

216	Virgibacillus si	<i>amensis</i> strain .	JCM 15395; 5 ,	, Virgibacillus	panthotenticus	strain LMG 7129	۱.
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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

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

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

Fatty acids	Name	1	2	3
C15:0 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
C15:0 iso	13-methyl-tetradecanoic acid	4.2	11.3	15.8
C14:0 iso	12-methyl-Tridecanoic acid	9.8	3.9	4.3
C _{16:00}	Hexadecanoic acid	23.3	1.5	5.2
C17:0 iso	15-methyl-Hexadecanoic acid	TR	1.5	2.8
C14:00	Tetradecanoic acid	NA	0.2	1.2

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

- **Table 3.** Genomic comparison of *Virgibacillus doumboii* strain Marseille-Q1616^T with its closely
- 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%	$20.8 \pm 4.7\%$	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*.



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



0.010

Figure 2: Phylogenetic tree highlighting the position of *Virgibacillus doumboii* sp. nov.,

strain Marseille-Q1616^T. The GenBank accession numbers for 16S rRNA genes are indicated

in parenthesis. Sequences were aligned using MUSCLE v7.0.26 with default parameters and

phylogenetic inferences were obtained using the maximum likelihood method with MEGA 7

software. Numbers at the nodes are percentages of bootstrap values obtained by repeating the

analysis 1000 times to generate a majority consensus tree. Only bootstrap values > 50% are
shown and the minimum value is 58%. The scale bar indicates a 1% nucleotide sequence

239 divergence.



Figure 3: Heatmap generated with OrthoANI values calculated using the OAT software

between *Virgibacillus doumboii* sp. nov., strain Marseille-Q1616^T and other closely related
 species with standing in nomenclature.