Virgibacillus doumboii sp. nov., a halophilic bacterium isolated from the stool of a healthy child in Mali

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Abstract

A moderately 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 3954 protein-coding genes including genes encoding phosphomycin resistance and *Listeria monocytogenes*, 16 rRNA genes and 64 tRNA genes. 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).

Keywords: Child malnutrition, culturomics, Mali, taxonogenomics, *Virgibacillus doumboii* sp. nov.
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Introduction

The instrumental role of the gut microbiota in severe acute malnutrition has been confirmed by recent studies that highlight the loss of methanogenic archaea [1], and the proliferation of *Proteobacteria* and *Bacteroidetes* [2] associated with a depletion of antioxidants in the gut environment [3]. To explore the gut microbiota of severely malnourished children, we applied the culturomics approach, which involves combining multiple culture conditions with sequencing (whole genome or 16S rRNA gene) and bacterial identification using matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (MALDI-TOF MS) [4,5]. In this context, we isolated the strain Marseille-Q1616^T, a species belonging to the *Virgibacillus* genus.

The genus *Virgibacillus*, belonging to the *Firmicutes* phylum, was first described by Heyndrickx *et al.* in 1998 to accommodate *Bacillus pantothenticus* [6] and transfer *Bacillus halodenitrificans* [7]. This genus includes 36 species validly published including three synonyms [8]. Members of the *Virgibacillus* genus are moderately or slightly halophilic and mostly isolated from the environment or fermented sea food [9]. In this study, phenotypic, phylogenetic and genomics characteristics were provided to describe *Virgibacillus doumboii* sp. nov., strain Marseille-Q1616^T, a moderate halophilic bacterium isolated from a healthy child under 5 years of age living in Mali.

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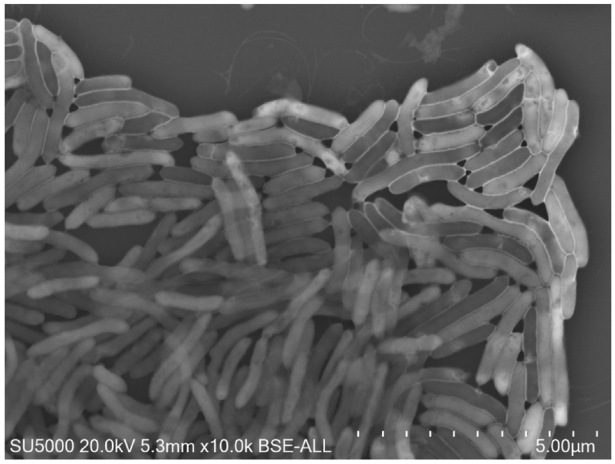

FIG. 1. Morphological structure of *Virgibacillus doumboii* sp. nov., strain Marseille-Q1616^T, obtained by scanning electron microscopy using Hitachi SU5000 instrument. Scale and parameters are shown.

Materials and methods

Stool sample collection

A stool sample was collected from a healthy boy under the age of 5 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 2014/46/CE/FMPOS on May 22nd, 2014.

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, *Virgibacillus siamensis* strain JCM 15395; and 5, *Virgibacillus pantothenticus* 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	+	—	+	+	+
Oxidase	—	—	+	+	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

Abbreviations: FA, facultative anaerobic; +, positive reaction; –, reaction; NF, data not available.

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 pantothenticus* 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

Abbreviations: TR, trace amounts <1%; –, not detected; NA, not applicable.

Stool salinity assessment

The salinity of the stool was measured using 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 2800 g; 100 µL of supernatant was then measured using the refractometer according to the manufacturer's instructions [10].

Strain isolation and growth conditions

The stool sample was inoculated into an aerobic blood culture bottle (Becton Dickinson, Le Pont-de-Claix, France) containing a halophilic medium prepared using a Columbia broth base (Sigma-Aldrich, Saint-Quentin-Fallavier, France) to which were added per litre: (1% (weight (w)/volume (v)) $MgSO_4$, 0.1% (w/v) $MgCl_2$, 0.4% (w/v) KCl, 0.1% (w/v) $CaCl_2$, 0.05% (w/v) $NaHCO_3$, 0.2% (w/v) glucose, 0.5% (w/v) yeast extract (Becton Dickinson) and 10% (w/v) NaCl depending on the salinity required. After a 2-day incubation at 37°C in an aerobic atmosphere, 50 µL of culture was inoculated on a Chapman medium (Malt Agar Salt medium) then incubated for an additional 48 hours. The capacity of the strain Marseille-Q1616^T to grow at different temperatures (25°C, 28°C, 37°C, 42°C and 56°C) and at varied pH (5 to 10) was tested. Growth of this strain was assessed concomitantly in different atmospheres, namely anaerobic, aerobic and microaerophilic. Cellular fatty acid methyl ester analysis was performed by gas chromatography/MS method as described previously [11].

Strain identification

Strain Marseille-Q1616^T was first tentatively described using MALDI-TOF MS, which is used for routine identification of bacteria and fungi in microbiology laboratories [12]. After an identification attempt using MALDI-TOF MS (score <1.7), the

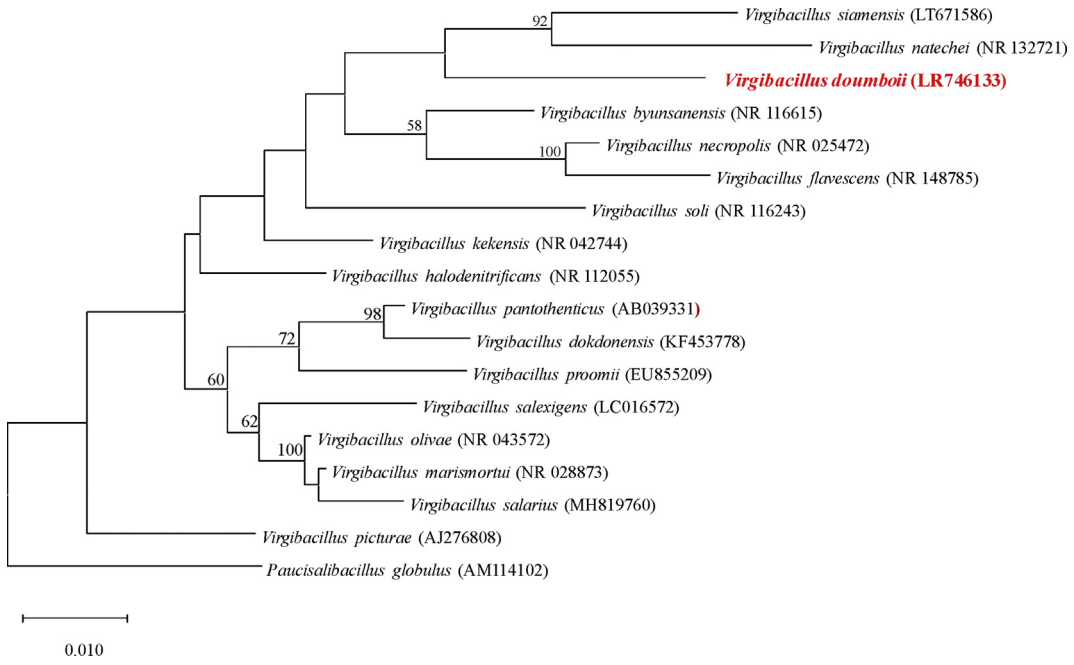

FIG. 2. Phylogenetic tree highlighting the position of *Virgibacillus doumboii* sp. nov., strain Marseille-Q1616^T. The GenBank accession numbers for 16S rRNA genes are indicated in parenthesis. Sequences were aligned using MUSCLE v7.0.26 with default parameters and phylogenetic inferences were obtained 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 divergence.

TABLE 3. Genomic comparison of *Virgibacillus doumboii* strain Marseille-Q1616^T with its closely related species using Genome-to-Genome Distance Calculator 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%

Abbreviations: dDDH, digital DNA–DNA hybridization; HSP, high scoring pair; Vdou, *Virgibacillus doumboii*; Vdok, *Virgibacillus dokdonensis*; Vhal, *Virgibacillus halodenitrificans*; Vpan, *Virgibacillus pantothenticus*; Vpro, *Virgibacillus promii*; Vpic, *Virgibacillus picturae*.

16S rRNA gene of the Marseille-Q1616^T strain was sequenced. Hence, the primer pair fD1 and rP2 (Eurogentec, Angers, France) and the Big Dye® Terminator v1.1 Cycle Sequencing Kit and 3500xLGenetic Analyser capillary sequencer (Thermofisher, Saint-Aubin, France) were used to sequence this gene as previously reported [12]. Codon Code Aligner software (http://www.codoncode.com) was subsequently used for assembly and correct sequences.

Genome sequencing, annotation and comparison

To extract genomic DNA, the EZ1 biorobot was used with the EZ1 DNA tissue kit (Qiagen, Hilden, Germany). Then, the MiSeq sequencer (Illumina Inc, San Diego, CA, USA) with the Nextera Mate Pair and Nextera XT Paired End (Illumina) sample preparation kit, allowed the sequencing of the complete genome of strain Marseille-Q1616^T, as previously described [13]. 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 factors was assessed using ABRICATE with the virulence and resistance database (VFDB, ResFinder, Megares). Genome analyses of this strain comparing it with the genomes of the closest species were performed by calculating digital DNA–DNA hybridization and studying genomic similarity with the OrthoANI software [15].

Results

Phenotypic and biochemical description

The strain Marseille-Q1616^T was a Gram-stain-positive, motile and non-spore forming bacterium. This strain was catalase

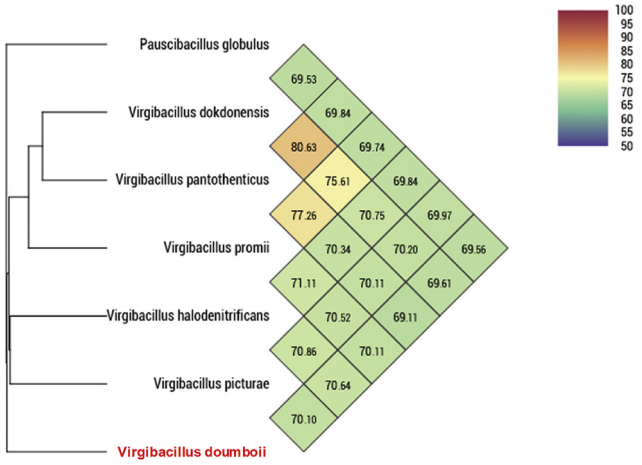

FIG. 3. Heatmap generated with OrthoANI values calculated using the OAT software between *Virgibacillus doumboii* sp. nov., strain Marseille-Q1616^T and other closely related species with standing in nomenclature.

positive but oxidase negative. Bacterial cells were elongated rod-shaped with a length ranging from 1.3 to 2.7 µm and a width ranging from 0.3 to 0.7 µm (Fig. 1). It was able to ferment glucose and D-turanose, hydrolyse esculin and also exhibited esterase and lipase activities (Table 1). Strain Marseille-Q1616^T was not able to grow in microaerophilic and anaerobic atmospheres but was able to withstand temperatures ranging from 28°C to 42°C with a pH ranging from 6.0 to 9.5 in an aerobic atmosphere. Analysis of cellular fatty acids revealed that the main fatty acids identified were 12-methyl tetradecanoic acid (49.0%), 14-methyl pentadecanoic acid (23%) and 12-methyl tridecanoic acid (10%) (Table 2). Using the E-test strips, the MIC was assessed for strain Marseille-Q1616^T: ceftazidime (0.075 µg/mL), ciprofloxacin (0.047 µg/mL), oxacillin (0.094 µg/mL), vancomycin (0.38 µg/mL), imipenem (0.064 µg/mL), clindamycin (0.023 µg/mL), doxycycline (4.000 µg/mL), erythromycin (0.5 µg/mL), rifampicin (0.004 µg/mL), daptomycin (0.047 µg/mL), benzylpenicillin (0.004 µg/mL) and linezolid (0.125 µg/mL). However, the strain showed a MIC >256 µg/mL for trimethoprim-sulfamethoxazole association and amikacin.

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

Genomic properties and comparison

The genome of strain Marseille-Q1616^T has a length of 3.91 Mbp with 39.79 mol% G+C content. It contains 3954 protein-coding genes, 16 rRNA genes and 64 tRNA genes. When compared with others shared genomes, it was smaller than that of *Virgibacillus massilliensis* Vm-5 (4.35 Mbp), but larger than those of *Virgibacillus pantothenticus* DSM 26T and *Virgibacillus senegalensis* SK-1 (4.75 and 3.92 Mbp, respectively). Overall, the distribution of genes into Clusters of Orthologous Groups of strain Marseille-Q1616^T is similar to that of *Virgibacillus halodenitrificans* strain Marseille P736. Using ABRICATE with the virulence and resistance database (VFDB, ResFinder, Megares), three genes were detected as resistance genes (fosB (fosfomycin resistance gene)) and virulence factors encoding genes *clpP* (ATP-dependent Clp protease proteolytic subunit (ClpP (VF0074)) (*Listeria monocytogenes* EGD-e)) and *clpC* (endopeptidase Clp ATP-binding chain C (ClpC (VF0072)) (*Listeria monocytogenes EGD*-e))). However; no heavy metal genes were found in the genome of strain Marseille-Q1616^T.

The Genome-to-Genome Distance Calculator online calculator (http://ggdc.dsmz.de/) with formula 2 was used to compute digital DNA–DNA hybridization between the genome of strain Marseille-Q1616^T and those of phylogenetically shared species. *Virgibacillus doumboii* sp. nov., strain Marseille-Q1616^T shard similarities of 25.1%, 20.4%, 18.2%, 22.0% and 19.5% with *Virgibacillus dokdonensis* strain DSW-10, *Virgibacillus halodenitrificans* strain 1806, *Virgibacillus pantothenticus* strain DSM 26, *Virgibacillus proomii* strain V–P and *Virgibacillus picturae* strain LMG 19492, respectively (Table 3). These values, all below the recommended DNA–DNA hybridization threshold value (<70%), suggest that *V. doumboii* strain Marseille-Q1616^T is different from the compared species.

In addition, the degree of genomic similarity of strain Marseille-Q1616^T with related species was estimated using the Orthologous Average Nucleotide Identity Tool (OAT) software [15]. Genomic analyses showed OrthoANI values ranging from 69.11% between *V. doumboii* and *V. pantothenticus* to 80.63%, between *V. pantothenticus* and *V. dokdonensis*. Additionally, 70.64% was the highest OrthoANI value shared by *V. doumboii* and *V. halodenitrificans* (Fig. 3). Given that all the

estimates of genomic similarity were below the 95% threshold delimiting new species, we confirm that *V. doumboii*, is a new species within the *Virgibacillus* genus.

Conclusion

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

Description of *Virgibacillus doumboii* sp. nov.

Virgibacillus doumboii (*do.um.bo'i.i* N.L. masc. adj. *doumboii* of Prof. Ogobara Doumbo, an eminent biologist who recently passed away, to whom we pay tribute for his numerous scientific works and the initiation of the project as a part of which strain Marseille-Q1616^T was isolated). Cells are Gram-stain positive rod-shaped bacteria with a mean length of 1.9 µm and a width of 0.5 µm. 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 optimum temperature at 37°C. The main cellular fatty acids found are 12-methyl-tetradecanoic acid (49.0 %), hexadecanoic acid (23.3 %) and the 14-methyl-hexadecanoic acid (9.4 %). Positive reactions were observed for esterase (C4), esterase lipase (C8), esculin ferric citrate, D-turanose, arginine dihydrolase, urease, β-glucosidase and protease. Strain Marseille-Q1616^T ferments glucose and reduces nitrates to nitrites. Its genome was 3.91 Mbp with 39.79 mol% of G+C content. The 16S rRNA and genome sequences of strain Marseille-Q1616^T were deposited in GenBank database under accession numbers LR746133 and CADCWQ000000000, respectively. The type strain Marseille-Q1616^T (= CSUR Q1616) was isolated from the stool of a healthy Malian child under 5 years of age.

Conflict of interest

None to declare.

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