Virgibacillus ihumii sp. nov., a new bacterium isolated from the stool of healthy African children

A. Camara[1,2,4], S. Konate[1,2,4], C. I. Lo[2,3], E. Kuete[1,2], M. Sarr[1,2], N. Amsrtong[1,2], S. Niare[4], M. A. Thera[4], F. Fenollar[2,3], D. Raoult[1,2] and M. Million[1,2]

1) *Aix-Marseille Université, IRD, APHM, MEΦI,* 2) *IHU-Méditerranée Infection,* 3) *Aix-Marseille Université, IRD, APHM, SSA, VITROME, Marseille, France and* 4) *Université des Sciences, des Techniques et des Technologies de Bamako, Bamako, Mali*

Abstract

During a case–control study on severe acute malnutrition, strain Marseille-Q1233 was isolated. It is a Gram-positive, rod-shaped and halophilic bacillus isolated from a stool sample of Malian child under the age of 5. The fatty acid profile of the strain consisted of C15:0-anteiso and C14:0-iso as major components. Digital DNA-DNA hybridization and average nucleotide identity calculation showed 23.10% and 80.81% similarity respectively between strain Marseille-Q1233 and *Virgibacillus siamensis* strain Marseille-P2607, the phylogenetically closely related species with standing in nomenclature. On the basis of these results, we report the description of *Virgibacillus ihumii* sp. nov. strain Marseille-Q1233 as a new bacterial species.

Keywords: Culturomics, Mali, severe acute malnutrition, taxonogenomics, *Virgibacillus ihumii* sp. nov.
Original Submission: 21 April 2020; **Revised Submission:** 13 October 2020; **Accepted:** 13 October 2020
Article published online: 17 October 2020

Corresponding author: M. Million, Institut Hospitalo-Universitaire Méditerranée-Infection, 19-21 Boulevard Jean Moulin, 13385, Marseille Cedex 05, France.
E-mail: matthieumillion@gmail.com

Introduction

Severe acute malnutrition (SAM) is a real public health problem that particularly affects children under the age of 5 [1]. Several factors are implicated, such as the increase in *Proteobacteria* [2], the involvement of intestinal microbes by anaerobic depletion [3] and the decrease in antioxidants [4], all of which are involved in the progressive establishment of SAM [5]. In this study, we used the culturomics approach, which consists of isolating bacteria under various culture conditions and identifying them using matrix-assisted laser desorption ionization—time-of-flight mass spectrometry (MALDI-TOF MS) [6,7].

Heyndrickx et al. [8] in 1998 described the genus *Virgibacillus* by analysing the diversified genus *Bacillus*. Since then, some new species have been discovered and many further species have been reclassified into this genus [9–11]. The genus *Virgibacillus* is composed of 40 validly published species with correct names [12]. Most of them were are isolated from different biotopes, such as seawater, animal, sediment or soil [13–17]. The members of the genus *Virgibacillus* are characterized by their motility and Gram-positive staining. In particular, *Virgibacillus* species possesses C15:0-anteiso as a major fatty acid and MK-7 as the main menaquinone [18].

Virgibacillus ihumii sp. nov., a new bacterial species, was characterized using a combination of genotypic and phenotypic criteria, following a previously reported taxonogenomics concept [19,20]. To this end, we performed a description of this new species belonging to the genus *Virgibacillus*, which was isolated from the stool of a healthy Malian child as part of the exploration of the intestinal microbiota in malnourished children.

Isolation and growth conditions

In 2019, a bacterial strain was isolated from the stool sample of Malian child as part of a case–control study on SAM. The growth

https://doi.org/10.1016/j.nmni.2020.100790

of this strain was obtained after 48 hours' incubation at 37°C on halophilic medium at pH 7.5. It was able to grow after 24 hours' incubation at temperatures ranging from 28°C to 42°C, with an optimal pH of 7.5 under aerobic conditions. However, no cell growth was observed in an anaerobic or microaerophilic atmosphere. After the identification protocol by MALDI-TOF MS as previously reported [20], no accurate identification was obtained for strain Marseille-Q1233 despite three tests performed. MALDI spectra generated with strain Marseille-Q1233 were imported using BioTyper 3.0 software (Bruker Daltonics, Bremen, Germany) and compared to those of the local URMS database, which was continuously incremented (https://www.mediterranee-infection.com/urms-data-base).

Phenotypic characteristics

Virgibacillus ihumii sp. nov. strain Marseille-Q1233 is a Gram-positive bacilli. It is motile and exhibits catalase-positive and oxidase-negative activities. Bacterial cells measured 1.5 µm in length and 0.3 µm in diameter. Scanning electron microscopic examination was performed on this strain with a Hitachi TM4000 instrument (Hitachi Group, Krefeld, Germany) (Fig. 1). Using the API ZYM strip (bioMérieux, Marcy l'Etoile, France), positive reactions were observed for alkaline phosphatase, esterase (C4), esterase lipase (C8) and naphthol-AS-BI-phosphohydrolase. Using the API 50 CH strip, only esculin ferric citrate and D-turanose were positive. Negative reactions with these analytical profile index (API) strips are noted in Table 1. The strain Marseille-Q1233 is sensitive to tobramycin, rifampicin, oxacillin, vancomycin, tetracycline, erythromycin, amoxicillin, benzylpenicillin, ciprofloxacin, clindamycin, gentamicin, linezolid, ceftazidime, ampicillin and daptomycin but resistant to amikacin, doxycycline, imipenem and mezlocillin.

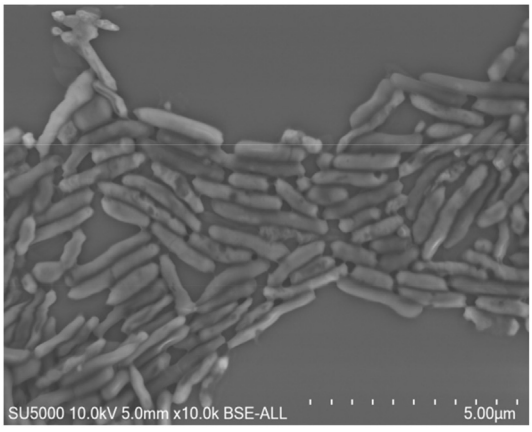

FIG. 1. Scanning electron micrograph of *Virgibacillus ihumii* sp. nov. strain Marseille-Q1233 obtained via Hitachi TM4000 microscope.

Some discriminatory criteria for distinguishing the Marseille-Q1233 strain from its closely related species are presented in Table 2. Gas chromatography/mass spectrometry testing was

TABLE 1. Biochemical tests performed on *Virgibacillus ihumii* sp. nov. strain Marseille-Q1233 using API strips ZYM and 50 CH

Characteristic	Result
API ZYM	
Alkaline phosphatase	+
Esterase (C4)	+
Esterase lipase (C8)	+
Lipase (C14)	—
Leucine arylamidase	—
Valine arylamidase	—
Cystine arylamidase	—
Trypsin	—
α-Chymotrypsin	—
Acid phosphatase	—
Naphthol-AS-BI-phosphohydrolase	+
α-Galactosidase	—
β-Galactosidase	—
β-Glucuronidase	—
α-Glucosidase	—
β-Glucosidase	—
N-Acetyl-β-glucosaminidase	—
α-Mannosidase	—
α-Fucosidase	—
API 50 CH	
Control	—
Glycerol	—
Erythritol	—
D-Arabinose	—
L-Arabinose	—
D-Ribose	—
D-Xylose	—
L-Xylose	—
D-Adonitol	—
Methyl-βD-xylopyranoside	—
D-Galactose	—
D-Glucose	—
D-Fructose	—
D-Mannose	—
L-Sorbose	—
L-Rhamnose	—
Dulcitol	—
Inositol	—
D-Mannitol	—
D-Sorbitol	—
Methyl-αD-mannopyranoside	—
Methyl-αD-glucopyranoside	—
N-acetyl-glucosamine	—
Amygdalin	—
Arbutin	—
Esculin ferric citrate	+
Salicin	—
D-Cellobiose	—
D-Maltose	—
D-Lactose	—
D-Melibiose	—
Sucrose	—
D-Trehalose	—
Inulin	—
D-Melezitose	—
D-Raffinose	—
Starch	—
Glycogen	—
Xylitol	—
Gentiobiose	—
D-Turanose	+
D-Lyxose	—
D-Tagalose	—
D-Fucose	—
L-Fucose	—
D-Arabitol	—
L-Arabitol	—
Potassium gluconate	—
Potassium 2-ketogluconate	—
Potassium 5-ketogluconate	—

API, analytical profile index.

carried out to reveal the existing cellular fatty acid in this strain, as previously reported [21]. The only abundant fatty acid from strain Marseille-Q1233 was 12-methyl-tetradecanoic acid (52.2%). Minor amounts of unsaturated, branched and other saturated fatty acids were also detected (Table 3).

Strain identification

To identify the strain Marseille-Q1233, the 16S ribosomal RNA (rRNA) gene was amplified using the primer pair fD1 and rP2 (Eurogentec, Angers, France) and sequenced using the Big Dye Terminator v1.1 Cycle Sequencing Kit and 3500xLGenetic Analyzer capillary sequencer (Thermo Fisher, Saint-Aubin, France), as previously described [22]. The 16S rRNA nucleotide sequences were assembled and corrected using CodonCode Aligner software (http://www.codoncode.com). Strain Marseille-Q1233 had a 99.28% 16S rRNA similarity with *Virgibacillus siamensis* strain MS3-4 (GenBank accession no. NR_112738.1), the phylogenetically closest species with standing in nomenclature (Fig. 2). Thus, this result, which is in disagreement with the obtained phenotypic and biochemical characteristics, led us to compare genomic data with *V. siamensis*, the closest phylogenetic neighbour with standing in nomenclature.

Genome sequencing

Genomic DNA was extracted using the EZ1 biorobot with the EZ1 DNA tissue kit (Qiagen, Hilden, Germany), then sequenced on a MiSeq sequencer (Illumina, San Diego, CA, USA) with the Nextera Mate Pair sample prep kit and Nextera XT Paired End (Illumina), as previously described [23]. The assembly was performed using a pipeline containing several software packages (Velvet [24], Spades [25] and Soap Denovo [26]) and trimmed (MiSeq and Trimmomatic [27] software) or untrimmed (only MiSeq software) data. GapCloser software [28] was used to decrease assembly gaps. Scaffolds that had a nucleotide number of <800 bp and scaffolds that had a depth value lower than 25% of the mean depths were removed. The best assembly was selected using different criteria (number of scaffolds, N50, number of N). The genome of strain Marseille-Q1233 was 3 813 121 bp long with 41.1% G + C content. Whole genome sequence analysis by BLAST (Basic Local Alignment Search Tool) in the GenBank database revealed that strain Marseille-Q1233 was 90.30% similar to *V. siamensis*. In addition, digital DNA-DNA hybridization (dDDH) values were

TABLE 3. Cellular fatty acid profiles (%) of 1, *Virgibacillus ihumii* sp. nov. strain Marseille-Q1233^T compared to 2, *Virgibacillus salinus* strain DSM 21756; 3, *Virgibacillus phasianinus* strain LM2416; 4, *Virgibacillus necropolis* strain DSM 14866

Fatty acid	Name	1	2	3	4
15:0 anteiso	12-Methyl-tetradecanoic acid	52.2	32.0	73.0	64.7
14:0 iso	12-Methyl-tridecanoic acid	15.2	17.1	3.1	2.5
5:0 iso	3-Methylbutanoic acid	5.3	ND	ND	ND
17:0 iso	15-Methyl-hexadecanoic acid	5.3	ND	0.7	0.9
15:0 iso	13-Methyl-tetradecanoic acid	4.9	9.4	5.3	3.1
16:00	Hexadecanoic acid	—	20.5	0.9	2.2
13:0 anteiso	10-Methyl-dodecanoic acid	TR	TR	TR	—
15:00	Pentadecanoic acid	TR	TR	TR	—

TR, trace amounts <1%; −, not detected; ND, not detected.

TABLE 2. Biochemical characteristics of 1, *Virgibacillus ihumii* sp. nov. strain Marseille-Q1233 compared to 2, *Virgibacillus siamensis* strain JCM 15395 [16]; 3, *Virgibacillus salinus* strain DSM 21756 [10]; 4, *Virgibacillus phasianinus* strain LM2416 [17]; 5, *Virgibacillus necropolis* strain DSM 14866 [18]

Property	1	2	3	4	5
Cell diameter (µm)	0.3–1.5	0.5–0.7	0.9	0.3–0.4	0.5–0.7
Oxygen requirement	+	+	+	+	+
Gram stain	+	+	+	+	+
Salt tolerance	+	+	+	+	+
Motility	+	+	+	+	+
Endospore formation	−	+	+		+
Alkaline phosphatase	+	NA	NA	+	+
Catalase	+	+	+	+	+
Oxidase	−	+	−	−	NA
Nitrate reductase	−	−	+	+	+
Urease	+	−	NA	−	−
β-Galactosidase	−	NA	NA	+	NA
N-Acetyl-glucosamine	−	NA	NA	+	w
Arabinose	−	−	+	+	−
Esterase lipase (C8)	−	NA	NA	+	NA
Mannose	−	−	−	+	−
Mannitol	−	−	−	+	−
Sucrose	−	−	+	+	−
D-Glucose	−	+	+	+	+
D-Fructose	−	−	+	+	w
D-Maltose	−	+	+	+	−
Source	Human stool sample	Fermented fish	Saline lake	Faecal sample	Deteriorated mural paintings

+, positive result; −, negative result; w, weakly positive result; NA, data not available.

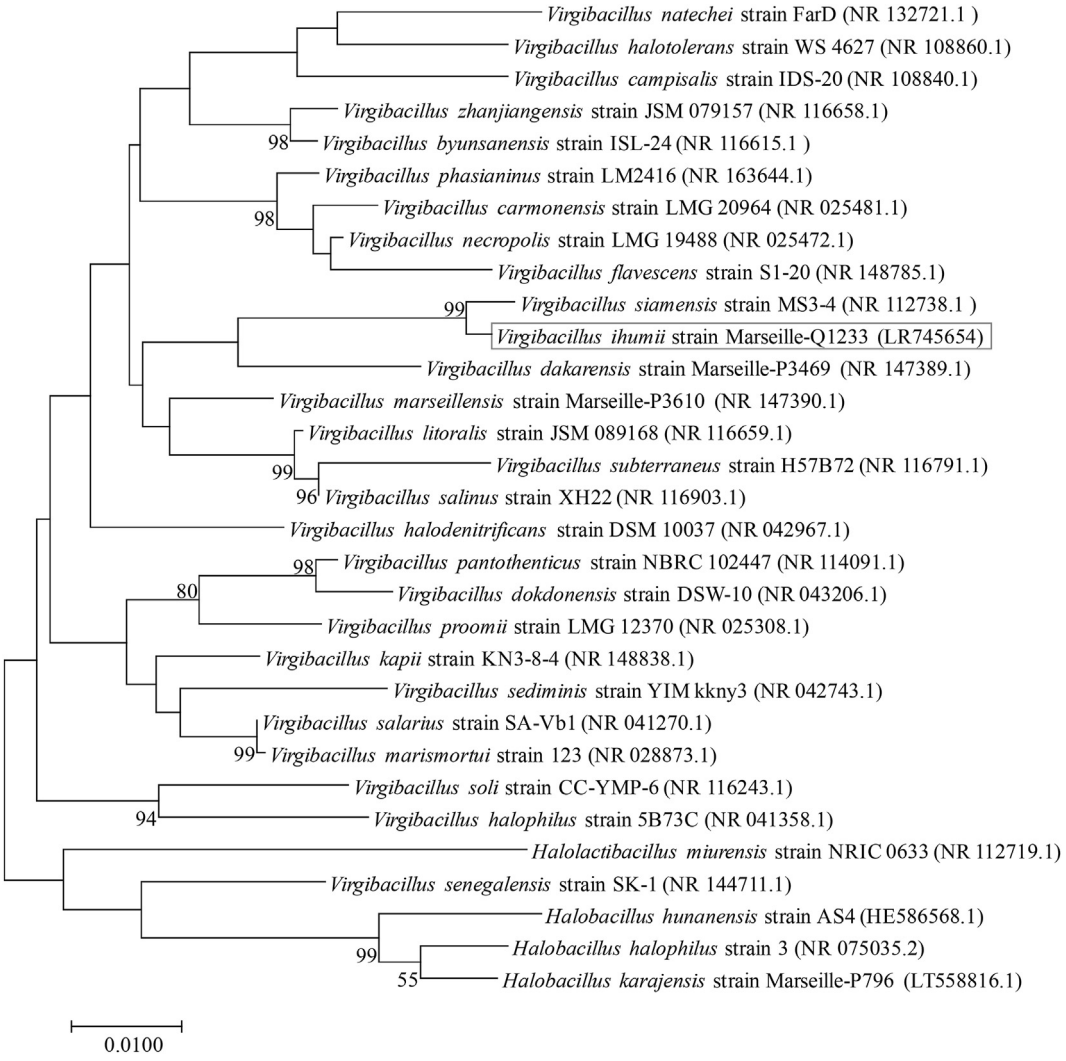

FIG. 2. Phylogenetic tree showing position of *Virgibacillus ihumii* sp. nov. strain Marseille-Q1233. GenBank accession numbers for 16S rRNA genes are indicated in parentheses. Sequences were aligned using MUSCLE 7.0.26 with default parameters; phylogenetic inferences were obtained using maximum likelihood method with MEGA 7 software. Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 1000 times to generate majority consensus tree. Only bootstrap values >70% were retained. Scale bar indicates 1% nucleotide sequence divergence.

below the recommended threshold value of >70% (Table 4). Indeed, the highest value from the Genome-to-Genome Distance Calculator 2.1 (http://ggdc.dsmz.de) was 23.10% between strain Marseille-Q1233 and *V. siamensis* strain Marseille-P2607. This strongly suggests that this strain is a new member of the genus *Virgibacillus* [29,30]. The degree of genomic similarity of the strain with closely related species was calculated using OAT software [31]. OrthoANI values among *Virgibacillus* species (Fig. 3) ranged from 66.14% between *V. soli* and *V. ihumii* to 79.67% between *V. ihumii* and *V. siamensis*. In addition, when the average nucleotide identity was calculated using an ANI calculator (http://enve-omics.ce.gatech.edu/ani/index), we found 80.81% similarity with *V. siamensis* strain Marseille-P2607. This result is below the cutoff value of 92% [32] recommended to delineate new bacterial species.

Despite the high similarity in the 16S sequence with the closest neighbour (92%), careful characterization confirmed the identification of a new species on the basis of phenotypic (endospore formation, oxidase, urease, metabolism of D-glucose and D-maltose) and genomic (dDDH 23%, OrthoANI 80%, ANI 81%) data. Indeed, nowadays, 16S similarity is not the only criterion to delineate a new species. Phenotypic and genomic characteristics should be taken into account with different thresholds. For dDDH, the threshold is 70%. Here, the dDDH is much lower than this threshold (23%). For OrthoANI (average nucleotide identity based on orthologous genes), the threshold was set at

TABLE 4. **Genome comparison between *Virgibacillus ihumii* sp. nov. strain Marseille-Q1233 and closely related species**

	V. ihumii	*V. dokdonensis*	*V. halodenitrificans*	*V. necropolis*	*V. phasianinus*	*V. salinus*	*V. siamensis*	*V. soli*
V. ihumii	100%	20.90%	21.50%	21.10%	21.10%	19.60%	23.10%	21.00%
V. dokdonensis		100%	23.20%	24.40%	28.50%	21.90%	22.50%	32.10%
V. halodenitrificans			100%	23.40%	23.00%	20.70%	21.70%	27.20%
V. necropolis				100%	22.90%	21.00%	22.60%	29.00%
V. phasianinus					100%	20.70%	21.30%	34.40%
V. salinus						100%	18.80%	22.70%
V. siamensis							100%	41.00%
V. soli								100%

Comparison made using Genome-to-Genome Distance Calculator (GGDC) and formula 2 (digital DNA-DNA hybridization estimates based on identities over high-scoring segment pair length).

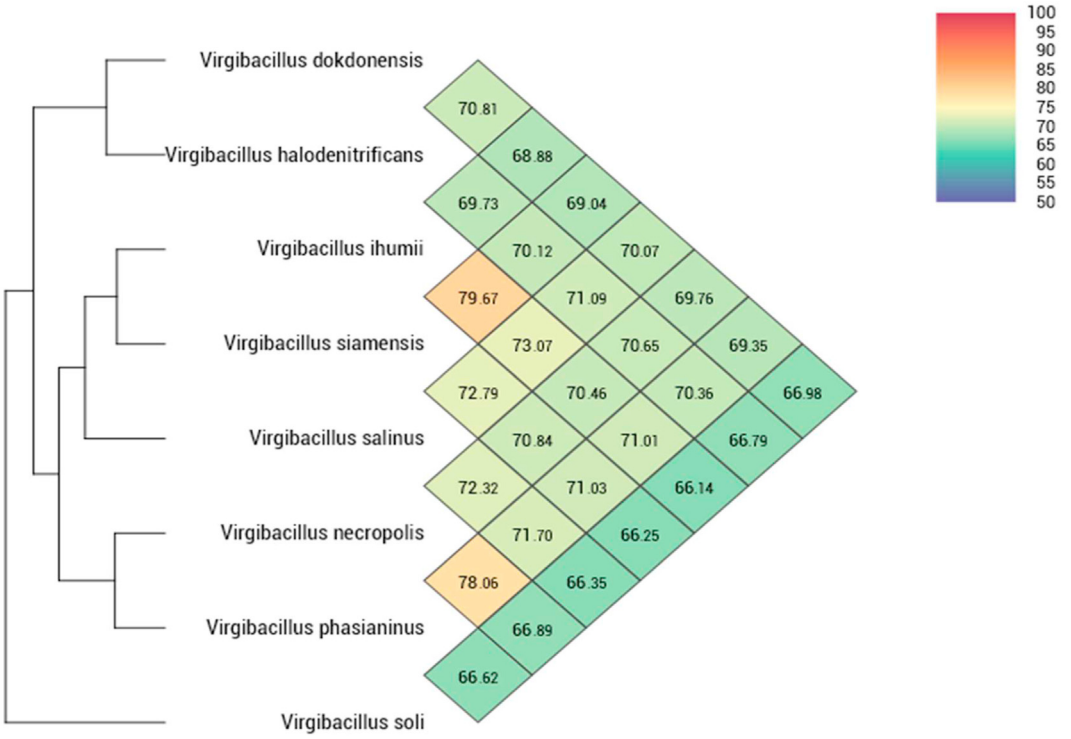

FIG. 3. Heat map generated with OrthoANI values calculated using OAT software between *Virgibacillus ihumii* sp. nov. strain Marseille-Q1233 and other closely related species with standing in nomenclature.

95%. Here, the OrthoANI is as low as 80%. Finally, for the average nucleotide identity, the usual threshold is 92%. Here, the ANI is 81%, well below this threshold. All these results confirm the description of a new species.

Conclusion

On the basis of unique phenotypic, phylogenetic and genomic features, we formally propose strain Marseille-Q1233 as the type strain of *V. ihumii* sp. nov., a new species within the genus *Virgibacillus*.

Description *Virgibacillus ihumii* sp. nov.

Virgibacillus ihumii (i.hu.mii, L. fem. adj. *ihumii*, the French acronym for Institut Hospitalo-Universitaire (IHU) Méditerranée Infection (MI) of Marseille, where the type strain was first isolated) is a motile rod-shaped bacterium 1.5 µm in diameter which grows on a halophilic medium at 100 g/L after 24 hours' incubation under aerobic conditions. Colonies are translucent and whitish. Positive reactions were observed with alkaline phosphatase, esterase (C4), esterase lipase (C8), naphthol-AS-BI-phosphohydrolase, esculin ferric citrate and D-turanose. However, glycerol,

arabinose, ribose, xylose, D-adonitol, galactose, glucose, fructose and mannose are not produced. The 16S rRNA gene sequence and whole genome shotgun sequence of Marseille-Q1233 were deposited in GenBank under accession numbers LR745654 and CACVAN000000000 respectively. The type strain is Marseille-Q1233 (= CSURQ1233), which was isolated from a stool sample from a Malian child under 5 years of age.

Funding sources

This work was supported by the Institut Hospitalo-Universitaire (IHU) Méditerranée Infection, and the Agence Nationale de Recherche (ANR) under the program " Investissements d'avenir", reference ANR-10-IAHU-03, the Région Provence Alpes Côte d'Azur and European funding FEDER PRIMI.

Conflict of interest

None declared.

Acknowledgements

We thank Aurelia Caputo for submitting the genomic sequences to GenBank.

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