Halobacillus ihumii sp. nov., a new bacterium isolated from stool of healthy children living in Mali

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Abstract

Strain Marseille-Q1234^T is a new species from the genus *Halobacillus* that was isolated in 2019 from a stool sample in a healthy Malian child <5 years old. Cells are Gram-positive and strictly halophilic bacilli. Strain Marseille-Q1234^T exhibits 98.46% 16S rRNA gene sequence similarity to *Halobacillus naozhouensis* strain JSM 071068^T (NR_116505.1), the phylogenetically closely related species with standing in nomenclature. Based on the phenotypic and phylogenetic evidence, OrthoANI values and results of the biochemical tests, the new species is named *Halobacillus ihumii* sp. nov., for which strain Marseille-Q1234^T (= CSURQ1234) is proposed as the type strain.

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Introduction

Severe acute malnutrition remains a public health problem in children under 5 years of age [1]. Beyond diet, the cause remains unclear but the involvement of gut microbes through anaerobic depletion [2], increasing *Proteobacteria* [3] and decreasing antioxidants [4] suggests a key role of this complex ecosystem in severe acute malnutrition [5]. More investigation based on the culturomics approach may bring better understanding of the microbiota composition. This is a new method that involves isolation of bacteria under diverse culture conditions and uses matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to identify them [6,7].

The genus *Halobacillus* was first described by Spring et al. in 1996 [8] to accommodate two novel species, *Halobacillus litoralis* and *Halobacillus trueperi*, and the transfer of *Sporosarcina halophila* [9] to *Halobacillus* as *Halobacillus halophilus*. Currently there are 21 species belonging to this genus with validly published names [10]. Members of the genus *Halobacillus* tolerating high salt concentrations are isolated predominantly from saline soils [9,11], salt lakes [12–14], mangrove environments [15,16] and sea anemone [17].

The new species, for which we propose the name *Halobacillus ihumii* sp. nov., was characterized using a combination of genotypic and phenotypic characteristics, following a previously described taxonogenomics strategy [18,19]. In this study, we perform a description of a new species within the genus *Halobacillus*, isolated from the stool of a healthy Malian child as part of an exploration of the gut microbiota in malnourished children.

Isolation and growth conditions

In 2019, as part of a case–control study on severe acute malnutrition, a strain was isolated from the stool of a healthy

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child of 19 months of age living in Mali. The growth of the strain Marseille-Q1234^T was observed after a 48-hour incubation at 37°C on halophilic medium (pH 7.5) under aerobic conditions. It grows after 24 hours of incubation at 28, 30 and 37°C with a pH ranging from 5.5 to 9 under aerobic conditions. However, no growth was noted under microaerophilic or anaerobic conditions. Screening with MALDI-TOF MS was attempted thrice without any identification for this strain. The assay was carried out on a Microflex LT spectrometer (Bruker Daltonics, Bremen, Germany) as previously described [6]. Spectra obtained were imported and analysed using the BIOTYPER 3.0 software against the Bruker database that was permanently improving with the local URMS database (https://www.mediterranee-infection.com/urms-data-base).

Phenotypic characteristics of strain Marseille-Q1234

Colonies of this strain were pink and circular seen on blood agar plate. Gram-staining showed Gram-positive rod-shaped bacilli. Strain Marseille-Q1234 presents catalase-positive and oxidase-negative activities. Bacterial cells measured 1.6 µm in length and 0.5 µm in diameter. Using TM4000 instrument (Hitachi Group, Krefeld, Germany) a scanning electron microscope (SEM) examination was performed on this strain (Fig. 1). Indeed, one pure colony was retrieved from agar and pushed into a 2.5% glutaraldehyde fixative solution. Then, a drop of the suspension was directly deposited on a poly-L-lysine-coated microscope slide for 5 minutes and treated with 1% phosphotungstic acid aqueous solution (pH 2.0) for 2 minutes to increase SEM image contrast. The slide was washed in water, air-dried and examined in a tabletop TM4000 SEM. To study the enzymatic activity and the use of carbohydrates of this strain, we used the API ZYM and API 50 CH strips (bioMérieux, Marcy l'Étoile, France), respectively. With the API ZYM strip, positive reactions were observed for β-galactosidase, esterase, esterase lipase and α-glucosidase whereas negative reactions were obtained for alkaline phosphatase, phosphatase acid, leucine arylamidase, β-glucosidase, naphthol-AS-BI-phosphohydrolase, α-fucosidase, β-galactosidase, α-galactosidase, β-glucuronidase, α-mannosidase, *N*-acetyl-β-glucosaminidase, α-chymotrypsin and cystine arylamidase. Esculin hydrolysis and fermentation of turanose were the only positive reactions obtained using API 50 CH strips. On the other hand, D-glucose, D-maltose, D-melibiose, D-trehalose, D-mannitol, D-saccharose, D-raffinose, D-xylose, inositol, D-fructose, D-sorbitol, L-xylose, D-adonitol, methyl β-D-xylopyranoside, glycerol, D-mannose, D-melezitose, inulin and starch were negative. The strain Marseille-Q1234 is sensible to ceftriaxone, ciprofloxacin, oxacillin, vancomycin, amoxicillin, amoxicillin-clavulanic acid, imipenem, clindamycin, doxycyclin, erythromycin, fosfomycin, gentamicin, penicillin, trimethoprim-sulfamethoxazole and rifampicin, but resistant to metronidazole and ofloxacin. In addition, comparative study of the biochemical discrepancies between strain Marseille-Q1234 and those of other species in the genus *Halobacillus*, is presented in Table 1. Gas chromatography/mass spectrometry was performed to analyse cellular

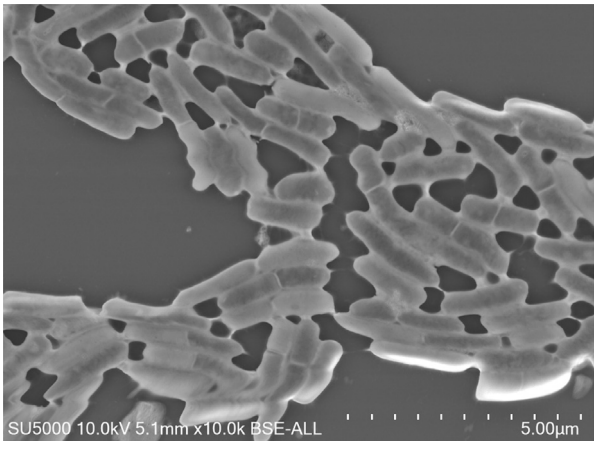

FIG. 1. Morphological structure of *Halobacillus ihumii* sp. nov., strain Marseille-Q1234, obtained by scanning electron microscopy using Hitachi TM4000 instrument. Scale and used parameters are shown in figure.

TABLE 1. Differential characteristics of *Halobacillus* species

Properties	1	2	3	4	5	6
Cell diameter (µm)	0.5	0.5–0.8	1.5	1.6–1.8	0.7–1.1	0.5–1
Oxygen requirement	+	+	+	+	+	+
Gram stain	+	+	+	+	+	+
Salt requirement	+	+	+	+	+	+
Motility	+	+	–	–	+	+
Endospore formation	–	+	+	+	+	+
Alkaline phosphatase	+	+	ND	+	–	ND
Catalase	+	+	+	+	+	+
Oxidase	–	+	+	–	+	+
Nitrate reductase	–	–	–	–	–	–
Urease	+	–	–	–	–	–
β-Galactosidase	+	–	ND	+	ND	+
N-acetyl-glucosamine	–	–	ND	–	ND	-
Arabinose	–	–	ND	ND	ND	ND
Lipase (C8)	–	–	ND	–	ND	–
Pyrrolidonyl arylamidase	–	–	ND	ND	ND	ND
Mannose	–	–	+	–	ND	–
Mannitol	–	–	ND	+	+	–
Sucrose	–	–	+	+	+	–
D-Glucose	–	+	+	+	+	–
D-Fructose	–	–	+	+	+	–
D-Maltose	–	–	+	+	+	–
Source	human stool	sea anemone	salt lake	soil	salt lake	leaves of tree

(1), *Halobacillus ihumii* sp. nov., compared with (2), *Halobacillus naozhouensis* [17]; (3), *Halobacillus alkaliphilus* [14]; (4), *Halobacillus faecis* [15]; (5), *Halobacillus litoralis* [8] and (6), *Halobacillus mangrovi* [16].
+, positive reaction; –, negative reaction; ND, not found in original paper.

TABLE 2. Cellular fatty acid profiles (%) of Halobacillus species

Fatty acids	Names	1	2	3	4
14:00	Tetradecanoic acid	TR	TR	TR	–
16:00	Hexadecanoic acid	1.1	5.7	2.1	1.5
15:0 anteiso	12-methyl-tetradecanoic acid	72.1	52.3	50.0	22.8
16:0 anteiso	13-methyl-pentadecanoic acid	3.5	TR	TR	–
17:0 anteiso	14-methyl-hexadecanoic acid	6.4	11.1	15.0	4.5
5:0 iso	3-methyl-butanoic acid	10.9	TR	TR	–
14:0 iso	12-methyl-tridecanoic acid	1.4	2.3	2.5	17.1
15:0 iso	13-methyl-tetradecanoic acid	3.8	10.4	13.8	14.8
17:0 iso	15-methyl-hexadecanoic acid	TR	2.6	2.9	4.4

1, *Halobacillus ihumii* sp. nov., strain Marseille-Q1234^T compared with 2, *Halobacillus naozhouensis* strain JSM 071068; 3, *Halobacillus faecis* strain IGA7-4; and 4, *Halobacillus mangrovi* strain MS10.
–, not detected; TR = trace amounts <1%.

fatty acids as previously described [20]. The only abundant fatty acid from strain Marseille-Q1234 was 12-methyl-tetradecanoic acid (72%). Minor amounts of unsaturated, branched and other saturated fatty acids were also detected (Table 2).

Strain identification

In order to identify the strain Marseille-Q1234, the 16S 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 (Thermofisher, Saint-Aubin, France) as previously reported [21]. The 16S rRNA nucleotide sequences were assembled and corrected using CodonCode Aligner software (http://www.codoncode.com). Strain Marseille-Q1234 exhibited a 98.46% 16S rRNA similarity with *Halobacillus naozhouensis* strain JSM 071068^T (GenBank Accession number: NR_116505.1), the phylogenetically closest species with standing in nomenclature (Fig. 2). This suggested that strain Marseille-Q1234 is a new member within the genus *Halobacillus* [22,23] and remains by far different to the nearest species with validly confirmed name [10].

Genome sequencing and comparison

Genomic DNA was extracted using the EZ1 biorobot with the EZ1 DNA tissue kit (Qiagen, Hilden, Germany) and then sequenced on a MiSeq sequencer (Illumina Inc., San Diego, CA, USA) with the Nextera Mate Pair sample prep kit and Nextera XT Paired End (Illumina), as previously described [24]. The assembly was performed using a pipeline containing several softwares (Velvet [25], Spades [26] and Soap Denovo [27]), and trimmed data (MiSeq and Trimmomatic [28] softwares) or untrimmed data (only MiSeq software). GapCloser software [29] was used to decrease assembly gaps. Scaffolds that had a nucleotide number <800 bp and scaffolds with a depth

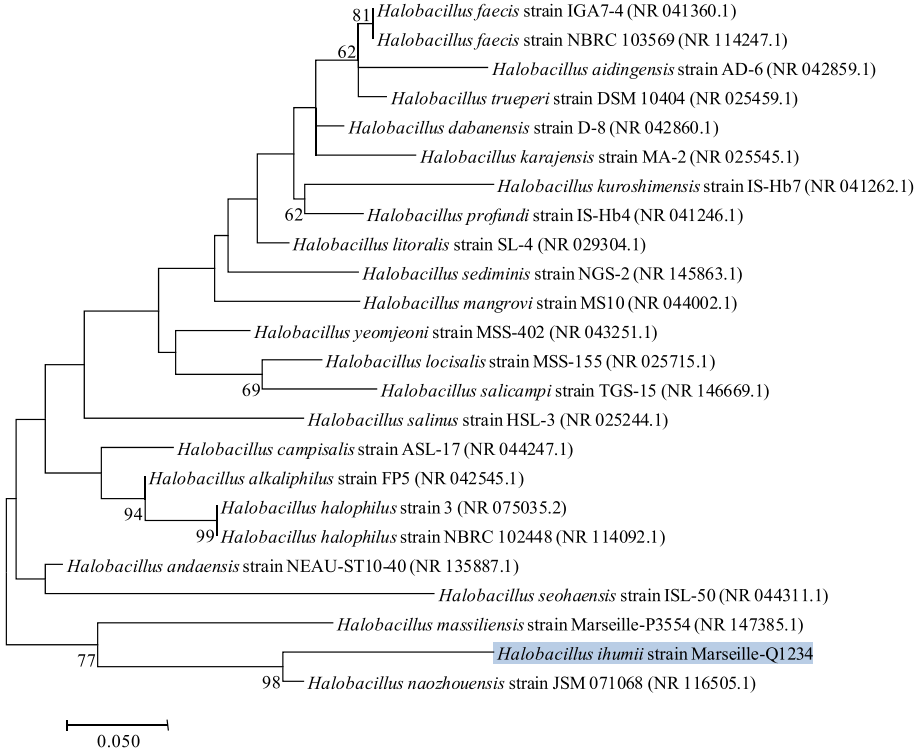

FIG. 2. Phylogenetic tree inferred from 16S rRNA sequence analysis highlighting the position of *Halobacillus ihumii* sp. nov., with regard to other closely related species. As the genome had more copies of 16S RNA sequences, we chose only four copies as shown in the figure. Phylogenetic inferences were obtained using the maximum likelihood method and the MEGA 7 software. Bootstrap values obtained by repeating the analysis 1000 times to generate a majority consensus tree are indicated at the nodes. The scale bar indicates a 5% sequence variance.

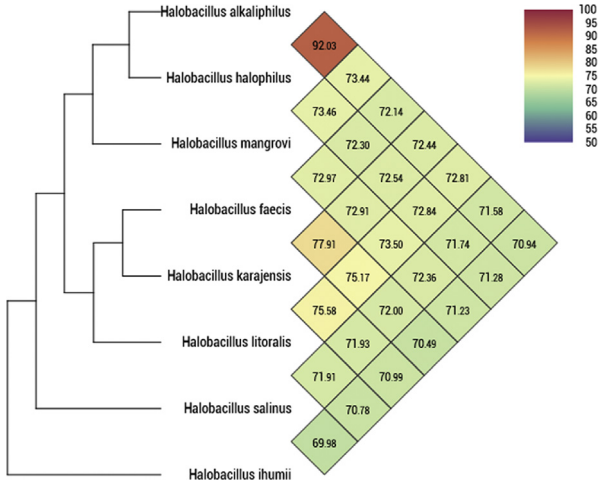

FIG. 3. Heatmap generated with OrthoANI values calculated using the OAT software between *Halobacillus ihumii* sp. nov., and other closely related species with standing in nomenclature.

value < 25% of the mean depths were removed. The best assembly was selected by using different criteria (number of scaffolds, N50, number of N). The genome of strain Marseille-Q1234 was 4 293 027 bp long with a 41.1% of G + C content. The degree of genomic similarity of the strain with closely related species was calculated using OAT software [30]. OrthoANI values among *Halobacillus* species (Fig. 3) ranged from 69.98% between *H. salinus* and *H. ihimii* to 92.03% between *H. alkaliphilus* and *H. halophilus*. It should also be noted that strain Marseille-Q1234 shared its greatest OrthoANI value (71.28%) with *H. mangrovi*. Furthermore, it should be noted that at the time of writing, there was no genome available for *Halobacillus naozhouensis*. Therefore, it is not included in the genomic comparison.

Conclusion

On the basis of unique phenotypic features, 16S rRNA sequence similarity and OrthoANI value < 98.7% and <95%, respectively, with the phylogenetically closest species with standing in nomenclature, we formally proposed strain Marseille-Q1234 as type strain of *Halobacillus ihumii* sp. nov., a new species within the genus *Halobacillus*.

Description of *Halobacillus ihumii* sp. nov.

Halobacillus ihumii sp. nov., (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 Gram-positive, motile and spore-forming bacterium. Bacterial cells were rod-shaped with a length ranging from 1.4 to 1.9 µm and a width ranging from 0.4 to 0.6 µm. Cells exhibit catalase-positive and oxidase-negative activities. Colonies of strain Marseille-Q1234 have a mean diameter of 0.5 mm with regular edges and a pinkish aspect on 5% sheep blood Columbia agar (bioMérieux). The strain grows under aerobic conditions at 37°C in halophilic media. The potential pathogenicity of the type strain Marseille-Q1233 (= CSUR Q1234) is unknown. This strain has a genome size of 4 293 027 bp long with a 41.1 mol% G + C content. The 16S rRNA gene sequence and whole-genome shotgun sequence of Marseille-Q1234 were deposited in GenBank under Accession numbers LR745662 and CACVAO010000000, respectively. Strain Marseille-Q1234 is the type strain of *Halobacillus ihumii* sp. nov., isolated from stool of healthy Malian child.

Conflict of interest

None to declare.

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Ethics and consent

The study was approved by the ethics committee of the Faculté de Médecine, de Pharmacie et d'OdontoStomatologie of Mali under the reference N2014/46/CE/FMPOS. The child's guardian gave a written consent.

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