Clostridiisalibacter paucivorans gen. nov., sp. nov., a novel moderately halophilic bacterium isolated from olive mill wastewater

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A moderately halophilic, strictly anaerobic bacterium, designated 37HS60^T, was isolated from an olive mill wastewater in southern Morocco (Marrakesh). The cells were straight, motile and stained Gram-positive, forming spherical and terminal spores and with an atypical thick and stratified multilayered cell wall. Major fatty acid components were iso-C17 : 1ω10*c* or anteiso-C17 : 1ω3*c* (19.3 %), C14 : 0 (14.3 %), C16 : 1ω7*c* (9.9 %), C16 : 1ω7*c* DMA (8.5 %) and C16 : 0 (7.6 %). Strain 37HS60^T grew from 20 to 50 °C with an optimum at 40–45 °C, and growth was observed from pH 5.5 to 8.5 with an optimum of 6.8. The salinity range for growth was 10–100 g NaCl l^{-1} with an optimum at 50 g NaCl l^{-1}. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain 37HS60^T fell within the evolutionary radiation enclosed by cluster XII of the *Clostridium* subphylum. Strain 37HS60^T exhibited highest 16S rRNA gene sequence similarity of 92.0 % with *Caloranaerobacter azorensis* and 90.6 % with *Clostridium purinilyticum.* Moreover, 37HS60^T did not grow on basal medium with hexose or pentose sugars as carbon and energy sources. Pyruvate, fumarate and succinate were the best substrates for 37HS60^T growth with 1.0 g yeast extract l^{-1}. The DNA G+C content was 33.0 mol%. Due to its phenotypic and genotypic characteristics, isolate 37HS60^T is proposed as a novel species of a new genus, *Clostridiisalibacter paucivorans* gen. nov., sp. nov. The type strain is 37HS60^T (=JCM 14354^T=CCUG 53849^T).

Olive mill wastewater (OMW) is a major environmental problem in the Mediterranean region, where more than 3×10^7 m^3 of this residue is produced annually. OMW presents a high organic load, including sugars, tannins, polyphenols, polyalcohols, pectins, lipids and a wide variety of simple aromatic compounds resulting from olive cell-wall degradation during the oil extraction process (Balice & Cera, 1984; Hamdi, 1993; Labat *et al.*, 2000; Lesage-Meesen *et al.*, 2001). This effluent is discharged directly into sewage systems and water streams, despite the fact that such disposal methods are prohibited in many Mediterranean countries. This is due to the current lack of appropriate alternative technologies to treat OMW properly, mainly in the south of the region. In many countries, such as Morocco, OMW is directly rejected into evaporation ponds without any complementary treatment.

Microbiological studies on OMW or digesters treating these wastes have revealed the presence of metabolically diverse anaerobic bacteria (Mechichi *et al.*, 1998, 1999a, b, 2000; Chamkha *et al.*, 2001a, b; Thabet *et al.*, 2004), all of which are anaerobic species belonging to the order *Clostridiales* and affiliated to the genus *Clostridium*. This genus has been defined as containing Gram-positive, anaerobic, rod-shaped and spore-forming bacteria unable to carry out dissimilatory sulfate reduction (Cato *et al.*, 1986; Hippe *et al.*, 1992). Collins *et al.* (1994) used rDNA phylogenetic analysis to define 15 clusters within the genus *Clostridium*, and they subsequently proposed five new

Abbreviation: OMW, olive mill wastewater.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of 37HS60^T is EF026082.

genera: *Caloramator*, *Filifactor*, *Moorella*, *Oxobacter* and *Oxalophagus*. Phylogenetically, however, the genus still embraces a high number of organisms from many different genera that are not defined by *Clostridium*-specific phenotypes (Cato & Stackebrandt, 1989; Lawson *et al.*, 1993; Rainey & Stackebrandt, 1993; Rainey *et al.*, 1993; Collins *et al.*, 1994; Stackebrandt & Rainey, 1997; Stackebrandt *et al.*, 1999; Stackebrandt & Hippe, 2001).

Despite some reclassification, the genus *Clostridium* remains a phylogenetically heterogeneous entity, containing a core cluster (I) of more than 60 species, while the remaining species form several independent phylogenetic lineages, in which species are sometimes closely related to organisms which are classified in different genera. Cluster XII, for example, recognized as being phenotypically incoherent, contains four *Clostridium* species, closely related to other genera such as *Tissierella* (Farrow *et al.*, 1995), *Thermohalobacter* (Cayol *et al.*, 2000), *Caloranaerobacter* (Wery *et al.*, 2001), *Sporanaerobacter* (Hernandez-Eugenio *et al.*, 2002), *Sedimentibacter* (Breitenstein *et al.*, 2002), *Soehngenia* (Parshina *et al.*, 2003) and *Garciella* (Miranda-Tello *et al.*, 2003). These genera, isolated from diverse environments, include non-spore-forming, Gram-negative and -positive bacteria. In cluster XII, the species are either mesophilic or thermophilic, and even halophilic.

Here, we describe a novel species of a novel genus belonging to the order *Clostridiales*, cluster XII, which is an anaerobic, mesophilic, halophilic, heterotrophic, spore-forming bacterium, isolated from an OMW evaporation pond located in Marrakesh, Morocco. Formerly, these ponds were old underground galleries, called khettaras, which brought water for irrigation of the desert. Nowadays they are used as reserve ponds for different agroindustrial effluents.

Samples were collected at a depth of 6 m, using a single tube with a specific stem to collect each sample of sediment. The mud was transferred (i) into 50 ml serum vials closed with butyl rubber stoppers then stored at 4 °C, and (ii) into 1.8 ml cryotubes containing 2 % glycerol (v/v), added as a cryoprotectant, then stored at −20 °C until processing.

The Hungate technique (Hungate, 1969) was used throughout the isolation procedure and the physiological and metabolic characterization. The basal medium contained (l^{-1}): 0.3 g KH_2PO_4, 0.3 g K_2HPO_4, 1.0 g NH_4Cl, 50 g NaCl, 0.1 g KCl, 0.1 g $CaCl_2.2H_2O$, 10 ml trace mineral element solution (Balch *et al.*, 1979) and 1 ml 0.1 % (w/v) resazurin. The pH was adjusted to 7.2 with 10 M KOH. The basal medium was boiled under a stream of O_2-free N_2 gas, cooled to room temperature, and 5 ml aliquots were distributed into Hungate tubes under a stream of O_2-free N_2 gas. The N_2 gas phase was replaced with N_2/CO_2 (80 : 20) and the tubes were autoclaved. Prior to inoculation, 0.05 ml $Na_2S.9H_2O$ (2 %, w/v), 0.1 ml $NaHCO_3$ (10 %, w/v) and 0.1 ml $MgCl_2.6H_2O$ (150 g l^{-1}) were added. To initiate enrichment cultures, a small part of the sediment sample was inoculated into the growth medium, followed by incubation at 37 °C without agitation. Positive enrichment cultures were obtained after 7 days incubation at 37 °C, and microscopic examination revealed the presence of motile, rod-shaped bacteria. The culture was then purified by repeated use of the Hungate roll tube method (Hungate, 1969). Single colonies (0.8–1.0 mm diam.), which developed in the roll tubes after 96 h incubation at 37 °C, were picked up and tenfold serial dilutions were performed in the roll tubes at least twice before the culture was considered to be pure. Several axenic cultures were obtained using this procedure, and one strain, named 37HS60^T, was used for further characterization.

Strain 37HS60^T was a straight, thin, long rod (0.5 μm × 3.0–8.0 μm), motile by means of peritrichous flagella (Fig. 1a), that stained Gram-positive and formed spherical and terminal spores (Fig. 1b) which appeared mainly in old cultures. However, ultrathin sections obtained as described by Fardeau *et al.*, (1997) showed an atypical, well-contrasted, Gram-positive-type cell wall. It exhibited a thick and stratified cell wall composed of dense multilayers, including a cytoplasmic membrane and an atypical thick and multilayered peptidoglycan, covered by an S-layer (Fig. 1c). Fatty acid methyl esters (FAMEs) were analysed at the CCUG (Göteborg, Sweden) using the Microbial Identification System (Microbial ID), and strain 37HS60^T was compared to the available fatty acid database. For FAME analysis, bacteria were grown on marine agar at 37 °C. 37HS60^T has an original cellular fatty acid profile, containing large amounts of saturated and unsatured fatty acids. The fatty acids detected were iso-C17 : 1ω10*c* or anteiso-C17 : 1ω3*c* (19.3 %), C14 : 0 (14.3 %), C16 : 1ω7*c* (9.9 %), C16 : 1ω7*c* DMA (8.5 %), C16 : 0 (7.6 %) and a weak proportion of diverse fatty acids (Table 1). A large proportion of both the major and weak fatty acids, as well as unknown fatty acids which are listed in Table 1, are rarely encountered and this atypical profile was unexpected for a Gram-positive bacterium.

Optimal growth conditions for strain 37HS60^T were determined in duplicate experiments performed in basal medium containing yeast extract (0.1 g l^{-1}) as described by Fardeau *et al.* (2000). Strain 37HS60^T was strictly anaerobic. The optimal temperature for growth was 42 °C (range 25–50 °C); growth was not observed at 20 or 55 °C. For pH studies, the medium was adjusted to the desired pH using anaerobically prepared stock solutions of $NaHCO_3$ (10 %) or Na_2CO_3 (8 %). The optimum pH was 6.8 (range 5.6–8.4). For studies of NaCl requirements, NaCl was weighed directly into the tubes for concentrations ranging from 0 to 200 g NaCl l^{-1} before dispensing basal medium without NaCl. Strain 37HS60^T required salt for growth (range 10–100 g NaCl l^{-1}), with an optimum of 50 g l^{-1}.

Substrate utilization was studied in the presence of 0.1 g yeast extract l^{-1} added to the basal medium. Only three organic acids (20 mM) were used as carbon and energy sources: succinate, fumarate and pyruvate. Succinate was

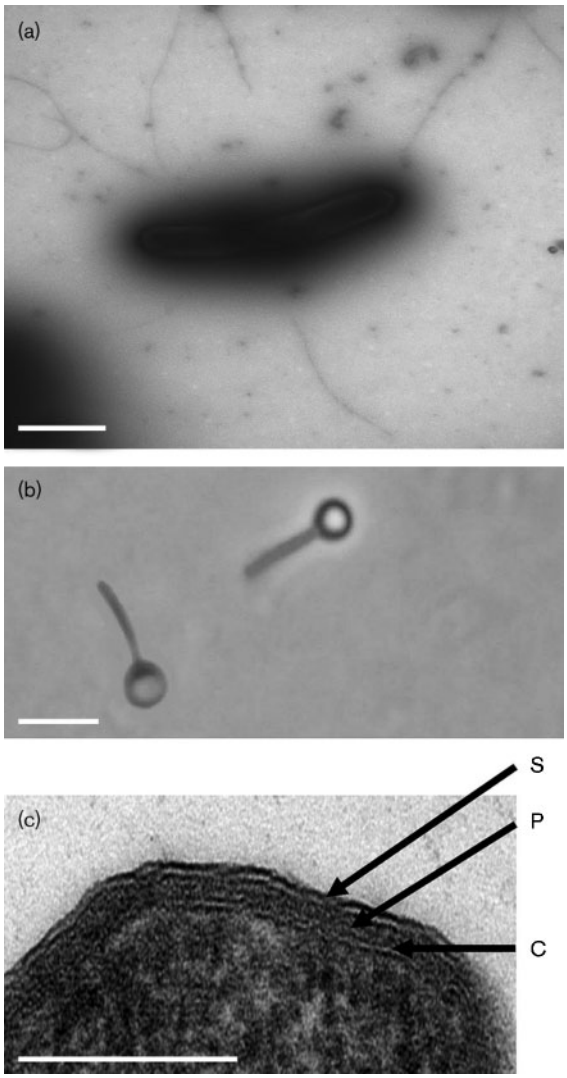

Fig. 1. (a) Electron micrograph of a negatively stained culture of strain 37HS60^T, showing peritrichous flagella. Bar, 1 μm. (b) Phase-contrast microphotograph of cells of strain 37HS60^T, showing terminal spherical spores swelling the sporangia. Bar, 5 μm. (c) Electron micrograph of an ultrathin section of strain 37HS60^T, showing the atypical Gram-positive cell wall structure composed of dense and stratified multiple layers. C, Cytoplasmic membrane; P, peptidoglycan layer; S, S-layer. Bar, 0.2 μm.

totally transformed into propionate and fumarate was slowly reduced to succinate. End products from pyruvate fermentation were acetate, H_2 and CO_2. Cellobiose, cellulose, fructose, galactose, glucose, lactose, maltose, mannose, ribose, sucrose, xylose, glycerol, mannitol, ethanol, methanol, acetate, propionate, lactate, citrate, xylan and starch were not used.

Strain 37HS60^T grew heterotrophically on peptone, biotrypticase, and Casamino acids (1.0 g l^{-1}). The Stickland reaction was negative, but four amino acids (20 mM) were used: acetate was formed from cystein, lysine and serine, and isobutyrate was formed from valine. Other amino acids, such as threonine, glycine, proline, tyrosine, phenylalanine, leucine, isoleucine, alanine, arginine, tryptophan, asparagine, aspartic acid, glutamine glutamic acid, methionine and histidine did not support growth.

Sulfate, thiosulfate, sulfite and elemental sulfur were separately added to the growth medium at final concentrations of 20 mM, 20 mM, 2 mM and 0.1 % (w/v), respectively, for potential use as an electron sink. Nitrate and nitrite utilization tests were carried out in triplicate, in butyl-capped Hungate tubes filled with 5 ml pre-reduced medium (without resazurin and Na_2S) distributed under a nitrogen atmosphere. $NaNO_3$ (10 mM) or $NaNO_2$ (10 mM) were added to the tubes immediately before inoculation (10 %, v/v). These tests were realized under similar conditions without pre-reducing the medium and without a nitrogen atmosphere to measure oxygen utilization.

Sulfate (20 mM), thiosulfate (20 mM), elemental sulfur (0.1 %), sulfite (2 mM), nitrate (20 mM) nitrite (2 mM) and oxygen were not used as electron acceptors. The sulfur test was realized photometrically as collodial CuS (Fardeau *et al.*, 1997), and the nitrate or nitrite reduction were assayed by using specific sticks (Quantofix; Machelez Nagel).

Growth of strain 37HS60^T was inhibited by the addition of three different classes of antibiotic compounds: chloramphenicol (25 μg ml^{-1}), penicillin G (300 μg ml^{-1}) and streptomycin (150 μg ml^{-1}).

The DNA G+C content, determined by the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) using the method of Mesbah *et al.* (1989), was 33.0 mol%.

Genomic DNA of 37HS60^T was extracted using the Wizard Genomic DNA Purification kit, according to the manufacturer's protocol (Promega). The universal primers Fd1 (5′CAGAGTTTGATCCTGGCTCAG-3′) and R6 (5′-TACGGTTACCTTGTTACGAC-3′) were used to amplify the SSU rRNA gene. The nucleotide sequence (1430 bases) was manually aligned, using the sequence alignment editor BioEdit (Hall, 1999). Reference sequences were obtained from the Ribosomal Database Project II (Maidak *et al.*, 2001) and GenBank databases (Benson *et al.*, 1999). The pairwise evolutionary distances based on 1281 unambiguous nucleotides were computed by the Jukes & Cantor (1969) method. The phylogenetic tree obtained by the neighbour-joining method (Saitou & Nei, 1987) is shown in Fig. 2. Its topology was also supported by using the maximum-parsimony and maximum-likelihood algorithms.

The most closely related sequences were found in members of cluster XII within the order *Clostridiales* (Collins *et al.*, 1994). Most of the related species originated from different environments. The closest phylogenetically described

Table 1. Cellular fatty acid content of strain 37HS60^T

Fatty acids	Percentage (w/w) of total fatty acids
Unknown 9.730	7.0
iso-C13 : 0 2-OH FAME	3.2
C14 : 0 FAME	14.3
iso-C15 : 1ω7*c* FAME	2.2
iso-C13 : 0 3-OH FAME	1.3
iso-C15 : 0 FAME	6.6
anteiso-C15 : 0 FAME	1.5
Unknown 14.776	3.8
Unknown 14.949	4.5
C16 : 1ω9*c* FAME	2.5
C16 : 1ω7*c* FAME	9.9
C16 : 0 FAME	7.6
C16 : 1ω9*c* dimethyl acetyl	1.8
C16 : 1ω7*c* dimethyl acetyl	8.5
anteiso-C17 : 1ω9*c* FAME	2.2
C17 : 1 ω10c or anteiso-C17 : 1ω3*c* FAME	19.3
C17cyc FAME	2.2
C17cyc dimethyl acetyl	1.6

strains were *Caloranaerobacter azorensis* DSM 13643^T (92.0 % similarity), isolated from a deep-sea hydrothermal vent (Wery *et al.*, 2001), and *Clostridium purinilyticum* DSM 1384^T, isolated from soils exposed to chicken manure and from sewage sludge enriched with adenine (90.6 % similarity). Other related species that exhibited <90 % similarity were *Clostridium acidurici* ATCC 7906^T and *Thermohalobacter berrensis* CNCM 105955^T (89.7 and 89.1 %, respectively). Apart from these species, only an undescribed strain 'Bacterium HY42-22', retrieved from a mud flat (accession no. DQ286650), exhibited a higher level of similarity (99.0 %). According to its phylogenetic position and the value accepted as the criterion for the delineation of different species (Stackebrandt & Goebel, 1994), our novel isolate probably represents a novel species and a novel genus in cluster XII of the *Clostridiales*, since its phylogenetic distance was equal to or lower than 92.0 % from *Caloranaerobacter azorensis* DSM 13643^T and *Clostridium purinilyticum* DSM 1384^T.

This is strongly supported by important differences in genetic and physiological traits (Table 2). First, strain 37HS60^T is an atypical Gram-positive bacterium, whereas *Caloranaerobacter azorensis* has a characteristic Gram-negative cell wall, demonstrating a specific difference between the structure of their respective envelopes. In addition, the DNA G+C content of *Caloranaerobacter azorensis* (27.0 mol%) is 6 mol% lower than strain 37HS60^T. Equally, strain 37HS60^T differs physiologically from *Caloranaerobacter azorensis* in terms of carbohydrates used, temperature and NaCl concentration ranges for growth. Strain 37HS60^T was able to grow at temperatures up to 50 °C and is thermotolerant (optimum 42 °C), although it was unable to grow at 55 °C, while *C. azorensis* has an optimal temperature for growth of 65 °C. In addition, unlike *Caloranaerobacter azorensis*, strain 37HS60^T was unable to grow on starch or any other sugar and unable to produce H_2S from sulfur.

Secondly, strain 37HS60^T differed from *Clostridium purinilyticum* (Dürre *et al.*, 1981) in terms of purine use, temperature range and NaCl concentration for growth. Strain 37HS60^T was unable to grow without NaCl, whereas *Clostridium purinilyticum* is not known to require sea salt or NaCl. Moreover, the optimum growth temperature of 37HS60^T (42 °C) is the maximal temperature for *Clostridium purinilyticum*. Hence, *Clostridium purinilyticum* is a mesophilic, non-halophilic species, whereas strain 37HS60^T is thermotolerant and moderately halophilic. In addition, the DNA G+C content of *Clostridium purinilyticum* (29.0 %) is 4 mol% lower than strain 37HS60^T. By contrast, strain 37HS60^T is not purinolytic and degrades some amino acids, unlike *Clostridium purinilyticum* which degrades only glycine.

Therefore, on the basis of the data reported here, we propose that strain 37HS60^T represents a novel species of a

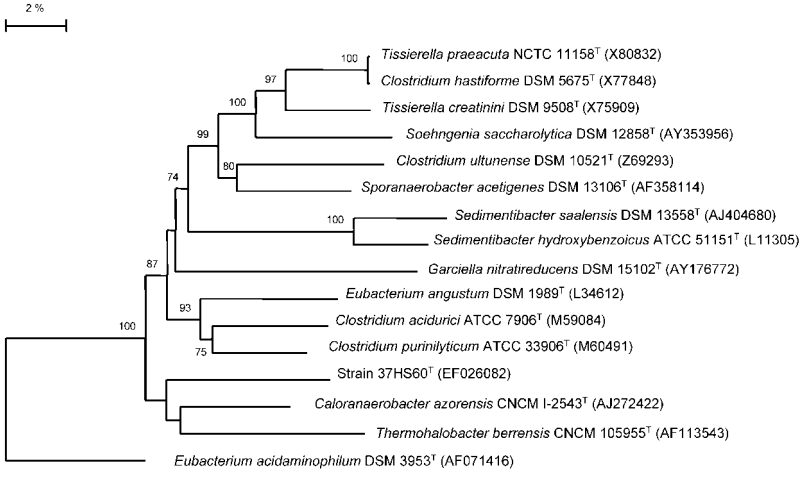

Fig. 2. Phylogenetic dendrogram based on 1281 unambiguous base pairs of the 16S rRNA gene sequence, showing the position of the novel genus and species *Clostridiisalibacter paucivorans* 37HS60^T. Bootstrap values (expressed as percentages of 100 replications) greater than 70 % are shown at branch points. Bar, 2 % sequence divergence. Numbers in parentheses are GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences.

Table 2. Differential phenotypic characteristics of strain *Clostridiisalibacter paucivorans* from phylogenetically related species

Taxa: 1, Strain 37HS60^T (present study); 2, *Caloranaerobacter azorensis* DSM 13643^T (Wery *et al.*, 2001); 3, *Clostridium purinolyticum* DSM 1384^T (Dürre *et al.*, 1981); ND, not determined. All three strains were negative for the Stickland reaction, reduction of $S_2O_3^{2-}$ and utilization of acetate. All three strains were positive for utilization of pyruvate.

Character	1	2	3
Width × length (μm)	0.5 × 3.0–8.0	0.3–0.5 × 0.5–2.0	1.4 × 6.2
Gram type	Positive	Negative	Positive
Temperature for growth (°C):			
Range	20–50	45–65	20–45
Optimum	42	65	36
pH growth:			
Range	5.5–8.5	5.5–9.0	6.5–9.0
Optimum	6.8	7.0	7.5
NaCl concentration for growth (g l^{-1}):			
Range	10–100	6.5-65	ND
Optimum	50	20	0
G+C content of DNA (mol%)	33.0	27.0	29.0
Reduction of S°	−	+	−
Substrates used:			
Purine	−	ND	+
Glycine	−	ND	+
Fructose	−	+	−
Galactose	−	+	−
Glucose	−	+	−
Mannose	−	ND	−
Ribose	−	+	−
Xylose	−	+	−
Glycerol	−	ND	−
Starch	−	+	−
Xylan	−	+	−
Fumarate	+	−	−
Succinate	+	−	−

new genus, namely *Clostridiisalibacter paucivorans* gen. nov., sp. nov.

Description of *Clostridiisalibacter* gen. nov.

Clostridiisalibacter (Clos.tri.di.i.sal′i.bac.ter. N.L. n. *Clostridium*, a bacterial genus name; L. n. *sal*, salt; N.L. masc. n. *bacter*, a rod; N.L. masc. n. *Clostridiisalibacter*, a halophilic rod, belonging to the *Clostridium* subphylum).

Rod-shaped, Gram-positive bacterium, forming spherical and terminal spores. Moderate halophilic and thermotolerant. Anaerobic, chemo-organotroph able to grow on yeast extract, peptone and Casamino acids. Sulfur, sulfate, thiosulfate, nitrate and nitrite are not necessary for growth. The major cellular fatty acids are iso-C17 : 1ω10*c* or anteiso-C17 : 1ω3*c* (19.3 %), C14 : 0 (14.3 %) and C16 : 1ω7*c* (9.9 %). The DNA G+C content is 33.0 mol%. 16S rDNA sequence comparisons locate *Clostridiisalibacter* in the lineage of the low-G+C content Gram-positive bacteria, within cluster XII of the *Clostridium* subphylum. The type species is *Clostridiisalibacter paucivorans*.

Description of *Clostridiisalibacter paucivorans* sp. nov.

Clostridiisalibacter paucivorans (pau.ci.vor′ans. L. adj. *paucus* little; L. v. *vorare* to eat; N.L. part. adj. *paucivorans* eating little, relating to the observation that the organism utilizes few fatty acids and few amino acids).

Cells are strictly anaerobic rods, 0.5 × 3.0-8.0 μm, occurring singly. Motile and peritrichous. Electron microscopy of cell sections exhibits a thick, multilayered, atypical Gram-positive-type cell wall. The fatty acids detected were iso-C17 : 1ω10*c* or anteiso-C17 : 1ω3*c* (19.3 %), C14 : 0 (14.3 %), C16 : 1ω7*c* (9.9 %), C16 : 1ω7*c* DMA (8.5 %), C16 : 0 (7.6 %) and a weak proportion of diverse fatty acids. Growth occurs between 25 and 50 °C (optimum 42 °C). Growth occurs in the presence of NaCl at 10–100 g l^{-1} (optimum 50 g l^{-1}). The optimum pH for growth is 6.8, but growth occurs between pH 5.5 and 8.5. Heterotrophic. Grows on yeast extract, peptone, Bio-trypticase and Casamino acids. Cells are unable to use sugars as carbon sources. Only the following substrates are fermented in the presence of yeast

extract (0.1 g l^{-1}): pyruvate, succinate, fumarate. Stickland reaction is negative. The following amino acids are used in the presence of yeast extract (0.1 g l^{-1}): cysteine, serine, lysine (oxidized to acetate) and valine (oxidized to isobutyrate). Does not use elemental sulfur, sulfate, thiosulfate, sulfite, nitrate, nitrite or oxygen as electron acceptors. The following tests are negative: β-galactosidase, arginine dihydrolase, lysine and ornithine decarboxylases, Simmons' citrate, H_2S production, urease, tryptophan deaminase, and indole and acetoin production (Voges–Proskauer reaction). The DNA G+C content of the type strain is 33.0 mol%.

The type strain, 37HS60^T (=JCM 14354^T=CCUG 53849^T), was isolated from OMW stored in an evaporation pond in southern Morocco (Marrakesh).

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