Marinobacter vinifirmus sp. nov., a moderately halophilic bacterium isolated from a wine-barrel-decalcification wastewater

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A halophilic, Gram-negative, motile, non-sporulating bacterium designated strain FB1^T was isolated from a wine-barrel-decalcification wastewater. The organism comprises straight rods and has a strictly respiratory metabolism with O_2. Strain FB1^T grows optimally at 20–30 °C and 5–6 % NaCl. The predominant fatty acids were found to be $C_{18:1}\omega9c$ (30·4 %), $C_{16:0}$ (25·7 %), $C_{12:0}$ 3-OH (10·3 %), $C_{16:1}\omega9c$ (9·7 %) and $C_{16:1}\omega7c$ (8·4 %). A phylogenetic analysis based on 16S rRNA gene sequences revealed that the strain forms a coherent cluster within the genus *Marinobacter*. The highest level of 16S rRNA gene sequence similarity (97·9 %) exhibited by strain FB1^T was with the type strain of *Marinobacter excellens*. However, the level of DNA–DNA relatedness between the novel strain and *M. excellens* CIP 107686^T was only 31·2 %. The DNA G+C content of strain FB1^T was 58·7 mol%. On the basis of phenotypic and genotypic characteristics, and also phylogenetic evidence, strain FB1^T is considered to represent a novel species of the genus *Marinobacter*, for which the name *Marinobacter vinifirmus* sp. nov. is proposed. The type strain is FB1^T (=DSM 17747^T=CCUG 52119^T).

Halophilic organisms can inhabit hypersaline environments because of their capacity to balance the osmotic pressure and resist the denaturing effect of salts. Of this group, bacteria can be loosely classified as slightly, moderately or extremely halophilic, depending on their requirement for NaCl (Kushner & Kamekura, 1988).

Various genera and species have been described for slightly to extremely halophilic organisms, including the genus *Marinobacter*; this genus was initially described by Gauthier *et al.* (1992) as containing a single species, *Marinobacter hydrocarbonoclasticus*. A second species, *Marinobacter aquaeolei*, originally described by Nguyen *et al.* (1999), was recently reclassified as a later heterotypic synonym of *M. hydrocarbonoclasticus* (Marquez & Ventosa, 2005). To date, the genus *Marinobacter* contains nine further species with validly published names: *Marinobacter excellens* (Gorshkova *et al.*, 2003), *M. litoralis* (Yoon *et al.*, 2003), *M. lutaoensis* (Shieh *et al.*, 2003), *M. lipolyticus* (Martín *et al.*, 2003), *M. flavimaris* and *M. daepoensis* (Yoon *et al.*, 2004), *M. bryozoorum* and *M. sediminum* (Romanenko *et al.*, 2005) and *M. maritimus* (Shivaji *et al.*, 2005).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain FB1^T is DQ235263.

These *Marinobacter* species comprise Gram-negative, aerobic, motile, rod-shaped bacteria. Phylogenetic analyses based on 16S rRNA gene sequences have shown that the genus falls within the *Gammaproteobacteria* (Yakimov *et al.*, 1998; Nguyen *et al.*, 1999; Anzai *et al.*, 2000). Moreover, all previously described members of this genus were characterized as having $C_{16:0}$, and $\omega9c$ isomers of $C_{16:1}$ and $C_{18:1}$ as the predominant fatty acids (Nguyen *et al.*, 1999; Gorshkova *et al.*, 2003) and as having DNA G+C contents ranging between 53 and 59·6 mol% (Gauthier *et al.*, 1992; Nguyen *et al.*, 1999; Yoon *et al.*, 2004; Romanenko *et al.*, 2005). In this study, we present the characterization of a novel strain affiliated to the genus *Marinobacter*.

Isolates were obtained from a wastewater-evaporation pond resulting from the decalcification of wine tanks. The purpose of this decalcification step is to remove the tartar that accumulates slowly in Inox storage tanks. Various strains were isolated on a basal medium, using the dilution-plating technique. Petri dishes were routinely incubated for 7 days at 37 °C. The medium contained the following compounds (l^{-1}): 1·0 g yeast extract, 0·6 g KH_2PO_4, 0·6 g K_2HPO_4, 60 g NaCl, 0·5 g KCl, 0·05 g NH_4Cl, 0·1 g KNO_3 and 1 ml trace-element mineral solution (Widdel & Pfennig, 1981). When required, 2 % (w/v) agar was added. The pH was adjusted to 7·2 with 10 M KOH.

After sterilization, the medium was supplemented with a solution containing 9·5 g $MgCl_2.6H_2O$ l^{-1} and 1·0 g $CaCl_2.2H_2O$ l^{-1}. The purity of the strains and their shape, size and mobility were analysed by using a photomicroscope (Eclipse E600; Nikon) with an oil-immersion objective (× 100). The 16S rRNA gene sequences of the isolates were compared by using amplified ribosomal DNA restriction analysis profiles: three of the isolates (FB1^T, FB1′ and FB2) showed similar profiles (data not shown). One of them, strain FB1^T, was selected for further characterization. For phenotypic characterization, strain FB1^T was routinely grown on either marine agar 2216 (Difco) or basal medium. Strain FB1^T was compared with other *Marinobacter* strains grown on the same media.

Standard tests (Gram staining and oxidase, catalase, amylase, caseinase, chitinase and gelatinase activities) were performed as described previously (Smibert & Krieg, 1994). Growth was monitored on basal medium at various NaCl concentrations ranging from 0 to 30 % (w/v). The temperature range for growth was tested at 5 °C intervals between 5 and 55 °C on marine agar 2216 and the pH range for growth was tested between pH 4 and 12 on basal medium.

Further biochemical analyses were performed by inoculating API 20NE, API 50 CH and Biotype100 strips (bioMérieux) according to the manufacturer's instructions, except that the cultures were resuspended in 6 % (w/v) NaCl solution.

Results obtained with the API tests were confirmed individually by testing different carbohydrates (20 mM) as sole carbon and energy sources, in aerobic cotton-capped tubes filled with 5 ml basal medium without yeast extract. The following carbohydrates were tested: D-glucose, D-fructose, sucrose, D-galactose, L- and D-arabinose, D-mannose, D-maltose, D-mannitol, D-sorbitol, D-trehalose, D-ribose, D-cellobiose, L-rhamnose, D-xylose, starch, gelatin, acetate, lactate and succinate. In addition, the resulting acid production was determined as described previously (Fardeau *et al.*, 2000). Similarly, the ability of the strain to reduce nitrate and nitrite was investigated both with the API 20NE system and in tubes. For these assays, the medium was pre-reduced and 5 ml aliquots were distributed under a nitrogen atmosphere in butyl-capped Hungate tubes. Immediately prior to inoculation (10 %, v/v) of the tubes, $NaNO_3$ (10 mM) or $NaNO_2$ (10 mM) was added. The API ZYM system (bioMérieux) was used to determine the main enzyme activities present (Humble *et al.*, 1977).

The susceptibility of strain FB1^T to antibiotics was determined on Mueller–Hinton agar (Difco no. 0252; Becton Dickinson) supplemented with 6 % (w/v) NaCl, using the standard antibiotic disc diffusion method (Bauer *et al.*, 1966). Inhibition diameters were recorded after 24 and 48 h incubation at 30 °C under aerobic conditions. The classification of the strain (susceptible, resistant or of intermediate susceptibility) was made according to the disc-manufacturer's instructions (bioMérieux), and based on the directives of the Comité de l'antibiogramme de la Société Française de Microbiologie (Cavallo *et al.*, 2004).

Fatty acid methyl esters were analysed using the standard procedure of the Microbial Identification System (Microbial ID), using bacteria grown on blood agar at 30 °C, and were compared with those in the fatty acid database.

Genomic DNA from strain FB1^T was extracted using the Wizard Genomic DNA purification kit according to the instructions of the manufacturer (Promega) and stored at −20 °C until use. The 16S rRNA gene sequence was amplified with universal primers Fd1 (5′-AGAGTTTGA-TCCTGGCTCAG-3′) and R6 (5′-TACGGCTACCTTGTT-ACG-3′) to obtain a PCR product of approximately 1·5 kb, corresponding to bases 8–1494 (based on *Escherichia coli* numbering of the 16S rRNA gene; Brosius *et al.*, 1981). The reaction mixture, prepared in a 0·2 ml test tube, was as follows: 0·5 µl (50 pmol μl^{-1}) each primer, as appropriate (Fd1, R6), 0·5 µl (10 mM) dNTPs, 5 µl 10 × *Taq* buffer, 0·5 µl *Taq* DNA polymerase (5 U μl^{-1}) (Promega), 5 µl $MgCl_2$ (25 mM) and 50 ng genomic DNA. The total volume was adjusted to 50 µl with sterile water. This amplification was performed with a T3 thermocycler (Whatman Biometra). After initial heating at 96 °C for 3 min, 30 cycles consisting of 96 °C for 20 s, 55 °C for 30 s and 72 °C for 2 min were performed. This program was completed with a final elongation step of 5 min at 72 °C.

PCR products were cloned into pGEM-T Easy plasmid (Promega), according to the manufacturer's protocol. The clone library was screened by direct PCR amplification from colonies by using the vector-specific primers SP6 (5′-ATTT-AGGTGACACTATAGAA-3′) and T7 (5′-TAATACGAC-TCACTATAGGG-3′) and the following reaction conditions: 2 min at 96 °C, 40 cycles of 30 s at 94 °C, 1 min at 55 °C and 3 min at 72 °C, with a final extension comprising 10 min at 72 °C. Plasmids containing the correct length of insert were isolated using the Wizard Plus SV Minipreps DNA purification system (Promega) according to the manufacturer's protocol.

Purified plasmids were subsequently sequenced to determine the full sequence of the 16S rRNA gene (Genome Express). The 16S rRNA gene sequence was then aligned with reference sequences from the GenBank database (Maidak *et al.*, 1996) by using the Ribosomal Database Project Sequence Aligner program; the alignment was verified manually as conforming to the 16S rRNA secondary-structure model (Winker & Woese, 1991). Pairwise evolutionary distances, based on 1409 unambiguous nucleotides, were computed by using the method of Jukes & Cantor (1969), and a dendrogram was constructed from these distances by using the neighbour-joining method (Saitou & Nei, 1987). Confidence in the dendrogram topology was determined by means of a bootstrap analysis using 100 resamplings of the sequences (Felsenstein, 1985). All of the programs used form part of the PHYLIP package (Felsenstein, 1993).

The G+C content and the level of binding of the DNA of the novel strain were determined at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany). The DNA was isolated and purified by chromatography on hydroxyapatite (Cashion *et al.*, 1977) and the G+C content was determined by HPLC of deoxyribonucleosides by using the method of Mesbah *et al.* (1989). DNA–DNA hybridization between strain FB1^T and the *M. excellens* type strain, CIP 107686^T, was carried out at the DSMZ as described by De Ley *et al.* (1970), with the modification described by Huß *et al.* (1983) and Escara & Hutton (1980), using a Gilford system model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992).

Strain FB1^T is a rod-shaped, aerobic, motile bacterium that stains Gram-negative. It is catalase-positive and weakly oxidase-positive and forms round, slightly convex colonies with regular margins. The colonies are white, opaque and have a smooth surface.

Analysis of the 16S rRNA gene sequence (1409 bases) of strain FB1^T indicated that it is a member of the *Gammaproteobacteria* (which includes, among others, the genera *Oceanospirillum*, *Marinobacter*, *Marinomonas*, *Halomonas* and *Pseudomonas*). Dendrogram analysis showed that strain FB1^T formed a coherent cluster with species of the genus *Marinobacter* (Fig. 1). Analysis of 16S rRNA gene sequences showed that strain FB1^T has a high level of sequence similarity with the type strain of *M. excellens* (97·9 %) and moderate degrees of similarity with other *Marinobacter* type strains such as *M. hydrocarbonoclasticus* ATCC 49840^T (96·6 %), *M. daepoensis* DSM 16072^T (96·5 %) and *M. litoralis* JCM 11547^T (96·4 %). The previously described *Marinobacter* species type strains (with the exception of *M. excellens*) therefore differed from the novel isolate at levels exceeding the value accepted for species delineation (Stackebrandt & Goebel, 1994).

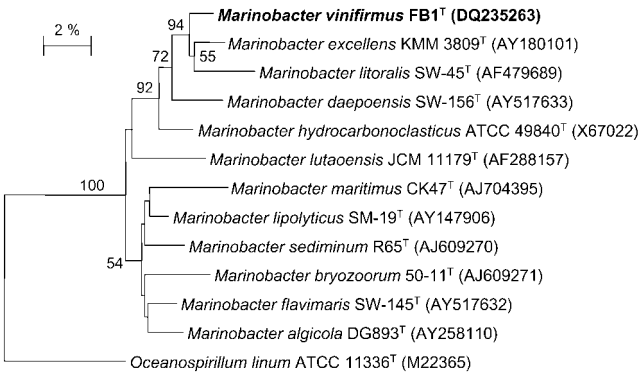

Fig. 1. Neighbour-joining phylogenetic dendrogram based on 1409 unambiguously aligned base pairs of the 16S rRNA gene sequence, showing the position of strain FB1^T. Bootstrap values (from 100 replications) greater than 50 are shown at branch points. GenBank/EMBL/DDBJ accession numbers are shown in parentheses. Bar, 2 % sequence divergence.

The relatedness between strain FB1^T and the type strain of *M. excellens* was also found in a dendrogram of distances obtained by using the neighbour-joining method. DNA–DNA hybridization was performed to elucidate the genetic relationships between strain FB1^T and the type strain of *M. excellens*: the DNA–DNA relatedness was 31·2 %. Therefore these strains are not related at the species level, since the value falls below the threshold (70 %) recommended for species definition by Wayne *et al.* (1987).

Physiological experiments were performed to compare strain FB1^T with the type strain of *M. excellens* and various bacteria of the same genus (Table 1). Strain FB1^T grew at temperatures in the range 15–45 °C, with an optimum at 20–30 °C. The optimal pH was 6·5–8·4 and no growth was observed at pH 4·5. Yeast extract was required for growth. Strain FB1^T grew in the presence of 0–20 % (w/v) NaCl and optimally at 3–6 % (w/v) NaCl. Strain FB1^T is strictly aerobic, heterotrophic and did not grow under anaerobic conditions on basal medium with acetate, citrate, succinate or glucose as carbon and energy sources. The strain has a cellular fatty acid profile containing large amounts of linear, unsaturated and hydroxy fatty acids. The major fatty acids detected in strain FB1^T (Table 2) were $C_{18:1}\omega9c$ (30·4 %), $C_{16:0}$ (25·7 %), $C_{12:0}$ 3-OH (10·3 %), $C_{16:1}\omega9c$ (9·7 %) and $C_{16:1}\omega7c$ (8·4 %). The fatty acid profile of strain FB1^T is similar in many respects to those of the type strains of previously described *Marinobacter* species, thus confirming its affiliation with the genus *Marinobacter*. However, important differences in the fatty acid profiles were detected. For example, $C_{16:1}\omega7c$, detected in strain FB1^T (8·4 %) as a major compound, was also found in *M. sediminum* DSM 15400^T (15·8 %) (Romanenko *et al.*, 2005) and *M. excellens* (6·0 %) (Gorshkova *et al.*, 2003). This observation may have resulted from differences in the experimental conditions, e.g. the cultivation conditions or analytical equipment.

Strain FB1^T has a number of distinct physiological and chemotaxonomic characteristics that distinguish it from all of the described species belonging to the genus *Marinobacter* (Table 1). Significant differences in the temperature, pH and NaCl optima and fatty acid compositions were observed. Firstly, strain FB1^T and *M. aquaeolei* share the ability to grow without NaCl. Secondly, although strain FB1^T is able to grow with 20 % NaCl, the three species closely related phylogenetically to strain FB1^T in the dendrogram are unable to grow under such conditions. Moreover, strain FB1^T is unable to use sugars (except fructose, weakly) as carbon sources. Its metabolic profiles for sugars and other metabolic compounds are similar only to those of *M. flavimaris* DSM 16070^T. *M. excellens* degrades D-maltose and D-fructose, whereas D-mannose and cellobiose are degraded only by *M. litoralis*, *M. hydrocarbonoclasticus* and *M. aquaeolei*. In addition, only strain FB1^T is unable to use L-proline. Finally, *M. excellens* and strain FB1^T differ in the use

Table 1. Phenotypic characteristics that can be used to differentiate strain FB1^T from phylogenetically related species

Strains: 1, strain FB1^T (data from the present study); 2, *M. excellens* CIP 107686^T (data from the present study and Gorshkova *et al.*, 2003); 3, *M. litoralis* JCM 11547^T (Yoon *et al.*, 2003); 4, *M. hydrocarbonoclasticus* ATCC 49840^T (Gauthier *et al.*, 1992); 5, *M. hydrocarbonoclasticus* DSM 11845 (type strain of *M. aquaeolei*) (Nguyen *et al.*, 1999; Marquez & Ventosa, 2005); 6, *M. flavimaris* DSM 16070^T (Yoon *et al.*, 2004); 7, *M. daepoensis* DSM 15400^T (Yoon *et al.*, 2004). All strains are positive for motility, oxidase, catalase, leucine arylamidase, *N*-acetyl-β-glucosidase, naphthol-AS-BI-phosphohydrolase, acid phosphatase, alkaline phosphatase, esterase, esterase lipase and the utilization of succinate and acetate. The following tests were negative for all species: cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase and the utilization of D-galactose, D-glucose, sucrose and D-xylose. +, Positive; −, negative; W, weakly positive; ND, not determined.

Characteristic	1	2	3	4	5	6	7
Cell size (μm)	0·5–0·7 × 1·0–2·0	0·6–1·4 × 1·0–8·0	0·5–0·8 × 1·5–3·0	0·3–0·6 × 2·0–3·0	0·4–0·5 × 1·4–1·6	0·6–0·9 × 1·5–3·0	0·6–0·8 × 1·5–3·0
Urease	−	+	+	−	+	−	−
Lipase	−	−	−	−	−	+	−
Valine arylamidase	+	+	−	W	+	−	−
Nitrate reduction to nitrite	−	+	+	+	+	+	−
Nitrite reduction to N_2	−	+	−	+	−	−	−
Hydrolysis of:							
Gelatin	−	−	−	−	+	−	−
Starch	−	+	−	−	ND	−	−
Tween 80	+	+	+	ND	+	+	+
Growth at/in:							
pH 5·0	−	−	+	−	+	−	−
0 % NaCl	+	−	−	−	+	−	−
20 % NaCl	+	−	−	+	+	+	−
4 °C	−	−	+	−	−	+	−
Utilization of:							
D-Fructose	W	+	−	−	−	+	−
D-Maltose	−	+	−	−	−	−	−
D-Mannose	−	−	+	+	+	−	−
D-Cellobiose	−	−	+	+	+	−	−
L-Glutamate	−	−	+	+	+	−	−
Citrate	−	−	ND	+	+	ND	ND
Lactate	+	−	ND	+	+	ND	ND
D-Mannitol	−	+	−	−	−	−	−
D-Sorbitol	−	+	−	−	−	−	−
L-Proline	−	+	ND	+	+	ND	ND
Temperature for growth (°C)							
Maximum	50	41	46	45	50	45	45
Optimum	20–30	20–25	30–37	32	30	37	30–37
DNA G+C content (mol%)	58	55–56	55	58	56	58	57

of D-mannitol, D-sorbitol and lactate and in terms of urease activity.

Lactate is the best substrate for growth of strain FB1^T. Strains FB1^T and *M. excellens* have different sensitivities to antibiotics. In addition, strain FB1^T is unable to reduce nitrate to nitrite, unlike many species of the genus *Marinobacter* (except *M. daepoensis*). The DNA–DNA relatedness value between strain FB1^T and the type strain of *M. excellens* fell far below the threshold value recommended for species delineation. Therefore, on the basis of the results obtained with regard to nutritional requirements, antibiotic susceptibilities and phylogeny, strain FB1^T should be placed in the genus *Marinobacter* within a novel species, for which we propose the name *Marinobacter vinifirmus* sp. nov.

Description of *Marinobacter vinifirmus* sp. nov.

Marinobacter vinifirmus [vi.ni.fir′mus. L. n. *vinum* wine; L. adj. *firmus* firm (as opposed to frail), stable, strong; N.L. masc. adj. *vinifirmus* wine-resistant, pertaining to the resistance to wine-barrel-decalcification wastewater, from which the type strain was isolated].

Cells are Gram-negative, weakly motile, non-spore-forming, pleomorphic rods, 0·5–2·0 μm long and 0·5–0·7 μm in diameter. Colonies are round and slightly convex with

Table 2. Cellular fatty acid compositions of strain FB1^T and some *Marinobacter* species

Strains: 1, strain FB1^T (data from the present study); 2, *M. excellens* CIP 107686^T (Gorshkova *et al.*, 2003); 3, *M. litoralis* JCM 11547^T (Yoon *et al.*, 2003); 4, *M. hydrocarbonoclasticus* ATCC 49840^T (Gauthier *et al.*, 1992); 5, *M. hydrocarbonoclasticus* DSM 11845 (type strain of *M. aquaeolei*) (Marquez & Ventosa, 2005); 6, *M. flavimaris* DSM 16070^T (Yoon *et al.*, 2004); 7, *M. daepoensis* DSM 16072^T (Yoon *et al.*, 2004). Values shown are percentages of total fatty acids. Differences in fatty acid composition may be the result of differences in experimental conditions. ND, Not detected.

Fatty acid	1	2	3	4	5	6	7
Saturated fatty acids							
$C_{10:0}$	1·0	ND	3·2	1·2	0·9	0·5	0·9
$C_{12:0}$	8·4	4·5	5·5	5·1	4·7	9·1	7·1
$C_{12:0}$ 3-OH	10·3	ND	9·6	8·4	7·6	10·5	9·4
$C_{14:0}$	1·7	2·3	3·0	3·1	2·8	1·1	1·9
$C_{15:0}$	0·3	0·5	0·7	1·0	1·4	0·7	1·1
$C_{16:0}$	25·7	26·0	25·0	25·3	29·9	26·7	24·8
$C_{17:0}$	0·6	3·5	2·5	1·9	3·6	3·7	2·6
$C_{18:0}$	1·9	5·4	2·9	2·0	3·2	3·3	3·1
Unsaturated fatty acids							
$C_{16:1}\omega5c$	0·4	ND	ND	0·5	ND	ND	ND
$C_{16:1}\omega7c$	8·0	6·0	2·6	5·1	ND	ND	ND
$C_{16:1}\omega9c$	9·7	11·3	10·6	7·6	8·0	10·2	12·8
$C_{17:1}\omega8c$	0·5	1·3	0·9	2·2	2·8	3·8	1·8
$C_{18:1}\omega9c$	30·4	36·7	30·3	32·5	26·3	17·4	24·3

regular margins, 1·0–3·0 mm in diameter after incubation for 2 days on marine agar 2216 and are white, opaque and have a smooth surface. They produce a homogeneous suspension. The optimum growth temperature is 25 °C. No growth is detected at 55 °C. The optimum pH for growth is 6·5. Optimal growth occurs in the presence of 6 % (w/v) NaCl and thus the strain could be classified as a moderately halophilic bacterium according to Kushner & Kamekura (1988). Anaerobic growth does not occur in the absence or presence of nitrate as the final electron acceptor. Catalase-positive and weakly oxidase-positive. Tween 80, chitin and aesculin are hydrolysed, whereas casein, gelatin, starch and agar are not. Nitrate is not reduced to nitrite. Acid is produced (weakly) only from fructose. Acid is not produced from the following sugars: D-glucose, sucrose, D-galactose, L- and D-arabinose, D-mannose, D-maltose, D-mannitol, D-sorbitol, D-trehalose, D-ribose, D-cellobiose, L-rhamnose and D-xylose. Acetate, lactate and succinate, but not citrate, are utilized as sole carbon and energy sources. Although phenolic compounds inhibit many micro-organisms, strain FB1^T displays a high degree of tolerance to a few of these substances. As an example, growth occurs with high levels (up to 10 mM) of phenol as the sole carbon source. Strain FB1^T is susceptible to ampicillin (10 μg), chloramphenicol (30 μg), polymyxin (25 μg) and penicillin (10 μg) but resistant to tetracycline (30 μg), kanamycin (30 μg), neomycin (30 μg) and streptomycin (10 μg). The following enzymes are present with the API ZYM system: esterase, esterase lipase, leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, *N*-acetyl-β-glucosamidase and acid phosphatase. Negative for urease, lipase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, α-glucosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase activities. On the basis of the 16S rRNA gene sequence, the species belongs to the genus *Marinobacter* within the *Gammaproteobacteria*. The DNA G+C content is 58·7 mol%.

The type strain, FB1^T (=DSM 17747^T=CCUG 52119^T), was isolated from wine-barrel-decalcification wastewater.

Acknowledgements

We gratefully acknowledge Marie-Laure Fardeau and Jean-Luc Cayol for useful discussions and Malick Mbengue for technical assistance.

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