Melitea salexigens gen. nov., sp. nov., a gammaproteobacterium from the Mediterranean Sea

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A novel aerobic, Gram-negative bacterial strain, designated 5IX/A01/131^T, was isolated from waters in the coastal north-western Mediterranean Sea. The cells were motile, straight rods, 1.6 μm long and 0.5 μm wide, and formed cream colonies on marine 2216 agar. The G+C content of the genomic DNA was 57 mol%. Phylogenetic analysis of the 16S rRNA gene sequence placed the strain in the class *Gammaproteobacteria*. On the basis of the 16S rRNA gene sequence comparisons and physiological and biochemical characteristics, strain 5IX/A01/131^T represents a novel genus and species, for which the name *Melitea salexigens* gen. nov., sp. nov. is proposed. The type strain of *Melitea salexigens* is 5IX/A01/131^T (=DSM 19753^T =CIP 109757^T =MOLA 225^T).

The genus *Microbulbifer* was originally proposed by Gonzales e*t al.* (1997) and at the time of writing includes five species: *Microbulbifer hydrolyticus* (Gonzalez *et al.*, 1997), *M. salipaludis* (Yoon *et al.*, 2003a), *M. maritimus* (Yoon *et al.*, 2004), *M. elongatus* (Humm, 1946; Palleroni, 1984; Yoon *et al.*, 2003b) and *M. celer* (Yoon *et al.*, 2007). Chemical markers for this genus include the presence of iso-15 : 0 and iso-17 : 1ω9*c* as major fatty acids and Q-8 as the major ubiquinone (Yoon *et al.*, 2004). *Microbulbifer* species have been isolated from various marine environments, including salt marshes, intertidal sediments and coastal waters. As part of a larger survey of the diversity of the seawater surface microlayer (Agogué *et al.*, 2004), several novel marine bacteria were discovered and described, including *Haliea salexigens* 3X/A02/235^T (Urios *et al.*, 2008) and an uncharacterized strain, designated 5IX/A01/131. Both of these strains are related to the genus *Microbulbifer* but, on the basis of 16S rRNA gene sequences and fatty acid compositions, are distinct taxonomically. The taxonomy of strain 5IX/A01/131 is defined in this study, leading to the proposal of a novel marine genus and species.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 5IX/A01/131^T is AY576729.

An electron micrograph of a negatively stained cell of 5IX/A01/131^T and graphs showing the effects of pH, salinity and temperature on growth are available as supplementary material with the online version of this paper.

Samples of seawater from the surface microlayer were collected in September 2001 from the bay of Barcelona (41° 24′ N 2° 16′ E) by submerging a metal screen (Agogué *et al.*, 2004). Subsamples were spread on MA plates and incubated at 25 °C for 2 weeks. Colonies were picked and purified after three subcultures. An isolate that formed cream-coloured colonies was obtained from among these colonies and was designated strain 5IX/A01/131^T (Agogué *et al.*, 2005).

Microscopic observations (AX70; Olympus) showed that cells from isolate 5IX/A01/131^T were motile rods, approximately 1.8 ± 0.2 μm long and 0.7 ± 0.1 μm wide. Cells were negatively stained for transmission electron microscopy (Raguénès *et al.*, 1997). Single polar flagella were observed (Supplementary Fig. S1, available in IJSEM Online) and the Ryu KOH reaction (Powers, 1995) led to immediate cell lysis that was confirmed by microscopy. This positive reaction indicated that the strain was Gram-negative.

The isolate was grown in marine broth 2216 medium (MB; Difco). To determine the range of salinities tolerated by strain 5IX/A01/131^T, MB was prepared according to the composition provided by the manufacturer, with the appropriate NaCl concentration. To test the pH range for growth, MES, PIPES, AMPSO or MOPS (Sigma) was added to MB to achieve the appropriate pH. Cultures were incubated at 30 °C under aerobic conditions. The methods

Table 1. Characteristics that serve to distinguish strain 5IX/A01/131^T from the type strains of related species

Strains: 1, *M. maritimus* JCM 12187^T (data from Yoon *et al.*, 2004); 2, *M. hydrolyticus* DSM 11525^T (Gonzalez *et al.*, 1997); 3, *M. salipaludis* KCCM 41586^T (Yoon *et al.*, 2003a); 4, *M. elongatus* DSM 6810^T (Yoon *et al.*, 2003b); 5, *M. celer* KCTC 12973^T (Yoon *et al.*, 2007); 6, *H. salexigens* OOB 286^T (Urios *et al.*, 2008); 7, strain 5IX/A01/131^T. All strains were positive for the following characteristics: alkaline phosphatase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. All strains possessed Q-8 as the major quinone. +, Positive; −, negative; (+), weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6	7
Sampling environment	Marine sediment	Salt marsh	Salt marsh	Sand and seawater	Marine saltern	Seawater	Seawater
DNA G+C content (mol%)	59.9	57.7	59	58.2	57.7	61.4	57.2
Motility	−	−	−	+	−	+	+
Temperature for growth (°C)							
Range	15–48	10–41	10–45	25–30	10–48	10–37	15–37
Optimum	37	37	37	25–30	37	25	30
Salinity for growth (g NaCl l^{-1})							
Range	2–10	6–60	2–10	ND	ND	7–70	7–70
Optimum	2–4	6–30	2–3	2–3	ND	42	28
pH for growth							
Range	5–7.5	6.5–8.5	5–8	ND	5–8	5–9	6–10
Optimum	6.5–7.5	7.5	7–8	ND	7–8	8	8
API ZYM tests							
Valine arylamidase	−	−	−	−	−	+	−
N-Acetyl-β-glucosaminidase	−	−	+	+	+	−	(+)
Esterase (C4)	+	+	+	+	+	−	−
Lipase (C8)	+	+	+	+	+	(+)	(+)
Substrates							
Arabinose	−	+	−	+	+	−	−
Cellobiose	+	+	+	+	−	−	(+)
Fructose	−	−	−	+	−	−	+
Galactose	−	−	−	+	−	−	−
Glucose	+	+	+	+	−	−	+
Lactose	−	−	−	+	+	−	(+)
Maltose	+	+	+	+	−	−	+
Mannose	−	−	−	+	−	(+)	+
Rhamnose	−	−	+	−	−	−	−
Raffinose	−	ND	−	−	−	−	+
Sucrose	−	−	−	+	−	−	+
Glycerol	ND	−	ND	−	ND	+	−
Acetate	+	+	ND	+	+	−	(+)
Aspartate	ND	−	ND	(+)	ND	+	−
Glutamate	−	+	ND	+	−	(+)	(+)
β-Hydroxybutyrate	ND	+	ND	ND	ND	+	−
Lactate	−	ND	ND	+	ND	−	−
Propionate	ND	+	ND	+	ND	−	−
Pyruvate	+	+	ND	ND	+	+	−
Succinate	+	+	ND	+	−	+	−
Alanine	ND	+	ND	(+)	ND	−	(+)
Leucine	ND	+	ND	+	ND	+	−
Proline	ND	+	ND	(+)	ND	+	−
Serine	+	+	ND	−	ND	−	−
Tween 80	ND	−	+	ND	+	+	−

used for the determination of growth parameters were as reported by Wery *et al.* (2001b). Growth was observed at 15–37 °C, with a clear optimum at 30 °C (Supplementary Fig. S2). The strain grew at NaCl concentrations ranging from 7 to 70 g l^{-1} and an optimum concentration could be defined as 42 g l^{-1} (Supplementary Fig. S2). Growth occurred at pH 6.0–10.0, with an optimum at pH 8.0. Growth decreased by 49 % at pH 6.0 in comparison with the value obtained at pH 7.0. Growth rates were the same at pH 7.0 and pH 9.0; a small difference (14 %) was observed between pH 9.0 and 10.0 (Supplementary Fig. S2). To investigate the possibility of growth under anaerobic conditions, cultures were placed in

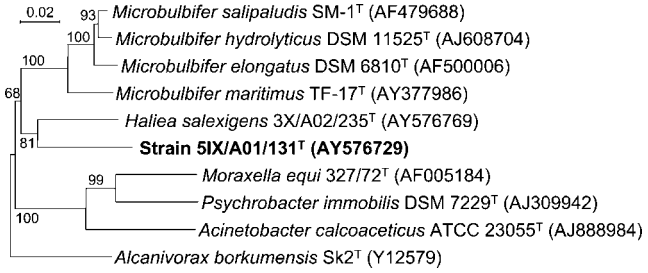

Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain 5IX/A01/131^T. The tree corresponds to an unrooted tree obtained using the neighbour-joining algorithm (with Kimura corrections). Accession numbers and type strains are indicated. Bootstrap percentages (based on 1000 resamplings) are shown at branch points. Bar, 0.02 substitutions per nucleotide position.

an anaerobic jar on MA plates at optimal conditions of temperature, pH and salinity (Supplementary Fig. S2). No growth was observed after 10 days and thus the strain should be considered as strictly aerobic.

The ability of isolate 5IX/A01/131^T to use various substrates was investigated using Biolog GN2 MicroPlates (Tang *et al.*, 1998) according to the manufacturer's instructions. Positive reactions are listed in the species description. A comparison between strain 5IX/A01/131^T and its closest relatives is presented in Table 1.

Enzyme activities were investigated using the API ZYM system (bioMérieux) according to the manufacturer's instructions. Positive reactions were obtained for alkaline phosphatase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Weakly positive signals were obtained for esterase (C8) and *N*-acetylglucosaminidase (Table 1).

Analysis of the fatty acid methyl esters was performed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). To allow comparisons between the fatty acid composition of strain 5IX/A01/131^T and those of its relatives, MA was used. Biomass was harvested from MA plates after cultivation at optimal conditions (Supplementary Fig. S2). The fatty acid composition (%) for isolate 5IX/A01/131^T was as follows: 17 : 1ω8*c* (34.1), 17 : 0 (13.0), 18 : 1ω7*c* (11.4), 16 : 1ω7*c*/iso-15 : 0 2-OH (9.0), 15 : 0 (5.3), 11 : 0 3-OH (4.6), iso-11 : 0 3-OH (4.3), 16 : 0 (3.9), iso-15 : 0 (2.0), 9 : 0 (1.9), 10 : 0 3-OH (1.7), iso-17 : 0 (1.3), iso-13 : 0 (1.0), iso-11 : 0 (0.9), 14 : 0 (0.8), 11 : 0 (0.7), 17 : 1ω6*c* (0.6), 18 : 0 (0.5), 12 : 1 3-OH (0.5), 13 : 0 (0.4), 12 : 0 (0.3) and 9 : 0 3-OH (0.3). Fatty acids 9 : 0, iso-13 : 0, 9 : 0 3-OH and 12 : 1 3-OH were only detected in strain 5IX/A01/131^T. Fatty acids 11 : 0, 12 : 0, 13 : 0, 17 : 1ω6*c* and 11 : 0 3-OH were found in strains 5IX/A01/131^T and *H. salexigens* DSM 19537^T but not in *Microbulbifer* species. Fatty acids iso-11 : 0, iso-15 : 0 and iso-17 : 0 were found in strain 5IX/A01/131^T and in *Microbulbifer* species but not in *H. salexigens* DSM 19537^T. The major fatty acid found in all *Microbulbifer* strains, iso-15 : 0, was 10-fold less abundant in strain 5IX/A01/131^T and iso-17 : 1ω9*c*, one of the other major fatty acids of *Microbulbifer* strains, was not detected in the novel strain. Fatty acids 12 : 0 3-OH and 15 : 1ω6*c*, one of the most abundant fatty acids in *H. salexigens* DSM 19537^T, were not found in strain 5IX/A01/131^T.

Analyses of respiratory quinones and polar lipids were carried out by the Identification Service of the DSMZ.

Table 2. Cellular fatty acid compositions (%) of strain 5IX/A01/131^T and related type strains in the genus *Microbulbifer*

Strains: 1, *M. maritimus* JCM 12187^T (data from Yoon *et al.*, 2004); 2, *M. hydrolyticus* DSM 11525^T (Gonzalez *et al.*, 1997); 3, *M. salipaludis* KCCM 41586^T (Yoon *et al.*, 2003a); 4, *M. elongatus* DSM 6810^T (Yoon *et al.*, 2003b); 5, *M. celer* KCTC 12973^T (Yoon *et al.*, 2007); 6, *H. salexigens* OOB 286^T (Urios *et al.*, 2008); 7, strain 5IX/A01/131^T. –, Not detected/not reported.

Fatty acid	1	2	3	4	5	6	7
9 : 0	–	–	–	–	–	–	1.9
10 : 0	1.3	1.7	2.4	1.6	1.6	–	–
11 : 0	–	–	–	–	–	1.0	0.7
12 : 0	–	–	–	–	–	1.6	0.3
13 : 0	–	–	–	–	–	1.3	0.4
14 : 0	1.0	1.2	2.6	0.7	0.9	1.3	0.8
15 : 0	1.6	1.5	1.7	0.9	1.5	4.5	5.3
16 : 0	8.7	11.4	16.3	7.1	12.6	2.0	3.9
17 : 0	1.3	2.9	2.2	2.5	2.6	9.3	13.0
18 : 0	–	1.6	1.4	1.2	0.8	–	0.5
17 : 0 cyclo	2.3	5.7	–	–	6.3	–	–
19 : 0ω8*c* cyclo	1.4	1.0	–	–	5.3	–	–
iso-11 : 0	10.0	5.7	4.8	6.5	6.7	–	0.9
iso-13 : 0	–	–	–	–	–	–	1.0
iso-15 : 0	25.9	24.4	19.4	20.7	21.7	–	2.0
iso-15 : 1	0.8	1.0	0.7	1.0	0.4	–	–
iso-16 : 0	–	–	–	0.5	0.3	–	–
iso-17 : 0	6.9	10.4	5.5	9.9	10.0	–	1.3
anteiso-17 : 0	–	–	–	0.8	0.3	–	–
iso-17 : 1ω9*c*	12.6	10.1	9.5	11.3	8.6	–	–
15 : 1ω6*c*	–	–	–	–	–	5.8	–
17 : 1ω6*c*	–	–	–	–	–	2.7	0.6
17 : 1ω8*c*	–	0.5	1.0	1.8	0.5	23.9	34.1
18 : 1ω5*c*	–	–	0.7	–	–	–	–
18 : 1ω7*c*	5.6	8.9	11.8	16.3	6.5	17.5	11.4
9 : 0 3-OH	–	–	–	–	–	–	0.3
10 : 0 3-OH	1.7	1.0	1.2	1.6	1.4	1.8	1.7
11 : 0 3-OH	–	–	–	–	–	3.3	4.6
12 : 0 3-OH	–	–	–	–	–	1.1	–
12 : 1 3-OH	–	–	–	–	–	–	0.5
16 : 0 2-OH	–	–	0.9	–	–	–	–
iso-11 : 0 3-OH	14.2	6.2	5.7	7.7	8.9	3.3	4.3
iso-17 : 0 3-OH	–	–	0.9	–	–	–	–
16 : 1ω7*c*/iso-15 : 0 2-OH	2.2	2.7	7.1	6.0	2.4	21.2	9.0

Strain 5IX/A01/131^T contained Q-8 and the major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and an undefined aminophospholipid.

Genomic DNA was extracted as described by Wery *et al.* (2001a). The G+C content was determined by means of thermal denaturation, using the method of Marmur & Doty (1962) and the conditions described by Raguénès *et al.* (1997). The G+C content of the genomic DNA of strain 5IX/A01/131^T was 57.2±0.2 mol%. The 16S rRNA gene was amplified and sequenced as described by Agogué *et al.* (2005). The sequence was analysed as described by Urios *et al.* (2006). Strain 5IX/A01/131^T was shown to be phylogenetically affiliated to the family *Alteromonadaceae* in the class *Gammaproteobacteria* (Fig. 1). The closest relative was *H. salexigens* 3X/A02/235^T, with a sequence similarity of 91 %.

Differences in several phenotypic properties, as shown in Tables 1 and 2, serve to distinguish strain 5IX/A01/131^T from its closest phylogenetic relatives. On the basis of these differences, we propose that strain 5IX/A01/131^T should be assigned to a novel species and genus. Because of the marine origin of strain 5IX/A01/131^T and its salinity requirement, the name *Melitea salexigens* gen. nov., sp. nov. is proposed.

Description of *Melitea* gen. nov.

Melitea (Me.li′te.a. N.L. fem. n. *Melitea* named after Melite, a nymph of the sea in Greek mythology, referring to the marine origin).

Cells are motile, Gram-negative rods. The major fatty acids are 17 : 1ω8*c*, 17 : 0, 18 : 1ω7*c*, 16 : 1ω7*c*, 15 : 0, 11 : 0 3-OH, iso-11 : 0 3-OH, 16 : 0, iso-15 : 0, 9 : 0, 10 : 0 3-OH, iso-17 : 0 and iso-13 : 0. The ubiquinone is Q-8 and the polar lipids are diphosphatidylglycerol and phosphatidylglycerol. Phylogenetically affiliated to the class *Gammaproteobacteria*. The type species is *Melitea salexigens*.

Description of *Melitea salexigens* sp. nov.

Melitea salexigens (sa.le′xi.gens. L. n. *sal*, *salis* salt, seawater; L. v. *exigo* to demand; N.L. part. adj. *salexigens* seawater-demanding).

Displays the following properties in addition to those described for the genus. Cream colonies are formed on MA medium. Cells are 1.8±0.2 μm long and 0.7±0.1 μm wide with single polar flagella. The DNA G+C content of the type strain is 57 mol%. Growth occurs at 15–37 °C (optimally at 30 °C), at pH 6.0–10.0 (optimally at pH 8.0) and at salinities in the range 7–70 g NaCl l^{-1} (optimally at 42 g l^{-1}). Positive reactions with Biolog GN2 plates are obtained for D-fructose, D-glucose, maltose, D-mannose, D-psicose, raffinose, sucrose, trehalose, turanose, α-ketoglutaric acid, α-ketovaleric acid, succinamic acid, L-glutamic acid and L-serine. Positive API ZYM reactions for enzyme activities are obtained for alkaline phosphatase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Oxidase- and catalase-positive.

The type strain, 5IX/A01/131^T (=DSM 19753^T =CIP 109757^T =MOLA 225^T), was isolated from seawater sampled from the surface microlayer in the bay of Barcelona (41° 24′ N 2° 16′ E).

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