Martelella mediterranea gen. nov., sp. nov., a novel α -proteobacterium isolated from a subterranean saline lake

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A bacterial strain was isolated from the water of Lake Martel in Mallorca (Spain). The isolate, designated MACL11^T, was halotolerant and strictly aerobic. The cells were non-motile, non-spore-forming, Gram-negative short rods. Comparative 16S rRNA gene sequence analysis revealed that MACL11^T represents a separate line of descent within the order 'Rhizobiales' of the class 'Alphaproteobacteria'. Strain MACL11^T was most closely related to the genera *Rhizobium* (93.3 % sequence similarity to *Rhizobium rhizogenes*), *Aurantimonas* (90.3 % sequence similarity to *Aurantimonas corallicida*) and *Fulvimarina* (90.3 % sequence similarity to *Fulvimarina pelagi*). Chemotaxonomically, strain MACL11^T was characterized by the presence of Q-10 as the major respiratory lipoquinone. The major fatty acids detected were C_{19:0} cyclo ω 8c, C_{18:1} ω 7c, C_{16:0} and 11-methyl C_{18:1} ω 7c. The G + C content of the DNA was 57.4 mol%. Oxidase and catalase activities were present. Growth with many different carbohydrates as the sole carbon source was observed. The data from this polyphasic study suggest that this bacterium belongs to a novel genus of the order 'Rhizobiales' and is not associated with any of the known families of this order. It is proposed that isolate MACL11^T should be classified in a novel genus and species, *Martelella mediterranea* gen. nov., sp. nov., with MACL11^T (=LMG 22193^T=CECT 5861^T) as the type strain.

In recent years, increasing interest in micro-organisms from saline environments has led to the discovery of novel species and genera belonging to the order 'Rhizobiales', such as *Roseibium* (Suzuki *et al.*, 2000), *Aurantimonas* (Denner *et al.*, 2003) and *Fulvimarina* (Cho & Giovannoni, 2003). They were all retrieved from marine environments; none of them was isolated from saline lakes. Here, we describe the characterization of a strain belonging to a novel genus of the order 'Rhizobiales' that was isolated from water collected at Lake Martel, a subterranean saline lake in Mallorca, Spain. Lake Martel is 177 m long, approximately 30 m wide and approximately 5–12 m deep and contains semi-salt water. The temperature of the water remains constant at 18 °C and its pH is about 7.5.

Strain MACL11^T was isolated under aseptic conditions from water samples taken from Lake Martel at a depth of 10 cm. A 200 ml sample was filtered under vacuum in

sterile conditions through a membrane filter (Millipore) with a pore diameter of 45 μ m. The membrane was placed on a plate containing YED medium (0.5 % yeast extract, 0.7 % glucose, 2 % agar) supplemented with 5 % (w/v) NaCl and was incubated at 28 °C. The colony morphology was examined in cultures grown on YED medium supplemented with 5 % (w/v) NaCl.

Strain MACL11^T was grown on YED medium (0.5 % yeast extract, 0.7 % glucose, 1.5 % agar) for 48 h to check for motility by phase-contrast microscopy (Axioskop 2; Zeiss). Gram staining was carried out using the procedure described by Doetsch (1981). Cells were gently suspended in sterile distilled water, stained with 0.2 % uranyl acetate and examined at 80 kV using a Zeiss EM 209 transmission electron microscope (Peix *et al.*, 2003).

Cells of strain MACL11^T were Gram-negative, non-motile short rods (1.0–1.1 $\times$ 1.3–1.5 μ m) (Fig. 1). Colonies were white- to cream-coloured on YED medium. They were smooth and mostly flat. Morphologically, strain MACL11^T was different from related genera (Table 1).

Physiological and biochemical tests were performed using

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The GenBank/EMBL/DDBJ accession number for the almost-complete 16S rRNA gene sequence of strain MACL11^T is AY649762.

Fig. 1. Electron micrograph of strain MACL11^T, grown on nutrient agar (Difco/Becton Dickinson) (48 h, 22 °C), showing coccobacillary morphology. Bar, 0.57 μm.

the API 20NE, API 20E and API ZYM strips (bioMérieux), according to the manufacturer's instructions and were incubated at 28 °C (testing must be performed in conventional format and for at least 72 h). Tests of enzyme activity

for caseinase, catalase and oxidase were performed as described previously (Rivas *et al.*, 2003). The temperature range for growth was determined by incubating cultures in YED medium between 4 and 45 °C. The pH range was determined in YED medium with a final pH between 4.0 and 10.0. Salt tolerance was studied in YED medium containing 0–20 % (w/v) NaCl.

Differentiating phenotypic characteristics of strain MACL11^T with respect to closely related genera are shown in Table 1. According to these data, this strain differs from members of the genus *Rhizobium* as regards growth at 40 °C, hydrolysis of aesculin and growth with 5 % NaCl, D-arabinose, citrate, D-mannose, D-sorbitol and sucrose. Strain MACL11^T is different from members of the genus *Aurantimonas* in terms of growth without NaCl, hydrolysis of aesculin and growth with D-arabinose, citrate, mannitol, D-mannose, D-sorbitol and sucrose. Finally, isolate MACL11^T differs from the genus *Fulvimarina* in terms of growth at 40 °C, hydrolysis of aesculin and growth with 10 % NaCl, D-arabinose, D-mannose and sucrose. Strain MACL11^T grew optimally at 28 °C (growth range 4–37 °C; no growth at 40 °C) and pH 7.0 (pH range 5–8.5). Strain MACL11^T grew optimally at NaCl concentrations

Table 1. Characteristics that differentiate the genus *Marteella* gen. nov. from related genera

Genera: 1, *Marteella* gen. nov.; 2, *Rhizobium*; 3, *Aurantimonas*; 4, *Fulvimarina*. Data are from Tighe *et al.* (2000), Young *et al.* (2001), Cho & Giovannoni (2003), Denner *et al.* (2003) and this study. Symbols: +, positive; –, negative; V, variable; ND, not detected.

Characteristic	1	2	3	4
Morphology	Short rods	Rods	Rods	Short rods
Division type	Binary fission	Binary fission	Branching division	Binary fission
Motility	–	+	+	–
Growth without NaCl	+	+	–	+
Growth at/in:				
40 °C	–	V	–	+
5 % NaCl	+	–	ND	+
10 % NaCl	–	–	ND	+
Hydrolysis of aesculin	+	V	–	–
Production of pigments	–	–	+	+
Utilization of carbohydrates:				
D-Arabinose	–	+	–	+
Citrate	–	+	–	ND
Mannitol	+	+	–	+
D-Mannose	–	+	+	+
D-Sorbitol	–	+	–	–
Sucrose	–	V	–	+
Predominant cellular fatty acids	C _{19:0} cycloω8c (41.4 %), C _{18:1} ω7c (21.6 %), C _{16:0} (12 %), 11-methyl C _{18:1} ω7c (8.8 %)	Summed feature 7* (24–68 %), C _{19:0} cycloω8c (3–50 %)	C _{18:1} ω7c (76.9 %), C _{19:0} cycloω8c (10.5 %)	C _{18:1} ω7c (82.9 %), C _{18:0} (8.2 %)
Other minor fatty acids	C _{18:0} , 10-methyl C _{19:0} , C _{20:2} ω6,9c, C _{18:0} 3-OH, C _{16:0} 3-OH	C _{16:0} , C _{16:0} 3-OH, C _{17:0} , C _{18:0} , C _{20:3} ω6,9,12c	C _{16:0} , C _{16:1} ω7c, C _{18:0} , C _{17:0} , C _{20:1} ω7c, C _{18:1} 2-OH	C _{16:0} , C _{16:1} ω7c, C _{18:1} 2-OH, C _{18:0} 3-OH, C _{19:0} ω8c, C _{20:1} ω7c
DNA G+C content (mol%)	57.4	57–66	66.3	57.6–59.9

*Summed feature 7 comprises 18:1ω7c/ω9t/ω12t and/or 18:1ω7c/ω9c/ω12t.

of up to 2% and it also grew in the presence of NaCl concentrations up to 5% (w/v), although salt was not essential for growth.

Determinations of lipoquinones and cellular fatty acid composition were performed as described by Zimmermann *et al.* (1998).

Ubiquinones were the only respiratory lipoquinones detected: Q-10 predominated (98%), although Q-9 was present in minor amounts (2%). This quinone profile is characteristic of most species within the class '*Alpha-proteobacteria*' (Collins & Jones, 1981; Yokota *et al.*, 1992; Busse *et al.*, 1999).

The cellular fatty acid profile of MACL11^T was characterized by 15 different fatty acids. The major fatty acids detected in strain MACL11^T were C_{19:0} cyclo ω 8c (41.44%), C_{18:1} ω 7c (21.66%), C_{16:0} (12.04%) and 11-methyl C_{18:1} ω 7c (8.84%). Additional fatty acids detected included summed feature 2 (7.68%; comprising C_{14:0} 3-OH, iso-C_{16:1} I, an unidentified fatty acid with an equivalent chain-length of 10.928 and/or C_{12:0} ALDE), C_{18:0} (4.29%), 10-methyl C_{19:0} (1.08%), C_{20:2} ω 6,9c (0.70%), C_{18:0} 3-OH (0.68%), summed feature 3 (0.63%; comprising C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH), C_{16:0} 3-OH (0.52%) and an unidentified fatty acid with an equivalent chain-length of 14.959 (0.44%). According to published data, significant amounts of a C_{19:0} cyclo ω 8c fatty acid are typical of members of the order '*Rhizobiales*' (Wilkinson, 1988; Moreno *et al.*, 1990; Jarvis *et al.*, 1996; Dunfield *et al.*, 1999; Kämpfer *et al.*, 1999; Tighe *et al.*, 2000). The fatty acid C_{20:3} ω 6,9,12c, which is detected in all species of the genus *Rhizobium*, is not present in strain MACL11^T. Another difference between strain MACL11^T and members of the genus *Rhizobium* is the presence of 11-methyl C_{18:1} ω 7c in the former. The fatty acids 11-methyl C_{18:1} ω 7c and 10-methyl C_{19:0} detected in strain MACL11^T are not present in members of the genera *Aurantimonas* and *Fulvimarina*. Moreover, strain MACL11^T was clearly differentiated from members of the genera *Rhizobium*, *Aurantimonas* and *Fulvimarina* in terms of the proportions of several fatty acids (Tighe *et al.*, 2000; Cho & Giovannoni, 2003; Denner *et al.*, 2003).

DNA for G+C content determination was prepared according to the method of Chun & Goodfellow (1995). The G+C content of the DNA (mol%) was determined using the thermal denaturation method (Mandel & Marmur, 1968): the value for strain MACL11^T was found to be 57.4 mol%. This value is similar to those obtained for members of the genera *Fulvimarina* and *Rhizobium*.

For 16S rRNA gene sequencing, DNA extraction was carried out as described previously (Rivas *et al.*, 2001). 16S rRNA gene amplification and sequencing were performed according to the methods described previously (Rivas *et al.*, 2003). An almost-complete 16S rRNA gene sequence was obtained and then compared with those deposited in the GenBank

database. Sequences were aligned using CLUSTAL X software (Thompson *et al.*, 1997). Distances were calculated according to the methods of Jukes & Cantor (1969), Kimura (1980), Tamura & Nei (1984) and Tajima & Nei (1993). Phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987), minimum evolution (Rzhetsky & Nei, 1993) and parsimony analysis (Felsenstein, 1983). Bootstrap analysis was based on 1000 resamplings. The MEGA2 package (Kumar *et al.*, 2001) was used for all analyses.

On the basis of the 16S rRNA gene sequence analysis, the organism under study belongs to the order '*Rhizobiales*' of the class '*Alphaproteobacteria*'. A comparison against the sequences held in GenBank indicated that the strain was most closely related to the genera *Rhizobium* (93.3% similarity to *Rhizobium rhizogenes* IFO 13257^T), *Aurantimonas* (90.4% similarity to *Aurantimonas coralicida* DSM 14790^T) and *Fulvimarina* (90.3% similarity to *Fulvimarina pelagi* HTCC2506^T). Fig. 2 shows the phylogenetic placement of strain MACL11^T within the order '*Rhizobiales*'. The same results were obtained when the phylogenetic distances were calculated using the Jukes–Cantor one-parameter, the Kimura two-parameter, the Tamura–Nei three-parameter and the Tajima–Nei four-parameter methods. The three methods for obtaining phylogenetic trees (using the four above-mentioned methods with each to calculate phylogenetic distances), i.e. neighbour-joining, minimum evolution and parsimony analyses, also afforded the same results (data not shown). As shown in the phylogenetic tree (Fig. 2), strain MACL11^T formed a separate branch within the order '*Rhizobiales*' that was not significantly associated with any of the known families of this order; this relationship was supported by a high bootstrap value (98%). Thus, phylogenetically, this novel genus probably belongs to a novel family within the order '*Rhizobiales*'.

Overall, the results of the present study, i.e. the low similarity value for the 16S rRNA sequence together with differences found in chemotaxonomic, morphological and physiological analyses, indicate that isolate MACL11^T should be classified within a novel genus as a novel species, for which we propose the name *Martellella mediterranea* gen. nov., sp. nov.

Description of *Martellella* gen. nov.

Martellella (Mar.tel'ell.a. N.L. fem. dim. n. *Martellella* in honour of the French explorer E. Martel, who, in 1896, discovered Lake Martel inside the caves of Drach in Mallorca, the site where this micro-organism was isolated).

Cells are Gram-negative, non-spore-forming short rods. Strictly aerobic. Oxidase- and catalase-positive. Phylogenetically related to members of the order '*Rhizobiales*'. The only respiratory lipoquinones present are ubiquinones, Q-10 predominating. The most abundant fatty acids are C_{19:0} cyclo ω 8c, C_{18:1} ω 7c, C_{16:0} and 11-methyl C_{18:1} ω 7c. The other minor fatty acids are C_{18:0}, 10-methyl C_{19:0},

Fig. 2. Neighbour-joining tree based on nearly-complete 16S rRNA gene sequences of *Martelella mediterranea* MACL11^T and other related organisms of the order 'Rhizobiales'. The significance of each branch is indicated by a bootstrap percentage calculated for 1000 subsets. Bar, 1 substitution per 100 nt.

C_{20:2}ω_{6,9}C, C_{18:0} 3-OH and C_{16:0} 3-OH. The DNA G+C content of the type species is 57.4 mol%. The type species of the genus is *Martelella mediterranea*.

Description of *Martelella mediterranea* sp. nov.

Martelella mediterranea [me.di.ter.ra.ne'a. L. fem. adj. *mediterranea* inland (used to refer to the Mediterranean Sea), referring to the fact that the type strain was isolated from a Mediterranean island].

Displays the following properties in addition to those given in the genus description. Cells are non-motile coccobacillary rods, 1.0–1.1 × 1.3–1.5 µm in size. Colonies on YED medium supplemented with 5% (w/v) NaCl are circular, smooth, opaque, white- to cream-coloured pigmented colonies that are usually 1–3 mm in diameter within 5 days at 28 °C. Grows in the presence of NaCl concentrations up to 5% (w/v), although salt is not essential for growth. The temperature range for growth is 4–37 °C (optimal growth occurs at 28 °C) and the pH range for growth is 5–8.5 (optimal growth occurs at pH 7). Urease-positive. Indole is not produced. Aesculin is hydrolysed. Positive in the Voges–Proskauer reaction and for nitrate reduction. The type strain utilizes gluconate, glucose, malate, maltose and mannitol as sole carbon sources, and does not grow in adipate, amygdalin, arabinose, caproate, citrate, inositol, mannose, melibiose, *N*-acetylglucosamine, phenylacetate, rhamnose, sorbitol or sucrose. The type strain actively produces β-galactosidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, α-galactosidase, β-glucuronidase, α-gluconidase, β-gluconidase and *N*-acetyl-β-glucosaminidase. It does not produce gelatinase, caseinase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-mannosidase, α-fucosidase or H₂S.

The type strain is MACL11^T (=LMG 22193^T=CECT

5861^T), isolated from a water sample from a subterranean saline lake on Mallorca, Spain.

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