Photobacterium halotolerans sp. nov., isolated from Lake Martel in Spain

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A halotolerant bacterium was isolated from a saline lake located in Mallorca, Spain. Cells of the strain, designated MACL01^T, were Gram-negative, rod-shaped and motile by means of polar flagella. Colonies of strain MACL01^T were white to cream in TSA medium, turning brown after 7 days of incubation; they were blue in thiosulphate/citrate/bile salts/sucrose agar medium. A neighbour-joining phylogenetic analysis based on 16S rRNA gene sequences showed that strain MACL01^T belongs to the genus *Photobacterium*, in which it forms a distinct lineage together with *Photobacterium rosenbergii* and *Photobacterium ganghwense* (showing 96.9 and 96.2 % similarity, respectively). The most closely related taxon according to phylogenetic analysis of the *rpoA* gene is also *P. rosenbergii* (90 % similarity). The *recA* gene also showed low similarity (83.7, 83.4 and 82.4 %, respectively) with respect to those of *Vibrio proteolyticus* LMG 3772^T, *Photobacterium leiognathii* LMG 4228^T and *P. rosenbergii* LMG 2223^T. Neighbour-joining phylogenetic analysis of the *rpoA* and *recA* genes confirms that strain MACL01^T belongs to the genus *Photobacterium*, forming a branch together with *P. rosenbergii*. Strain MACL01^T was able to grow in 0–8 % NaCl. Growth occurred between 4 and 37 °C (optimum, 28 °C) and at pH 5–8.5. Luminescence was negative on marine agar. Strain MACL01^T was found to be sensitive to the vibriostatic agent O/129. It reduced nitrate to nitrite, produced β -galactosidase and hydrolysed gelatin, but did not produce arginine dihydrolase, indole or acetoin. Strain MACL01^T used several carbohydrates and fermented glucose, L-arabinose and sucrose. The most abundant fatty acids were summed feature 3 (32.6 %; comprising C_{16:1} ω 7c and/or C_{15:0} iso 2-OH), C_{16:0} (21.2 %) and C_{18:1} ω 7c (19.9 %). The G + C content of the genomic DNA was 49.8 mol%. On the basis of genotypic, phenotypic, chemotaxonomic and phylogenetic results, strain MACL01^T (= LMG 22194^T = CECT 5860^T) should be classified as the type strain of a novel species of the genus *Photobacterium*, for which the name *Photobacterium halotolerans* sp. nov. is proposed.

There are currently 12 accepted *Photobacterium* species with validly published names, and these were isolated from aquatic and marine environments. The genus belongs to the family *Vibrionaceae* together with other genera that also contain species isolated from these habitats. In this study, we report the characterization of a novel isolate from the genus *Photobacterium* that was isolated from water collected from Lake Martel, a subterranean saline lake in Mallorca, Spain.

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The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain MACL01^T is AY551089.

An electron micrograph, neighbour-joining trees based on partial *recA* and *rpoA* gene sequences and a table of the differential phenotypic characteristics of strain MACL01^T are available as supplementary material in IJSEM Online.

Lake Martel was formed by filtration from the Mediterranean Sea through underground rocks and is one of the largest subterranean lakes in the world, being approximately 177 m in length; the width is approximately 30 m and the depth is 5–12 m. The water of Lake Martel contains about 0.4 % NaCl and maintains a constant temperature of about 18 °C throughout the year. The bacterial diversity of this ecosystem remains unknown, although a species from a novel genus, *Marteella mediterranea*, was recently isolated from this environment (Rivas *et al.*, 2005).

Strain MACL01^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 (yeast extract, 0.5 %; glucose, 0.7 %; agar, 2 %) supplemented with 1.5 % (w/v) NaCl and

was incubated at 28 °C. Colony morphology was examined in cultures grown on TSA medium (BD Difco) supplemented with 1.5 % (w/v) NaCl. To test the ability to grow in TCBS, strain MACL01^T was tested for growth on thiosulphate/citrate/bile salts/sucrose agar (TCBS; BD Difco) plates by incubating them 48 h at 28 °C.

Isolate MACL01^T was observed under a phase-contrast microscope after 48 h growth in YED medium at 22 °C to check for cell shape and motility. The cells were also stained according to the classic Gram procedure described by Doetsch (1981). For electron microscopy, cells were grown on nutrient agar for 2 days at 22 °C. Cells were gently suspended in sterile water and then stained with 0.2 % uranyl acetate and examined at 80 kV with a Zeiss EM 209 transmission electron microscope (Peix *et al.*, 2003).

Extraction and amplification of genomic DNA for 16S rRNA sequence analysis were carried out as described previously (Rivas *et al.*, 2003) and the *recA* and *rpoA* genes were amplified and sequenced as described by Thompson *et al.* (2005a, b). The sequences of these genes were compared against the sequences available from GenBank using the BLASTN programme (Altschul *et al.*, 1990) and were aligned using CLUSTAL X software (Thompson *et al.*, 1997). Distances were calculated according to Kimura's method (Kimura, 1980). Phylogenetic trees were inferred using the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis was based on 1000 resamplings. The MEGA2 package (Kumar *et al.*, 2001) was used for all analyses.

DNA for the determination of G + C content was prepared according to Chun & Goodfellow (1995); the value was obtained by using the thermal denaturation method (Mandel & Marmur, 1968).

For fatty acids analyses, strain MACL01^T was cultivated for 24 h at 28 °C in TSB (BD Difco) amended with 1.5 % agar. The cellular fatty acids of strain MACL01^T were analysed as methyl esters by GLC analysis at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) according to the instructions of the Microbial Identification System (MIDI). Physiological and biochemical tests were performed using various API strips (bioMérieux). The API 20NE strip (Seo *et al.*, 2005) was inoculated according to the manufacturer's instructions, without salt addition, with a suspension of strain MACL01^T in sterile water containing 1.5 % NaCl. For the inoculation of API ZYM strips (Thompson *et al.*, 2005b), suspensions of strain MACL01^T incubated for 48 h in TSA plus 1.5 % NaCl were obtained according to the manufacturer's instructions. Finally, for the inoculation of the API 50CH strip, a suspension in AUX medium plus 1.5 % NaCl was used. The API 20NE, API 20E and API 50CH strips were incubated for 48 and 72 h: the results were identical for both of these incubation times. 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 and 10. Salt tolerance was studied in YED medium

containing 0–12 % (w/v) NaCl. Luminescence was observed in the dark on MA (BD Difco), in agreement with Macián *et al.* (2001). Standard phenotypic tests were performed as described previously (Baumann & Schubert, 1984; Farmer & Hickman-Brenner, 1992; Thompson *et al.*, 2002a, b, 2005b). Resistance to the vibriostatic agents O/129 (10 and 150 µg; Oxoid) and novobiocin (5 µg; Oxoid) was tested on MA medium.

Strain MACL01^T comprised cells that were Gram-negative, rod-shaped (1.7 × 0.7 µm) and motile by means of two polar flagella (see Supplementary Fig. S1 available in IJSEM Online).

According to the 16S rRNA gene sequence analysis, the organism under study belongs to the genus *Photobacterium* and forms a branch within this genus together with *Photobacterium rosenbergii* and *Photobacterium ganghwense* (Fig. 1). Strain MACL01^T shows 96.9 and 96.2 % 16S rRNA gene sequence similarity with *P. rosenbergii* LMG 22223^T and *P. ganghwense* FR1311^T, respectively. These values are close to the cut-off value of 97 % for bacterial species definition (Stackebrandt & Goebel, 1994) and, hence, even though they suggested that the novel isolate could belong to a novel species of the genus *Photobacterium*, we analysed other housekeeping genes useful in the differentiation of *Vibrio* and related species (Thompson *et al.*, 2004, 2005a, b).

The *rpoA* gene sequence analysis supports the phylogenetic position of strain MACL01^T within the genus *Photobacterium* as it shows 90.0 % similarity with respect to *P. rosenbergii* LMG 22223^T (Supplementary Fig. S2). The remaining species of the genus *Photobacterium* showed less than 88 % similarity. This result is in agreement with those obtained by 16S rRNA gene sequence analysis and coincides with those obtained by other workers, and indicates that *rpoA* gene sequences correlate well with 16S rRNA gene sequences (Zeigler, 2003; Thompson *et al.*, 2005b). In the bacterial genomes sequenced to date, the *rpoA* gene is a ubiquitous single-copy gene that seems to be resistant to lateral gene transfer (Lerat *et al.*, 2003; Zeigler, 2003; Gevers *et al.*, 2004). Accordingly, it has recently been proposed as an alternative marker for bacterial classification and as a chronometer in *Vibrio*-like species (Thompson *et al.*, 2005a).

The *recA* gene has been also proposed as an alternative marker in the family *Vibrionaceae*, and its sequences seem to be more discriminatory than those of the 16S rRNA gene in the genus *Vibrio* (Thompson *et al.*, 2004). A pairwise analysis of the *recA* sequence of strain MACL01^T also revealed low levels of similarity between this strain and several species from the genera *Vibrio* and *Photobacterium*. For example, the analysis pointed to 83.7 % similarity with *Vibrio proteolyticus* LMG 3772^T, 83.4 % similarity with *Photobacterium leiognathii* LMG 4228^T and 82.4 % similarity with *P. rosenbergii* LMG 22223^T. Nevertheless, neighbour-joining phylogenetic analysis of the *recA* gene from strain MACL01^T showed that this strain clustered with several species of the genus *Photobacterium* (Supplementary Fig. S3). The results of the *recA* analysis confirm those

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences of strain MACL01^T and other related organisms of the family *Vibrionaceae*. The significance of each branch is indicated by a bootstrap percentage calculated for 1000 subsets. Bar, 2 nucleotide substitutions per 100 nucleotides.

obtained by other workers (Thompson *et al.*, 2004, 2005a, b), and show that *Photobacterium* and *Vibrio* are two different phylogenetic groups, although *Photobacterium* species did not form a homogeneous group.

According to Thompson *et al.* (2005a), there is variation of about 0.4 and 5.5% among strains from *P. rosenbergii* species in *rpoA* and *recA* sequences, respectively. The lower similarity values found between the sequences of these two genes of *P. rosenbergii* and MACL01^T confirm that this strain does not belong to *P. rosenbergii*. Therefore, analysis of the 16S rRNA, *rpoA* and *recA* genes indicates that strain MACL01^T belongs to a novel species of the genus *Photobacterium*.

The DNA G + C content of strain MACL01^T is 49.8 mol%, which is slightly higher than those reported for the remaining species of the genus *Photobacterium*.

The results of the fatty acid methyl ester analysis are given in the species description below. The fatty acid composition

of strain MACL01^T displayed qualitative and quantitative differences with respect to the species to which it is most closely related phylogenetically, namely *P. rosenbergii* and *P. ganghwense*. The main differences between strain MACL01^T and its closest relatives were as follows. Some fatty acids detected in *P. rosenbergii* were not detected in strain MACL01^T, e.g. C_{15:0}, C_{15:0} iso, C_{15:0} iso 3-OH and C_{17:1}ω9c iso. The fatty acid C_{16:0} was detected in larger amounts in strain MACL01^T and *P. ganghwense* (21%) than in *P. rosenbergii* (10–15%). In contrast, the amount of fatty acids detected from summed feature 3 (comprising C_{16:1}ω7c and/or C_{15:0} iso 2-OH) was larger in *P. rosenbergii* (41–44%) than in strain MACL01^T (32.6%) and *P. ganghwense* (27.8%). The amount of fatty acid C_{18:1}ω7c (29.6%) was larger in *P. ganghwense* than in *P. rosenbergii* (23%) and strain MACL01^T (20%). Finally, some fatty acids that were detected in amounts greater than 1% in strain MACL01^T, such as C_{16:1}ω9c (2.5%), C_{16:0} iso (1%) and an unknown fatty acid at 12.484 (1.2%), were not found for *P. rosenbergii* or *P. ganghwense*.

The phenotypic characteristics of strain MACL01^T are given in the species description below. The strain differed from *P. rosenbergii* and *P. ganghwense* in several physiological and biochemical respects. Colonies of strain MACL01^T on TCBS plates were blue, those of *P. rosenbergii* were yellow and those of *P. ganghwense* were green. In contrast with *P. rosenbergii*, strain MACL01^T grew at 4 °C and was able to grow at 8 % NaCl. Strain MACL01^T produced gelatinase and fermented L-arabinose whereas *P. rosenbergii* did not. Strain MACL01^T did not produce arginine dihydrolase, esterase, esterase lipase, valine arylamidase or naphthol-AS-BI-phosphohydrolase, whereas these compounds were produced by *P. rosenbergii*. Strain MACL01^T did not ferment melibiose, L-rhamnose or amygdalin and did not assimilate cellobiose, galactose, mannose, melibiose, raffinose or methyl α -D-glucoside. These tests were positive for *P. rosenbergii*. Strain MACL01^T differed from *P. ganghwense* in terms of growth at 4 °C. Indole and arginine dihydrolase were not produced by strain MACL01^T but were assimilated or produced by *P. ganghwense*. Strain MACL01^T produced β -galactosidase and fermented sucrose and L-arabinose, unlike *P. ganghwense*. Several differential phenotypic characteristics of strain MACL01^T and the remaining *Photobacterium* species are shown in Supplementary Table S1.

In conclusion, the overall results of the present study indicate that isolate MACL01^T should be classified within a novel species, for which the name *Photobacterium halotolerans* sp. nov. is proposed.

Description of *Photobacterium halotolerans* sp. nov.

Photobacterium halotolerans (ha.lo.to'le.rans. Gr. n. *hals* salt; L. part. adj. *tolerans* tolerating; N.L. part. adj. *halotolerans* referring to the ability to tolerate high salt concentrations).

Cells are rod-shaped, Gram-negative and motile by means of polar flagella. Colonies on TSA supplemented with 1.5 % (w/v) NaCl are circular, smooth, white to cream in colour, opaque and usually 2–4 mm in diameter within 2 days at 28 °C. The isolate grows on TCBS medium, producing blue colonies. Oxidase- and catalase-positive. The DNA G+C content of the type strain is 49.8 mol%. Aerobic or facultatively anaerobic, chemo-organotrophic, mesophilic and halotolerant. Able to ferment carbohydrates. Negative Voges–Proskauer reaction and negative for indole production and H₂S production. Does not produce arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, caseinase, urease, esterase, esterase lipase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, phosphohydrolase or glucuronidase. Gelatin is hydrolysed and the hydrolysis of aesculin is weak. The strain produces amylases, β -galactosidase, acid and alkaline phosphatase, leucine arylamidase and β -glucosaminidase. Positive for reduction of nitrate to nitrite; tryptophan deaminase production is weak. Negative for luminescence on marine agar. Grows in the presence of NaCl concentrations up to 8 % (w/v) and optimally in the presence of 1.5 % (w/v) NaCl at 28 °C,

although salt is not essential for growth. Growth occurs at 4–37 °C (optimal growth occurs at 28 °C), at pH 5–8.5 (optimal growth occurs at pH 7). Shows sensitivity to the vibriostatic agent O/129 and is novobiocin-positive. The most abundant fatty acids are summed feature 3 (32.6 %; comprising C_{16:1} ω 7c and/or C_{15:0} iso 2-OH), C_{16:0} (21.2 %), C_{18:1} ω 7c (19.9 %), C_{12:0} 3-OH (6.6 %) and C_{12:0} (5.9 %). The following fatty acids were detected in small amounts: summed feature 2 (3.0 %; comprising C_{14:0} 3-OH, C_{16:1} iso I, an unidentified fatty acid with an equivalent chain length of 10.928 and/or C_{12:0} ALDE), C_{16:1} ω 9c (2.5 %), an unidentified fatty acid with an equivalent chain length of 12.484 (1 %), C_{16:0} iso (1 %) and C_{14:0} (1 %). Ferments glucose, mannitol, sucrose and L-arabinose. Uses N-acetylglucosamine, citrate, adipate, glucose, malate, maltose, mannitol, phenylacetate, sucrose, fructose and trehalose as sole carbon sources. Utilization of L-arabinose, D-ribose, D-xylose, gentiobiose, starch and glycogen is weak. Does not use amygdalin, caproate, D-arabinose, L-xylose, L-rhamnose, galactose, sorbose, D-mannose, melibiose, cellobiose, lactose, melezitose, raffinose, turanose, lyxose, tagatose, D-fucose, L-fucose, methyl α -D-xyloside, methyl α -D-mannoside, methyl α -D-glucoside, arbutin, salicin, inulin, adonitol, inositol, sorbitol, dulcitol, erythritol, xylitol, arabinitol, glycerol and 2-ketogluconate or 5-ketogluconate as carbon sources.

The type strain, MACL01^T (= LMG 22194^T = CECT 5860^T), was isolated from Lake Martel, Mallorca, Spain.

Acknowledgements

This work was supported by the CAICYT-DGES and the JCyL (Spanish Government). We are grateful to the staff at Deutsche Sammlung von Mikroorganismen und Zellkulturen for the chemotaxonomic analyses. We also thank N. Skinner for correction of the English version of the manuscript.

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