Marinobacter lipolyticus sp. nov., a novel moderate halophile with lipolytic activity

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In the course of a screening programme in hypersaline habitats of southern Spain to isolate halophilic bacteria that are able to produce different extracellular enzymes, a novel, moderately halophilic bacterium (strain SM19^T) that displays lipolytic activity has been isolated and characterized. Strain SM19^T is a Gram-negative rod that grows optimally in culture media that contain 7.5 % NaCl. The DNA G + C content was 57.0 mol%. According to phenotypic and genotypic data, this strain was assigned to the genus *Marinobacter*. However, 16S rDNA sequence similarity between strain SM19^T and species of the genus *Marinobacter* was <96.7 %; this value is sufficiently low to propose its designation as a novel species. In addition, DNA–DNA hybridization with reference strains of close phylogenetic relatives was between 11 and 19 %. On the basis of these data, the inclusion of strain SM19^T in the genus *Marinobacter* as a novel species is proposed, with the name *Marinobacter lipolyticus* sp. nov. The type strain of the novel species is SM19^T (= DSM 15157^T = NCIMB 13907^T = CIP 107627^T = CCM 7048^T).

Isolation and characterization of extracellular hydrolytic enzymes that show optimal activity at different salt concentrations constitutes an interesting research field with potential biotechnological applications. Availability of such enzymes would facilitate different industrial processes that require activity at high salt concentrations.

Moderately halophilic bacteria have adapted to live in a wide range of salt concentrations (3–15 % NaCl) and constitute an interesting group of micro-organisms that could be used as a source of such salt-adapted enzymes (Ventosa *et al.*, 1998). A screening programme to detect hydrolytic activities, such as protease, amylase, cellulase, pullulanase and lipase, has recently been performed in different hypersaline environments of southern Spain (Sánchez-Porro *et al.*, 2003). This study revealed a wide diversity of moderately halophilic bacteria with the potential to hydrolyse a range of structurally non-related polymers. Among the isolates, 207 moderately halophilic bacteria that display lipolytic activity have been detected. Applications of lipolytic enzymes, which comprise mainly esterases and lipases, are widely found in the food, detergents, pharmaceutical and chemical industries (Jaeger *et al.*, 1999; Pandey *et al.*, 1999). A large number of microbial lipolytic enzymes has been identified and characterized to date; however, no lipolytic enzymes have so far been characterized from moderate halophiles. Taxonomic/phylogenetic studies performed on

these halophilic, lipolytic enzyme producers accommodate them within different genera, such as *Salinivibrio*, *Halomonas*, *Chromohalobacter*, *Salibacillus*, *Marinococcus* and *Marinobacter*. In this study, the most promising isolate among the lipolytic producers, strain SM19^T, was assigned to the genus *Marinobacter*. This genus was proposed by Gauthier *et al.* (1992), belongs to the γ -subclass of the class *Proteobacteria* and, at the time of writing, includes two species with validly published names: *Marinobacter hydrocarbonoclasticus* (Gauthier *et al.*, 1992) and *Marinobacter aquaeolei* (Nguyen *et al.*, 1999). Species of this genus are able to degrade hydrocarbons and some crude oil components.

In this work, we present a taxonomic/phylogenetic study of strain SM19^T, a moderately halophilic bacterium that shows lipolytic activity with potential industrial applications, in order to establish its exact taxonomic position. We show that it constitutes a novel species of the genus *Marinobacter*, for which we propose the name *Marinobacter lipolyticus* sp. nov.

Strain SM19^T was isolated from saline soil in Cádiz, Spain. The following reference strains were used for comparative purposes: *M. hydrocarbonoclasticus* DSM 8798^T, *M. hydrocarbonoclasticus* DSM 50418 and *Marinobacter aquaeolei* DSM 11845^T. *M. hydrocarbonoclasticus* DSM 50418 was the type strain of the former species *Pseudomonas nautica* (Baumann *et al.*, 1972), which was transferred to the genus *Marinobacter* following a polyphasic taxonomic approach (Spröer *et al.*, 1998) and, consequently, it has been included

The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of *Marinobacter lipolyticus* SM19^T is AY147906.

in this study. These strains were cultured on a saline medium (SW-7.5) with a total salt concentration of 7.5 % (w/v), supplemented with 0.5 % (w/v) yeast extract (Ventosa *et al.*, 1982). All cultures were cultivated at 37 °C in an orbital shaker (New Brunswick Scientific) at 200 r.p.m. When necessary, solid media were prepared by adding 20 g Bacto-agar l⁻¹ (Difco).

Standard phenotypic tests were performed to characterize this new isolate, including Gram reaction, cell morphology, motility, growth under anaerobic conditions, catalase and oxidase production, hydrolysis of gelatin, starch and Tween 80, as well as other tests that are included in the species description. Procedures for all tests have been described previously (Ventosa *et al.*, 1982; Quesada *et al.*, 1984; Garcia *et al.*, 1987). Unless otherwise indicated, tests were performed in media that contained 7.5 % (w/v) salt, at pH 7.5 and incubated at 37 °C in sealed containers.

Strain SM19^T is a Gram-negative, motile rod that grows optimally in media that contain approximately 7.5 % (w/v) salt and does not grow on nutrient agar without NaCl. This isolate can be considered as a moderately halophilic bacterium according to Kushner & Kamekura (1988). *M. hydrocarbonoclasticus* is an extremely halotolerant species, whereas *M. aquaeolei* is a moderately halophilic bacterium with optimal growth at 5 % NaCl. Strain SM19^T grows at 15–40 °C and pH 5–10; optimal growth occurs at 37 °C and pH 7.5. The new isolate is strictly aerobic and catalase- and oxidase-positive.

Ability to utilize 95 different compounds was tested by using the Biolog GN automatic identification system. Strains were grown on isolate medium at 37 °C for 24 h and suspended in pre-warmed sterile saline medium (3 % NaCl) within the density range specified by the manufacturer (determined with a Biolog model 21101 photometer). Immediately after suspending the cells in saline solution, suspensions were transferred into sterile multichannel pipetter reservoirs and the Biolog GN MicroPlates were inoculated with 125 µl cell suspension per well by means of an eight-channel repeating pipetter. Inoculated Biolog GN plates were incubated at 37 °C for 24 h and the results

were read with a MicroPlate reader, using MicroLog 3.59 computer software to perform automated reading and identification. The results obtained are included below in the description of the species.

In this work, the almost-complete 16S rDNA sequence (approx. 1443 nt) of strain SM19^T was obtained. For this purpose, DNA was extracted and precipitated by following the CTAB (cetyltrimethylammonium bromide) protocol for bacterial genomic DNA preparation (Wilson, 1987) and PCR amplification of the 16S rRNA gene was carried out by using methods that have been described previously in detail (Mellado *et al.*, 1995). 16S rDNA sequence analyses were performed with the ARB software package (Ludwig & Strunk, 1996) as reported by Arahal *et al.* (2002). Based on this analysis, the phylogenetic position of strain SM19^T was determined to be within the genus *Marinobacter* (Fig. 1). With respect to the sequences of the type species of the genus, *M. hydrocarbonoclasticus*, similarities were between 96.4 % (with GenBank sequence no. X67022) and 96.7 % (with AB021372, Y16735 and AB019148). X67022 corresponds to strain ATCC 49840^T, the type strain of the species, whereas AB021372 and AB019148 are equivalent sequences of *M. hydrocarbonoclasticus* ATCC 27132 (DSM 50418), which was the type strain of *Pseudomonas nautica* (Spröer *et al.*, 1998). Although information about the strain used to obtain sequence Y16735 is not available, it is almost coincident with AB021372.

Sequence similarities of strain SM19^T with respect to *M. aquaeolei* are 96.7 % (GenBank no. AJ000726, sequence of the type strain VT8^T) and 97.7 % (AF173969, sequence of strain KT02ds19). However, the study in which the latter sequence was reported (Eilers *et al.*, 2000) is an ecological article that is related to the abundance of pelagic bacteria in the North Sea and does not follow polyphasic taxonomic criteria. In our opinion, it would be more appropriate to consider strain KT02sd19 as *Marinobacter* sp.

Highest similarity values to the sequence of strain SM19^T (98.0–98.8 %) corresponded to partially determined sequences, which are not significant for taxonomic purposes. '*Marinobacter marinus*' has low sequence similarity to

Fig. 1. Phylogenetic tree based on 16S rRNA sequences, showing the relationship of *Marinobacter lipolyticus* SM19^T to other members of the genus *Marinobacter*. Root not shown. Sequences corresponding to the type strains of *Marinobacter* species are shown in bold. Accession numbers of the sequences used in this study are shown in parentheses after the strain designation. Bar, 1 % sequence divergence.

strain SM19^T (94.8 %) and '*Marinobacter arcticus*' shows 98.0 % sequence similarity to strain SM19^T. However, these names have not yet been validly published. Moreover, the article in which the latter organism is studied (Button & Robertson, 2001) does not report any taxonomic description of this species. In conclusion, the results of the phylogenetic analyses clearly indicate that strain SM19^T belongs to the genus *Marinobacter* but is sufficiently distant (with similarity values of <97 %) to other members of the genus, giving support to its proposal as a novel species.

Cellular fatty acids of strain SM19^T and the type strains of *M. aquaeolei* and *M. hydrocarbonoclasticus* were analysed with the MIDI Microbial Identification system. Cells were cultured in SW-7.5 medium at 37 °C for 24 h. Results of the cellular fatty acid analysis are shown in Table 1. The predominant fatty acids of strain SM19^T were C_{16:0}, C_{18:1}ω9c, C_{12:0} 3-OH and C_{16:1}ω9c. The fatty acid profile is very similar to those of other species of the genus *Marinobacter* (Spröer *et al.*, 1998; Nguyen *et al.*, 1999).

Table 1. Cellular fatty acid compositions of strain SM19^T and related species of the genus *Marinobacter*

Species: 1, *M. lipolyticus* SM19^T; 2, *M. aquaeolei* DSM 11845^T; 3, *M. hydrocarbonoclasticus* DSM 8798^T. Values are percentages of total fatty acids; values <1 % are not shown.

Fatty acid	1	2	3
C _{10:0}	1.5	0.9	1.0
C _{12:0}	8.3	4.7	6.5
C _{14:0}	—	2.8	2.8
C _{15:0}	1.0	1.4	1.7
C _{16:0}	28.5	29.9	30.3
C _{17:0}	3.6	3.6	3.5
C _{18:0}	2.7	3.2	1.8
10-methyl C _{16:0}	4.0	1.0	1.0
C _{16:1} ω9c	10.5	8.0	9.4
C _{17:1} ω8c	2.9	2.8	2.8
C _{18:1} ω7c	2.3	1.0	0.48
C _{18:1} ω9c	13.9	26.3	19.6
C _{12:0} 3-OH	11.3	7.6	10.3

For determination of the DNA base composition of strain SM19^T, DNA was extracted and purified by the method of Marmur (1961) and its G+C content was determined according to Marmur & Doty (1962) by using the equation of Owen & Hill (1979). The DNA G+C content of strain SM19^T was 57.0 mol%. This value is similar to those described for *M. hydrocarbonoclasticus* (57.5 mol%; Spröer *et al.*, 1998) and *M. aquaeolei* (55.7 mol%; Nguyen *et al.*, 1999).

DNA–DNA hybridization was studied by the competition procedure of Johnson (1994), described in detail elsewhere (Arahal *et al.*, 2001). Hybridization experiments were carried out under optimal conditions at a temperature of 54.5 °C, which is within the limits of validity for the filter method (De Ley & Tijtgate, 1970). Hybridization (%) was calculated as described by Johnson (1994). Hybridization levels between strain SM19^T and *M. hydrocarbonoclasticus* and *M. aquaeolei* are shown in Table 2. The low hybridization values (11–19 %) obtained in this study clearly demonstrate that isolate SM19^T represents a novel species of the genus *Marinobacter*.

Lipolytic activity of the new isolate was detected by screening for zones of hydrolysis around colonies growing on SW-10 plates that contained 1 % Tween 80, after incubation for 48 h. Substrate specificity of the enzyme was studied by performing a spectrophotometric assay that used different *p*-nitrophenyl esters (Winkler & Stuckmann, 1979). Highest activity was observed with *p*-nitrophenyl caprilate (100 %). Activity was lower towards *p*-nitrophenyl esters with a longer chain, such as *p*-nitrophenyl laureate (42 %), *p*-nitrophenyl palmitate (17 %) and *p*-nitrophenyl stearate (8 %). In addition, the production of extracellular lipolytic activity during growth of strain SM19^T was determined. Strain SM19^T was able to grow in saline media that contained 2–15 % total salt, with optimum growth at 7.5 % salt. To determine the influence of salt on lipolytic activity, different saline media were used for bacterial growth. The highest level of activity in the supernatant was reached after 8 h cultivation in medium SW-7.5 at 37 °C and pH 7.2. Activity was slightly lower in saline medium that contained 5 % NaCl; however, activity decreased severely when SW-10 was used.

Table 2. DNA G+C contents and levels of DNA–DNA relatedness for *Marinobacter lipolyticus* and related species of the genus *Marinobacter*

Source of unlabelled DNA	DNA G+C content (mol%)	Hybridization with ³ H-labelled DNA from <i>M. lipolyticus</i> SM19 ^T (%)
<i>M. lipolyticus</i> SM19 ^T	57.0	100
<i>M. hydrocarbonoclasticus</i> DSM 8798 ^T	57.3*	11
<i>M. hydrocarbonoclasticus</i> DSM 50418	57.7*	19
<i>M. aquaeolei</i> DSM 11845 ^T	55.7*	13

*Data from Spröer *et al.* (1998) and Nguyen *et al.* (1999).

Overall, our results show that strain SM19^T represents a novel species of the genus *Marinobacter*, for which the name *Marinobacter lipolyticus* sp. nov. is proposed.

Description of *Marinobacter lipolyticus* sp. nov.

Marinobacter lipolyticus (li.po.ly'ti.cus. Gr. n. *lipos* fat; N.L. adj. *lyticus* from Gr. adj. *lytikos* dissolving; N.L. adj. *lipolyticus* fat-dissolving).

Gram-negative, rod-shaped cells that are 0.3–0.5 × 2.5–3.5 µm in size and occur singly, in pairs or in short chains. Motile and non-spore-forming. Colonies on SW-7.5 medium are circular, convex, slightly elevated, 2–3 mm in diameter and cream-pigmented. Growth occurs in the presence of 1–15% (w/v) total salt; optimal growth occurs in the presence of 7.5% (w/v) total salt. No growth occurs in the absence of salt. Grows occurs at 15–40 °C (optimal temperature, 37 °C) and at pH 5.0–10.0 (optimal pH, 7.5). Strictly aerobic. Catalase and oxidase are produced. Acid is produced from D-glucose, maltose and D-mannitol. Acid is not produced from glycerol, D-lactose or D-melibiose. Tween 80 is hydrolysed, but DNA, gelatin and starch are not. Indole, methyl red, phosphatase, Voges–Proskauer, phenylalanine deaminase, arginine dihydrolase and lysine and ornithine decarboxylase tests are negative. Simmons' citrate test is positive. Nitrate and nitrite are not reduced. The following compounds are utilized as sole carbon and energy sources: dextrin, glycogen, Tween 40, Tween 80, N-acetyl-D-glucosamine, D-fructose, D-glucose, maltose, D-mannitol, D-trehalose, methyl pyruvate, D-gluconic acid, inosine and thymidine. The following compounds are not utilized as sole carbon and energy sources: α-cyclodextrin, N-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, cellobiose, i-erythritol, L-fucose, D-galactose, m-inositol, α-D-lactose, lactulose, D-mannose, D-melibiose, methyl β-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, turanose, xylitol, monomethyl succinate, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuronamide, alaninamide, D-alanine, L-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, urocanic acid, uridine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, DL-α-glycerol phosphate, glucose 1-phosphate and glucose 6-phosphate. The G + C content of the DNA is 57.0 mol% (*T_m* method).

The type strain is SM19^T (= DSM 15157^T = NCIMB 13907^T = CIP 107627^T = CCM 7048^T). Habitat: saline soil.

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