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 SM19T) that displays lipolytic activity has been isolated and characterized. Strain SM19T 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 SM19T 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 SM19T 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 SM19T (=DSM 15157T =NCIMB 13907T =CIP 107627T =CCM 7048T).

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, Halo- monas, Chromohalobacter, Salibacillus, Marinococcus and Marinobacter. In this study, the most promising isolate among the lipolytic producers, strain SM19T, 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 SM19T, 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 SM19T was isolated from saline soil in Cádiz, Spain. The following reference strains were used for comparative purposes: M. hydrocarbonoclasticus DSM 8798T, M. hydrocarbonoclasticus DSM 50418 and Marinobacter aquaeolei DSM 11845T. 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
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 Bactoagar 1−1 (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 SM19T 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 SM19T 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 MicroPlates 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 SM19T 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 SM19T 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 49840T, 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 SM19T with respect to M. aquaeolei are 96·7 % (GenBank no. AJ000726, sequence of the type strain VT8T) 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 KT02ds19 as Marinobacter sp.

Highest similarity values to the sequence of strain SM19T (98·0–98·8 %) corresponded to partially determined sequences, which are not significant for taxonomic purposes. ‘Marinobacter marinus’ has low sequence similarity to...
strain SM19<sup>T</sup> (94·8 %) and ‘Marinobacter arcticus’ shows 98·0 % sequence similarity to strain SM19<sup>T</sup>. 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<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> were C<sub>16 : 0</sub>, C<sub>18 : 1ω9c</sub>, C<sub>12 : 0 3-OH</sub> and C<sub>16 : 1ω9c</sub>. The fatty acid profile is very similar to those of other species of the genus Marinobacter (Spröer et al., 1998; Nguyen et al., 1999).

For determination of the DNA base composition of strain SM19<sup>T</sup>, 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<sup>T</sup> 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 (Arahah 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 & Tijtgat, 1970). Hybridization (%) was calculated as described by Johnson (1994). Hybridization levels between strain SM19<sup>T</sup> 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<sup>T</sup> 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 caprate (100 %). Activity was lower towards p-nitrophenyl esters with a longer chain, such as p-nitrophenyl laurate (42 %), p-nitrophenyl palmitate (17 %) and p-nitrophenyl stearate (8 %). In addition, the production of extracellular lipolytic activity during growth of strain SM19<sup>T</sup> was determined. Strain SM19<sup>T</sup> 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 supernant 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 1. Cellular fatty acid compositions of strain SM19<sup>T</sup> and related species of the genus Marinobacter

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tr>
<td>C&lt;sub&gt;10 : 0&lt;/sub&gt;</td>
<td>1·5</td>
<td>0·9</td>
<td>1·0</td>
</tr>
<tr>
<td>C&lt;sub&gt;12 : 0&lt;/sub&gt;</td>
<td>8·3</td>
<td>4·7</td>
<td>6·5</td>
</tr>
<tr>
<td>C&lt;sub&gt;14 : 0&lt;/sub&gt;</td>
<td>2·8</td>
<td>2·8</td>
<td>2·8</td>
</tr>
<tr>
<td>C&lt;sub&gt;15 : 0&lt;/sub&gt;</td>
<td>1·0</td>
<td>1·4</td>
<td>1·7</td>
</tr>
<tr>
<td>C&lt;sub&gt;16 : 0&lt;/sub&gt;</td>
<td>28·5</td>
<td>29·9</td>
<td>30·3</td>
</tr>
<tr>
<td>C&lt;sub&gt;17 : 0&lt;/sub&gt;</td>
<td>3·6</td>
<td>3·6</td>
<td>3·5</td>
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<tr>
<td>C&lt;sub&gt;18 : 0&lt;/sub&gt;</td>
<td>2·7</td>
<td>3·2</td>
<td>1·8</td>
</tr>
<tr>
<td>10-methyl C&lt;sub&gt;16 : 0&lt;/sub&gt;</td>
<td>4·0</td>
<td>1·0</td>
<td>1·0</td>
</tr>
<tr>
<td>C&lt;sub&gt;16 : 1ω9c&lt;/sub&gt;</td>
<td>10·5</td>
<td>8·0</td>
<td>9·4</td>
</tr>
<tr>
<td>C&lt;sub&gt;17 : 1ω8c&lt;/sub&gt;</td>
<td>2·9</td>
<td>2·8</td>
<td>2·8</td>
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<td>C&lt;sub&gt;18 : 1ω7c&lt;/sub&gt;</td>
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<td>1·0</td>
<td>0·48</td>
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<td>C&lt;sub&gt;18 : 1ω9c&lt;/sub&gt;</td>
<td>13·9</td>
<td>26·3</td>
<td>19·6</td>
</tr>
<tr>
<td>C&lt;sub&gt;12 : 0 3-OH&lt;/sub&gt;</td>
<td>11·3</td>
<td>7·6</td>
<td>10·3</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Source of unlabelled DNA</th>
<th>DNA G+C content (mol%)</th>
<th>Hybridization with &lt;sup&gt;3&lt;/sup&gt;H-labelled DNA from M. lipolyticus SM19&lt;sup&gt;T&lt;/sup&gt; (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. lipolyticus SM19&lt;sup&gt;T&lt;/sup&gt;</td>
<td>57·0</td>
<td>100</td>
</tr>
<tr>
<td>M. hydrocarbonoclasticus DSM 8798&lt;sup&gt;T&lt;/sup&gt;</td>
<td>57·3*</td>
<td>11</td>
</tr>
<tr>
<td>M. hydrocarbonoclasticus DSM 50418</td>
<td>57·7*</td>
<td>19</td>
</tr>
<tr>
<td>M. aquaeolei DSM 11845&lt;sup&gt;T&lt;/sup&gt;</td>
<td>55·7*</td>
<td>13</td>
</tr>
</tbody>
</table>

*Data from Spröer et al. (1998) and Nguyen et al. (1999).
Overall, our results show that strain SM19<sup>T</sup> represents a novel species of the genus *Marinobacter*, for which the name *Marinobacter lipolyticus* sp. nov. is proposed.

**Description of Marinobacter lipolyticus sp. nov.**


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, phosphate, 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, hydrogen, Tween 40, Tween 80, N-acetyl-D-glucosamine, D-fructose, D-galactose, methanol, D-mannitol, D-trehalose, methyl pyruvate, D-gluconic acid, inosine and thymidine. The DNA is 57 mol% (λ, C) and at pH 5.0–10.0 (optimal pH, 7.5).

**References**


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