Halovivax ruber sp. nov., an extremely halophilic archaeon isolated from Lake Xilinhot, Inner Mongolia, China

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A Gram-negative, pleomorphic, extremely halophilic archaeon, designated strain XH-70T, was isolated from the saline Lake Xilinhot, in Inner Mongolia, China. It formed small (0.9–1.5 mm), red-pigmented, elevated colonies on agar medium. The strain required at least 2.5 M NaCl and 5 mM Mg²⁺ for growth. The 16S rRNA gene sequence analysis indicated that strain XH-70T belongs to the family Halobacteriaceae, showing 99.5 % similarity to the type strain of Halovivax asiaticus and 94.7 and 94.6 % similarity, respectively, to the type strains of Natronococcus amylolyticus and Natronococcus occultus. Polar lipid analysis supported the placement of strain XH-70T in the genus Halovivax. DNA–DNA hybridization studies (32 % with Halovivax asiaticus CGMCC 1.4248T), as well as biochemical and physiological characterization, allowed strain XH-70T to be differentiated from Halovivax asiaticus. A novel species, Halovivax ruber sp. nov., is therefore proposed to accommodate this strain. The type strain is XH-70T (= CGMCC 1.6204T = DSM 18193T = JCM 13892T).

The family Halobacteriaceae includes a total of 22 genera and a large number of species (Ventosa, 2006). They have typical archaeal characteristics and are extremely halophilic aerobes, able to grow at high salt concentrations (Grant et al., 2001). In the course of a study of extremely halophilic microorganisms from the saline Lake Ejinor, in Inner Mongolia (China), Castillo et al. (2006) isolated a haloarchaeon, strain EJ-46T, from a sediment sample. On the basis of 16S rRNA gene sequence, salt requirements and chemotaxonomic and physiological characteristics, they proposed to classify it in a new archaeal genus, Halovivax, with only one species, Halovivax asiaticus. This archaeon is an aerobic, non-motile, pleomorphic rod. In this study, we describe an extremely halophilic micro-organism isolated from Lake Xilinhot in Inner Mongolia, and we propose it as the type strain of a second species of the genus Halovivax.

Strain XH-70T was isolated from a water sample collected in September 2003 from the saline Lake Xilinhot (43° 55’ N 115° 37’ E). The water of the lake had a temperature of 23.6 °C, a pH of 8.5 and a conductivity of 185 mS cm⁻¹. The isolate was grown and maintained aerobically at 37 °C in a medium containing (l⁻¹): NaCl, 195 g; MgCl₂·6H₂O, 32.5 g; MgSO₄·7H₂O, 50.8 g; CaCl₂, 0.8 g; KCl, 5 g; NaHCO₃, 0.16g; NaBr, 0.6 g; and yeast extract, 5 g. The pH was adjusted to 8 with 1 M NaOH. A pure culture was obtained by repeated plating on the same medium with agar added until clonal purity was obtained.

Phenotypic tests were performed according to the proposed minimal standards for the description of new taxa of the order Halobacteriales (Oren et al., 1997). The optimal conditions for growth were determined in media containing 0.9–5.2 M NaCl or 0.005–1 M Mg²⁺. The pH range for...
growth was assayed from pH 6.0 to 10.0 at intervals of 0.5 in liquid medium. The temperature range for growth of strain XH-70\textsuperscript{T} was determined in a medium at pH 7.5 with optimal NaCl and Mg\textsuperscript{2+} concentrations. Cell morphology and motility were examined by using an Olympus BX41 microscope equipped with phase-contrast optics. For photography, drops of exponentially growing liquid cultures were mixed on a microscope slide with an equal volume of melted 1% agarose containing 20% NaCl and then covered with a cover slip. Colony morphology was observed under optimal growth conditions on agar medium after incubation at 37\textdegree C for 10 days. Cells of strain XH-70\textsuperscript{T} are pleomorphic, from rods to squares or disc-shaped (Fig. 1). Colonies are red, smooth, circular and elevated. Anaerobic growth was tested in the presence of 5 g nitrate or L-arginine l\textsuperscript{-1} in filled stoppered tubes. The following characteristics were tested as described by Oren et al. (1997): hydrolysis of starch, gelatin, DNA, aesculin, casein and Tween 80, nitrate reduction, production of indole and H\textsubscript{2}S, catalase and oxidase activities and utilization of sugars, alcohols, amino acids and organic acids as sources of carbon and energy or carbon, nitrogen and energy. Appropriate positive and negative controls were included in all tests. Susceptibility to antibiotics was determined on agar medium plates by using antibiotic discs with the following amounts of antibiotic: ampicillin (10 \mu g), bacitracin (10 U), chloramphenicol (30 \mu g), erythromycin (15 \mu g), gentamicin (10 \mu g), nalidixic acid (30 \mu g), neomycin (10 \mu g), novobiocin (30 \mu g), penicillin G (10 U), rifampicin (30 \mu g), streptomycin (10 \mu g) and tetracycline (30 \mu g). The results of the phenotypic tests and antibiotic susceptibility are included in the species description and in Table 1.

Chromosomal DNA of strain XH-70\textsuperscript{T} was isolated and purified according to the methods described by Wilson (1987) and Marmur (1961). The 16S rRNA gene of strain XH-70\textsuperscript{T} was amplified by PCR using three universal primers as described previously (Lopez-Garcia et al., 2001; Arahal et al., 1996) and an almost-complete nucleotide sequence (1379 bp) was determined. The ARB software package (Ludwig et al., 2004) was used for 16S rRNA gene sequence analysis. 16S rRNA gene sequences were aligned and the alignment was confirmed and checked against both primary and secondary structure of the 16S rRNA molecule using the alignment tool of the ARB software package. The aligned sequences were subjected to different phylogenetic methods integrated in the ARB software for phylogenetic inference. These methods included maximum-likelihood, maximum-parsimony and neighbour-joining algorithms. Base-frequency filters were applied in the sequence comparison analysis and the effects on the results were evaluated. The 16S rRNA gene sequence phylogenetic analysis, performed based on the neighbour-joining method (Saitou & Nei, 1987), clearly showed the position of strain XH-70\textsuperscript{T} within the genus Halovivax. Maximum-parsimony- and maximum-likelihood-based trees using the full dataset or a selection of sequences were also obtained showing the same phylogenetic position of strain XH-70\textsuperscript{T} within the genus Halovivax (data not shown). The 16S rRNA gene sequence similarities between strain XH-70\textsuperscript{T} and the type strains Halovivax asiaticus EJ-46\textsuperscript{T}, Natronococcus amylolyticus Ah-36\textsuperscript{T} and Natronococcus occultus NCIMB 2192\textsuperscript{T} were 99.5, 94.7 and 94.6\%, respectively. Phylogenetic analysis based on 16S rRNA gene sequence comparison showed that strain XH-70\textsuperscript{T} formed a coherent cluster with the type strain of Halovivax asiaticus (Fig. 2). The G+C content of genomic DNA was determined from the mid-point (T_m) of the thermal denaturation profile (Marmur & Doty, 1962), using the equation of Owen & Hill (1979) as described previously (Ventosa et al., 2004). The DNA G+C content of strain XH-70\textsuperscript{T} was 65.0 mol\%, which is higher than that

### Table 1. Some characteristics that distinguish strain XH-70\textsuperscript{T} from Halovivax asiaticus

Data from Castillo et al. (2006) and this study.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain XH-70\textsuperscript{T}</th>
<th>Halovivax asiaticus CGMCC 1.4248\textsuperscript{T}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony pigmentation</td>
<td>Red</td>
<td>Pale pink</td>
</tr>
<tr>
<td>Mg\textsuperscript{2+}  requirement</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Acid from D-xylose</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Mannose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ribose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Acetate</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fumarate</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sensitivity to:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin (10 \mu g)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Gentamicin (10 \mu g)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Neomycin (10 \mu g)</td>
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<td>-</td>
</tr>
<tr>
<td>Rifampicin (30 \mu g)</td>
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<td>-</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>65.0</td>
<td>60.3</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Phase-contrast photomicrograph of cells of strain XH-70\textsuperscript{T}. Bar, 10 \mu m.
reported for *Halovivax asiaticus* (60.3 mol%; Castillo et al., 2006).

The polar lipids were extracted with chloroform/methanol as described previously (Kamekura, 1993). TLC was performed using Merck HPTLC silica gel 60 plates (art. 5641) with the solvent system chloroform/methanol/acetic acid/water (85 : 22.5 : 10 : 4, by vol.). Glycolipids were detected with the solvent system chloroform/methanol/acetic acid/as described previously (Kamekura, 1993). TLC was performed using Merck HPTLC silica gel 60 plates (art. 5641) with the solvent system chloroform/methanol/acetic acid/water (85 : 22.5 : 10 : 4, by vol.). Glycolipids were detected with the solvent system chloroform/methanol/acetic acid/water (85 : 22.5 : 10 : 4, by vol.).

DNA–DNA hybridization analyses was performed as described by Arahal et al. (2001), according to the competition procedure of the membrane method by Johnson (1994). The hybridization temperature was 56 °C, which is within the limit of validity for the filter method (De Ley & Tijtgat, 1970), and the percentage of hybridization was calculated according to Johnson (1994). Three independent determinations were carried out for each experiment and the results reported are mean values. The DNA–DNA hybridization level of strain XH-70T to *Halovivax asiaticus* CGMCC 1.4248T was 32%. Comparison of phenotypic properties (Table 1) also indicated differences between strain XH-70T and *Halovivax asiaticus*. Strain XH-70T requires Mg²⁺ for growth, whereas *Halovivax asiaticus* does not. In addition, strain XH-70T could be distinguished from *Halovivax asiaticus* by its sensitivity to ampicillin, gentamicin, neomycin and rifampicin, by its ability to use D-mannose, ribose, starch and fumarate, but not acetate, as sole carbon and energy sources and by its inability to produce acid from D-xylose (Table 1).

On the basis of the phylogenetic, genotypic, chemotaxonomic and phenotypic data, it is proposed that strain XH-70T should be classified as the type strain of a novel species within the genus *Halovivax*, as *Halovivax ruber* sp. nov.

**Description of *Halovivax ruber* sp. nov.**

*Halovivax ruber* (ru’ber. L. masc. adj. ruber red).

Cells are non-motile and Gram-negative. Pleomorphic, with rods as well as irregular cells, 3.5–4.5 μm long by 0.6–0.8 μm wide. The cells are lysed in distilled water. Colonies are red, smooth, circular and elevated. At least 2.5 M NaCl is required for growth, with optimal growth at 3.4 M NaCl. The Mg²⁺ concentration required for growth is 0.005–1 M in solid medium, but the optimum Mg²⁺ concentration is around 0.15 M (on medium containing 2.5 M NaCl). The pH range for growth is 6.0–9.0, with optimum growth at pH 7.0–7.5. Grows within the temperature range 25–45 °C (optimum at 37 °C). Catalase- and oxidase-positive. Does not produce acid from the following sugars: D-arabinose, D-fructose, D-galactose, D-glucose, lactose, maltose, maltose, D-mannose, sucrose, D-trehalose and D-xylose. Indole, methyl red, Voges–Proskauer, citrate and phosphatase tests are negative. Anaerobic growth does not occur with nitrate or arginine. Neither nitrate nor nitrite is reduced. Gelatin, Tween 80 and casein are hydrolysed. Aesculin, DNA and starch are not hydrolysed. H₂S is produced from cysteine. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are not produced. The following compounds are used as sole carbon and energy sources: lactose, D-mannose, raffinose, ribose, starch, trehalose, xylose, fumarate, glutamate and propionate. The following compounds are not used as sole carbon and energy sources: L-arabinose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, sucrose, D-trehalose and D-xylose. Indole, methyl red, Voges–Proskauer, citrate and phosphatase tests are negative. Anaerobic growth does not occur with nitrate or arginine. Neither nitrate nor nitrite is reduced. Gelatin, Tween 80 and casein are hydrolysed. Aesculin, DNA and starch are not hydrolysed. H₂S is produced from cysteine. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are not produced. The following compounds are used as sole carbon and energy sources: lactose, D-mannose, raffinose, ribose, starch, trehalose, xylose, fumarate, glutamate and propionate. The following compounds are not used as sole carbon and energy sources: L-arabinose, D-fructose, D-galactose, D-glucose, lactose, mannitol, sorbitol, maltose, malate and succinate. The following compounds are not used as sole carbon, nitrogen and energy sources: L-asparagine, glycine, L-threonine, isoleucine, L-lysine and L-serine. Susceptible to ampicillin (10 μg), bacitracin (10 μg), gentamicin (10 μg), neomycin (10 μg), novobiocin (30 μg) and rifampicin (30 μg) and resistant to chloramphenicol (30 μg), erythromycin (15 μg), nalidixic acid (30 μg) and penicillin G (10 μg). Polar lipids include phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester and a pattern of glycolipids similar to that of *Halovivax asiaticus*. The G+C content of the DNA of the type strain is 65.0 mol% (Tm method).
The type strain is XH-70T (=CGMCC 1.6204T = DSM 18193T = JCM 13892T), isolated from the saline Lake Xilinhot in Inner Mongolia, China.

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References


