Halorhabdus tiamatea sp. nov., a non-pigmented, extremely halophilic archaeon from a deep-sea, hypersaline anoxic basin of the Red Sea, and emended description of the genus Halorhabdus

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An extremely halophilic archaeon was isolated from a sample of the brine–sediment interface of the Shaban Deep in the northern Red Sea. Phylogenetic analysis of the 16S rRNA gene sequence revealed a close proximity to Halorhabdus utahensis (99.3 %), the sole species of the genus Halorhabdus. Strain SARL4BT formed non-pigmented colonies and showed optimum growth at 45 °C, in 27 % (w/v) NaCl and at pH 6.5–7.0. This organism utilized a few complex substrates, such as yeast extract and starch, for growth. Strain SARL4BT grew under anaerobic and microaerophilic conditions but grew extremely poorly under aerobic conditions. The ether lipids were diphytanyl derivatives. The DNA G+C content of the type strain was 61.7 mol%. On the basis of the phylogenetic data and physiological and biochemical characteristics, strain SARL4BT represents a novel species of the genus Halorhabdus, for which the name Halorhabdus tiamatea is proposed. The type strain is SARL4BT (=DSM 18392T =JCM 14471T). An emended description of the genus Halorhabdus is also proposed.

An ever increasing diversity of extremely halophilic archaea is being uncovered from practically every type of hypersaline biotope, but no strain has ever been described from a deep-sea, hypersaline, anoxic basin. These deep-sea brines are unusual athalassohaline environments created by the evaporation, and subsequent exposure and flooding, of ancient seas, and are relatively stable as a result of their higher density (DasSarma & Arora, 2001). All of the known deep-sea brines are associated with tectonic activity, though found in regions around the world that represent entirely different geodynamic environments, namely divergent and convergent plate boundary settings (Red Sea and Mediterranean Sea, respectively) and the salt tectonics of the Gulf of Mexico (Degens & Ross, 1969; Scientific Staff of Cruise Bannock 1984–12, 1985; Wiesenburg et al., 1985).

The first deep-sea brines to be discovered were in the Red Sea, but only a few microbiological studies have been performed on these brines and, consequently, only a few micro-organisms (all of which are bacteria) have been isolated from these unusual biotopes (Antunes et al., 2003, 2007; Eder et al., 2001; Fiala et al., 1990; Trüper, 1969). Nonetheless, data from phylogenetic and biochemical studies have shown that a diverse archaeal population exists in these deep-sea brines (Eder et al., 1999, 2001, 2002; Michaelis et al., 1990).

New samples for microbiological studies were retrieved from the northern-most brine-filled depths of the Red Sea during Cruise 52/3 of RV Meteor in 2002 (Antunes, 2003). Strain SARL4BT was isolated from the brine–sediment interface of the Shaban Deep as the result of a subsequent microbial diversity assessment study that relied on phylogenetic targeting of members of the Archaea. A phylogenetic analysis based on 16S rRNA gene sequencing revealed a close relationship between strain SARL4BT and Halorhabdus utahensis, the sole recognized species of the genus Halorhabdus. We propose that, on the basis of
physiological, biochemical and phylogenetic evidence, isolate SARL4B<sup>T</sup> represents a novel species of the genus *Halorhabdus*.

Strain SARL4B<sup>T</sup> was isolated from a sample of the brine–sediment interface of the eastern basin of the Shaban Deep in the Red Sea (station no. 133.1-2; 26° 13.9' N 35° 21.3' E) taken at a depth of 1447 m, a pH of 6.0, a salinity of 24.4% and an in situ temperature of 24.1°C. Serum flasks (120 ml) were filled with 20 ml anaerobic HBM liquid medium (Halobacteria medium; DSMZ medium 372) containing the following (l<sup>-1</sup>): 5.0 g yeast extract, 5.0 g Casamino acids, 5.0 g sodium citrate, 2.0 g KCl, 3.0 g sodium glutamate, 2.0 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 200.0 g NaCl, 36.0 mg FeCl<sub>2</sub>·4H<sub>2</sub>O and 0.36 mg MnCl<sub>2</sub>·4H<sub>2</sub>O. The liquid medium was dispensed, stoppered and sealed inside an anaerobic tent and the gas phase exchanged with N<sub>2</sub> (300 kPa) prior to autoclaving. The medium was inoculated with 0.2 ml of this sample and then incubated at 42°C for up to 9 days (API 20NE) or 6 h (API ZYM). Nitrate reduction was tested using the method described by Smibert & Krieg (1981). Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activities were determined in minimal medium supplemented with 0.05% (w/v) NH<sub>4</sub>Cl, 0.1% (w/v) yeast extract and 0.5 or 1% (w/v) arginine, lysine or ornithine. pH values were monitored throughout growth, the results being considered positive when an increase in pH was detected. The Voges–Proskauer reaction was performed in minimal HBM medium supplemented with 0.05% (w/v) NH<sub>4</sub>Cl and 0.5% (w/v) maltose. At the end of the growth period, the reaction was tested as described elsewhere (Smibert & Krieg, 1981). Methyl red test reactions were determined by measuring the final pH of the medium described above and were considered positive if the pH was ≤4.2.

Anaerobic growth in the presence of S<sup>0</sup>, Na<sub>2</sub>SO<sub>4</sub>, Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> and KNO<sub>3</sub> (0.5%, w/v) was tested in HBM and in HBM minimal medium supplemented with 0.5% (w/v) maltose, and in both media with MgSO<sub>4</sub>·7H<sub>2</sub>O having been replaced by an equimolar amount of MgCl<sub>2</sub>·6H<sub>2</sub>O. Hydrogen sulphide was detected using lead(II) acetate paper (Merck).

Single-carbon-source assimilation was tested for 20 days at 42°C in minimal HBM medium supplemented with 0.05% (w/v) NH<sub>4</sub>Cl and the substrate at 0.2, 0.5 or 0.7% (w/v). Gas production from sugars was confirmed by measuring the pressure inside the anaerobic flasks at the end of the growth period. Sensitivity to antimicrobial agents was tested by the addition of filter-sterilized antibiotics (25 and 50 μg ml<sup>-1</sup>) to HBM anaerobic liquid medium (Oren et al., 1997).

Polar lipids and diether core lipids were extracted and analysed by using TLC as described previously (Ross & Grant, 1985). Lipoquinones were extracted from freeze-dried cells and purified by using TLC according to the method of Tindall (1989).

DNA for the determination of the G+C content was isolated as described by Nielsen et al. (1995). The G+C content of the DNA was determined by using HPLC according to the method of Mesbah et al. (1989). The extraction of genomic DNA, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out as described previously (Dyall-Smith, 2001; Eder et al., 2001). The novel 16S rRNA gene sequence was integrated into an alignment of approximately 30 000 sequences.
full and partial 16S rRNA sequences available in public databases using the corresponding automated tools of the ARB software package (Ludwig et al., 2004). The resulting alignment was checked manually. The neighbour-joining phylogenetic tree was constructed using the methods available in the ARB software package. The phylogenetic distance was determined by distance matrix analyses (Jukes & Cantor correction) also included in the ARB software package.

DNA–DNA hybridization was carried out at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA–DNA hybridization was carried out as described by De Ley et al. (1970) with the modifications described by Huß et al. (1983) using a Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 x 6 multiccil changer and a temperature controller with an in situ temperature probe (Varian).

16S rRNA gene sequence analysis indicated that isolate SARL4B$^T$ was a member of the family Halobacteriaceae. Phylogenetic analysis showed that *Hrd. utahensis*, the sole species of the genus *Halorhabdus*, is the closest relative of isolate SARL4B$^T$, having a sequence similarity value of 99.3% (Fig. 1). The sequence similarity values for SARL4B$^T$ with respect to all other recognized members of the family Halobacteriaceae were found to be below 91.0%. The mean levels of DNA–DNA hybridization between strain SARL4B$^T$ and *Hrd. utahensis* were 41.2%. This value is well below the threshold value of 70% DNA–DNA relatedness generally accepted for the definition of a novel species (Wayne et al., 1987).

The cells of isolate SARL4B$^T$ were pleomorphic, ranging from rods to coccoid or irregular forms. The pleomorphism of the culture increased with age, as is often observed for members of the family *Halobacteriaceae*.

The results of the physiological and chemotaxonomic characterization are given in Table 1 and in the species description. The polar lipid profile of strain SARL4B$^T$ was found to be similar to that of *Hrd. utahensis*, having phosphatidylglycerol, methylated phosphatidylglycerophosphate, triglycosyl diether and sulfated diglycosyl diether as the major lipids, while phosphatidylglycerolsulfate was absent (see Supplementary Fig. S1 in IJSEM Online). The antibiotic-sensitivity profiles of the novel strain and *Hrd. utahensis* were also largely similar.

Several physiological and biochemical characteristics, however, served to distinguish strain SARL4B$^T$ from the type strain of the sole species of the genus *Halorhabdus* (Table 1). One of the most striking differences was the absence of pigmentation in strain SARL4B$^T$; perhaps pigmentation was lost during evolution in a light-deprived environment. Another significant difference concerns the ability of strain SARL4B$^T$ to utilize some complex substrates for growth, such as yeast extract and starch. The inability of *Hrd. utahensis* to use these substrates was referred to as being unique within the family Halobacteriaceae and was suggested as a distinguishing feature (Wainø et al., 2000). This characteristic trait was subsequently observed in the phylogenetically related genus *Halosimplex* (Vreeland et al., 2002). Isolate SARL4B$^T$ was able to grow under strictly anaerobic, microaerophilic and aerobic conditions. However, this organism, unlike *Hrd. utahensis*, exhibited extremely poor aerobic growth, having a clear preference for anaerobic conditions. Such a

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**Fig. 1.** Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strain SARL4B$^T$ within the family *Halobacteriaceae*. Bar, 10% estimated difference in nucleotide sequence.
**Table 1.** Characteristics that distinguish strain SARL4Bᵀ from *Hrd. utahensis*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SARL4Bᵀ</th>
<th><em>Hrd. utahensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C) for growth</td>
<td>Range 15–55</td>
<td>17–55*</td>
</tr>
<tr>
<td></td>
<td>Optimum 44–46</td>
<td>50*</td>
</tr>
<tr>
<td>pH for growth</td>
<td>Range 5.5–8.5</td>
<td>5.5–8.5*</td>
</tr>
<tr>
<td></td>
<td>Optimum 6.5–7.0</td>
<td>6.7–7.1*</td>
</tr>
<tr>
<td>NaCl (% w/v) for growth</td>
<td>Range 10–30</td>
<td>9–30*</td>
</tr>
<tr>
<td></td>
<td>Optimum 26–28</td>
<td>27*</td>
</tr>
<tr>
<td>MgSO₄ range (% w/v)</td>
<td>0–25</td>
<td>0.05–20*</td>
</tr>
<tr>
<td>Gas production from sugars</td>
<td>+</td>
<td>–*</td>
</tr>
<tr>
<td>Hydrogen sulphide production</td>
<td>–</td>
<td>+*</td>
</tr>
<tr>
<td>Methyl red test</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Nitrite reduction</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>+</td>
<td>–*</td>
</tr>
<tr>
<td>Presence of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidase</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Esterase (C4)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Lipase (C14)</td>
<td>–</td>
<td>+*</td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Utilization of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Starch</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Colour</td>
<td>Non-pigmented</td>
<td>Red</td>
</tr>
<tr>
<td>Lipoquinones</td>
<td>MK-8(VIII H₂)</td>
<td>MK-8, MK-8 (VIII H₂)</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>61.7</td>
<td>64.0*</td>
</tr>
</tbody>
</table>

*Data from Waino et al. (2000).

Both species used glucose, fructose and xylose, showed β-glucosidase activity, reduced nitrate and were capable of anaerobic growth with S⁰ or KNO₃. +, Positive; –, negative; w, weak reaction.

Strain SARL4Bᵀ represents the first described archaeon isolated from a deep-sea hypersaline anoxic basin. Interestingly, recent phylogenetic studies performed on the deep-sea hypersaline anoxic basins of the eastern Mediterranean Sea have revealed a high percentage of archaeal clone sequences similar to that of *Hrd. utahensis* (van der Wielen et al., 2005). The presence of members of the genus *Halorhabdus* in at least two deep-sea, anoxic brine pools located in different geographical locations suggests a high degree of evolutionary success in their adaptation to this type of extreme biotope.

The physiological and biochemical characteristics of isolate SARL4Bᵀ were very distinctive, despite the phylogenetic proximity to *Hrd. utahensis*.

Our results show that strain SARL4Bᵀ is sufficiently different from the single species currently recognized as belonging to the genus *Halorhabdus* to warrant the classification of this isolate as a novel species of the genus, for which we propose the name *Halorhabdus tiamatea*. In view of some notable differences with respect to the type species of the genus, we also propose the emendation of the description of the genus *Halorhabdus* so as to accommodate this novel species.

**Emended description of the genus *Halorhabdus***

The main characteristics of the genus *Halorhabdus* are as previously described (Waino et al., 2000). In addition, strains of some species are non-flagellated, non-motile, produce non-pigmented colonies and show a clear preference for anaerobic conditions. The DNA G+C content ranges from 61.7 to 64.0 mol%. The type species of the genus is *Halorhabdus utahensis*.

**Description of *Halorhabdus tiamatea* sp. nov.**

*Halorhabdus tiamatea* (ti.a.m.a.te.a. N.L. fem. adj. tiamatea belonging to, or related to, Tiamat, the ancient Mesopotamian goddess of ‘the primal abyss’ and salty water).

Gram-negative, pleomorphic cells ranging from rods to coccoid or irregular forms (0.5–1 × 1–8 μm). Flagella are not observed. Colonies on BHM agar are round, non-pigmented and 2–3 mm in diameter. Extremely halophilic: growth occurs at NaCl concentrations between 10 and 30 % (w/v), with optimum growth occurring at approximately 27 % (w/v) NaCl. Growth occurs with MgSO₄ .7H₂O at 0–25 % (w/v). The temperature range for growth is between 15 and 55 °C (optimal temperature, 45 °C) and the pH range is between approximately 5.5 and 8.5 (optimal pH, 6.5–7.0). Grows under anaerobic and microaerophilic conditions; shows extremely poor aerobic growth. Produces acid from maltose and yeast extract. Oxidase-negative and catalase-positive. Methyl red test is positive and Voges–Proskauer test is negative. Indole is not produced. Poly-β-hydroxybutyrate is produced. Gelatin and asesculin are hydrolysed; starch is weakly hydrolysed. Casein and Tween 80 are not hydrolysed. Maltose and yeast extract are used for growth. Glucose, xylose, fructose, galactose, and proline are weakly assimilated. Amylase, l-arabinose, lactose, sucrose, sodium acetate, sodium citrate, sodium formate, sodium glucuronate, sodium lactate, sodium pyruvate, N-acetylglucosamine, alanine, betaine, lysine, phenylalanine, serine, acetamide, ethanol, glycerol, methanol, sorbitol, glycogen and peptone are not utilized. α-Glucosidase and β-glucosidase are present. Weak reactions also detected for α-galactosidase, β-galactosidase and...
\(\beta\)-glucuronidase. Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, \(\varepsilon\)-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, \(\alpha\)-acetyl-\(\beta\)-glucosaminidase, \(\varepsilon\)-mannosidase and \(\varepsilon\)-fucosidase are not detected. Cells are resistant to ampicillin, cephalosporin C, chloramphenicol (25 \(\mu\)g ml\(^{-1}\)), erythromycin, neomycin, penicillin G and tetracycline, but susceptible to anisomycin, aphidicolin, bacitracin, chloramphenicol (50 \(\mu\)g ml\(^{-1}\)), novobiocin and rifampycin B. The ether lipids are diphytanyl derivatives. MK-8(VIII-H\(_2\)) is the only respiratory lipoquinone present. The major polar lipids present are phosphatidylglycerol, methylated phosphatidylglycerophosphate, triglycosyl diether, sulfated diglycosyl diether and an unknown component. Phosphatidylglycerol sulphate is absent. The DNA G+C content of the type strain is 61.7 mol%.

The type strain, SARLAB\(^T\) (\(=\)DSM 18392\(^T\)=JCM 14471\(^T\)), was isolated from the brine–sediment interface of the Shaban Deep in the Red Sea.

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