Halomicroarcula pellucida gen. nov., sp. nov., a non-pigmented, transparent-colony-forming, halophilic archaeon isolated from solar salt

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A novel halophilic strain, BNERC31T, was isolated from solar salt, ‘Sel marin de Guérande’, imported from France. Colonies on agar medium containing soluble starch, sodium citrate, sodium glutamate and inorganic salts were non-pigmented and transparent, while cells obtained by centrifuging liquid cultures were red-pigmented. Cells of strain BNERC31T were non-motile, pleomorphic, stained Gram-negative and lysed in distilled water. Growth occurred with 20–30 % (w/v) NaCl (optimum, 25 %, w/v), with 0–500 mM MgCl2 (optimum, 10 mM), at pH 6.0–8.5 (optimum, pH 7.0) and at 25–55 °C (optimum, 40 °C). Growth was dependent on soluble starch, and inhibited completely by 0.5 % organic nutrients, such as Casamino acids or yeast extract. The DNA G + C content was 64.1 mol%. Strain BNERC31T possessed at least two heterogeneous 16S rRNA genes, and the sequence of the orthologous gene (preceded by the dihydroorotate oxidase gene, pyrD) showed the highest similarity (96.5 %) to that of Haloarcula marismortui JCM 8966T. The RNA polymerase subunit B′ gene sequence showed the highest similarity (91.7 %) to that of Haloarcula amylolytica JCM 13557T. The polar lipids of strain BNERC31T were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, diglycosyl diether and sulfated diglycosyl diether, similar to those of species of the genus Halomicrobium. The phenotypic and phylogenetic characteristics showed that strain BNERC31T differed from species of the genera Haloarcula and Halomicrobium and indicated that it represents a novel species in a new genus, for which the name Halomicroarcula pellucida gen. nov., sp. nov. is proposed. The type strain of the type species is BNERC31T (≡JCM 17820T ≡CECT 7537T).

This study was initiated several years ago in an attempt to isolate extreme halophiles that produced amylase in simple synthetic media containing 1 % soluble starch. We isolated an extremely halophilic strain, designated BNERC31T, from a solar salt sample imported from France, which formed non-pigmented transparent colonies on agar containing sodium glutamate as the sole nitrogen source. The strain was extraordinary in requiring soluble starch for growth, and that growth was inhibited completely by 0.5 % Casamino acids or yeast extract. The 16S RNA gene sequences indicated that the strain BNERC31T was closely related to species of the genus Haloarcula of the family Halobacteriaceae, followed by species of the genus Halomicrobium. However, the polar lipid composition of strain BNERC31T differed from those of
species of the genus *Haloarcula* but was similar to those of members of the genus *Halomicrobium*. The genus *Haloarcula* (Torreblanca *et al.*, 1986) comprises nine recognized species at the time of writing. Members of the genus *Haloarcula* are pleomorphic rods ranging from irregular discs to occasional squares and triangles and form orange- to red-pigmented colonies. The genus *Halomicrobium* was described by Oren *et al.* (1997) as *Halomicrobium mukohataei*. At the time of writing, the genus *Halomicrobium* comprises three recognized species which also form orange- to red-pigmented colonies. In the present study, we report on the phenotypic and phylogenetic characterization of strain BNERC31<sup>T</sup>, which exhibited many features differentiated from species of the genera *Haloarcula* and *Halomicrobium*. We propose that the strain represents a novel species in a new genus of the family *Halobacteriaceae*.

A medium SS contained (g l<sup>−1</sup>): 10.0 soluble starch, 0.3 sodium citrate, 0.1 sodium glutamate, 200 NaCl, 2.0 KCl, 20.0 MgSO<sub>4</sub>·7H<sub>2</sub>O, 7H<sub>2</sub>O, 36 mg FeCl<sub>2</sub>·4H<sub>2</sub>O, 0.36 MnCl<sub>2</sub>·4H<sub>2</sub>O and 20 Bacto agar; adjusted to pH 7.2 with 40 % (w/v) KOH. Approximately 0.5 g salt sample was spread directly on an agar plate with a spatula, and incubated at 37 °C for 3 weeks in a plastic bag to prevent desiccation. In an early stage of our survey, we noticed that a few non-pigmented, transparent, large colonies appeared, together with red-pigmented, smaller colonies, on a plate spread with ‘Sel marin de Guérande’ imported from France. We have never observed similar non-pigmented transparent colonies from the 136 species of 40 genera of the family *Halobacteriaceae* kept in our laboratory. Some species of the genera *Halarchaeum*, *Halorhabdus* and *Natrialba* are non-pigmented, but their colonies are not transparent. Although the strain was amylase-negative (no halo was observed surrounding the colonies after Lugol’s staining), we decided to isolate the strain by plating serial dilutions and repeated transfers on agar. A few years were spent trying to find strains that formed similar colonies from 637 salt samples. However, we could not encounter any similar colonies, and no 16S rRNA gene sequences similar to those of strain BNERC31<sup>T</sup> (GenBank accession numbers AB766179 and AB766180) had been deposited in the databases. Therefore, we used the single strain BNERC31<sup>T</sup> for further study. All physiological, morphological and chemotaxonomic analyses were carried out following the minimum standard tests for the description of a new taxon in the order *Halobacteriales* (Oren *et al.* 1997). The physiological tests were conducted using liquid or solidified SS medium at 37 °C, otherwise stated. Reference strains, *Haloarcula amylolytica* JCM 13557<sup>T</sup>, *Haloarcula argentinensis* JCM 9737<sup>T</sup>, *Haloarcula hispanica* JCM 8911<sup>T</sup>, *Haloarcula japonica* JCM 7785<sup>T</sup>, *Haloarcula marismortui* JCM 8966<sup>T</sup>, *Haloarcula quadrata* JCM 11048<sup>T</sup>, *Haloarcula salinarum* JCM 15795<sup>T</sup>, *Haloarcula tradensis* JCM 15760<sup>T</sup>, *Haloarcula vallismortis* JCM 8877<sup>T</sup>, *Halomicrobium katesii* CECT 7257<sup>T</sup>, *Halomicrobium mukohataei* JCM 9738<sup>T</sup> and *Halomicrobium zhouni* JCM 17095<sup>T</sup> were also used in this study.

Colony formation on agar medium was rich (Fig. S1, available in IJSEM Online), while growth in liquid medium was poor, with a maximum <i>A<sub>660</sub></i> of 0.1. After centrifugation of a liquid culture, a tiny red cell pellet was obtained at the bottom of a tube, suggesting that non-pigmented transparent colonies might be formed by a small number of red cells scattered in the huge amount of extracellular materials secreted by the cells. No growth was observed in SS medium without soluble starch, either corn, potato, rice, or wheat. Strain BNERC31<sup>T</sup> showed growth on agar without sodium glutamate as the sole nitrogen source. However, no growth was observed without sodium glutamate either in liquid media, or on agar plates prepared with Difco Noble agar, suggesting some nitrogenous compounds in Bacto agar served as a nitrogen source.

Cell morphology was studied using a phase-contrast microscope (Axiovert 135; Zeiss). Gram-staining was performed according to Dussault (1955). The cells of strain BNERC31<sup>T</sup> were motile and pleomorphic, approximately 1.5–2.0 × 1.5–2.0 μm, and stained Gram-negative. Colonies of strain BNERC31<sup>T</sup> were non-pigmented and transparent (Fig. S1), approximately 1.5–2.0 mm in diameter. Cells lyed in distilled water.

The range of NaCl concentrations for growth was determined in modified SS liquid media containing 10 mM MgCl<sub>2</sub> and 0–30 % (w/v) NaCl (in 1 % intervals), pH 7.2. The range of MgCl<sub>2</sub> concentrations was determined at 0–500 mM MgCl<sub>2</sub> (in 10 mM intervals) and 25 % (w/v) NaCl, pH 7.2. The pH range for growth was determined at pH 5.0–11.0 (in 0.5 pH intervals), adjusted by 10 % (w/v) H<sub>2</sub>SO<sub>4</sub> or 10 % (w/v) KOH, at 25 % (w/v) NaCl and 10 mM MgCl<sub>2</sub>. The temperature range for growth was determined from 10–70 °C (in 5 °C intervals) at 25 % (w/v) NaCl, 10 mM MgCl<sub>2</sub> and pH 7.2. Strain BNERC31<sup>T</sup> grew in the range of 20–30 % (w/v) NaCl, 0–500 mM MgCl<sub>2</sub>, pH 6.0–8.5 and 25–55 °C. Optimum growth was observed at 25 % (w/v) NaCl, 10–20 mM MgCl<sub>2</sub>, pH 7.0 and 40 °C. Anaerobic growth with KNO<sub>3</sub> was observed in an anaerobic jar after incubation for 7 days at 40 °C, but no growth was observed with arginine, DMSO or TMAO (trimethylamine N-oxide).

Tests for catalase and oxidase activities, sole carbon source utilization, hydrolysis of starch, casein, gelatin and Tween 80, indole and H<sub>2</sub>S production, reduction of nitrate and antibiotic sensitivity were performed according to the procedures of Smibert & Krieg (1994), Oren *et al.* (1997) and Minegishi *et al.* (2010a). The results are included in the species description.

Utilization of organic nutrients for growth was determined in liquid SS medium containing 0.5 % of each complex carbon or nitrogen sources; Bacto Casamino acids, Bacto neopeptone, Bacto peptone, Bacto tryptone, Bacto yeast extract and Oxoid peptone. All these organic nutrients, at 0.5 % concentrations, inhibited the growth of the strain BNERC31<sup>T</sup> completely. A lower concentration of 0.1 % was suppressive on the growth. In order to see if the cells lysed in the media, a loopful of strain BNERC31<sup>T</sup> was used to inoculate SS liquid media (1.0 ml) with and without 0.5 %
Casamino acids. After incubation for 12 h at 37 °C, a loopful of the two cultures were streaked on plain SS agar without Casamino acids, and both showed good growth. There was no difference in the cell numbers of two cultures examined microscopically, suggesting that cells were not killed in the medium containing Casamino acids. The five reference strains (Har. marismortui JCM 8966T, Har. argentinensis JCM 9737T, Har. hispanica JCM 8911T, Hmc. mukohataei JCM 7938T and Hmc. katesii CECT 7257T) showed good growth in the SS medium with 0.5 % organic nutrients.

Total DNA was extracted by the method of Cline et al. (1989). The G + C content of the genomic DNA of strain BNERC31T, determined by the HPLC method of Tamaoka & Komagata (1984), was 64.1 mol%. The 16S rRNA genes were amplified with a primer set designed from the 5’- and 3’-terminals and sequenced as described by Minegishi et al. (2010a). The sequences of 20 clones suggested that strain BNERC31T possessed two 16S rRNA genes (GenBank accession numbers AB766179 and AB766180, 1471 bp), with a sequence similarity as low as 94.8 %. The nine species of the genus Halococcus and the three species of the genus Halomicrobium also possess multiple heterogeneous 16S rRNA genes. The 16S rRNA genes of the genus Halococcus are divergent at 4.8–5.6 % (Cui et al., 2010). The 16S rRNA genes of strain BNERC31T and all species of the genera Halococcus and Halomicrobium are orthologous, and designed a PCR primer set to amplify DNA fragments encompassing a region from the conserved region of the pyrD gene to a conserved region of the 23S rRNA gene. The use of the orthologous 16S rRNA gene sequence present immediately downstream of the pyrD gene resolved the difficulty in the phylogenetic analysis of species of genera Halococcus and Halomicrobium.

Of the two 16S rRNA genes detected from strain BNERC31T (AB766179 and AB766180), AB766179 was shown to be orthologous by Minegishi’s method. Of the three 16S rRNA genes of Hmc. katesii (GenBank accession numbers JN120801, JN120802 and JN120803) and Hmc. zhouii (HM063952, HQ215546 and HQ215547), JN120801 (=AB663391) and HM063952 (=AB771432), respectively, were shown to be orthologous in this study. Sequence similarities of AB766179 of strain BNERC31T with the orthologous genes of Hmc. mukohataei JCM 9738T, Hmc. katesii CECT 7257T and Hmc. zhouii JCM 17095T were 93.1 %, 93.1 %, 93.6 %, respectively, as calculated by GENETYX-MAC version 15.0.5 (GENETYX Corporation). These data suggested that the strain BNERC31T was distinguishable from species of the genus Halomicrobium at the genus level. AB766179 of strain BNERC31T was most closely related to the orthologous genes of Har. marismortui (AB663354), Har. argentinensis (AB663351) and Har. hispanica (AB663352) with similarities of 96.5 %, 96.4 %, 96.4 %, respectively. The sequences of related strains retrieved from the DNA Data Bank of Japan (Miyazaki et al., 2003) were aligned using CLUSTAL X 2.1.0 (Larkin et al., 2007). The neighbour-joining tree was reconstructed using the method of Saitou & Nei (1987), and was evaluated by bootstrap sampling methods (Felsenstein, 1985), based on 1000 replicates. The maximum-likelihood analyses were performed with RAxML 7.0.4 using GTR + Γ model (Stamatakis et al., 2005), and support values were obtained by bootstrapping (1000 replicates) using CONSENSE in PHYLIP software (Felsenstein, 2002). The neighbour-joining tree (Fig. 1) and maximum-likelihood tree (Fig. S2) showed that strain BNERC31T was clearly separated from species of the genus Haloarcula as well as from species of the genus Halomicrobium. Alignment of the orthologous 16S rRNA gene sequence of strain BNERC31T with those from nine species of the genus Haloarcula and three species of the genus Halomicrobium showed the presence of as many as 21 signature bases, which differentiate strain BNERC31T from species of the genera Haloarcula and Halomicrobium (Fig. S3).

The determination of DNA-dependent RNA polymerase subunit B’ gene sequence (rpoB’) and its analysis were performed according to Minegishi et al. (2010b). The rpoB’ gene sequence of strain BNERC31T (1827 bp, AB771578) was most closely related to that of Har. amyloytica JCM 13557T (91.7 %), followed by Har. hispanica JCM 8911T (91.6 %) and Har. japonica JCM 7785T (91.3 %). The maximum-likelihood tree (Fig. S4) showed that the strain BNERC31T formed a branch supported by a 99.8 % bootstrap value, definitely separated from the sequence cluster of the nine species of the genus Haloarcula.

Polar lipids were extracted with chloroform/methanol and developed as described previously (Kamekura, 1993). Strain BNERC31T contained phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, diglycosyldiether and sulfated diglycosyldiether. The polar lipid composition was distinguished from species of the genus Haloarcula, in that strain BNERC31T did not possess triglycosyldiether (TGD), the signature glycolipid of species of the genus Haloarcula but was similar to the two species of the genus Halomicrobium (Fig. S5).

Table 1 summarizes the phenotypic characteristics of strain BNERC31T and all species of the genera Haloarcula and Halomicrobium. In addition to the non-pigmented colony formation and growth inhibition by organic nutrients as described above, it was noteworthy that the optimum Mg2+ concentration of BNERC31T was the lowest, 10–20 mM, while species of the genera Haloarcula and Halomicrobium showed higher optimum concentrations. The absence of catalase and oxidase activities in strain BNERC31T was also remarkable compared to all species of
the genera *Haloarcula* and *Halomicrobiun* that were catalase- and oxidase-positive. Furthermore, the polar lipid profile distinguished strain BNERC31T from species of the genus *Haloarcula*, in that strain BNERC31T did not possess TGD, the signature glycolipid of species of the genus *Haloarcula*. The polar lipid profile of strain BNERC31T was similar to those of species of the genus *Halomicrobiun*, which were distinguishable phylogenetically based on sequences of orthologous 16S rRNA and polymerase subunit B' genes.

According to comparison of the phenotypic and phylogenetic characteristics, strain BNERC31T could be distinguished from species of the genera *Haloarcula* and *Halomicrobiun* and represents a novel species in a new genus, for which the name *Halomicroarcula pellucida* gen. nov., sp. nov. is proposed.

**Description of *Halomicroarcula* gen. nov.**

*Halomicroarcula* (Ha.lo.mi.cro.ar’cu.la. Gr. n. hals, halos salt; Gr. adj. mikros small; L. fem. n. arcula a box; N.L. fem. n. Halomicroarcula salt small box).

Cells are Gram-negative, motile and pleomorphic. Extremely halophilic, neutrophilic, mesophilic and aerobic. Catalase- and oxidase-negative. The polar lipids are phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate, diglycosyl diether and sulfated diglycosyl diether. The DNA G+C content of the type species is 64.1 mol%. The type species is *Halomicroarcula pellucida*.

**Description of *Halomicroarcula pellucida* sp. nov.**

*Halomicroarcula pellucida* (pel.lu’ci.da. L. fem. adj. pellu-cida transparent, referring to the transparent colonies).

Exhibits the following characteristics in addition to those given in the genus description. Colonies formed on agar are non-pigmented and transparent. Cells lyse in distilled water. Growth occurs with 20–30% (w/v) NaCl, with optimum growth at 25% (w/v) NaCl. The MgCl2 range for growth is 0–500 mM, with optimum growth at 10–20 mM. Growth occurs at pH 6.0–8.5, with optimum growth at pH 7.0. The temperature range for growth is 25–55 °C, with optimum growth at 40 °C. Grows anaerobically with KNO3; does not grow anaerobically with arginine, DMSO or TMAO. Gelatin is hydrolysed, but casein, starch and Tween 80 are not. Indole and H2S are not produced. Reduction of
Table 1. Differential characteristics of strain BNERC31\textsuperscript{T} and species of the genera *Haloarcula* and *Halomicrobium*

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nitrate and gas formation are observed. D-Fructose, D-galactose, glycerol, maltose, D-sorbitol and D-xylene can be utilized as sole carbon sources but L-arabinose, cellobiose, D-glucose, lactose, D-mannitol, D-mannose, ribose, raffinose, sucrose and trehalose cannot be utilized. Sensitive to (μg disc⁻¹) anisomycin (50), bacitracin (25), novobiocin (25), pravastatin (50), rifampicin (50), streptomycin (100) and sulfisoxazole (250), but resistant to ampicillin (50), carbenicillin (100), chloramphenicol (25), clindamycin (2), erythromycin (25), gentamicin (50), kanamycin (50), neomycin (25) and penicillin G (25), tetracycline (50) and vancomycin (25). Growth is inhibited by organic nutrients such as Bacto Casamino acids, Bacto neopeptone, Bacto peptone, Bacto tryptone, Bacto yeast extract and Oxoid peptone at a concentration of 0.5%.

The type strain is BNERC31 T (=JCM 17820 T = CECT 7537 T), isolated from solar salt ‘Sel marin de Guérande’ imported from France. The DNA G+C content of the type strain is 64.1 mol%.

References


