Halosimplex pelagicum sp. nov. and Halosimplex rubrum sp. nov., isolated from salted brown alga Laminaria, and emended description of the genus Halosimplex

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Two halophilic archaeal strains, R2T and R27T, were isolated from the brown alga Laminaria produced at Dalian, Liaoning Province, China. Both had pleomorphic cells that lysed in distilled water, stained Gram-negative and formed red-pigmented colonies. They grew optimally at 42 °C, pH 7.0 and in the presence of 3.1–3.4 M NaCl and 0.03–0.5 M Mg2+. The major polar lipids of the two strains were phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and four major glycolipids chromatographically identical to those of Halosimplex carlsbadense JCM 11222T. 16S rRNA gene analysis revealed that each strain had two dissimilar 16S rRNA genes and both strains were phylogenetically related to Halosimplex carlsbadense JCM 11222T (92.7–98.8 % similarities). The rpoB gene similarities between strains R2T and R27T and between these strains and Halosimplex carlsbadense JCM 11222T were 95.7 %, 96.1 % and 95.8 %, respectively. The DNA G+C contents of strains R2T and R27T were 62.5 mol% and 64.0 mol%, respectively. The DNA–DNA hybridization values between strains R2T and R27T and between the two strains and Halosimplex carlsbadense JCM 11222T were 43 %, 52 % and 47 %, respectively. It was concluded that strain R2T (CGMCC 1.10586T = JCM 17263T) and strain R27T (CGMCC 1.10591T = JCM 17268T) represent two novel species of the genus Halosimplex, for which the names Halosimplex pelagicum sp. nov. and Halosimplex rubrum sp. nov. are proposed. An emended description of the genus Halosimplex is also presented.

Most halophilic archaea, members of the family Halobacteriaeae within the order Halobacterales, can grow on complex media containing yeast extract, peptones, amino acids and carbohydrates (Kocur & Hodgkiss, 1973; Colwell et al., 1979). However, certain members of the order Halobacterales have been reported to grow on defined Halobacteriales media with a single carbon and energy source. Haloquadratum walsbyi grows best on pyruvate as sole carbon source (Burns et al., 2002). Based on the nutritional requirements, the members of family Halobacteriaeae are more diverse than has been previously recognized.

The genus Halosimplex was proposed to accommodate the species Halosimplex carlsbadense described based on strain 2-9-1T isolated from unsterilized salt crystals taken from the 250-million-year-old Salado formation in southeastern New Mexico (Vreeland et al., 2002). The organism was distinctly different from other members of the family Halobacteriaeae and was completely unable to use complex nutrients, possessed two unknown glycolipids and had two distinct 16S rRNA genes (Boucher et al., 2004). During our surveys on the microbiological nature of the peculiar reddening of salted Laminaria, we isolated two halophilic archaeal strains most closely phylogenetically related to Halosimplex carlsbadense (with 92.7–98.8 % 16S rRNA gene similarities). In this study, we characterize these two strains, R2T and R27T.

Strains R2T and R27T were isolated from the red brine of salted Laminaria produced at Dalian, Liaoning Province, pyruvate, or pyruvate alone (Vreeland et al., 2002). Based on the nutritional requirements, the members of family Halobacteriaeae are more diverse than has been previously recognized.

Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony; PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; S-DGD-1, sulfated mannosyl glucosyl diether; S-TeGD, sulfated tetracyglycosyl diether; S2-DGD, disulfated mannosyl glucosyl diether.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains R2T and R27T are HM159602 (rrnA) and KF434756 (rrnB) and HM159603 (rrnA) and KF434757 (rrnB), respectively. Those for the rpoB gene sequences of strains R2T and R27T are KF434759 and KF434760, respectively.

Two supplementary figures are available with the online version of this paper.

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China. The brine had a pH of 7.5 and a total salinity of 293 g l⁻¹. Neutral oligotrophic halocarchael medium (NOM) was used for the isolation procedure and contained the following ingredients (g l⁻¹): yeast extract (Oxoid) 0.05, fish peptone (Sinopharm Chemical Reagent) 0.25, sodium pyruvate 1.0, KCl 3.4, K₂HPO₄ 0.3, CaCl₂ 0.25, NH₄Cl 0.25, MgSO₄ 7H₂O 26.8, MgCl₂ 6H₂O 23.0, NaCl 184.0 (pH adjusted to 7.0–7.2 with 1 M NaOH solution). The medium was solidified with 2.0% agar. The strains were routinely grown aerobically at 37 °C in NOM medium.

Determination of morphology and growth characteristics, nutrition, miscellaneous biochemical tests and sensitivity to antimicrobial agents were performed for all species in the same basic medium, NOM, according to the proposed minimal standards for description of novel taxa in the order Halobacteriales (Oren et al., 1997). The NaCl range for growth was determined by incubating each strain at NaCl concentrations of 0.9, 1.4, 1.7, 2.1, 2.6, 3.1, 3.4, 3.9, 4.3, 4.8 and 5.1 M. The pH range for growth was determined at pH 5.0–10.0 (with intervals of 0.5) using following buffers: MES (pH 5.5–6.7), PIPES (pH 6.1–7.5), MOPS (pH 6.5–7.9), HEPES (pH 6.8–8.2), Tricine (pH 7.4–8.8) and CHES (pH 8.6–10.0) at a concentration of 25 mM. The temperature range for growth was determined by incubating each strain at 10, 15, 20, 25, 30, 37, 40, 42, 45, 50, 55 and 60 °C. Halosimplex carlsbadense JCM 11222T, Halomicrobium mukohataei JCM 9738T, Halobacterium jilantaiense CGMCC 1.5337T and Haloterrigena longa CGMCC 1.5334T were selected as reference strains for phenotypic tests. These reference strains were routinely grown aerobically at 37 ºC in NOM medium.

Polar lipids were extracted using a chloroform/methanol system and analysed using one- and two-dimensional TLC, as described previously (Cui et al., 2010). Merck silica gel 60 F₂₅₄ aluminium-backed thin-layer plates were used for TLC analyses. In two-dimensional TLC, the first solvent was chloroform/methanol/water (65:25:4, by vol.) and the second solvent was chloroform/methanol/acetic acid/water (80:12:15:4, by vol.). The latter solvent mixture was also used in one-dimensional TLC. Two specific detection spray reagents, phosphate stain reagent for phospholipids and z-naphthol stain for glycolipids, were used. The general detection reagent, sulfuric acid/ethanol (1:2, v/v), was also used to detect total polar lipids. The presence of phospholipids and glycolipids on the two-dimensional TLC was confirmed by comparison with one-dimensional TLC on which the polar lipid profiles of reference strains were developed.

Genomic DNA from halophilic archaeal strains was prepared as described previously (Cui et al., 2011). The 16S rRNA genes were amplified, cloned and sequenced according to the previously used protocol (Cui et al., 2009). PCR-mediated amplification and sequencing of the rpoB' genes were performed as described previously (Minegishi et al., 2010). Multiple sequence alignments were performed using the CLUSTAL W program integrated in the MEGA 5 software (http://www.megasoftware.net/). Phylogenetic trees were reconstructed using neighbour-joining, maximum-parsimony (MP) and maximum-likelihood (ML) algorithms in the MEGA 5 software (Tamura et al., 2011). Gene sequence similarity among halophilic archaea was calculated using the Pairwise-Distance computing function of MEGA 5. The DNA G+C content was determined from the mid-point value (Tm) of the thermal denaturation method (Marmur & Doty, 1962) at 260 nm with a DU800 spectrophotometer (Beckman-Coulter) equipped with a high-performance temperature controller. Halomicrobium mukohataei JCM 9738T was selected as the reference strain for these analyses, and the formula G+C mol%reference strain = G+C mol%unknown strain + 2.08 × (Tm unknown strain – Tm reference strain) was used to calculate the G+C content from the known Tm value (Owen & Pitcher, 1985). DNA–DNA hybridizations were performed in a DU800 spectrophotometer equipped with a high performance temperature controller and were carried out according to the thermal denaturation and renaturation method (De Ley et al., 1970; Huß et al., 1983). DNA–DNA hybridizations were carried out in 2× SSC at 79 °C and each determination was carried out in triplicate.

Cells of strains R2T and R27T were motile and pleomorphic when grown in NOM-3 liquid medium (Fig. S1 available in IJSEM Online). They stained Gram-negative and their colonies were red-pigmented. Strain R2T was able to grow at 25–45 °C (optimum 42 °C), in the presence of 1.4–4.8 M NaCl (optimum 3.4 M NaCl), with 0.05–0.7 M MgCl₂ (optimum 0.5 M MgCl₂) and at pH 5.5–9.0 (optimum pH 7.0), while strain R27T was able to grow at 25–45 °C (optimum 42 °C), in the presence of 0.9–3.4 M NaCl (optimum 3.1 M NaCl), with 0.0–0.7 M MgCl₂ (optimum 0.03 M MgCl₂) and at pH 5.5–9.5 (optimum pH 7.0). The cells of both isolates lysed in distilled water and the minimum NaCl concentrations that prevented cell lysis were 80 g l⁻¹ for strain R2T and 50 g l⁻¹ for strain R27T. The two strains did not grow under anaerobic conditions using nitrate, DMSO or L-arginine, did not reduce nitrate to nitrite and did not produce gas from nitrate. They did not produce indole from tryptophan and did not hydrolyse starch, gelatin, casein or Tween 80. The main phenotypic characteristics differentiating strains R2T and R27T from Halosimplex carlsbadense JCM 11222T were: optimum NaCl for growth, growth requirement for Mg²⁺, utilization of specific carbon sources and H₂S formation (Table 1). More detailed results of phenotypic tests and nutritional features of strain R2T and strain R27T are given in the species descriptions.

The major polar lipids of strain R2T and strain R27T were phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me) and four major glycolipids (GL1–GL4), in a pattern chromatographically identical to the polar lipid profile of Halosimplex carlsbadense JCM 11222T (Fig. S2). Among these four glycolipids, two of them (GL1 and GL4) were chromatographically identical to disulfated mannosyl glucosyl diether (S2-DGD)
Sulfated mannosyl glucosyl diether (S-DGD-1), respectively, the remaining two glycolipids (GL2 and GL3) were unidentified. This result is somewhat inconsistent with that of Vreeland et al. (2002), in which sulfated tetraglycosyl diether (S-TeGD) was one of the four major polar lipids. In this study, no polar lipid chromatographically identical to S2-DGD and S-DGD-1, respectively, the remaining two glycolipids are unidentified. The major polar lipids are PG, PGP-Me and four major glycolipids, two of them sulfated mannosyl glucosyl diether (S-DGD-1), respectively, the remaining two glycolipids (GL2 and GL3) were unidentified. This result is somewhat inconsistent with that of Vreeland et al. (2002), in which sulfated tetraglycosyl diether (S-TeGD) was one of the four major polar lipids. In this study, no polar lipid chromatographically identical to S2-DGD and S-DGD-1, respectively. Ten complete 16S rRNA gene sequences of strain R2T and eight complete 16S rRNA gene sequences of strain R27T were obtained. Sequence comparisons indicated that both strain R2T and strain R27T had two dissimilar 16S rRNA gene sequences, rRNA and rRN. In strain R2T, rRNA (1473 nt, HM159602) showed 95.7% similarity to rRNA (1472 nt, KF343756) while in strain R27T, rRNA (1473 nt, HM159603) was 93.3% similar to rRNA (1473 nt, KF343757). The rRNA sequence of strain R2T displayed 96.8% and 95.5% similarities to the rRNA and rRB sequences of strain R2T while the rRB sequence of strain R2T displayed 93.1% and 99.3% similarities to the rRN and rRB sequences of strain R27T, respectively. Strain R2T and strain R27T were related to Halosimplex carlsbadense JCM 11222T (92.7–98.8% similarities). Phylogenetic analysis using the neighbour-joining algorithm revealed that all the dissimilar 16S rRNA genes of strain R2T and strain R27T formed a tight clade clustered with Halosimplex carlsbadense JCM 11222T (Fig. 1). The phylogenetic position was also confirmed in other trees generated using the maximum-parsimony and maximum-likelihood algorithms (data not shown).

The rpoB' genes of both strains were sequenced and found to be identical in length (1827 bp), and the nucleotide sequences were 95.7% similar to each other. The rpoB' gene similarities between strains R2T and R27T and Halosimplex carlsbadense JCM 11222T were 96.1% and 95.8%, respectively. Strain R2T and strain R27T clustered with Halosimplex carlsbadense JCM 11222T and the three taxa formed a monophyletic group separated from the other genera of the family Halobacteriaceae. The phylogenetic position was also confirmed in the trees generated using the MP and ML algorithms (data not shown).

The 16S rRNA gene-based and rpoB' gene-based phylogenetic analysis results supported the placement of strain R2T and strain R27T in the genus Halosimplex. The DNA G+C contents of strains R2T and R27T were 62.5 and 64.0 mol%, respectively. These values were lower than that of Halosimplex carlsbadense JCM 11222T (64.4 mol%) (Vreeland et al., 2002). The DNA–DNA hybridization values between strains R2T and R27T and between the two strains and Halosimplex carlsbadense JCM 11222T were 43%, 52% and 47%, respectively, much lower than the accepted threshold value (70%) used to separate two species (Stackebrandt & Goebel, 1994).

It was concluded that strain R2T and strain R27T represent two novel species of the genus Halosimplex, for which the names Halosimplex pelagicum sp. nov. and Halosimplex rubrum sp. nov. are proposed. Characteristics that distinguish strains R2T and R27T from Halosimplex carlsbadense JCM 11222T are listed in Table 1.

**Emended description of the genus Halosimplex**

**Vreeland et al. 2002**

Cells are rod or pleomorphic under optimal growth conditions and Gram-stain-negative. Cells lyse in distilled water. Aerobic heterotrophs. The colonies are pink, red, small, circular, opaque, smooth, shiny and raised. Extremely halophilic, temperatures between 25 and 50 °C and pH values between 5.5 and 9.0 support growth. Can utilize pyruvate as a sole carbon source in defined medium. Can utilize glycerol as a carbon source but may require pyruvate or acetate to be present in addition. Members of the genus may metabolize sugars. The major polar lipids are PG, PGP-Me and four major glycolipids, two of them chromatographically identical to S2-DGD and S-DGD-1, the remaining two glycolipids are unidentified. The genomic DNA G+C contents are between 62.5 mol% and 64.4 mol%.

The type species is Halosimplex carlsbadense.

### Table 1. Differential characteristics between strain R2T, strain R27T and Halosimplex carlsbadense JCM 11222T

<table>
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<th>Characteristic</th>
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<td>Optimum NaCl (M)</td>
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<td>4.3</td>
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<tr>
<td>Mg²⁺ required</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Utilization of:</td>
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<tr>
<td>d-Glucose</td>
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<td>+</td>
<td>−</td>
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<tr>
<td>d-Mannose</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>d-Galactose</td>
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<td>−</td>
</tr>
<tr>
<td>Sucrose</td>
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<td>−</td>
</tr>
<tr>
<td>Lactose</td>
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<td>+</td>
<td>−</td>
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<td>−</td>
</tr>
<tr>
<td>d-Sorbitol</td>
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<td>+</td>
<td>−</td>
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<tr>
<td>Acetate</td>
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<tr>
<td>DL-Lactate</td>
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<tr>
<td>Succinate</td>
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<td>L-Malate</td>
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<td>Fumarate</td>
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<td>Citrate</td>
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<tr>
<td>L-Alanine</td>
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<tr>
<td>L-Arginine</td>
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<td>L-Glutamate</td>
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<tr>
<td>L-Ornithine</td>
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<tr>
<td>H₂S formation</td>
<td>−</td>
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<td>+</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>62.5</td>
<td>64.0</td>
<td>64.4</td>
</tr>
</tbody>
</table>
**Description of Halosimplex pelagicum sp. nov.**

*Halosimplex pelagicum* (pe.la'gi.cum. L. neut. adj. pelagicum of or belonging to the sea).

Cells are motile, pleomorphic under optimal growth conditions and Gram-stain-negative. Colonies on agar plates containing 3.4 M NaCl are red, elevated and round. Chemo-organotrophic and aerobic. Growth occurs at 25–45 °C (optimum 42 °C), at 1.4–4.8 M NaCl (optimum 3.4 M), at 0.05–0.7 M MgCl₂ (optimum 0.5 M) and at pH 5.5–9.0 (optimum pH 7.0). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis is 80 g l⁻¹. Catalase and oxidase are positive. Does not grow under anaerobic conditions with nitrate, arginine or DMSO. Nitrate reduction to nitrite and gas formation from nitrate are not observed. H₂S was not produced from sodium thiosulfate. Indole formation is negative. Does not hydrolyse starch, gelatin, casein or Tween 80. The following substrates are utilized as single carbon and energy sources for growth: D-glucose, D-mannose, D-galactose, sucrose, lactose, glycerol, D-mannitol, D-sorbitol, acetate, pyruvate, DL-lactate, succinate, L-malate, fumarate and citrate. The following substrates are utilized as single carbon, nitrogen or energy sources for growth: L-alanine, L-arginine, L-aspartate and L-ornithine. No growth occurs on D-fructose, L-sorbose, D-ribose, D-xylose, maltose, starch, glycine, L-glutamate or L-lysine. Acid is produced from D-glucose, D-mannose, D-galactose, sucrose and lactose. Sensitive to the following antimicrobial compounds (µg per disc, unless otherwise indicated): novobiocin (30), bacitracin (0.04 IU per disc), rifampicin (5), mycostatin (100), nitrofurantoin (300) and norfloxacin (10). Resistant to the following antimicrobial compounds: trimethoprim (5), erythromycin (15), penicillin G (10 IU per disc), ampicillin (10), chloramphenicol (30), neomycin (30), ciprofloxacin (5), streptomycin (10), kanamycin (30), tetracycline (30), vancomycin (30), gentamicin (10) and nalidixic acid (30). The major polar lipids are PG, PGP-Me and four major glycolipids, two of them chromatographically identical to S₂-DGD and S-DGD-1, the remaining two glycolipids are unidentified.

The type strain is R2T (=CGMCC 1.10586T=JCM 17263T) and was isolated from salted brown alga *Laminaria* produced at Dalian, Liaoning Province, China. The DNA G+C content of strain R2T is 62.5 mol% (Tₘ).
Description of Halosimplex rubrum sp. nov.

**Halosimplex rubrum** (ru’brum. L. neut. adj. rubrum red).

Cells are motile, pleomorphic under optimal growth conditions and Gram-stain-negative. Colonies on agar plates containing 3.1 M NaCl are red, elevated and round. Chemo-organotrophic and aerobic. Growth occurs at 25–45 °C (optimum 42 °C), at 0.9–3.4 M NaCl (optimum 3.1 M), at 0–0.7 M MgCl₂ (optimum 0.03 M) and at pH 5.5–9.5 (optimum pH 7.0). Cells lyse in distilled water and the minimal NaCl concentration to prevent cell lysis is 50 g L⁻¹. Catalase and oxidase are positive. Does not grow under anaerobic conditions with nitrate, arginine or DMSO. Nitrate reduction to nitrite and gas formation from nitrate are not observed. H₂S is produced from nitrate. H₂S is produced from DMSO. Nitrate reduction to nitrite and gas formation under anaerobic conditions with nitrate, arginine or DMSO.

The type strain is R27 T (CGMCC 1.1059T = JCM 7268T) and was isolated from salted brown alga *Laminaria* produced at Dalian, Liaoning Province, China. The DNA G+C content of strain R27T is 64.0 mol% (**Tₘ**).

Acknowledgements

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References


