Halomicronuclea limicola sp. nov., isolated from a marine solar saltern, and emended description of the genus Halomicronuclea

Wen-Jiao Zhang and Heng-Lin Cui
School of Food and Biological Engineering, Jiangsu University, Zhenjiang 212013, PR China

Halophilic archaeal strain YGHS32\textsuperscript{T} was isolated from the Yinggehai marine solar saltern near Sanya city of Hainan Province, China, we obtained a halophilic archaeal isolate, YGHS32\textsuperscript{T}, which was most closely related to Halomicronuclea pellucida JCM 17820\textsuperscript{T}, as judged from 16S rRNA gene sequences. The genus Halomicronuclea was first proposed by Echigo et al. to accommodate the species Halomicronuclea pellucida, which is a non-pigmented, transparent-colony-forming, halophilic archaeon isolated from a solar salt sample imported from France (Echigo et al., 2013). Halomicronuclea pellucida, the type species and only member of the genus Halomicronuclea, harbours two different 16S rRNA gene copies in its genome, with 5.1 % nucleotide divergence. 16S rRNA gene analysis revealed that Halomicronuclea pellucida JCM 17820\textsuperscript{T} was closely related to the species of the genus Haloarcula of the family Halobacteriaceae, followed by the species of genus Halomicrobium. Halomicronuclea pellucida JCM 17820\textsuperscript{T} contained phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS), sulfated mannosyl glucosyl diether (S-DGD-1) and sulfated mannosyl glycolipid diether (DGD-1), similar to those of species of the genus Halomicrobium. In contrast, members of the genus Haloarcula contained glucosyl mannosyl glucosyl diether (TGD-2) and an unknown diglycosyl diether (DGD-2) (Namwong et al., 2011; Oren et al., 2009; Yang & Cui, 2012). In this study, we characterized strain YGHS32\textsuperscript{T} as a representative of a novel species of the genus Halomicronuclea.

During our surveys of the halophilic archaeal diversity of Yinggehai marine solar saltern near Sanya city of Hainan Province, China, we obtained a halophilic archaeal isolate, YGHS32\textsuperscript{T}, which was most closely related to Halomicronuclea pellucida JCM 17820\textsuperscript{T}, as judged from 16S rRNA gene sequences. The genus Halomicronuclea was first proposed by Echigo et al. to accommodate the species Halomicronuclea pellucida, which is a non-pigmented, transparent-colony-forming, halophilic archaeon isolated from a solar salt sample imported from France (Echigo et al., 2013).

Halomicronuclea pellucida, the type species and only member of the genus Halomicronuclea, harbours two different 16S rRNA gene copies in its genome, with 5.1 % nucleotide divergence. 16S rRNA gene analysis revealed that Halomicronuclea pellucida JCM 17820\textsuperscript{T} was closely related to the species of the genus Haloarcula of the family Halobacteriaceae, followed by the species of genus Halomicrobium. Halomicronuclea pellucida JCM 17820\textsuperscript{T} contained phosphatidylglycerol (PG), phosphatidylglycerol phosphate methyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS), sulfated mannosyl glucosyl diether (S-DGD-1) and sulfated mannosyl glycolipid diether (DGD-1), similar to those of species of the genus Halomicrobium. In contrast, members of the genus Haloarcula contained glucosyl mannosyl glucosyl diether (TGD-2) and an unknown diglycosyl diether (DGD-2) (Namwong et al., 2011; Oren et al., 2009; Yang & Cui, 2012). In this study, we characterized strain YGHS32\textsuperscript{T} as a representative of a novel species of the genus Halomicronuclea.

Strain YGHS32\textsuperscript{T} was isolated from a brine sample from a crystallizer pond of Yinggehai marine solar saltern near Sanya city of Hainan Province, China (18° 31’ 52” N 108° 43’ 39” E; elevation, sea level) and the sample was stored at 4 °C during transport to the laboratory in 2010. The pH of the brine was pH 7.2 and the salinity was 226 g L\textsuperscript{-1}. Neutral haloarchaeal medium (NHM) was used for the isolation procedure, and contained the following ingredients (g L\textsuperscript{-1}): yeast extract (Oxoid) 0.05, fish peptone

**Abbreviations:** DGD-1, mannosyl glucosyl diether; DGD-2, unknown diglycosyl diether; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol phosphate methyl ester; PGS, phosphatidylglycerol sulfate; S-DGD-1, sulfated mannosyl glucosyl diether; TGD-2, glucosyl mannosyl glucosyl diether.

The GenBank/EMBL/DDBJ accession numbers for the rrnA and rrnB 16S rRNA gene and rpoB\textsuperscript{+} gene sequences of strain YGHS32\textsuperscript{T} are KF082944, JQ237128 and KF582946, respectively.

Four supplementary figures are available with the online version of this paper.
Halomicrobium Positive; JCM 17820T and the genera Halomicroarcula pellucida JCM 17820T and Haloarcula amylolytica JCM 9738T and Haloarcula pellucida CGMCC 1.5335T were selected as reference strains for a phospholipids and detection spray reagents, phosphate stain reagent for was also used in one-dimensional TLC. Two specific water (80 : 12 : 15 : 4, by vol.). The latter solvent mixture the second solvent was chloroform/methanol/acetic acid/ was chloroform/methanol/water (65 : 25 : 4, by vol.) and TLC analyses. In two-dimensional TLC, the first solvent was described previously (Cui et al., 2010). The type strains Halomicroarcula pellucida JCM 17820T, Halomicr- bium mukohataei JCM 9738T and Haloarcula amylolytica CGMCC 1.5335T were selected as reference strains for phenotypic tests. These reference strains were routinely grown aerobically at 37 °C in NHM medium. Determination of morphology and growth characteristics, nutrition, miscellaneous biochemical tests and sensitivity to antimicrobial agents were performed according to the proposed minimal standards for description of new taxa in the order Halobacteriales (Oren et al., 1997). The type strains Halomicroarcula pellucida JCM 17820T, Halomicr- bium mukohataei JCM 9738T and Haloarcula amylolytica CGMCC 1.5335T were selected as reference strains for phenotypic tests. These reference strains were routinely grown aerobically at 37 °C in NHM 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 2-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 in the two- dimensional TLC was confirmed by comparing with one- dimensional TLC in 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 a previous protocol (Cui et al., 2009). PCR-mediated amplification and sequencing of the rpoB genes were performed as described by 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 (NJ), 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 Beckman-Coulter DU800 spectrophotometer equipped with a high-performance temperature controller. DNA–DNA hybridizations were carried out according to the thermal denaturation and renaturation method (De Ley et al., 1970; Huß et al., 1983).

Cells of strain YGHS32T were motile and pleomorphic when grown in NHM liquid medium (Fig. S1, available in the online Supplementary Material). Cells stained Gram-negative and the colonies were red-pigmented. Strain YGHS32T was able to grow at 20–50 °C (optimum 37 °C), in the presence of 0.9–4.8 M NaCl (optimum 2.1 M NaCl), with 0.005–1.0 M MgCl2 (optimum 0.3 M MgCl2) and at pH 6.0–8.5 (optimum pH 7.5). The cells of strain YGHS32T lyed in distilled water and the minimum NaCl concentration that prevented cell lysis was 5 % (w/v). Strain YGHS32T grew under anaerobic conditions using nitrate, DMSO or l-arginine, but did not produce H2S from sodium thiosulfate,
did not produce indole from tryptophan and did not hydrolyse casein, gelatin, starch or Tween 80. The remarkable phenotypic characteristics differentiating strain YGHS32T from \textit{Halomicroarcula pellucida} JCM 17820T and members of the genera \textit{Halomicrobium} and \textit{Haloarcula} were: colony pigmentation, optimum NaCl concentration for growth, Mg\textsuperscript{2+} requirement, lack of gas formation from nitrate, utilization of specific substrates for growth and lack of gelatin hydrolysis (Table 1). More detailed results of phenotypic features of strain YGHS32T are given in the species description.

Fig. 1. NJ phylogenetic tree reconstructions based on 16S rRNA gene (a) and \textit{rpoB} gene (b) sequences, showing the relationships between strain YGHS32\textsuperscript{T} and related members of the family \textit{Halobacteriaceae}. Bootstrap values (%) are based on 1000 replicates and are shown for branches with more 65% bootstrap support. Bars, expected changes per site.
accession no. KF582944) showed 95.9 % similarity to the rrsB sequence (1471 nt; JQ237128). Strain YGHS32T was closely related to *Halomicroarcula pellucida* JCM 17820T (92.9–96.3 % sequence similarity). Phylogenetic tree reconstruction using the NJ algorithm revealed that the rrsA and rrsB sequences of strain YGHS32 clustered tightly with those of *Halomicroarcula pellucida* JCM 17820T and the type I and type II genes of the genus *Haloarcula* (Fig. 1a). Their phylogenetic position was also confirmed in other trees generated using the MP and ML algorithms (Figs S3a and S3b).

The rpoB gene of strain YGHS32T was sequenced and found to be 1827 bp in length. It was very similar to the corresponding gene of *Halomicroarcula pellucida* JCM 17820T (91.7 %). In the reconstructed phylogenetic tree, strain YGHS32T clustered tightly with *Halomicroarcula pellucida* JCM 17820T (Fig. 1b). This phylogenetic position was also confirmed in other trees generated using the MP and ML algorithms (Figs S3b and S4b).

The 16S rRNA gene-based and rpoB gene-based phylogenetic analysis results supported the placement of strain YGHS32T in the genus *Halomicroarcula*.

The major polar lipids of strain YGHS32T were PG, PGP-Me, PGS, and four major glycolipids (GL1, GL2, GL3 and GL4) chromatographically identical to S-DGD-1, DGD-1, TGD-2 and DGD-2 (Fig. S2). The polar lipid profile of strain YGHS32T seemed to be a combination of those of the genera *Halomicrobium* and *Haloarcula* (Namwong et al., 2011; Yang & Cui, 2012), which indicated that the polar lipid profiles of the members of the genus *Haloarcula* may be diverse. Since both strain YGHS32T and *Halomicroarcula pellucida* JCM 17820T shared the S-DGD-1 and DGD-1, it is reasonable to assign strain YGHS32T to the genus *Halomicroarcula*.

The DNA G+C content of strain YGHS32T was 64.0 mol%. The DNA–DNA hybridization value between strain YGHS32T and *Halomicroarcula pellucida* JCM 17820T was 45 %, much lower than the accepted threshold value (70 %) to separate two species (Stackebrandt & Goebel, 1994).

The phenotypic, chemotaxonomic and phylogenetic properties suggested that strain YGHS32T represents a novel species of the genus *Halomicroarcula*, for which the name *Halomicroarcula limicola* sp. nov. is proposed. Characteristics that distinguish strain YGHS32T from *Halomicroarcula pellucida* JCM 17820T are shown in Table 1.

### Emended description of the genus *Halomicroarcula* Echigo et al. 2013

Cells are pleomorphic under optimal growth conditions and stain Gram-negative. Cells lysed in distilled water. Aerobic heterotrophs forming small, non-pigmented or red-pigmented and transparent colonies on agar. Chemooorganotrophic and halophilic. The major polar lipids are PG, PGP-Me, PGS, S-DGD-1 and DGD-1. Two other glycolipids, TGD-2 and DGD-2, may be present in some species. Genomic DNA G+C contents are between 64.0 and 64.1 mol%. The type species is *Halomicroarcula pellucida*. Recommended three-letter abbreviation: Hma.

### Description of *Halomicroarcula limicola* sp. nov.

*Halomicroarcula limicola* [li.mi.co.la. L. n. limus mud; L. suff. n. -cola (from L. n. incola) dweller; N.L. masc. or fem. n. limicola mud-dweller].

Cells are motile, pleomorphic under optimal growth conditions and stain Gram-negative. Colonies on agar plates containing 2.1 M NaCl are red, elevated and round. Chemo-organotrophic and aerobic. Grows at 20–50 °C (optimum 37 °C), in the presence of 0.9–4.8 M NaCl (optimum 2.1 M NaCl), with 0.005–1.0 M MgCl2 (optimum 0.3 M MgCl2) and at pH 6.0–8.5 (optimum pH 7.5). Cells lyse in distilled water and the minimal NaCl
concentration to prevent cell lysis is 5% (w/v). Catalase and oxidase are positive. Grows under anaerobic conditions with nitrate, arginine or DMSO. Nitrate reduction to nitrite is positive but gas formation from nitrate is not observed. H₂S is not produced from sodium thiosulfate. Indole formation is negative. Does not hydrolyse casein, starch, gelatin or Tween 80. The following substrates are used as single carbon and energy sources for growth: starch, gelatin or Tween 80. The following substrates are utilized as single carbon and energy sources for growth: sucrose, glycerol, D-mannitol, D-sorbitol, acetate, pyruvate, DL-lactate and L-malate. No growth occurs on D-glucose, D-mannose, D-galactose, D-fructose, L-sorbose, D-ribose, D-xylene, maltose, lactose, starch, succinate, fumarate, citrate, glycine, L-alanine, L-arginine, L-aspartate, L-glutamate, L-lysine or L-ornithine. Sensitive to the following antimicrobial compounds: novobiocin, bacitracin, rifampicin and nitrofurantoin. Resistant to the following antimicrobial compounds: mycostatin, trimethoprim, erythromycin, penicillin G, ampicillin, chloramphenicol, neomycin, norfloxacin, ciprofloxacin, streptomycin, kanamycin, tetracycline, vancomycin, gentamicin and nalidixic acid. The major polar lipids are PG, PGP-Me, PGS, S-DGD-1, DGD-1, TGD-2 and DGD-2.

The type strain, YGHS32T (=CGMCC 1.12129T =JCM 18640T), was isolated from the Yinggehai marine solar saltern near Sanya city of Hainan Province, China. The DNA G+C content of strain YGHS32T is 64.0 mol% (Tm).

Acknowledgements

This work was supported by the National Natural Science Foundation of China (no. 31370054), the grant from China Ocean Mineral Resources R & D Association (COMRA) Special Foundation (DY125-15-R-03), the Qingshan Project of Jiangsu Province and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). We are grateful to Dr Akinobu Echigo for kindly providing strain Halomicroarcula pellucida BNERC31T (=JCM 17820T).

References


http://ijs.sgmjournals.org