Taxonomic study of the genera *Halogeometricum* and *Halosarcina*: transfer of *Halosarcina limi* and *Halosarcina pallida* to the genus *Halogeometricum* as *Halogeometricum limi* comb. nov. and *Halogeometricum pallidum* comb. nov., respectively

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Members of the haloarchaeal genera *Halosarcina* and *Halogeometricum* (family *Halobacteriaceae*) are closely related to each other and show 96.6–98 % 16S rRNA gene sequence similarity. This is higher than the accepted threshold value (95 %) to separate two genera, and a taxonomic study using a polyphasic approach of all four members of the two genera was conducted to clarify their relationships. Polar lipid profiles indicated that *Halogeometricum rufum* RO1-4ᵀ, *Halosarcina pallida* BZ256ᵀ and *Halosarcina limi* RO1-6ᵀ are related more to each other than to *Halogeometricum borinquense* CGMCC 1.6168ᵀ. Phylogenetic analyses using the sequences of three different genes (16S rRNA gene, *rpoB* and *EF-2*) strongly supported the monophyly of these four species, showing that they formed a distinct clade, separate from the related genera *Halopelagius*, *Halobellus*, *Haloquadratum*, *Haloferax* and *Halogranum*. The results indicate that the four species should be assigned to the same genus, and it is proposed that *Halosarcina pallida* and *Halosarcina limi* be transferred to the genus *Halogeometricum* as *Halogeometricum pallidum* comb. nov. (type strain, BZ256ᵀ=KCTC 4017ᵀ=JCM 14848ᵀ) and *Halogeometricum limi* comb. nov. (type strain, RO1-6ᵀ=CGMCC 1.8711ᵀ=JCM 16054ᵀ).

The genus *Halogeometricum* was proposed in 1998 to accommodate a pleomorphic strain, *Halogeometricum borinquense* PR3ᵀ, isolated from the solar salterns of Cabo Rojo, Puerto Rico (Montalvo-Rodríguez et al., 1998). The phenotypic features and its phylogenetic position indicated that it was distinct from related genera, and a major glycolipid GLb (Cui et al., 2010c) detected in *Halogeometricum borinquense* PR3ᵀ served as the characteristic glycolipid for describing this genus. In 2008, the novel genus *Halosarcina* was proposed to accommodate a sarcina-shaped strain, BZ256ᵀ, that showed 96.8 % 16S rRNA gene sequence similarity to *Halogeometricum borinquense* PR3ᵀ and contained a major glycolipid (S-DGD-1) that was chromatographically different from GLb (Savage et al., 2008; Cui et al., 2010b). The species *Halogeometricum rufum* RO1-4ᵀ and *Halosarcina limi* RO1-6ᵀ were described two years later, and emended descriptions of the genera *Halogeometricum* and *Halosarcina*, including the polar lipid profiles, were reported (Cui et al., 2010b; Cui et al., 2010c). However, the four members of the genera *Halosarcina* and *Halogeometricum* are closely related to each other, showing 96.6–98 % 16S rRNA gene sequence identity, a level that is higher than the ‘lower cut-off’ value (95 %) reported by Yarza et al., (2008) and that generally marks genus-level boundaries in prokaryotes. To elucidate the taxonomy of these genera, the type strains *Halogeometricum borinquense* CGMCC 1.6168ᵀ, *Halogeometricum rufum* RO1-4ᵀ, *Halosarcina pallida* BZ256ᵀ and *Halosarcina limi* RO1-6ᵀ were directly compared in a polyphasic taxonomic study.

Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Halogeometricum rufum* RO1-4ᵀ, *Halosarcina pallida* BZ256ᵀ and *Halosarcina limi* RO1-6ᵀ are EU887286, HM185493 and EU931577, respectively. Those for the *rpoB* sequences are JN180928, JN180932 and JN180931, respectively. Those for the *EF-2* gene sequences of *Halogeometricum rufum* RO1-4ᵀ and *Halosarcina limi* RO1-6ᵀ are KC906701 and KC854795, respectively.

A supplementary figure and a supplementary table are available with the online version of this paper.
The strains were routinely grown aerobically at 37 °C in NOM medium (Cui et al., 2011a). Phenotypic tests were performed according to the proposed minimal standards for description of new taxa in the order Halobacteriales (Oren et al., 1997). *Halobacterium jilantaiense* NG4^T^, *Halofex rufum* vulcanii CGMCC 1.2150^T^ and *Halaoarucla marismortui* CGMCC 1.1784^T^ were used as reference strains. Various tests relating to morphology and growth characteristics, nutrition, miscellaneous biochemical activities and sensitivity to antimicrobial agents were performed as described previously (Cui et al., 2012).

Other than the type strains of genera *Halogoeometricum* and *Halosarcina*, three other strains belonging to the *Halogeoeometricum/Halosarcina* cluster, strains RO3-11, HO1-4 and GSL-24, were also included in the analysis of polar lipid composition. Polar lipids were extracted using a chloroform/methanol system and analysed using one- and two-dimensional TLC, as described previously (Cui et al., 2009a). Merck silica gel 60 F254 aluminium-backed thin-layer plates were used in TLC analysis. 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 was also used in one-dimensional TLC. Two specific detection spray reagents were used; phosphate stain reagent for phospholipids and naphthol stain for glycolipids. The general detection reagent, sulfuric acid/ethanol (1 : 2, v/v) was used to detect total polar lipids.

Genomic DNAs from halophilic archaeal strains were prepared as described previously (Cui et al., 2011b). The 16S rRNA genes were amplified, cloned and sequenced according to a previously described protocol (Cui et al., 2009). PCR-mediated amplification and sequencing of the *rpoB* genes were carried out as described previously (Minegishi et al., 2010). The EF-2 genes were amplified and sequenced using the primer pair EF-2f (5'-ATGGYMGACGHAAAGA-3') and EF-2r (5'-GCGBGRRCCRGGTGGAT-3'). These primers were designed (this study) using aligned genomic sequences encoding the EF-2 genes from 26 genera of the family *Halobacteriaceae* (sequences downloaded from the GenBank database). 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 the neighbour-joining, maximum-parsimony (MP) and maximum-likelihood (ML) algorithms in the MEGA 5 software. Gene sequence similarity values were calculated using the pairwise-distance computing function of MEGA 5.

*Halogeometricum borinquense* CGMCC 1.6168^T^, *Halogeometricum rufum* RO1-4^T^, *Halosarcina pallida* BZ256^T^ and *Halosarcina limi* RO1-6^T^ shared similar phenotypic features. They stained Gram-negative, required Mg^{2+} for growth, grew best at mesophilic temperatures (25–45 °C) and over the pH range 6–8. They did not hydrolyse starch, did not produce H_{2}S from sodium thiosulfate, were sensitive novobiocin, bacitracin, rifampicin, mycostatin and nitrofurantoin and resistant to trimethoprim, erythromycin, ampicillin, penicillin G, chloramphenicol, neomycin, ciprofloxacin, streptomycin, kanamycin, vancomycin, norfloxacin, tetracycline, gentamicin and nalidixic acid. They utilized D-glucose, D-galactose, sucrose, glycerol, acetate, pyruvate, L-alanine and L-glutamate, but did not use D-ribose, D-mannitol, citrate, L-aspartate or L-ornithine.

The main phenotypic characteristics differentiating the four species from each other were: cell shape, motility, colony colour, optimum NaCl, optimum Mg^{2+}, growth temperature and pH, anaerobic growth with nitrate, gas formation from nitrate, nitrate reduction, indole formation, casein hydrolysis, gelatin liquefaction and utilization of specific carbon sources (Table 1). The differential phenotypic characteristics of the four species clearly distinguish between them at the species level but they are not helpful regarding genus-level relationships.

The lipids of all strains were examined by one- and two-dimensional TLC, and the results are presented in Fig. S1 and summarized in Table S1 available in IJSEM Online. The common polar lipids phosphatidylglycerol and phosphatidylglycerol-phosphate methyl ester were found in all four strains, as were glycolipids GL1 and GL3. Three other glycolipids (GL2, GL4 and GL5) were present in three of the strains (*Halogeometricum rufum* RO1-4^T^, *Halosarcina pallida* BZ256^T^ and *Halosarcina limi* RO1-6^T^) but not in *Halogeometricum borinquense* CGMCC 1.6168^T^. The glycolipid GLb is the major polar lipid of *Halosarcina limi* RO1-6^T^ and a chromatographically identical lipid was detected (at low levels) in two other strains (*Halogeometricum rufum* RO1-4^T^ and *Halosarcina limi* RO1-6^T^: Fig S1(i,iii)). Two other lipids, P1 and P2, are distributed in an overlapping pattern across the strains, with *Halogeometricum rufum* RO1-4^T^ having both lipids, while each of the other strains has only one of them. The polar lipids profiles of strains RO3-11, HO1-4 and GSL-24 are similar to that of *Halosarcina pallida* BZ256^T^ [Fig. S1(iv)]. The summary table (Table S1) more clearly indicates those lipids shared by all strains (GL1 and GL3), those shared by three of the strains (GLb, GL2, GL4, GL5 and P1), or only two of the strains (P2). The shared lipids observed in these strains would be consistent with them being members of the same genus, although phylogenetic methods are needed to accurately determine this.

The 16S rRNA gene sequence similarities of the seven species ranged from 96.4 % to 99.5 %. All of these values are above the recently described threshold of 95 % for delineating prokaryotic genera (Yarza et al., 2008; Tindall et al., 2010). Phylogenetic tree reconstructions based on 16S rRNA gene sequences revealed that these species formed a tight cluster, with high bootstrap confidence and were distinct from the related genera, *Halobellus*, *Haloquadrum*, *Halopegalus* and *Halofex* (Fig. 1a). The members of the genera *Halogeometricum* and *Halosarcina* did not branch as distinct monophyletic clades related to their currently assigned
genera, but specifically clustered together as a paraphyletic group, indicating that these taxa are phylogenetically related at the genus level. This phylogenetic position was also supported by tree reconstructions generated using the MP and ML algorithms (not shown).

A recent taxonomic study of the Halobacteriaceae has proposed that a similarity value less than 86.2% between rpoB genes can be used to distinguish genera (Minegishi et al., 2010). The rpoB genes of the four type strains, *Halogeometricum borinquense* CGMCC 1.6168<sup>T</sup>, *Halogeometricum rufum* RO1-4<sup>T</sup>, *Halosarcina pallida* BZ256<sup>T</sup> and *Halosarcina limi* RO1-6<sup>T</sup>, +, Positive; −, negative.

| Characteristic | 1 | 2 | 3 | 4 |
|----------------|---|---|---|---|
| Cell shape     | Pleomorphic | Pleomorphic | Coccus | Pleomorphic |
| Motility       | + | + | − | + |
| Colony colour  | Pink | Red | Pink | Red |
| Optimum NaCl (M) | 3.4–4.3 | 3.9 | 3.1 | 3.9 |
| Optimum Mg<sup>2+</sup> (M) | 0.04–0.08 | 0.3 | 0.1–0.3 | 0.3 |
| Optimum temperature (°C) | 40 | 40–42 | 30 | 37 |
| Optimum pH     | 7.0 | 7.0 | 6.5 | 7.0 |
| Anaerobic growth with nitrate | + | − | − | − |
| Gas formation from nitrate | + | − | − | − |
| Nitrate reduction | + | + | − | + |
| Indole formation | + | + | + | − |
| Casein hydrolysis | + | − | − | − |
| Gelatin liquefaction | + | − | − | − |
| Utilization of: | | | | |
| D-Mannose | + | + | − | + |
| D-Fructose | + | + | − | − |
| D-Xylose | + | − | − | − |
| Malose | + | + | + | − |
| Lactose | + | + | − | + |
| DL-Lactate | + | + | − | + |
| DNA G+C content (mol%) | 59.1 | 64.9 | 65.4 | 61.2 |

This polyphasic taxonomic study has provided clear evidence that the four species are sufficiently related that they should belong to the same genus. On the basis of these results, we propose that *Halosarcina pallida* and *Halosarcina limi* be transferred to the genus *Halogeometricum* as *Halogeometricum pallidum* comb. nov. (type strain, BZ256<sup>T</sup>=KCTC 4017<sup>T</sup>=JCM 14848<sup>T</sup>) and *Halogeometricum limi* comb. nov. (type strain, RO1-6<sup>T</sup>=CGMCC 1.8711<sup>T</sup>=JCM 16054<sup>T</sup>).

**Description of *Halogeometricum limi* comb. nov.**

*Halogeometricum limi* (li’mi. L. gen. n. *limi* of/from mud).

Basonym: *Halosarcina limi* Cui et al. 2010.

The description is identical to that of *Halosarcina limi* given by Cui et al. (2010b). The type strain is RO1-6<sup>T</sup> (=CGMCC 1.8711<sup>T</sup>=JCM 16054<sup>T</sup>).

**Description of *Halogeometricum pallidum* comb. nov.**

*Halogeometricum pallidum* (pal’li.dum. L. neut. adj. *pallidum* pale).
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(a) Halogeometricum rufum RO1-4T (EU887286)
   Strain GSL-24 (KF286650)
   Strain RO3-11 (FJ944014)
   Halogeometricum borinquense DSM 11551T (NC_014729)
   Halosarcina pallida B2256T (HM185493)
   Strain HO1-4 (KF286651)
   Halosarcina limi RO1-6T (EU931577)
   Halobellus clavatus TNN18T (GQ282620)
   Halopelagius inordinatus RO5-2T (EU887284)
   Haloferax volcanii DS2T (NC_013967)
   Halogranum rubrum RO2-11T (EU887283)
   Haloplanus natans JCM 14081T (JN206648)
   Haloquadratum walsbyi C23T (HM165235)
   Halorubrum saccharovorum JCM 8865T (U17364)
   Methanospirillum hungatei JF-1T (NC_007796)

(b) Halogeometricum rufum RO1-4T (JN180928)
   Strain GSL-24 (KF318212)
   Strain RO3-11 (KF318214)
   Halogeometricum borinquense DSM 11551T (NC_014729)
   Halosarcina limi RO1-6T (JN180931)
   Halosarcina pallida B2256T (JN180932)
   Strain HO1-4 (KF318213)
   Halopelagius inordinatus RO5-2T (JN180929)
   Haloferax volcanii JCM 8879T (AB477170)
   Halobellus clavatus TNN18T (JN180927)
   Haloplanus natans JCM 14081T (JN180923)
   Haloquadratum walsbyi C23T (JN180930)
   Halorubrum saccharovorum JCM 8865T (AB477184)
   Haloplanus natans JCM 14081T (AB477174)
   Halopelagius inordinatus RO5-2T (JN180929)
   Haloplanus natans JCM 14081T (AB477174)
   Halorubrum saccharovorum JCM 8865T (AB477184)
   Methanospirillum hungatei JF-1T (NC_007796)

(c) Halogeometricum rufum RO1-4T (KC906701)
   Strain GSL-24 (KF318215)
   Strain RO3-11 (KF318216)
   Halosarcina pallida JCM 14848T (AOIV01000026)
   Strain HO1-4 (KF318217)
   Halogeometricum borinquense DSM 11551T (NC_014729)
   Halosarcina limi RO1-6T (KC854795)
   Halopelagius inordinatus RO5-2T (KC906702)
   Haloferax volcanii DS2T (NC_013967)
   Halogranum rubrum RO2-11T (KC906703)
   Haloplanus natans JCM 14081T (KC906705)
   Halorubrum saccharovorum JCM 8865T (AOJE01000057)
   Halobellus clavatus TNN18T (KC906704)
   Haloquadratum walsbyi C23T (NC_017459)
   Methanospirillum hungatei JF-1T (NC_007796)
Halosarcina pallida Savage et al. 2008.

The description is identical to that of Halosarcina pallida given by Savage et al. (2008). The type strain is BZ256T (=KCTC 4017\(^\text{T}\))=JCM 14848\(^\text{T}\).

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