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Halococcoides cellulosivorans gen. nov., sp. nov., an extremely halophilic cellulose-utilizing haloarchaeon from hypersaline lakes

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Running title: *Halococcoides cellulosivorans* gen. nov., sp. nov.

The GenBank accession number of the whole genome sequences of strain HArcel1^T is CP028858

An extremely halophilic euryarchaeon, strain HArcel1^{T}, was enriched and isolated in pure culture from the surface brines and sediments of hypersaline athalassic lakes in the Kulunda Steppe (Altai region, Russia) using amorphous cellulose as the growth substrate. The colonies of HArcel1^{T} are pale-orange, and form large zones of cellulose hydrolysis around them. The cells are nonmotile cocci of variable size with a thin monolayer cell wall. The isolate is an obligate aerobic heterotroph capable of growth with only 3 substrates: various forms of insoluble cellulose, xylan and cellobiose. Strain HArcel1^{T} is an extremely halophilic neutrophile, growing within the salinity range from 2.5 to 5 M NaCl (optimum at 3.5-4 M). The core archaeal lipids are dominated by C_{20}-C_{20} and C_{25}-C_{20} dialkyl glycerol ethers (DGE), in approximately 6:1 proportion. The phylogenetic analysis based on 16S rRNA gene, *rpo*B' gene and the ribosomal proteins indicated that strain HArcel1^{T} forms a separate genus-level lineage within the family *Haloarculaceae*, order *Halobacteriales*, with the genera *Halorhabdus* and *Halopricus* as closest relatives. This is also in line with the ANI and DDH values being far below the intragenus level. On the basis of the unique phenotypic properties and distinct phylogeny based on multiple conservative markers, it is suggested that strain HArcel1^{T} is classified into a new genus and species, *Halococcoides cellulosivorans* gen. nov., sp. nov. (JCM 31941^{T}=UNIQEM U975^{T}).

Abbreviations

DGE, Dialkyl glycerol ether
MGE, monalky glycerol ether
PG, phosphatidyl glycerol
PGS, phosphatidyl glycerol sulfate
PGP-Me, Phosphatidylglycerophosphate methylester
DG, diglycosyl diether
TGD, triglycosyl diether

Extremely halophilic euryarchaea of the class *Halobacteria* form dense blooms in inland salt lakes and sea solar salterns with salt concentrations close to saturation. Most of the cultured species are aerobic heterotrophs, utilizing simple soluble organic monomers, such as sugars and organic acids, or complex rich amino acid-based substrates, such as various peptons and yeast extract [1-6].

The polymer mineralizing function at hypersaline conditions is usually attributed to halophilic bacteria [3-4]. There are only few published examples of the utilization of polymeric substances, such as starch, proteins or olive oil, as growth substrates among the haloarchaeal species [7-11]. In particular, nearly nothing is known about the ability of haloarchaea to hydrolyze and utilize insoluble recalcitrant polysaccharides, such as cellulose or chitin, for growth. The glycosidase genes encoding putative cellulases (GH family 3, 5 and 9) are present in many haloarchaeal genomes (*Haloarcula, Halobacterium, Halalkalicoccus, Haloferax, Halorhabdus, Halovivax, Halostagnicola, Haloterrigena-Natrinema* group*, Natronococcus)*, while the presence of functional beta-1,4 endoglucanases has been, to date, demonstrated only in two genera of neutrophilic haloarchaea, i.e. *Haloarcula and Halorhabdus* [12-14]. However, it remains to be investigated whether these haloarchaea are actually capable of using native forms of cellulose as carbon and energy source.

So far, only two studies have focused on the functional aspect of cellulose degradation by haloarchaea [15-16]. In those works we were able, for the first time, to enrich and isolate in pure culture a number of haloarchaeal strains utilizing various forms of native insoluble cellulose as carbon and energy source both in neutral and alkaline saturated salt brines. The cellulotrophic natronoarchaea from hypersaline alkaline lakes included 2 subgroups: two strains with relative weak cellulase activity, belonging to a known species *Natronolimnobius baerhaense* (for which the capacity for cellulose hydrolysis had not previously been demonstrated) [15] and six strains with high cellulose-degrading capacity described recently as *Natronobiforma cellulositropha* gen. nov., sp. nov. [16]. The group of neutrophilic cellulotrophic haloarchaeal isolated from various hypersaline chloride-sulfate lakes, included *Halomicrobium* sp. strain HArel3, *Halosimplex* sp. strain HArcel2 and a novel lineage, strain HArcel1^T [15]. In this paper we describe the phenotypic and phylogenetic properties of strain HArcel1^T and suggest its assignment into a novel genus and species *Halococcoides cellulosivorans*.

Surface sediments and near-bottom brines from 3 hypersaline lakes in Kulunda Steppe (Altai region, Russia) with salt concentration of 280-350 g l^{-1} and pH from 7.5-8.1 were used to enrich for cellulotrophic haloarchaea [15]. The brine-sediment slurries from three lakes were mixed, homogenized by vortexing and the resulting mix was briefly centrifuged at low speed to remove the course sediment fraction, while the remaining colloidal fraction was used as an inoculum.

The basic mineral medium used for the enrichment and cultivation of haloarchaea contained (in g l^{-1}): 240 NaCl, 5 KCl, 0.25 NH_4Cl and 3 of K_2HPO_4/KH_2PO_4, pH 6.8. After sterilization, the base was supplemented with vitamin and trace metal mix [17], 1 mM $MgSO_4$, 20 mg l^{-1} yeast extract and 10 mM filter-sterilized $NaHCO_3$. Various forms of insoluble cellulose obtained from Sigma or synthesized as described previously (amorphous cellulose, [15]) were used as the only carbon and energy source at a final concentration of 1 g l^{-1}. For the enrichment, 1 ml of colloidal sediments was used to inoculate 20 ml medium containing 1 g l^{-1} of amorphous cellulose in 100 ml closed serum bottles placed on a rotary shaker at 37°C and at 120 rpm. The development of cells was monitored by the visual extent of cellulose degradation, the appearance of pink-orange color and by microscopy. After visible cellulose degradation and cell growth (30-40 days), the culture was serially diluted in the same medium and the maximal positive dilutions were plated onto a solid medium prepared by mixing the liquid medium (with additional solid NaCl addition to compensate for dilution with agar) and 5% extensively washed agar 3:2 at 55^{o}C. The plates were incubated at 37^{o}C in closed plastic bags for 40-60 days. The appearance of colored colonies with large clearance zones was used as an indicator of growth of cellulolytic haloarchaea. It needs to be stressed here, that such colonies were never dominating on the plates, even obtained from final positive serial dilutions, indicating a presence of high proportion of satellites probably feeding on the cellulose hydrolysis products. The cellulolytic colonies (**Fig. 1a**) were transferred to the liquid medium with amorphous cellulose and the positive cultures were further purified by several rounds of plating-liquid culture cultivation with amorphous cellulose. This yielded 3 pure cultures of cellulotrophic haloarchaea with identical 16S-rRNA gene sequence, of which strain HArcel1^T was chosen for further characterization.

The phase contrast microscopy was done using the Zeiss Axioplan Imaging 2 microscope (Göttingen, Germany). For the electron microscopy of thin sections, the cells of strain HArcel1^T grown with amorphous cellulose were fixed in 1% (w/v) OsO_4 containing 3.0 M NaCl for 1 week at 4^{o}C, washed and resuspended in 3 M NaCl, stained overnight with 1% (w/v) uranyl acetate, dehydrated in ethanol series, and embedded in Epon resin. After thin sectioning, the preparations were post-stained with 1% (w/v) lead citrate and examined using the JEOL-100 model of TEM (Japan),

Cells of HArcel1^T were non-motile cocci of variable size from 0.8 to 3 μm (**Fig. 1b**). During the first stage of growth on insoluble celluloses most of the cells aggregated with cellulose particles/fibres (**Fig. 1c**), while free cells appeared only after massive cellulose hydrolysis. Electron microscopy revealed the presence of a large nucleoid and a thin, single layer cell wall, typical for many haloarchaeal species (**Fig. 1d**). The cells lyzed after resuspension in solutions containing less than 10% NaCl.

Genomic DNA was isolated by ISOLATE II Genomic DNA Kit (Bioline Reagents, UK) according to manufacturer’s instructions. Fragment genomic libraries were prepared from 1 μg of genomic DNA with NEBNext Ultra DNA library preparation kit (New England Biolabs, Ipswich, MA, USA) according to manufacturer's instructions to obtain mean library size of 600 - 700 bp. The library was sequenced with MiSeq™ Illumina Inc. (Illumina Inc., San Diego, CA, USA) using paired-end 250-bp reads. After sequencing all reads were subjected to stringent quality filtering and trimming with CLC Genomics Workbench 10.0 (Qiagen, Germany). Sequencing adapters were trimmed with SeqPrep tool (https://github.com/jstjohn/SeqPrep). Finally, 925,497 read pairs were used for *de novo* assembly. Reads were assembled with SPADES 3.10.0 [18]. Initial assembly consisted of 166 scaffolds of total length 2,793,855 nt and N50 of 2,525,738 nt. In parallel, reads were assembled with MIRA 4.0.2 genome assembler [19], resulting in assembly of total length 2,726,789 nt and N50 43612 nt. After manual curation and comparison of two assemblies using CLC Genomics Workbench 10.0 software (Qiagen, Germany) circular ungapped chromosome of strain HArcel1^T was obtained. Total length of the strain HArcel1^T chromosome is 2,723,120 bp, GC-content is 65.74%. Validation of an assembly was performed by analysis of mapping of all obtained reads back to chromosome sequence performed with CLC Genomics Workbench (Qiagen, Germany). 99.76% of reads were mapped resulting in final genome coverage of 88.3 ± 22.6 x. Additionally, integrity of the assembly was checked by the analysis of unaligned read ends with InDel analysis tool of CLC Genomics Workbench (Qiagen, Germany). No regions, significantly enriched by partially aligned reads were found. Due to these results our genomic assembly can be considered as finalized complete genome sequence. Annotation with IMG/ER server pipeline [20] resulted in prediction of 2,641 protein-coding genes, 60 tRNA genes and one complete rRNA operon. Genomic assembly and related metadata have been deposited in NCBI database under accession numbers XCP028858, PRJNA449302, SAMN08826612 for the genomic assembly, Bioproject and Biosample, respectively.

16S rRNA and rpo*B′* gene sequences were obtained from the draft genome assemblies of strain HArcel1^T. The phylogenetic analysis was performed in Mega 7 package [21]. The 16S rRNA gene sequences of all species of the *Halobacteriales* order with validly described names obtained from the Genbank were aligned together with the complete sequence of strain HArcel1^T using G-INS-i method in MAFFT server v7 [22]. The phylogenetic analysis was performed using Maximum Likelihood algorithm and the General Time Reversible (GTR) model (*G+I*, 4 categories) [23]. The *rpo*B'-based phylogenetic analysis, was performed the same way as for 16S rRNA gene. For ribosomal proteins phylogenetic analysis of 17 single-copy conserved ribosomal protein sequences (S2, S3, S11, S12, S17, S19, L3, L4, L5, L10, L11, L13, L14, L15, L23, L24. L29) were obtained from 39 available in IMG/M-ER [20] genomes of *Halobacteriales* representatives with *Natronomonas* as an outgroup. The protein sequences were aligned in MAFFT v7 [22] using L-INS-i algorithm and then concatenated using FaBox joiner alignment [24]. Phylogenetic tree based on concatenated alignment of the proteins was constructed using Maximum Likelihood method and the LG model (G + *I*, 4 categories) [25].

BLAST of strain HArcel1^T 16S rRNA gene against nucleotide sequences from cultured haloarchaeal species revealed *Halorhabdus* species and *Halapricum salinum* being the closest relatives with 94.0-92.9 and 92.5 % sequence identity, respectively. This level of relation indicates a separate genus status. Further phylogenetic analysis based of the 16S rRNA gene comparison demonstrated that strain HArcel1^T forms a separate lineage within the family *Haloarcelaceae* [26] with the genera *Halorhabdus* and *Halapricus* as the closest relatives (**Fig. 2 a**). Since the divergence point of “strain HArcel1-*Halorhabdus*” and *Halapricum* clusters was not supported by bootstrap test, the additional markers (*rpoB*` gene and ribosomal proteins) were used to infer phylogenetic position of strain HArcel1^T (**Fig 2 b, c**). The results support a separation of strain HArcel1^T, *Halorhabdus* and *Halapricum* in a distinct cluster, whereby strain HArcel1^T forms a longest branch suggesting its novel genus level.

Pairwise ANI comparison was performed using IMG built-in tool [27]. The calculated ANI values were 74.1 % between strain HArcel^T and *Halapricum salinum*; 74.8 % between strain HArcel^T and *Halorhabdus utahensis*; 75.1 % between strain HArcel^T and *Halorhabdus tiamatea*

(**Table 1**). For digital DDH we used the Genome-to-Genome Distance Calculator 2.1 (GGDC) [28]. BLAST+ was selected as local alignment tool and three formula were used: 1 – length of all HSPs divided by total genome length, 2 – sum of all identities found in HSPs divided by overall HSP length (recommended) and 3 - sum of all identities found in HSPs divided by total genome length The average *in silico* DDH values calculated from the 3 formulas between strain HArcel1^T and *Halapricum salinum*, *Halorhabdus utahensis* and *Halorhabdus tiamatea* were 15.7, 16.4 and 16.6 %, respectively (**Table 1**). Thus the calculated values of both ANI and DDH were significantly below the recognized species separation (96% and 70%, respectively), [29].

Taken together, the phylogenetic analysis and genome-based comparison demonstrated a separate genus-level status of strain HArcel1^T within the *Haloarculaceae* family.

The core membrane lipids were obtained by acid hydrolysis (5% HCl in methanol by reflux for 3 h) of the freeze-dried cells and subsequent analysis by HPLC-MS for GDGTs and archaeol derivatives according to [30]. Intact polar lipids were obtained by Bligh Dyer extraction of freeze-dried cells and subsequent HPLC-MS analysis as described in [31].

The core membrane lipids were dominated by archaeol [C_{20}-C_{20} dialkyl glycerol ether (DGE), 81% of the total] with lesser amounts of extended archaeol (C_{20}-C_{25} DGE, 13% of the total). Traces of the monoglycerol ether (MGE) lipids (1-C_{20} MGE, 2-C_{20} MGE, and 2-C_{25} MGE) were also detected. The intact polar lipid profile (identified using multistage mass spectrometry) was quite complex, including (in order of abundance) phosphatidylglycerophosphate methylester (PGP-Me), phosphatidylglycerol (PG), a sulfophospholipid with an unknown sulfur-containing headgroup, a diglycosyl (2GL), phosphatidylglycerophosphate (PGP) and phosphatidylglycerosulfate (PGS) (**Supplementary Fig. S1**). When compared with the two closest phylogenetic neighbours (**Table 2**), only first two most abundant lipids were present in all 3 species: phosphatidylglycerophosphate methylester (PGP-Me) and phosphatidylglycerol (PG). These phospholipids are most common in the members of *Halobacteria* and, in particular, the domination of the PGP-Me is considered to be related to extreme salt tolerance [32]. The less abundant lipids in strain HArcel1^T included a glycolipid phosphatidyldiglycoside (2GL) and 2 sulfolipids. Lipids belonging to the glycolipid and sulfolipid classes are also present in the two closest relatives of HArcel^T. For example, the closest relative, *Halorhabdus tiamatea*, contains a three glycosyl (3GL) glycolipid and a monosulfated diglycosyl diether (S1-DGD) sulfolipid. It is probable that the structurally homologues different glyco- and sulfolipids play a similar function in maintaining membrane homeostasis at extreme salinity [33-34] (Kates 1992; Oger 2013). Sulfolipids are also commonly found in neutrophilic haloarchaea, and in particular in the members of the family *Haloarculaceae* [26].

Strain HArcel1^T is an obligately aerobic saccharolytic haloarchaeon. Anaerobic growth with cellobiose as substrate was tested in 10 ml liquid cultures placed into 23 ml serum bottles, closed with butyl rubber stoppers and made anoxic by sterile evacuation-flushing with argon. The results were negative either for fermentation, or with elemental sulfur, thiosulfate, DMSO, TMA and nitrate as *e*-acceptors. During aerobic growth, strain HArcel1^T utilized only three substrates as their carbon and energy source: insoluble celluloses with different degree of crystallinity, including an amorphous form, Sigma celluloses, filter paper; xylan (from birch wood) and cellobiose. Weak and irregular growth was noticed with lichenan (beta-1,4/-1,3 glycan). No growth was detected with the following polysaccharides: CMC, beta 1,3/1,6 and alpha glucans, beta-mannan, beta-galactan, chitin, chitosan, pectin; heteropolysaccharides, such as beta gluco- and galacto- mannans, alginate. The soluble sugar compounds tested negative included glucose, fructose, galactose, mannose, arabinose, rhamnose, N-acetylglucosamine, glucosamine, glucuronic and galacturonic acids, maltose, lactose, trehalose, melibioze, melizitose, xylose, ribose, sorbitol, mannitol and glycerol. Likewise, no growth was observed with organic acids (C_2-C_{10} fatty acids, lactate, pyruvate, malate, succinate, fumarate) and complex organic amino acid substrates, such as various peptons and yeast extract. The extremely narrow specialization on cellulose polymers of the neutraphilic haloarchaeon HArcel1^T is only a second example among known species of haloaerchae, resembling its recently described alkaliphilic counterpart *Natronobiforma cellulositropha* found in various hyperslaine soda lakes [16].

Recommended enzymatic activity tests [35] included plate assays for amylase (soluble starch), protease (casein, gelatin), esterase (tributyrin) and lipase (emulsified olive oil) using a low background of cellobiose (1 mM). Amylase activity was detected by flooding the plate with Lugol solution, for protease activity the plate was flooded with 10% TCA to denature undegraded protein, while esterase and lipase activities are evident from the visual clearance of turbid background around the colonies. All of these activities were negative. Strain HArcel1^T was strongly catalase positive (colony test with 3% H_2O_2), but only weak-positive in the oxidase activity (colony test with 1% tetramethylphenyldiamine hydrochloride on filter paper). Sulfide formation from thiosulfate or sulfur during aerobic growth with cellobiose (lead acetate paper test) and indole formation from tryptophan (Kovac's reagent test, [36]) were all negative. While growing with cellobiose, strain HArcel1^T used only ammonium salts as the N-source (urea, nitrate, nitrite were negative).

The salt profile for growth in strain HArcel1^T culture was investigated using cellobiose as the substrate in medium buffered at pH 7 with potassium phosphate buff in liquid culture incubated at 37^{o}C. Growth was observed within NaCl range from 2.5 to 5 M with an optimum at 3.5-4 M. The pH for growth with cellobiose at 4 M NaCl was investigated within the range from 5 to 9 using a combination of HEPES (4 g l^{-1}) and potassium phosphates (5 g l^{-1} in total) as buffers for the pH range from 5 to 8 and a combination of potassium phosphates and 0.5 M Na_2CO_3 for the pH 8.5-9. The pH during growth was also maintained either by adding CO_2 into the gas phase (to decrease the actual pH) or 1 M filter-sterilized $NaHCO_3$ (to increase the actual pH). Strain HArcel1^T was able to grow within the pH range of 6.5-8.0 with an optimum at 7.0-7.2. Based on the data, the isolate can be classified as an extremely halophilic neutrophile. At pH 7 and 4 M NaCl, the strain grew equally well at Mg concentrations from 1 to 20 mM, thus belonging to a low Mg-requiring type. The temperature profiling during growth on cellobiose at pH 7 and 4 M NaCl was done starting from 20 and up to 60^{o}C with an increment of 5^{o}C. The growth was possible from 25 to 50^{o}C with an optimum between 40 and 45^{o}C.

Antibiotic resistance of strain HArcel1^T was tested at optimal growth conditions in liquid culture using cellobiose as substrate. The following antibiotics (100 mg l^{-1}) did not inhibit growth: penicillin G, ampicillin, kanamycin, streptomycin, erythromycin, gentamicine and vancomicin. No growth was observed in presence of chloramphenicol and rifampicin at concentrations above 50 and 30 mg l^{-1}, respectively.

A phenotypic comparison of strain HArcel1^T with the closest haloarchaeal relatives from *Haloarcelaceae* is shown in **Table 2.** Interestingly, the closest relatives of HArcel1^T, the *Halorhabdus species*, are apparent polysaccharide degraders, according to the presence of multiple GH genes in the genome and activity tests in *H. tiamatea* [14, 37] and the proven ability of *H. utahensis* to grow with xylan [38]). Our tests with the type strain of *H. tiamatea* JCM 14471^T and also with our own isolates closely related to this species demonstrated that these haloarchaea are, indeed, potent polysaccharide degraders capable of growth with a range of glycans as sole source of carbon and energy (**Table 2**). Especially interesting is the ability (albeit weak with never a complete utilization) of *H. tiamatea* to grow with beta-1,4 mannan. So far, only two such cases have been found among the extremely halophilc euryarchaea - in *Natronoarchaeum mannanilyticum* and recently described cellulose-utilizing *Natronobiforma cellulositropha* [16, 39]. However, the major difference between the *Halorhabdus* species and strain HArcel1^T is the ability of the latter to use cellulose as growth substrate : none of the tested forms of insoluble celluloses with different degree of crystallinity, including amorphous, four types of Sigma celluloses, filter paper and Avicell, supported growth of *H. tiamatea*. On the other hand, tests on CMC plates showed a presence of beta-1,4 endoglucanase activity in colonies of *H. tiamatea*. This is another demonstration, that what is often claimed on the basis of test with soluble artificial analogue of cellulose (CMC) as the ability to grow with cellulose should not be considered as valid. Since the genome of another closest relative of strain HArcel1^T, *Halapricum salinum* [40], completely lacks genes encoding the GH-family glycosidases, it might be concluded, that it differs significantly in its key physiological specialization, most probably being an ordinary saccharolytic utilizing products of polymer hydrolysis. Taking into account that three other members of the family *Haloarculaceae* - the genera *Haloarcula*, *Halomicrobium* and *Halosimplex* do have species with confirmed ability to degrade glycans, including cellulose [12-13, 15] and chitin (*Halomicrobium*) [15], it might be speculated that such potential has already been acquired in the common ancestor of this radiation of *Halobacteria* but lost later on in some members, such as *Halapricum*, and proliferated in the others, of which strain HArcel1^T seems to be the most narrowly specialized. Further phylogenomic reconstructions might be able to substantiate this interesting question.

In conclusion, strain HArcel1^T is the first example of an extremely halophilic euryarchaeon directly enriched and isolated from hypersaline lakes using insoluble celluloses as the growth substrate. Taking into account its unique phenotypic properties and distant phylogenetic position, as inferred from the robust phylogenetic reconstruction based on 19 conservative markers, and ANI and *in silico* DDH values far below the recognized intragenus levels, we propose to classify strain HArcel1^T in a novel genus and species *Halococcoides cellulovorans*.

Description of *Halococcoides* gen. nov.

Ha.lo.coc.co'i.des. [Gr. n. *hals*, halos salt of the sea; N.L. masc. n. *coccus* (from Gr. masc. n. *kokkos*, grain, seed), coccus; L. suff. *-oides* (from Gr. suff. -eides, from Gr. n. *eidos*, that which is seen, form, shape, figure), resembling, similar; L. suff. *-oides*, resembling, similar; N.L. neutral. n. *Halococcoides*, coccus-shaped holophile].

Extremely halophilic euryarchaeon, a member of the family *Haloarculacea*, order *Halobacteriales*, class *Halobacteria*, found in hypersaline athalassic lakes. Specialized in utilization of cellulose as growth substrate. The type species is *Halococcoides cellulosivorans.* The recommended three-letter abbreviation for this genus is Hcd.

Description of *Halococcoides cellulosivorans* sp. nov.

Halococcoides cellulosivorans (cel.lu.lo.si.vo'rans N.L. neutral n. *cellulosum*, cellulose; L. pres. part. *vorans*, devouring; N.L. part. adj. *cellulosivorans*, cellulose devouring)

Cells are non-motile cocci, 0.8-3 μm, with a thin monolayer cell wall. The colonies on amorphous cellulose agar are flat, up to 1 mm, soft and slightly orange. It is a strictly aerobic (catalase/oxidase positive) saccharolytic specialized on utilization of native forms of insoluble cellulose and xylan. Cellobiose is the only soluble sugar utilized for growth. The nitrogen source is ammonium. Nitrate and urea are not utilized. Does not grow anaerobically either by fermentation or anaerobic respiration. Does not utilize organic acids or organic nitrogen compounds as carbon and energy source. High Mg is not required for growth. Proteolytic and lipolytic activity are absent. Strain HArcel1^T is an extremely halophilic neutrophile, with the NaCl range for growth between 3 and 5 M (optimum at 3.5-4 M) and the pH range from 6.5 to 8.0 (optimum at pH 7.0-7.2). The maximum growth temperature at 4 M NaCl with cellobiose as substrate is 50°C (optimum at 40-42^{o}C). The core membrane lipids are dominated by C_{20}-C_{20} and C_{25}-C_{20} DGE with 1-C_{25} MGE and 2-C_{20} MGE as minor components. The identified intact membrane polar lipids include phosphatidylglycerophosphate methylester (PGP-Me) and phosphatidylglycerol (PG) as dominant and diglycosyl diether glycolipid (2GL) and phosphatidylglycerol sulfate (PGS) sulfolipid as minor components. The G + C content of the genomic DNA in the type strain is 65.74 mol% (genome).

The habitat is hypersaline lakes with near-neutral pH. The type strain (HArcel1^T=JCM 31939^T=UNIQEM U972^T). The full genome accession number in the GenBank is CP028858.

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Conflict of interest:

The authors declare that there is no conflict of interests.

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Table 1. Average pairwise genomic Nucleotide Identity (ANI-P) and digital DNA-DNA hybridization analyses (% similarity) of strain HArcel1$^{\mathrm{T}}$ with the nearest phylogenetic relatives from the family *Haloarculaceae*.

Compared with:	**ANI-P**				**Digital DDH** (average from 3 formulas)
	Strain HArcel1$^{\mathrm{T}}$	*Halorhabdus tiamatea*	*Halothabdus utahensis*	*Halapricum salinum*	**Strain HArcel1$^{\mathrm{T}}$**
Halorhabdus tiamatea SARL4B$^{\mathrm{T}}$	**75.1**		85.6	75.7	16.6
Halorhabdus utahensis AX-2$^{\mathrm{T}}$	**74.8**	85.6		75.3	16.4
Halapricum salinum CBA1105$^{\mathrm{T}}$	**74.1**	75.7	75.2		15.7

Table 2. Comparative property of cellulotrophic haloarchaeon strain HArcel1^T with the nearest phylogenetic relatives in *Haloarculaceae*: *Halorhabdus tiamatea* [14, 37], *Halopricum salinum* [40].

Feature	**Strain HArcel1^T**	*Halorhabdus tiamatea* JCM 14471^T	*Halapricum salinum* CBA1105^T
Cell morphology	Non-motile coccoids	Pleomorphic, non-motile	Pleomorphic cocci, non-motile
Pigmentation	Pale orange	-	Red
Growth substrates:			
polymers	Insoluble celluloses, xylan	pullulan[#], starch, xyloglycan[#], xylane[#], arabinoxylane", glycomannan[#], beta-mannan (weak)[#]	-
sugars	Cellobiose	Galactose, maltose, mannose[#], xylose[#]	Glucose, mannose maltose, sucrose
others			glutamate
Number of cellulase genes (GH families) in the genome	GH5 (24); GH9 (3); GH12 (2)	GH5 (6); GH9 (1); GH12 (1)	none
Anaerobic growth	-	+ (fermentative, denitrification)	-
Esterase/lipase	- (tributyrin/ olive oil)	+ (C8)/nd	Tweens/nd
Protease activity	- (casein, gelatin)	+ (gelatin)	-
Oxidase/catalase	weak/+	-/+	+/-
Salinity range (opt.) M NaCl	2.5-5 (3.5-4.0)	1.6-5 (4.5)	2.5-6.0 (3.2)
pH range (opt.)	6.5-8.0 (7.0-7.2)	6.0-8.5 (7.0-7.5)	7.0-8.0 (7.0)
Temperature (°C)	max. 50 (opt. 43)	max. 55 (opt. 45)	max. 45 (37)
Core lipids	C_{20}-C_{20}, C_{25}-C_{20}	DGE (undefined)	nd
Intact membrane polar lipids	PGP-Me, PG, DGD, PGP, PGS; unknown sulfolipid	PG, PGP-Me, TGD, S_1-DGD	PG, PGP-Me, 3 unidentified glycolipids
DNA G+C (mol%)	65.7 (genome)	61.7 (T_m)	66.0 (T_m)
Habitat	Hypersaline salt lakes in *s-w* Siberia	Deep-sea hypersaline brines (Red Sea)	Solar saltern

Phospholipids: (PGP-Me) phosphatidylglycerophosphate methylester, (PG) phosphatidylglycerol, (GL-PG) phosphatidylglycose, (DGD) diglycosyl glycerol diether, (PGS) phosphatidylglycerol sulfate, (PGP) phosphatidylglycerophosphate; glycolipids: (S_1-DGD) monosulfated diglycosyl diether, TGD (triglycosyl glycerol diether).

[*] based on the genomic data and activity measurements but not yet validated by growth experiments

[#]determined in this work; negative results for *H. tiamatea* included amylopectin, dextrans, inulin, galactan, galactomannan, beta-1,3 glycans, arabinan, arabinogalactan and various forms of native insoluble cellulose

Legends to the figures

Fig. 1 Morphology of strain HArcel1^T growing at 4 M total NaCl and 37^{o}C. (**a**) colonies on amorphous cellulose plates forming large hydrolysis zones; (**b**) phase contrast microphotograph of cells grown with amorphous cellulose in liquid culture; (**c**) phase contrast microphotograph of cells forming biofilm on a cellulose fiber; **(d)** electron microscopy of thin sections of cells grown with amorphous cellulose. **CW**, cell wall; **CM**, cytoplasmic membrane; **N**, nucleoid.

Fig. 2. Phylogeny of strain HArcel1^T.

(**a**) Maximum Likelihood 16S rRNA gene sequence-based phylogenetic tree showing position of HArcel^T (in bold) within the order *Halobacteriales*. Branch lengths (see scale) correspond to the number of substitutions per site with corrections, associated with the model (GTR, G + *I*, 4 categories). All positions with less than 95% site coverage were eliminated. Totally 1435 positions were used in the alignment of 119 sequences. Numbers at nodes indicate bootstrap values of 1000 repetitions, bootstrap values below 50% are not shown. *Halomarina* genus was used as an outgroup.

(**b**) Maximum Likelihood *rpoB'* gene sequence-based tree showing position of strain HArcel1^T (in bold) within the order *Halobacteriales*. All parameters were the same as in 16S rRNA gene-based phylogeny. Totally 1827 positions were used in the alignment of 81 sequences. *Halomarina* genus was used as an outgroup.

(c) Maximum Likelihood tree based on 17 ribosomal proteins alignment showing position of strain HArcel1^T (in bold) within the order *Halobacteriales*. Branch lengths (see scale) correspond to the number of substitutions per site with corrections, associated with the model (LG, G + *I*, 4 categories). All positions with less than 95% site coverage were eliminated. Totally 2938 positions were used in the alignment of 40 amino acid sequences. *Natronomonas* genus was used as an outgroup

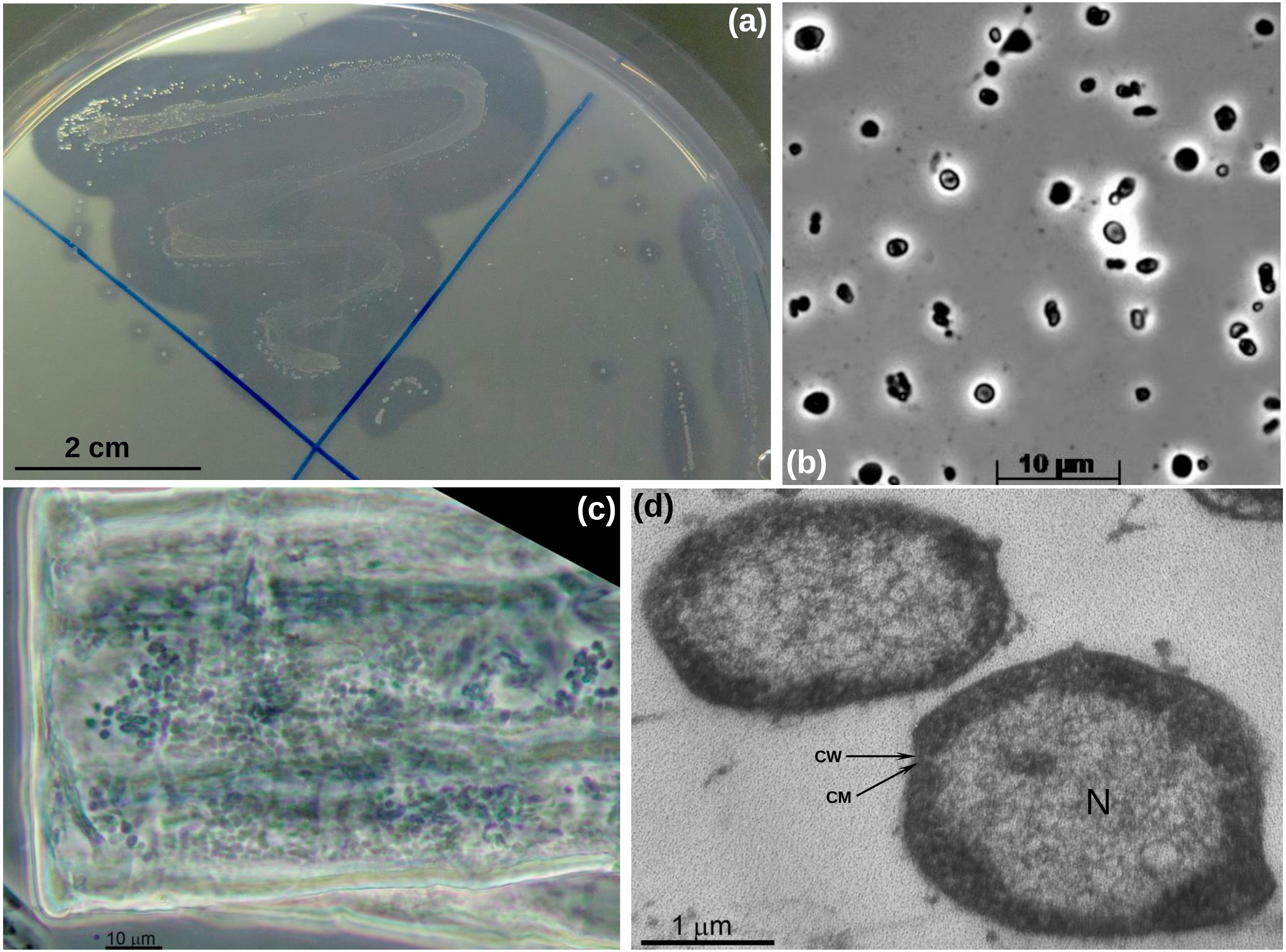
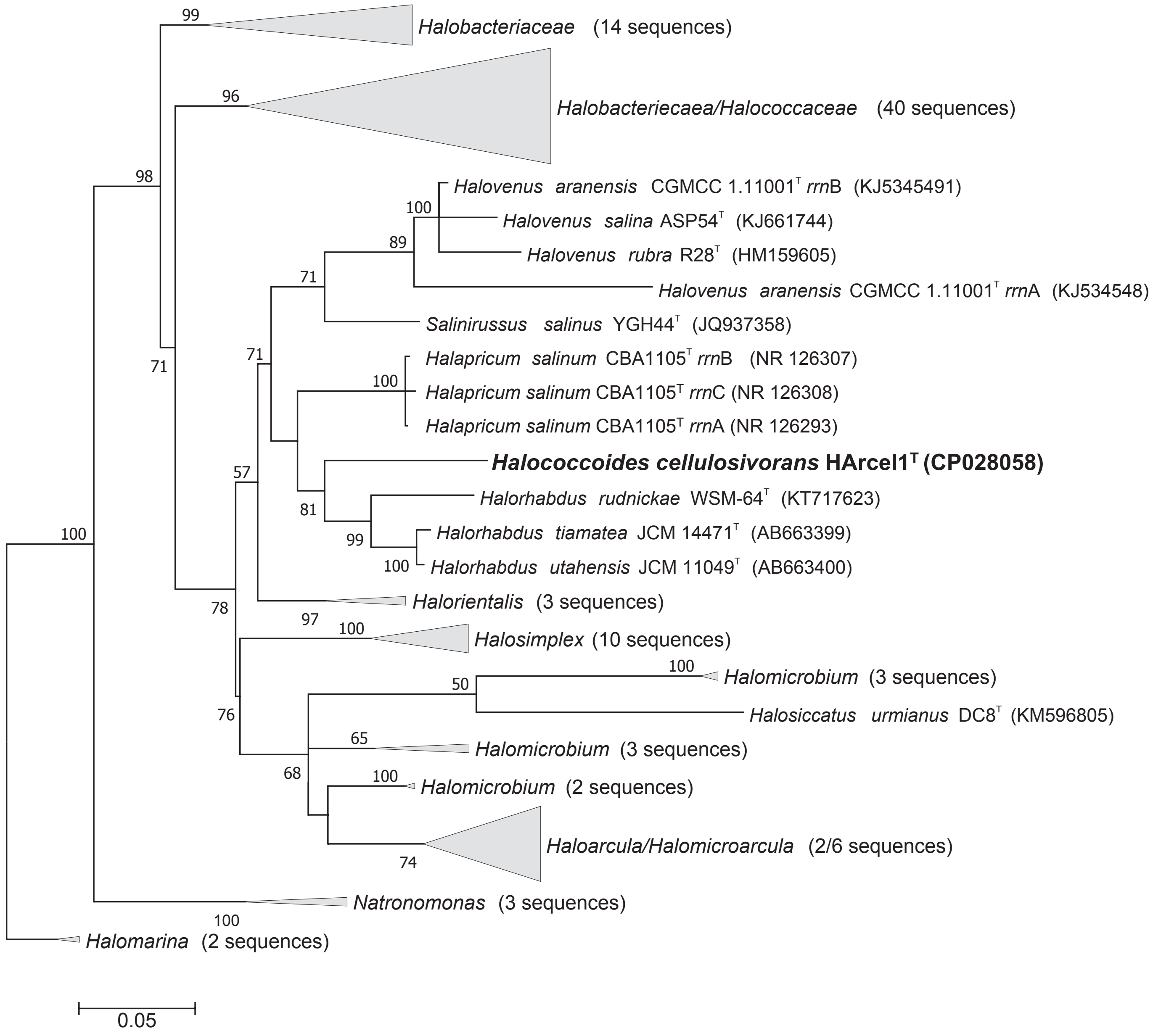


Fig.1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Halobacteriaceae (14 sequences)
96
Halobacteriecaea/Halococcaceae (40 sequences)
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100
Halovenus salina ASP54T (KJ661744)
89
Halovenus rubra R28T (HM159605)
71
Halovenus aranensis CGMCC 1.11001T rrnA (KJ534548)
Salinirussus salinus YGH44T (JQ937358)
71
71
Halapricum salinum CBA1105T rrnB (NR 126307)
100
Halapricum salinum CBA1105T rrnC (NR 126308)
Halapricum salinum CBA1105T rrnA (NR 126293)
57
Halococcoides cellulosivorans HArcel1T (CP028058)
Halorhabdus rudnickae WSM-64T (KT717623)
81
99
Halorhabdus tiamatea JCM 14471T (AB663399)
100
100
Halorhabdus utahensis JCM 11049T (AB663400)
Halorientalis (3 sequences)
78
97
100
Halosimplex (10 sequences)
100
Halomicrobium (3 sequences)
50
76
Halosiccatus urmianus DC8T (KM596805)
65
Halomicrobium (3 sequences)
68
100
Halomicrobium (2 sequences)
Haloarcula/Halomicroarcula (2/6 sequences)
74
Natronomonas (3 sequences)
100
Halomarina (2 sequences)
0.05

Fig. 2a

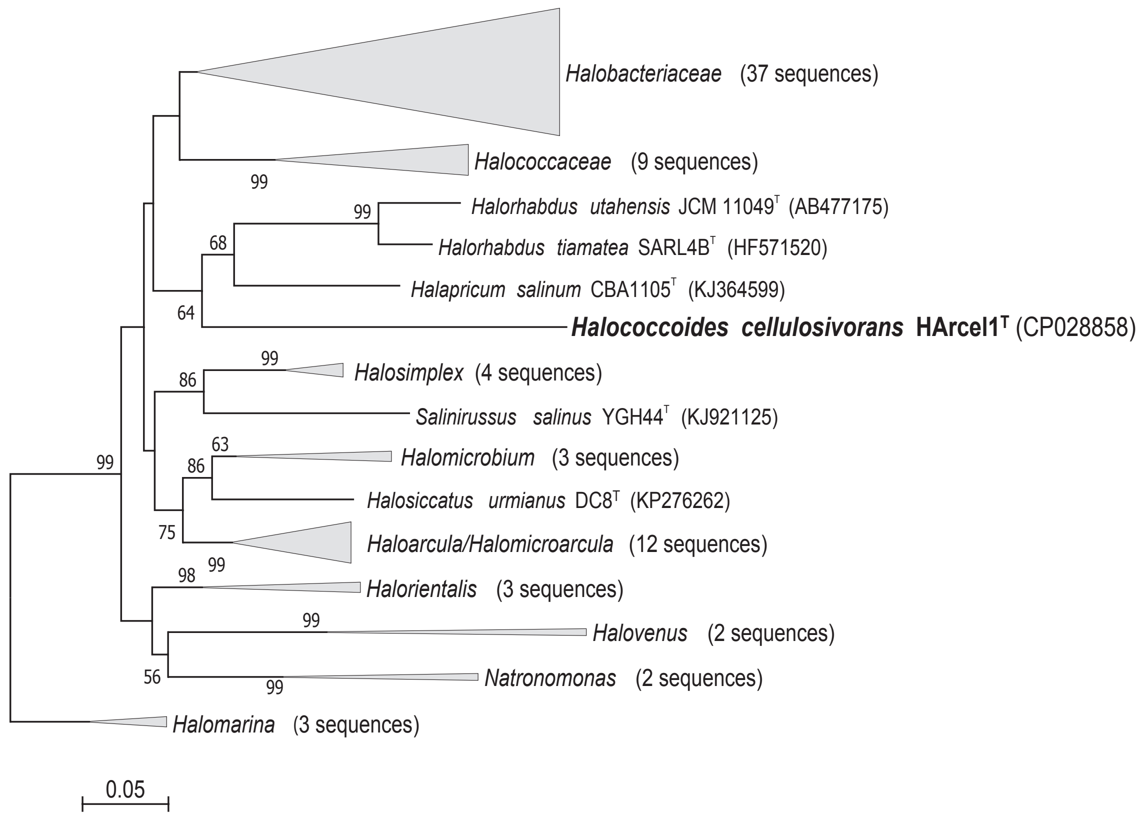
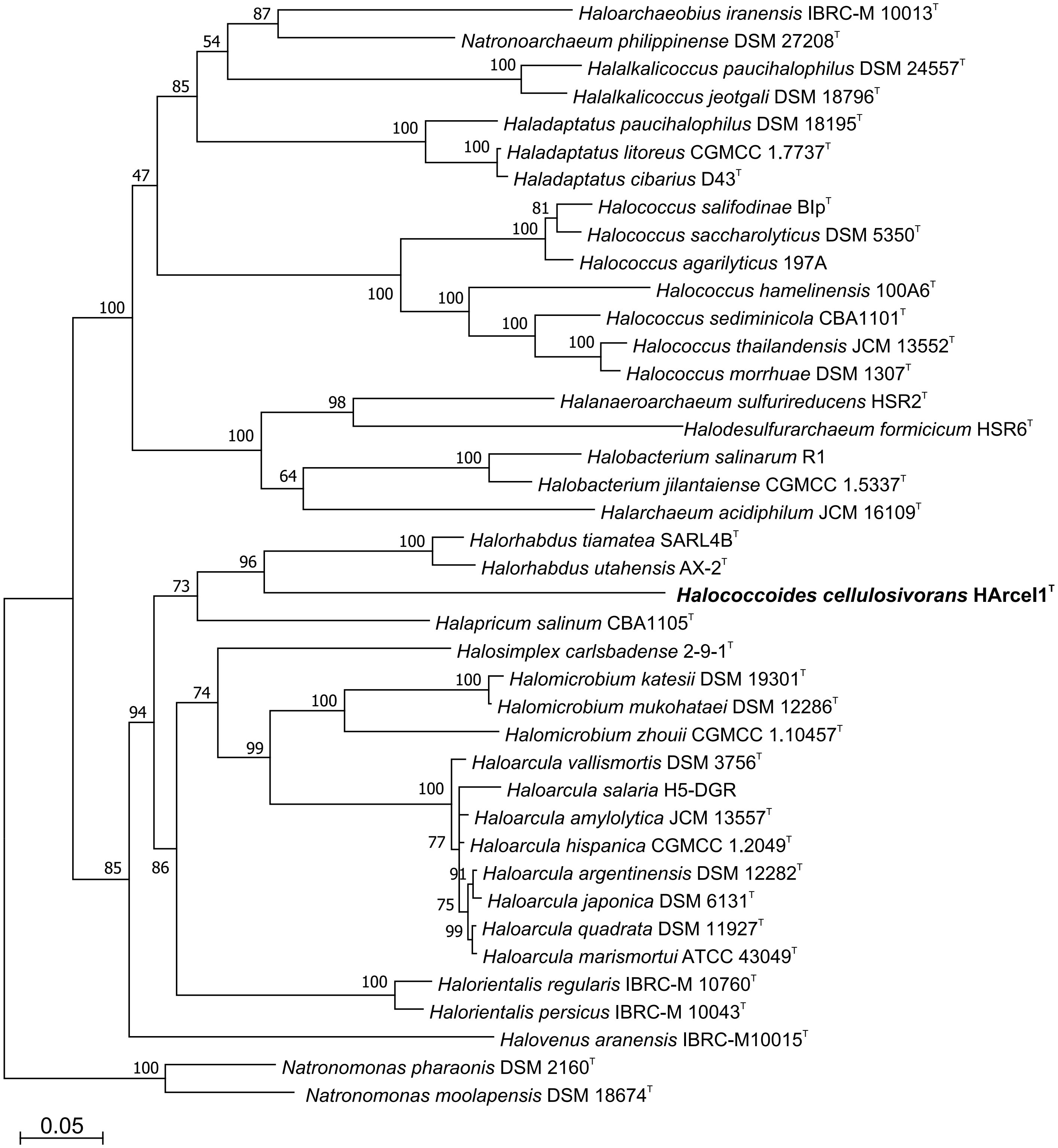

Halobacteriaceae (37 sequences)
Halococcaceae (9 sequences)
99
99
Halorhabdus utahensis JCM 11049T (AB477175)
Halorhabdus tiamatea SARL4BT (HF571520)
68
Halapricum salinum CBA1105T (KJ364599)
64
Halococcoides cellulosivorans HArcel1T (CP028858)
99
86
Halosimplex (4 sequences)
Salinirussus salinus YGH44T (KJ921125)
63
86
Halomicrobium (3 sequences)
99
Halosiccatus urmianus DC8T (KP276262)
75
Haloarcula/Halomicroarcula (12 sequences)
98
99
Halorientalis (3 sequences)
99
Halovenus (2 sequences)
56
99
Natronomonas (2 sequences)
Halomarina (3 sequences)
0.05

Fig. 2b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Haloarchaeobius iranensis IBRC-M 10013T
Natronoarchaeum philippinense DSM 27208T
54
100
Halalkalicoccus paucihalophilus DSM 24557T
85
Halalkalicoccus jeotgali DSM 18796T
100
Haladaptatus paucihalophilus DSM 18195T
100
Haladaptatus litoreus CGMCC 1.7737T
Haladaptatus cibarius D43T
47
81
Halococcus salifodinae BIpT
100
Halococcus saccharolyticus DSM 5350T
Halococcus agarilyticus 197A
100
100
Halococcus hamelinensis 100A6T
100
100
Halococcus sediminicola CBA1101T
100
Halococcus thailandensis JCM 13552T
Halococcus morrhuae DSM 1307T
98
Halanaeroarchaeum sulfurireducens HSR2T
100
Halodesulfurarchaeum formicicum HSR6T
100
Halobacterium salinarum R1
64
Halobacterium jilantaiense CGMCC 1.5337T
Halarchaeum acidiphilum JCM 16109T
100
Halorhabdus tiamatea SARL4BT
96
Halorhabdus utahensis AX-2T
73
***Halococcoides cellulosivorans* HArcel1T**
Halapricum salinum CBA1105T
Halosimplex carlsbadense 2-9-1T
100
Halomicrobium katesii DSM 19301T
74
100
Halomicrobium mukohataei DSM 12286T
94
Halomicrobium zhouii CGMCC 1.10457T
99
Haloarcula vallismortis DSM 3756T
100
Haloarcula salaria H5-DGR
Haloarcula amylolytica JCM 13557T
77
Haloarcula hispanica CGMCC 1.2049T
85
86
91
Haloarcula argentinensis DSM 12282T
75
Haloarcula japonica DSM 6131T
99
Haloarcula quadrata DSM 11927T
Haloarcula marismortui ATCC 43049T
100
Halorientalis regularis IBRC-M 10760T
Halorientalis persicus IBRC-M 10043T
Halovenus aranensis IBRC-M10015T
100
Natronomonas pharaonis DSM 2160T
Natronomonas moolapensis DSM 18674T
0.05

Fig. 2c

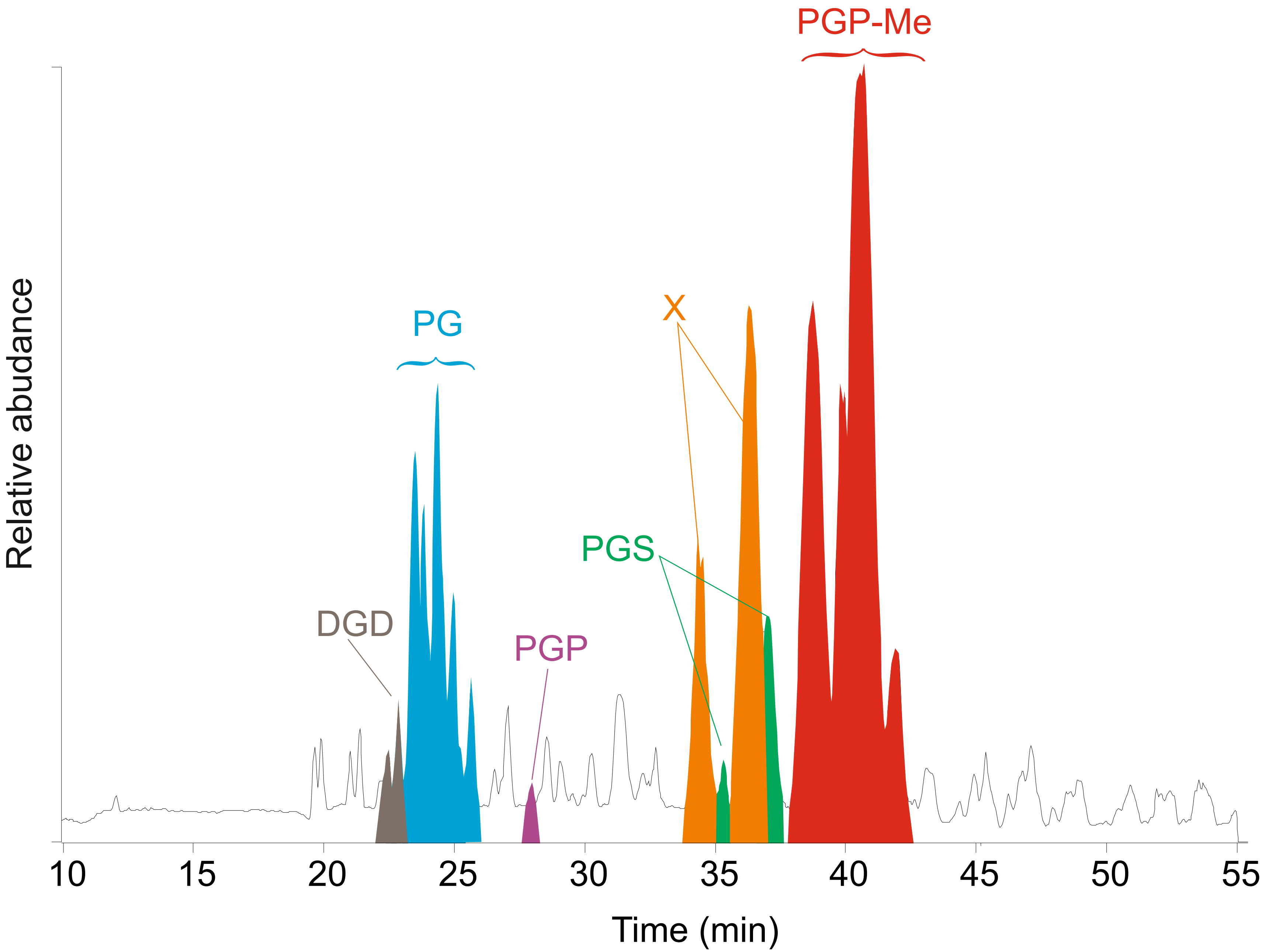
Supplementary data file

Halococcoides cellulosivorans gen. nov., sp. nov., an extremely halophilic cellulose-utilizing haloarchaeon from hypersaline lakes

Dimitry Y. Sorokin, Tatiana V. Khijniak, Nadezhda A. Kostrikina, Alexander G. Elcheninov, Stepan V. Toshchakov, Nicole J. Bale, Jaap S. Sinninghe Damsté, Ilya V. Kublanov

Supplementary Figure S1

Partial base peak chromatogram (Gaussian smoothed) of the HPLC-ESI/MS analysis of intact polar lipids in the cell extract of strain Harcel1^T. Peak labels: PGP-Me = phosphatidylglycerophosphate methylester, PG = phosphatidylglycerol, DGD = diglycosyl diether, X = unknown sulfur containing headgroup, PGP = phosphatidylglycerophosphate and PGS = phosphatidylglycerosulfate. Double or multiple peaks are due to the presence of the polar head group with both the archaeol core (C_{20}-C_{20} dialkyl glycerol ether) and the extended archaeol core (C_{20}-C_{25}) as well as their unsaturated homologs.



Supplementary Figure S1