Halanaeroarchaeum sulfurireducens gen. nov., sp. nov., the first obligately anaerobic sulfur-respiring haloarchaeon, isolated from a hypersaline lake

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Abstract:	Anaerobic enrichments with acetate as e-donor and carbon source and elemental sulfur as electron acceptor at 4 M NaCl using anaerobic sediments and brines from several hypersaline lakes in Kulunda Steppe (Altai, Russia) resulted in isolation in pure culture of four strains of obligately anaerobic haloarchae growing exclusively by sulfur respiration. Such metabolism has not yet been demonstrated in any known species of Halobacteria and in the whole archaeal kingdom the acetate oxidation with sulfur as acceptor was not previously demonstrated. The four isolates had nearly identical 16S rRNA gene sequences and formed a novel genus-level branch within the family Halobacteraceae. The strains had a restricted substrate range limited to acetate and pyruvate as e-donors and elemental sulfur as e-acceptor. In contrast to aerobic haloarchaea, the biomass of anaerobic isolates completely lacked the typical red pigments. The growth with acetate+sulfur was observed between 3-5 M NaCl and at a pH range from 6.7 to 8.0. The membrane core lipids were dominated by archaeols. On the basis of distinct physiological and phylogenetic data, it is proposed that the sulfur-respiring isolates represent a novel genus and species Halanaeroarchaeaum sulfurireducens gen. nov., sp. nov. (type strain HSR2T=JCM 30661T=UNIQEM U935T).

Halanaeroarchaeum sulfurireducens gen. nov., sp. nov., a first obligately anaerobic sulfur-respiring haloarchaeon from hypersaline lakes

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The 16S-rRNA gene sequences of the strains HSR strains described here have been deposited in the GenBank under the numbers KM875608 and KM875610-KM875612.

Anaerobic enrichments with acetate as e-donor and carbon source and elemental sulfur as electron acceptor at 4 M NaCl using anaerobic sediments and brines from several hypersaline lakes in Kulunda Steppe (Altai, Russia) resulted in isolation in pure culture of four strains of obligately anaerobic haloarchae growing exclusively by sulfur respiration. Such metabolism has not yet been demonstrated in any known species of *Halobacteria* and in the whole archaeal kingdom the acetate oxidation with sulfur as acceptor was not previously demonstrated. The four isolates had nearly identical 16S rRNA gene sequences and formed a novel genus-level branch within the family *Halobacteraceae*. The strains had a restricted substrate range limited to acetate and pyruvate as *e*-donors and elemental sulfur as *e*-acceptor. In contrast to aerobic haloarchaea, the biomass of anaerobic isolates completely lacked the typical red pigments. The growth with acetate+sulfur was observed between 3-5 M NaCl and at a pH range from 6.7 to 8.0. The membrane core lipids were dominated by archaeols. On the basis of distinct physiological and phylogenetic data, it is proposed that the sulfur-respiring isolates represent a novel genus and species *Halanaeroarchaeaum sulfurireducens* gen. nov., sp. nov. (type strain HSR2^T=JCM 30661^T=UNIQEM U935^T).

Key words: hypersaline lakes, haloarchaea, sulfur reduction, anaerobic

Our recent study on the microbiology of reductive sulfur cycling in hypersaline habitats resulted in the discovery of a novel functional group of haloarchaea in anaerobic sediments of hypersaline lakes growing exclusively by dissimilatory elemental sulfur respiration (Sorokin *et al*., 2016). This metabolic type was previously unknown among the haloarchaea, but even more surprising anaerobic acetate oxidation with a low-potential electron acceptor such as elemental sulfur has not yet been demonstrated in the whole archaeal kingdom. This makes the newly discovered group of obligately anaerobic haloarchaea truly unique. The previous work was mostly focused on the genomic properties of the type strain HSR2^T and its functional annotation. Here we provide a formal taxonomic description of the novel taxon as *Halanaeroarchaeum sulfurireducens* gen. nov., sp. nov.

Sources of inocula were brines and anaerobic sulfidic surface sediments (2-10 cm) obtained from hypersaline chloride-sulfate lakes (see Sorokin *et al*., 2012 for a detailed description) in the Kulunda Steppe (Altai, Russia) in 2009-2013. The enrichment and isolation procedures, the medium composition and cultivation conditions have been described previously (Sorokin *et al*., 2016). Overall, anaerobic enrichments using acetate as *e*-donor/C source and elemental sulfur as *e*-acceptor at 4 M NaCl, pH 7 and 37^{o}C resulted in isolation of four strains of haloarchaea designated HSR2^T, HSR3, HSR4 and HSR5. The cell morphology of the isolates was typical for haloarchaea, i.e. flat coccoids and board-like rods, non-motile (**Fig. 1, a-d**). On the other hand, the cell mass lack any detectable red pigments characteristic of haloarchaea. Flagella were not observed in negatively stained cells. For thin sectioning, the cell pellets were fixed in 1% (w/v) OsO_4 containing 3.0 M NaCl for 48 h at room temperature, washed, stained overnight with 1% (w/v) uranyl acetate, dehydrated in an increasing ethanol series, and embedded in Epon resin. Thin sections were stained with 1% (w/v) lead citrate. The cells of HSR2^T had a thin monolayer proteinaceous cell wall and extended nucleoid (**Fig. 1, e**) and the cells lyzed immediately when the salt concentration dropped below 1.0 M.

The core membrane lipid analysis were performed by a method described in Weijers *et al*. (2009). The core lipids of strain HSR2^T consisted of two major diether components, archaeol, and extended archaeol (i.e. C20-C25) in nearly equal proportion (47 and 53%, respectively), both common in haloarchaea (e.g. Villanueva *et al*., 2014). The polar phospholipids were analysed with an LC/MS^n method described in Sinninghe Damsté *et al*. (2011). They are dominated by phosphatidylglycerolsulfate (PGS) and phosphatidylglycerolphosphate methyl ether (PGP-Me), while three other components, phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), and an unknown complex phospholipid, were less abundant. All phospholipids were present with an archaeol and an extended archaeol core.

The 16S-rRNA gene sequences of the *Haa. sulfurireducens* strains were aligned with those of validly named related species of the order *Halobacteriales* (Gupta *et al*., 2015) using the SILVA Incremental Aligner (Prüesse et al., 2012). The phylogenetic neighbours and pairwise sequence similarities were determined using EzTaxon-e (Kim *et al*., 2012) and the phylogenetic trees were constructed with MEGA5 (Tamura *et al*. 2011) using the neighbour-joining (NJ) (Saitou & Nei, 1987), maximum-parsimony (MP) (Fitch, 1971) and maximum likelihood (ML) (Felsenstein, 1981) algorithms with 1,000 randomly selected bootstrap replicates. Phylogenetic analyses of the 16S rRNA genes of the four isolates revealed that they are closely related to each other (at least 99% 16S rRNA gene similarity) and, in fact, represent a single genetic species. These strains were quite distant from the nearest described members of the family *Halobacteraceae*, forming a separate genus-level lineage together with some cloned sequences from various hypersaline habitats (**Fig. 2**).

The novel isolates were clearly different from all previously described haloarchaea in respect of their metabolism. First, all strains were obligately anaerobic respirers. Next, their metabolism was extremely narrow, limited to acetate and pyruvate as *e*-donors/C source and

elemental sulfur as *e*-acceptor. The details of anaerobic growth kinetics have been described previously (Sorokin *et al*., 2016). In general, the cultures growing with acetate produced more sulfide (up to 9 mM in one month) and less biomass than the cultures grown on pyruvate. Apart from sulfide, trace amounts of volatile organic sulfur were detected in stationary culture of strain HSR2, including carbon disulfide and methanthiol. To our knowledge, the formation of these reduced sulfur compounds had never been previously observed in known sulfur-reducing prokaryotes. The optimal growth occurred at 4 M NaCl and within the range from 3 to 5 M and at optimal temperature of 37-40ºC.

This type of catabolism has not been demonstrated previously in any pure culture of haloarchaea and the discovery of such haloarchaea has a broad implication on the possible ecological role of extreme halophiles. Together with the recent demonstration of the ability of haloarchaea to oxidize CO (King, 2015), to participate in dissimilatory arsenic cycling (Rascovan *et al*., 2015) and to actively mineralize such insoluble polymers as chitin and cellulose (Sorokin *et al*., 2015), it significantly shifts our perception of haloarchaea as an important biogeochemical actor in hypersaline habitats.

Overall, on the basis of phenotypic and genetic differences, the novel extremely halophilic and obligately anaerobic sulfur-respiring isolates are suggested to be placed into a new genus and species within the halobacteria for which a name *Halanaeroarchaeum sulfurireducens* is proposed.

Description of Halanaeroarchaeum **gen. nov.**

[hal.an.ae.ro.ar.chae'um Gr.n. *hals, halos* salt of the sea; Gr. pref. *an*, not; Gr. n. *aer aeros*, air; N.L. neut. n. *archaeum* archaeon from Gr. adj. archaios-ê-on ancient; N.L. neut. n. *Halanaeroarchaeum* - anaerobic halophilic archaeon]

Obligately anaerobic haloarchaea with the ability to grow by sulfur-dependent respiration on acetate. Extremely halophilic, neutrophic members of the family *Halobacteraceae*. The cells are irregularly shaped, flattened, nonmotile. Recommended three-letter abbreviation: Haa.

***Description of Halanaeroarchaeum sulfurireducens sp*. nov.**

[sul.fu.ri.re.du′cens L. n. *sulfur*, L. part. adj. *reducens* leading back, reducing, N.L. part. adj. *sulfurireducens* reducing sulfur]

The cells are angled flattened nonmotile coccoids to board-like rods, 0.5-1.5x1-2 μm. The cell wall consists of a thin proteinaceous layer. The cells lyze in hypotonic solutions below 1 M NaCl. Red pigments are absent. The core membrane diether lipids are composed of C20-C20 DGE (archaeol) and C20-C25 DGE (extended archaeol) in equal proportion. The polar phospholipids included (in the order of abundance) phosphatidylglycerolsulfate (PGS), phosphatidylglycerolphosphate methyl ether (PGP-Me), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). Obligately anaerobic growing by elemental sulfur respiration with either acetate or pyruvate as *e*-donor/C source. Ammonium is utilized as N-source. Optimum growth temperature is 37°C (maximum at 46ºC). Extremely halophilic with a range of NaCl for growth from 3 to 5 M (optimum at 4 M) and neutrophilic with a pH range for growth with acetate and sulfur from 6.5 to 8 (optimum at 7.0-7.5). The G + C content of the DNA is 62.8 mol% (genome). Habitat - hypersaline lakes. The type strain (HSR2^T=JCM 30661^T=UNIQEM U935^T) was isolated from mixed anaerobic sediments of hypersaline chloride-sulfate lakes in Kulunda Steppe (Altai, Russia).

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Legends to the figures

Fig. 1 Cell morphology of sulfur-respiring haloarchaea grown anaerobically at 4 M NaCl. (**a-d)**, phase contrast microscopy. (**a**), HSR2^T grown with acetate; (**c-d**), HSR3, HSR4 and HSR5 grown with pyruvate. (**e**), thin section electron microscopy of strain HSR2^T.

Fig. 2. Phylogenetic position of novel anaerobic sulfur-respiring haloarchaeae based on the 16S rRNA gene within the order *Halobacterales* (Gupta *et al*., 2015). The numbers on the nodes indicate the bootstrap values (>75%) calculated using the NJ algorithm probabilities. The tree was rooted with *Natronomonas moolapensis* (AB576127), *Natronomonas pharaonis* (CR936257) and *Halomarina oriensis* (AB519798) sequences. *Methanohalophilus halophilus* (FN870068) sequence served as the outgroup. The bar represents 0.05 accumulated changes per nucleotide.

Figure1

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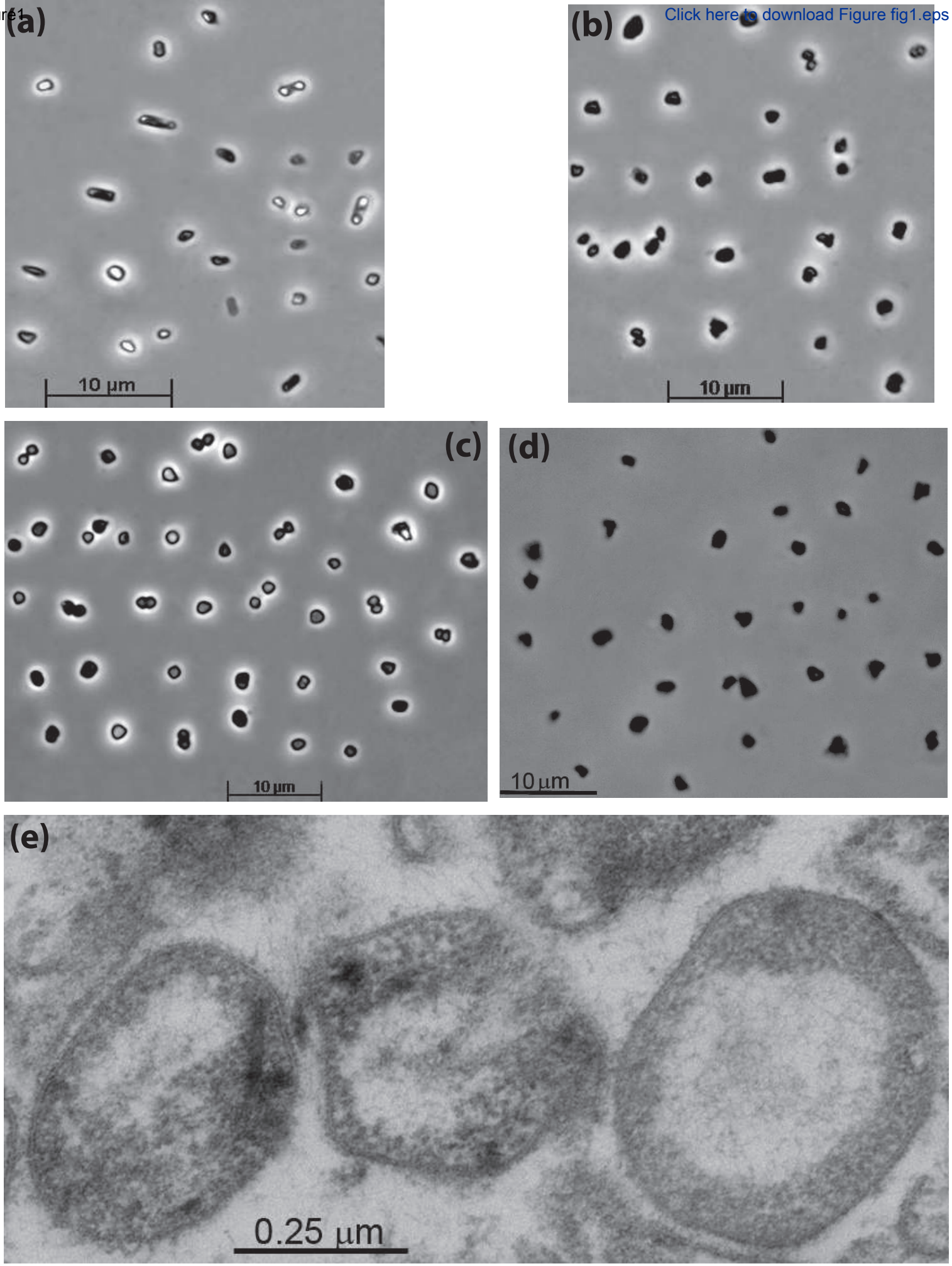

fig.1

Figure2

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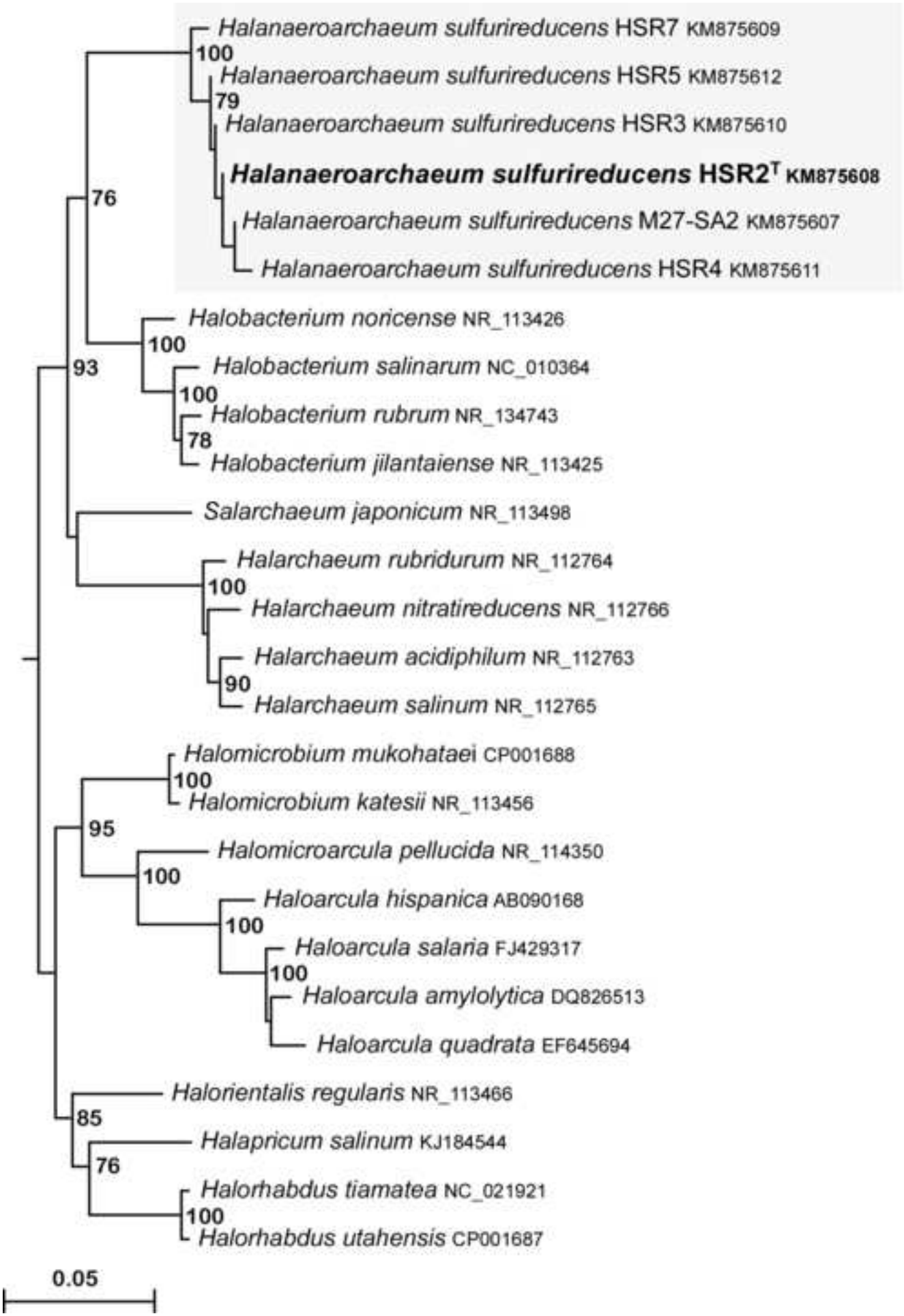

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