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

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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 Halanaeroarchaeum sulfurireducens gen. nov., sp. nov. (type strain HSR2T=JCM 30661T=UNIQEM U935T).

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

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32 Running title: *Halanaeroarchaeum sulfurireducens* gen. nov., sp. nov.

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34 Category: new taxa - Archaea

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

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

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

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

74 Sources of inocula were brines and anaerobic sulfidic surface sediments (2-10 cm)
75 obtained from hypersaline chloride-sulfate lakes (see Sorokin *et al.*, 2012 for a detailed
76 description) in the Kulunda Steppe (Altai, Russia) in 2009-2013. The enrichment and
77 isolation procedures, the medium composition and cultivation conditions have been described
78 previously (Sorokin *et al.*, 2016). Overall, anaerobic enrichments using acetate as *e*-donor/C
79 source and elemental sulfur as *e*-acceptor at 4 M NaCl, pH 7 and 37°C resulted in isolation of
80 four strains of haloarchaea designated HSR2^T, HSR3, HSR4 and HSR5. The cell morphology
81 of the isolates was typical for haloarchaea, i.e. flat coccoids and board-like rods, non-motile
82 (**Fig. 1, a-d**). On the other hand, the cell mass lack any detectable red pigments characteristic
83 of haloarchaea. Flagella were not observed in negatively stained cells. For thin sectioning, the
84 cell pellets were fixed in 1% (w/v) OsO₄ containing 3.0 M NaCl for 48 h at room temperature,
85 washed, stained overnight with 1% (w/v) uranyl acetate, dehydrated in an increasing ethanol
86 series, and embedded in Epon resin. Thin sections were stained with 1% (w/v) lead citrate.
87 The cells of HSR2^T had a thin monolayer proteinaceous cell wall and extended nucleoid (**Fig.**
88 **1, e**) and the cells lysed immediately when the salt concentration dropped below 1.0 M.

89 The core membrane lipid analysis were performed by a method described in Weijers *et*
90 *al.* (2009). The core lipids of strain HSR2^T consisted of two major diether components,
91 archaeol, and extended archaeol (i.e. C20-C25) in nearly equal proportion (47 and 53%,
92 respectively), both common in haloarchaea (e.g. Villanueva *et al.*, 2014). The polar
93 phospholipids were analysed with an LC/MSⁿ method described in Sinninghe Damsté *et al.*
94 (2011). They are dominated by phosphatidylglycerolsulfate (PGS) and
95 phosphatidylglycerolphosphate methyl ether (PGP-Me), while three other components,
96 phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), and an unknown complex
97 phospholipid, were less abundant. All phospholipids were present with an archaeol and an
98 extended archaeol core.

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

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

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

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

129
130 Overall, on the basis of phenotypic and genetic differences, the novel extremely halophilic
131 and obligately anaerobic sulfur-respiring isolates are suggested to be placed into a new genus
132 and species within the halobacteria for which a name *Halanaeroarchaeum sulfurireducens* is
133 proposed.

134

135 ***Description of Halanaeroarchaeum gen. nov.***

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

139

140 Obligately anaerobic haloarchaea with the ability to grow by sulfur-dependent respiration on
141 acetate. Extremely halophilic, neutrophic members of the family *Halobacteraceae*. The cells
142 are irregularly shaped, flattened, nonmotile. Recommended three-letter abbreviation: Haa.
143

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

145 [sul.fu.ri.re.du'cens L. n. *sulfur*, L. part. adj. *reducens* leading back, reducing, N.L. part. adj.
146 *sulfurireducens* reducing sulfur]
147

148 The cells are angled flattened nonmotile coccoids to board-like rods, 0.5-1.5x1-2 µm. The cell
149 wall consists of a thin proteinaceous layer. The cells lyze in hypotonic solutions below 1 M
150 NaCl. Red pigments are absent. The core membrane diether lipids are composed of C20-C20
151 DGE (archaeol) and C20-C25 DGE (extended archaeol) in equal proportion. The polar
152 phospholipids included (in the order of abundance) phosphatidylglycerolsulfate (PGS),
153 phosphatidylglycerolphosphate methyl ether (PGP-Me), phosphatidylglycerol (PG) and
154 phosphatidylethanolamine (PE). Obligately anaerobic growing by elemental sulfur respiration
155 with either acetate or pyruvate as *e*-donor/C source. Ammonium is utilized as N-source.
156 Optimum growth temperature is 37°C (maximum at 46°C). Extremely halophilic with a range
157 of NaCl for growth from 3 to 5 M (optimum at 4 M) and neutrophilic with a pH range for
158 growth with acetate and sulfur from 6.5 to 8 (optimum at 7.0-7.5). The G + C content of the
159 DNA is 62.8 mol% (genome). Habitat - hypersaline lakes. The type strain (HSR2^T=JCM
160 30661^T=UNIQEM U935^T) was isolated from mixed anaerobic sediments of hypersaline
161 chloride-sulfate lakes in Kulunda Steppe (Altai, Russia).
162

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238 **Legends to the figures**

239

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

243

244 **Fig. 2.** Phylogenetic position of novel anaerobic sulfur-respiring haloarchaeae based on the
245 16S rRNA gene within the order *Halobacteriales* (Gupta *et al.*, 2015). The numbers on the
246 nodes indicate the bootstrap values (>75%) calculated using the NJ algorithm probabilities.
247 The tree was rooted with *Natronomonas moolapensis* (AB576127), *Natronomonas pharaonis*
248 (CR936257) and *Halomarina oriensis* (AB519798) sequences. *Methanohalophilus halophilus*
249 (FN870068) sequence served as the outgroup. The bar represents 0.05 accumulated changes
250 per nucleotide.

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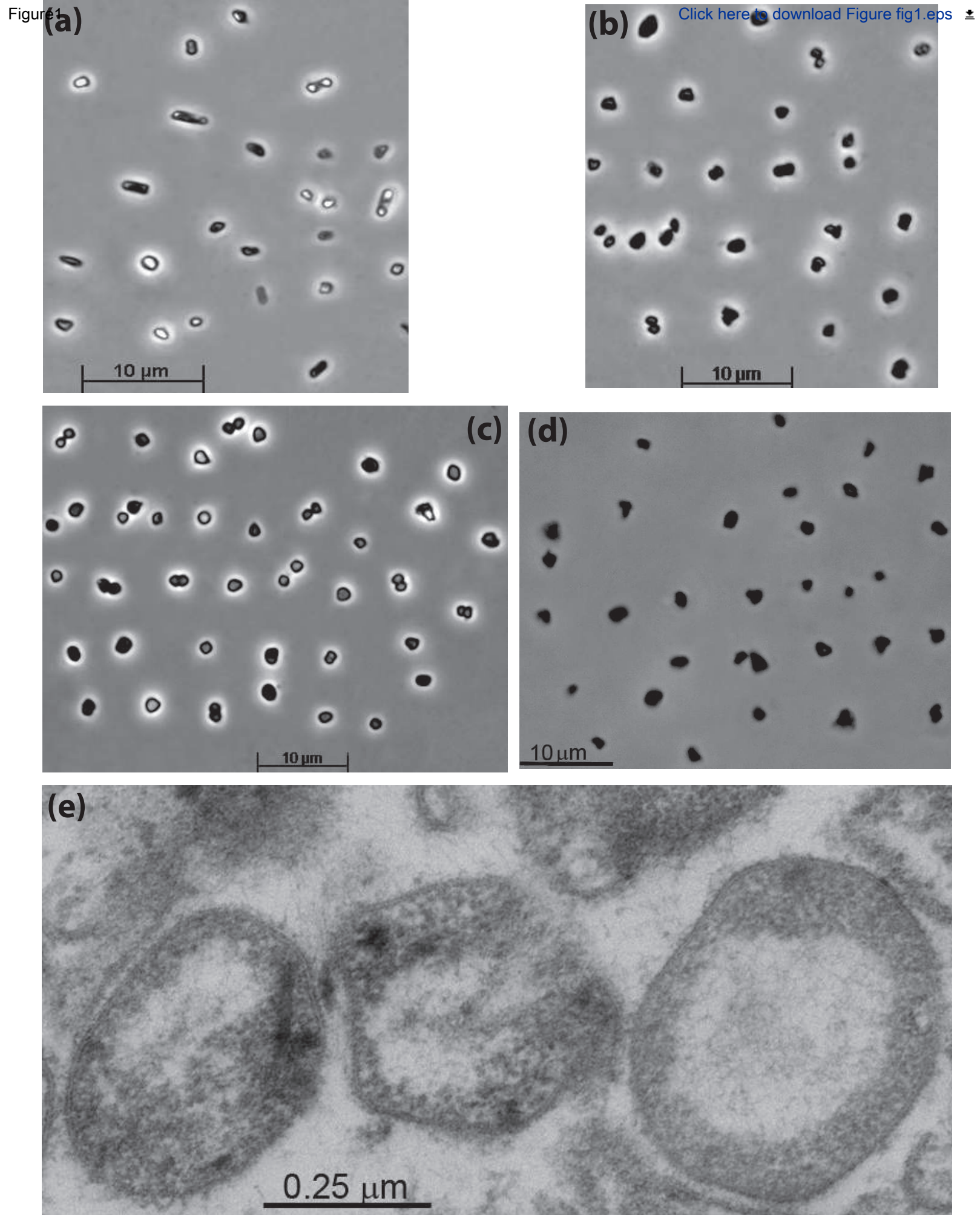
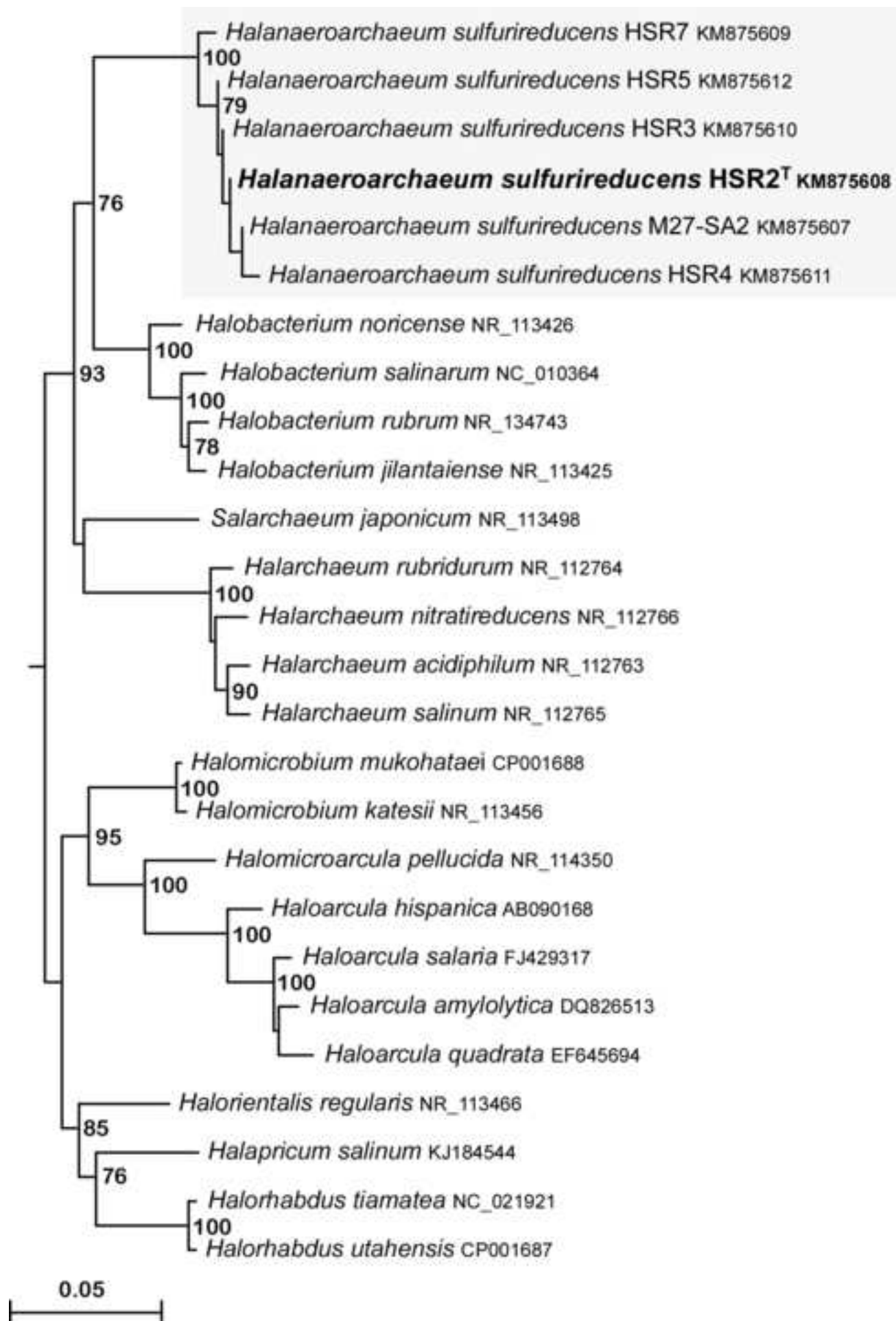
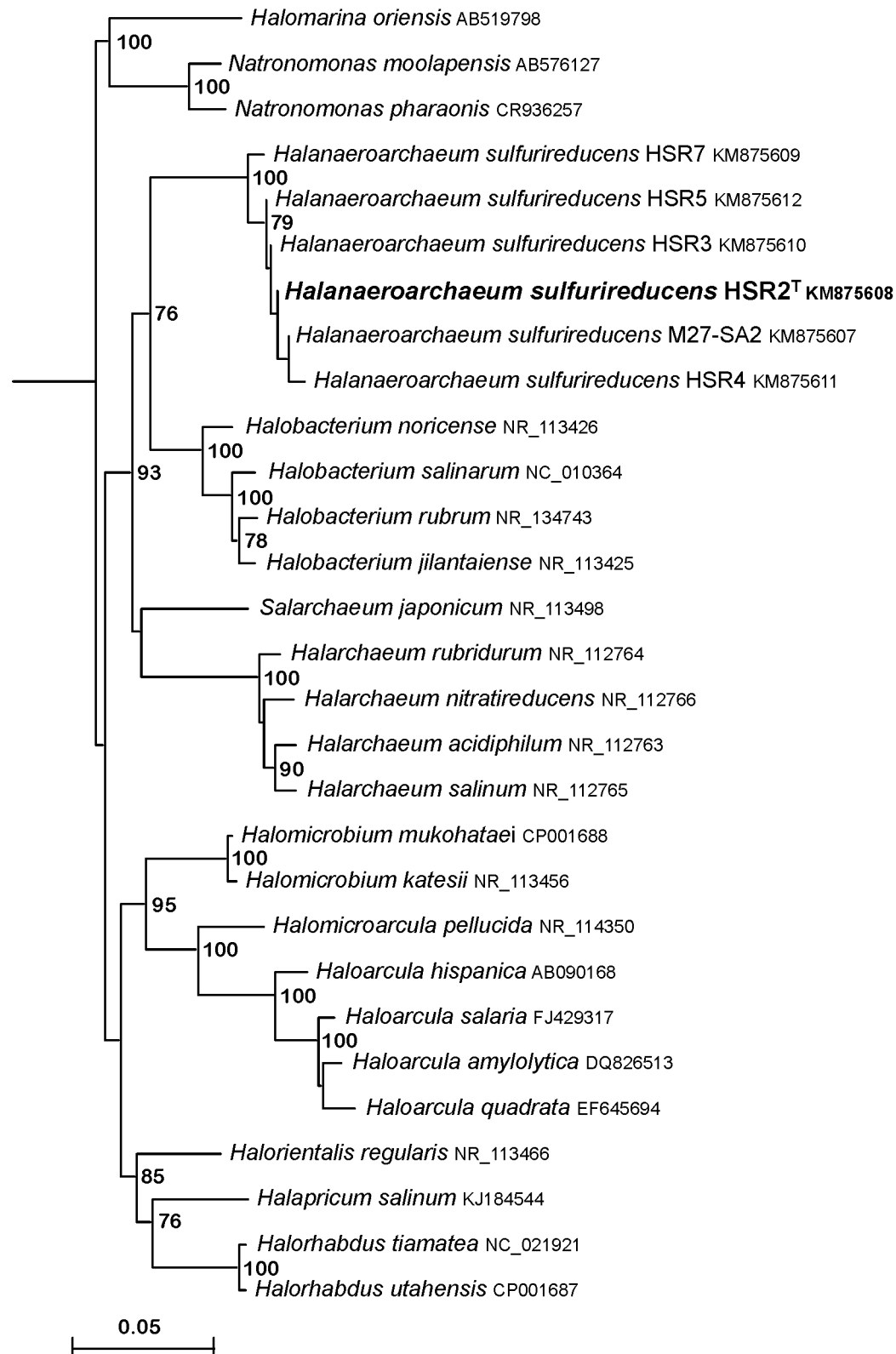


fig.1







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