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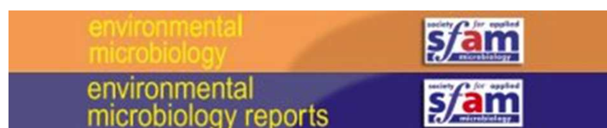

Syntrophic associations from hypersaline soda lakes converting organic acids and alcohols to methane at extremely haloalkline conditions

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2 # Syntrophic associations from hypersaline soda lakes converting organic
3 # acids and alcohols to methane at extremely haloalkline conditions

4

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19 Originality-Significance:

20 For the first time a possibility of anaerobic conversion of VFA and primary alcohols to methane
21 at haloalkaline conditions has been demonstrated and the highly enriched syntrophic associations
22 responsible for these conversions have been obtained and microbiologically characterized. These
23 results demonstrate that, despite a low energy yield of the overall conversions, the haloalkaliphilic
24 anaerobes, working in syntrophy, can still overcome the additional energetic costs of double
25 extreme conditions of high salt-high pH.

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27 Summary

28 **Until now anaerobic oxidation of VFA at high salt-pH have only been demonstrated**
29 **at sulfate-reducing conditions. Here we present results of microbiological**
30 **investigation of anaerobic conversion of organic acids and alcohols at methanogenic**
31 **conditions by syntrophic associations enriched from hypersaline soda lakes in**
32 **Central Asia. Sediment incubation experiments showed active, albeit very slow,**
33 **methane formation from acetate, propionate, butyrate and C_2-C_4 alcohols at pH 10**
34 **and various levels of salinity. Enrichments of syntrophic associations using**
35 **hydrogenotrophic members of the genus *Methanocalculus* from soda lakes as**
36 **partners resulted in several highly purified cultures converting acetate, propionate,**
37 **butyrate, benzoate and EtOH to methane. Most syntrophs belonged to *Firmicutes*,**
38 **while the propionate-oxidizer formed a novel lineage within the family**
39 ***Syntrophobacteraceae* in the *Deltaproteobacteria*. The acetate-oxidizing syntroph was**
40 **identified as '*Ca*. Syntrophonatronum acetioxidans' previously found to convert**
41 **acetate at sulfate-reducing conditions up to very high salt concentrations. Two**
42 **butyrate-utilizing and a benzoate-utilizing syntrophs represent novel genus-level**
43 **lineages in *Syntrophomonadales* which are proposed as Candidatus taxons**
44 **'Syntrophobaca', 'Syntrophocurvum' and 'Syntropholuna'. Overall, despite a very**
45 **slow growth, the results indicated the presence of a functionally competent**
46 **syntrophic community in hypersaline soda lakes, capable of efficient oxidation of**
47 **fermentation products to methane at extremely haloalkaline conditions.**
48

49 **Keywords** syntrophic, methanogenic, haloalkaliphilic, hypersaline soda lakes,
50 *Methanocalculus*, *Syntrophobacteraceae*, *Syntrophomonadales*
51

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52 **Introduction**

53 Thermodynamically unfavorable anaerobic oxidation of fatty acids and alcohols driven
54 by the interspecies electron transfer, either in a classical form of H_2/formate or recently
55 discovered DIET (direct interspecies electron transfer) (Rotaru et al., 2014; Lohner et al.,
56 2014) is a well established phenomenon of microbial metabolism "at the edge of a knife",
57 i.e. proceeding near thermodynamic equilibrium (reviewed by: Schink 2002; Schink and
58 Stams, 2006; Hattori 2008; McInerney et al., 2008; 2009; Sieber et al., 2012; Warm et al.,
59 2010; 2014). The essence of the process is that two or more organisms can do what each
60 of them can not separately, acting in syntrophy. That is possible when a second partner,
61 normally hydrogenotrophic methnogens or SRB, or acetoclastic methanogens, remove the
62 reducing equivalents very efficiently from the reaction medium shifting the reaction
63 equilibrium to the right side. And yet, the total available energy which also has to be
64 shared by two or more organisms, is so little that it is barely enough to sustain growth,
65 which makes this kind of conversions a bottleneck in anaerobic organic matter
66 degradation both in natural systems and industrial anaerobic digestion processes.
67 Therefore, this type of conversions is easily upset by nonoptimal conditions imposing an
68 additional adaptational demands on energetics of the consortia. High salt concentrations
69 and high pH, for sure, are such burdens, since they impose high energy demands (Oren,
70 1999; 2011). There are practically no data available on syntrophy already for high salt
71 neutral conditions. And when high salt and high pH combine in hypersaline soda lakes, it
72 appears that syntrophic metabolism has no chance to exist. Indeed, until now, there was
73 no evidence of syntrophic methanogenesis at haloalkaline conditions of soda lakes. The
74 only reported cases of syntrophy were observed at sulfate-reducing conditions (with

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75 higher total energy output than at methanogenic conditions) for acetate oxidation (Zhilina
76 et al., 2005; Sorokin et al., 2014 a).

77 Hypersaline soda lakes are a special type of athalassic salt lakes with a substantial
78 fraction of sodium carbonates in the brines resulting in stable pH around 9.5-10.5 due to
79 molar soluble alkalinity. They are present world wide in rain-shadow (North America,
80 Africa) and dry steppe regions (Central Asia). Despite harsh conditions, diverse
81 communities of Prokaryotes, apparently well adapted to high salt-high pH conditions,
82 have been detected in various soda lakes, including hypersaline examples (Humayoun *et*
83 *al.*, 2003; Rees *et al.*, 2004; Ma *et al.*, 2004; Mesbah *et al.*, 2007; Mwrichia et al., 2010;
84 Sorokin et al., 2014 b; 2015 a). Sulfur-rich anoxic sediments of soda lakes are shown to
85 harbor active and diverse populations haloalkaliphilic bacteria performing sulfur-
86 dependent respiration (sulfate, thiosulfate and elemental sulfur as *e*-acceptors) resulting in
87 often accumulation of millimolar concentrations of free sulfide (Sorokin et al., 2010;
88 2011 a).

89 On the other hand, in some of these lakes the sediments also contain high
90 concentrations of methane. Methanogenesis is known to occur in alkaline saline lakes and,
91 until recently, "noncompetive" methylotrophic pathway was supposed to be responsible
92 for the process (Iversen et al., 1987; Oremland and Des Marais, 1983; Oremland et al.,
93 1982; 1987; 1993; Oremland and Miller, 1993; Kulp et al., 2007; McGenity 2010).
94 However, recent research has provided evidences that a unique group of
95 hydrogenotrophic methanogens belonging to the genus *Methanocalculus* can be active at
96 conditions of hypersaline soda lakes (Zhilina et al., 2013) at a much higher total salt
97 concentration than has previously been considered as a limit for this process (i.e. 2 M
98 NaCl; Oren 1999; 2011). Our recent focused investigation of methanogenes in

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99 hypersaline soda lakes in south-western Siberia confirmed the early data on the North
100 American soda lakes but, also provided more details on the composition of methanogenic
101 archaea living in soda lakes and also resulted in several surprising discoveries which
102 substantially change the whole picture of methanogenesis in hypersaline habitats (Nolla-
103 Ardèvol et al., 2012; Sorokin et al., 2015 b). First, we have discovered that lithotrophic
104 methanogenesis in hypersaline soda lakes can be active up to soda-saturating conditions
105 (pH 10, 4 M total Na^+) which increase the previously accepted border two times. The
106 organisms responsible for this activity have recently been described as *Methanocalculus*
107 *natronophilus* and *Methanocalculus alkaliphilus* (Zhilina et al., 2013; Sorokin et. al.,
108 2015 c). Secondly, we have discovered a possibility of acetoclastic methanogenesis at
109 low salt alkaline conditions and enriched the responsible organism *Methansaeta* strain
110 Mx capable of growth at pH up to 10 and marine salinity. And the last, an activity of
111 conversion of fatty acids and alcohols to methane has been shown in sediments
112 incubations and primary enrichments of syntrophic associations of acetogens and
113 natronophilic *Methanocalculus*. The detailed analysis of the latter is reported in the
114 results below.
115
116

117 **Results**

118
119 *Potential methanogenic activity in the sediments*
120
121 Addition of acetate, propionate, butyrate, benzoate and C_2-C_4 alcohols to sediment
122 slurries in the presence of 5 mM sodium molybdate (to inhibit SRB) resulted in methane

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123 formation in all incubations with variable rates (**Fig. 1**). The activity of methanogenesis
124 with alcohols and butyrate were lower, but in the same order of magnitude as measured
125 for the direct methanogenic substrate formate (Sorokin et al., 2015 b), while for other
126 substrates the rates were much lower. Assuming extreme salinity level in all of the
127 Kulunda soda lakes in 2011-2012 (above 100 g/l), the latter is not surprising. Despite this,
128 the positive results still allowed us to perform the next step in investigating which
129 bacteria were behind the conversions.
130

131 *Enrichment of syntrophic cultures and their molecular analysis*
132

133 The sediment slurries at the end of incubation experiments showing methane production
134 in the range of several % in the gas phase were used as a source for enrichment cultures
135 (1:100) using soda buffer-based media with pH 9.5 and salt concentration ranged from
136 0.6 to 3 M total Na^+. Positive enrichments producing methane in the range from 10 to
137 50% in the gas phase were obtained for all substrates, but at different salinities.
138 Propionate, benzoate and 2-PrOH were only positive at lowest salinity used (0.6 M Na^+,
139 equal to marine salt concentration), while other substrates gave positive results up to 3 M
140 total Na^+, although at salinity above 1 M those cultures developed extremely slow except
141 for EtOH and BuOH. Since all attempts to obtain growth on solid media in the presence
142 of two pure cultures of *Methanocalculus* failed, dilution series in liquid media was the
143 only way to minimize the consortia. Finally, stable cultures free of initial sediment
144 material were obtained for the following conditions: acetate - at 0.6, 1, 2 and 3 M Na^+;
145 butyrate - at 0.6, 2 and 3 M Na^+; propionate, benzoate, 1-and 2-PrOH - at 0.6 M Na^+;
146 EtOH and BuOH - at 2 M Na^+. Together with the sediment incubation experiments, this

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147 have already proven an existence of previously unsuspected haloalkaliphilic syntrophy
148 with broad substrate spectrum at methanogenic conditions.

149 Further work was aimed for the minimization of syntrophic consortia by dilution
150 to extinction technique using *Mc. alkaliphilus* for moderate salt conditions (0.6-1.5 M
151 Na^+) and *Mc. natronophilus* for high salt (2-3.5 M Na^+). It must be mentioned, that,
152 because of extremely slow growth with a very long lag phase in most of the case, this
153 work took a very long time and only in two cultures, so far, resulted in obtaining binary
154 consortia of a syntrophic bacterium and a methanogen - for propionate and for butyrate at
155 0.6 M Na^+. Nevertheless, in most of the other cases the number of bacterial members,
156 apart from the obviously dominant syntrophs, were substantially reduced (1 - 3) in
157 comparison with the initial cultures. The composition of bacteria in those final cultures
158 was analyzed by DGGE.

159 Analysis of various cultures converting acetate to methane at salinities from 0.6
160 and up to 3.5 M Na^+ at pH 9.5-10 demonstrated a domination of an extremely high salt-
161 tolerant alkaliphilic anaerob which has previously been found to oxidize acetate at
162 sulfate-reducing conditions with *Desulfonatronospira* sp. as a partner and described as
163 '*Ca*. Syntrophonatronum acetioxidans' - a novel member of the clostridial order
164 *Syntrophomonadales* (Sorokin et al., 2014 b) (Supplementary Fig. 1a; **Fig. 2a; Fig 3 a**).
165 Apparently this organism is the only one capable of reverse acetogenic acetate oxidation
166 at combined hypersaline and hyperalkaline conditions in syntrophy with high salt tolerant
167 natronophilic lithotrophic SRB or methanogens.

168 The dominant syntrophic members in butyrate converting cultures belonged to
169 two different novel groups (one at 0.6 M, and another - at 2 and 3 M Na^+) within the
170 family *Syntrophomonadacea* most closely related the members of the genus

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171 *Syntrophomonas* with 94 % 16S rRNA gene sequence similarity to each other and 92-
172 94% to the known species of *Syntrophomonas* (Supplementary Fig. 1b**; Fig. 2 b-e; Fig 3**
173 **a**). The culture at 0.6 M Na^+ was purified from contaminants but all our attempts to grow
174 it alone with more oxidized substrates than butyrate or with butyrate + *e*-acceptors failed
175 and, therefore, we suggest to consider it as an obligate syntroph with a candidatus status
176 '*Ca*. Syntrophofaba alkaliphila'. This organism was also able to grow in syntrophy with a
177 low salt-tolerant alkaliphilic SRB *Dnsv. magnus* (Supplementary Fig. 2).

178 The high salt-tolerant culture converting butyrate at 2-3 M Na^+, apart from the
179 dominant syntroph, still contained 2 other bacteria from bacteroidetes and a close relative
180 of *Haloanaerobium hydrogeniformans*. The latter was also present in natronophilic
181 syntrophic associations converting acetate at sulfate-reducing conditions, but its role
182 remains obscure since it was described as a sugar fermenter (Brown et al., 2011). In
183 some of the cultures, for example at pH 9, a formation of prospores were observed
184 (Supplementary Fig. 2). However, attempts to use pasterization to obtain a binary culture
185 failed, probably because of the absence of mature endospores. The high salt-tolerant
186 syntroph converting butyrate is suggested to classify as '*Ca*. Syntrophocurvum
187 alkaliphilum'.

188 The culture converting propionate to methane at low salt (0.6 M Na^+) contained a
189 single major bacterium identified as a member of the family *Syntrophobacteracea* in
190 *Deltaproteobacteria* - one of the few taxons containing dedicated propionate-oxidizing
191 syntrophs (Supplementary Fig. 1 c; **Fig. 2 f-g; Fig 3 b**). However its 16S rRNA sequence
192 was less than 95% similar to any members of the family indicating a possible new genus.
193 It was the only VFA-oxidizing syntroph from soda lakes capable of growth without

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194 *Methanocalculus* in presence of sulfate and, therefore, can be validly described as a new
195 genus and species, but is outside of scope of this work.
196 The dominant bacterial component in the culture converting benzoate to methane
197 at 0.6 M Na^+ was identified as a novel deep lineage in the order *Syntrophomonadales*
198 clustering with the '*Ca*. Syntrophonatronum' lineage (Supplementary Fig. 1 d; **Fig. 2 h-i;**
199 **Fig 3 a**). It is suggested to be classify as '*Ca*. Syntropholuna alkaliphila'.
200 The 2-PrOH utilizing methanogenic culture contained two dominant bacterial
201 components (Supplementary Fig. 1 e; **Fig. 2 j**), both within *Syntrophomonadales* (**Fig. 3**
202 **a**): one was closely related to sulfur/thiosulfate reducing lithotroph from soda lakes
203 *Dethiobacter alkaliphilus* (Sorokin et al., 2008), another - to '*Ca*. Contubernalis
204 alkaliaceticus', a low salt tolerant syntrophic acetate oxidizer from soda lakes (Zhilina et
205 al., 2005). Since the latter was also shown to oxidize 2-PrOH in syntrophy with SRB, it is
206 plausible to assume that this organism played a key role in the methanogenic culture as
207 well.
208 The bacterial part of the cultures developing on primary C_2-C_4 alcohols at 0.6-2 M
209 Na^+ was dominated by acetogens belonging to the haloalkaliphilic genus *Tindallia*
210 (Supplementary Fig. 1 f; **Fig. 2 k-l; Fig. 3 a**). The known species of this genus can not
211 oxidize alcohols, but, apparently, might be able to do it in the presence of electron-
212 consuming syntrophic partners, such as lithotrophic methanogens. Interestingly, two
213 closely related *Tindallia* strains were present in the EtOH culture at 2 M Na^+ (**Fig. 2 k**).
214 We have tried to see if their relative abundance was influenced by pH-salinity variation.
215 One of the two, closely related to "Clostridium elmenteita", a misplaced member of the
216 genus *Tindallia*, was indeed more dominant at high pH and salinity (Supplementary Fig.
217 1 g**).**

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218 *Growth kinetics of selected syntrophic associations*

219

220 The acetate-converting methanogenic associations were obtained from 4 soda different
221 soda lakes at pH 10 and salt concentration up to 3 M total Na^+, which was unexpected
222 given a very low energy yield of the conversion (Suppl. Table 1). However, the culture
223 growth was extremely slow (**Fig. 4 a**) and it took in average 1-2 years to obtain growth in
224 final dilutions. The acetate was converted to methane close to stoichiometry of the
225 reaction (close to 50 % carbon). We were also able to prove that acetate-converting '*Ca*.
226 Syntrophonatronum' was able to switch to sulfidogenic conditions in presence of sulfate
227 and *Desulfonatronospira* sp. ASO3-2, originally isolated from the acetate-converting
228 association with '*Ca*. Syntrophonatronum' (Sorokin et al., 2014 b).

229 Growth dynamics of the propionate-converting culture was unpredictable,
230 normally with only a single positive culture out of 3-4 inoculated, reflecting a difficulty
231 of such a conversion even at moderate salinity and high pH (actually, we did not find in
232 the available literature any evidences on influence of salinity and high pH on syntrophic
233 propionate oxidation at methanogenic conditions). **Fig. 4 b** represents an example of
234 growth on propionate of two different cultures with significantly different rate of methane
235 formation between them. The most active culture converted 30 mM propionate into 26
236 mM acetate and 22 mM methane, which is not far from a theoretical stoichiometry (Suppl.
237 Table 1). Since the dominant organism belonged to the family *Syntrophobacteracea*
238 which members can grow on propionate with sulfate as *e*-acceptor, this possibility was
239 also tested for the soda lake association, and indeed, we were able to cultivate the
240 dominant bacterial component independent from the methanogen. Eventually a pure
241 culture, strain APr1, was obtained and it is currently under investigation.

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242 The culture selected on butyrate at moderate salinity was the most fast-growing
243 among the organic acids-converting soda lake consortia, although, similar to most of the
244 others, it suffered from fast inactivation during even short-term storage, which typically
245 resulted in a very long lag phase in transferes (**Fig. 4 c**). 20 mM butyrate was converted
246 to acetate and methane close to theoretical stoichiometry (Suppl. Table 1**)**. This culture
247 was eventually minimized to a single bacterial partner and it was shown to be able to
248 oxidize C_4-C_{12} straight chain fatty acids and iso-butyrate in consortium with *Mcc.*
249 *alkaliphilus* at pH 10. In this culture a biomass ratio (calculated as approximate
250 biovolumes) between the bacterial and archaeal partners varied from 2.4 (early
251 exponential) to 1.7 (late exponential growth phase), indicating that the bacterial partner
252 obtained more energy from the overall conversion.

253 On the other hand, the association selected on butyrate at extreme salinity of 2-3
254 M total Na^+ was very difficult to maintain in active culture and its growth was extremely
255 sporadic and unpredictable. Nevertheless, the kinetic data were obtain in a few successful
256 cultures (**Fig. 4 d**). The graph demonstrates methane formation in 3 different cultures at 2
257 M total Na^+ with practically incomparable rates. The most efficient culture converted
258 butyrate to acetate and methane close to stoichiometry of incomplete butyrate oxidation.

259 The benzoate-converting culture was the most slow and poorly-growing. In most
260 of the cases it would start to produce methane with some growth of biomass and then
261 stopped after only 1-2 mM of benzoate out of 10 provided was converted. In some cases,
262 shown on **Fig. 3 e** a steady methane formation was maintained by periodic addition of
263 1 % (v/v) of freshly grown culture *Mcc. alkaliphilus*. Acetate and methane were the final
264 products of benzoate conversion. However, since the benzoate consumption was not
265 analyzed, it is not possible to make a conclusion about the stoichiometry of the

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266 conversion. Shifting to sulfate-reducing conditions with *Dnsv. magnus* as a partner
267 slightly improved the growth reproducibility and the density of the culture
268 (Supplementary Fig. 2). Currently we use these condition to obtain a binary culture.

269 The EtOH-converting culture was the fastest and most robust among the high-salt
270 tolerant consortia from soda lakes, although it also suffered from storage inactivation and
271 had a lag phase at low cell density in the beginning of growth (**Fig. 4 f**). EtOH was
272 converted to acetate and methane close to the theoretical stoichiometry (Suppl. Table 1).

273

274 *Influence of pH and salinity on growth and activity of methanogenic associations from*
275 *soda lakes*

276

277 The key environmental parameters setting hypersaline soda lakes apart from any other
278 habitats are high salt concentration, mostly in the form of sodium carbonates, which, in
279 turn, result in two other extreme parameters - molar range of soluble alkaline buffering
280 capacity creating stable pH around 10. To test the response of the selected syntrophic
281 associations to salinity in the form of sodium carbonates and to pH optimal salinity the
282 salt and pH profiling was performed both for growing cultures (**Fig. 5 a-b**) and for
283 washed (resting) cells (catabolic activity; **Fig. 5 c-d**). The obtained profiles are a sum of
284 the response of the bacterial and archaeal components. From the previous work with the
285 two *Methanocalculus* species isolated from the same lakes, which served as the
286 hydrogenotrophic partners in the studied association, we knew that they are functional
287 within the pH range from 8 to 10.5 and cover the whole salinity range being tested.
288 Therefore, the archaeal part of the associations mast have not limited the response of the
289 whole associations. The obtained profiles were more or less similar to the profiles for the

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290 methanogens, i.e. with the range for active growth starting from 8.5 and up to 10.2 with
291 few cases extending to pH up to 10.5 and an optimum from 9 to 10 (**Fig. 5 a-b**). In
292 general, the activity of washed cells extended a bit more into the neutral zone in
293 comparison with the growing cultures and, in case of acetate, behind pH 10.5, while the
294 pH optimum remained within the same range except for the culture growing on butyrate
295 at high salt. There an unusual high pH shift of 1 unit for the optimum was observed in
296 resting cells in comparison to the growing culture.

297 The salt response of both growing and resting cells clearly correlated with the salt
298 concentration at which the certain association was enriched and isolated (**Fig. 5 c-d**). The
299 most salt sensitive association was the benzoate-converting, which lost viability and
300 activity already at salinity above 1 M total Na^+. The growth of propionate- and the
301 butyrate-converting cultures enriched at 0.6 M Na^+ was also barely possible at salinity
302 above 1 M total Na^+, however, the metabolic activity was still observed up to 1.5-1.7 M.
303 The cultures enriched at 2-5 times higher salinity, converting acetate, butyrate and EtOH,
304 were able to grow close to soda-saturating conditions (4 M total Na^+). Particularly
305 remarkable was the ability of acetate-converting association from Bitter-1 hypersaline
306 soda lake to grow above 3 M total Na^+ despite an extremely low growth rates.
307 Interestingly, after a step-wise adaptation to 3 M Na^+, this culture eventually proliferated
308 at 3.75 M - the highest value at which its methanogenic partner *Mcc. natronophilus* was
309 able t reach in pure culture with formate as substrate. Overall, the pH-salinity profiling
310 demonstrated the obligate natronophilic (soda-loving) nature of the syntrophic
311 methanogenic association enriched from soda lakes.

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314 **Discussion**

315

316 The main significance of the obtained results is in uncovering a previously unrecognized
317 potential for syntrophic methanogenic degradation of VFA and alcohols at double
318 extreme conditions dominating in soda lakes. Because of a very low energy yield, in
319 general, of such conversions, one would expect that they can not be realized at conditions
320 imposing additional high energy cost, such as high salinity and maintenance of
321 unfavorable pH gradient. Nevertheless, we found that in conditions of hypersaline soda
322 lakes all tested substrates were converted, albeit at extremely slow rates, even those
323 which have the lowest energy yield, such as acetate and propionate oxidation **(Suppl.**
324 **Table 1)**. Apparently, there are mechanisms to overcome the unfavorable
325 thermodynamics. We recalculated the Gibbs energy change for the reactions in
326 hypersaline alkaline lakes in comparison with typical neutrophilic anaerobic digester
327 conditions. Calculations were conducted assuming thermodynamic equilibrium between
328 the different inorganic carbon species and the formate lyase reaction to bicarbonate and
329 molecular hydrogen. A total salt concentration of 1 M and a pH of 10 were assumed for
330 the soda lake conditions. The results demonstrate that energy distribution in syntrophic
331 communities between formate/hydrogen producing and consuming strains change in
332 comparison with neutrophilic conditions. Evidently, the high pH makes proton producing
333 reactions more favorable, but inorganic carbon production makes the reaction less
334 favorable. It should be noted that a specific value for the hydrogen partial pressure was
335 assumed in the calculations, but it may very well be that the actual redox state is different
336 enabling more equal distribution of Gibbs energy between syntrophic partners.

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337 Another possible explanation for the obviously surprising possibility of such low
338 energy-yielding conversions in double extreme conditions of hypersaline soda lakes
339 might be rooted in the so far poorly recognized fact of a dramatic difference between
340 alkaline sodium carbonates and neutral NaCl brines as the live media for Prokaryotes.
341 The osmotic pressure of NaCl (strong electrolyte, high osmotic pressure) brines at 2-4 M
342 Na^+ is two times higher than in sodium carbonate (weak electrolytes, low osmotic
343 pressure) brines with the same sodium concentration, as was previously demonstrated for
344 haloalkaliphilic sulfur-oxidizing bacteria from soda lakes (Sorokin et al., 2015 a). That
345 would mean two times less energy demands for the osmoprotection, which are extremely
346 high, especially in case of organic osmotic strategy (Oren, 1999; 2011).

347 The overview of diversity and of properties of the obtained syntrophic
348 associations are presented in **Table 1**. The bacterial partners in 4 out of 9 obtained
349 cultures fell into novel lineages of the genus level. The propionate-converting syntroph
350 was the only one which grew separately without a hydrogenotrophic partner and will be
351 described elsewhere as a new taxon within the family *Syntrophobacteracea*. The other 3
352 syntrophs, converting butyrate and benzoate are proposed here to form 3 Candidate
353 taxons within the class *Clostridia* , which formal diagnoses are presented below.

354

355

356 Description of '***Candidatus*** **Syntrophofaba'** gen. nov.

357 [Syn.tro.pho.fa′ba] Gr. prep. *syn*, in company with, together with; Gr. n. *trophos*, one who
358 feeds; L. fem. n. *faba*, a bean; N.L. fem. n. *Syntrophofofaba*, a bean-shaped syntroph.
359

360 Gram-positive curved rods. Obligate anaerobic, oxidizing fatty acids in syntrophy with
361 the H_2-consuming partners. Obligate haloalkaliphiles. Habitat - soda lakes. A member of

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362 the family *Syntrophomonadaceae* (order *Syntrophomonadales*, class *Clostridia*). Type
363 species – *S. alkaliphila.*
364
365 Description of '***Ca.* Syntrophofaba alkaliphila'** sp. nov.
366 N.L. n. *alkali*, soda ash (from Arabic *al-qalyi*, the ashes of saltwort); N.L. adj. *philus -a -*
367 *um* (from Gr. adj. *philos -ê -on*), friend, loving; N.L. fem. adj. *alkaliphila*, alkali-loving.
368
369 Gram-positive rods, 0.5-0.6 x 1-1.5 µm, motile by peritrichous flagella located mostly at
370 the inside part of the cell arcs. Obligately anaerobic, oxidizing C_4-C_{12} straight chain fatty
371 acids and *iso*-butyrate to acetate in the presence of haloalkaliphilic hydrogenotrophic
372 partners, such as *Methanocalculus alkaliphilus* or *Desulfonatronovibrio magnus*. Do not
373 grow alone on pyruvate, crotonate or fumarate and on butyrate in presence of sulfate.
374 Obligately haloalkaliphilic with the pH range for growth in syntropic culture from 9.3 to
375 10.2 (optimum at 9.5) and sodium carbonate concentrations from 0.3 to 1.0 (optimum at
376 0.6) M total Na^+. Growth is optimal at 33-35°C. The type strain B(0.6M)^T is maintained
377 in a syntrophic coculture with the natronophilic methanogenic partner *Methanocalculus*
378 *alkaliphilus* deposited in the DSMZ culture collection under the number DSM XXXX
379 and in the UNIQEM culture collection under the number U997. Isolated from anaerobic
380 sediments of hypersaline soda lakes in Kulunda Steppe (Altai, Russia). The GenBank 16S
381 rRNA gene sequence accession number of the type strain B(0.6M)^T is KU681302.
382
383 Description of '***Candidatus* Syntrophocurvum'** gen. nov.
384 [Syn.tro.pho.cur′vum] Gr. prep. *syn*, in company with, together with; Gr. n. *trophos*, one
385 who feeds; *curvum*: N.L. neut. substantive from L. neut. adj. *curvum*, curved.
386 *Syntrophocurvum*, an arc-shaped syntroph.
387
388 Gram-positive curved rods. Obligate anaerobic, oxidizing butyrate in syntrophy with the
389 H_2-consuming partners. Extremely salt-tolerant and alkaliphilic. Habitat - soda lakes. A

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390 member of the family *Syntrophomonadaceae* (order *Syntrophomonadales*, class
391 *Clostridia*). Type species – *S. alkaliphilum.*
392
393 Description of '***Ca.* Syntrophocurvum alkaliphilum'** sp. nov.
394 N.L. n. *alkali*, soda ash (from Arabic *al-qalyi*, the ashes of saltwort); N.L. adj. *philus -a -*
395 *um* (from Gr. adj. *philos -ê -on*), friend, loving; N.L. neut. adj. *alkaliphilum*, alkali-loving.
396
397 Gram-positive curved rods, 0.4 x 4-8 μm, motile by peretrichous flagella. Obligately
398 anaerobic, oxidizing butyrate to acetate in the presence of haloalkaliphilic
399 hydrogenotrophic partners, such as *Methanocalculus natrophilus* or *Desulfonatronovibrio*
400 *thiodismutans*. Do not grow alone on pyruvate, crotonate or fumarate and on butyrate in
401 presence of sulfate. Obligately alkaphilic and extremely salt tolerant. Grows in syntropic
402 methanogenic cultures within the pH range from 8.5 to 10 (optimum at 9) and sodium
403 carbonate concentrations from 1 to 3 (optimum at 1.5) M total Na^+. The optimal
404 temperature is between 30 and 37°C. The type strain $B(2M)^T$ in a syntrophic coculture
405 with the natronophilic methanogenic partner *Methanocalculus natronophilus* is deposited
406 in the UNIQEM culture collection under the number U998. The culture was obtained
407 from anaerobic sediments of hypersaline soda lakes in Kulunda Steppe (Altai, Russia).
408 The GenBank 16S rRNA gene sequence accession number of the type strain $B(2M)^T$ is
409 KU681303.
410
411 Description of '***Candidatus* Syntropholuna'** gen. nov.
412 [Syn.tro.pho.lu′na] Gr. prep. *syn*, in company with, together with; Gr. n. *trophos*, one who
413 feeds; *luna,* L. fem. n. *luna*, a crescent, lune; N.L. fem. n. *Syntropholuna*, a crescent-
414 shaped syntroph.
415
416 Gram-positive curved rods. Obligate anaerobic, oxidizing benzoate in syntrophy with the
417 H_2-consuming partners. Moderately salt-tolerant and alkaliphilic. Habitat - soda lakes. A

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418 member of the order *Syntrophomonadales* (class *Clostridia*). Type species – *S.*
419 *alkaliphila.*
420
421 Description of '***Ca.* Syntropholuna alkaliphila'** sp. nov.
422 N.L. n. *alkali*, soda ash (from Arabic *al-qalyi*, the ashes of saltwort); N.L. adj. *philus -a -*
423 *um* (from Gr. adj. *philos -ê -on*), friend, loving; N.L. neut. adj. *alkaliphila*, alkali-loving.
424
425 Gram-positive curved rods, 0.8-1 x 2.5-5 µm, motile by a single thick flagellum.
426 Obligately anaerobic, oxidizing benzoate to acetate in the presence of haloalkaliphilic
427 hydrogenotrophic partners, such as *Methanocalculus alkaliphilus* or
428 *Desulfonatronovibrio magnus*. Do not grow alone on pyruvate, crotonate or fumarate and
429 on benzoate or butyrate in presence of sulfate. Obligately alkaphilic and moderately salt
430 tolerant. Grows in syntropic methanogenic cultures within the pH range from 8.5 to 10.2
431 (optimum at 9.5) and sodium carbonate concentrations from 0.3 to 1.2 (optimum at 0.6)
432 M total Na^+. The optimal temperature is 30-33°C. The type strain Be(0.6M)^T in a
433 syntrophic coculture with the natronophilic methanogenic partner *Methanocalculus*
434 *alkaliphilus* is deposited in the UNIQEM culture collection under the number U996. The
435 culture was obtained from anaerobic sediments of hypersaline soda lakes in Kulunda
436 Steppe (Altai, Russia). The GenBank 16S rRNA gene sequence accession number of the
437 type strain B(2M)^T is KU681304.
438

439 Experimental procedures

440

441 *Potential methanogenic activity in sediment slurries*

442

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443 The samples of top 10 cm anaerobic sediments in 5 hypersaline soda lakes in Kulunda
444 Steppe (Altai, Russia) were taken in July 2011-2012. The lakes characteristics and the
445 details of field measurements and are given in the previous publication (Sorokin et al.,
446 2015 b). The potential for syntrophic methanogenic conversions in the sediments were
447 measured by monitoring methane formation in sediment slurries prepared by 1:1 mixing
448 with anoxic brines and incubated at 30°C. The substrates were supplied at 10 mM C and 5
449 mM sodium molybdate was added to inhibit activity of SRB. The sediments without
450 substrate addition served as an endogenous control. The incubations were performed in
451 duplicates in 25 ml serum bottles filled with 10 ml slurries capped with butyl rubber
452 stoppers and made anoxic by 3 cycles of evacuation-flushing with Ar gas. 0.1 ml gas
453 samples were periodically taken for methane analysis.
454

455 *Enrichment and cultivation of haloalkaliphilic syntrophic associations*
456

457 Sediment slurries showing potential methanogenic activity were used as a source for
458 further enrichment and purification of haloalkaliphilic methanogenic associations. For
459 this, the sodium carbonate-based buffered at 9.5-10 containing 0.6-4.0 M of total Na^+ and
460 1 g/L of K_2HPO_4 was used. After sterilization, the medium was supplemented with (final
461 concentration): 4 mM NH_4Cl, 1 mM $MgCl_2$, acidic trace metals (1 ml/L) and vitamins (1
462 ml/L) (Pfennig & Lippert, 1966), basic Se/W solution (1 ml/L) (Plugge, 2004) and yeast
463 extract (10 mg/l). Organic carbon substrates were supplied at concentrations 10-20 mM.
464 The medium was dispensed either into 30 ml serum bottles (20 ml) for primary
465 enrichments, 120 ml bottles (80 ml) for growth dynamic experiments or into 15 ml
466 Hungate tubes (10 ml) for serial dilutions. Na_2S (1 M) was added from a filter-sterilized

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467 anaerobic stock solution as a reductant and oxygen was removed by five cycles of
468 flushing with argon gas. Final reduction of the medium was done by adding a drop of
469 10% dithionite solution in 1 M $NaHCO_3$. At the later stages of culture purification by
470 serial dilutions, fully grown cultures of 4 haloalkaliphilic hydrogenotrophic partners
471 isolated earlier from the same source were being added at the start of incubation at 5%
472 (v/v): *Methanocalculus alkaliphilus* AMF2 and *Mc. natronophilus* AMF5 at salinities
473 0.6-1 and 2-3.5 M total Na^+, respectively (Sorokin et al., 2015 c) or *Desulfonatronovibrio*
474 *magnus* and *Desulfonatronovibrio thiodismutans* at 0.6-1.0 M and 2-3 M Na^+,
475 respectively (Sorokin et al., 2011 b).
476 The salinity effect was studied by using sodium carbonate/bicarbonate buffer with pH
477 9.5-10 and a Na^+ concentration ranging from 0.2 to 4 M. The pH effect was determined
478 by using HEPES/$NaHCO_3$ buffer for the pH range from 6 to 8 and a $NaHCO_3$/Na_2CO_3
479 buffer for pH 8.5-11. The profilings were performed both in growing cultures and for
480 methanogenic activity of washed cells, obtained from 0.5 L cultures harvested at the end
481 of exponential growth phase. Since the pH after incubation often deviated substantially
482 from the initial value, the final pH values were measured and they are indicated in the
483 results. All experiments were done in duplicate and, in some cases, in triplicates.
484
485 **Molecular and phylogenetic analyses**
486 The DNA was extracted from the cells using the UltraClean Microbial DNA Isolation kit
487 (MoBio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions.
488 The nearly complete 16S rRNA gene was obtained from finally diluted associations using
489 standard molecular cloning procedure with general bacterial primers 11f-1492r (Lane,
490 1991). The PCR products were purified using the Wizard SV-gel and PCR Clean-Up

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491 System (Promega), ligated into plasmids using pGEM-T Easy Vector Systems (Promega)
492 and the plasmids were then electroporated into the competent cells of *E. coli* strain
493 DH10B. The DNA from positive clones (25) was extracted with Wizard MiniPreps
494 (Promega). Community analysis of syntrophic associations was performed by using 16S-
495 rRNA gene-based DGGE according to Schäfer and Muyzer (2001). For the 16S-rRNA
496 analysis the primer pair was bacterial 341f + GC clamp/907r with the gel gradient from
497 20 to 70%. Phylogenetic analysis was reconstructed using ARB software. The sequences
498 were aligned using Codoncode aligner (CodonCode Corp., Dedham USA). Sequences
499 were aligned with complete length sequences of closest relatives from the orders
500 *Syntrophomonadales* and the family *Syntrophobacteraceae* obtained using the ARB
501 FastAligner utility. The maximum likely hood method, RAxML (implemented in ARB),
502 was used to calculate the resulting phylogenetic trees.
503
504 **Analytical procedures and microscopy**
505 Concentrations of methane in the gas phase were measured by GC (Chromateck Crystall
506 5000 (Ufa, Russia); Column Hayesep 80-100 mesh, 2 m x 3 mm, 40°C; Detector: FID,
507 200°C; carrier gas: argon, 25 ml/min). Concentrations of VFA and alcohols were
508 measured in filtrated brines after their dilution to 0.5 M total Na^{+} and neutralization to pH
509 7 by titration with 2 M HCl. The HPLC parameters were as follow: Animex HPX-87H
510 column at 60°C, eluent 5 mM H_2SO_4 at 0.6 ml min^{-1}, UV and RI detectors.
511 Phase contrast microscopy and microphotographs were done with a Zeiss
512 Axioplan Imaging 2 microscope (Göttingen, Germany). The biovolume estimation for the
513 binary syntrophic culture converting butyrate at 0.6 M Na^{+} was done by counting the
514 cells of the bacterial (average biovolume = 0.5 μm^{3} as a cylinder) and the methanogen

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515 (average biovolume = 0.35 μm^3 as a sphere) member in 20 randomly selected fields. For
516 electron microscopy, the cells were prefixed in 2.5% glutaraldehyde added directly to the
517 culture aliquots for 1 h at 4oC. Then the cells were centrifuged and resuspended in the
518 same volume of NaCl solution with the same sodium molarity as in the original cultures.
519 The cells were positively stained by 2% uranyl acetate.

520 The thermodynamic calculations of the syntrophic conversions taking into
521 account the soda lake conditions are presented in Supplementary Table 2.

522

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525

526

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528

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642 **Table 1** Characteristics of haloalkalphilic syntrophic methanogenic associations enriched from hypersaline soda lakes

Characteristics	**Acetate**	**Butyrate**		**Propionate**	**Benzoate**	**EtOH**	**2-PrOH**
Enriched at (M Na^+)	0.6-3	0.6	2.0	0.6	0.6	2.0	0.6
Fermenting syntroph	'*Ca.* Syntrophonatronum acetioxidans'	'*Ca.* Syntrophofaba alkaliphila'	'*Ca.* Syntrophocurvum alkaliphilum'	*Syntrophobacteracea* strain APr1	'*Ca.* Syntropholuna alkaliphila'	*Tindallia* spp.	*Dethiobacter* '*Ca.*Contubernalis alkaliaceticus '
Methanogenic partner	*Methanocalculus natronophilus*	*Methanocalculus alkaliphilus*	*Methanocalculus natronophilus*	*Methanocalculus alkaliphilus*	*Methanocalculus alkaliphilus*	*Methanocalculus natronophilus*	*Methanocalculus alkaliphilus*
Phylogenetic status	New genus	New genus	New genus	New species	New genus	Known species	Known species
Obligate syntrophy	+	+	+	Can grow with propionate+sulfate	+	nd	nd
Ability to grow with SRB as partner	+ (*Desulfonatrono spira*)	+ (*Desulfonatrono vibrio*)	+ (*Desulfonatrono vibrio*)	Can grow alone as an SRB	+ (*Desulfonatrono vibrio*)	nd	nd
Final products	CH_4	Acetate+CH_4	Acetate+CH_4	Acetate+CH_4	Acetate+CH_4	Acetate+CH_4	CH_4
pH profile : growth	8.9-10.2 (opt. 9.7)	9.3-10.2 (opt. 9.5)	8.5-10 (opt. 9.0)	8.4-10.2 (opt. 9.5)	8.5-10.2 (opt.9.5)	8.0-10.4 (opt.10.0)	
pH profile: activity	8.5-10.7 (opt .9.5)	9-10.4 (opt.9.5)	9.0-10.3 (opt. 9.5)	8.0-10.5 (opt. 9.5)	8.6-10.15 (9.3-9.5)	nd	
Salinity profile: growth (Na^+, M)	0.5-3.0 (opt. 0.3-1.0)	0.3-1.0 (opt. 0.6)	1.0-3.0 (opt.1.0)	0.3-1.5 (opt. 0.6)	0.3-1.2 (opt.0.6)	0.3-3.75 (opt. 0.6-1.0)	
Salinity profile: activity (Na^+, M)	0.3-3.0 (opt.0.6-1.5)	0.2-1.0 (opt. 0.4)	1.0-3.0 (opt.1.5)	0.3-1.5 (opt.0.2-0.4)	0.2-1.5 (opt.0.2-0.4)	nd	
Lag-phase for growth (inoculum 2%)	3-4 weeks	1 week	2-3 weeks	1-3 weeks	4-8 weeks	1-2 weeks	1-4 weeks
μ_{max} (d^{-1}) (on the basis of CH_4 accumulation at optimal pH-salt)	0.07	0.50	0.10	0.10	0.035	0.11	0.06

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643 **Figure legends**

644

645 **Fig. 1** Potential for methanogenic conversions of VFA and alcohols in anaerobic sediments
646 from hypersaline soda lakes in Kulunda Steppe (Altai, Russia) taken in 2011-2012. The lakes
647 salinity was from 100 to 400 g/l and pH was around 10. The acetate conversion was tested in 3
648 individual lakes from 2012, while the rest of the substrates were tested in mixed sediments from 5
649 lakes sampled in 2011. 5 mM sodium molybdate was added to inhibit sulfidogenesis. The data
650 represent the average from two independent replicates with standard deviation within the range of
651 5 to 30%. The endogenous rates (without substrate addition) were subtracted.

652

653 **Fig. 2** Cell morphology of purified syntrophic methanogenic associations from hypersaline soda
654 lakes grown at pH 9.5-10. **a**, acetate at 1 M Na^+; **b-c**, butyrate at 0.6 M Na^+; **d-e**, butyrate at 2 M
655 Na^+; **f-g**, propionate at 0.6 M Na^+; **h-i**, benzoate at 0.6 M Na^+; **j**, 2-PrOH at 0.6 M Na^+; **k**, 1-PrOH
656 at 0.6 M Na^+; **l**, EtOH at 2.0 M Na^+.

657

658 **Fig. 3** 16S r-RNA-based phylogeneny of bacterial members in syntrophic methanogenic
659 associations from hypersaline soda lakes. **a**, propionate-converting syntroph in *Syntrophobacterales*
660 (*Deltaproteobacteria*); **b**, soda lake syntrophs in *Syntrophomonadales* (*Firmicutes*).

661

662 **Fig. 4** Growth kinetics of purified syntrophic methanogenic associations from hypersaline soda
663 lakes at pH 9.5-10. **a**, acetate at 1 M Na^+; **b**, propionate at 0.6 M Na^+; **c**, butyrate at 0.6 M Na^+; **d**,
664 butyrate at 2 M Na^+; **f**, benzoate at 0.6 M Na^+; **e**, EtOH at 2.0 M Na^+. In case of acetate and EtOH
665 the data represent mean from two parallel cultures with standard deviations less than 10%. For other
666 cultures methane formation is presented for 2-3 parallels, while the other data are given for the
667 fastest cultures.

668

669 **Fig. 5** Influence of pH (**a-b**) and salinity in the form of sodium carbonates (**c-d**) for growth (**a, c**)
670 and methanogenic activity of washed cells (**b, d**) in selected syntrophic methanogenic
671 associations from soda lakes. The data are mean from two independent experiments. The
672 maximum rates of methane formation (VCH_4) were calculated during exponential growth for
673 growing cultures or from the linear plots for washed cell suspension experiments.

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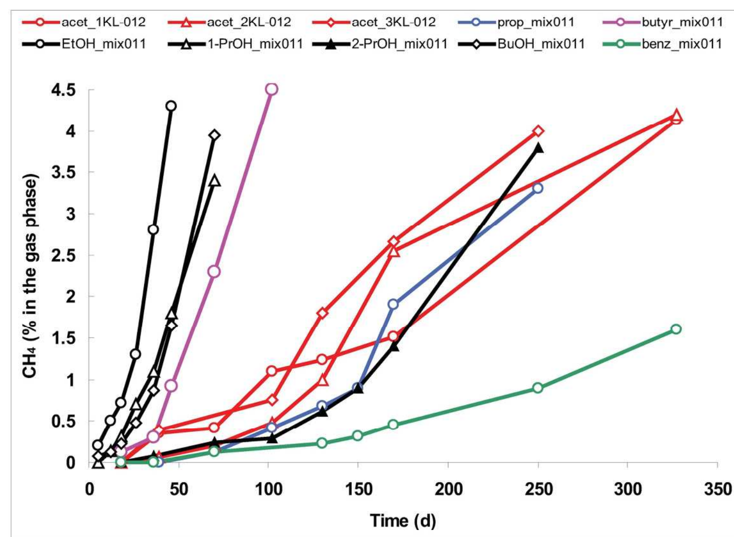

Fig. 1

Fig.1
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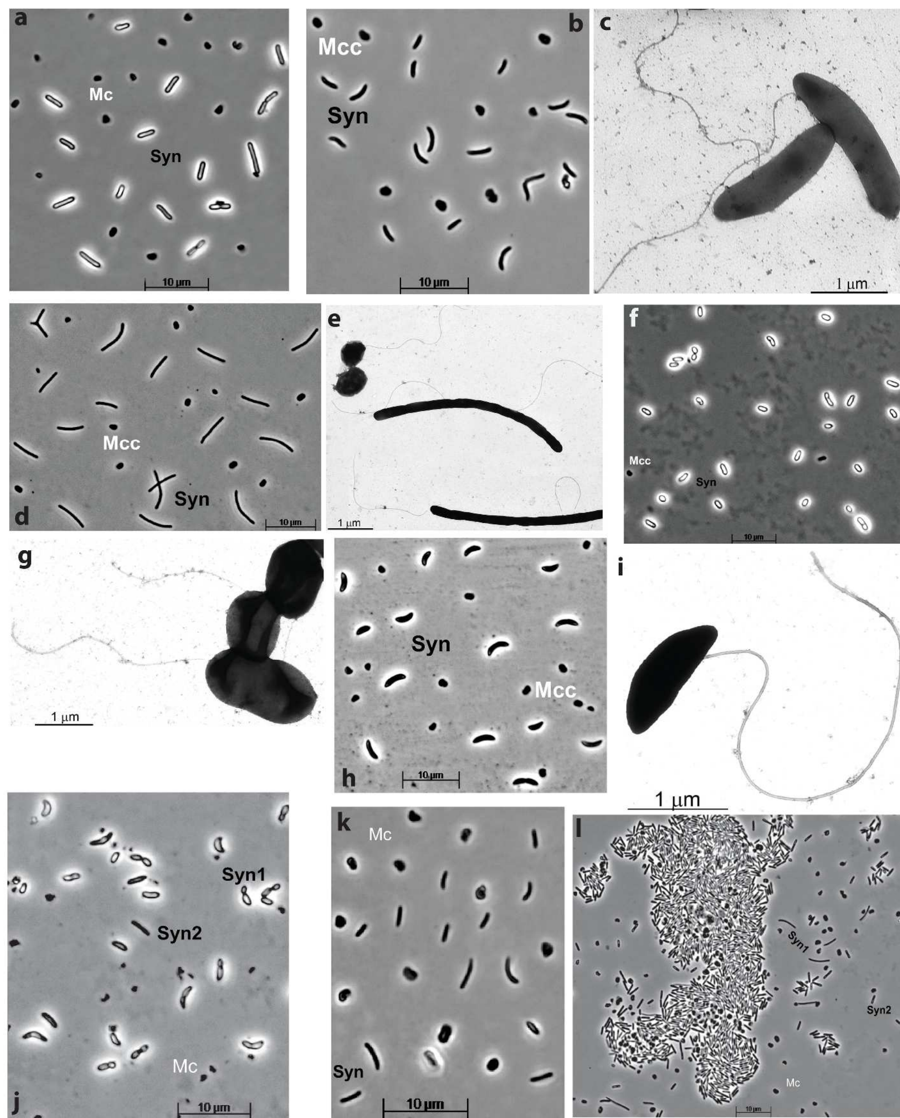

Fig.2

Fig.2
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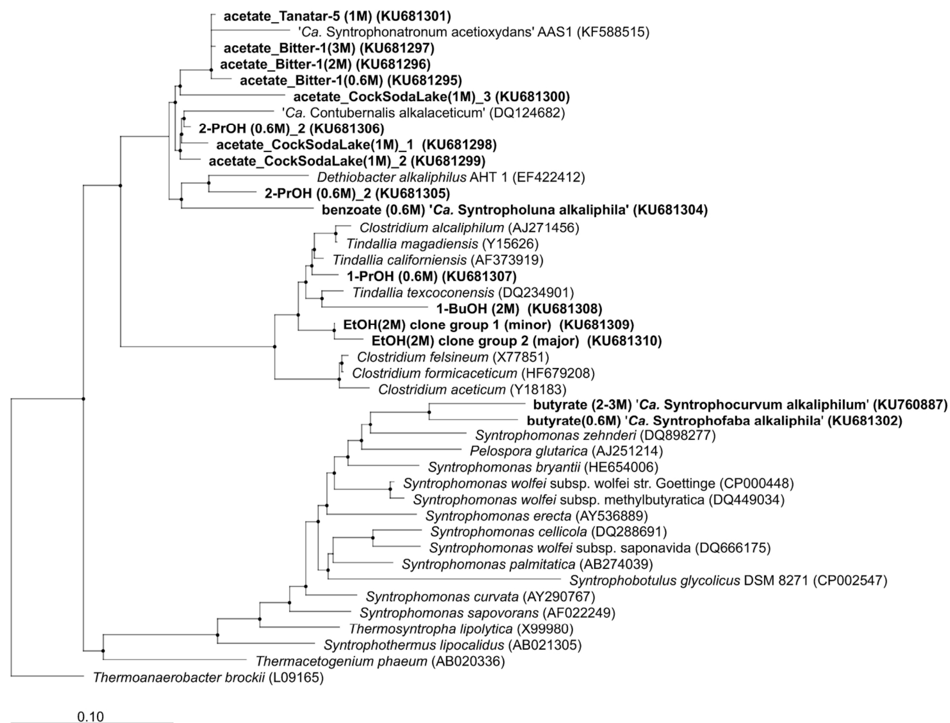

fig.3a
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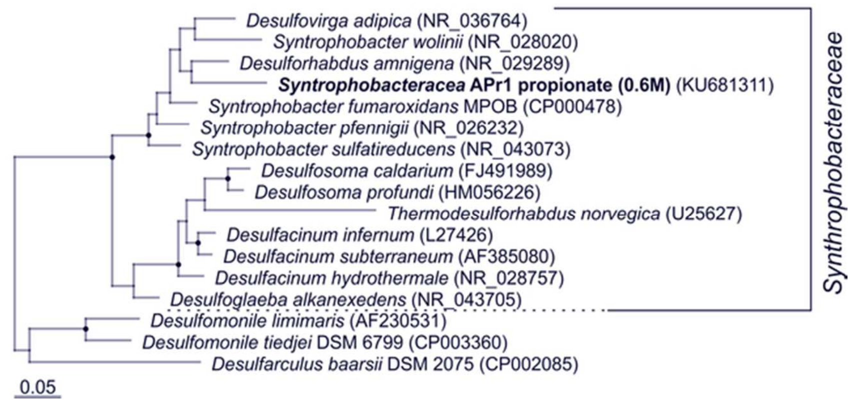

fig.3b
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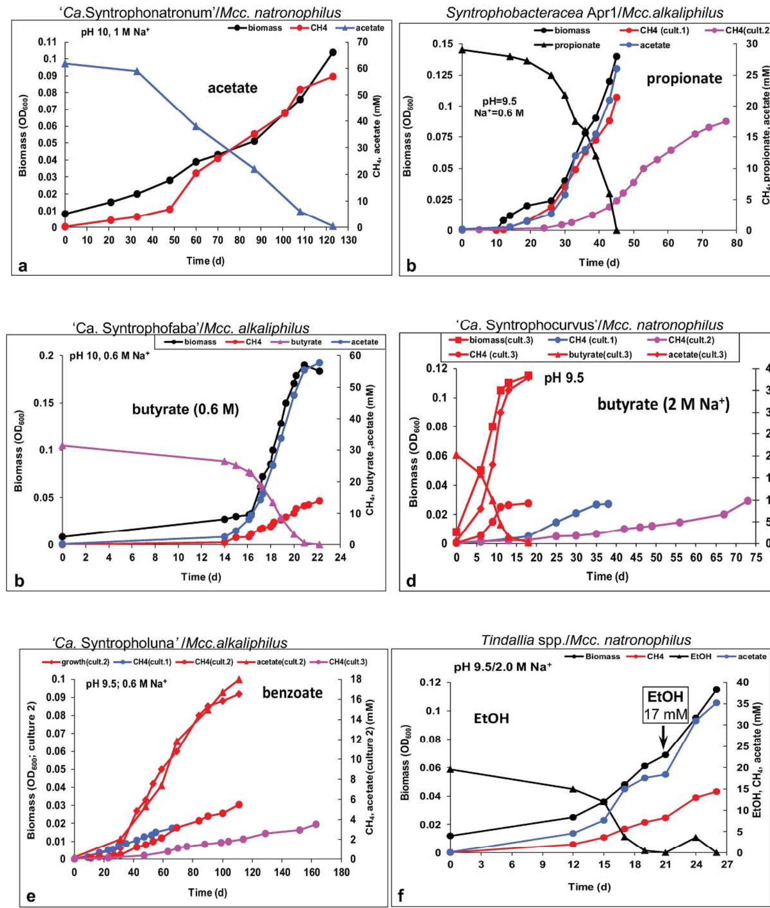

Fig. 4

fig.4
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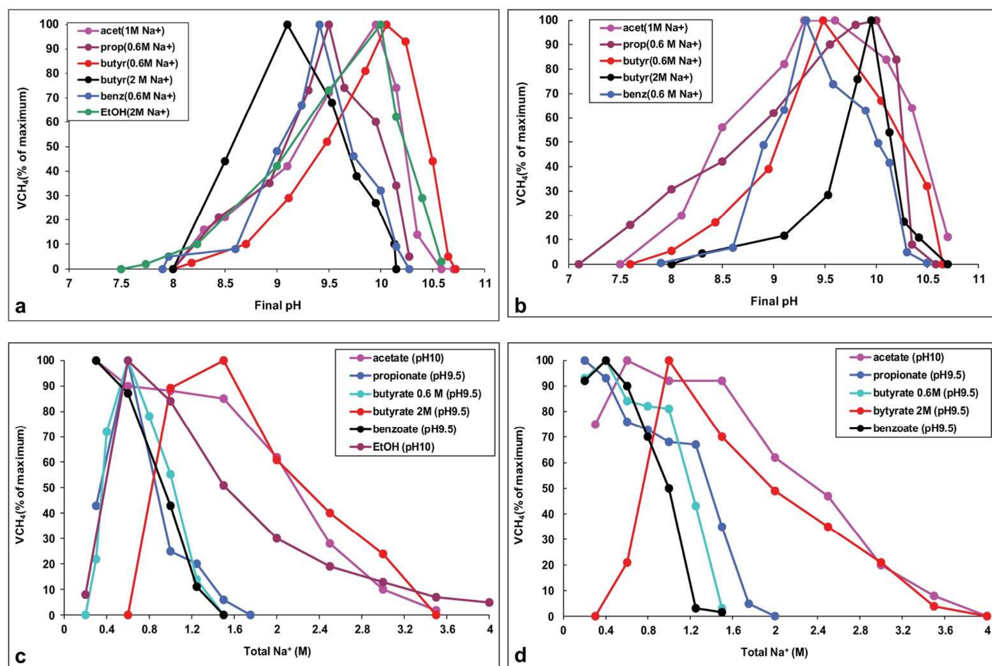

Fig. 5

fig.5
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