

1 **Title: Model communities hint to promiscuous metabolic linkages**
2 **between ubiquitous free-living freshwater bacteria**

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21

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23 **Abstract**

24 Free-living microorganisms with streamlined genomes are very abundant in the environment.
25 Genome streamlining results in losses in the cell's biosynthetic potential generating
26 physiological dependencies between microorganisms. However, there exists no consensus
27 on the specificity of these microbial associations. To verify specificity and extent of these
28 associations, mixed cultures were established from three different freshwater environments.
29 These cultures contained free-living streamlined organisms lacking multiple biosynthetic
30 pathways. Among the co-occurring members of the mixed cultures, there was no clear
31 recurring pattern of metabolic complementarity and dependencies. This, together with weak
32 temporal co-occurrence patterns observed using time-series metagenomics, suggests that
33 free-living freshwater bacteria form loose and unspecific cooperative loops. Comparative
34 genomics suggests that the proportion of accessory genes in populations of streamlined
35 bacteria allows for flexibility in interaction partners. Altogether this renders these free-living
36 bacterial lineages functionally versatile despite their streamlining tendencies.

37

38 **Introduction**

39 Microorganisms can cooperate in many different ways and their relationships range from
40 facultative to obligate dependencies ¹. On the far end of the dependency spectrum are
41 endosymbionts (and endoparasites). These show obligate dependencies where genome
42 reduction and associated loss of essential biosynthesis pathways are widespread. In most
43 cases, a defined host - endosymbiont specificity is established. At the other end of the
44 spectrum, free-living bacteria are considered to be widely autonomous. However, this image
45 is now changing rapidly with frequent reports of reduced microbial genomes in the
46 environment resulting in unique and singular auxotrophies ².
47 In natural aquatic environments where nutrients generally occur in low concentrations,
48 microbes produce many compounds that are costly, but promote survival and reproduction
49 not only for themselves but also for neighboring cells ³. In fact, auxotrophy for amino acids
50 and vitamins has recently been shown for numerous free-living bacteria ⁴⁻⁶, pointing to
51 critically important metabolic dependencies from other community members as exemplified in
52 a simple model community ⁷. Therefore, it would not be surprising that most natural aquatic

53 systems allow for frequent and complex metabolic interactions, for example, via continuous
54 mixing of the environment and molecular-scale diffusion facilitating distribution of public
55 goods. Hence, even when abundant environmental bacteria are seen as free-living, their
56 streamlined genomes render them tightly linked and dependent on other microorganisms⁸ in
57 the community. The sheer number of bacteria and hence genetic variability in a population
58 could allow for high metabolic flexibility at the population level with an endless number of
59 metabolic interactions possible among free-living bacterial communities. However, the
60 character of these associations still remains largely unknown.

61 To investigate the specificity of the dependencies between free-living freshwater
62 microorganisms, we established dilution mixed cultures as model communities. These
63 cultures are a small subsample of the complex natural community⁹ with about 12 initial cells.
64 About 600 of such mixed cultures were established and screened for a group of cosmopolitan
65 yet streamlined freshwater organisms from the Actinobacterial lineage *acl*. Here, we describe
66 six of these model communities and resolve their metabolic interaction potentials using
67 metagenomics. Finally, for a single mixed culture, we calculate co-occurrence patterns across
68 a metagenomics time-series in a lake to test if the observed dependencies are specific or if
69 the interaction-partners are flexible in more complex natural communities.

70

71 **Results and Discussion**

72 *Mixed cultures as model communities and their genomic reconstructions*

73 Ubiquitous freshwater Actinobacteria of the *acl* lineage were cultivated by diluting different
74 lake samples with triple-filtered sample water incubated in 96-well liquid culture plates. In
75 total, seven culture plates were set up and around forty-five wells contained *acl*. Of these,
76 only 6 cultures successfully propagated with densities comparable to those observed in the
77 environment (i.e. 10^6 cells ml^{-1}) for subsequent transfers and growth in larger volumes. This
78 resulted in 6 dilution-to-extinction mixed cultures: FNEF8, FNEB6, FNEB7, FNED7, FSWF8
79 and TBE6 (FNE refers to Northeast basin of Lake Grosse Fuchskuhle, FSW to Southeast
80 basin of Lake Grosse Fuchskuhle and TB to Trout Bog Lake). Characterizations of FNEF8
81 have been published, previously^{7,10}.

82 DNA was extracted from 4 L-cultures, and reads obtained by shotgun sequencing were
83 assembled. Contigs > 1000 bp were considered for further analysis. In total, 77 metagenome
84 assembled genomes (MAGs) were obtained from the assemblies (Supplemental). Of these,
85 31 MAGs each recruited more than 1% of the reads in the original metagenome. These
86 MAGs have an average completeness of 83.5% as calculated by CheckM (Supplemental). It
87 has been previously observed that because CheckM relies on lineage-specific marker genes,
88 the completeness of genomes without lineage representation can often be underestimated^{5,7}.
89 However, since these MAGs have extremely high coverage and were assembled from model
90 communities (low diversity of starter cells) we are confident of their high quality.

91

92 *Some free-living microorganisms fulfill their metabolic needs from those they happen to*
93 *encounter*

94 Populations represented by the top 31 MAGs were assumed to be the dominant model
95 community members and were analyzed further (mapping rates in Supplemental). All six
96 cultures yielded at least one MAG of the cosmopolitan freshwater Actinobacteria acI
97 (recruiting between 15% and 40% of reads), four cultures yielded a MAG of the freshwater
98 Actinobacteria acIII (between 3% and 9% of the reads), two cultures yielded yeast MAGs (1
99 and 3% of the reads), two cultures featured MAGs affiliated with Bacteroidetes (2% and 6% of
100 the reads), two cultures yielded a Polynucleobacter MAG (3% and 55% of the reads), two
101 cultures yielded a Spirochaetes MAG (8% and 22% of the reads), one culture yielded an
102 Alphaproteobacteria MAG (1% of the reads) and one an Acidimicrobiales MAG (48% of the
103 reads). Knowing that acI Actinobacteria do not grow in pure culture¹¹, we now have strong
104 support that the free-living acI is highly dependent on interactions with other microorganisms.
105 Results from previous co-occurrence studies support the idea that free-living streamlined
106 bacteria have very high connectivity in their environments and are critically dependent on
107 metabolites that might be provided by the surrounding members of the planktonic
108 communities^{8,12}. Moreover, by creating an initial bottleneck¹³ of approximately 12 cells, we
109 obtained a first evidence that these plankton community partners do represent a diverse sets
110 of community members. This hints towards a non-specific metabolic dependence of acI on
111 other abundant freshwater bacteria, as no consistent interaction patterns were observed.

112 Since six mixed cultures might be not sufficient to generalize the character of the observed
113 associations, we correlated the abundance of the mixed culture TBE6 MAGs with those
114 obtained from a nine-year time-series shotgun metagenome from its source environment,
115 Trout Bog Lake ¹⁴. For this, we also used MAGs reassembled from this time-series data. The
116 observed correlations indicate that *acl* interacts promiscuously and randomly (Figure 1,
117 Figure S1 and S2). That is, no strong exclusive correlations can be discerned, either with the
118 members of TBE6 or MAGs from the time-series, strengthening the hypothesis that *acl* is
119 promiscuous in its interactions.

120

121 *Tight metabolic dependencies between different free-living microorganisms*

122 Canonical central metabolic pathways such as glycolysis, the pentose phosphate pathway,
123 the citric acid cycle, ammonia assimilation and oxidative phosphorylation are observed in
124 each of the abundant MAGs of the microbial model communities, suggesting a mainly aerobic
125 heterotrophic lifestyle (Supplemental). When looking at all extant *acl* MAGs and single-cell
126 genomes (SAGs) ^{15,16}, auxotrophies for amino acids and vitamins were observed (Figure 2A).
127 However, in the mixed cultures, at least one of the main members in each community was
128 able to synthesize each of these metabolites, hinting to a high degree of metabolic
129 dependency within the members of each model community of relatively low complexity. For
130 example, in culture FNEB6, Bacteroidetes is the only producer of asparagine and so
131 potentially supplies it to the other community members (see more examples in Figure 2B).
132 Interactions at the level of amino acids and vitamins have been previously observed and
133 described in several aquatic study systems ⁴⁻⁶. Furthermore, one or two members of each
134 model community had the ability to reduce sulfate, consistent with prior findings of the transfer
135 of redox reaction products known as “metabolic handoffs” ¹⁷. These kinds of observed
136 dependencies merely represent a few examples of the many interactions that likely occur in
137 the complex natural environment. In this respect, it was interesting that they also formed
138 cross-domain linkages to two ubiquitous yeasts. We consider this as an indication that these
139 interactions potentially can cross boundaries to eukaryotic plankton.

140

141 *High degree of genomic diversity might support flexibility in interaction partners*

142 Our findings from analysis of genome content combined with prior evidence of high genome-
143 level diversity within *acl*¹⁵ prompted us to examine the variability in gene content among *acl*
144 genomes. Interestingly, even when our cultures harbor a reduced diversity, all of our cultures
145 contained more than one genotype of *acl*. This likely reflects the large genomic plasticity of
146 this common and dominant free-living freshwater bacterium. This genomic plasticity, however,
147 cannot be resolved with the short 16S rRNA gene fragments obtained in normal amplicon
148 sequencing (Supplemental). Using all 33 *acl* Actinobacteria MAGs from our 6 mixed cultures
149 in addition to the 14 available *acl* single cell amplified genomes (SAGs) (Figure S3), ~8000
150 orthologous gene clusters (OGCs) were found. The distribution of gene clusters in the
151 genomes revealed that ~800 OGCs form the core genome of *acl* (Figure S4 and S5).
152 Assuming around 1600 genes per *acl*¹⁵, this means that about half of the whole *acl* genome
153 belongs to the flexible genome. This is around the same proportion as SAR11¹⁸. Thus, both
154 *acl* and SAR11 have quite larger flexible genomes than other ubiquitous free-living aquatic
155 bacteria like *Prochlorococcus*¹⁹. The paradox of *acl*'s streamlining tendencies is that their
156 high number of auxiliary genes is likely to render populations of this free-living bacterial
157 lineage functionally versatile.

158

159 In summary, as is the case for other streamlined free-living aquatic bacteria, *acl*
160 Actinobacteria depend on numerous other abundant microorganisms for metabolic handouts.
161 This kind of dependence seems to be non-taxa-specific or promiscuous since no consistent
162 interaction patterns could be observed. These observations add to the knowledge on the
163 structure of cooperation and dependencies between free-living aquatic microbes.

164

165 **Methods**

166 *Mixed culture and DNA extraction*

167 Mixed cultures that included the abundant and ubiquitous freshwater *Actinobacteria* were
168 established in 2012 and 2013. Samples were collected from 5 different lakes: Lake Grosse
169 Fuchskuhle (North East basin and South West basin), Lake Stechlin, Lake Dagow, Lake
170 Mendota and from Trout Bog Lake (metadata reported in Supplementary Table). In total,
171 seven plates were set up, two for Northeast basin (Lake Grosse Fuchskuhle) and one for

172 each of the other lakes (Southwest basin Lake Grosse, Lake Stechlin Lake, Lake Dagow,
173 Lake Mendota and Trout Bog Lake. Cultivation method, media preparation, aqPCR assays,
174 general observations and DNA extractions were as previously described for mixed culture
175 FNE-F8¹⁰. Selected mixed cultures (based on cell density) were scaled up to a four-liter
176 culture and DNA was extracted. DNA was sent to JGI and to Research and Testing
177 Laboratory (RTL) (<http://researchandtesting.com/>) for shotgun metagenomic sequencing and
178 16S rRNA gene amplicon sequencing, respectively.

179

180 *16S rRNA gene amplicon sequencing*

181 RTL performed PCR amplification and MiSeq sequencing of the 16S rRNA gene amplicons.
182 The bacterial primers (*E. coli* positions) were 357wF (5'-CCTACGGGNGGCWGCAG-3') and
183 926wR (5'-CCGTC AATTYMTTTRAGTTT-3'). All downstream analysis was done using the
184 RTL standard pipeline from 2014. In brief all sequences were clustered at 4% divergence into
185 OTUs using the UPARSE²⁰ algorithm. The centroid sequence from each cluster was then run
186 against either the USEARCH global alignment algorithm or the RDP Classifier against a
187 database of high quality sequences derived from the NCBI database. The output was then
188 analyzed using an RTL internally developed python program that assigns taxonomic
189 information to each sequence and then computes and writes the final analysis files.

190

191 *Library preparation and sequencing*

192 JGI performed the library preparation and the sequencing under Community Sequencing
193 Project 1289. First, 100 ng of genomic DNA was sheared to 270 bp using a focused-
194 ultrasonicator (Covaris). The sheared DNA fragments were size selected using SPRI beads
195 (Beckman Coulter). The selected fragments were then end-repaired, A-tailed, and ligated of
196 Illumina compatible adapters (IDT, Inc.) using KAPA-Illumina library creation kit (KAPA
197 biosystems). The prepared sample library was quantified using KAPA Biosystem's next-
198 generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR
199 instrument. The quantified library was then prepared for sequencing on the Illumina HiSeq
200 sequencing platform utilizing a TruSeq paired-end cluster kit, v3, and Illumina's cBot
201 instrument to generate a clustered flowcell for sequencing. Sequencing of the flowcell was

202 performed on the Illumina HiSeq2000 sequencer using Illumina TruSeq SBS sequencing kits,
203 v3, following a 2x150 indexed high-output run recipe.

204

205 *Genome assembly, annotations and metabolic features analysis*

206 After reads had been filtered based on their quality scores using sickle (version 1.210)²¹, the
207 reads were digitally normalized using khmer 1.4²² and assembled with megahit²³. Coverage
208 was computed by mapping back the reads competitively to the assembly using bbmap 35.40
209 (sourceforge.net/projects/bbmap/) using default parameters. Mapped reads were indexed and
210 sorted using SAMtools 1.3²⁴ while we removed duplicates using picard-tools (version 1.101).
211 Bedtools (version 2.18.2)²⁵ was used for computing coverage. For the binning metaBAT²⁶
212 was used. Tribe/taxonomical affiliation were defined using whole genome information and the
213 public database using PhyloPhlAn²⁷ and using previously published genomes and SAGs¹⁶
214 as references. If the MAGs belong to one of the freshwater tribes²⁸ they have the name of the
215 tribe, otherwise the taxonomic name after NCBI is given.

216 For bacterial genomes, gene prediction analysis was performed within the Integrated
217 Microbial Genomes (IMG)^{29,30} platform developed by the Joint Genome Institute, Walnut
218 Creek, CA, USA (<http://img.jgi.doe.gov>). Specific KEGG biosynthetic maps were inspected for
219 completeness (<http://www.genome.jp/kegg/mapper.html>) counting number of missing
220 enzymes from the most complete pathway. MAG completeness was calculated using CheckM
221³¹.

222 For fungal genomes, ab initio gene prediction was performed using augustus (v 2.5.5) with
223 *Aspergillus nidulans* (FSWF8-4) or *Cryptococcus neoformans* (FNED7-22) models. Gene
224 model ORFs were then annotated using matches to Pfam 30.0 (June 2016) by hmmscan
225 (HMMER 3.1b2). Functional annotation was done using predicted aminoacids in the KAAS
226 platform³².

227

228 *Core genome computation*

229 Clusters of orthologous genes were identified using OrthoMCL³³ using default options
230 following an established protocol. Core genome of the *acl* was computed using all 33 *acl*
231 Actinobacteria MAGs from our 6 mixed cultures in addition to the 14 available *acl* single cell

232 amplified genomes (SAGs). Due to the incompleteness SAGs and MAGs, common methods,
233 such as picking all the OGCs present in at least 90% of the genomes, are unsafe. To reduce
234 the noise generated by incompleteness, a semi-supervised approach has been chosen. For
235 this we compute a distance matrix based on the genomes that contain the different OGCs.
236 Two OGCs will have a distance of 0.0 if they are found in the same genomes, and a distance
237 of 1.0 if none of the genomes containing one also contains the other. The overlap between
238 the genome sets of each cluster were computed and normalized to the smallest genome set.
239 OGCs contained in less than 4 genomes were removed. The obtained distances are then
240 used with a hierarchical clustering to compute a heatmap (Supplemental Figure acl core).
241 This heatmap clearly shows a set of OGCs that overlaps significantly with all other OGCs, this
242 cluster is selected as core. Core OGCs should have a strong overlap with all other OGCs
243 irrelevant of their position in the heatmap.

244

245 *Metagenome recruitment to MAGs*

246 A total of 113 metagenome samples from Trout Bog lake¹⁴ were used in this study. As above,
247 after assembly and binning, bbmap was used to compute the coverage of all the MAGs (from
248 the time-series as well as the cultures), for all the time-points. All coverages have been
249 normalized to sequencing depth. Mean coverage vectors of all MAGs are then correlated
250 using Spearman correlation. For the heatmaps, MAGs from Trout Bog lake were only kept if
251 they correlate with at least 0.6 to at least one MAG from our mixed culture TBE6 (positive or
252 negative correlation). MAGs assembled from Trout Bog lake have not been further analyzed
253 as they might represent an average population genome.

254

255 **Competing financial interests**

256 The authors declare no conflict of interest

257

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273

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275

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- 367

368 **Data accessibility**

369 The raw shotgun metagenome reads are publicly available in the JGI portal and the assembly
370 is available in IMG/MER under the ER submission IDs 26656, 26658, 26650, 29729, 29808,
371 50227. The bacterial metagenome-assembled genomes (MAGs) are also available through
372 IMG. The fungal MAGs have been deposited at DDBJ/ENA/GenBank. For taxon OID or
373 accession number check supplementary material.

374

375 **Author contribution**

376 SLG and FW conceived the research. SLG and HPG collected and prepared the samples.
377 SLG, MB, JJH, AE, CW and MAR analyzed the data. All authors wrote and/or revised the
378 manuscript.

379

380 **Conflict of interest Statement:** The authors declare no conflict of interest.

381

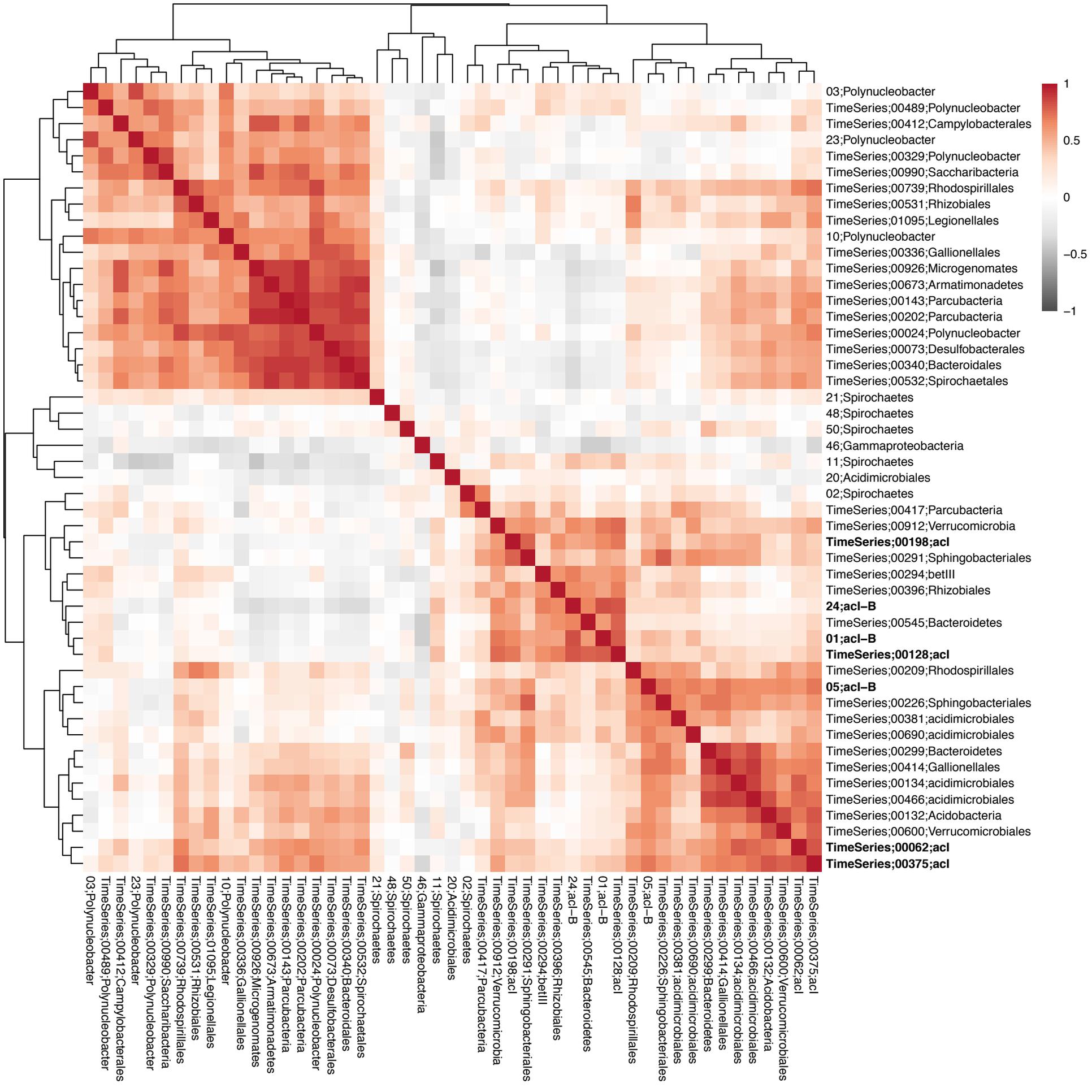
382 **Titles and legends to figures**

383

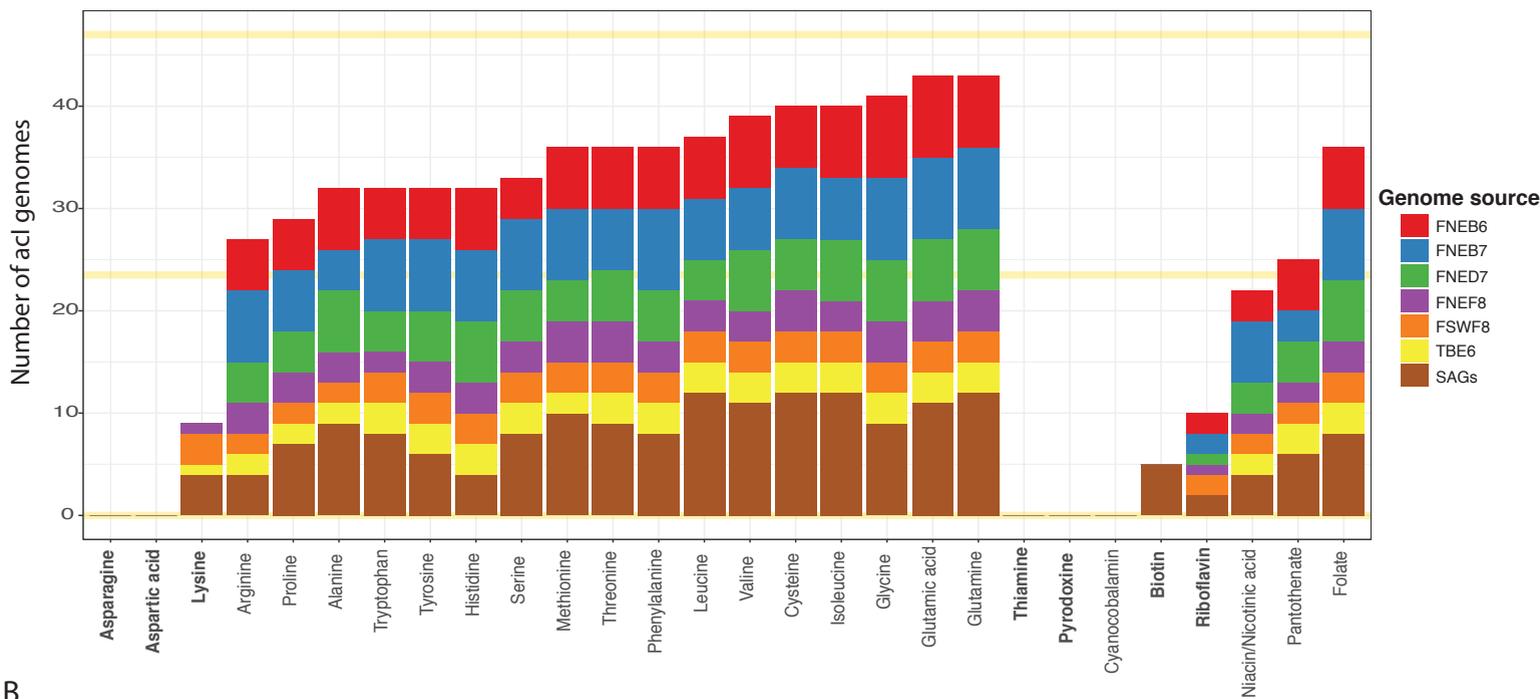
384 **Figure 1.** Spearman correlation on normalized relative abundance between MAGs from TBE6
385 and taxa in the epilimnion of the environment of origin (Trout Bog Lake). Metagenome
386 samples from time-points from all years were used (45 unique dates). Correlation is displayed
387 via hierarchical clustering. MAG names that start with a number refers to a MAG from the
388 mixed culture (for details on these MAGs look at supplemental tables). MAG names that start
389 with the words “time series” were previously binned from the Trout Bog nine year
390 metagenome series¹⁴. The acI MAGs are highlighted in bold. To look into the correlations of
391 hypolimnion and both combined look into the supplemental.

392

393 **Figure 2. A.** Visualization of the amino acid and vitamin biosynthetic potential of all acI MAGs
394 and SAGs analyzed. It includes acI information from all MAGs binned from each culture in this
395 study plus the previously published SAGs. Yellow lines indicate 0%, 50% and 100% of
396 genomes analyzed. Completeness of a metabolic pathway was considered if 80% of the
397 pathway was present. **B.** Potential metabolic complementarity among major members of each
398 of the cultures. Circle plots are displayed for metabolites highlighted in bold in panel A plus
399 potential to assimilate sulfate and sulfite. Colors indicate each mixed culture using same color
400 scheme as in panel A. Arrow shows the direction in which the metabolite would be “shared”.
401 Alpha – Alphaproteobacterium. Acidi – Acidimicrobium.



A



B

