# High-Throughput Metabolic Network Analysis and Metatranscriptomics of a Cosmopolitan and Streamlined Freshwater Microbial Lineage

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## Abstract

An explosion in the number of available genome sequences obtained through metagenomics and single-cell genomics has enabled a new view of the diversity of microbial life, yet we know surprisingly little about how microbes interact with each other or their environment. In fact, the majority of microbial species remain uncultivated, while our perception of their ecological niches is based on reconstruction of their metabolic potential. In this work, we demonstrate how the “seed set framework”, which computes the set of compounds that an organism must acquire from its environment (Borenstein *et al.*, 2008), enables high-throughput, computational analysis of metabolic reconstructions, while providing new insights into a microbe’s metabolic capabilities, such as nutrient use and auxotrophies. We apply this framework to members of the

ubiquitous freshwater Actinobacterial lineage acI, confirming and extending previous experimental and genomic observations implying that acI bacteria are heterotrophs reliant on peptides and saccharides. We also present the first metatranscriptomic study of the acI lineage, featuring high expression of transport proteins and the light-harvesting protein actinorhodopsin, and confirming predictions of nutrients and essential metabolites while providing additional support to the hypothesis that members of the acI are photoheterotrophs.

## Introduction

Natural microbial communities have central roles in the biosphere, ranging from mediators of nutrient cycling to agents of human health and disease (Falkowski *et al.*, 2008; Blaser *et al.*, 2016). However, the majority of microbial species remain uncultivated, a feature that poses a significant challenge to our understanding of their physiology and metabolism. Recent advances in sequencing technology and bioinformatics have enabled assembly and analysis of reference genomes for a wide range of hitherto uncultured community members from diverse environments (Sangwan *et al.*, 2016) that can be explored for inferring links between the genome content of an individual microbe and its metabolic traits, a concept referred to as “reverse ecology” (Levy and Borenstein, 2012).

Reverse ecological analyses can be performed using metabolic network reconstructions (Feist *et al.*, 2009; Thiele and Palsson, 2010), which are structured summaries of the metabolic capabilities of an organism as defined by its enzymes and their coupled biochemical reactions. These reconstructions can then be analyzed using metabolic network graphs, mathematical objects in which biochemical reactions are

46 represented as connections between substrates and products (Levy and Borenstein,
47 2012). One such graph-based, reverse ecology approach is to compute an organism's
48 *seed set*, the set of compounds that the organism cannot synthesize on its own and
49 must exogenously acquire from its environment (Borenstein *et al.*, 2008). These
50 compounds may represent both *auxotrophies* for essential metabolites for which
51 biosynthetic routes are missing, and *nutrients* for which degradation but not synthesis
52 routes are present in the genome. This *seed set framework* offers potential advantages
53 over other reconstruction-based approaches, as 1) metabolic network graphs can be
54 rapidly analyzed computationally, 2) a network-centric approach makes no *a priori*
55 assumptions about which metabolic pathways may be important for an organism's
56 niche, and 3) identification of seed compounds facilitates a focused analysis by
57 identifying those compounds that an organism must obtain from its environment.

58 Freshwater lake microbiomes are ideal systems for applying the seed set
59 framework, as long-term monitoring has revealed the ecology of dominant bacterial
60 lineages (Newton *et al.*, 2011), and reference genomes for these key lineages are now
61 readily available (Martinez-Garcia *et al.*, 2012; Garcia *et al.*, 2013, 2015; Ghai *et al.*,
62 2014; Ghylin *et al.*, 2014; Tsementzi *et al.*, 2014; Bendall *et al.*, 2016). Among the
63 freshwater bacteria, uncultivated Actinobacteria of the acI lineage are among the most
64 abundant (Zwart *et al.*, 1998, 2002; Glöckner *et al.*, 2000). The acI have been
65 phylogenetically divided into three clades (acI-A, acI-B, and acI-C) and thirteen tribes on
66 the basis of their 16S rRNA gene sequences (Newton *et al.*, 2011). The abundance and
67 ubiquitous distribution of these free-living ultramicrobacteria suggests they have central
68 roles in nutrient cycling in diverse freshwater systems (Glöckner *et al.*, 2000; Newton *et*

69 *al.*, 2006, 2007; Wu *et al.*, 2006, 2007; De Wever *et al.*, 2008; Humbert *et al.*, 2009;
70 Ghai *et al.*, 2012).

71 To identify the metabolic processes that these bacteria mediate, the metabolism
72 of the acI lineage has been the focus of a number of recent studies. Surveys using
73 fluorescent *in situ* hybridization (FISH) and catalyzed reporter deposition (CARD) or
74 microautoradiography (MAR) reveal that the acI are capable of consuming amino acids
75 (Salcher *et al.*, 2010, 2013), glucose (Buck *et al.*, 2009; Salcher *et al.*, 2013), N-
76 acetylglucosamine (NAG) (Beier and Bertilsson, 2011; Eckert *et al.*, 2012, 2013), the
77 deoxynucleoside thymidine (Pérez *et al.*, 2010; Salcher *et al.*, 2013), and acetate (Buck
78 *et al.*, 2009). Furthermore, metabolic reconstructions of single-cell genomes (SAGs) and
79 metagenome-assembled genomes (MAGs) have further expanded the view of substrate
80 uptake capabilities for members of clades acI-A and acI-B. These studies indicate
81 members of these clades are capable of consuming a wide array of N-containing
82 compounds, including ammonium, branched-chain amino acids, polyamines, di- and
83 oligo-peptides, and cyanophycin (Ghylin *et al.*, 2014; Garcia *et al.*, 2015). Members of
84 these two clades also seem to be capable of consuming numerous mono-, poly-, and
85 oligo-saccharides (Garcia *et al.*, 2013, 2015; Ghylin *et al.*, 2014). Finally, a recent study
86 of a metagenome-assembled genome from clade acI-B predicted that some members
87 of the clade are unable to synthesize a number of essential vitamins and amino acids
88 (Garcia *et al.*, 2015).

89 In the present study, we present a computational pipeline to automate the
90 calculation of an organism's substrate utilization capabilities using the seed set
91 framework, thereby facilitating high-throughput analysis of genomic data. To do so, we

92 developed a Python package to predict seed compounds, using metabolic network
93 reconstructions generated from KBase (Arkin *et al.*, 2016). We expand existing
94 analyses of the acI lineage by applying the seed set framework to a reference genome
95 collection of 36 freshwater acI genomes covering all three acI clades. The seed
96 compounds predicted by our analysis are in agreement with previous experimental and
97 genomic observations, confirming the ability of our method to predict an organism's
98 auxotrophies and nutrient sources. To complement these predictions, and to understand
99 which pathways dominate active metabolism of acI in its natural environment, we
100 conducted the first *in situ* metatranscriptomic analysis of gene expression in the acI
101 lineage. Knowledge of seed compounds enhanced interpretation of the
102 metatranscriptome results by facilitating a focused analysis of acI metabolism.
103 Additional analyses show that the acI express a diverse array of transporters that we
104 hypothesize may contribute to their observed dominance and widespread distribution in
105 a variety of aquatic systems.

106 **Materials and Methods**

107 *A Freshwater Reference Genome Collection*

108 This study relies on an extensive collection of freshwater bacterial genomes,
109 containing MAGs obtained from two metagenomic time-series from two Wisconsin lakes
110 (Bendall *et al.*, 2016; Garcia *et al.*, 2016), as well as SAGs from three lakes in the
111 United States (Martinez-Garcia *et al.*, 2012). Additional information about this genome
112 collection can be found in the Supplemental Online Material.

113 *Metatranscriptome Sampling and Sequencing*

114 This study used four metatranscriptomes obtained as part of a 24-hour sampling
115 experiment designed to identify diel trends in freshwater microbial communities.
116 Additional information about these samples can be found in the Supplemental Online
117 Material. All protocols and scripts for sample collection, RNA extraction, rRNA depletion,
118 sequencing, and bioinformatic analysis can be found on Github
119 (https://github.com/McMahonLab/OMD-TOILv2, DOI:######). Metadata for the four
120 samples used in this study can be found in Table S1, and the raw RNA sequences can
121 be found on the National Center for Biotechnology Information (NCBI) website under
122 BioProject PRJNA362825.

123 *Identification of acI SAGs and Actinobacterial MAGs*

124 acI SAGs were identified within a previously-published genome collection
125 (Martinez-Garcia *et al.*, 2012) and classified to the tribe level using partial 16S rRNA
126 genes and a reference taxonomy for freshwater bacteria, as described in the
127 Supplemental Online Material. Actinobacterial MAGs were identified within two
128 metagenomic time-series (Bendall *et al.*, 2016; Garcia *et al.*, 2016) using taxonomic
129 assignments from a subset of conserved marker genes, as described in the
130 Supplemental Online Material. Phylogenetic analysis of acI SAGs and Actinobacterial
131 MAGs was performed using a concatenated alignment of single-copy marker genes
132 obtained via Phylosift (Darling *et al.*, 2014). Maximum likelihood trees were generated
133 using RAxML (Stamatakis, 2014) using the automatic protein model assignment option
134 (PROTGAMMAAUTO) and 100 bootstraps.

135 *Genome Annotation, Metabolic Network Reconstruction, and Computation and*
136 *Evaluation of Seed Compounds*

137 In the seed set framework, an organism's metabolism is represented via a
138 metabolic network graph, in which nodes denote compounds and edges denote
139 enzymatically-encoded biochemical reactions linking substrates and products (Jeong *et*
140 *al.*, 2000). Allowable biochemical transformations can be identified by drawing paths
141 along the network, in which a sequence of edges connects a sequence of distinct
142 vertices. In our implementation of the seed set framework, metabolic network graphs
143 were generated as follows.

144 Genome annotations were performed and metabolic network reconstructions
145 were built using KBase. Contigs for each genome were uploaded to KBase and
146 annotated using the "Annotate Microbial Contigs" method with default options, which
147 uses components of the RAST toolkit for genome annotation (Brettin *et al.*, 2015;
148 Overbeek *et al.*, 2014). Metabolic network reconstructions were obtained using the
149 "Build Metabolic Model" app with default parameters, which relies on the Model SEED
150 framework (Henry *et al.*, 2010) to build a draft metabolic model. These reconstructions
151 were then pruned (currency metabolites and highly-connected compounds) and
152 converted to metabolic network graphs (Figure S1 and Supplemental Online Material).
153 Many of the individual acI genomes are incomplete (see Results). Therefore, composite
154 metabolic network graphs were constructed for each tribe and clade, to increase the
155 accuracy of seed identification by means of a more complete metabolic network (Figure
156 S2 and Supplemental Online Material).

157 Formally, the seed set of the network is defined as the minimal set of compounds
158 that cannot be synthesized from other compounds in the network, and whose presence
159 enables the synthesis of all other compounds in the network (Borenstein *et al.*, 2008).
160 Seed compounds for each composite metabolic network graph were calculated using a
161 new Python implementation of the seed set framework (Borenstein *et al.*, 2008) (Figure
162 S3 and the Supplemental Online Material). Because seed compounds are computed
163 from a metabolic network, it is important to manually evaluate all predicted seed
164 compounds to identify those that may be biologically meaningful, and do not arise from
165 errors in the metabolic network reconstruction. Examples of this process are given in
166 the Supplemental Online Material.

167 All computational steps were implemented using Python scripts, freely available
168 as part of the reverseEcology Python package developed for this project
169 (https://pypi.python.org/pypi/reverseEcology/, DOI:######).

170 *Identification of Transported Compounds*

171 For each genome, we identified all transport reactions present in its metabolic
172 network reconstruction. Gene-protein-reaction associations (GPRs) for these reactions
173 were manually curated to remove unannotated proteins, group genes into operons (if
174 applicable), and to identify missing subunits for multi-subunit transporters. These genes
175 were then mapped to their corresponding COGs, and grouped accordingly. Finally, the
176 most common annotation for each COG was used to identify likely substrates for each
177 of these groups.

178 *Protein Clustering, Metatranscriptomic Mapping, and Clade-Level Gene Expression*

179 OrthoMCL (Li *et al.*, 2003) was used to identify clusters of orthologous groups
180 (COGs) in the set of acI genomes. Both OrthoMCL and BLAST were run using default
181 options (Fischer *et al.*, 2011). Annotations were assigned to protein clusters by
182 choosing the most common annotation among all genes assigned to the respective
183 cluster. Trimmed and merged metatranscriptomic reads from each of the four biological
184 samples were then mapped to a single reference fasta file containing all acI genomes
185 using BBMap (https://sourceforge.net/projects/bbmap/) with the *ambig=random* and
186 *minid=0.95* options. The 95% identity cutoff was chosen as this represents a well-
187 established criterion for identifying microbial species using average nucleotide identity
188 (ANI) (Konstantinidis and Tiedje, 2005), while combining the *ambig* option with
189 competitive mapping using pooled acI genomes as the reference ensures that reads
190 map only to a single genome. These results were then used to compute the expression
191 of each COG in each clade.

192 Next, HTSeq-Count (Anders *et al.*, 2014) was used to count the total number of
193 reads that map to each gene in our acI genome collection. After mapping, the list of
194 counts was filtered to remove those genes that did not recruit at least one read in all
195 four samples. Using the COGs identified by OrthoMCL, the genes that correspond to
196 each COG were then identified.

197 Within each clade, gene expression for each COG was computed on a Reads
198 Per Kilobase Million (RPKM) basis (Mortazavi *et al.*, 2008), while also accounting for
199 different gene lengths within a COG and numbers of mapped reads for each genome
200 within a clade. That is, the RPKM value for a single COG represents the sum of RPKM

201 values for each gene within that COG, normalized to the appropriate gene length and
202 total number of mapped reads. RPKM counts were then averaged across the four
203 metatranscriptomes and normalized to the median level of gene expression within that
204 clade.

205 *Availability of Data and Materials*

206 All genomic and metatranscriptomic sequences are available through IMG and
207 NCBI, respectively. A reproducible version of this manuscript is available at
208 https://github.com/joshamilton/Hamilton_acI_2016 (DOI:######).

209 **Results**

210 *Phylogenetic Affiliation of acI Genomes*

211 From a reference collection of freshwater bacterial genomes, we identified 17
212 SAGs and 19 MAGs from members of the acI lineage. A phylogenetic tree of these
213 genomes is shown in Figure 1. Previous phylogenetic analysis using 16S rRNA gene
214 sequences have revealed that the acI lineage can be grouped into three distinct
215 monophyletic clades (Newton *et al.*, 2011). In this study, the phylogenetic tree built from
216 37 concatenated marker genes also identified three monophyletic branches, enabling
217 MAGs to be classified as clade acI-A or acI-B based on the taxonomy of SAGs within
218 each branch. Note that three MAGs formed a monophyletic group separate from clades
219 acI-A and acI-B; we assume these genomes belong to clade acI-C as no other acI
220 clades have been identified to date.

221 *Estimated Completeness of Tribe- and Clade-Level Composite Genomes*

222 Metabolic network reconstructions created from acI SAGs and MAGs will likely
223 be missing reactions, as the underlying genomes are incomplete (Table 1). Previous
224 studies have examined the effect of genome incompleteness on the predicted seed set
225 (Borenstein *et al.*, 2008). Using the formal (mathematical) definition of a seed
226 compound, this showed that the percentage of correct seed compounds (true positives)
227 is approximately equal to the completeness of the reaction network, and the number of
228 false positives is approximately equal to the incompleteness of the network. Thus, we
229 constructed composite genomes at higher taxonomic levels (e.g., tribe and clade) to
230 increase genome completeness for more accurate seed identification at that taxonomic
231 level.

232 Using 204 conserved single-copy marker genes (Parks *et al.*, 2015), we
233 estimated the completeness of tribe- and clade-level composite genomes to determine
234 the finest level of taxonomic resolution at which we could confidently compute seed
235 compounds, using genome completeness as a proxy for metabolic reaction network
236 completeness (Figure 2). Because CheckM relies on lineage-specific marker genes, the
237 completeness of genomes without representation in the CheckM database can be
238 underestimated (Garcia *et al.*, 2015). As a result, we deemed genomes to be complete
239 if they contained 95% of the lineage-specific marker genes. With the exception of tribe
240 acI-B1, tribe-level composite genomes are estimated to be incomplete (Figure 2A). At
241 the clade level, clades acI-A and acI-B are estimated to be complete, while the acI-C
242 composite genome remains incomplete, as it only contains 75% of the 204 marker
243 genes (Figure 2B). As a result, seed compounds were calculated for composite clade-

244 level genomes, with the understanding that some true seed compounds for the acI-C
245 clade will not be predicted.

246 *Computation and Evaluation of Potential Seed Compounds*

247 Seed compounds were computed for each clade, using the composite metabolic
248 network graph for that clade (Figure 3, and Figures S1 to S3). A total of 125 unique
249 seed compounds were identified across the three clades (Table S2). Additional details
250 are available in the Supplemental Online Material.

251 Seed compounds were predicted using the results of an automated annotation
252 pipeline, and as such are likely to contain inaccuracies (e.g., due to missing or incorrect
253 annotations). As a result, we screened the set of predicted seed compounds to identify
254 those that represented biologically plausible auxotrophies and nutrients, and manually
255 curated this subset to obtain a final set of auxotrophies and nutrient sources.
256 Compounds involved in fatty acid and phospholipid biosynthesis pathways were
257 removed during curation, as these pathways are often organism-specific and unlikely to
258 be properly annotated by automatic metabolic reconstruction pipelines. Seed
259 compounds related to currency metabolites were also removed, as reactions for the
260 synthesis of these compounds may have been removed during network pruning. Of 125
261 unique compounds, 39 (31%) passed this screening and were deemed biologically
262 plausible.

263 The Supplemental Online Material contains a series of brief vignettes explaining
264 why select compounds were discarded based on the afore-mentioned considerations,
265 and provides examples of additional curation efforts applied to biologically plausible
266 compounds. For a plausible auxotrophy, we screened the genomes for the canonical

267 biosynthetic pathway(s) for that compound, and retained those compounds for which
268 the biosynthetic pathway was incomplete. For a plausible nutrient source, we screened
269 the genomes for the canonical degradation pathway(s) for that compound, and retained
270 those compounds for which the degradation pathway was complete. Of the 39
271 compounds deemed biologically plausible auxotrophies and nutrients, 31 (79%) were
272 retained in the final set of proposed auxotrophies and nutrients. Tables S6 and S7
273 contain this final set of compounds for clades acI-A, acI-B, and acI-C, and Figure 4
274 shows the auxotrophies and nutrients these compounds represent.

275 *Making Sense of Seed Compounds via Protein Clustering and Metatranscriptomic*
276 *Mapping*

277 With regards to seed compounds representing nutrient sources, genes
278 associated with the consumption of these compounds should be expressed. However,
279 because seed compounds were computed from each clade's composite metabolic
280 network graph, genes associated with the consumption of seed compounds may be
281 present in multiple genomes within the clade. To facilitate the linkage of
282 metatranscriptome measurements to seed compounds, we decided to map
283 metatranscriptome samples to clusters of orthologous groups (COGs) within each
284 clade. We used OrthoMCL (Li *et al.*, 2003) to identify COGs in the set of acI genomes,
285 and counted each COG as present in a clade if that COG was present in at least one
286 genome belonging to that clade. We then used BBMap to map metatranscriptome reads
287 to our reference genome collection, and counted the unique reads which map to each
288 Actinobacterial COG.

289 Sequencing of cDNA from all four rRNA-depleted metatranscriptome samples
290 yielded approximately 160 billion paired-end reads. After merging, filtering, and further
291 *in-silico* rRNA removal, approximately 81 billion, or 51% of the reads remained (Table
292 S1). OrthoMCL identified a total of 5013 protein clusters across the three clades (Table
293 S3). The COGs were unequally distributed across the three clades, with clade acI-A
294 genomes containing 3175 COGs (63%), clade acI-B genomes containing 3459 COGs
295 (69%), and clade acI-C genomes containing 1365 COGs (27%). After mapping the
296 metatranscriptomes to our acI genomes (Table S4), we identified 650 COGs expressed
297 in clade acI-A, 785 in clade acI-B, and 849 in clade acI-C (Table S5). Among expressed
298 genes, the median log2 average RPKM value was 10.3 in clade acI-A, 10.2 in clade acI-
299 B, and 9.0 in clade acI-C. Thus, despite differential abundance of each clade within the
300 lake, median gene expression within each clade was similar.

301 *Auxotrophies and Nutrient Sources of the acI Lineage*

302 Seed set analysis yielded seven auxotrophies that could be readily mapped to
303 ecophysiological attributes of the acI lineage (Figure 4a). In all three clades, beta-
304 alanine was identified as a seed compound, suggesting an auxotrophy for pantothenic
305 acid (Vitamin B5), a precursor to coenzyme A formed from beta-alanine and pantoate.
306 In bacteria, beta-alanine is typically synthesized via aspartate decarboxylation, and we
307 were unable to identify a candidate gene for this enzyme (aspartate 1-decarboxylase,
308 E.C. 4.1.1.11) in any acI genome. Pyridoxine 5'-phosphate and 5'-pyridoxamine
309 phosphate (forms of the enzyme cofactor pyridoxal 5'-phosphate, Vitamin B6) were also
310 predicted to be seed compounds, and numerous enzymes in the biosynthesis of these
311 compounds were not found in the genomes.

312 Clades within the acI lineage also exhibited distinct auxotrophies. Clade acI-A
313 was predicted to be auxotrophic for the cofactor tetrahydrofolate (THF or Vitamin B9),
314 and numerous enzymes for its biosynthesis were missing. This cofactor plays an
315 important role in the metabolism of amino acids and vitamins. In turn, clade acI-B was
316 predicted to be auxotrophic for adenosylcobalamin (Vitamin B12), containing only a
317 single reaction from its biosynthetic pathway. Finally, acI-C was predicted to be
318 auxotrophic for the nucleotide uridine monophosphate (UMP, used as a monomer in
319 RNA synthesis) and the amino acids lysine and homoserine. In all cases multiple
320 enzymes for the biosynthesis of these compounds were not found in the acI-C
321 genomes. However, with the exception of adenosylcobalamin, we did not identify
322 transporters for any of these compounds. Furthermore, because the acI-C composite
323 genome was estimated to be around 75% complete, we cannot rule out the possibility
324 that the missing genes might be found in when additional genomes are recovered.

325 A number of seed compounds were predicted to be nutrients, compounds which
326 can be degraded by members of the acI lineage (Figure 4B). Both clades acI-A and acI-
327 B were predicted to use D-altronate and trans-4-hydroxy proline as nutrients, and acI-B
328 was additionally predicted to use glycine betaine. These compounds indicate that the
329 acI may participate in the turnover of plant- and animal-derived organic material in
330 freshwater systems: glycine betaine is an important osmolyte in plants (Ashraf and
331 Foolad, 2007), D-altronate is produced during degradation of galacturonate, a
332 component of plant pectin (Mohnen, 2008), and trans-4-hydroxy-L-proline is a major
333 component of animal collagen (Eastoe, 1955).

334 Finally, all three clades were predicted to use di-peptides and the sugar maltose
335 as nutrients. Clades acI-A and acI-C were also predicted to consume the
336 polysaccharides stachyose, manninotriose, and cellobiose. In all cases, these
337 compounds were associated with reactions catalyzed by peptidases or glycoside
338 hydrolases (Table S8 and S9), which may be capable of acting on compounds beyond
339 the predicted seed compounds. Thus, we used these annotations to define nutrient
340 sources, rather than using the predicted seed compounds themselves. Among these
341 nutrient sources were di- and polypeptides, predicted to be released from both
342 cytosolic- and membrane-bound aminopeptidases. As discussed below, we identified a
343 number of transport proteins capable of transporting these released residues. In Lake
344 Mendota, these aminopeptidases were expressed in clades acI-A and acI-B at around
345 70% of the median gene expression levels, while they were expressed at up to twice the
346 median in clade acI-C (Table S8). These findings agrees with MAR-FISH and CARD-
347 FISH studies that confirm the ability of acI bacteria to consume a variety of amino acids
348 (Salcher *et al.*, 2010, 2013).

349 All three clades were predicted to encode an alpha-glucosidase, which in Lake
350 Mendota was expressed most strongly in clade acI-C, at approximately 116% of the
351 median (Table S9). Clades acI-A and acI-C also encode a beta-glucosidase, but it was
352 not expressed, at least under prevailing environmental conditions. Both of these
353 enzymes release glucose monomers, which acI is known to consume (Buck *et al.*, 2009;
354 Salcher *et al.*, 2013). Furthermore, these two clades encode an alpha-galactosidase
355 and multiple maltodextrin glucosidases (which free maltose from maltotriose), but these
356 were only expressed in clade acI-C during our sampling period. The alpha-

357 galactosidase had a log2 average RPKM expression value of 2.5 times the median,
358 while the maltodextrin glucosidases were expressed at approximately 20% of the
359 median (Table S9).

360 *Compounds Transported by the acI Lineage*

361 Microbes may be capable of transporting compounds that are not strictly required
362 for growth, and comparing such compounds to predicted seed compounds can provide
363 additional information about an organism's ecology. Thus, we used the metabolic
364 network reconstructions for the acI genomes to systematically characterize the transport
365 capabilities of the acI lineage.

366 All acI clades encode for and expressed a diverse array of transporters (Figure 5,
367 Tables S10 and S11, and the Supplemental Online Material). Consistent with the
368 presence of peptidases, all clades contain numerous genes for the transport of peptides
369 and amino acids, including multiple oligopeptide and branched-chain amino acid
370 transporters, as well as two distinct transporters for the polyamines spermidine and
371 putrescine. All clades also contain a transporter for ammonium. As averaged over the
372 24-hour sampling period, the ammonium, branched-chain amino acid, and oligopeptide
373 transporters had expression values above the median, with expression values for the
374 substrate-binding protein (of the ATP-binding cassette (ABC) transporters) ranging from
375 2 to 325 times the median (Table S10). In contrast, while all clades expressed some
376 genes from the polyamine transporters, only clade acI-B expressed the
377 spermidime/putrescine binding protein, at approximately 75 times the median (Table
378 S10). Additionally, clade acI-A contains a third distinct branched-chain amino acid
379 transporter, composed of COGs not found in clades acI-B or acI-C. This transporter was

380 not as highly expressed as the shared transporters, with the substrate-binding protein
381 not expressed at all (Table S10). Finally, clades acI-A and acI-B also contain a
382 transporter for glycine betaine, which was only expressed in clade acI-A, at
383 approximately 35 times the median (Table S10). However, because these observations
384 were made at a single site at a single point in time, we cannot rule out the possibility
385 that the expression of these transporters changes with space and time.

386 All clades also strongly expressed transporters consistent with the presence of
387 glycoside hydrolases, including transporters for the sugars maltose (a dimer of glucose)
388 and xylose, with expression values for the substrate-binding protein ranging from 3 to
389 144 times the median (Table S10). Clades acI-A and acI-B also contain four distinct
390 transporters for ribose, although the substrate-binding subunit was not expressed at the
391 time of sampling (Table S10).

392 Representatives from the acI lineage also encode and expressed a number of
393 transporters that do not have corresponding seed compounds, including a uracil
394 permease, and a xanthine/uracil/thiamine/ascorbate family permease, both of which are
395 expressed at levels ranging from 11 to 127 times the median (Table S10) during the
396 sampling period. Clades acI-A and acI-B also contain a
397 cytosine/purine/uracil/thiamine/allantoin family permease, even if it was only expressed
398 in clade acI-B at the time of sampling (Table S10). Though not strictly annotated as
399 such, all three of these transporters may be responsible for the uptake of the seed
400 compound UMP. In addition, clade acI-A contains but did not express a transporter for
401 cobalamin (Vitamin B12), and both clades acI-A and acI-B contain but did not express
402 transporters for thiamin (Vitamin B1) and biotin (Vitamin B7) (Table S10). Despite

403 predicted auxotrophies for Vitamins B5 and B6, we were unable to find transporters for
404 these two compounds. However, as identification and annotation of transport proteins is
405 an active area of research (Saier *et al.*, 2014), transporters for these vitamins may yet
406 be present in the genomes.

407 Finally, all three clades expressed actinorhodopsin, a light-sensitive protein that
408 functions as an proton efflux pump (Sharma *et al.*, 2008). In all clades, actinorhodopsin
409 was among the top seven most highly-expressed genes at the time of sampling (Table
410 S4), with expression values in excess of 300 times the median in all three clades (Table
411 S4). Given that many of the transport proteins are ABC transporters, we speculate that
412 actinorhodopsin may facilitate maintenance of the proton gradient necessary for ATP
413 synthesis. Above-median expression of the ATP synthase genes is consistent with this
414 hypothesis. Coupled with high expression levels of diverse transporters, this result
415 strongly suggests that acI functioned as a photoheterotroph and was actively pumping
416 protons during our sampling period. However, it remains to be seen if this behavior is a
417 general feature of acI ecology or restricted to the specific conditions of the lake and our
418 sampling period.

419 **Discussion**

420 This study introduces the use of high-throughput metabolic network
421 reconstruction and the seed set framework to predict auxotrophies and nutrient sources
422 of uncultivated microorganisms from incomplete genome sequences. By leveraging
423 multiple genomes from closely related populations, we were able to construct composite
424 genomes for individual acI clades. Obviously this masks differences among tribes as
425 well as smaller populations and individual cells, and may sometimes overestimate the

426 metabolic capabilities of a clade or group. However, it provides a framework that can be
427 used to generate new hypotheses about the substrates used by members of a defined
428 phylogenetic group, provided multiple closely related genomes are available. As
429 metagenomic assembly and binning techniques and single cell sequencing methods
430 improve and complete genomes become available, we anticipate our approach being
431 applied to individual microbial genomes.

432 Our predictions of substrate use capabilities of the acI lineage are largely
433 congruent with previous genome-based studies based on smaller but manually curated
434 genome collections, indicating that the use of automatic metabolic network
435 reconstructions yields similar predictions to manual metabolic reconstruction efforts,
436 while being both high-throughput and focused on an organism's substrate utilization
437 capabilities. In particular, this study predicts that the consumption of N-rich compounds
438 is a universal feature of the acI lineage, with all three clades predicted to consume
439 ammonium, branched-chain amino acids, and di- and oligopeptides. We provide new
440 evidence for further specialization within each clade, identifying unique substate binding
441 proteins for some of their amino acid and peptide transporters (see Supplemental
442 Online Material), and the expression of a transporter for the polyamines spermidine and
443 putrescine in clade acI-B. Furthermore, we confirm the ability of all three clades to
444 consume xylose and maltose, and of clades acI-A and acI-B to consume ribose.
445 However, despite the presence and expression of alpha-glucosidases in all three
446 clades, and beta-glucosidases in clades acI-A and acI-B, no obvious glucose transport
447 system was found in the genomes. Our analysis also made novel predictions, including

448 the presence of beta-glucosidases, as well as alpha- and beta-galactosidases, in clades
449 acI-A and acI-C.

450 This study also suggests that auxotrophies for some vitamins may be universal
451 features of the acI lineage, as we predict all clades to be auxotrophic for pantothenic
452 acid and pyridoxal 5'-phosphate (Vitamins B5 and B6). We also predict new
453 auxotrophies within the acI lineage, including THF (clade acI-A), and lysine,
454 homoserine, and UMP (clade acI-C). While our acI-C composite genome remains
455 incomplete, these results nonetheless provide additional support to the hypothesis that
456 distributed metabolic pathways and metabolic complementarity may be common
457 features of freshwater bacterial communities (Garcia *et al.*, 2015; Garcia, 2016).

458 Combined, these results indicate that acI are photoheterotrophs, making a living
459 on a diverse array of N-rich compounds, sugars, oligo- and poly-saccharides, and light.
460 We hypothesize that the acI obtain peptides from the products of cell lysis, and may
461 participate in the turnover of high molecular weight dissolved organic compounds, such
462 as starch, glycogen, and cellulose. The acI lineage does not appear to be metabolically
463 self-sufficient, relying on other organisms for the production of essential nutrients.

464 This study also presents the first combined genomic and metatranscriptomic
465 analysis of a freshwater microbial lineage. Transport proteins were among the most
466 highly expressed in the acI genomes, and the expression of multiple amino acid
467 transporters may facilitate uptake of these labile compounds. We also observed
468 differences in the relative expression of these transporters, which may point to clade-
469 specific differences in the affinity for these substrates. Finally, the actinorhodopsin

470 protein was highly expressed, and may facilitate synthesis of the ATP needed to drive
471 acI's many ABC-type transporters.

472 A close comparison of our predictions to previous studies of the acI lineage
473 reveals some important limitations of the seed set framework and automatic metabolic
474 reconstructions. First, the seed set framework only identifies compounds that the
475 metabolic network **must** obtain from its environment, and will fail to identify compounds
476 that the organism can acquire from its environment but can also synthesize itself. For
477 example, members of clades acI-A and acI-B are capable of consuming branched-chain
478 amino acids (Ghylin *et al.*, 2014; Garcia *et al.*, 2015), but can also synthesize them.
479 Thus, these compounds were not identified as seed compounds. However, transport
480 reactions for branched-chain amino acids were identified in the genomes, and our
481 metatranscriptomic found them to be highly expressed.

482 Second, automatic metabolic network reconstructions may not fully capture an
483 organism's metabolic network (e.g., due to missing or incorrect genome annotations).
484 For example, previous genome-based studies have suggested that members of the acI
485 lineage harbor cyanophycinase, an enzyme that allows them to hydrolyze the
486 cyanobacterial peptide cyanophycin (Garcia *et al.*, 2013; Ghylin *et al.*, 2014). Manual
487 inspection revealed that KBase annotated this putative enzyme as a hypothetical
488 protein, and we could not identify transporters for cyanophycin in the metabolic network
489 reconstruction. As biochemical characterization of hypothetical proteins and automatic
490 gene and protein annotation are active areas of research, we anticipate that advances
491 in these fields will continue to improve the accuracy of automatic metabolic network
492 reconstructions.

493 **Conclusions**

494 In this study, we examined the ecological niche of uncultivated acI bacteria using
495 automatic metabolic network reconstructions and the seed set framework combined
496 with metatranscriptomics. Predicted seed compounds include peptides and
497 saccharides, many of which acI have been observed to consume *in situ*, as well as
498 newly predicted auxotrophies for vitamins and amino acids. Metatranscriptomic analysis
499 in a lake with abundant acI members suggests many of these compounds are
500 consumed by acI bacteria in their natural environment. Our high-throughput approach
501 easily scales to 100s of genomes, and enables a focused metabolic analysis by
502 identifying those compounds through which an organism interacts with its environment.
503 Finally, the seed set framework enables additional reverse ecological analyses, which
504 promise to predict the interactions among microbial species in complex environments
505 (Levy and Borenstein, 2012).

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521 **Conflict of Interest**

522 The authors declare no conflict of interest.

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680 and Europe indicate the existence of clades of globally distributed freshwater
681 bacteria. *Systematic and Applied Microbiology* **21**: 546–556.
682

683 **Figure Captions**

684 *Figure 1*

685

686 Phylogenetic placement of the genomes used in this study within the acI lineage.
687 The tree was built using RAxML (Stamatakis, 2014) from a concatenated alignment of
688 protein sequences from 37 single-copy marker genes (Darling *et al.*, 2014). The order
689 Actinomycetales forms the outgroup. Vertical black bars indicate groups of genomes
690 belonging to defined tribes/clades within the acI lineage, as determined using 16S rRNA
691 gene sequences (for SAGs and bin FNEF8-2 bin_7 acI-B only) and a defined taxonomy
692 (Newton *et al.*, 2011). SAGs are indicated with italic text. Supplemental Figure S5
693 shows the position of the acI lineage relative to other orders within the class
694 Actinobacteria.

695

696 *Figure 2*

697

698 Mean estimated completeness of tribe-level (clade-level) population genomes as
699 a function of the number of sampled genomes. For each tribe (clade), genomes were
700 randomly sampled (with replacement) from the set of all genomes belonging to that tribe
701 (clade). Completeness was estimated using 204 single-copy marker genes from the
702 phylum Actinobacteria (Parks *et al.*, 2015). Error bars represent the 95% confidence
703 interval estimated from 1000 iterations.

704

705 *Figure 3*

706

707 Overview of the seed set framework and metatranscriptomic mapping, using
708 three genomes from the acI-C clade as an example. **(A)** Metabolic network graphs are
709 created for each genome belonging to clade acI-C. In these graphs, metabolites are
710 represented as nodes (circles) and reactions by arcs (arrows). Grey nodes and edges
711 indicate components of the composite graph missing from that genome graph.
712 Additional information on this step of the workflow is available in Figure S1. **(B)** A
713 composite network graph is created for each clade by joining graphs for all genomes
714 from that clade, and seed compounds (red) are computed for the composite graph.
715 Additional information on this step of the workflow is available in Figures S2, S3, and
716 S4. **(Inset)** Three seed compounds which indicate an auxotrophy for L-homoserine, a
717 methionine precursor. **(C)** Metatranscriptomic reads are mapped to each individual
718 genome using BBMap. Orthologous gene clusters are identified using OrthoMCL (Li *et*
719 *al.*, 2003). For each cluster, unique reads which map to any gene within that cluster are
720 counted using HTSeq (Anders *et al.*, 2014). The relative gene expression is computed
721 using RPKM (Mortazavi *et al.*, 2008).

722

723 *Figure 4*

724

725 Seed compounds of members of the acI lineage. **(A)** Auxotrophies and nutrient
726 sources, not including peptides and glycosides. **(B)** Peptides and glycosides. These
727 compounds represent those inferred from genome annotations, rather than the seed

728 compounds themselves. In panel (B), the intensity of the color indicates the average
729 log2 RPKM of the encoding gene cluster. For compounds acted upon by multiple gene
730 clusters, the percentile of the most highly-expressed cluster was chosen.
731

732 *Figure 5*

733

734 Transporters that are actively expressed by members of the acl lineage, as
735 inferred from consensus annotations of genes associated with transport reactions
736 present in metabolic network reconstructions. The intensity of the color indicates the
737 average log2 RPKM of the encoding gene cluster. For multi-subunit transporters, the
738 RPKM of the substrate-binding subunit was chosen.
739

740 *Supplementary Figure 1*

741

742 Converting an unannotated genome to a metabolic network graph, for a
743 simplified genome containing only glycolysis. **(A)** Microbial contigs are annotated using
744 KBase, and a metabolic network reconstruction is built from the annotations. The
745 reconstruction provides links between protein-encoding genes in the genome and the
746 enzymatic reactions catalyzed by those proteins. **(B)** The metabolic network
747 reconstruction represents metabolism as a hypergraph, in which metabolites are
748 represented as nodes and reactions as hyperedges. In this representation, an edge can
749 connect more than two nodes. For example, a single hyperedge (denoted by a heavy
750 black line) connects the metabolites glucose and ATP to glucose-6P, ADP, and Pi. For

751 clarity, protons are not shown. **(C)** However, the algorithm used by the seed set
752 framework requires metabolism to be represented as a metabolic network graph, in
753 which an edge can connect only two nodes. In this representation, a reaction is
754 represented by a set of edges connecting all substrates to all products. For example,
755 the heavy hyperedge in (B) is now denoted by six separate edges connecting glucose to
756 ADP, glucose to Pi, glucose to glucose-6P, ATP to ADP, ATP to Pi, and ATP to
757 glucose-6P (again denoted by heavy black lines). Of these, only one (glucose to
758 glucose-6P) is biologically meaningful. The dotted line surrounds the currency
759 metabolites. **(D)** The metabolic network graph is then pruned, a process which removes
760 all currency metabolites and any edges in which those metabolites participate. Of the
761 six heavy edges in (C), only the biologically meaningful one is retained, connecting
762 glucose to glucose-6P (again denoted by a heavy black line). The images in (B) and (C)
763 are modified from (Ma and Zeng, 2003).
764

765 *Supplementary Figure 2*

766

767 Construction of composite metabolic network graph for clade acI-C. Beginning
768 with metabolic network graphs for genomes Actinobacterium_10 and ME00885, nodes
769 and edges unique to ME00885 are identified (in blue). These nodes and edges are
770 added to the Actinobacterium_10 graph, giving the composite metabolic network graph
771 for these two genomes (Actinobacterium_10 + ME00885). Then, this graph is compared
772 to the graph for ME03864, and nodes and edges unique to ME03864 are identified (in

773 blue). These nodes and edges are added to the Actinobacterium_10 + ME00885
774 metabolic network graph, giving the composite metabolic network graph for clade acI-C.
775

776 *Supplementary Figure 3*

777

778 Identifying seed compounds in metabolic networks, using the same metabolic
779 network as in Supplemental Figure S1. **(A)** To identify seed compounds, the metabolic
780 network graph is first decomposed into its strongly connected components (SCCs), sets
781 of nodes such that each node in the set is reachable from every other node. Here, each
782 set of circled nodes corresponds to a unique SCC. **(B)** SCC decomposition enables
783 seed sets to be identified from source components (components with no incoming
784 edges) on the condensation of the original graph. In the condensation of the original
785 graph shown here, each node corresponds to a unique SCC. This network has a single
786 seed set, SCC_1, enclosed in a dotted circle. **(C)** Seed compounds can be found from
787 the mapping between SCCs and their constituent metabolites. In this example, glucose
788 is the sole seed compound. While this particular result is probably intuitive, real
789 metabolic networks are considerably more complex. Note: The visual representations
790 shown here are intended to illustrate the metabolic network reconstruction process, and
791 are not indicative of the data structures used by our pipeline.
792

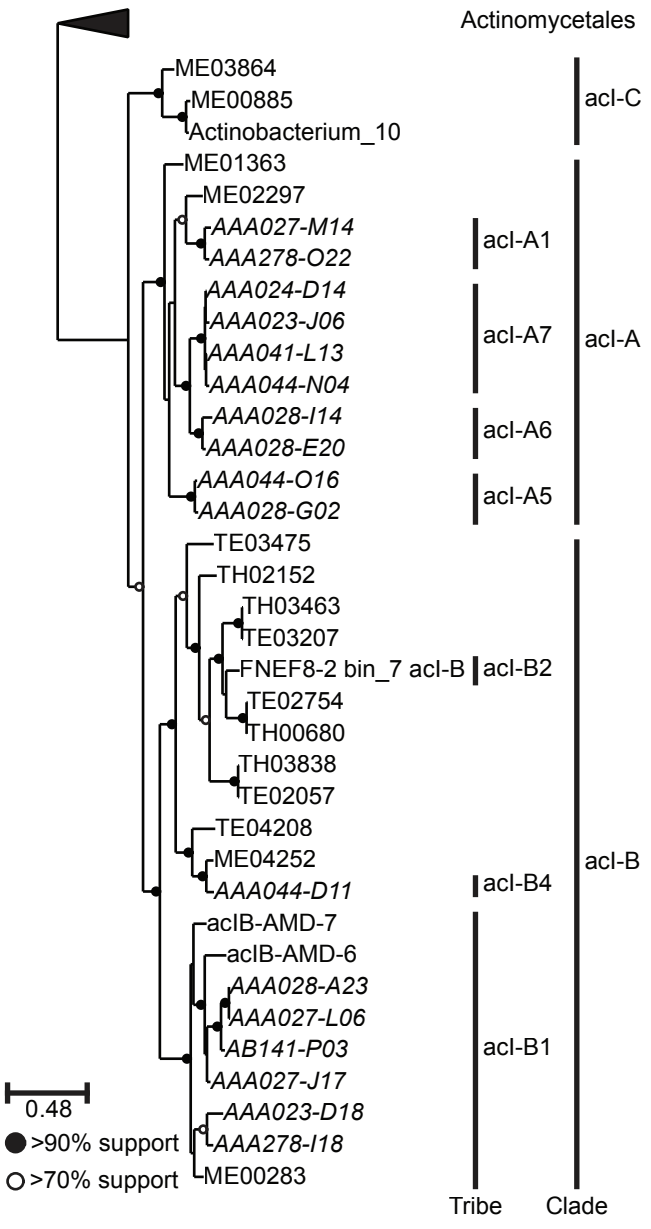
793 *Supplementary Figure 4*

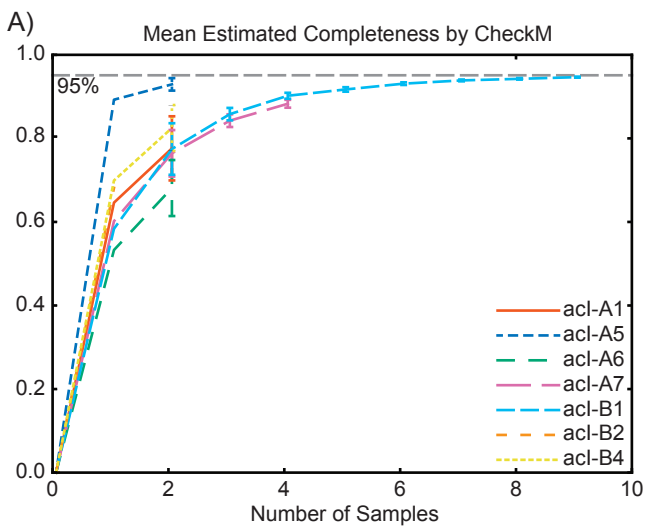
794

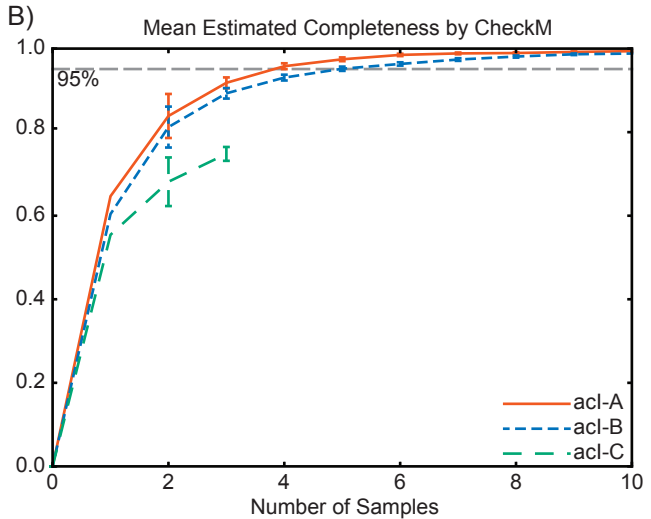
795 Complete composite metabolic network graph for clade acI-C, showing
796 disconnected components (gray) and the single largest component (green and black).
797 Disconnected components are dropped prior to computing the network's seed sets
798 because these groups of nodes are not connected to the bulk of the network. Within the
799 single largest component, the giant strong component contains a substantial fraction of
800 the compounds (green nodes), giving rise to a bow-tie structure in the metabolic
801 network graph.
802

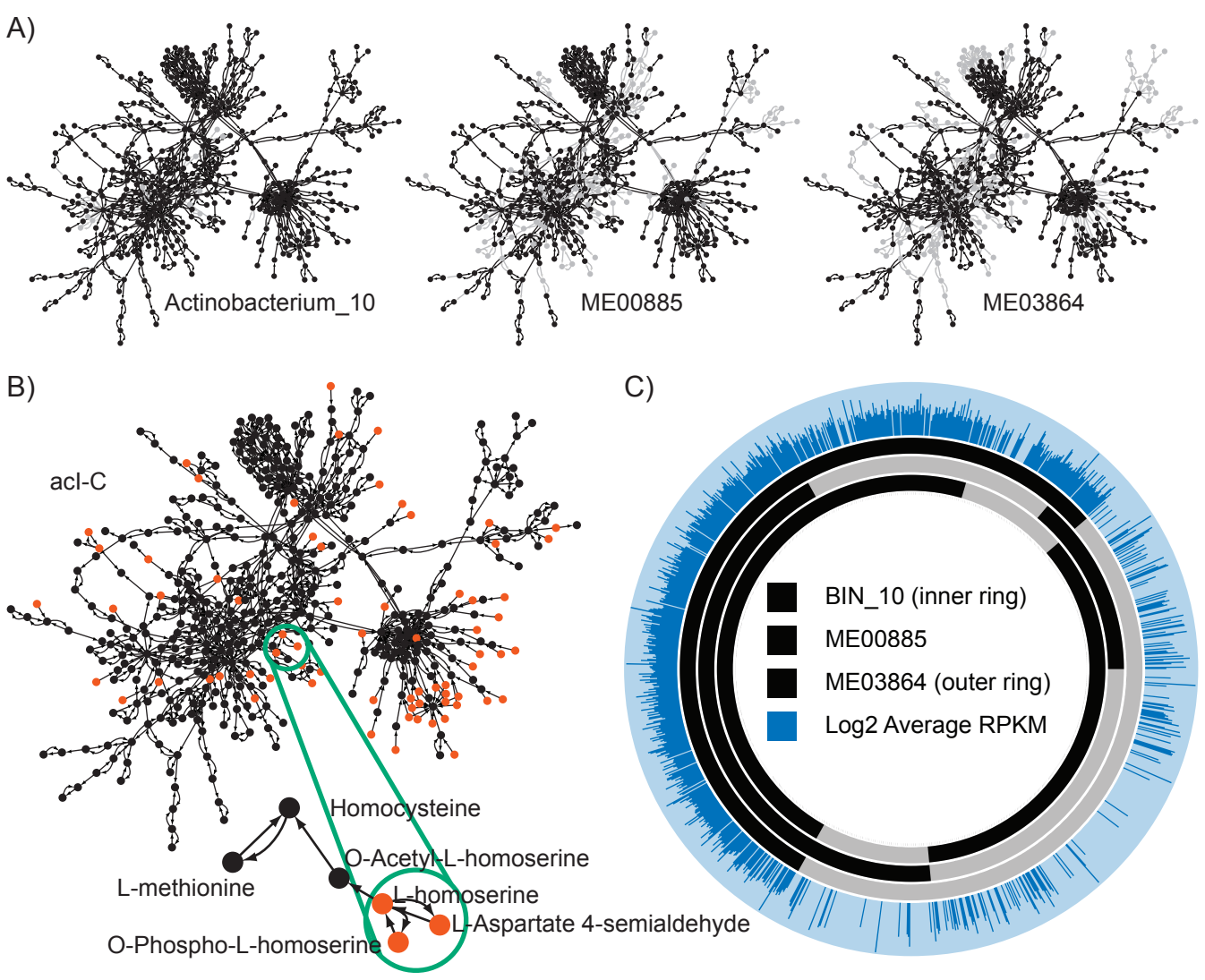
803 *Supplementary Figure 5*
804

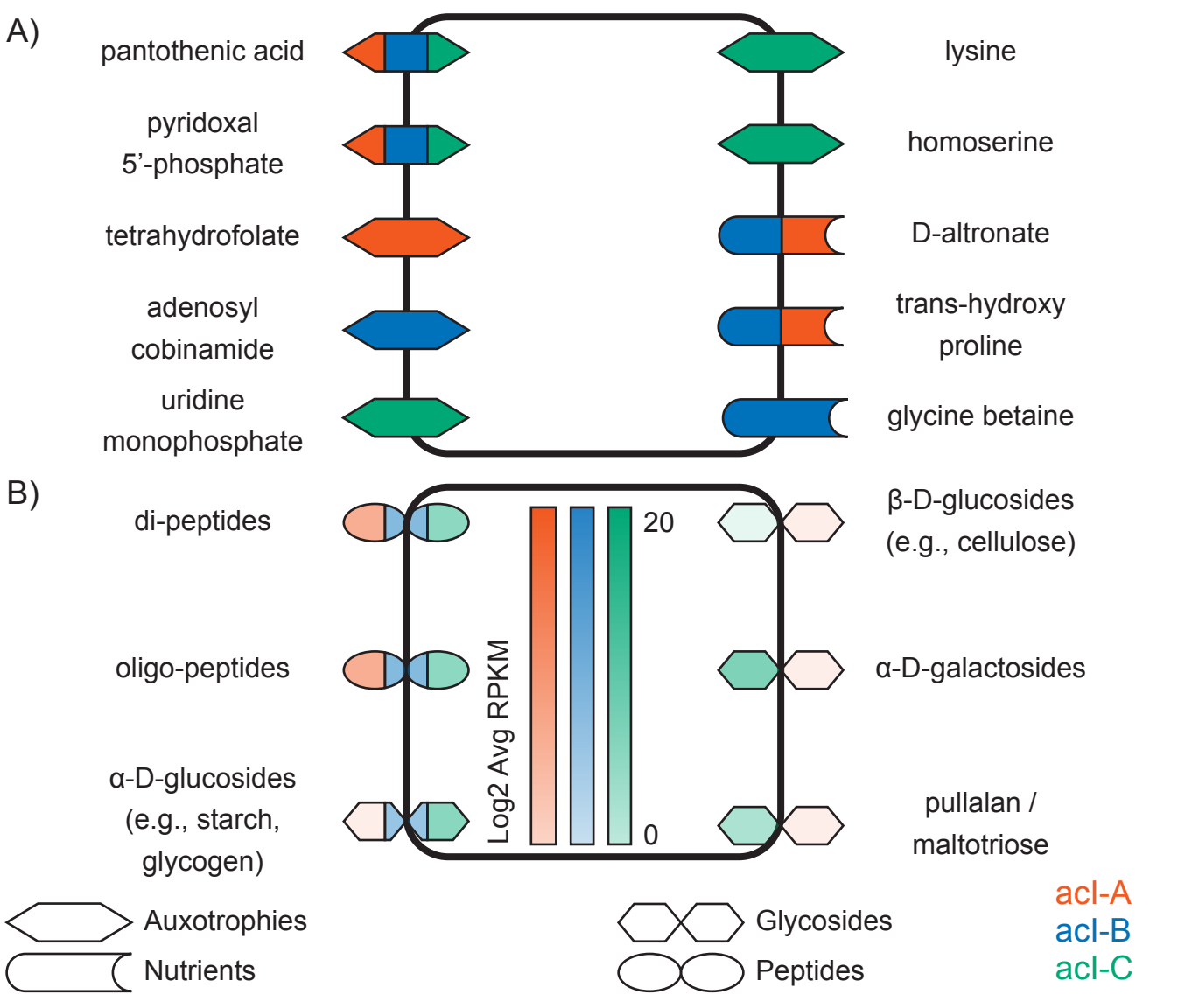
805 Phylogenetic placement of the genomes used in this study within the acI lineage,
806 relative to other sequenced actinobacterial genomes in the class Actinobacteria (Gao
807 and Gupta, 2012) (Table S17). The tree was built using RAxML (Stamatakis, 2014) from
808 a concatenated alignment of protein sequences from 37 single-copy marker genes
809 (Darling *et al.*, 2014). The class Acidimicrobiia forms the outgroup. Vertical black bars
810 indicate groups of genomes belonging to defined tribes/clades within the acI lineage, as
811 determined using 16S rRNA gene sequences (for SAGs and bin FNEF8-2 bin_7 acI-B
812 only) and a defined taxonomy (Newton *et al.*, 2011). SAGs are indicated with italic text.

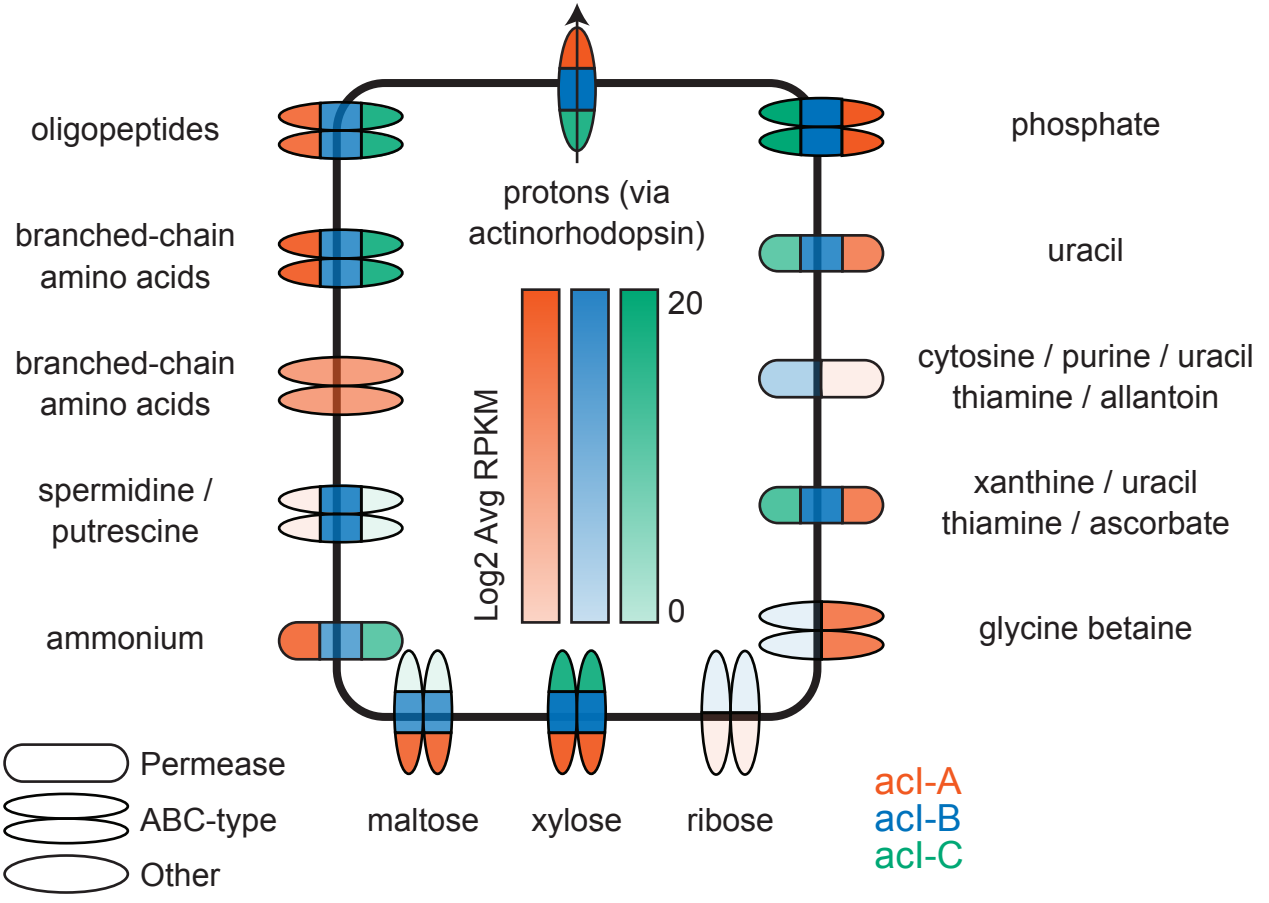

Actinomycetales
ME03864
ME00885
Actinobacterium_10
acI-C
ME01363
ME02297
AAA027-M14
AAA278-O22
acI-A1
AAA024-D14
AAA023-J06
AAA041-L13
AAA044-N04
acI-A7
acI-A
AAA028-I14
AAA028-E20
acI-A6
AAA044-O16
AAA028-G02
acI-A5
TE03475
TH02152
TH03463
TE03207
FNEF8-2 bin_7 acI-B
acI-B2
TE02754
TH00680
TH03838
TE02057
TE04208
ME04252
AAA044-D11
acI-B4
acI-B
acIB-AMD-7
acIB-AMD-6
AAA028-A23
AAA027-L06
AB141-P03
AAA027-J17
acI-B1
AAA023-D18
AAA278-I18
ME00283
0.48
>90% support
>70% support
Tribe
Clade


A)
Mean Estimated Completeness by CheckM
95%
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Number of Samples
acl-A1
acl-A5
acl-A6
acl-A7
acl-B1
acl-B2
acl-B4


B)
Mean Estimated Completeness by CheckM
95%
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Number of Samples
acl-A
acl-B
acl-C


A)
Actinobacterium_10
ME00885
ME03864
B)
acl-C
Homocysteine
O-Acetyl-L-homoserine
L-methionine
L-homoserine
L-Aspartate 4-semialdehyde
O-Phospho-L-homoserine
C)
BIN_10 (inner ring)
ME00885
ME03864 (outer ring)
Log2 Average RPKM


A)
pantothenic acid
pyridoxal 5'-phosphate
tetrahydrofolate
adenosyl cobinamide
uridine monophosphate
lysine
homoserine
D-altronate
trans-hydroxy proline
glycine betaine
B)
di-peptides
oligo-peptides
α-D-glucosides (e.g., starch, glycogen)
Log2 Avg RPKM
20
0
β-D-glucosides (e.g., cellulose)
α-D-galactosides
pullalan / maltotriose
Auxotrophies
Nutrients
Glycosides
Peptides
acl-A
acl-B
acl-C

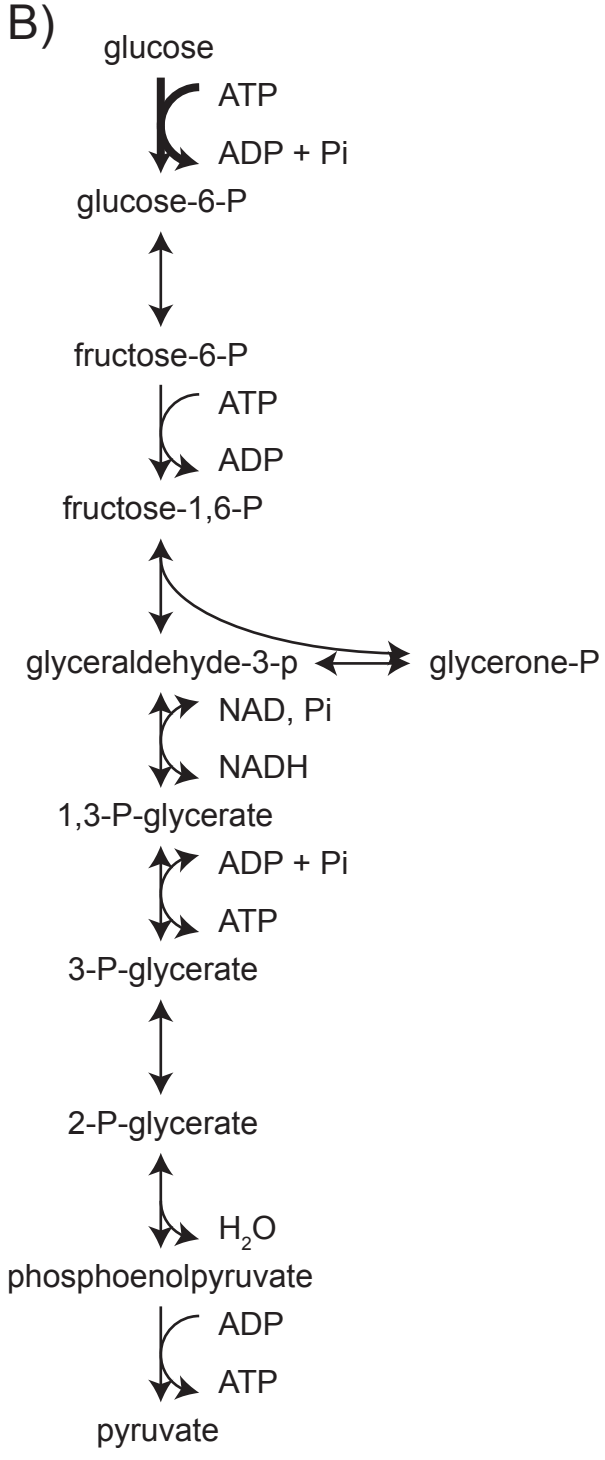

oligopeptides
branched-chain amino acids
branched-chain amino acids
spermidine / putrescine
ammonium
protons (via actinorhodopsin)
Log2 Avg RPKM
20
0
phosphate
uracil
cytosine / purine / uracil thiamine / allantoin
xanthine / uracil thiamine / ascorbate
glycine betaine
maltose
xylose
ribose
Permease
ABC-type
Other
acl-A
acl-B
acl-C

A)

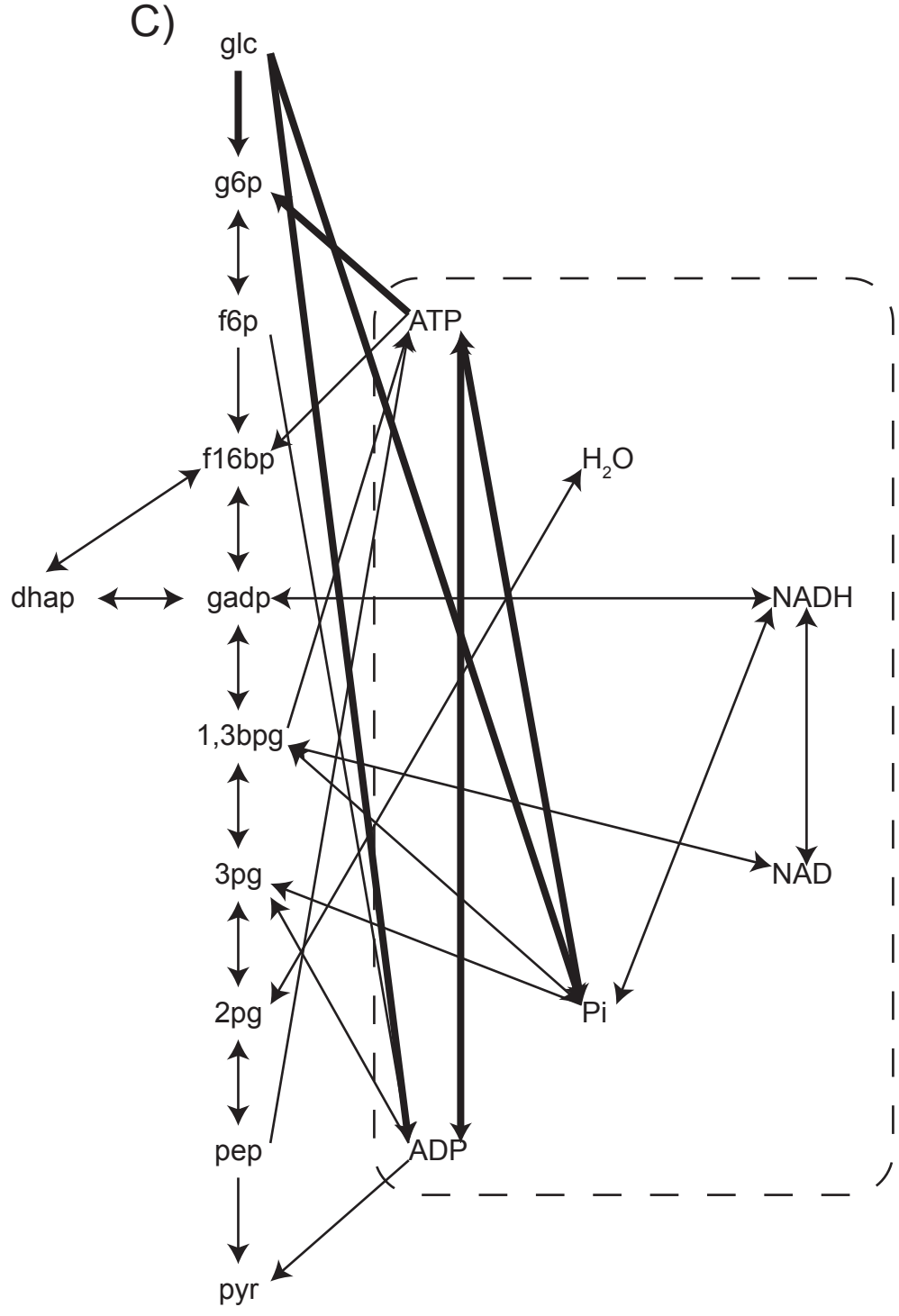
```
>TE02754.contig.1
TCACCAAGTGAATAC
CAGCAGCACGGGCG
AGCTGAGTGATGCGA
ACGATGGACTCTTCTA
CTTCGCGAGCCGCAA
CCATCATTAGATCTGC
TAATTCATCGATGATC
ACAAGTAGGTAGGGA
TATGGTTCGAGAATTC
GCTCGCTGCCTT...
```

| Reaction | Genes | Equation |
|---|---|---|
| rxn00216 | TE02754.1703 | glucose + ATP -> gluose-6-P + ADP + Pi |
| rxn00558 | TE02754.1688 | glucose-6-P <--> fructose-6-P |
| rxn00545 | TE02754.2419 | fructose-6-P + ATP --> fructose-1,6-P + ADP |
| rxn00786 | TE02754.1795 OR TE02754.2367 | fructose-1,6-P <--> glyceraldehyde-3-P +glycerone-P |
| rxn00747 | TE02754.1685 | glycerone-P <--> glyceraldehyde-3-P |
| rxn00781 | TE02754.1683 | glyceraldehyde-3-P + NAD + Pi <--> 1,3-P-glycerate + NADH |
| rxn01100 | TE02754.1684 | 1,3-P-glycerate + ADP + Pi <--> 3-P-glycerate + ATP |
| rxn01106 | TE02754.1899 | 3-P-glycerate <--> 2-P-glycerate |
| rxn00459 | TE02754.1568 OR TE02754.1842 | 2-P-glycerate <--> phosphoenolpyruvate + H2O |
| rxn00148 | TE02754.1665 | phosphoenolpyruvate + ADP --> pyruvate + ATP |

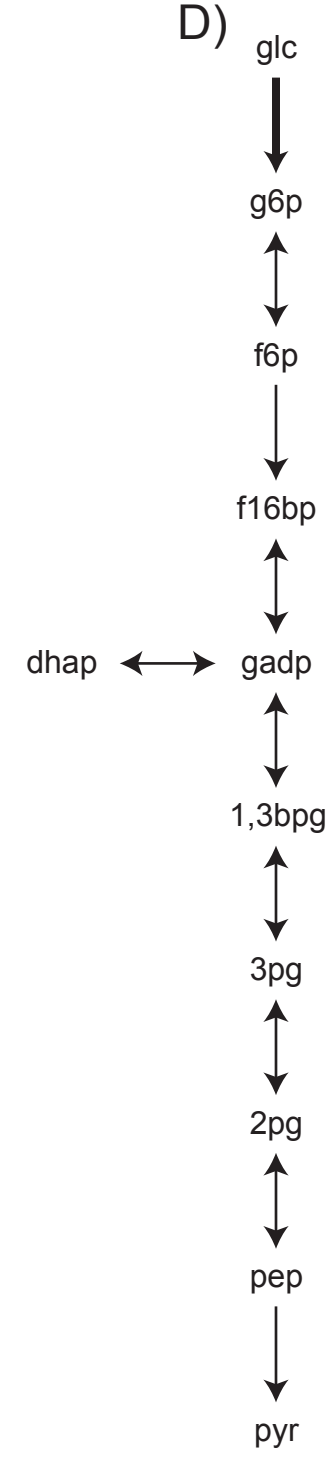
B)

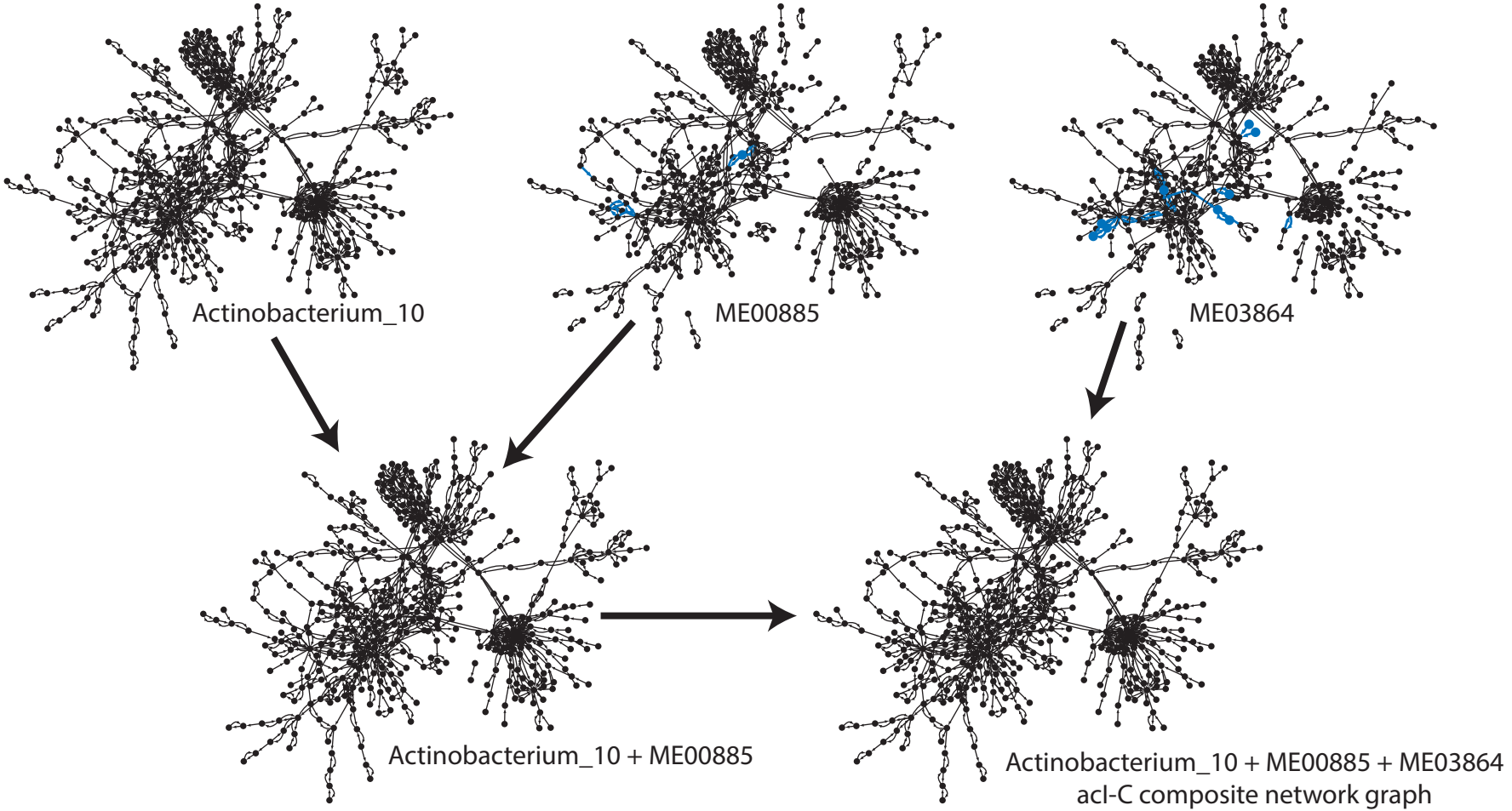


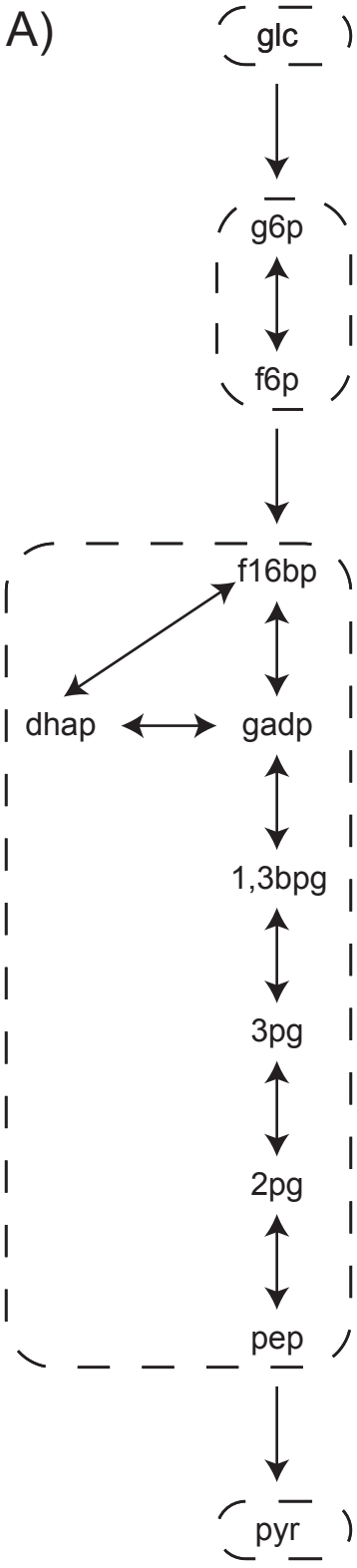

C)

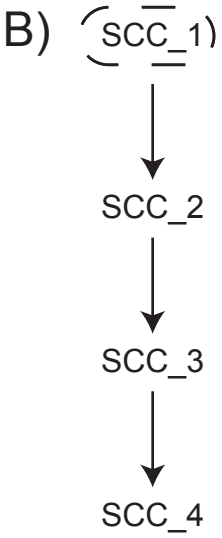



D)




Actinobacterium_10
ME00885
ME03864
Actinobacterium_10 + ME00885
Actinobacterium_10 + ME00885 + ME03864
acl-C composite network graph

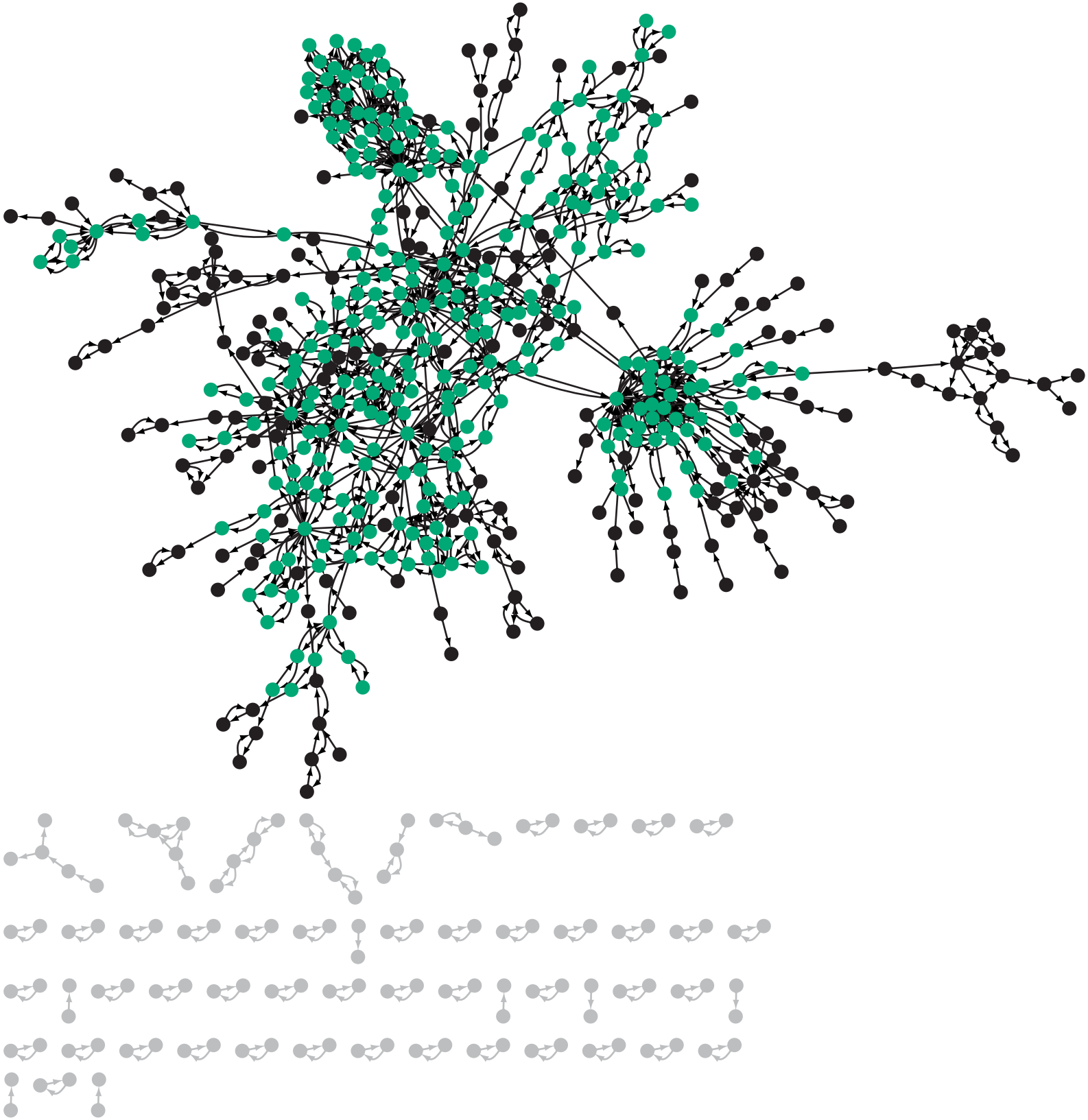


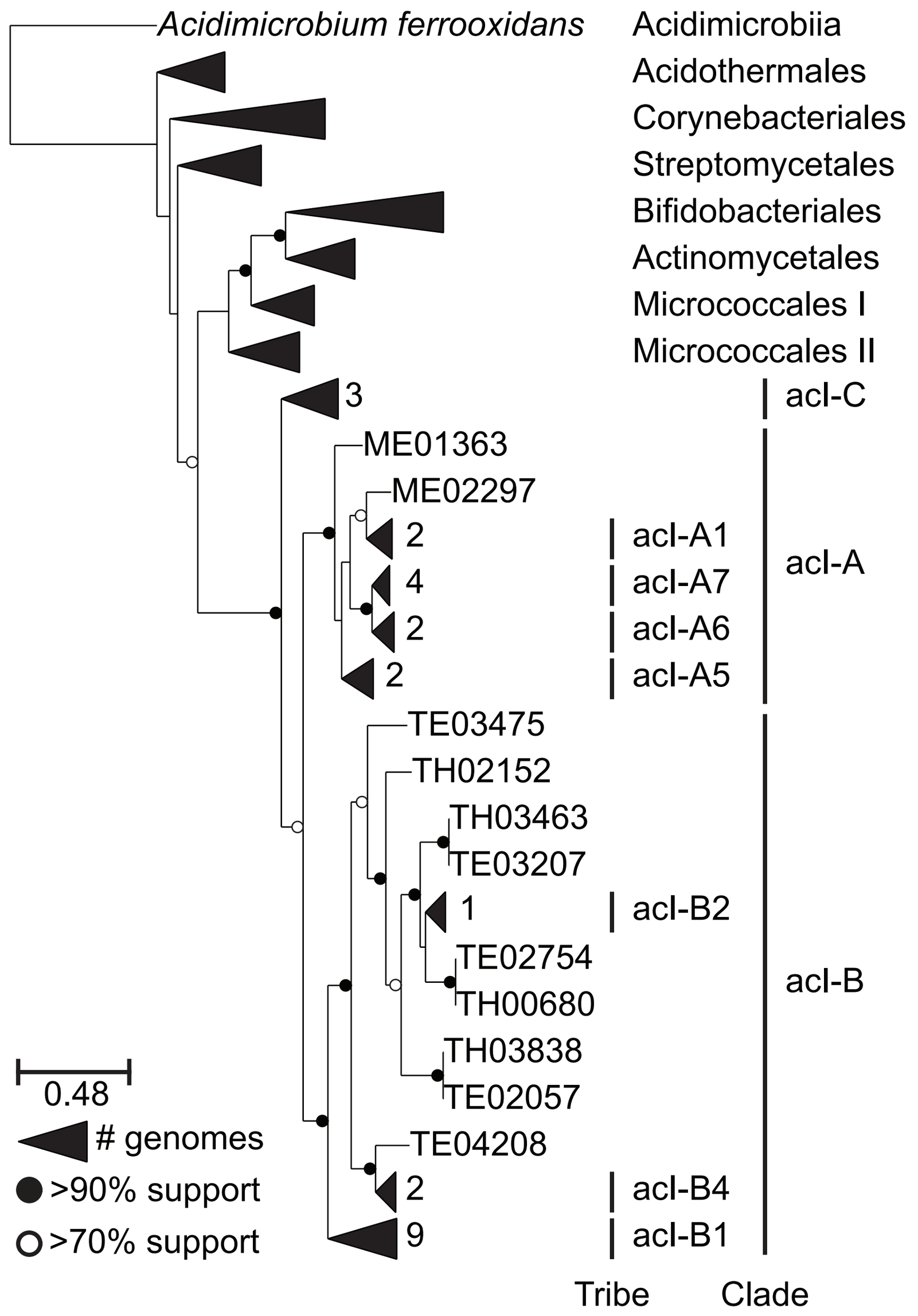




| SCC | In | Out | Seed |
|---|---|---|---|
| SCC_1 | 0 | 1 | Y |
| SCC_2 | 1 | 1 | N |
| SCC_3 | 1 | 1 | N |
| SCC_4 | 1 | 0 | N |

C)

| SCC | Metabolites |
|---|---|
| SCC_1 | glc |
| SCC_2 | g6p, f6p |
| SCC_3 | f16bp, gadp, dhap, 1,3bpg,3pg, 2pg, pep |
| SCC_4 | pyr |




Acidimicrobium ferrooxidans
Acidimicrobiia
Acidothermales
Corynebacteriales
Streptomycetales
Bifidobacteriales
Actinomycetales
Micrococcales I
Micrococcales II
3
acI-C
ME01363
ME02297
2
acI-A1
4
acI-A7
2
acI-A6
2
acI-A5
acI-A
TE03475
TH02152
TH03463
TE03207
1
acI-B2
TE02754
TH00680
acI-B
TH03838
TE02057
TE04208
2
acI-B4
9
acI-B1
0.48
# genomes
>90% support
>70% support
Tribe
Clade