Culturing the ubiquitous freshwater actinobacterial acI lineage by supplying a biochemical 'helper' catalase

Suhyun Kim†, Ilnam Kang†, Ji-Hui Seo, and Jang-Cheon Cho*

Department of Biological Sciences, Inha University, Incheon 22212, Republic of Korea

† These authors contributed equally to this work.

*E-mail: chojc@inha.ac.kr

Corresponding author information:

Address: Department of Biological Sciences, Inha University, Inharo 100, Namgu, Incheon 22212, Republic of Korea

Tel: +82-32-860-7711

Unlike the ocean from which abundant microorganisms with streamlined genomes such as *Prochlorococcus*[1], *Pelagibacter*[2], and *Nitrosopumilus*[3] have been isolated, no stable bacterial cultures are available for the ubiquitous freshwater actinobacterial acI lineage[4]. The acI lineage is among the most successful limnic bacterioplankton found on all continents[5], often representing more than half of all microbial cells in the lacustrine environment[6,7] and constituting multiple ecotypes[5,8]. Dilution-to-extinction culturing followed by whole-genome amplification recently yielded 20 complete acI genomes from lakes in Asia[9] and Europe[10]. However, the establishment of stably growing cultures has failed despite various cultivation efforts[9-13] using growth factors predicted from genome information[14]. Here, we report two pure cultures of the acI lineage successfully maintained by supplementing catalase into the growth media. Catalase was critical for stabilizing growth by degrading hydrogen peroxide irrespective of the genomic presence of the catalase-peroxidase (*katG*) gene, making the acI strains the first example of Black Queen Hypothesis[14] reported for freshwater bacteria. The two strains, representing two novel species, displayed differential phenotypes and distinct preferences for reduced sulfurs and carbohydrates, some of which were difficult to predict based on genomic information. Our results suggest that culturing the hitherto-uncultured freshwater bacteria can be facilitated by the simple catalase-supplement method and indicate that genome-based metabolic prediction can be complemented by physiological analyses.

In contrast to the typical terrestrial *Actinobacteria* found in soil and sediments, planktonic acI *Actinobacteria* have streamlined genomes[15] with 1.2–1.6 Mb genome sizes and are predicted to show auxotrophy for vitamin B compounds, some amino acids, and reduced sulfurs[8-10,16]. Despite the rich genomic data obtained from putatively axenic cultures and various cultivation efforts driven by genomic information, all initial cultures have been lost and no growing cultures are currently available[9,10], preventing further ecological studies on the ubiquitous acI lineage. In the present study, we successfully cultivated the acI lineage by catalase supplementation to remove hydrogen peroxide.

Strain IMCC25003 of the acI-A1 tribe and strain IMCC26103 of the acI-A4 tribe[4,5] were isolated from Lake Soyang in Korea by dilution-to-extinction culturing[9]. Strains IMCC25003 and IMCC26103 were most closely related to the recently proposed Candidatus species '*Ca*. Planktophila sulfonica' MMS-IA-56 (100%) and '*Ca*. Planktophila lacus' MMS-21-148 (99.9%)[10], respectively, based on their 16S rRNA gene phylogenetic

analyses (Supplementary Fig. 1). According to the average nucleotide identity (ANI)[17] with their closest relatives (74–86%)[10], the two strains each represent novel species of the genus '*Ca*. Planktophila'.

Because the two strains were among the few acI isolates and the ability to maintain stably-growing acI strains is prerequisite to studying the phenotypic characteristics of the acI lineage, we first attempted to resuscitate both strains from glycerol stocks. IMCC26103 did not grow for 21 days after reviving from the original frozen glycerol stocks but showed an abrupt increase in cell density (Supplementary Fig. 2a), which was found to be due to the growth of contaminating strains (Supplementary Fig. 2b). IMCC25003 began growing without a lag from the original frozen stocks and reached a cell density of 7.6×10^5 cells ml^{-1} in 5 days (Supplementary Fig. 2c; upper panel), but showed an atypical growth curve when the revived culture was transferred into the same medium on day 12 (Supplementary Fig. 2c; middle panel). When the first-transferred culture was inoculated again on day 15, no growth was detected until ~70 days (Supplementary Fig. 2c; lower panel). Because maintenance of the growing cultures of the acI strains was not successful in the media used for their original isolation, further attempts to obtain stable cultures were made by introducing various modifications to the media composition. These attempts were possible only for IMCC25003, as many glycerol stocks could be stored using the firstly revived cultures of IMCC25003. Modification of media composition was based on the putative metabolic features and growth requirements of the acI lineage predicted by approaches such as microautoradiography combined with fluorescence *in situ* hybridization (FISH) and single-cell genomics[8,18]. Culture media contained various supplements of FAMV medium (*see* Supplementary Table 1 for media composition) such as carbon substrates, proteinogenic amino acids, peptone, and yeast extract and different basal media including artificial freshwater medium (AFM)[19] and spent medium of *Limnohabitans* sp. IMCC26003 (Supplementary Table 2). However, growth of IMCC25003 was not detected under any culture conditions tested.

Failure in establishing stable acI bacterial cultures by supplementation with defined or undefined nutrients led us to hypothesize that controlling the putative growth inhibitors may be more crucial than supplying growth promoters. Because the growth of *Prochlorococcus* and ammonia-oxidizing archaea (AOA) was previously found to be promoted by removing hydrogen peroxide (H_2O_2)[20-22], we examined whether catalase, an enzyme that degrades H_2O_2, could facilitate the cellular growth of IMCC25003, although the IMCC25003 genome contains *katG*, which codes for a bifunctional catalase-peroxidase. IMCC25003 showed good growth and reached a maximum cell density of 2.7×10^7 cells ml^{-1} in catalase (10 U ml^{-1})-amended medium

(FAMV+CM+AA, *see* Methods for media composition), while no growth was detected in the medium devoid of catalase (Supplementary Fig. 3a). IMCC26103, which does not contain *katG*, was also successfully revived from the last-remained glycerol stocks in the catalase-amended medium, attaining a maximum cell density of 5.2 × 10^7 cells ml^{-1} (Supplementary Fig. 3b). Because both strains exhibited robust and reproducible growth upon repeated transfers in the media supplemented with catalase, catalase was considered a critical supplement in acI strain cultivation.

Before further experiments, dilution-to-extinction culturing of the successfully revived strains was performed twice to ensure the purity of the cultures, resulting in the establishment of stably growing pure cultures. The purity of the established cultures was verified by FISH (Fig. 1a), transmission electron microscopy (Fig. 1b), and whole genome sequencing followed by read mapping. Both strains produced reddish cell pellets colored by carotenoids and actinorhodopsin in 4 L cultures (Supplementary Fig. 4a), which were subsequently used for genome sequencing. The genome sequences were identical (1 base pair difference) to those obtained by multiple displacement amplification (MDA) in our previous study[9], with more even sequencing coverages (Supplementary Fig. 4b). For both strains, more than 99.9% of sequencing reads were mapped to the complete genomes, demonstrating the purity of the cultures.

To reconfirm the catalase-dependent growth, the two acI strains were cultivated with various concentrations of catalase (0–20 U ml^{-1}). Growth of the strains was dependent on the presence of catalase, as no growth was detected in the absence of catalase, while 0.5 U ml^{-1} catalase increased the cell density to ~10^7 cells ml^{-1} (Fig. 2a, b). Cell densities and H_2O_2 concentrations were simultaneously monitored in a catalase-spiked growth experiment to determine whether the pivotal role of catalase in promoting growth is mediated by degradation of H_2O_2. Without catalase, no cellular growth was detected (Fig. 2c, d) and the H_2O_2 concentration was maintained at the initial level of ~60 nM (Fig. 2e, f). In contrast, when 10 U ml^{-1} catalase was added at the time of inoculation, the H_2O_2 concentration decreased promptly to less than 10 nM and the acI strains began growing, reaching maximum cell densities in 6–9 days. When catalase was spiked at 7 or 13 days for IMCC25003 and 5 or 12 days for IMCC26103, the H_2O_2 concentration rapidly decreased from ~60 nM to ~10 nM (Fig. 2e, f). The acI strains, which showed no growth before catalase addition, began growing following the decrease in H_2O_2 concentration immediately after catalase addition, indicating that H_2O_2 removal by catalase was critical for maintaining the growth of the acI strains.

107 Among the several traits explained by the Black Queen Hypothesis (BQH)[14] such as auxotrophy for
108 vitamins or amino acids[15,23], the dependency on external H_2O_2 scavengers is the first-reported and among the
109 most representative cases of the BQH, discovered in two abundant marine autotrophic prokaryotic groups,
110 *Prochlorococcus* and AOA[20-22]. The dependency of cellular growth on the external H_2O_2 scavenger found in this
111 study makes the acI lineage the first freshwater bacterial example supporting the BQH, which explains the
112 evolution of metabolic dependency of genome-streamlined oligotrophic bacteria on external 'helpers' in nutrient-
113 depleted environments[15,24]. This crucial role of catalase in cultivating the acI strains was rather unexpected
114 because all culture media used in cultivation trials contained pyruvate, a well-known chemical scavenger of
115 H_2O_2[25,26] and because acI bacteria failed to resuscitate from the original dilution cultures regardless of the
116 presence of *katG*[9,10]. In this context, we first assumed that some unknown growth factors would be required for
117 laboratory cultivation rather than H_2O_2 scavengers and tested various nutrients and growth factors to revive the
118 acI strains. However, only catalase treatment resulted in successful cultivation of the acI strains, while all other
119 attempts failed. Our cultivation results suggest that acI bacteria grow only at very low concentrations of H_2O_2
120 attainable by catalase addition under laboratory conditions, regardless of the presence of *katG*, suggesting that
121 the metabolic dependency of bacterial groups cannot be predicted based solely on genome information.
122 Although *katG* was found only in IMCC25003, the two acI strains showed similar growth responses to
123 H_2O_2: no growth at a high concentration of H_2O_2 but good growth at minute concentration of H_2O_2 lowered by
124 catalase. This result indicated that the *katG* gene of IMCC25003 or its gene product, bifunctional catalase-
125 peroxidase (KatG), did not function properly in defense against H_2O_2 in the culture expriments. Therefore, we
126 first examined expression of *katG* in IMCC25003 by qPCR. The qPCR results showed that *katG* was expressed
127 and the relative expression level increased slightly with increasing H_2O_2 concentrations (Supplementary Fig. 5).
128 Next, *katG* of IMCC25003 was cloned and expressed in *E. coli* and the gene product was purified by affinity
129 chromatography (Supplementary Fig. 6). The native molecular weight of IMCC25003 KatG was ~165 kDa
130 (Supplementary Fig. 6d) and the molecular weight of the subunit analyzed by SDS-PAGE was ~83 kDa
131 (Supplementary Fig. 6a), indicating that IMCC25003 KatG formed a homodimer composed of two subunits. In
132 an in-gel enzyme activity assay, IMCC25003 KatG showed both catalase and peroxidase activities but its
133 catalase activity was much lower than that of bovine catalase (KatE), belonging to the monofunctional catalase
134 group, which was supplemented into the culture media in this study (Supplementary Fig. 7). To more accurately

135 assay catalase activity of IMCC25003 KatG, decomposition of H_2O_2 was plotted using varying amounts of
136 enzymes (Supplementary Fig. 8). All enzymatic parameters including specific activity (U mg^{-1}) and catalytic
137 efficiency (k_{cat}/K_m) showed that IMCC25003 KatG displayed lower catalase activities than those of other
138 microbial KatGs reported previously as well as bovine catalase (KatE) assayed in this study (Supplementary
139 Table 3), suggesting that IMCC25003 KatG does not have an activity sufficient to overcome the growth
140 inhibition by H_2O_2 present in the culture media.

141 We investigated the distribution of *katG* among the acI genomes available, as only one (IMCC25003) of
142 four acI strains isolated from Lake Soyang contains *katG*[9]. *katG* was found in 14 of 20 complete acI genomes
143 (Supplementary Table 4). When single amplified genomes were included, 20 of 35 genomes carried *katG*. The
144 distribution of *katG* in the acI lineage was biased toward acI-A compared to acI-B. Of the 25 acI-A genomes 19
145 carried *katGs*, while only one of 10 acI-B genomes contained *katG* (Supplementary Table 4), which prompted us
146 to analyze phylogenetic features of acI KatG proteins. Unexpectedly, phylogenetic analyses including diverse
147 bacterial KatG proteins[27] indicated that KatGs of the acI lineage belonged to two distinct groups that are
148 separated widely in the entire KatG tree (Fig. 3a). Further, this KatG phylogeny of the acI lineage was not
149 consistent with a phylogenomic tree of the lineage based on conserved proteins (Fig. 3b). KatGs of the acI-A
150 sublineage were divided into two different KatG groups depending on the tribes. acI-A1 and A2 KatGs formed
151 group A, while acI-A4, A5, A6, and A7 KatGs formed group B together with KatG of acI-B4 (Fig. 3). These
152 results indicate that complicated evolutionary processes occurred for KatG in the acI lineage. Because KatG of
153 IMCC25003 belonging to the group A showed a low activity, it would be interesting to see determine KatGs
154 belonging to group B also exhibit low catalase activity, as isolates possessing group B KatG were also reported
155 not to grow in lake water-based medium[10].

156 Establishment of stable pure cultures by catalase addition enabled the first phenotypic characterization of
157 the acI lineage which may have been impossible without cultures, including detailed morphology (Fig. 1b),
158 temperature preference (Supplementary Fig. 9), fatty acid profile (Supplementary Table 5), growth requirement,
159 and substrate utilization (Fig. 4), leading to the proposal of two novel species named as '*Ca*. Planktophila rubra'
160 for IMCC25003 and '*Ca*. Planktophila aquatilis' for IMCC26103 (*see* Supplementary Text). Actively growing
161 cells of the two strains were curved rods of very small sizes and biovolumes of 0.041 (for IMCC25003) and
162 0.061 μm^3 (for IMCC26103), representing one of the smallest ultramicrobial (<0.1 μm^3) cells among known

163 cultivated freshwater bacteria, but 1.6–2.4-fold larger cell volumes than those of other acI bacteria estimated by
164 epifluorescence microscopy[10] (Supplementary Table 6).

165 The cell densities of IMCC25003 were increased by amino acids but not by carbon mixtures (Fig. 4a).
166 Growth of IMCC26103 was enhanced by both amino acids and carbon mixtures, but the enhancement by carbon
167 mixtures was observed only in the presence of amino acids (Fig. 4b). These results indicated that the growth of
168 both strains was mainly limited by at least one of 20 amino acids and that IMCC26103 (but not IMCC25003)
169 utilized at least one of the carbon sources added. In each carbon compound-amended experiment, no single
170 carbon compound affected the growth of IMCC25003 (Fig. 4c), but ribose and glucose enhanced the growth of
171 IMCC26103 (Fig. 4d). These results were supported by genomic information showing that the IMCC26103
172 genome contained genes for D-ribose pyranase, ribokinase, and glucose ABC transporter, while none of these
173 genes were found in the IMCC25003 genome. In each amino acid-amended test, methionine and cysteine
174 increased the growth of both strains (Fig. 4e, f), supporting the genome-based metabolic prediction that acI
175 strains lacked the assimilatory sulfate reduction pathway and thus require reduced sulfur compounds for growth[9].
176 Interestingly, the two strains showed different preferences for reduced sulfur compounds. IMCC25003 preferred
177 methionine (optimum at 250 nM) over cysteine (Fig. 4e, g), while IMCC26103 preferred cysteine (optimum at
178 10 nM) over methionine (Fig. 4f, 4h), which was difficult to predict from genome information. Although a
179 limited number of substrates was tested, these differential preferences of different acI tribes on growth substrates
180 may lead to niche differentiation, underlying the tribe-specific spatiotemporal dynamics of the acI lineage found
181 in several cultivation-independent studies[16,28-30].

182 In conclusion, we successfully maintained actively-growing pure cultures of the acI lineage, the most
183 abundant freshwater bacterioplankton group, by supplementing catalase into the culture media to lower H_2O_2
184 concentration, enabling further analysis of the physiological properties that could not be inferred from genome
185 sequences alone. It is expected the simple catalase-supplement method can accelerate the cultivation of
186 bacterioplankton with streamlined genomes and thus contribute to studies on the ecological roles of ubiquitous
187 and abundant freshwater oligotrophic bacteria.

188

189

190 **Methods**

Initial isolation of the acI stains, medium preparation, and revival experiments

Freshwater collection, initial isolation, and identification of strains IMCC25003 and IMCC26103 are described in our previous study[9]. Briefly, both strains were initially isolated as liquid cultures by high-throughput culturing (HTC) method based on dilution-to-extinction in 0.2 μm-filtered and autoclaved freshwater media supplemented with very low levels of carbon compounds, amino acids mixture, and vitamin mixture. Two cryovials each containing 200 μl of initial HTC cultures suspended in 10% (v/v) sterile glycerol were stored at -80°C and used for further revival experiments. As shown in Supplementary Fig. 2, the axenic culture of IMCC25003 was obtained from the revival experiments, which was further used in the following experiments to establish a stably-maintainable pure culture of IMCC25003.

Culture media for reviving IMCC25003 were prepared based on natural freshwater (*see* Supplementary Table 1 for culture media ingredients). Natural freshwater collected at a depth of 1 m from the Dam station (37°56′50.6″ N, 127°49′7.9″ E) of Lake Soyang in February 2016 was filtered through a 0.2 μm pore-size membrane filter (Supor, Pall Corporation), autoclaved for 1.5 h, and aerated for 3 h. The filtered-autoclaved-aerated medium was supplemented with 10 μM NH_4Cl, 10 μM KH_2PO_4, and trace metal mixture[31], which was designated FAM (filtered and autoclaved freshwater medium). The FAM medium supplemented with the vitamin mixture[32] (Supplementary Table 1), designated FAMV, was used as a basal medium that did not contain extra-amended organic compounds. The FAMV supplemented with a carbon mixture (CM; 50 μM pyruvate, 5 μM D-glucose, 5 μM N-acetyl-D-glucosamine, 5 μM D-ribose, and 5 μM methyl alcohol) and amino acid mixture (AA; 20 proteinogenic amino acids, 100 nM each) were prepared and used as the basal heterotrophic growth medium.

As the first attempt to obtain a stable culture of IMCC25003 (Supplementary Table 2), 100 μl glycerol stock of IMCC25003 was inoculated into 20 ml of FAMV, FAMV amended with CM (FAMV+CM), FAMV amended with AA (FAMV+AA), and FAMV amended with CM and AA (FAMV+CM+AA). Different concentrations (0.5×, 1×, 5×, and 10×) of CM were added to FAMV+AA and the growth of IMCC25003 was tested. A 0.1 μm-filtered but non-autoclaved freshwater medium (FM) and artificial freshwater medium (AFM)[19] each supplemented with vitamin mixture, CM, and AA were also tested. In the second attempt, the following organic nutrients were individually supplemented into FAMV+CM+AA: 20 μM acetate, 20 μM oxaloacetate, 20 μM putrescine, 20 μM glycerol, 20 μM xylose, 1 mg l^{-1} proteose peptone, and 1 mg l^{-1} yeast extract. In the third attempt, the spent medium of the genus *Limnohabitans* which is often considered as a co-occurring bacterium

219 with acI bacteria, was added to the growth test medium. *Limnohabitans* sp. IMCC26003 grew to 3.1×10^6 cells
220 ml^{-1} in 100 ml of FAMV+CM+AA at 25°C for 2 weeks. After the *Limnohabitans* culture was filtered through a
221 0.2 µm pore-size membrane followed by a 0.1 µm pore-size membrane, 1 ml of the filtrate of the spent medium
222 and 100 µl of glycerol stock of IMCC25003 were inoculated into 20 ml of FAMV+CM+AA. In the fourth
223 attempt, catalase (from bovine liver, C9322, Sigma-Aldrich) stock solution (10^5 U ml^{-1} in 10 mM PBS, pH 7.4)
224 was added to FAMV+CM+AA at a final concentration of 10 U ml^{-1} and 100 µl glycerol stock of IMCC25003
225 was inoculated. All reviving tests were performed at 18°C for 5 weeks.
226
227 **Measurement of bacterial cell densities**
228 Bacterial cell densities in all growth experiments were determined by flow cytometry (Guava easyCyte
229 Plus, Millipore) as described previously[31,33]. After 200 µl of bacterial cultures were stained with SYBR Green I
230 (5× final concentration, Invitrogen) for 1 h, each stained sample was run for 10 s or until total cell counts
231 reached 5000. To accurately measure the cell counts in the samples with high cell density, the stained sample was
232 diluted to contain less than 200 cells μl^{-1}.
233
234 **Culture maintenance and confirmation of culture purity**
235 Bacterial cultures of strains IMCC25003 and IMCC26103 revived first from glycerol stocks by catalase
236 amendment were purified twice by dilution-to-extinction culturing using FAMV+CM+AA containing 10 U ml^{-1}
237 of catalase. Cells were diluted to 5, 1, or 0.1 cells ml^{-1} and dispensed into 48-well plates (1 ml per each well).
238 After incubation, growth-positive wells (>10^5 cells ml^{-1}) from the most diluted inoculum (0.1 cells ml^{-1}) were
239 used for next round of dilution-to-extinction, resulting in the establishment of stably-growing pure cultures.
240 To confirm the purity of the established pure cultures by FISH, 5 ml of exponentially grown cells were
241 fixed with 2% paraformaldehyde (in PBS, pH 7.4) and filtered through 0.2-µm polycarbonate filters (Isopore,
242 Millipore). Hybridization was performed at 35°C for 6 h in hybridization buffer [900 mM NaCl, 20 mM Tris (pH
243 7.4), 0.01% SDS, 15% formamide] with Cy3-labeled oligonucleotide probes (AcI-852 and AcI-1214; 2 ng μl^{-1}
244 each) and helper probes (AcI-852-H1 and AcI-852-H2; 2 ng μl^{-1} each) targeting the acI clade[6]. After washing the
245 membranes twice with washing buffer [150 mM NaCl, 20 mM Tris (pH 7.4), 6 mM EDTA, and 0.01% SDS] at

246 55°C for 10 min followed by DAPI (4,6-diamidino-2-phenylindole) staining, Cy3-positive and DAPI-positive
247 cells were visualized with a confocal laser scanning microscope (LSM 510 META, Carl Zeiss).
248 For purity confirmation by genome sequencing, the two strains were cultivated in 4 L of FAMV+CM+AA
249 supplemented with 10 U ml^{-1} of catalase. Cells were harvested by centrifugation at 20,000 ×*g* for 120 min and
250 the cell pellets (Supplementary Fig. 4a) were used for genomic DNA extraction. Sequencing libraries were
251 constructed using the Nextera library preparation kit. Genome sequencing was performed on an Illumina MiSeq
252 platform (2 × 300 bp) by ChunLab, Inc (Seoul, Republic of Korea). Raw sequencing data were assembled using
253 SPAdes 3.9.0[34], resulting in a circularly closed complete genome for both strains. Comparison of the genome
254 sequences obtained in this study to those obtained by MDA[9] was performed using BLASTn. Calculation of
255 sequencing coverages and estimation of culture purity based on reads mapping were performed as described by
256 Kang *et al.*[9], using the 'depth' and 'flagstat' options in samtools.
257 After purification, both strains were stored as 10% (v/v) glycerol suspensions at -80°C and working
258 cultures were maintained in FAMV+CM+AA supplemented with 1 U ml^{-1} of catalase at 25°C. Throughout this
259 study, the peak shape of the cultures in the flow cytometry, morphology of SYBR Green I-stained cells in
260 epifluorescence microscopy, FISH images obtained using acI-specific probes, and electropherograms from 16S
261 rRNA gene sequencing were routinely examined to evaluate culture purity.
262
263 **Characterization of catalase-dependent growth properties**
264 The effects of catalase concentration on the growth of strains IMCC25003 and IMCC26103 were tested in
265 triplicate in FAMV+CM+AA supplemented with 0.5, 1, 5, 10, and 20 U ml^{-1} of catalase at 25°C. Bacterial cell
266 densities were measured by flow cytometry every 2 d.
267 To confirm the effect of catalase on the enhancement of cellular growth and decrease of H_2O_2
268 concentration, 10 U ml^{-1} of catalase was added to IMCC25003-inoculated culture medium (FAMV+CM+AA)
269 that had been maintained for 0, 7, and 13 days without catalase treatment. Similarly, for strain IMCC26103, 10 U
270 ml^{-1} of catalase was spiked at 0, 5, and 12 days. Cultures maintained without catalase were used as controls. For
271 all cultures, cell densities were monitored every day by flow cytometry. At the same time, the concentration of
272 H_2O_2 in the culture medium was measured using a modified acridinium ester chemiluminescence method[35]
273 described below.

274

275 **Determination of hydrogen peroxide concentration**

276 A modified acridinium ester chemiluminescence method[35] was applied to determine H_2O_2 concentrations in
277 the culture medium. To measure H_2O_2 concentrations at the nanomolar level, acridinium NHS ester (A63063;
278 Cayman Chemical) reagent was used as an indicator and the resulting chemiluminescence was estimated using a
279 SpectraMax L microplate Reader (Molecular Devices). A standard curve for H_2O_2 was prepared in the range of
280 0.02–2.0 μM by diluting 30% (v/v) of H_2O_2 solution (H1009; Sigma-Aldrich) with ddH_2O. The acridinium ester-
281 H_2O_2 response signal was integrated for 13 s after sequential injections of 20 μl 2 M Na_2CO_3 (pH 11.3) followed
282 by 80 μl of 2.2 mg L^{-1} acridinium ester (in 1 mM phosphate buffer, pH 3) at 300 μl s^{-1} injection flow speed and
283 0.1-s interval. All measurements were conducted in triplicate at 20°C.

284

285 **Morphological and physiological characterization of acI strains**

286 Cell morphology was examined by transmission electron microscopy (CM200, Philips) using whole cell
287 staining and thin-sectioning. For whole cell staining, 20 ml cultures were gently filtered using a 0.2-μm pore size
288 polycarbonate membrane on which formvar/carbon-coated copper grids were placed, followed by staining of the
289 grids with 0.5% uranyl acetate. To prepare thin-sectioned samples, cell pellets were harvested by centrifugation
290 of 2 L cultures at 20,000 ×*g* for 120 min. Cell pellets that were primary fixed with 2.0% Karnovsky's fixative and
291 secondary fixed with 1.0% osmium tetroxide were dehydrated using ethanol series from 35% to 100% and Epon
292 812 resin was infiltrated. Ultrathin sections (70 nm) were prepared with an ultramicrotome using a diamond
293 knife, placed on formvar/carbon-coated copper grids, and double-stained with 2% uranyl acetate and 0.5% lead
294 oxide.

295 The temperature range for growth of the two acI strains was monitored at 10–35°C in FAMV+CM+AA
296 supplemented with 10 U ml^{-1} of catalase. After the optimum temperature had been determined, all growth
297 experiments were performed in triplicate at 25°C in different growth media supplemented with 10 U ml^{-1} of
298 catalase. The growth curves in FAMV, FAMV+CM, FAMV+AA, and FAMV+CM+AA were determined to
299 evaluate the effects of carbon compound mixtures and amino acids mixtures on cellular growth. To identify
300 which amino acids are required for growth, 20 individual amino acids were supplemented in the FAMV medium
301 at 100 nM and cellular growth was monitored. Similarly, 5 individual carbon sources (50 μM pyruvate, 5 μM D-

302 glucose, 5 μM *N*-acetyl-D-glucosamine, 5 μM D-ribose, and 5 μM methyl alcohol) were supplemented into
303 FAMV+AA and the growth curves were determined. To determine the optimal concentration of reduced sulfur
304 compounds, growth of the acI strains was measured at 0–10 μM of methionine (for IMCC25003) and cysteine
305 (for IMCC26103).
306 Cellular fatty acid profiles were determined using the cells harvested from 2 L cultures grown in
307 FAMV+CM+AA supplemented with 10 U ml^{-1} of catalase. Fatty acid methyl esters were extracted from cell
308 pellets using the standard protocol for the Sherlock Microbial Identification System (MIDI, Inc.A) and analyzed
309 by gas chromatography (Agilent 7890 GC) based on MIDI version 6.1 with the TSBA6 database[36].
310
311 **Measurement of *katG* expression by qPCR**
312 Strain IMCC25003 was grown in triplicate in 4 L of FAMV+CM+AA supplemented with 1 U ml^{-1} of
313 catalase until the early stationary phase. Each 4-L bacterial culture was harvested by centrifugation at 20,000 ×*g*
314 for 120 min. To examine the expression of IMCC25003 *katG* and compare relative level of gene expression
315 according to H_2O_2 concentrations, harvested cells were treated with different concentrations of H_2O_2 (10, 50, and
316 100 μM) for 30 min and untreated cells were used as a control. RNA was extracted using TRIzol (Sigma-
317 Aldrich). Reverse transcription was performed with 1 μg of RNA using qPCRBIO cDNA Synthesis Kit
318 (PCRBIO Systems) and real-time qPCR was conducted using qPCRBIO SyGreen Blue Mix Lo-ROX (PCRBIO
319 Systems) in a real-time thermal cycler (Rotor-Gene 3000, Corbett). The *katG* primer set (forward, 5′-
320 CATGGCGATGAATGATGAAG-3′; reverse, 5′-GCTGTTCTTCCAGCCAAGTC-3′) for targeting *katG* of
321 IMCC25003 was used to evaluate gene expression and the GAPDH gene of IMCC25003 was employed as a
322 housekeeping gene with the GAPDH primer set (forward, 5′-GTTCAGCGACAGACCTCACA-3′; reverse, 5′-
323 TGGTGAGCTGTGAATCGAAG-3′).
324
325 **Expression, purification, and characterization of KatG from IMCC25003**
326 The gene encoding catalase-peroxidase (KatG) of IMCC25003 was amplified by PCR using the following
327 primers: forward, 5′-CATATGATGACTCAAGAATCAACTCC-3′; reverse, 5′-
328 CTCGAGTTACTTCTTCTTTGAC-3′. The PCR product was inserted into the pET-15b vector (Novagen) and
329 expressed in *Escherichia coli* BL21 (DE3) using 1 mM isopropyl-*β*-D-thiogalatoside (Sigma-Aldrich). The

330 expressed KatG-His recombinant protein was purified using a gravity-flow Ni^{2+}-nitrilotriacetic acid affinity
331 column (Novagen). The high concentration of salts used for elution in the affinity column was removed using a
332 PD-10 desalting column (GE Healthcare). To increase protein purity, the eluted fractions were applied to a
333 Superose-12 FPLC column (10 × 300 mm, GE Healthcare) equilibrated with 50 mM potassium phosphate buffer
334 (pH 7.0). The purified recombinant proteins were concentrated to approximately 10 mg ml^{-1} in Centricon tubes
335 (MWCO 10,000 Da; Millipore) and stored at 4°C. All purification steps were carried out at 4°C or on ice.
336 The native molecular weight of IMCC25003 KatG was determined by size exclusion chromatography on a
337 Superose-12 FPLC column (10 × 300 mm, GE Healthcare) equilibrated with 50 mM potassium phosphate buffer
338 (pH 7.0). The molecular weights of the subunits were determined by discontinuous sodium dodecyl sulfate-
339 polyacrylamide gel electrophoresis (SDS-PAGE) according to the standard Laemmli method. Catalytic activity
340 of recombinant IMCC25003 KatG was determined using a spectrophotometric assay by measuring the
341 decomposition of H_2O_2 at 240 nm. Catalase-specific activity was quantified by allowing varying amounts of
342 enzyme (0–1.0 µg for bovine catalase; 0–5.0 µg for IMCC25003 KatG) to react with 5 mM H_2O_2 in 50 mM
343 potassium phosphate buffer (pH 7.0) at 25°C. The absorption coefficient at 240 nm, pH 7.0, and 25°C for H_2O_2
344 was determined to be 49.8 M^{-1} cm^{-1}. Kinetic parameters were determined in triplicate from initial linear reaction
345 rates of H_2O_2 ranging from 1 to 10 mM. The apparent Km (mM) and kcat (s^{-1}) values at these substrate
346 concentrations were determined from Lineweaver-Burk plots. Catalase from bovine liver was used as a positive
347 control. In SDS-PAGE, staining for catalase activity was performed with the ferricyanide negative staining
348 method using 2% (w/v) ferric chloride and 2% (w/v) potassium ferricyanide solution, and peroxidase activity
349 was detected by double-staining with 3,3′,5,5′-tetramethylbenzidine[37].
350

351 **Phylogenetic analyses based on 16S rRNA gene, whole genome, and KatG protein**

352 The 16S rRNA gene sequences of the acI genomes[8-10] were downloaded from the IMG database and
353 GenBank, aligned using SINA online aligner, and imported into the ARB-SILVA database (SSURef NR 99,
354 release 123). Multiple alignments of the imported sequences and other reference sequences of the acI lineage
355 were exported with the 'ssuref:bacteria' filter and used to construct a maximum-likelihood tree in RAxML 8.2.7
356 with the GTRGAMMA model. Phylogenetic assignment of the sequences was performed as described by
357 Newton *et al.*[5] and the recently proposed names for the two *Candidatus* genera[10] .

358 To build phylogenomic trees, protein sequences predicted in 4 completed acI genomes[9] and single-cell
359 genomes[8] and 16 recently published acI genomes[10] were downloaded from the IMG database and NCBI RefSeq
360 database. Downloaded protein sequences were processed using CheckM[38], which produces concatenated
361 alignment of 43 conserved proteins. This concatenated alignment was used to build a maximum-likelihood tree
362 using RAxML 8.2.7 with the PROTGAMMAAUTO model.
363 KatG proteins in the acI genomes (listed in Supplementary Table 4) were searched by BLASTp using the
364 KatG sequence of IMCC25003 as a query and the acI protein sequences downloaded above as a search database,
365 which resulted in the identification of a total of 20 acI KatG proteins. Phylogenetic trees of KatG proteins were
366 constructed to identify the phylogenetic positions of the KatGs found in the acI genomes. Sequences collected
367 for tree construction included the following: 20 acI KatG proteins, 16 KatG proteins showing high similarities to
368 acI KatG proteins in BLASTp against the nr database of GenBank, 19 actinobacterial KatG proteins searched
369 from the genomes representing diverse taxonomic groups of the phylum *Actinobacteria*[9], and >300 KatG
370 proteins downloaded from PeroxiBase[39] (http://peroxibase.toulouse.inra.fr; Category 'Catalase peroxidase' of
371 'Class I peroxidase superfamily' under 'Non Animal peroxidase'). After sequence collection, several rounds of
372 alignment and tree building were performed, and some sequences were excluded because of their short length,
373 poor alignment, or unstable positioning. Finally, 303 KatG proteins were selected, aligned with Muscle[40]
374 implemented in the MEGA 6 program, and used to construct a maximum likelihood tree using RAxML (version
375 8.2.7), with automatic model selection based on the aic criterion (-m PROTGAMMAAUTO --auto-prot=aic).
376 The selected model was LG likelihood with empirical base frequencies. Grouping of the KatG proteins was
377 performed as described by Zamocky *et al.*[27]
378
379 **Data availability**
380 The complete genome sequences of strains IMCC25003 and IMCC26103 obtained from cell pellets using
381 Illumina MiSeq platform have been deposited in GenBank with the accession numbers CP029557 for IMCC25003
382 and CP029558 for IMCC26103.
383
384 **Acknowledgements**

This study was supported by the Mid-Career Research Program through the National Research Foundation (NRF) funded by the Ministry of Sciences and ICT (to J-CC, NRF-2016R1A2B2015142) and partially by the Basic Science Research Program funded by the Ministry of Education, Republic of Korea (to IK, NRF-2016R1A6A3A11934789; to J-HS, NRF-2016R1A6A3A11935361).

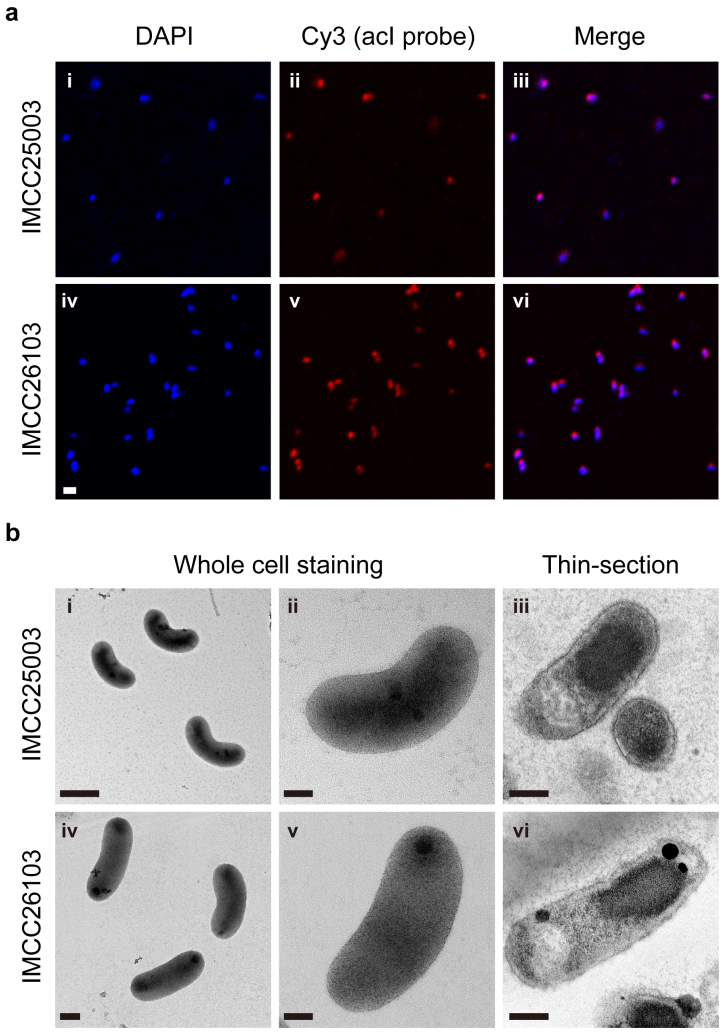
Author contributions

S.K isolated and physiologically characterized strains IMCC25003 and IMCC26103. S.K and I.K analyzed the genome sequences and phylogeny. J.-H.S performed the experiments for the expression and characterization of KatG from IMCC25003. J.-C.C. and I.K designed and supervised the study. S.K, I.K., J.-H.S, and J.-C.C. wrote the manuscript.

Competing interests

The authors declare no competing interests.

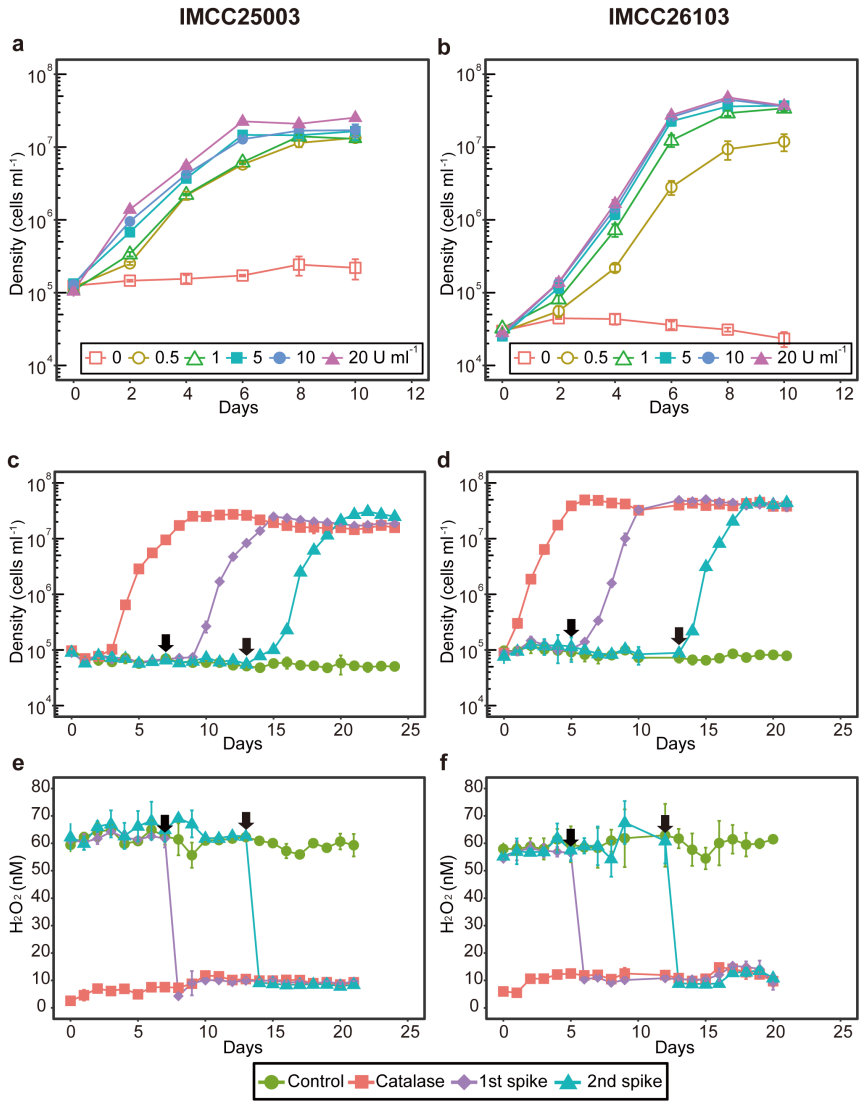
399 **Figures**



400

401 **Figure 1.** Microscopic examination of the two acI strains for purity evaluation and morphological
402 characterization. (a) Demonstration of culture purity of strains IMCC25003 (upper; i, ii, and iii) and IMCC26103
403 (lower; iv, v, and vi) by FISH analysis. The acI cells were dual-stained with DAPI (i and iv) and Cy3-conjugated
404 oligonucleotide probes specific to the acI lineage (AcI-852 and AcI-1214) (ii and v). Merged images are shown
405 in iii and vi. Bar, 1 μm. (b) Cell morphology of strains IMCC25003 (upper; i, ii, and iii) and IMCC26103 (lower;
406 iv, v, and vi) examined by transmission electron microscopy. The acI cells were stained with uranyl acetate (i, ii,
407 iv, and v) or double stained with uranyl acetate and lead oxide after thin-sectioning (iii and vi). Scale bars; 0.5
408 μm (i), 0.2 μm (iv), and 0.1 μm (ii, iii, v, and vi).

409

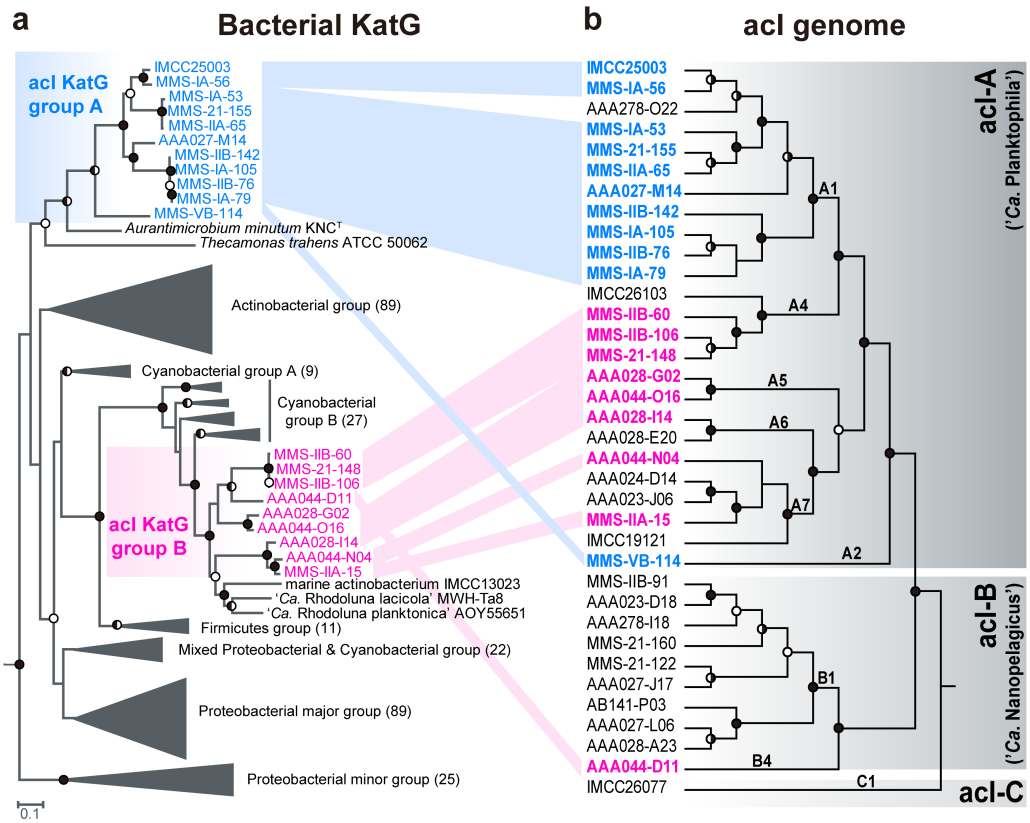


410

411 **Figure 2.** Catalase-dependent growth of the two acI strains. (a and b) The effect of various concentrations (0–20
412 U ml^{-1}) of catalase on the growth of strains IMCC25003 (a) and IMCC26103 (b). (c, d, e, and f) The effect of
413 catalase addition on cell densities (c and d) and H_2O_2 concentrations (e and f) during cultivation of strains
414 IMCC25003 (c and e) and IMCC26103 (d and f). Catalase (10 U ml^{-1}) was added to the culture media at day 0
415 ('Catalase') or days indicated by black arrows ('1st spike' and '2nd spike'). No catalase was added to the cultures
416 designated 'Control'. All experiments were performed in triplicate using the culture medium FAMV+CM+AA
417 (Supplementary Table 1). Error bars indicate standard deviation. Note that error bars shorter than the size of the
418 symbols are hidden.

419

420



421

422 **Figure 3.** Comparison between a phylogenetic tree of bacterial KatG proteins and phylogenomic tree of
423 members of the acI lineage. (a) A maximum-likelihood tree of bacterial KatG protein sequences including those
424 predicted in the acI genomes. The acI KatG proteins are indicated in color (blue and red for acI KatG group A
425 and B, respectively), while other bacterial KatG proteins were grouped following the scheme proposed by
426 Zamocky *et al.*[27]. The number of proteins included in each KatG group are indicated within parentheses at the
427 end of group names. The KatG sequences of *Rhodopirellula baltica* and *Pirellula staleyi* (both from the phylum
428 *Planctomycetes*) were used as the outgroup. (b) A phylogenomic tree of members of the acI lineage constructed
429 using concatenated alignment of conserved marker proteins. The acI strains containing *katG* are indicated in
430 color according to the grouping in the left KatG tree. The SAG AAA027-D23 (belonging to the acSTL-A1
431 tribe)[16] was set as an outgroup. Only the tree topologies are shown, and branch lengths do not represent
432 phylogenetic distances. For both trees, bootstrap values (from 100 and 250 replicates for the KatG tree and
433 phylogenomic tree, respectively) are shown at the nodes as filled circles (≥90%), half-filled circles (≥70%), and
434 empty circles (≥50%).

435

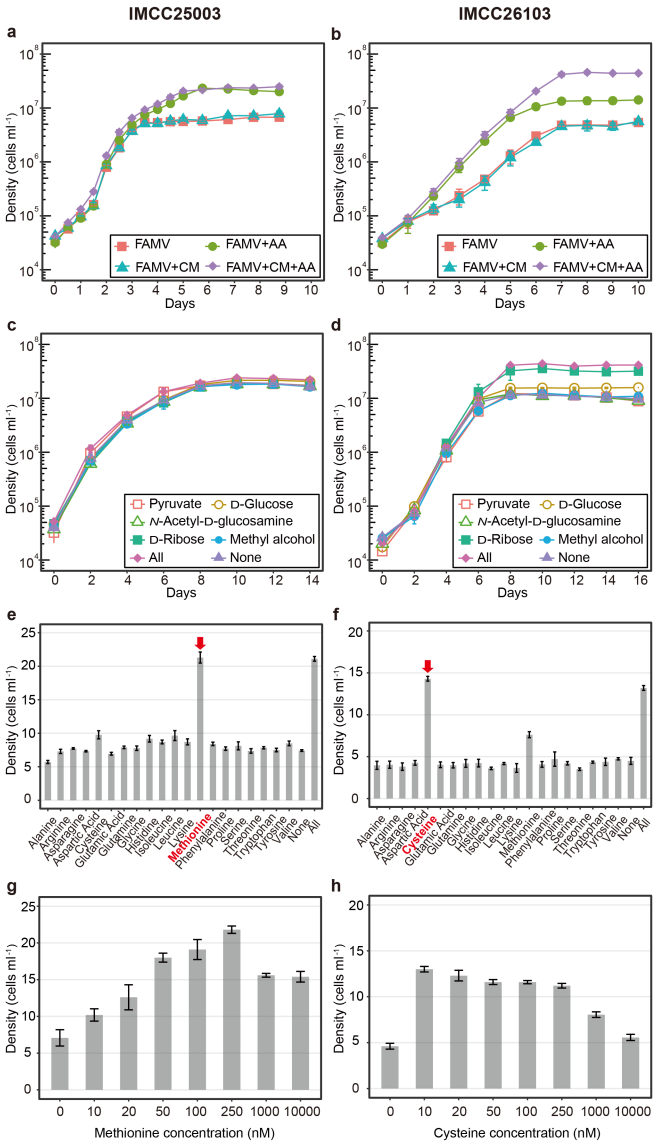


Figure 4. Effects of carbon sources and amino acids on the growth of the acI strains IMCC25003 (figures on the left: a, c, e, and g) and IMCC26103 (figures on the right: b, d, f, and h). (a and b) The effects of amino acid mixture (AA) and/or carbon source mixture (CM). AA and CM were added to the FAMV medium separately or together. Detailed medium composition is presented in Supplementary Table 1. (c and d) The effects of individual carbon compound. Each of 5 carbon compounds [5 μM, except for pyruvate (50 μM)] was added to the FAMV+AA medium. All, 5 carbon substrates were added together; None, no carbon source was added. (e and f) The effects of individual amino acids. Each of 20 proteinogenic amino acids was added at a concentration of 100 nM to the basal medium FAMV. All, 20 amino acids were added together; None, no amino acid was added. (g and h) Optimal concentrations of L-methionine (for IMCC25003) and L-cysteine (for IMCC26103). Various concentrations (0–10,000 nM) of L-methionine or L-cysteine were added to the basal medium FAMV.

448 ## References

449 1. Moore, L. R., Rocap, G. & Chisholm, S. W. Physiology and molecular phylogeny of coexisting
450 *Prochlorococcus* ecotypes. *Nature* **393**, 464-467 (1998).
451 2. Rappe, M. S., Connon, S. A., Vergin, K. L. & Giovannoni, S. J. Cultivation of the ubiquitous SAR11
452 marine bacterioplankton clade. *Nature* **418**, 630-633 (2002).
453 3. Konneke, M. et al. Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* **437**, 543-
454 546 (2005).
455 4. Warnecke, F., Amann, R. & Pernthaler, J. Actinobacterial 16S rRNA genes from freshwater habitats
456 cluster in four distinct lineages. *Environ. Microbiol.* **6**, 242-253 (2004).
457 5. Newton, R. J., Jones, S. E., Eiler, A., McMahon, K. D. & Bertilsson, S. A guide to the natural history of
458 freshwater lake bacteria. *Microbiol. Mol. Biol. Rev.* **75**, 14-49 (2011).
459 6. Warnecke, F., Sommaruga, R., Sekar, R., Hofer, J. S. & Pernthaler, J. Abundances, identity, and growth
460 state of Actinobacteria in mountain lakes of different UV transparency. *Appl. Environ. Microbiol.* **71**,
461 5551-5559 (2005).
462 7. Allgaier, M. & Grossart, H.-P. Diversity and seasonal dynamics of *Actinobacteria* populations in four
463 lakes in northeastern Germany. *Appl. Environ. Microbiol.* **72**, 3489-3497 (2006).
464 8. Ghylin, T. W. et al. Comparative single-cell genomics reveals potential ecological niches for the
465 freshwater acl Actinobacteria lineage. *ISME J* **8**, 2503-2516 (2014).
466 9. Kang, I., Kim, S., Islam, M. R. & Cho, J.-C. The first complete genome sequences of the acI lineage,
467 the most abundant freshwater *Actinobacteria*, obtained by whole-genome-amplification of dilution-to-
468 extinction cultures. *Sci. Rep.* **7**, 42252 (2017).
469 10. Neuenschwander, S. M., Ghai, R., Pernthaler, J. & Salcher, M. M. Microdiversification in genome-
470 streamlined ubiquitous freshwater Actinobacteria. *ISME J* **12**, 185-198 (2018).
471 11. Jezbera, J., Sharma, A. K., Brandt, U., Doolittle, W. F. & Hahn, M. W. '*Candidatus* Planktophila
472 limnetica', an actinobacterium representing one of the most numerically important taxa in freshwater
473 bacterioplankton. *Int. J. Syst. Evol. Microbiol.* **59**, 2864-2869 (2009).
474 12. Garcia, S. L., McMahon, K. D., Grossart, H.-P. & Warnecke, F. Successful enrichment of the ubiquitous
475 freshwater acI *Actinobacteria*. *Environ. Microbiol. Rep.* **6**, 21-27 (2014).

476 13. Garcia, S. L. et al. Auxotrophy and intrapopulation complementary in the 'interactome' of a cultivated
477 freshwater model community. *Mol. Ecol.* **24**, 4449-4459 (2015).
478 14. Morris, J. J., Lenski, R. E. & Zinser, E. R. The Black Queen Hypothesis: evolution of dependencies
479 through adaptive gene loss. *mBio* **3**, e00036-12 (2012).
480 15. Giovannoni, S. J., Cameron Thrash, J. & Temperton, B. Implications of streamlining theory for
481 microbial ecology. *ISME J* **8**, 1553-1565 (2014).
482 16. Garcia, S. L. et al. Contrasting patterns of genome-level diversity across distinct co-occurring bacterial
483 populations. *ISME J* **12**, 742-755 (2018).
484 17. Konstantinidis, K. T. & Tiedje, J. M. Genomic insights that advance the species definition for
485 prokaryotes. *Proc. Natl. Acad. Sci. USA.* **102**, 2567-2572 (2005).
486 18. Salcher, M. M., Posch, T. & Pernthaler, J. In situ substrate preferences of abundant bacterioplankton
487 populations in a prealpine freshwater lake. *ISME J* **7**, 896-907 (2013).
488 19. Kim, S., Kang, I. & Cho, J. C. Genomic analysis of a freshwater actinobacterium, "*Candidatus*
489 Limnosphaera aquatica" strain IMCC26207, isolated from Lake Soyang. *J. Microbiol. Biotechnol.* **27**,
490 825-833 (2017).
491 20. Morris, J. J., Kirkegaard, R., Szul, M. J., Johnson, Z. I. & Zinser, E. R. Facilitation of robust growth of
492 *Prochlorococcus* colonies and dilute liquid cultures by "Helper" heterotrophic bacteria. *Appl. Environ.*
493 *Microbiol.* **74**, 4530-4534 (2008).
494 21. Morris, J. J., Johnson, Z. I., Szul, M. J., Keller, M. & Zinser, E. R. Dependence of the cyanobacterium
495 *Prochlorococcus* on hydrogen peroxide scavenging microbes for growth at the ocean's surface. *PLoS*
496 *ONE* **6**, e16805 (2011).
497 22. Kim, J. G. et al. Hydrogen peroxide detoxification is a key mechanism for growth of ammonia-
498 oxidizing archaea. *Proc. Natl. Acad. Sci. USA.* **113**, 7888-7893 (2016).
499 23. D'Souza, G. & Kost, C. Experimental evolution of metabolic dependency in bacteria. *PLoS Genet.* **12**,
500 e1006364 (2016).
501 24. Morris, J. J., Papoulis, S. E. & Lenski, R. E. Coexistence of evolving bacteria stabilized by a shared
502 Black Queen function. *Evolution* **68**, 2960-2971 (2014).

503 25. Desagher, S., Glowinski, J. & Premont, J. Pyruvate protects neurons against hydrogen peroxide-induced
504 toxicity. *J. Neurosci.* **17**, 9060-9067 (1997).
505 26. Varma, S. D. & Hegde, K. R. Lens thiol depletion by peroxynitrite. Protective effect of pyruvate. *Mol.*
506 *Cell. Biochem.* **298**, 199-204 (2007).
507 27. Zamocky, M., Gasselhuber, B., Furtmuller, P. G. & Obinger, C. Molecular evolution of hydrogen
508 peroxide degrading enzymes. *Arch. Biochem. Biophys.* **525**, 131-144 (2012).
509 28. Berry, M. A. et al. Cyanobacterial harmful algal blooms are a biological disturbance to Western Lake
510 Erie bacterial communities. *Environ. Microbiol.* **19**, 1462-2920 (2017).
511 29. Okazaki, Y. & Nakano, S.-I. Vertical partitioning of freshwater bacterioplankton community in a deep
512 mesotrophic lake with a fully oxygenated hypolimnion (Lake Biwa, Japan). *Environ. Microbiol. Rep.* **8**,
513 780-788 (2016).
514 30. Newton, R. J. & McLellan, S. L. A unique assemblage of cosmopolitan freshwater bacteria and higher
515 community diversity differentiate an urbanized estuary from oligotrophic Lake Michigan. *Front.*
516 *Microbiol.* **6**, 1028 (2015).
517 31. Carini, P., Steindler, L., Beszteri, S. & Giovannoni, S. J. Nutrient requirements for growth of the
518 extreme oligotroph '*Candidatus* Pelagibacter ubique' HTCC1062 on a defined medium. *ISME J* **7**, 592-
519 602 (2013).
520 32. Davis, H. C. & Guillard, R. R. L. Relative value of ten genera of micro-organisms as food for oyster
521 and clam larvae. *Fish. Bull. (Wash. D. C.).* **58,** 293–304 (1958).
522 33. Tripp, H. J. et al. SAR11 marine bacteria require exogenous reduced sulphur for growth. *Nature* **452**,
523 741-744 (2008).
524 34. Bankevich, A. et al. SPAdes: a new genome assembly algorithm and its applications to single-cell
525 sequencing. *J. Comput. Biol.* **19**, 455-477 (2012).
526 35. King, D. W. et al. Flow injection analysis of H_2O_2 in natural waters using acridinium ester
527 chemiluminescence: method development and optimization using a kinetic model. *Anal. Chem.* **79**,
528 4169-4176 (2007).
529 36. Sasser, M. *Identification of bacteria by gas chromatography of cellular fatty acids.* MIDI Technical
530 Note 101. Newark, DE: MIDI Inc. (1990).

531 37. Thomas, P. E., Ryan, D. & Levin, W. An improved staining procedure for the detection of the
532 peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. *Anal.*
533 *Biochem.* **75**, 168-176 (1976).
534 38. Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P. & Tyson, G. W. CheckM: assessing the
535 quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res.* **25**,
536 1043-1055 (2015).
537 39. Fawal, N. et al. PeroxiBase: a database for large-scale evolutionary analysis of peroxidases. *Nucleic*
538 *Acids Res.* **41**, D441-444 (2013).
539 40. Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic*
540 *Acids Res.* **32**, 1792-1797 (2004).