Genome-scale fitness profile of *Caulobacter crescentus* grown in natural freshwater

Kristy L. Hentchel[1], Leila M. Reyes Ruiz[1], Patrick D. Curtis[2], Aretha Fiebig[1*], Maureen L. Coleman[3*], Sean Crosson[1*]

[1]Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60637, USA.

[2]Department of Biology, University of Mississippi, University, MS 38677, USA.

[3]Department of the Geophysical Sciences, University of Chicago, Chicago, IL 60637, USA.

[*]To whom correspondence should be addressed: E-mail: aretha@uchicago.edu; mlcoleman@uchicago.edu; scrosson@uchicago.edu; 929 E. 57th St. Chicago, IL 60637, USA. Phone: (+1) 773-834-1926

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ABSTRACT

Bacterial genomes evolve in complex ecosystems and are best understood in this natural context, but replicating such conditions in the lab is challenging. We used transposon sequencing to define the fitness consequences of gene disruption in the bacterium *Caulobacter crescentus* grown in natural freshwater, compared to axenic growth in common laboratory media. Gene disruptions in amino acid and nucleotide biosynthesis pathways and in metabolic substrate transport machinery impaired fitness in both lake water and defined minimal medium relative to complex peptone broth. Fitness in lake water was enhanced by insertions in genes required for flagellum biosynthesis and reduced by insertions in genes involved in biosynthesis of the holdfast surface adhesin. We further uncovered numerous hypothetical and uncharacterized genes for which disruption impaired fitness in lake water, defined minimal medium, or both. At the genome scale, the fitness profile of mutants cultivated in lake water was more similar to that in complex peptone broth than in defined minimal medium. Microfiltration of lake water did not significantly affect the terminal cell density or the fitness profile of the transposon mutant pool, suggesting that *Caulobacter* does not strongly interact with other microbes in this ecosystem on the measured timescale. Fitness of select mutants with defects in cell surface biosynthesis and environmental sensing were significantly more variable in lake water than in defined medium, presumably owing to day-to-day heterogeneity in the lake environment. This study reveals genetic interactions between *Caulobacter* and a natural freshwater environment, and provides a new avenue to study gene function in complex ecosystems.

42 **INTRODUCTION**

43 Environments inhabited by microbial cells have significant microscale heterogeneity. This is well
44 recognized in biofilms, soils, and host-associated habitats (1-3). Free-living aquatic bacteria often
45 encounter chemical gradients that can appear as ephemeral patches, arising from algal exudates, sinking
46 particles, or lysis events (4, 5) and they may have to cope with prolonged periods of nutrient scarcity. In
47 addition, these bacteria face interspecies interactions, protistan predators, and viruses, as well as
48 fluctuations in physical conditions such as temperature and light. These biotic and abiotic factors have
49 driven myriad adaptations that enable survival and reproduction in natural environments.

50 In contrast with this natural complexity, studies on microbial physiology and gene function have
51 traditionally relied on simplified experimental conditions. Thus it is not surprising that a large fraction
52 of bacterial genes remain uncharacterized. Recently developed transposon sequencing (Tn-Seq)
53 approaches (6) now enable rapid phenotypic assessment of thousands to millions of distinct mutant
54 strains, and these methods have been used to interrogate gene function in a variety of in vitro and host-
55 associated conditions (7, 8). More recently, transposon mutagenesis approaches in which each
56 transposon carries a unique 20-bp barcode sequence have been developed (9); each insertion site is
57 associated with a short barcode, and the abundance of all mutant strains in the pool can be assessed by
58 simple amplicon sequencing.

59 Here, we used a barcoded Tn-Seq approach to identify genes affecting fitness in *Caulobacter*
60 *crescentus* strain CB15, cultivated in natural freshwater from Lake Michigan, Illinois, USA. As a well-
61 characterized and genetically tractable bacterium originally isolated from a pond in California in 1960
62 (10), this strain is well suited for this study. Briefly, *C. crescentus* is among a group of dimorphic
63 prosthecate (i.e. stalked) bacteria that attach to surfaces, often forming epibiotic interactions with algae
64 and plant material (11). More broadly, members of the genus *Caulobacter* are common in soil
65 ecosystems, where they likely play an important role in plant matter decomposition (12). In aquatic

66 systems, *Caulobacter* interactions with substrates contribute to biopolymer mineralization, and have
67 been proposed to enhance productivity of aquatic ecosystems (11, 13). However, *C. crescentus* (hereafter
68 referred to simply as *Caulobacter*) is typically grown in an artificial medium consisting of dilute peptone
69 and yeast extract (PYE) or in a defined medium consisting of mineral salts and a single carbon source
70 such as xylose (M2X) (14), neither of which adequately represents natural freshwater. PYE is replete
71 with peptides, amino acids, and a range of carbon sources, while M2X requires *Caulobacter* to synthesize
72 all cellular building blocks from salts and a simple sugar. Natural freshwaters, by contrast, contain an
73 undefined, complex mixture of organic and inorganic nutrient sources (15). In many freshwater systems,
74 essential nutrients including phosphorus and labile carbon do not accumulate to high concentrations (16,
75 17). We predicted that genes that are dispensable in PYE (18) or M2X medium would be important for
76 fitness in complex natural freshwater, and that these genes would offer insights into *Caulobacter*
77 physiology in a *bona fide* freshwater system.
78

79 **MATERIALS AND METHODS**

80 *Bacterial strains and primers*. Strains and primers used in this study are listed in **Table S1**. All primers
81 were synthesized by Integrated DNA Technologies (Coralville, IA, USA).
82

83 *Growth media*. *Caulobacter crescentus* strain CB15 (10) was grown in PYE medium [0.2% peptone
84 (Fisher Scientific), 0.1% yeast extract (Fisher Scientific), 0.5 mM $MgSO_4$, 0.5 mM $CaCl_2$] or M2X
85 minimal defined medium [6.1 mM Na_2HPO_4, 3.9 mM KH_2PO_4, 9.3 mM NH_4Cl, 0.5 mM $MgSO_4$, 10 μM
86 $FeSO_4$ (EDTA chelate; Sigma Chemical Co.), 0.25 mM $CaCl_2$] supplemented with 0.15% xylose (14).
87 *Escherichia coli* strains were grown in LB broth (1% peptone, 0.5% yeast extract, 0.5% NaCl). Solid
88 growth media included 1.5% agar.
89

90 *Lake water collection*. Water from Lake Michigan was collected at Promontory Point, Chicago,
91 Illinois, USA (Latitude: 41.794, Longitude: -87.579), on four dates in 2016 (Nov 30, Dec 6, Dec 9, and
92 Dec 12). We measured water temperature, phosphate and nitrate/nitrite level (Aquacheck Water
93 Quality Test Strips; Hach), and pH (pH indicator strips, Fisher Scientific) at the time of collection
94 (**Table 1)**. Lake water was filtered using Nalgene™ Rapid-Flow™ Sterile Disposable 0.1 µm Filter
95 Units with PES Membrane (Thermo Scientific).
96
97 *Measurement of* Caulobacter *growth in lake water*. Colonies of *Caulobacter* were inoculated into 2 mL
98 of PYE in glass culture tubes (13 × 100 mm) and grown overnight at 30°C with shaking at 200 rpm, for
99 a total of five biological replicates. At saturation, 1 mL of culture was centrifuged at 8,000 ×*g* and washed
100 twice in 1 mL of filtered lake water (see **Figure 2** for information on lake water). The washed pellet was
101 resuspended in filtered lake water to a final OD_{660} of 0.1, and 0.5 µL (approximately 0.5–1 × 10^5 cells)
102 was inoculated into 5 mL of filtered lake water in a glass culture tube (20 mm × 150 mm) in technical
103 duplicate. Cultures were grown at 30°C with shaking at 200 rpm. To monitor growth, 20 µL of culture
104 was removed at various time points, serially diluted, and titered onto PYE agar plates, which were
105 incubated at 30°C for 2 days. Growth was monitored by enumeration of colony forming units (CFUs).
106
107 *Construction of barcoded Tn-Himar mutant library*. The recipient strain (*Caulobacter*) was grown
108 overnight in 2 mL of PYE at 30°C with shaking at 200 rpm. This starter culture was used to inoculate
109 20 mL of PYE and grown at 30°C overnight with shaking at 200 rpm until saturated. The donor *E. coli*
110 strain (APA752, gift from Adam Deutschbauer, University of California-Berkeley, USA), carrying the
111 pKMW3 (kanamycin resistant) Himar transposon vector library (9), was inoculated into 20 mL of LB
112 containing kanamycin (30 µg mL^{-1}) and diaminopimelate (DAP; 300 µM) and grown overnight at 37°C
113 with shaking at 200 rpm; the *E. coli* Himar donor strain is a DAP auxotroph, and thus requires addition
114 to the medium. To conjugate the barcoded transposon pool into *Caulobacter*, the recipient strain and

115 donor strains were each centrifuged at 8,000 ×*g* for 2 min and resuspended in a total volume of 500 µL
116 of PYE medium. The cultures were combined at a 10:1 ratio of recipient to donor and mixed by gentle
117 pipetting. The mixed culture was centrifuged again at 8,000 ×*g*, and the supernatant decanted. The cells
118 were resuspended in 30 µL of PYE, spotted onto a PYE agar plate containing diaminopimelate (300
119 µM), and incubated overnight at 30°C. After growth, the mating spot was scraped from the plate and
120 resuspended in 6.5 mL of PYE. This suspension was spread evenly (500 µL per plate) over 14 large (150
121 × 15 mm) PYE agar plates containing 25 µg mL^{-1} kanamycin and incubated for approximately 3 days at
122 30°C. Cells were harvested from all the plates and inoculated into 400 mL of PYE containing 5 µg mL^{-}
123 ^{1} kanamycin. This cell mixture was grown at 30°C with shaking at 200 rpm for three doublings. Cells
124 were centrifuged at 8,000 ×*g*, resuspended in 70 mL of PYE containing 15% glycerol, and stored as 1
125 mL aliquots at -80°C.
126
127 *Mapping of the sites of Tn-Himar insertion in the* Caulobacter *BarSeq library* (see Fig. S1 for graphical
128 overview). Genomic DNA was extracted using guanidium thiocyanate as previously described (19). The
129 DNA was sheared (~300 bp fragments), cleaned with a standard bead protocol, end-repaired and A-
130 tailed, and a custom double-stranded Y adapter was ligated. The custom adapter was prepared by
131 annealing Mod2_TS_Univ and Mod2_TruSeq (**Table S1**) as described (9). The sheared fragments
132 containing transposons were enriched by PCR using the primers Nspacer_BarSeq_pHIMAR and
133 P7_MOD_TS_index1 using GoTaq® Green Master Mix according to the manufacturer's protocol in a
134 100-µL volume with the following cycling conditions: 94°C for 2 min, 25 cycles at 94°C for 30 s, 65°C
135 for 20 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. After a second bead cleanup,
136 the *Caulobacter* library was sequenced using a standard Illumina sequencing primer on an Illumina
137 HiSeq2500 at the University of Chicago Genomics Facility with a 150-bp single-end read. The locations
138 of Himar transposon insertions were aligned and mapped using BLAT (20), and unique barcode
139 sequences were associated with their corresponding genome insertion location using a custom Perl script

140 (MapTnSeq.pl). Sets of barcodes that consistently map to one location in the genome were identified
141 using a custom Perl script (DesignRandomPool.pl). This ensures that each unique barcode is properly
142 assigned to a single insertion site. These scripts have been described by Wetmore and colleagues (9) and
143 are available at https://bitbucket.org/berkeleylab/feba. For all analyses, reads were mapped to the *C.*
144 *crescentus* NA1000 genome (accession CP001340) (21), which is more comprehensively annotated (22)
145 than the highly-related CB15 parent strain.
146
147 *Cultivation of the Tn-Himar library*. An aliquot of the *Caulobacter* library (2 mL) from a glycerol stock
148 was inoculated into 18 mL of PYE, split into two tubes (20 × 150 mm) with 10 mL each, and grown in
149 a cell culture roller drum (Fisher Scientific) at 30°C for 4 h. The tubes were then moved to a 30°C
150 incubator with shaking at 200 rpm for an additional 2 h. Cultures were combined and centrifuged for 20
151 min at 3,000 ×*g* at 4°C. The cell pellet was resuspended and washed in 10 mL of filtered lake water, and
152 centrifuged again at 3,000 ×*g* for 20 min at 4°C. The resulting pellet was resuspended in 5 mL of filtered
153 lake water, and the OD_{660} measured. Flasks containing filtered or unfiltered lake water (7.5 L total
154 volume per condition, divided over 3 flasks) were inoculated with the washed library with the aim of an
155 initial starting concentration of approximately 2.5×10^7 total cells per flask (**Fig. S2**). Flasks were
156 incubated at 30°C with shaking at 150 rpm. At 0 and 64 h, an aliquot of culture was removed from each
157 flask for CFU enumeration on PYE agar plates (**Fig. S3**). After ~64 h of growth, cells from all three
158 flasks were collected by filtration using an Express Plus Membrane 0.22 µm filter (Millipore). Filters
159 were stored at -80°C until needed. To mimic saturating conditions with the same number of doublings
160 in defined M2X and complex PYE laboratory medium as in lake water, we inoculated cultures at a
161 concentration that after five doublings (the estimated number of doublings in lake water), the cultures
162 reached saturation. Cells were pelleted at 10,000 ×g for 1 min and stored at -20°C. Genomic DNA from
163 all samples was extracted using guanidium thiocyanate as previously described (19), with the exception

164 that the lake water samples were lysed directly from the filters they were collected on. DNA quality and
165 quantity was measured using a NanoDrop^{OneC} (Thermo Scientific).
166
167 *Amplification and sequencing of Tn-Himar barcodes*. PCR amplification for each sample was performed
168 as previously described (9) (**Fig. S1**) using a standard reaction protocol for Q5 DNA polymerase (New
169 England BioLabs) with the primers BarSeq_P1 and 1 of 16 forward primers (BarSeq_P2_IT001 to
170 BarSeq_P2_IT016; **Table S1**) containing unique 6-bp TruSeq indexes that were sequenced using a
171 separate index primer. Cycling conditions were as follows: 98°C for 4 min followed by 25 cycles of 30s
172 at 98°C, 30s at 55°C, and 30s at 72°C, followed by a final extension at 72°C for 5 min. PCR products
173 were purified using GeneJET PCR Purification Kit (Thermo Scientific). Purified samples were run on a
174 2.5% agarose gel to confirm correct product size (~200 bp). A total of 10 µL per purified PCR product
175 was pooled, assessed for quality, and quantified using a Bioanalyzer. The amplified barcodes from the
176 reference (PYE) and treatment (M2X, unfiltered lake water, and filtered lake water) were sequenced on
177 an Illumina HiSeq4000 at the University of Chicago Genomics Facility, multiplexing all 16 samples in
178 one lane with 50-bp single-end reads. All sequence data have been deposited in the NCBI Sequence
179 Read Archive under BioProject accession PRJNA429486; BioSample accession SAMN08348121; SRA
180 accession SRP128742.
181
182 *Analysis of Tn-Himar strain fitness*. We followed the fitness calculation protocol of Wetmore and
183 colleagues (9), using scripts available at https://bitbucket.org/berkeleylab/feba. Briefly, the total count
184 of each barcode in each sample was calculated using a Perl script (MultiCodes.pl) and, from this table
185 of barcodes, strain fitness was calculated using an R script (FEBA.R). The fitness of each strain was
186 calculated as a normalized $\log_2$ ratio of barcode counts in the treatment sample to counts in the PYE
187 reference sample. The fitness of genes was calculated as the weighted average of strain fitness values,
188 the weight being inversely proportional to a variance metric based on the total number of reads for each

189 strain; this weighting is fully described by Wetmore and colleagues (9). Successful gene fitness
190 calculations required at least 3 reads per strain and 30 reads for each of the 16 samples. Insertions in the
191 first 10% or last 10% of a gene were not considered in gene fitness calculations. The complete data set
192 of fitness values for each condition is listed in **Table S2**.

193 To assess the distribution of fitness scores, we calculated the standard deviation for each
194 condition using the frequency distribution of the mean fitness value of each gene (filtered lake water =
195 0.41, unfiltered lake water = 0.40, defined medium = 1.1). When the outlier region of the defined medium
196 dataset (< -2.5) was removed, the calculated standard deviation was 0.36; therefore, a standard deviation
197 of 0.4 was chosen and applied to all conditions. Genes with a mean fitness value approximated at ± 3σ
198 from the mean (less than -1.2 and greater than +1.2) were selected for further examination. We also
199 examined t-values, the fitness value of a gene divided by a variance metric, based on the total number of
200 reads for each gene (as previously described (9)), to provide a metric to assess the significance of fitness
201 values (**Table S3**).

202 To identify genes showing differential fitness across lake water samples, we fit a linear model
203 with two factors, sampling day and filtration treatment (filtered or unfiltered). The model was
204 implemented using the functions *lmfit, eBayes, and topTable* in the R package *limma* (23). Genes were
205 identified as having differential fitness across either sampling days or filtration treatment, with a false
206 discovery rate threshold of 0.05.

207

208 *Analysis of* Caulobacter *Tn5-seq fitness.* A *Caulobacter* Tn5 insertion library containing an estimated 3
209 $\times 10^5$ clones was constructed as previously described (24). The lake water fitness experiment for the Tn5
210 library (from Lake Michigan water collected in April 2016) was performed similarly to the Tn-Himar
211 library experiments with the following modifications: A total of 200 µL of the *Caulobacter* Tn5 library
212 was inoculated into 20 mL of PYE for the initial outgrowth for 5 h, which was then inoculated into 2 L
213 for the PYE and unfiltered lake water treatments, and 2 replicates of 2 L each for filtered lake water.

214 Lake water cultures were harvested by filtration after 60 h of growth, and the PYE condition was filtered
215 after 12 h to approximate the same number of doublings. However, our PYE cultures achieved over 6
216 doublings, versus 4 doublings for lake water.

217 A nested PCR approach was used to specifically amplify transposon-containing DNA fragments
218 for sequencing. A low cycle PCR amplification for each sample was first performed using a standard
219 reaction protocol for KOD Xtreme™ Hot Start Polymerase with 5% DMSO and 0.3 μM primer using
220 the primers F1 and P7 (24) (**Table S1**). Cycling conditions were as follows: 95°C for 90 sec; 5 cycles of
221 95°C for 15 sec, 68°C for 30 sec, and 72°C for 30 sec; 13 cycles of 95°C 15 sec, 55°C 30 sec and 72°C
222 30 sec, followed by a final extension at 72°C for 5 min. Samples were treated with ExoSAP-IT™ PCR
223 product cleanup reagent (Thermo Fisher Scientific) according to manufacturer's protocol. A second PCR
224 step was performed with the transposon specific primer containing the adapter sequence using KOD
225 Xtreme™ Hot Start Polymerase with 5% DMSO and 0.3 μM primer in a 62.5-μL reaction volume using
226 the primers Tn5-left and P7 (24) (**Table S1**). Cycling conditions were as follows: 95°C for 3 min, 12
227 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, followed by a final extension at 72°C
228 for 5 min. Product size (~200 bp) was confirmed on a 1% agarose gel. After standard bead cleanup and
229 Illumina library preparation, samples were sequenced at the University of Chicago Genomics Facility
230 using a custom sequencing primer (24) (**Table S1**).

231 Fitness analysis was performed as previously described (25) using the TRANSIT software
232 (available at https://github.com/mad-lab/transit). We used the permutation test in TRANSIT to quantify
233 differences in sequencing read counts between our PYE and lake water conditions (25). The complete
234 Tn5 dataset (**Tables S4 & S5**), genes with differential fitness ($p<0.01$; **Tables S5 & S6**), and genes
235 shared between the Tn5 and Tn-Himar datasets (**Table S7**) are listed in Supplemental Information. Raw
236 Tn-seq data are deposited in the NCBI sequence read archive under BioProject accession PRJNA429486;
237 BioSample accession SAMN08348191; SRA accession SRP128742.

238

239 **RESULTS**

240 *Growth of* Caulobacter *in natural freshwater*

241 As a prerequisite to measuring strain fitness, we first sought to demonstrate *Caulobacter* growth in
242 natural freshwater. We collected nearshore water from Lake Michigan, representing a typical
243 oligotrophic freshwater system inhabited by *Caulobacter* spp. (26, 27). With no additional
244 supplementation, filtered (0.1 μm) lake water supported *Caulobacter* growth to a maximal density of
245 approximately 5×10^5 CFU/mL (**Fig. 1A**), from an initial inoculum of 2.5×10^4 CFU/mL. *Caulobacter*
246 doubled 4–5 times at a rate of 0.14 hr^{-1} (doubling time 5 hr). Similar growth rates were observed in
247 unfiltered lake water. Supplementation with 0.1% xylose increased the maximal density by about 10-
248 fold, while addition of 23 μM K_2HPO_4 had no effect (**Fig. 1A**), implying that carbon, but not phosphorus,
249 limits *Caulobacter* growth in Lake Michigan water. For comparison, we also assayed *Caulobacter*
250 growth in water collected from Lake Superior and found a similar growth yield (**Fig. 1B**).
251 Supplementation with either 23 μM K_2HPO_4 or 0.1% xylose did not significantly enhance *Caulobacter*
252 growth, but together 0.1% xylose and 23 μM K_2HPO_4 enhanced growth by more than 10-fold, suggesting
253 that both carbon and phosphorus limit growth in Lake Superior. By comparison, *Caulobacter* reached a
254 density of 3×10^9 CFU/mL in PYE broth or in defined M2X medium (**Fig. 1C**). Notably, cell density
255 was stable for one week in lake water but declined by 2-3 orders of magnitude after 2 days of cultivation
256 in artificial media (**Fig. 1**). This finding is consistent with a report by Poindexter describing *Caulobacter*
257 isolates that tolerated prolonged nutrient scarcity with little loss of viability (11). Based on our results,
258 we chose to perform our genetic analysis in unsupplemented water from Lake Michigan.

259

260 *A global Tn-sequencing approach identifies* Caulobacter *mutants with altered fitness in lake water*

261 We sought to identify genes required for *Caulobacter* growth in natural freshwater, compared to defined
262 M2X or complex PYE medium. To this end, we constructed mutant libraries (**Table 2**) using two

263 different transposons: Tn5, which inserts randomly, and Tn-Himar, which inserts specifically at TA
264 dinucleotides, which occur on average every 82 bp in the *Caulobacter* genome. Each transposon in the
265 pool of Himar transposons contains a unique 20-bp barcode sequence, which is mapped once to a specific
266 insertion site in the genome and thereafter can be quantified by simple amplicon sequencing (9), see **Fig.**
267 **S1**. Both transposon libraries were constructed by growing cells in PYE; hence insertions in genes
268 essential for growth in PYE are not represented in either library.

269 We cultivated the *Caulobacter* Tn5 pool in PYE and filtered lake water (0.1 µm). Although Tn5
270 is capable of insertion at almost every position in the genome, our Tn5 library had lower site saturation
271 than our Tn-Himar library, which limited the statistical power to identify significant fitness effects (25,
272 28). We calculated mutant fitness and gene essentiality for all genes (**Tables S5 & S6)** and identified 55
273 genes for which Tn5 disruption significantly diminished or enhanced growth in lake water relative to
274 PYE (adjusted p-value cutoff < 0.01). Given the limited power of the Tn5 dataset, we focused our
275 analyses on the Tn-Himar dataset, but include the Tn5 data in the supplemental material as they provide
276 useful validation of the Tn-Himar data discussed hereforward.

277 The *Caulobacter* Tn-Himar library contained an estimated 2×10^6 clones, of which 7×10^4
278 passed the criteria for barcode mapping (9). Considering there are only ~ 5×10^4 TA insertion sites in
279 the *Caulobacter* genome, it is clear that in this population we hit some sites more than once with unique
280 barcodes. We cultivated this library in four conditions: 1) complex PYE medium, 2) defined M2X
281 medium, 3) filtered lake water, and 4) unfiltered lake water (**Fig. S2**). To ensure that we started the
282 experiment with sufficient mutant strain diversity, we inoculated the same total number of cells ($2.5 \times$
283 10^7) in each treatment and aimed for 4–5 doublings into the late exponential phase of growth (**Fig. 1 and**
284 **S2**). For PYE and M2X treatments, cells were grown in 1.5 mL volumes for 10 and 20 h, respectively.
285 For lake water treatments cells were grown in three flasks each containing 2.5 L for 64 hours. By varying
286 culture volume, we ensured an equal number of cell divisions across a similar phase of the growth curve.
287 This approach required cultivation at different cell densities between conditions. After harvest, barcodes

288 were analyzed as described (9), and strain fitness was calculated as the $\log_2$ of the ratio of barcode
289 abundance in lake water (or M2X) to the control condition (PYE) (9). Given the 20–30-fold increase in
290 cell number of the mutant pool, a Tn-Himar insertion strain that did not grow at all should have a fitness
291 score around -4 to -5; more extreme (lower) fitness scores indicate strains that did not survive cultivation.
292 The most extreme negative fitness scores in this dataset (i.e. < -4) likely reflect genes that are essential
293 in a particular condition (**Fig. 2A & 2D; Table S2**). The distributions of fitness scores in defined medium
294 and the two lake water conditions are presented in **Figs. 2B & 2C**.

295 To validate our approach, we examined the fitness consequences of disrupting xylose utilization
296 genes in the M2-xylose (M2X) growth condition. Genes in the *xylXABCD* operon are required for xylose
297 utilization (29, 30). As expected, insertions in these genes generated fitness scores of -3.6 to -6.6 when
298 the pool was cultivated in M2X (**Fig. 3A, Table S2**). Disruption of *xylR*, which functions as a
299 transcriptional repressor of the xylose operon (29), resulted in a positive fitness score in M2X relative to
300 PYE, indicating that derepression of the xylose utilization genes is advantageous when xylose is the sole
301 carbon source. Disruption of the *xylXABCD* genes had little effect on fitness in lake water, which
302 contains a range of carbon sources beyond xylose; disruption of *xylR* resulted in a modest fitness decrease
303 in lake water relative to PYE (**Fig. 3A)**, suggesting a cost to constitutive expression of unused genes.

304

305 *Increased variability of fitness scores in lake water*

306 Compared to defined M2X medium, lake water is more heterogeneous over time and space. Our four
307 lake water experiments used water collected on four days over a 2-week period and showed greater
308 variability in strain fitness scores than our four independent M2X replicates (**Figs. 2A, 3B & 4**). In
309 addition, we fit a linear model to test the effects of two factors, sampling day and filtration condition, on
310 strain fitness scores, and found a number of genes that differed significantly across days, including genes
311 related to cell surface carbohydrate biosynthesis and environmental sensing and gene regulation (**Table**
312 **S8**). This variability likely reflects day-to-day differences in temperature, mixing, and biotic factors such

313 as phage dynamics, though we cannot completely rule out technical day-to-day variations in sample
314 processing. Future work with additional temporal replicates could discriminate genes whose functions
315 are consistently important from genes that are exploited under transient conditions in the lake.

316 Surprisingly, filtration (0.1μm) had little effect on the global fitness profile of *Caulobacter* (**Fig.**
317 **4**), and our linear model approach did not identify any genes with differential fitness between filtered
318 and unfiltered lake water. This result implies that particulates and other microorganisms present in the
319 lake water did not affect strain growth, suggesting that *Caulobacter* is not in strong competition with
320 other microbes for "common goods" in this system on the time scale of our experiment.

321

322 *Fitness defects are more extreme in defined medium than in lake water*

323 Transposon disruption of genes required for amino acid biosynthesis, nucleotide biosynthesis,
324 lipopolysaccharide biosynthesis, and nucleotide sugar biosynthesis resulted in extreme (fitness score < -
325 4) growth defects in M2X (**Fig. 3, Tables S2–S3**); these fitness scores provide evidence that strains
326 harboring disruptions of these genes did not grow at all in M2X and thus likely comprise a strain/gene
327 set that is essential in this defined condition. This result is not surprising, considering that growth in
328 M2X medium requires de novo biosynthesis of diverse monomers and intermediates, many of which are
329 supplied exogenously in the reference PYE condition. In many cases, strains with severe fitness defects
330 in M2X also had reduced growth in lake water, but the fitness costs were less severe (**Tables S2 & S9-**
331 **S10**). We controlled the number of doublings (approximately 4–5) across all conditions, so the more
332 pronounced fitness costs in defined medium compared to lake water cannot be explained by differences
333 in the number of doublings. Instead, these results imply that lake water is more similar to the reference
334 condition PYE than M2X is to PYE, in terms of the metabolic demands it imposes on cells. This
335 inference is supported by principal component analysis across all growth conditions (**Fig. 3B**). Indeed,
336 we expect that natural freshwater supplies diverse metabolites and growth substrates that may render
337 some genes dispensable, whereas defined media provides fewer exogenous resources.

338

339 *Pathways conferring differential fitness in natural freshwater and artificial media*

340 To further explore the selective pressures faced by *Caulobacter* across these conditions, we focused on
341 genes whose disruption induced large fitness effects, namely fitness scores less than -1.2 and greater
342 than +1.2 (this approximates a ±3σ cutoff). Based on this criterion, we identified 83 and 82 genes in the
343 filtered and unfiltered lake water conditions, respectively, and 213 genes in the defined M2X medium
344 (**Table S9**). Genes with significant fitness values across all three conditions based on the t-statistic of
345 Wetmore and colleagues (9) are outlined in **Table S10**. Broad functional patterns in our Tn-Himar
346 dataset were assessed using clusters of orthologous group (COG) annotations (31) (**Fig. 5**). A full
347 comparison of genes for which Tn-Himar disruption results in a specific advantage or disadvantage in
348 M2X defined medium, but not in filtered or unfiltered Lake Michigan water (relative to complex PYE
349 medium), and vice versa, are presented in **Tables S11-S12**. Genes that were not hit by Tn-Himar, and
350 thus not included in any of our analyses are included in **Table S13**. Many of these genes have been
351 previously defined as essential (18). A clustered heatmap that contains genes with fitness scores less
352 than -1.2 and greater than +1.2 from either the filtered or unfiltered lake water conditions is presented in
353 Fig. S6.

354 Not surprisingly, the most negative fitness scores were observed for genes in amino acid and
355 nucleotide biosynthesis (discussed above), and in genes required for transport of metabolic substrates
356 into the cell (**Tables S2 and S9**); we observed similar defects in our Tn5 dataset (**Tables S4-S6**). In
357 addition, disruption of genes encoding catabolic enzymes in the glycine cleavage pathway and in
358 branched amino acid degradation led to an apparent enhancement of fitness in both M2X medium and
359 in lake water relative to PYE, in both the Tn-Himar and Tn5 experiments (**Fig. 4; Tables S2 & S4-S7**).
360 This result likely reflects the higher cost of deleting these catabolic genes in the reference PYE condition
361 compared to M2X or lake water, and is consistent with transcriptional data showing that select amino

362 acid degradation pathways — including glycine cleavage, histidine, branched chain, and phenylalanine
363 degradation — are upregulated in PYE compared with M2X (32).

364 Surprisingly, we found enhanced fitness for strains with disruptions in motility genes in lake
365 water relative to PYE (**Fig. 4 & 5**). We more carefully examined the fitness scores of genes involved in
366 synthesis and assembly of the flagellum (**Fig. 6**). The flagellum is assembled in a regulated hierarchy of
367 stages, which is well described in *Caulobacter* (33-35). Class II genes encode the inner components of
368 the flagellum, including the export apparatus, and regulatory proteins that activate expression of class
369 III and IV genes. Class III genes encode the basal body and hook structures. Completion of class III
370 structures activates translation of class IV genes, which encode the subunits of the flagellar filament.
371 Thus, defects in each class prevent expression of subsequent classes. Within each class of flagellar genes,
372 we observed consistent fitness patterns, demonstrating the power of this method to capture even modest
373 effects of gene disruption. Disruption of class II flagellar genes conferred an advantage that was
374 significantly greater in lake water than in M2X compared to PYE (**Fig. 6B & S2**). Disruption of class III
375 genes followed similar trends, but with smaller magnitude effects. *Caulobacter* encodes six redundant
376 class IV flagellin genes (36), three of which are represented in our Tn-Himar pool and whose disruption,
377 not surprisingly, had no effect on fitness. Disruption of the motor stator gene *motA* or *motB*, which results
378 in a fully assembled but paralyzed flagellum (37, 38), did not affect fitness under our cultivation
379 conditions. Together, these results suggest that the fitness advantage of flagellar gene disruption is not
380 derived from energy saved in powering the flagellum, but rather in energy or resources saved in
381 synthesizing and assembling the flagellum. In the lake water cultivations, we observed appreciable day-
382 to-day variation in the fitness of each class of flagellar gene mutants (**Fig. S2**), which was particularly
383 pronounced for class III genes. Patterns in this day-to-day variability were consistent across members of
384 each class, suggesting that this variability is driven by environmental factors rather than technical factors.

385 Fitness was also affected by the ability to synthesize the polar extracellular adhesin known as the
386 holdfast (**Fig. 6A & S2**). We systematically analyzed genes involved in synthesis, secretion, and

387 attachment of the holdfast. Most holdfast genes yield partial to complete defects in holdfast development
388 when disrupted (39); we categorized these genes as 'unique functions' genes. However, two sets of
389 holdfast biosynthesis genes have redundant functions: two Wzy-family polymerase genes function in
390 holdfast development and three paralogs of the HfsE glycosyltransferase have genetically redundant
391 activities in holdfast synthesis (39). Disruption of genes in these redundant sets had no effect on fitness.
392 Disruption of genes in the unique function group resulted in a modest but consistent fitness advantage
393 in M2X and a fitness disadvantage in both filtered and unfiltered lake water, relative to PYE (**Fig. 6A &**
394 **S2A**). For the group of all unique genes, the fitness consequence for loss of holdfast was significantly
395 different between growth conditions ($p < 0.0001$) (**Fig. 6A**).
396
397 *Genes of unknown function contribute to fitness in natural freshwater*
398 We hypothesized that many genes of unknown function play important roles in natural environmental
399 contexts but not in typical laboratory media. Of all genes showing large fitness effects ($\pm 3\sigma$),
400 hypothetical genes or genes of unknown function accounted for 16% (13/83) in filtered lake water, 15%
401 (12/82) in unfiltered lake water, and 7% in defined medium (15/213) (**Table 3**). Across these three
402 conditions, five hypothetical genes were shared. *CCNA_03860* was the only hypothetical gene for which
403 disruption provided a fitness benefit across all three conditions relative to PYE. CCNA_03860 contains
404 a conserved domain belonging to the YkuD superfamily, which has been shown to have L,D-
405 transpeptidase catalytic activity, providing an alternate pathway for peptidoglycan cross-linking (40, 41).
406 Disruption of *CCNA_01724*, *CCNA_03864*, *CCNA_03909*, and *CCNA_00375* resulted in reduced fitness
407 across all three conditions relative to PYE. Hence using natural growth conditions may be critical for
408 understanding the functions of many uncharacterized bacterial genes.
409
410 **DISCUSSION**

411 *Tn-seq fitness scores provide a window into cell-environment interactions*

412 Bacterial genomes carry relatively little noncoding DNA. Genes that confer no fitness benefit tend to
413 decay over time (42) implying that genes that are maintained are beneficial at least under some
414 circumstances. Yet traditional microbial cultivation approaches often fail to yield discernable mutant
415 phenotypes for many genes. One approach to overcome this challenge is to interrogate gene function in
416 more relevant ecosystem contexts, embracing physicochemical complexity. The genome-scale fitness
417 analysis of *Caulobacter* transposon mutants reported in this study provides new understanding of genes
418 that affect growth in a *bona fide* freshwater environment. Disruption of genes involved in biosynthesis
419 of non-aromatic amino acids, lipopolysaccharides, and nucleotide sugars results in large fitness defects
420 in natural freshwater compared to complex laboratory medium (PYE). Moreover, fitness effects were
421 variable across temporal lake water replicates; this variability likely reflects physicochemical and
422 biological variability in the lake and suggests an important role for transient response genes in fluctuating
423 environments.

424

425 *The fitness costs and benefits of motility and attachment in freshwater*

426 The energetic cost of flagellar biosynthesis and motility is well established (43, 44). Our data
427 indicate that transposon disruption of genes required for the synthesis of the single polar flagellum of
428 *Caulobacter* enhanced fitness in lake water relative to PYE medium (**Fig. 6B**). This is consistent with a
429 *Salmonella* Tn-Seq study that revealed a fitness advantage in strains with disrupted flagellar genes (45).
430 Notably, we found that fitness effects were not uniform across all flagellar genes: disruption of class II
431 genes, which has the greatest impact on flagellar gene expression, also led to greater effects on fitness,
432 compared to class III and IV genes. The fitness enhancement in lake water is not due to the energy
433 savings from motor rotation, as strains with insertions in the *motA* and *motB* stator genes, which assemble
434 a full but non-rotary flagellum (38), showed no fitness difference (**Fig. 6B).** We conclude that the relative
435 fitness advantage of flagellar gene disruption is related to the cost of biosynthesis of flagellar proteins.

436 It seems certain that over longer cultivation timescales, and in more spatially complex environments, the
437 *Caulobacter* flagellum provides a fitness advantage, as flagellar genes are maintained in natural
438 freshwater environments.

439 Our data reveal that disruption of genes required for holdfast biosynthesis is disadvantageous
440 when strains are cultivated in lake water relative to PYE. This fitness cost was evident in both filtered
441 (particle-free) and unfiltered lake water relative to PYE (**Fig. 6A**), suggesting that the effect is not due
442 to adhesion to particles in the medium. Instead, it is possible that the holdfast confers a growth advantage
443 by enabling adherence to the flask surface, where polymeric nutrients concentrate to form conditioning
444 films (46, 47). In defined M2X medium, disruption of holdfast biosynthesis genes confers a fitness
445 advantage (**Fig. 6A**). In this medium, all the components are salts or simple sugars, which do not
446 efficiently condition naïve surfaces (46, 47). In this case, surface attachment is apparently not
447 advantageous, and holdfast biosynthesis comes at a cost.

448

449 *Genetic evidence suggests a complex medium is a better freshwater analog than a defined mineral*
450 *medium*

451 Fitness defects of *Caulobacter* mutants were often more severe in a defined mineral xylose medium
452 (M2X) than in lake water, relative to PYE. Moreover, the overall fitness profile of *Caulobacter* mutants
453 cultivated in lake water more closely resembles that in PYE than in M2X, suggesting that dilute complex
454 medium is a better proxy for natural freshwater. *Caulobacter* belongs to a group of dimorphic prosthecate
455 (i.e. stalked) alphaproteobacteria that are often specialized for oligotrophic, dilute environments (10, 11).
456 Indeed, the inhibition of growth and stalk development due to excess nutrients was the first physiological
457 property of *Caulobacter* spp. to be described (48). Complex and defined media of varying compositions
458 have been outlined for cultivation of *Caulobacter* and related genera, but it is notable that dilute peptone
459 (less than 0.2% w/v) generally supports growth of all dimorphic prosthecate bacteria (11). This
460 observation supports the notion that the natural nutrient environment of this class of bacteria is best

461 captured by cultivation in a dilute complex medium that contains amino acids and other trace complex
462 biomolecular components. Our data also demonstrate that an M2-based medium exerts highly specific
463 metabolic constraints and is likely not an ecologically or physiologically relevant growth condition.
464
465 *An approach to study gene function in ecosystem context*
466 The explosion of bacterial genome sequence information has far outpaced our ability to
467 characterize gene function using traditional approaches, leading to the accumulation of thousands of
468 'unknown' protein families. Many of these families are conserved throughout the bacterial domain,
469 which is evidence that they confer a selective benefit in particular conditions. This leads to the following
470 question: under what circumstances do these conserved families provide a fitness advantage? At the
471 onset of this study, we hypothesized that many of these unknown protein families would prove to be
472 important in the natural ecological context of a bacterium. Among the genes whose disruption leads to
473 the greatest fitness effects (± 3σ) in filtered lake water relative to PYE, approximately 15% are
474 hypothetical or conserved genes of unknown function (**Tables 3, S8–S9**). The approach we describe here
475 indicates that these genes of unknown function play an important role in *Caulobacter* physiology in a
476 natural freshwater environment. Going forward, one can take advantage of lake-specific growth
477 phenotypes to begin to define the functions of these genes in an ecologically relevant context.
478

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Competing Interests

The authors declare no competing interests in relation to this work.

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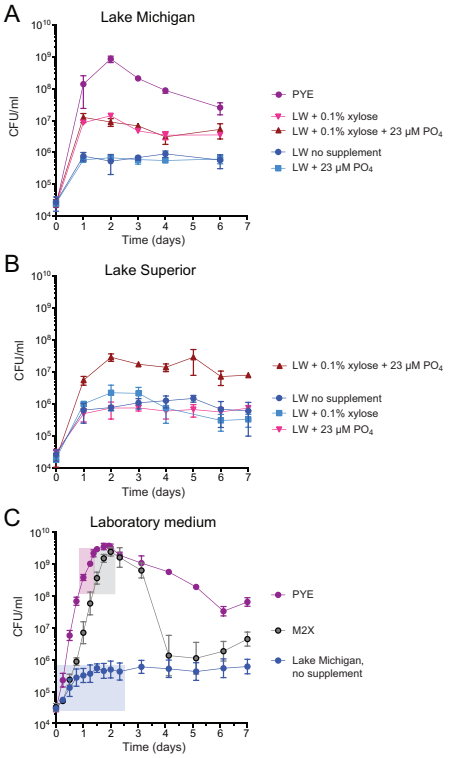

Figure 1. Growth of *Caulobacter* in laboratory medium, and supplemented or unsupplemented water from two Great Lakes. Overnight cultures washed with filtered lake water (LW) were inoculated into 5 mL of filtered water from Lake Michigan (A) or Lake Superior (B). Growth assays in water supplemented with carbon (0.1% w/v xylose) and/or phosphorus (23 μM K_2HPO_4) as indicated; growth was monitored every 24 hours by enumeration of colony forming units (CFUs) by dilution plating. Lake water growth is compared to growth in a laboratory peptone yeast extract (PYE) medium. Data represent mean ± standard deviation of 5 replicates per condition. (C) Fine scale growth of *Caulobacter* in PYE, M2-xylose defined medium (M2X), and filtered Lake Michigan water. Cells were grown as in A and B and monitored by enumerating CFUs. Data represent mean ± standard deviation of 5 replicates per condition. Boxes represent the approximate region of the growth curve (cell density and incubation time) in which the barcoded Tn-Himar mutant library was cultivated.

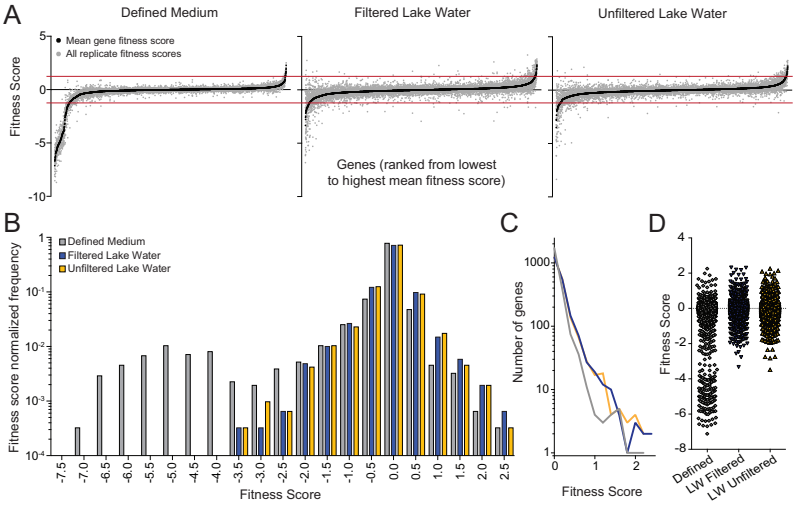

Figure 2. *Caulobacter* gene fitness score summary after cultivation in defined medium, filtered, or unfiltered Lake Michigan water. (A) Rank ordered mean fitness scores of each scorable *Caulobacter* gene across each of the four replicate experiments for each growth condition is plotted; black = mean fitness score; gray = independent replicate fitness scores. Red lines represent ± 3σ from the mean score of the entire dataset (which is approximately zero; genes with fitness scores less than -2.5 in M2X were excluded from this determination). (B) Distributions of mean gene fitness scores for each condition: defined M2X medium (gray), filtered Lake Michigan water (blue), and unfiltered Lake Michigan water (yellow). (C) Distribution of mean gene fitness values between 0 and +2.5 plotted for each condition; defined M2X medium (gray), filtered Lake Michigan water (blue), and unfiltered Lake Michigan water (yellow). (D) Genes fitness score distribution scores plotted for each of the three cultivation conditions: defined M2X medium (gray), filtered Lake Michigan water (blue), and unfiltered Lake Michigan water (yellow).

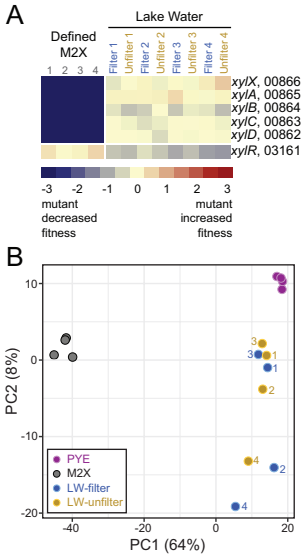

Figure 3. Functional validation of the barcoded Tn-Seq approach; PCA profile of Tn strain fitness in complex medium, minimal defined medium and Lake Michigan water. (A) Heatmap of gene fitness scores for six xylose utilization genes (*xylX*, *xylA*, *xylB*, *xylC*, *xylD*, *xylR*) from each replicate experiment of cultivation of the Tn-Himar mutant library in M2X defined medium, filtered lake water, and unfiltered lake water. Fitness score color scale bar is shown below the panel. (B) Principal component (PC) analysis (PCA carried out in ClustVis (49)) of genomic-scale fitness values for the barcoded *Caulobacter* Tn-Himar mutant library cultivated in complex peptone yeast extract (PYE) medium (reference set), defined M2X medium, filtered Lake Michigan water, and unfiltered Lake Michigan water. The plot shows PCA values for individual samples from each cultivation condition. Percent of variance contributed by the first two PCs is noted on the axes. PCA plot is based on all fitness score values for all genes in the Tn-Himar datasets (see Table S2).

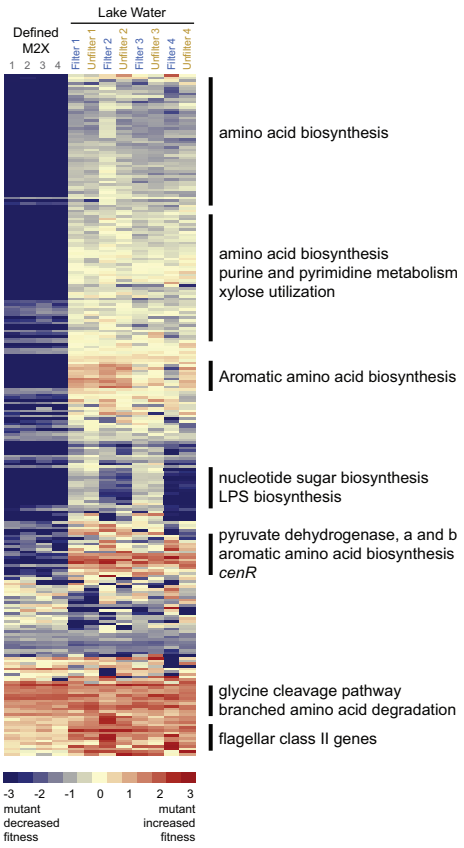

Figure 4. Functional summary of mutant strains with diminished or enhanced fitness in minimal defined medium and Lake Michigan water. Heatmap of fitness scores for genes with mean fitness scores higher than 1.2 or lower than -1.2 (approximates ± 3σ fitness score cutoff) in at least one cultivation condition. The sigma cutoff was based on the major fitness score distribution centered on zero (see Figure 2B). Genes are hierarchically clustered using Cluster 3.0 (average linkage) and visualized using TreeView, and fitness scores for each replicate experiment are color coded on scale bar shown below. General functions of genes within particular regions of this 256 gene cluster are noted; this entire figure is expanded and split into three fully annotated clusters in Fig. S5, with gene names included. The full cluster of genes with mean fitness scores higher than +1.2 or lower than -1.2 in the lake water conditions (excluding the M2X data) is presented in Fig. S6.

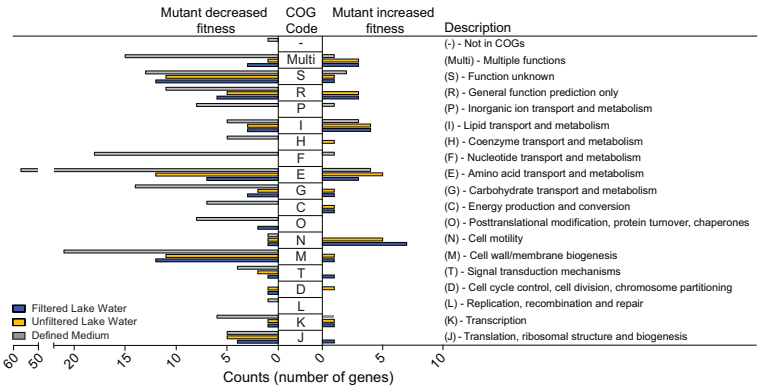

Figure 5. COG analysis of Tn-Himar gene fitness data. The analysis includes genes with fitness scores of absolute value greater than 1.2 (which approximates a ± 3σ fitness score cutoff) in each condition. Each gene was assigned a cluster of orthologous group (COG) functional category, obtained through the NCBI COG site (31). The number of genes in each COG category is plotted; genes with negative fitness values (left) and genes with positive fitness values (right).

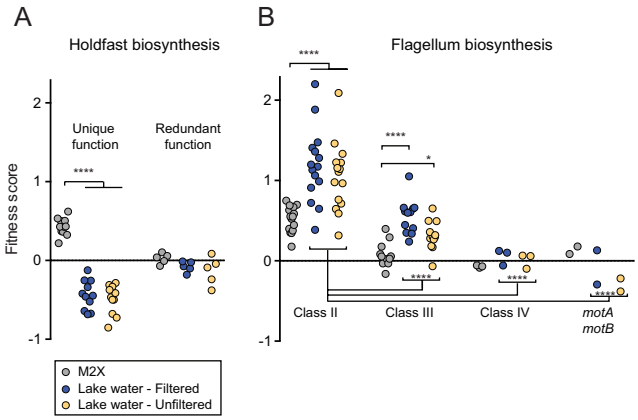

Figure 6. Genes with functions in flagellum and holdfast biosynthesis influence fitness in Lake Michigan water. Mean fitness scores of genes involved in (A) holdfast biosynthesis (17 genes) and (B) flagellum biosynthesis (38 genes) are plotted for each cultivation condition (four independent growth replicates): defined M2X medium (gray); filtered lake water (blue); and unfiltered lake water (yellow). Holdfast genes with unique function (i.e. single gene deletions have a holdfast defect) and redundant function (i.e. single mutants have no holdfast defect) are shown separately. Class II, Class III, and Class IV genes in the flagellar hierarchy and *motA*/*motB* stator complex genes are also shown separately. Clusters of holdfast and flagellum fitness score data, with individually annotated genes, are shown in Fig. S4. One-way ANOVA was applied to assess differences in fitness scores between marked groups; Tukey's post test (**** $p < 0.0001$; * $p < 0.05$).

679 # TABLES

680 **Table 1:** Analysis of Lake Michigan water used for barcoded Tn-Himar fitness experiments

Water collection[a]	Nov. 30	Dec. 6	Dec. 9	Dec. 12
Date of experiment	Dec. 2	Dec. 6	Dec. 9	Dec. 12
Water temperature (°C)	7.5	7	3	2
Air temperature (°C)	7.8	4.4	-3.3	-7.2
pH	5.8	5.8	5.8	5.8
Phosphate	5 ppm	5 ppm	5 ppm	5 ppm
Nitrate/Nitrite[b]	undetectable	undetectable	undetectable	undetectable

[a]Water collection occurred in 2016 at Promontory Point, Hyde Park, Chicago, Illinois, USA.
[b]Limit of detection: Nitrite 0.15 mg/L, Nitrate 1 mg/L

681

682 **Table 2:** Transposon library statistics

Library	Unique insertion sites	Total TA sites	Percent sites hit	Average transposons per ORF	Mean reads
Tn5[a]	115,788	N/A	2.9	30	90–150 (per Tn)
Tn-Himar[b] (BarSeq)	46,395	49,437	93.8	24	7612 (per gene)

683
684 [a]Tn5 can insert into any dinucleotide site. We identified 115,788 insertion sites in an initial
685 population of ~300,000 clones.
686 [b]TnHimar specifically inserts into TA dinucleotides.
687

688 **Table 3:** Average fitness scores for hypothetical genes and genes of unknown function with fitness
689 scores less than -1.2 and greater than +1.2 (bold and shaded) in at least one condition.
690

Locus number		Defined	Filtered LW	Unfiltered LW
CCNA_00375	No conserved domains	**-1.21**	**-2.57**	**-2.83**
CCNA_01724	COG4649, TPR_21 pfam09976	**-2.58**	**-1.25**	**-1.28**
CCNA_03860	COG3786, YkuD superfamily	**1.33**	**1.53**	**1.41**
CCNA_03864	DUF3576, pfam12100	**-1.71**	**-1.32**	**-1.57**
CCNA_03909	No conserved domains	**-1.20**	**-1.76**	**-1.84**
CCNA_00927	No conserved domains	**-2.56**	**-2.11**	-0.67
CCNA_02875	No conserved domains	**-1.54**	**-2.19**	-0.39
CCNA_00895	No conserved domains	-0.19	**-2.14**	**-1.44**
CCNA_00913	No conserved domains	-0.28	**-2.48**	**-1.21**
CCNA_00519	No conserved domains	**-1.66**	-0.37	-0.61
CCNA_01176	DUF2849, pfam11011	**-4.98**	-0.81	-0.84
CCNA_01178	DUF934, pfam06073, COG3749	**-4.36**	-0.60	-0.64
CCNA_01676	TamB, COG2911, pfam04357	**-6.40**	-0.52	-0.74
CCNA_01219	No conserved domains	**1.68**	-0.70	0.78
CCNA_02669	Uncharacterized membrane protein, DUF3422, pfam11902, COG4949	**-4.41**	0.38	0.20
CCNA_03273	COG4944, DUF1109, pfam06532 NrsF, anti-sigF	**-1.63**	-0.39	-0.27
CCNA_03692	COG1975, XdhC/CoxF family	**-4.06**	-0.98	-0.65
CCNA_02796	No conserved domains	-0.45	**-1.20**	-0.52
CCNA_03420	No conserved domains	0.40	**-1.46**	0.42
CCNA_03883	No conserved domains	0.05	**-1.25**	0.99
CCNA_03984	No conserved domains	0.01	**-1.22**	-0.25
CCNA_01286	No conserved domains	0.25	-0.25	**-1.27**
CCNA_02053	No conserved domains	-0.56	-0.74	**-1.34**
CCNA_02160	No conserved domains	0.26	-1.02	**-1.38**
CCNA_03015	No conserved domains	-0.15	0.57	**-1.46**
CCNA_03945	No conserved domains	-0.43	0.24	**-1.90**

691
692 LW, Lake Michigan water.
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