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Transcriptional Mechanisms of Thermal Acclimation in Prochlorococcus

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1 Transcriptional mechanisms of thermal acclimation in *Prochlorococcus*

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34 **Abstract**

35 Low temperature limits the growth and the distribution of the key oceanic primary
36 producer *Prochlorococcus*, which does not proliferate above ca. 40 degrees latitude. Yet,
37 the molecular basis of thermal acclimation in this cyanobacterium remains unexplored.
38 We analyzed the transcriptional response of the strain *Prochlorococcus marinus*
39 MIT9301 in long-term acclimations and in natural *Prochlorococcus* populations along a
40 temperature range enabling its growth (17 to 30°C). MIT9301 upregulated mechanisms
41 of the global stress response at the temperature minimum (17°C) but maintained the
42 expression levels of genes involved in essential metabolic pathways (e.g., ATP synthesis,
43 carbon fixation) along the whole thermal niche. Notably, their declining growth from the
44 optimum to the minimum temperature was coincident with a transcriptional suppression
45 of their photosynthetic apparatus and a dampening of its circadian expression patterns,
46 indicating a loss in their regulatory capacity under cold conditions. Under warm
47 conditions, the cellular transcript inventory of MIT9301 was strongly streamlined, which
48 may also induce regulatory imbalances due to stochasticity in gene expression. The day-
49 time transcriptional suppression of photosynthetic genes at low temperature was also
50 observed in MIT9301-like populations across the global ocean, implying that this
51 molecular mechanism may be associated with the restricted distribution of
52 *Prochlorococcus* to temperate zones.

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58 **Importance**

59 *Prochlorococcus* is a major marine primary producer with a global impact on atmospheric
60 CO₂ fixation. This cyanobacterium is widely distributed across the temperate ocean, but
61 virtually absent at latitudes above 40° for yet unknown reasons. Temperature has been
62 suggested as a major limiting factor, but the exact mechanisms behind *Prochlorococcus*
63 thermal growth restriction remain unexplored. This study brings us closer to
64 understanding how *Prochlorococcus* functions under temperature challenging conditions,
65 by focusing on its transcriptional response after long-term acclimation from its optimum
66 to its thermal thresholds. Our results indicate a detrimental role of oxidative stress on
67 *Prochlorococcus* fitness under cold conditions, as the drop in its growth rate was
68 paralleled by a transcriptional suppression of the photosynthetic machinery during day-
69 time, likely to mitigate the generation of ROS. Notably, warm temperature induced a
70 marked shrinkage of its cellular transcript inventory, which may induce regulatory
71 imbalances in the future functioning of this cyanobacterium.

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80 **Introduction**

81 *Prochlorococcus* is the most abundant photosynthetic organism on Earth (1), and a major
82 contributor to the oceanic primary production (2). Despite sustaining vast populations at
83 the global scale, *Prochlorococcus* exhibits an enigmatic distribution in the ocean, with a
84 sharp latitudinal barrier at ca. 45°N and 40°S (3, 4). While the ultimate reasons for such
85 restricted distribution are still unknown and may involve biotic interactions (5), temper-
86 atures in the range of 12°-15°C typically limit the growth of *Prochlorococcus* in culture
87 (3, 6, 7) and *in situ* (3), potentially representing critical thresholds for their metabolism.
88 Due to genome streamlining, *Prochlorococcus* is assumed to have a lower regulatory ca-
89 pacity than other phytoplankton groups, and a limited metabolic flexibility to adapt to
90 environmental disturbance, including temperature changes (8–10). However, a mechanis-
91 tic understanding of their temperature sensitivity at the molecular level is still lacking.

92 In general, addressing the impact of temperature on the functioning of any organism is
93 complex, as this parameter has overriding effects on virtually every aspect of cell
94 physiology. Temperature notably impacts the cellular size, the stability and conformation
95 of macromolecules, and the kinetics of biochemical reactions, altogether leading to
96 differences in cell growth (11). In the case of photosynthetic organisms such as
97 *Prochlorococcus*, growth is closely linked to their photosynthetic capacity, which is also
98 impacted by temperature (12–14). Under temperature conditions below the optimum, the
99 slow-down in carbon fixation rates constrains the replenishment of final acceptors of the
100 photosynthetic electron flow, producing an imbalance between photochemistry and
101 metabolism. Under these conditions, the excess of light energy absorbed can generate
102 cell-damaging reactive oxygen species (ROS), which need to be counterbalanced by
103 photoprotective mechanisms (15). Under heat-stress conditions (35-50°C) ROS are also

104 typically produced likely not as a result of an excess of energy absorbed, but of heat-
105 induced structural and functional changes in the photosystems and thylakoid membranes
106 (16). The decline in photosynthetic activity in phototrophs under moderate warm stress
107 has been associated with different processes, including the inhibition of *de novo* synthesis
108 of the photosystem II D1 protein by ROS (17), the inactivation of the oxygen-evolving
109 complex (18) and declines in electron transport (19).

110 At the expression level, a variety of compensatory mechanisms have been found to be
111 activated in several phytoplankton groups (i.e., *Synechococcus* and eukaryotic phyto-
112 plankton) to preserve cell functioning under thermal stress conditions (12–14, 20–23).
113 The cold-stress response network involves the upregulation of fatty acid desaturases to
114 offset decreases in membrane fluidity at low temperature, and RNA helicases and cellular
115 chaperones to facilitate proper folding of nucleic acids and proteins (21, 22, 24, 25). Low
116 temperature can also impact the expression of central components of the transcriptional
117 and translational machinery in eukaryotic phytoplankton, which are upregulated to com-
118 pensate for their reduced efficiency (21, 26), and even impact global regulatory networks
119 such as circadian rhythms in cyanobacteria (27). At elevated temperatures, a nearly uni-
120 versal response induces the expression of heat-shock proteins, which degrade or restruc-
121 ture denatured proteins and nucleic acids (28).

122 Some of the temperature compensatory mechanisms involve only short-term transcrip-
123 tional responses until cell functioning is restored (25). In other cases, the baseline expres-
124 sion of key enzymes is upregulated under long-term cold acclimations (26). Beyond
125 short-term temperature manipulation experiments, where cells are suddenly exposed to a
126 “thermal shock”, understanding mechanisms of long-term acclimation is particularly rel-
127 evant as they more accurately reflect responses to gradually changing thermal conditions.
128 Here, we performed a series of experiments on *Prochlorococcus marinus* MIT9301,

129 where cells were progressively exposed from a temperature close to their optimum to
130 their upper and lower thresholds of growth. This strain, which is a representative of the
131 *Prochlorococcus* dominant clade *in situ* (HLII, (29, 30)), was selected because it shares
132 the highest sequence similarity with environmental sequences (31) and enabled the iden-
133 tification of closely related transcripts in natural *Prochlorococcus* populations. We first
134 used a quantitative RNA-Seq approach (32, 33) to decipher the transcriptional response
135 of MIT9301 to temperature acclimations. Then, we addressed the environmental rele-
136 vance of one of the most conspicuous transcriptional responses observed in MIT9301,
137 involving highly expressed photosynthetic genes, in oceanic metatranscriptomes col-
138 lected along a comparable thermal gradient. Our objective was to analyse how sustained
139 growth at sub-optimal temperature reprograms the transcriptome of *Prochlorococcus* and
140 significantly advance in our understanding of which mechanisms underlie their growth
141 restriction under cold and warm conditions.

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143 **Results**

144 **Thermal acclimation experiments with *Prochlorococcus marinus* MIT9301: growth** 145 **rates and mRNA content.**

146 MIT9301 cultures were synchronized to a diel 12:12 light/dark cycle and long-term
147 acclimated to six temperatures along their thermal range (Figure 1, Figure S1). MIT9301
148 could not survive single-generation transfers below 17°C or above 30°C degrees and, thus,
149 these were considered thermal thresholds for this strain (T_{\min} and T_{\max} , hereinafter). The
150 growth rate of MIT9301 increased from 0.17 day⁻¹ at T_{\min} to ca. 0.61 day⁻¹ at 25°C
151 (hereinafter referred to as optimum growth temperature, T_{opt}) and, thereafter, entered a
152 warm-stress zone up to 30°C, where no further increases in growth rate were observed

153 (Figure 1A). The average size of MIT9301 cells also changed along the thermal gradient,
154 with maximum and minimum values at the T_{\min} and T_{\max} , respectively (Figure 1A).

155 After the acclimation period, RNA samples were collected at different temperature
156 conditions 3 hours after subjective sunrise and sunset (day- and night-time, respectively,
157 hereinafter). Large variations in the number of mRNA transcripts per cell were found in
158 replicate cultures acclimated to 17 and 20°C during day-time, while values were highly
159 constrained in temperatures close to the optimum (22 and 25°C, Figure 1B). A pattern of
160 decrease of mRNA transcripts with increasing temperature was found, with the average
161 number of mRNA transcripts per cell being positively correlated with cellular size in
162 night-time samples (Spearman $Rho = 0.89$, $p < 0.001$, Supplementary Figure S2).

163 **Quantitative global transcriptomic analysis of MIT9301 in experimental long-term** 164 **acclimations**

165 Top-expressed genes in MIT9301 were associated with photosynthetic components (e.g.,
166 *psbA*, *psbC*), the RuBisCO enzyme (*rbcL*) or an ammonium transporter (*amt*), which
167 reached average values above two mRNA transcripts per cell (Table S3). However, the
168 average abundance of most mRNA transcripts was at least one order of magnitude below
169 that value, which indicates that only a fraction of the population was actively expressing
170 them at any given time. Estimates of mRNA transcript abundance were normalized by
171 cell biovolume (i.e., transcripts per μm^3 or [mRNA], Table S4), to discard potential
172 indirect effects of cell size on transcript abundance in further analyses.

173 A substantial fraction of MIT9301 genes were differentially expressed along the
174 temperature gradient (i.e., exhibited significant variations in cellular [mRNA] estimates
175 at different temperatures). In total, 61% of protein-coding MIT9301 genes were
176 differentially expressed at night-time, while this number was reduced to 30% during day-

177 time (Kruskal-Wallis test, $p < 0.05$), in association with an increase in the variability of
178 gene expression levels; while [mRNA] values of individual genes in different biological
179 replicates were highly constrained at the T_{opt} , a large variation was found during day-time
180 at both thresholds of growth, and particularly at the T_{min} (Figures 2 and 3, Table S4). This
181 suggests a reduced ability of MIT9301 cells to maintain a tight transcriptional control
182 under challenging temperature conditions when cells are exposed to light. Notably, the
183 expression of some sigma factors and key regulatory proteins from different families also
184 showed this pattern (Figures 3A and 3B).

185 We identified five clusters of genes according to their patterns of day and night-time
186 expression at different temperatures (Figure 2, Table S5). More than 90% of MIT9301
187 protein-coding genes were assigned to one of these clusters with a probability score $>$
188 0.5), which indicates that these clusters were highly representative of the main thermal
189 gene expression responses in this strain. Clusters A and E were represented by genes
190 involved in core cellular and metabolic processes typically expressed in *Prochlorococcus*
191 during day- and night-time, respectively (34). Clusters C and D were associated with
192 mechanisms of cold-stress response, as they were characterized by a strong upregulation
193 at the T_{min} either during day-time or both day- and night-time, respectively. Finally, the
194 expression of genes in Cluster B showed a decreasing trend from the T_{opt} towards the T_{min}
195 during day-time (Figure 2), paralleling the pattern observed in growth rates along the
196 thermal niche (Figure 1A). Therefore, we hypothesize that the expression of Cluster B
197 genes is associated with metabolic processes limiting the growth of *Prochlorococcus*
198 when exposed to cold conditions.

199 Cluster A included genes related with C fixation and assimilation such as the RuBisCO
200 (*rbcLS*), CO₂ transporters (*csoS2*), carboxysome shell proteins (*ccmK*), the Calvin cycle
201 (e.g., *gap2*, *tktA*, *glpX*, *pgk*, *cbbA*) and glycogen synthesis (*glgABC*), consistently

202 expressed during day-time along the thermal gradient (Figure 2, Tables S4-S5). This
203 cluster also included ATP synthesis genes (*atpADE*) and a few components of the PS II
204 (*psbA*, *psbC* and *psbD*). Cluster E included genes related to catabolic consumption (*cyoB*,
205 *ndhD*), DNA replication (*dnaA*, *nrdJ*, *gyrB*), cell division (*ftsZYQ*) and the pentose
206 phosphate pathway (*tal*, *gnd*, *zwf*), all of them upregulated at night-time. Altogether, these
207 essential pathways likely represent a transcriptional core, which *Prochlorococcus*
208 *marinus* MIT9301 maintains at all temperature conditions.

209 Clusters C and D genes included different elements of the global stress response (e.g.,
210 cellular chaperones such as *groES/groES*, *dnaK*, *clpBCP* (35, 36)), mechanisms of DNA
211 repair (*recA*, *ruvB* (37, 38)), and the synthesis of antioxidant compounds such as carote-
212 noids (*pds*, *crtBH*) and rubredoxin (*rub*) (Table S5). Notably, the expression of the chap-
213 erones *groEL/groES*, *grpE* and *htpG* were strongly upregulated at the T_{\min} only during
214 day-time, suggesting a prioritization of their expression during the light-exposed period
215 (Figure 3C). Other metabolic processes upregulated at the T_{\min} were the mobilization of
216 energy storage (i.e., glycogen degradation, *glgP*), and the synthesis of proteins, as re-
217 flected by the increase in the [mRNA] of amino-acid synthesis genes (*glyA*, *serA*, *leuA*),
218 translation initiation factors (*infABC*) and N acquisition genes (Figures 2 and 3, Table
219 S4).

220 The strong upregulation of N acquisition mechanisms at the T_{\min} under light conditions
221 was observed not only for genes predominately expressed during day-time at the T_{opt} (i.e.,
222 the global nitrogen regulatory protein *ntcA*, *glnA*, and urea transporters), but also for
223 genes typically expressed at night-time in *Prochlorococcus*, such as the ammonium trans-
224 porter (*amt*) and urease genes (*ureABC*.) This likely reflects the large cellular demand of
225 N for protein synthesis during day-time under cold stress conditions. In the case of phos-
226 phosphate uptake, the high affinity ABC transporter *pstABC* genes were upregulated under

227 cold conditions at night, following the pattern of cluster E genes, possibly related to the
228 cellular demand of P for DNA replication. By contrast, both copies of the periplasmic
229 phosphate binding protein (*pstS*) showed maximum expression values around the T_{opt}
230 during day-time following the pattern of most photosynthetic genes (cluster B), which
231 highlights the complexity in the thermal response of nutrient acquisition genes (Figure
232 3D).

233 Finally, the expression of all components of the PS I complex (*psaABDEFKL*) and some
234 of the PS II (including *psbBJH* and the oxygen evolving complex protein *psbO*) showed
235 a gradual decrease in expression from a temperature close to the optimum to the T_{min} ,
236 (Figure 2) in correlation with MIT9301 growth rates (Figure 1). This expression pattern
237 was different from other PSII components (*psbACD*, see above), which were not
238 differentially expressed during day-time along the thermal niche (Kruskall-Wallis, $p >$
239 0.05, Figure 2). Similarly, many components of the photosynthetic electron transport
240 genes were assigned to either Cluster B (*petACGNM*) or Cluster A (*petBEDH*), implying
241 a non-uniform transcriptional thermal response of all components of the photosynthetic
242 apparatus (Figure 4). Yet, a general pattern of upregulation of photosynthetic genes
243 during night-time under cold conditions, inducing changes in their day-night log2fold
244 expression ratio was observed (Figure 2), suggesting a loss in the capacity of the cells to
245 regulate their circadian expression.

246 **Transcriptional response of photosynthetic genes in naturally occurring** 247 ***Prochlorococcus* populations**

248 With the aim of testing whether the transcriptional suppression of photosynthetic genes
249 at cold temperature observed in MIT931 was also found in natural *Prochlorococcus*
250 populations *in situ*, we identified reads closely related to MIT9301 in the *Tara* Oceans
251 metatranscriptome dataset (39) including samples distributed across different ocean

252 basins along a comparable thermal gradient (Table S6). The number of MIT9301-like
253 reads ranged from 6379 to 9.62 million in the different environmental samples (Table
254 S7). The DESeq2 normalized abundance of transcript counts from some of the most
255 actively expressed genes of the PSII (*psbA*, *psbD*) did not show any significant trend
256 along the *in situ* thermal gradient from 17 to 30°C (Spearman correlation, $p > 0.05$),
257 mirroring the response observed under culture conditions (Figure 5, Supplementary
258 Figure S3). By contrast, other PSII components (*psbJ*) were positively correlated with
259 temperature both in the experimental and *in situ* datasets (Spearman $R = 0.6$, $p < 0.01$),
260 indicating a downregulation at cold temperature (Figure 5). Similarly, the downregulation
261 of PS I genes at cold temperature was clearly observed both in the experimental and *in*
262 *situ* dataset (Figure 5), reinforcing the environmental relevance of this response.
263 Temperature had the strongest correlation to photosynthetic transcript counts in
264 comparison with all other tested environmental variables (Supplementary Figure S4),
265 excluding the possibility that these correlations were driven by co-varying environmental
266 parameters.

267 **Discussion**

268 There is a remarkable gap of fundamental knowledge on the molecular mechanisms of
269 thermal acclimation in *Prochlorococcus*, as previous studies have concentrated on their
270 cyanobacterial sister clade *Synechococcus* and eukaryotic phytoplankton (12–14, 20–22,
271 40, 41). Acquiring this knowledge would be crucial towards answering a long-standing
272 question: what limits the ability of *Prochlorococcus* to expand to high-latitude environ-
273 ments? The decrease in the light-harvesting capacity of phytoplankton under cold condi-
274 tions has been typically attributed to changes in the conformation of membrane lipids and
275 proteins of their photosynthetic apparatus (14, 42). Our quantitative gene expression

276 analysis shows that, in the case of *Prochlorococcus*, the inability to maintain their photo-
277 synthetic capacity under cold conditions is already critically compromised at the tran-
278 scriptional level, as we found a clear suppression of the expression of most photosynthetic
279 genes towards cold conditions. The same pattern was found for MIT9301-like reads in *in*
280 *situ* metatranscriptomes across the global ocean, suggesting that this molecular mecha-
281 nism may be relevant to explain the restricted distribution of *Prochlorococcus* to temper-
282 ate zones. Notably, this response differs from previous studies targeting diatom cultures
283 (26), or phytoplankton communities *in situ* (21, 39) , indicating fundamental differences
284 in the ability of *Prochlorococcus* to respond to cold temperature, which may be related
285 to their exceptional high sensitivity to oxidative stress (38, 43).

286 Photosystems are naturally sensitive to light damage, which is exacerbated under low
287 temperature (22). The cellular decision of shutting down their sunlight energy conversion
288 system at cold conditions likely arises from the inability of *Prochlorococcus* to cope with
289 uncontrolled redox chemistry when there is an imbalance between the production of ex-
290 cited electrons by light and their metabolic consumption. The downregulation of photo-
291 systems would represent an emergency mechanism to slow-down the electron flow and
292 prevent the production of cell-damaging ROS. Notably, the response was not equal for
293 all photosystem components, as previously observed in cultured *Prochlorococcus* strains
294 undergoing other types of stress, such as iron starvation (44), phage infection (45), or
295 high-light exposure (46). On the one hand, the high expression of *psbA* transcripts at all
296 temperature conditions is likely related to the need to maintain an exceptionally high turn-
297 over rate of this protein (47), being differentially regulated than other PS components (34,
298 48, 49). On the other hand, the selective downregulation of PSI probably arises from the
299 strong need to protect PSI from oxidative stress, as this photosystem typically lacks effi-
300 cient repair machinery, and therefore its damage may be practically irreversible (50).

301 Interestingly, MIT9301 also upregulated the plastoquinol terminal oxidase (PTOX) at the
302 T_{min} (Figure 2), which is thought to function as a safety valve in cyanobacteria to avoid
303 electron flow towards PSI (51).

304 In addition to the transcriptional suppression of photosynthetic genes, we found that under
305 light conditions, cold temperature induced a loss in the regulatory capacity of MIT9301
306 cells. This was reflected in the loss of the circadian day/night gene expression ratio and
307 the increased variability in the concentration of cellular mRNA transcripts among
308 biological replicates. In a previous study, it has been suggested that higher levels of
309 stochasticity in *Prochlorococcus*' gene expression during the transition from
310 photosynthesis to the use of internal energy is related to the accumulation of ROS (52).
311 Our transcriptomic results are consistent with the idea of oxidative stress impacting the
312 regulatory capacity of *Prochlorococcus*. Notably, at the T_{min} we observed a high
313 variability in the expression of regulatory proteins that modulate the cellular response to
314 daily light fluctuations in cyanobacteria, including sigma factors (*rpoD*) (53, 54), the
315 NblS-RpaB two-component system (55) and the SasA-RpaA clock output system (56, 57).
316 Assuming that mRNA transcript levels reflect the protein levels of these regulators, our
317 results would imply a critical impairment of their co-regulated networks. Other
318 mechanisms of transcriptional regulation in *Prochlorococcus* (i.e., RNA-based regulatory
319 strategies (58)), were likely also compromised at low temperature, as evidenced by the
320 upregulation of the ribonuclease *rne* gene at the T_{min} (Table S4).

321 Marked changes in the day/night gene expression ratio of some of *Prochlorococcus* key
322 functional genes along the thermal niche imply a deregulation of one of their fundamental
323 features, i.e., the coordination of transcriptional oscillations with the daily light/dark cy-
324 cle. This feature is key to optimize cellular processes through anticipating and synchro-
325 nizing transcription of photosynthetic genes with daylight hours (34, 59, 60). At T_{opt} , the

326 day/night expression *preferenda* of most key functional genes of MIT9301 matched those
327 previously observed in the model strain MED4 (34), reinforcing the idea that maintaining
328 this transcriptional choreography is a highly conserved and critical trait. The deregulation
329 observed at cold temperature led to the paradoxical situation where, after subjective sun-
330 rise, chilled *Prochlorococcus* MIT9301 cells contained several-fold less PSI transcripts
331 than after subjective sunset, which supports the idea of a malfunctioning regulatory net-
332 work. With only two samplings over the daily cycle, we cannot ensure whether the ob-
333 served changes were due to random misregulations or variations in the phasing or ampli-
334 tude of their diel gene expression patterns. Yet, considering that we were sampling the
335 two daily periods when most *Prochlorococcus* genes show maxima in expression (34),
336 the inversion of their day/night *preferenda* is remarkable and likely has an important im-
337 pact on its fitness.

338 By contrast to the pattern observed in day-time samples, genes related to *Prochlorococcus*
339 cell division cycle, typically expressed during night-time, showed rather constrained
340 [mRNA] values between replicates. We observed an increase in [mRNA] of those genes
341 towards cold conditions, which may be related to the predominant cell cycle phase of the
342 cells. As the timing of cell division is delayed in *Prochlorococcus marinus* MIT9301
343 when acclimated to cold temperature (61), our results likely reflect a situation where a
344 higher proportion of cells were still undergoing the S phase at 17°C and 20°C at the time
345 of sampling (i.e., 3 hours after subjective dusk), as compared to other acclimation tem-
346 peratures.

347 At the warm temperature threshold, the upregulation of a variety of cellular chaperones
348 (e.g., *groEL/groES*, *htpG*, *grpE*) reflected that cells were undergoing substantial stress.
349 However, no drastic changes were generally observed in the expression of most protein-
350 coding genes at the T_{max} . Accordingly, a previous study on the short-term response of

351 MED4 to warm stress found that the synthesis of new polypeptides was not induced (62).
352 We had previously shown that *Prochlorococcus* MIT9301 cells decrease in size after
353 long-term warm acclimation, in accordance with the temperature-size rule (61). Interest-
354 ingly, here, our quantitative transcriptomic approach showed a conspicuous streamlining
355 in the transcript inventory in the cells in parallel with the progressive reduction in cell
356 size. While at non-restricting growth temperature the average estimate of mRNA tran-
357 script abundance per cell was within the range reported for environmental marine bacteria
358 (i.e., ca. 85-255 transcripts per cell (32)), under warm conditions this average dropped
359 down to 30 mRNA transcripts of protein-coding genes per cell at night-time. Cellular
360 reactions involving small numbers of molecules are intrinsically noisy, being dominated
361 by fluctuations in concentration and stochasticity (63, 64). Thus, the decline in the tran-
362 script inventory under warm conditions invites the hypothesis that there is a critical
363 threshold in the number of mRNA copies beyond which cells lose regulatory capacity,
364 contributing to the growth arrest.

365 In summary, previous studies on phytoplankton have identified the cellular membrane
366 and translational apparatus as central elements associated with the thermal adaptation at
367 the transcriptional level (21, 26). Some of these mechanisms were also observed in
368 MIT9301, such as the upregulation of the translational machinery, likely to compensate
369 the general slow-down in protein synthesis rates under cold conditions (24). Yet, we
370 found that in the case of *Prochlorococcus*, oxidative stress emerged as a major factor
371 impacting its physiology when approaching cold temperature, as this source of stress re-
372 lates to both the need to protect the photosynthetic machinery and the ability of *Pro-*
373 *chlorococcus* to “reset” their daily cycles in the morning hours (65). Cold temperature
374 has been previously shown to nullify the circadian rhythm in different organisms, includ-
375 ing plants and different phytoplankton species, moving the circadian oscillation to a

376 damped oscillation (see references in (27)). As *Prochlorococcus* contains only a mini-
377 malist circadian system, their cell cycle is likely more easily perturbed than that from
378 organisms containing a complete *kaiABC* gene set.

379 The disruption of the transcriptional choreography in *Prochlorococcus* may be a differ-
380 entiating factor as compared to *Synechococcus* and eukaryotic phytoplankton, which do
381 not require maintaining such a finely tuned daily expression rhythm to maintain their
382 metabolism. The higher tolerance of *Synechococcus* to oxidative stress(38) may also im-
383 ply a more resilient photochemistry when exposed to cold conditions, enabling them to
384 colonize higher latitudes. Additionally, we postulate that increased levels of stochasticity
385 in gene expression at temperature challenging conditions may contribute to a decrease in
386 *Prochlorococcus* fitness. These remains as an interesting hypotheses to test in future stud-
387 ies, with implications for the functioning of this global primary producer and, thus, the
388 marine carbon biogeochemistry in future oceanic conditions.

389

390 **Materials and Methods**

391 **Growth of cultures and temperature acclimation**

392 We grew non-axenic cultures of *Prochlorococcus marinus* MIT9301 obtained from the
393 Roscoff Culture Collection (RCC) in PCR-S11 culture medium (66) based on Red Sea
394 Salt (Houston, TX, USA). Cultures were grown under a 12:12 hour photoperiod and
395 irradiance of $120 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ in polycarbonate flasks with vented caps. During
396 the acclimation, we maintained the cultures in exponential growth by performing serial
397 transfers before cell density reached 30% of the maximum yield. The acclimation started
398 from 22°C (i.e., temperature of maintenance in RCC) and temperature was progressively
399 changed towards the upper and lower thermal thresholds. At each acclimation step,

400 temperature was changed by a maximum of 2°C and down to 0.2°C when approaching
401 the temperature thresholds, to avoid lethal thermal stress (Figure S1). We considered that
402 the culture had been fully acclimated to a temperature treatment when growth rate stayed
403 stable in at least two consecutive growth curves after at least 8 generations. Flow
404 cytometry was used for sustained monitoring of the culture growth during the acclimation
405 process. Samples were fixed with glutaraldehyde (final concentration of 0.025%) for 10
406 minutes at room temperature and dark conditions, and frozen at -80°C until analysis in a
407 FACSCalibur flow cytometer (Becton Dickinson). Estimates of cell size were obtained
408 based on the natural logarithmic-transformed side scatter (SSC) using the calibration
409 provided in (67) and assuming a spherical cellular shape.

410 **RNA samples collection and extraction**

411 Once the cultures reached full acclimation to the temperatures selected (17, 20, 22, 25
412 and 30°C), we re-inoculated biological replicate batch cultures into fresh medium (160
413 mL) and collected samples for RNA-Seq analysis during exponential growth (with cell
414 density values ranging between 6 to 17 10^7 cells mL⁻¹, Table S1). For each temperature
415 and biological replicate (ranging from 3 to 7, depending on the experimental treatment,
416 Table S1), samples for RNA extraction were collected 3 hours after the onset and offset
417 of the photoperiod (day- and night-time, respectively). Exceptionally, for the acclimation
418 temperature 22°C, only day-time RNA samples were available. Samples were filtered on
419 0.22- μ m pore-size PES filters using a vacuum pump at a pressure of 5 psi. Immediately
420 after filtration, filters were snap frozen in liquid nitrogen and stored at -80°C. The time
421 elapsed from the start of the filtration until freezing was always lower than 2 minutes.

422 Quantitative benchmarked RNA extraction was performed following (33) using five RNA
423 standards from *Saccharolobus (Sulfolobus) solfataricus* P2 (NCBI Taxon ID 273057)
424 obtained by *in vitro* transcription of genomic templates of the isolate (standards 3, 6, 7,

425 13 and 14, as described in (33)). The RNA standards were individually spiked (10 to 28
426 μL) at a concentration of ca. $20 \text{ pg } \mu\text{L}^{-1}$ to the filters prior to the initiation of the RNA
427 extraction. Subsequently, RNA samples were extracted with the mirVana kit (Ambion),
428 and DNA was removed using the TURBO DNase (Ambion). Samples were depleted from
429 ribosomal RNA using the Ribo-Zero rRNA removal kit (Bacteria, Illumina) and quality
430 check was performed using a Bioanalyzer (Agilent). mRNA samples were concentrated
431 using the Zimo Clean & Concentrator kit, and cDNA libraries were constructed using the
432 TruSeq Stranded mRNA Sample Preparation Kit (Illumina). cDNA libraries were
433 sequenced as 75bp paired-end reads on an Illumina HiSeq v4 platform (CNAG, Spain).
434 Raw reads have been submitted to ENA under project accession number PRJEB54738.

435 **Quantitative gene expression analysis of *Prochlorococcus* MIT9301 under culturing**
436 **conditions**

437 Sequence read quality check was performed with the FastQC tool (68), and Trimmomatic
438 (69) was used to trim raw sequences (SLIDINGWINDOW:50:35 and MINLEN:50) and
439 pair those passing quality thresholds. rRNA sequences were removed using SortMeRNA
440 (70) and the remaining reads were mapped with Bowtie2 (71) (using the ‘–non-deterministic’
441 parameter) against the *Prochlorococcus marinus* MIT9301 genome (NCBI Taxon
442 ID 167546). The same procedure was done with the *S. solfataricus* genome to identify
443 RNA internal standard reads. Read count tables were obtained using HTSeq (72) with the
444 following parameters: ‘–stranded = reverse -a 10 -m intersection-nonempty’. Quantitative
445 estimates of individual transcript abundance (T_a) of MIT9301 protein-coding genes in
446 each RNA sample were obtained following the calculations described in (73):

447
$$T_a = (T_s \times S_a) / S_s$$

448 where T_a corresponds to the estimated number of transcripts of an individual MIT9301
449 protein-coding gene, T_s corresponds to the number of reads assigned to the corresponding

450 MIT9301 protein-coding gene, Sa corresponds to the molecules of internal RNA stand-
451 ards spiked to the RNA sample, and Ss corresponds to the number of reads assigned to *S.*
452 *solfataricus* internal standards. In this calculation, one of the standards (standard 14) was
453 removed from the analysis because it was consistently recovered in higher proportion
454 than other standards. Ta values were divided by total cell abundance or estimates of total
455 cell biovolume collected in the corresponding RNA filter to obtain estimates of transcript
456 abundance per cell and per volume (i.e., transcript concentration), respectively (Tables
457 S1- S3). This normalization is relevant because cell size, which varies over the thermal
458 gradient in this strain (see Figure 1A), can impact the number of cellular transcripts (74,
459 75).

460 **Bioinformatic analysis of transcriptional patterns of *Prochlorococcus*** 461 **photosynthetic genes in oceanic samples and culture conditions**

462 For comparing the expression patterns of *Prochlorococcus* photosynthetic genes along
463 the same thermal gradient under environmental and culturing conditions, two datasets
464 were produced using a common normalization method (DESeq2 (76)). The experimental
465 dataset was obtained from the raw read counts obtained by HTSeq2 in the long-term
466 thermal acclimation experiments, as explained above. For obtaining the environmental
467 database, a dataset of *Tara* Oceans metatranscriptomes (39) was initially selected based
468 on the latitude range where *Prochlorococcus* is found in the ocean (i.e., between 45° N
469 and S), the thermal range of MIT9301 (17°C to 30°C), and the time of sample collection
470 (between 6 and 12 am, when the expression of photosynthetic genes should be close to
471 their maximum). Fastq sequence files were quality filtered with fastp (77) using default
472 parameters, and rRNA sequences were removed with RiboDetector (78) with the option
473 "--ensure" selected. The remaining reads were filtered to remove reads not affiliated with
474 *Prochlorococcus*, using a custom made database with the complete genome sequences in

475 the MarRef database, which included 16 *Prochlorococcus* genomes (79), and eukaryotic
476 genes of interest extracted from Refseq (80) (i.e., *psaA*, *psaB*, *psaD*, *psaF*, *psaL*, *psbA*,
477 *psbD*, and *psbO*, searched with Entrez filter [Gene Name] AND ((protists[filter] OR
478 plants[filter]) AND refseq[filter]). Metatranscriptome sequences closest to
479 *Prochlorococcus* were identified based on Blastn v.2.12.0+ searches (identity \geq 98% and
480 bitscore \geq 30), and only those with highest bitscore to *Prochlorococcus* sequences were
481 considered further. Subsequently, we applied a two-step filtering strategy to obtain read
482 counts closely related to MIT9301. First, we aligned each metatranscriptome sample
483 against MIT9301 genome using BWA mem version 0.7.17-r1188 with default parameters
484 (81). Next, we discarded alignments which had a percent identity lower than 98%, a read
485 length lower than 50 bases, and a number of matches lower than 50% of the total length
486 of each aligned sequence. Percent identity was computed as $100 \times (N_m / (N_m + N_i))$,
487 where N_m corresponds to the number of matches in the alignment and N_i to the number
488 of mismatches. The number of matches was obtained by parsing the MD tag of the
489 alignment record, while the number of matches and mismatches was obtained from the
490 CIGAR string in the SAM file. Once alignments were filtered, we proceeded to count
491 aligned reads with HTSeq v.2.0. with default parameters (82). Next, we removed non-
492 coding genes from the count matrices as well as genes which had no counts across all
493 conditions, to facilitate the consequent normalization. We also discarded samples which
494 had counts in less than 10 genes other than the *psbA* gene. Finally, we normalized count
495 data using the default DESeq2 v.3.15 count normalization workflow (76). All analyses
496 were assisted with customized Python code (83) available at
497 <https://github.com/Robaina/prochlorococcus>.

498 **Softclustering and statistical analyses**

499 Clusters of differentially expressed genes that responded similarly over the thermal
500 gradient were identified using softclustering, following (84). A matrix of N genes x 9
501 treatments (4 temperatures at night-time and 5 temperatures at day-time) was used as
502 input data. Data for each gene was standardized to zero mean and unit variance. The
503 optimal value of the parameter m in the Mfuzz algorithm was estimated through
504 randomization following (85). The number of clusters was chosen to maximize the
505 functional enrichment of gene clusters (COGs) and the ClusterJudge method (86). The
506 standardized data was clustered by a generalized version of the fuzzy c-means algorithm.
507 Finally, statistically significant differences in cellular transcript concentration among
508 temperature regimes were determined by the Kruskal-Wallis test using R.

509

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798 **Figure legends**

799 **Figure 1.** Growth rates, cell size and number of mRNA transcripts per cell in samples of
800 *Prochlorococcus marinus* MIT9301 collected after long-term thermal acclimation.
801 Between 3 and 7 biological replicate samples are represented depending on the
802 temperature treatment and error bars report the standard deviation between replicates. On
803 the top of each plot, the minimum (T_{\min}), optimum (T_{opt}) and maximum temperature
804 (T_{\max}) are indicated and, additionally, in the upper left plot, temperature treatments where
805 RNA samples were collected are shown with arrows. (A) Growth rate and size of
806 MIT9301 cells along the thermal niche. (B) Estimates of mRNA transcripts per cell along
807 the thermal niche in samples collected at day-time (left panel) and night-time (right
808 panel). Letters denote statistical significant differences (ANOVA test, Tukey post-hoc
809 test, $p < 0.001$).

810 **Figure 2.** Clusters of *Prochlorococcus marinus* MIT9301 genes according to their pattern
811 of day- and night-time expression along the thermal niche. Within each cluster, right- and
812 left-side panels represent day- and night-time expression of the same genes, respectively.
813 Dot colours indicate local density at each point of the scatterplot, with red circles
814 indicating a high density of dots. Line colours within each softcluster indicate the
815 membership value assigned by the “Fuzzy c-means soft” clustering of each gene, ranging
816 from 1 (red, high score) to 0.5 (blue, low score). Genes with a membership value lower
817 than 0.5 are not plotted, neither included in the total number of genes for each cluster (but
818 they are included in Table S5). Below each cluster, the 20-top expressed genes are shown
819 in rank order plots, and the expression levels (measured as [mRNA]) and day/night log-
820 2-fold expression ratio) of a selection of representative genes are shown. Day-time values
821 appear as blue lines and night-time values as black lines. Between 3 and 7 biological
822 replicate samples are represented depending on the temperature treatment and error bars

823 report the standard deviation between replicates. Asterisks in the plots denote significant
824 differences in transcript concentration along the thermal gradient in day-time (blue
825 asterisks) or night time (black asterisks) according to Kruskal-Wallis test (* $p < 0.05$, **
826 $p < 0.01$, *** $p < 0.001$). In the log₂fold ratios, values above 0 represent preferential
827 expression during day-time (highlighted in blue), while values below 0 represent
828 preferential expression during night-time.

829 **Figure 3.** Cellular expression levels (measured as [mRNA]) in *Prochlorococcus marinus*
830 MIT9301 during day-time (blue lines) and night-time (black lines) along the thermal
831 niche of (A) RNA polymerase components including sigma factors, (B) Histidine kinases
832 and other regulatory proteins, (C) genes involved in the stress response, and (D) Nitrogen
833 and Phosphate acquisition genes. Asterisks denote significant differences in transcript
834 concentration along the thermal gradient in day-time (blue asterisks) or night time (black
835 asterisks) according to Kruskal-Wallis test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The
836 softcluster membership of each gene is shown only for those cases where the probability
837 score was > 0.80 .

838 **Figure 4.** Schematic diagram showing some of the main components of the
839 photosynthetic and carbon metabolism pathways in *Prochlorococcus*. Colours of genes
840 (or their corresponding protein complexes) follow the same code of their respective
841 softclusters in Figure 2.

842 **Figure 5.** Expression patterns of a selection of *Prochlorococcus* photosynthetic genes of
843 Photosystem II (*psbA*, *psbJ*) and Photosystem I (*psaA*, *psaB*, *psaF*) along the thermal
844 gradient 17 to 30°C in experimental acclimations (as analysed in *P. marinus* MIT9301 by
845 transcriptomics, left pannel) and *in situ* environmental conditions (as analysed in
846 MIT9301-like reads identified in metatranscriptomes of the *Tara* Oceans dataset, right
847 panel). In both cases, reads were normalized using DESeq2 and log-transformed. Linear

848 regression lines are shown for all genes with significant Spearman correlation coefficients
849 at a p -value < 0.05 (indicated in the plots).

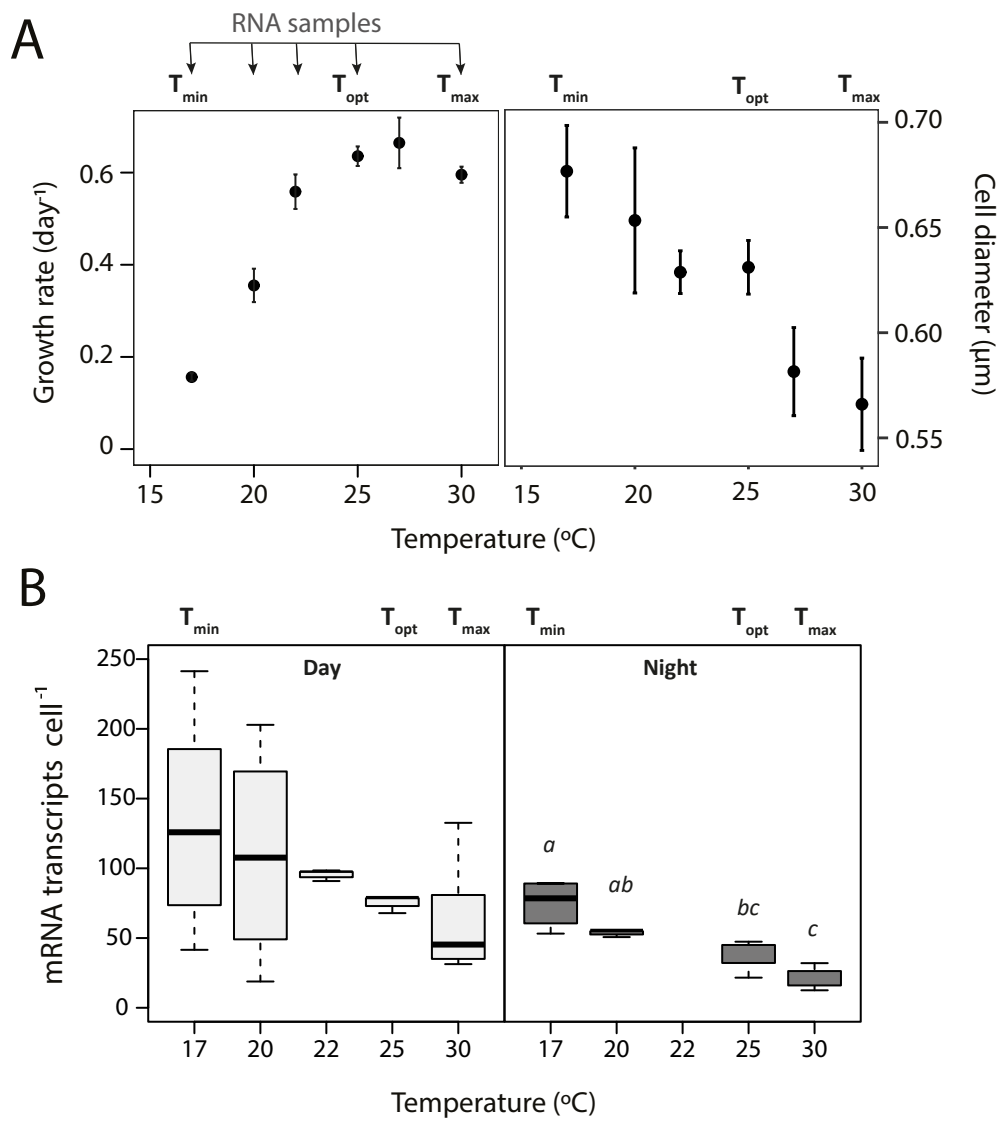


Figure 1

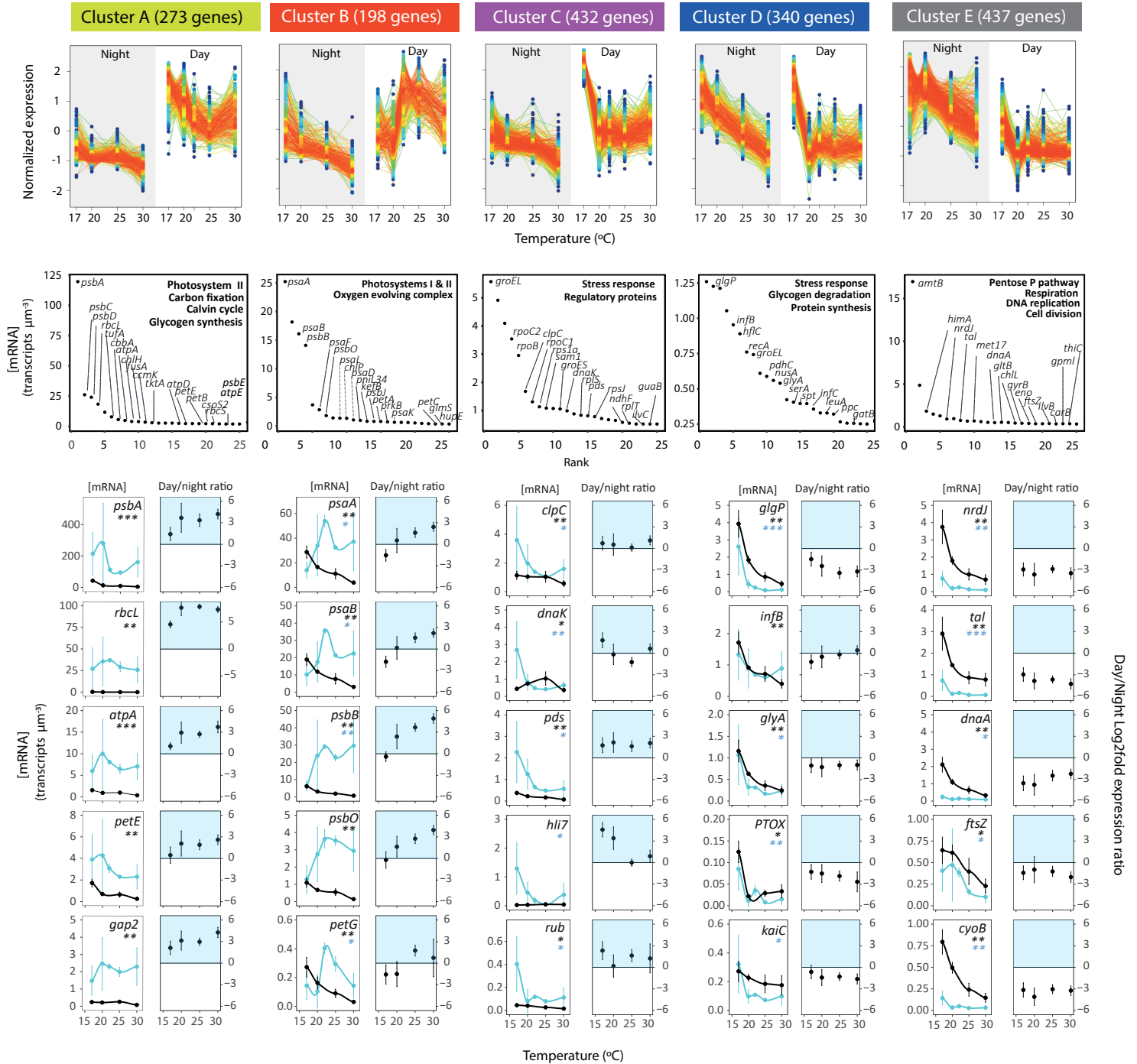


Figure 2

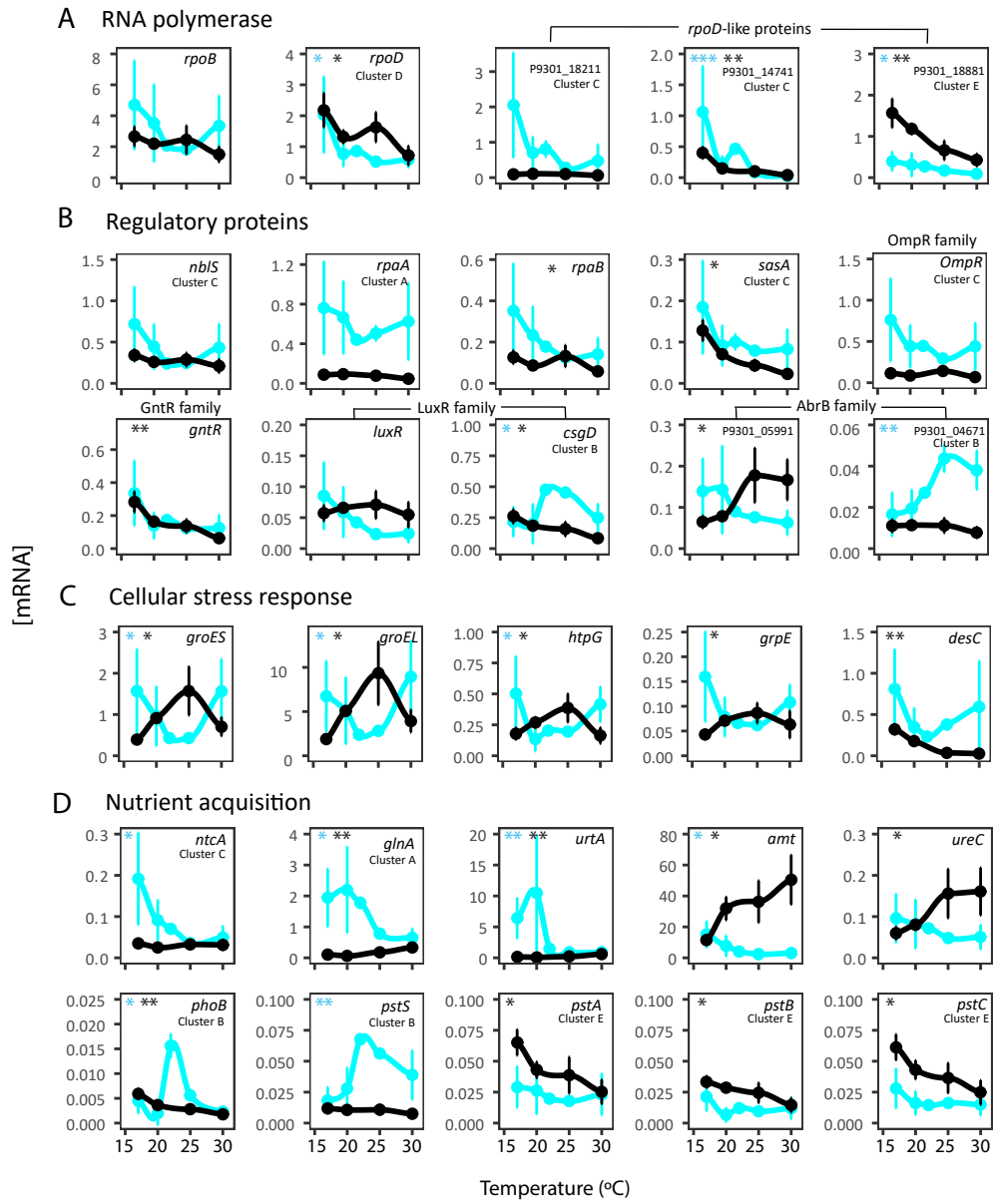


Figure 3

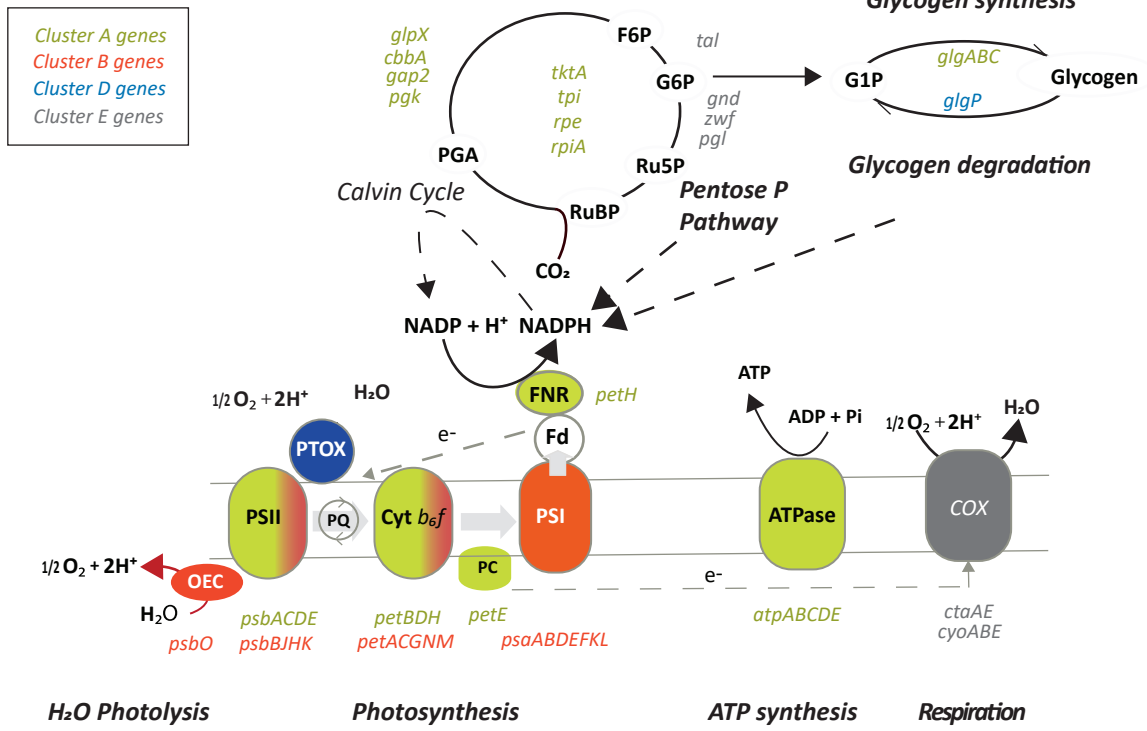


Figure 4

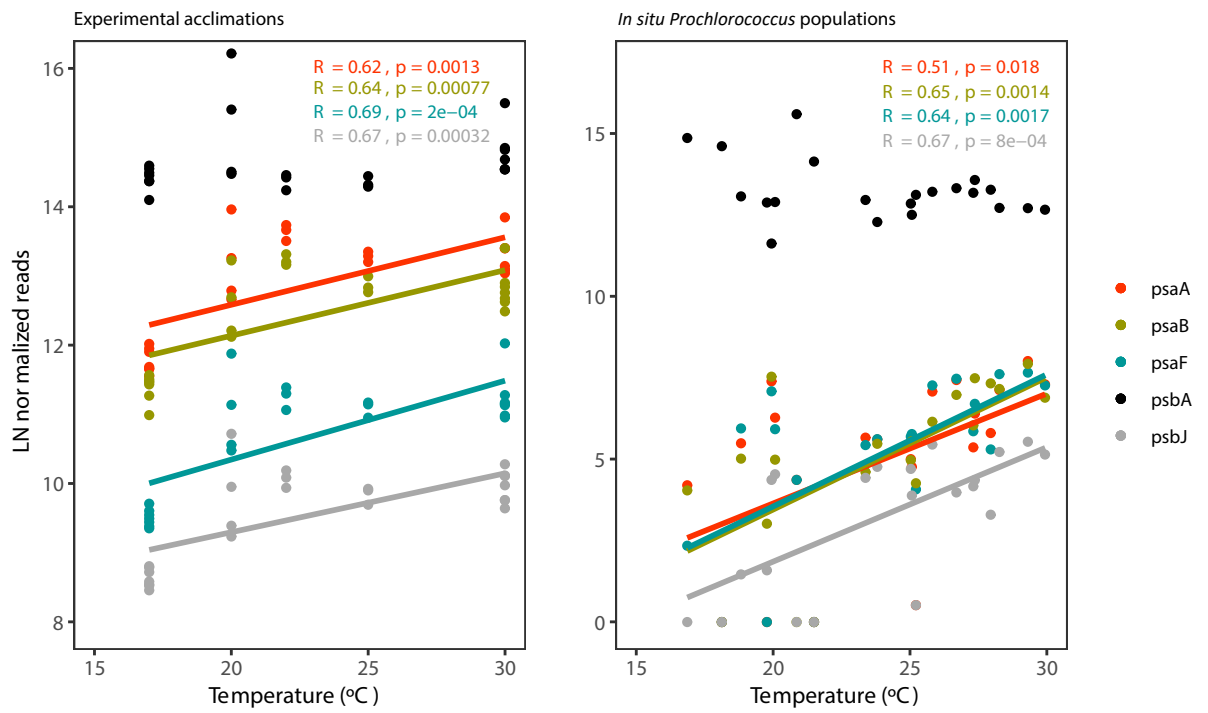


Figure 5