1 Sustained photobiological hydrogen production in the presence of N₂
2 by nitrogenase mutants of the heterocyst-forming cyanobacterium
3 *Anabaena*

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16
17 **Abstract**

18 Heterocyst-forming cells of the cyanobacterium *Anabaena* sp. strain PCC 7120 ΔHup,
19 lacking an uptake hydrogenase, photobiologically produce H₂ by nitrogenase. Under N₂-rich
20 atmosphere, the nitrogenase activity declines in a rather short time due to the sufficiency of
21 combined nitrogen. From the parental ΔHup strain, site-directed double-crossover variants,
22 dc-Q193S and dc-R284H, were created with amino acid substitutions presumed to be
23 located in the vicinity of the FeMo-cofactor of nitrogenase. Unlike the case for the ΔHup
24 strain, H₂ production activities of the variants were not decreased by the presence of high
25 concentrations of N₂ and they continuously produced H₂ over 21 days with occasional
26 headspace gas replacement. This property of N₂ insensitivity is a potentially useful strategy
27 for reducing the cost of the culture gas in future practical applications of sustainable biofuel
28 production. This *Anabaena* strain has only the Mo-containing nitrogenase which reduces
29 acetylene to ethylene, but the dc-Q193S variant also produced ethane at low but
30 measurable rates along with greater rates of ethylene production.

31
32 **Keywords:**

33 Cyanobacteria; *Anabaena*; hydrogen production; biofuel; nitrogenase; site-directed
34 mutagenesis

1. Introduction

Photobiological production of H₂ by cyanobacteria is an attractive source of renewable energy because these microorganisms have simple needs: readily available sunlight for energy and water as the electron donor [1-5]. H₂ is an efficient substrate for fuel cells that cleanly generate electrical and thermal power with water as the final product, in contrast to the situation for fossil fuels that also form green-house gases and pollute the environment.

H₂-producing enzymes in cyanobacteria include hydrogenases and nitrogenases[3, 5]. We have chosen to focus on nitrogenase as the enzyme for H₂ production because it catalyzes essentially unidirectional H₂ production and thus does not consume H₂ as a substrate. When the hydrogenase activity is eliminated by genetic engineering, the cyanobacterial mutants accumulate a high concentration of H₂ (about 7-30%) even in the presence of O₂[6, 7]. By contrast, wild-type nitrogen-fixing cyanobacteria accumulate significantly less of this product because their hydrogenase(s) catalyze the uptake of H₂[8].

H₂ is produced as an inevitable by-product of nitrogen fixation. The optimized reaction for nitrogen fixation can be expressed as:

In the absence of N₂ (e.g., under argon; Ar), all electrons are directed to H₂ production:

For the filamentous N₂-fixing cyanobacterium used here, *Anabaena* (also called *Nostoc*) sp. strain PCC 7120, nitrogen deficiency leads to the development of specialized cells called heterocysts that protect nitrogenase against inactivation by O₂[9], however, elevated O₂ partial pressures remain harmful to nitrogenase activity to some extent [6, 10-12]. The enzyme activity is induced during the late stages of heterocyst differentiation and then declines in a rather short time (usually less than several hours) under ambient air or N₂, because its product ammonia meets the nitrogen nutritional requirements of the cells and consequently suppresses *de novo* nitrogenase synthesis [9]. While the mechanism of ammonia inhibition of nitrogenase in cyanobacteria had been considered to be different from that in other bacteria [13], an ammonia “switch-off” of the enzyme (within a few tens of minutes) in the presence of O₂ was reported for other heterocyst-forming strains [9, 14]. However, the mechanism and the presence/absence of ammonia “switch-off” in cyanobacteria is still under debate [9]. For sustained, high-level production of H₂ by nitrogenase, Ar purging is frequently used to both decrease N₂ concentration (thus minimizing repression of nitrogenase activity due to the increased cellular N/C ratio) and alleviate the rise in O₂ partial pressure produced by photosynthesis (thus minimizing nitrogenase inactivation) [11, 15].

In a previous study, over 40 single-crossover (sc) mutants were created in *Anabaena* sp.

strain PCC 7120 Δ Hup in which the uptake hydrogenase (Hup) activity had been inactivated by genetic engineering [7, 16]. Each of these isolates contained a 9.4-kb DNA fragment inserted into the *nif* gene cluster along with distinct site-directed mutations in *nifD* (Fig. 1, top) resulting in separate amino acid substitutions presumed to be located in the vicinity of the catalytic FeMo-cofactor of nitrogenase. Some of the sc-mutants photobiologically produced H_2 under N_2 at rates comparable to those under Ar over the course of several days. These properties of the sc-mutants suggested that such a mutational strategy could decrease the cost of gases for use in photobiological H_2 production in the future. Thus, we have chosen two potentially useful sc-site-directed mutants and constructed the corresponding double-crossover (dc) strains which lack the inserted DNA fragment (Fig. 1, bottom) so that revertants can not be generated. Using the dc-mutants, we studied the effects of gas composition on the sustainability of photobiological H_2 production activity more closely, and found that they allow more efficient expression of nitrogenase activity than the sc-mutants under high N_2 atmosphere.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

Cyanobacterial strains were grown at 28°C in an 8-fold dilution of Allen and Arnon (AA/8) liquid medium [17] with or without added nitrate. The cultures were placed on a rotary shaker in air or bubbled with air under continuous illumination (using cool white fluorescent lamps at 30 or 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ of photosynthetically active radiation, PAR), unless otherwise indicated.

The chlorophyll *a* (Chl *a*) concentration of the cultures was determined by the method of Porra[18].

2.2. Construction of *Anabaena* double-crossover recombinants

The sc-variants sc-AnNif-Q193S- Δ Hup and sc-AnNif-R284H- Δ Hup (hereafter referred to as sc-Q193S and sc-R284S, respectively) containing site-directed mutations in *nifD* to provide the indicated variant proteins[7] were chosen for further analysis because they exhibited high levels of nitrogenase-based H_2 production that was not strongly inhibited by the presence of N_2 . To remove the 9.4-kb DNA insertion from the sc-variant chromosomes, they were grown in AA/8 liquid medium without nitrate for one week, and portions of the cultures were spread over AA agar solid medium containing nitrate and 5% sucrose for isolation of the dc-recombinants[19]. Among the sucrose resistant recombinants (due to loss of *sacB*), neomycin- and erythromycin-sensitive isolates (missing *Nm'* and *Em'*, respectively) were selected to identify fully segregated cells (Fig. 1). The *nifUHDK*-ORF region of the dc-variant genomes was confirmed by PCR with the primer pairs *nifSUH-F/nifSUH-R* and

109 ORFnifE-F/ORFnifE-R [7] (data not shown). These two dc-variants were designated
110 dc-Q193S and dc-R284H, respectively. In some experiments, the sc-AnNif Δ Hup strain
111 (previously denoted AnNif Δ Hup) containing the wild-type version of *nifD*[7] was used for
112 comparison.

113 **2.3. H₂ accumulation and determination of H₂ production and acetylene reduction** 114 **activities**

115 For assays of cumulative H₂ production, cells were grown in AA/8-plus-nitrate medium while
116 bubbling with air under illumination, washed, and suspended in BG11₀ medium (without
117 added nitrate) [20] that was supplemented with 5
118 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-KOH buffer (pH 8.2).
119 Portions of the cultures (8 ml, containing 30 or 60 μ g of Chl *a*) were transferred into 25-ml
120 serum bottles that were sealed with butyl rubber stoppers. The bottles were flushed with Ar
121 or N₂, and CO₂ was added to a final concentration of 5%. Where indicated, N₂ also was
122 added to the Ar-based gas to provide final concentrations of 1.2-24%. The flasks were
123 illuminated with PAR of 90-100 μ mol photons m⁻² s⁻¹ from cool white fluorescent lamps on a
124 rotary shaker (80-90 rpm) at 28°C. Triplicate samples were prepared for each assay of
125 different gas composition. The concentration of H₂ accumulated in the headspace was
126 measured by gas chromatography (GC) using a GC-2010 Plus (Shimadzu Co., Ltd., Kyoto,
127 Japan) equipped with a thermal conductivity detector and a Rt-Msieve 5A PLOT capillary
128 column (I.D. 0.53 mm x 50 m, Restek Co., Ltd., USA), and the headspace gas was renewed
129 to the initial gas composition every 3 to 4 days as indicated in Fig. 2 and 3.

130 Photobiological activities of proton reduction to H₂ and acetylene reduction to
131 ethylene and ethane were measured essentially as described previously [7]. Portions (2 ml)
132 of the culture samples grown in air were collected after combined-nitrogen step-down at the
133 day indicated in Tables and Figures, transferred to sealed vials (9 ml) and incubated with
134 PAR of 60 to 65 μ mol photons m⁻² s⁻¹ for 1 to 1.5 h. The headspace gases in the vials were
135 replaced with Ar or N₂ for assay of H₂ production or with 12% acetylene (C₂H₂) in Ar for
136 assay of the C₂H₂ reduction. After 2-3 h of incubation under illumination, the concentrations
137 of H₂, C₂H₄ and C₂H₆ in the headspace were measured by GC as described previously [7].

138

139 **3. Results and Discussion**

140 **3.1. Effect of varying N₂ concentrations on long-term H₂ production by the** 141 **parental Δ Hup cells**

142 Prior studies with *Anabaena* sp. strain PCC 7120 Δ Hup cells incubated in closed flasks
143 containing 5% CO₂ in Ar without gas replacement resulted in continuous production of H₂ for
144 several days, with the H₂ concentration leveling off after 5-7 days [7]. Such limited timing of

hydrogen production represents a major shortcoming associated with this culture method for photobiological production of H₂. Some investigators [21, 22] working with different cyanobacterial strains showed that H₂ production could be sustained for longer times by periodic gas replacement (Ar) to relieve increased pressure of O₂ accumulated in the closed flasks, resulting in increased cumulative amounts of H₂ produced. A similar effect of gas renewal was shown in the same Δ Hup strain immobilized in Ca²⁺-alginate thin films [23]. These findings raised the question of whether renewal of the headspace gas in suspension cultures of the *Anabaena* Δ Hup strain may similarly lead to sustained H₂ production activity.

Effects of varied concentrations of N₂ on the production of H₂ over 20 days by the parental Δ Hup with periodic replacement of the headspace gas every 3 to 4 days are shown in Fig. 2A. The sample lacking added N₂ in the gas phase evolved H₂ for a shorter time of about 10 days, whereas the cultures provided with 1.2-6% N₂ continuously produced H₂ throughout the 21 days of the experiment. More precisely, the samples containing 1.8%-3% N₂ produced the largest amounts of H₂, whereas cultures containing 6% or more N₂ exhibited smaller levels of H₂ production activity (Fig. 2A). The latter effects likely relate to the formation of N₂-derived nitrogenous products leading to decreased nitrogenase activity [9, 14]. It is also probable that at high N₂ concentrations more energy is consumed for nitrogen fixation and subsequent cell growth, thus decreasing the H₂ production [24]. Interestingly, the *K_m* values for N₂ of isolated nitrogenase of *Azotobacter vinelandii* [25, 26], *Clostridium pasteurianum* and *Klebsiella pneumoniae* [27] have been reported to be 0.122 or 0.136, 0.069, and 0.099 atm, respectively. Assuming that the *Anabaena* wild-type dinitrogenase, which shares more than 70% identity in amino acid sequences with those from the other bacteria, has a similar *K_m* value (between 0.069 and 0.136 atm), the enzyme activity with the parental Δ Hup cells at 1, 3, 6 and 79% (air) N₂ is calculated to be about 13, 30, 47, and 92% (*K_m* = 0.069 atm) or 7, 18, 27 and 85% (*K_m* = 0.136 atm) of the maximal velocity (*V_{max}*), respectively. From these calculations, it seems that when N₂ concentration exceeds 6%, the N₂ fixation activity operates at around 27-47% or higher of the maximum in the wild-type cells, which leads to a drastic decline of H₂ production activity due to the sufficiency of combined nitrogen produced by the culture's nitrogenase activity. Our results in Fig. 2A and 3 also indicate that the enzymatic provision of small amounts of combined nitrogen is required for sustaining nitrogenase activity at high levels for several weeks in this strain.

The status of the cultures on day 21 was assessed by quantification of Chl *a* content (Fig. 2B). When the N₂ concentrations were less than 3%, the Chl *a* contents were found to have decreased from the initial level. The colors of these cultures were consistent with some portions of the chlorophyll being degraded (data not shown), presumably due to the nutrients being recruited to the developing heterocysts as observed in

181 phycobiliprotein degradation following nitrogen starvation [28]. In contrast, for cultures
182 provided with more than 3% N₂ the Chl a contents increased over the initial level due to cell
183 growth. Considering that the cultures containing higher contents of chlorophyll absorbed
184 more light than those with less chlorophyll during incubation in the bottles under our
185 experimental conditions, the former cultures tend to yield apparently higher H₂ productivity
186 per bottle than the latter ones. Accordingly, the optimum N₂ concentration for efficient H₂
187 production by the cultures may be lower than 2.4-3.0%.

188 In an earlier study involving N₂-starved cultures of heterocystous *Anabaena cylindrica*
189 grown with continuous gassing by CO₂ in Ar [10], periodic additions of NH₄Cl (10⁻⁴ M) were
190 shown to sustain the H₂ production activity for 7 to 19 days. This strain exhibited similar
191 effects of N₂ supply (0.2-0.8% v/v N₂ and 0.5% CO₂ in Ar) on the sustainability of H₂
192 production, but with the peak rate of H₂ production decreasing to half within about 10 days
193 [29, 30]. The decline in H₂ production for that case may arise, at least in part, from H₂ uptake
194 by hydrogenase(s), which is not the case for the Δ Hup-derived mutants described here.
195 These earlier reports highlight the importance of supplying small amounts of combined
196 nitrogen to heterocystous cyanobacterial cultures for sustained H₂ production by
197 nitrogenase-based H₂ production. In agreement with those results, sustained H₂ production
198 by the *Anabaena* Δ Hup cells can be attained by optimizing the N₂ concentration in the
199 headspace gases (Fig. 2A). Using the same *Anabaena* Δ Hup mutant, Marques et al. (2011)
200 [31] reported the results of experiments extending over 20 days that the H₂ production
201 activity of the culture was kept at lower levels by daily addition of 1% N₂ and 1% CO₂ in Ar
202 (thus resulting in daily increasing concentrations of N₂ and CO₂) than by daily addition of
203 only 1% CO₂ in Ar. Their results seem to indicate that the increased N₂ concentrations
204 decrease the H₂ production activity of the Δ Hup culture to lower levels, which is partly in
205 harmony with our results in Fig. 2A, although the regime of experiments somewhat differ
206 between the two groups. We found that the presence of low concentrations (1.2-3%) of N₂
207 help sustain the H₂ production activity at higher levels than that of the culture under gas
208 devoid of N₂ (Fig. 2A).

209

210 **3.2. Long-term H₂ production by the dc-Q193S and dc-R284H nitrogenase** 211 **variants in an N₂-rich atmosphere**

212 The *Anabaena* Δ Hup cells exhibited the desired property of continuous H₂ production for
213 prolonged time periods; however, this required careful control of the N₂ concentration at low
214 levels by diluting with Ar. We reasoned that some nitrogenase mutants of this strain would
215 fix less nitrogen and may exhibit long-term H₂ production activities even in the presence of
216 high concentrations of N₂. Previous studies had characterized over 40 sc-nitrogenase

217 mutants of this strain and identified two that produced H₂ under N₂ at rates comparable to
 218 those under Ar over 7 days without periodic gas renewal[7]. In order to assess the effects of
 219 N₂ on the sustainability of H₂ production, the corresponding dc-variants, dc-Q193S and
 220 dc-R284H, were constructed and characterized for H₂ production under incubation with
 221 N₂-rich or Ar-rich gas (Fig. 3). Although the magnitudes of the activity fluctuated among
 222 different batches of the culture of the same strains, we have consistently observed that the
 223 desirable properties of the sc-variants were essentially maintained in the dc-counterparts;
 224 i.e., in contrast with the Δ Hup cultures, the dc-mutants produced H₂ when incubated with 5%
 225 CO₂ in N₂ at rates roughly comparable to those with 5% CO₂ in Ar over long time periods
 226 when the headspace gases were renewed every 3 to 4 days. Close examination revealed
 227 subtle differences between the respective sc- and dc-counterparts: The H₂ production
 228 activity of the dc-R284H culture under N₂-rich gas was about the same level with that under
 229 Ar-rich gas through 10 days, and gradually exceeded the latter thereafter.

230 The former activity also exceeded the activity of the parental Δ Hup culture under Ar-rich
 231 gas after 10 days. The dc-Q193S culture exhibited steady, long-term H₂ productivity under
 232 N₂ while it initially exhibited a greater rate of H₂ production under Ar, but the rate was slowed
 233 within about 10 days. Compared to the parental Δ Hup culture, it showed recognizably lower
 234 long-term H₂ productivity under Ar that could be attributed to the disturbed active site
 235 environment of the catalytic Fe-Mo cofactor of dinitrogenase caused by the amino acid
 236 substitution. In contrast, both sc-variant cultures were unable to sustain H₂ production for
 237 longer than 7 days and the accumulated levels of H₂ under N₂-rich gas for 7 days were
 238 recognizably less than what was observed for the corresponding dc-counterparts (Table S1
 239 in the supplementary data). Furthermore, the sc-variants were incapable of diazotrophic
 240 growth on agar plates [7] or in liquid medium (Fig. 4), whereas the dc-Q193S variant, but not
 241 the dc-R284H variant, could grow at very low rates in either medium as measured on the
 242 basis of Chl *a* content (Fig. 4). These results indicate that the nitrogen fixation activity of the
 243 dc-Q193S culture was slightly more active under N₂-rich gas than the corresponding
 244 sc-culture, allowing the culture to obtain the combined nitrogen which was required for
 245 maintenance of its activities.

246 Differences in H₂ production activity between the sc- and dc-Q193S cultures can be
 247 ascribed to their altered capacities for synthesis of active nitrogenase. Of likely relevance,
 248 transcription of *nifB-fdxN-nifSUHDK* in *Anabaena variabilis* ATCC 29413 requires two
 249 promoters that are located upstream of *nifB* and within *nifU*; these transcripts are cleaved in
 250 the *nifU-nifH* intergenic region by posttranscriptional processing producing the
 251 *nifB-fdxN-nifSU* and *nifHDK* transcripts [32]. Also, Northern blot analysis of the unicellular
 252 cyanobacterium *Synechococcus* sp. strain RF-1 showed *nifEN*, located downstream of the

253 *nifHDK*-ORF, is expressed as a separate transcript [33]. Finally, a stem-loop structure(s) is
 254 predicted to exist in the ORF-*nifE* intergenic region of *Anabaena* PCC 7120 [34] implying that
 255 the *nifEN* transcript also may be a cleavage product of the larger transcripts. On the basis of
 256 these lines of evidence, we speculate that the differences in H₂ production activities
 257 between sc- and dc-Q193S can be explained by differences in transcription of the various *nif*
 258 genes due to variation in the length of the *nifHDK*-ORF-*nifEN* region: this portion of the
 259 sc-variant is 9.4 kb longer than that of the dc-mutant (Fig. 1). The relatively unstable
 260 transcripts of *nifE* and *nifN* are degraded more rapidly than the other *nif* genes [33], yet the
 261 corresponding products are required for the biosynthesis of FeMo-cofactor [35], so the
 262 shorter transcript of the dc-variants could more efficiently synthesize active nitrogenase
 263 proteins than the sc-counterparts. These speculations remain to be investigated. The results
 264 obtained for the two dc-mutants in Fig. 3 indicate that a small amount of combined nitrogen
 265 is required for sustaining the nitrogenase-based H₂ production activity at higher levels
 266 presumably by maintaining cell metabolism as with the parental Δ Hup strain, although the
 267 details of how the fixed nitrogen helps sustain the activity remains to be elucidated. The N₂
 268 concentrations required for optimum H₂ production, although not yet investigated in detail,
 269 appear to be lower with the dc-Q193S than with the dc-R284H.

270 Prior studies of vanadium (V) nitrogenase-expressing and H₂-uptake-deficient *A.*
 271 *variabilis* PK84 in a photobioreactor demonstrated continuous H₂ production when placed
 272 under 2% CO₂ in air for 2.5 months in the laboratory [36] and 40 days outdoors [37]. It
 273 should be noted that these long-term H₂ production studies were achieved by
 274 chemostat-type operation by diluting the growing cell cultures with fresh medium. The H₂
 275 production activity of that culture under N₂ was about half of that under Ar due to its
 276 inhibition by N₂[36]. In contrast, our site-directed variants have a potential cost advantage in
 277 sustaining H₂ production at high rates by periodic gas renewal using the less expensive N₂
 278 gas without changing the culture medium.

279

280 **3.3. Short-term assays of H₂ production and acetylene reduction by the dc-Q193S** 281 **and dc-R284H variants**

282 The *in vivo* H₂ production activities of the parental strain and the cultures
 283 containing the two dc-variant nitrogenases were measured by short term incubation with
 284 aliquots of 2- and 3-day cultures after combined-nitrogen step-down with growth in air (Table
 285 1). For both samples of the Δ Hup strain the H₂-evolving activity when assayed under N₂ was
 286 about 27% of that assayed under Ar. On the other hand, the activities of the dc-R284H and
 287 dc-Q193S cultures assayed under N₂ were about 100% and 83% of those obtained when
 288 assayed under Ar, respectively. These values are similar to those reported previously for the

289 sc-variants [7]. From these results together with Fig. 4 we conclude that the N₂-fixing
290 activities of variant nitrogenases in dc-R284H or dc-Q193S were extremely low or absent,
291 and these substituted enzymes directed nearly all the electrons to H₂ production even in the
292 presence of N₂. This finding suggests that the N₂-fixing activity, especially in the dc-R284H
293 culture, may be too low to support diazotrophic growth of the mutant cells under the
294 conditions tested.

295 The two variants and the ΔHup strain were further characterized by examining the
296 products (C₂H₄, C₂H₆, and H₂) obtained after incubation in an acetylene gas mixture (12%
297 C₂H₂ in Ar) after growing under air for two or three days after nitrogen step-down (Table 2).
298 The acetylene reduction assay has been widely used as a convenient surrogate for
299 assessing the nitrogenase activity of the cultures. Although it is generally accepted that the
300 conventional molybdenum (Mo)-containing nitrogenase reduces acetylene only to ethylene,
301 while V-containing and Fe-only nitrogenases reduce acetylene to both ethylene and ethane
302 [38, 39], we had previously reported the production of ethane by several of the sc-mutants
303 [7]. The parental cyanobacterial strain *Anabaena* sp. strain PCC 7120 only encodes the
304 Mo-nitrogenase according to whole genome analysis [34]. Notably, the dc-Q193S culture
305 produced ethane at low but measurable rates along with greater rates of ethylene
306 production, while neither the ΔHup nor dc-R284H cultures produced measurable ethane.
307 The percentages of electron allocation to each gas product were similar for the 2-day and
308 3-day cultures grown under air within the respective strains. The parental ΔHup strain with
309 the normal protein environment surrounding the FeMo-cofactor reduced C₂H₂ at much
310 greater rates in air-grown cultures subjected to 2 days of nitrogen step-down, but lower rates
311 were observed in cultures after 3 days. By contrast, the dc mutants with alterations of the
312 protein environment near the FeMo-cofactor exhibited similar C₂H₂ reduction rates for both
313 periods of nitrogen deprivation. All strains produced H₂ at much lower rates when assayed in
314 the presence of C₂H₂ compared to those when assayed with N₂ (Table 1). However, it should
315 be noted that the proportions of electrons allocated to H₂ in the presence of C₂H₂ by the
316 dc-Q193S and dc-R284H cultures were greater than that of the ΔHup strain. This result
317 illustrates that the total nitrogenase activities of the variants are underestimated by the
318 conventional acetylene reduction assay. A comparison of the total electron utilization by
319 nitrogenase for the 2 day and 3 day samples assayed in the presence of C₂H₂ revealed that
320 the activity of the ΔHup strain declined rapidly, whereas electron utilization by the two
321 dc-variants was much more stable, as seen also in H₂ production activities under Ar and N₂.
322 This observation is explained by sufficiency and insufficiency of fixed-nitrogen in the
323 air-grown cultures of the ΔHup strain and the two variant cultures, respectively.

324

325 **4. Conclusions**

326 We have shown that two site-directed mutants of a heterocyst-forming cyanobacterium with
327 amino acid substitutions presumed to be located in the vicinity of the catalytic FeMo-cofactor
328 of nitrogenase rapidly produced H₂ under N₂-rich gas at higher sustained rates than under
329 Ar-rich gas over 3 weeks, in contrast to the parental Δ Hup strain which requires low N₂
330 concentration for prolonged H₂ production. Both of the sc- and dc-variants have desirable
331 properties in which their H₂ production activities under N₂ are comparable to those under Ar.
332 It is noteworthy that diazotrophic growth capability was seen in the dc-Q193S variant,
333 although at very low levels, but not in the sc-variant. In contrast, the dc-R284H variant did
334 not show any diazotrophic growth. The extremely low diazotrophic growth capability could
335 allow the dc-Q193S variant to supply itself with a small amount of combined nitrogen which
336 is required for maintenance of its cell metabolism, leading to long-term, higher H₂ production.
337 This property of N₂ insensitivity of the variants is a potentially useful strategy for reducing the
338 cost of the culture gas in future practical applications of sustainable biofuel production.

339

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346

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442

443 **Figure legends**

444 **Fig. 1.** Organization of the *Anabaena* sp. PCC 7120 *nif* gene cluster for sc- and dc-mutants
445 with site-directed substitutions in *nifD*. The sc-mutant chromosome contains 8.2-kb of
446 inserted sequence (line with arrows) and a 1.2-kb duplicated region (horizontal grey bars)
447 between *nifK* and *nifE*. These components were completely segregated in the dc-mutant
448 chromosome, thus eliminating the genes conferring sucrose sensitivity and antibiotic
449 resistance. The dots indicate gaps in the DNA for the two panels, with the lengths of the
450 omitted sequences provided. The wild-type *nif* gene cluster has two excision elements: a
451 59.4-kb element in *fdxN* and an 11.3-kb element in *nifD* [34]; however, the latter element is
452 absent in the parental Δ Hup strain [7]. Bent arrows show the putative transcription start
453 sites (upstream of *nifB* and within *nifU*) and their orientations of transcription [32].

454
455 **Fig. 2.** Cumulative H₂ production (A) and Chl *a* contents (B) of the parental Δ Hup culture in
456 the presence of varied N₂ concentration in Ar and 5% CO₂. The cultures were incubated
457 under continuous illumination (90-100 μ mol photons m⁻² s⁻¹ of PAR) at 28°C for 21 days
458 and the headspace gas was renewed with the respective initial gases every 3 to 4 days at
459 the times indicated by the vertical arrows. The initial Chl *a* content of the cultures at the
460 start of incubation was 30 μ g per vial, indicated by the horizontal dashed line in Fig. 2B,
461 and the contents shown were assessed after 21 days. Values are average $\pm$ the standard
462 deviation of triplicate samples.

463
464 **Fig. 3.** Effects of N₂ on sustained H₂ production by the two dc-variants and the parental
465 Δ Hup culture. Cultures of two site-directed dc-variants, dc-Q193S (squares) and dc-R284H
466 (triangles), and the parental Δ Hup strain (circles) were incubated under 5% CO₂ in Ar
467 (dashed line, open symbols) or under 5% CO₂ in N₂ (solid lines, closed symbols) for 21
468 days with periodic gas replacement at the times indicated by the vertical arrows. Values are
469 averages of two or three independent experiments, each performed with triplicate samples.
470 The initial Chl *a* contents of the cultures were 60 μ g per vial. H₂ production activities of the
471 two dc-variants under 5% CO₂ in N₂ were sustained at least for a total period of about one
472 month.

473
474 **Fig. 4 Diazotrophic growth curves after combined-nitrogen step-down.**

475 Cultures were inoculated from nitrate-grown cultures into AA/8 medium lacking combined
476 nitrogen and incubated on a rotary shaker under a light intensity of 20-30 μ mol photons m⁻²
477 s⁻¹ of PAR. The Chl *a* content was monitored as the cultures grew. The parental Δ Hup
478 strain (closed circle) along with the dc-Q193S (closed square) and ds-R284H (closed

479 triangle) variants are shown by solid lines while the sc-AnNif Δ Hup strain (open circle)
480 containing the wild-type version of *nifD* and the sc-Q193S (open square) and sc-R284H
481 (open triangle) variants are shown by dashed lines. A replicate experiment provided
482 analogous results.

Table 1 H₂ production rates of the two dc-variants and the parental Δ Hup strain assayed under Ar or N₂

Strain	H ₂ production activities ($\mu\text{mol mg of Chl } a^{-1} \text{ h}^{-1}$) $\pm$ SD			
	under Ar		under N ₂	
	2 days	3 days	2 days	3 days
Δ Hup	25 $\pm$ 8	8.6 $\pm$ 2.6	6.8 $\pm$ 2.3	2.3 $\pm$ 0.8
dc-Q193S	35 $\pm$ 5	36 $\pm$ 7	29 $\pm$ 5	30 $\pm$ 5
dc-R284H	29 $\pm$ 12	23 $\pm$ 8	29 $\pm$ 10	23 $\pm$ 9

Cultures were grown in air on a rotary shaker for 2 or 3 days after nitrogen deprivation and aliquots of the cultures were transferred to the closed flasks for assays under Ar or N₂ for 2-3 hours in the light. Values are averages of three to five independent experiments, each performed with duplicate samples.

Table 2 Composition of gas products when assayed in the presence of 12% C₂H₂ in Ar and the calculated total electron utilization for the two dc-variants and the parental ΔHup strain

Reduction activities in the presence of acetylene ($\mu\text{mol mg of Chl } a^{-1} \text{ h}^{-1}$) $\pm$ SD												Calculated total electron utilization		
Products														
C_2H_4				C_2H_6				H_2						
Strain	2 days		3 days		2 days		3 days		2 days		3 days		2 days	3 days
ΔHup	24 $\pm$ 9	(96%)	8.1 $\pm$ 2.5	(93%)	ND		ND		1.0 $\pm$ 0.5	(3.9%)	0.61 $\pm$ 0.06	(6.9%)	50	17
dc-Q193S	8.8 $\pm$ 0.6	(56%)	10 $\pm$ 2	(57%)	0.078 $\pm$ 0.008	(0.49%)	0.091 $\pm$ 0.016	(0.52%)	6.8 $\pm$ 1.2	(43%)	7.2 $\pm$ 1.0	(42%)	32	35
dc-R284H	17 $\pm$ 5	(81%)	13 $\pm$ 5	(80%)	ND		ND		4.0 $\pm$ 0.6	(19%)	3.1 $\pm$ 0.8	(20%)	42	32

Conditions of culture growth and activity measurements were as described in Table 1, except that gas phase was C₂H₂ (12%) in Ar for the acetylene reduction assay. Values are averages of three to five independent experiments, each performed with duplicate samples. For calculation of the electron utilization associated with each product, C₂H₄, C₂H₆, and H₂ require 2, 4, and 2 electrons, respectively, and the total utilization of electrons was calculated by summing all of these values. The percentages of the total electrons allocated to each product in the presence of 12% C₂H₂ in Ar are given in parentheses.

Figure 1

Single-crossover (sc) mutant chromosome with mutation in *nifD*

Double-crossover (dc) mutant chromosome with mutation in *nifD*

Figure 2

A

B

Figure 3

Figure 4