1 **Disrupting Calvin cycle phosphoribulokinase activity in *Rhodopseudomonas***
2 ***palustris* increases the H₂ yield and specific production rate proportionately**

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4 Alexandra L. McCully and James B. McKinlay#

5 Department of Biology, Indiana University, Bloomington, Indiana

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7 Address: 1001 E 3rd Street, Jordan Hall, Bloomington, IN 47405, USA

8

9 #Corresponding author:

10 James B. McKinlay

11 jmckinla@indiana.edu

12 Ph: 812-855-0359

13 Fax: 812-855-6705

ABSTRACT

Anoxygenic phototrophs, like *Rhodopseudomonas palustris*, can convert light energy and electrons from organic waste into H₂ gas, a potential biofuel. During phototrophic growth on organic compounds the CO₂-fixing Calvin cycle competes against H₂ production for electrons. Here we address why genetically disrupting the CO₂-fixing enzyme ribulose 1,5-bisphosphate carboxylase (Rubisco), increases the H₂ yield but not the specific H₂ production rate. We hypothesized that remaining upstream phosphoribulokinase (PRK) activity negatively impacts growth and thereby the specific H₂ production rate, likely due to the accumulation of ribulose-1,5-bisphosphate, the substrate for Rubisco. In agreement with our hypothesis, deletion of PRK resulted in proportional increases to both the H₂ yield and the specific production rate. Thus, even though Rubisco is traditionally a more common target to eliminate Calvin cycle activity we propose PRK as a favorable alternative to avoid undesirable pleiotropic effects.

Keywords: hydrogen, *Rhodopseudomonas palustris*, Calvin cycle, Rubisco, phosphoribulokinase

1. INTRODUCTION

Hydrogen gas (H₂) is an important commodity chemical and an attractive fuel that can be produced from renewable resources using microbes (1–3). Purple nonsulfur bacteria, like *Rhodopseudomonas palustris*, can convert light energy and electrons from organic compounds into H₂ using the enzyme, nitrogenase (Eq. 1) (2). In the absence of N₂, nitrogenase serves as an ATP-powered hydrogenase, producing pure H₂ gas (Eq 2).

39 Normally, fixed nitrogen compounds, such as NH_4^+ , inhibit nitrogenase expression and
40 therefore H_2 production, making it difficult to achieve Eq. 2. However, *R. palustris* NifA*
41 strains express nitrogenase and produce H_2 constitutively due to mutations in the Q-
42 linker region of NifA, the master transcriptional activator of nitrogenase genes (4, 5).
43 Thus, *R. palustris* NifA* strains produce H_2 via Eq. 2 when incubated under an argon
44 headspace with NH_4^+ as the nitrogen source (4, 5).

45 When *R. palustris* grows phototrophically on organic substrates
46 (photoheterotrophic growth), it reduces electron carriers like NAD(P)H using many of the
47 same pathways found in non-photosynthetic heterotrophs (e.g., the TCA cycle). To
48 maintain electron balance, photoheterotrophs must couple electron carrier oxidation to
49 reductive pathways such as biosynthetic reactions, CO_2 fixation via the Calvin cycle, and
50 H_2 production via nitrogenase (Fig. 1). All electrons consumed are accounted for in
51 biomass and H_2 (5, 6). The Calvin cycle can compete against H_2 production for
52 electrons, making it a logical target for disruption to divert more electrons to H_2 (6).
53 Indeed, genetic disruption of the CO_2 -fixing Calvin cycle enzyme, ribulose 1,5-
54 biphosphate carboxylase (Rubisco) resulted in higher H_2 yields (H_2 produced per
55 substrate consumed) from multiple organic substrates during photoheterotrophic growth
56 by *R. palustris* (6). These H_2 yield increases could be predicted from the Calvin cycle
57 fluxes in the parent strain, which were determined using ^{13}C -labeling experiments (6).
58 However, this $\Delta\text{Rubisco}$ mutant did not show a similar increase in the specific H_2
59 production rate (H_2 produced per cell per h; from hereon simply referred to as H_2
60 production rate) (5). Our hypothesis at the time for the disparate effects on H_2 yield and
61 rate was that a low nitrogenase catalytic rate could not fully compensate for the loss of
62 electron carrier oxidation via the Calvin cycle (Fig. 1, Model 1).

63 $\Delta\text{Rubisco}$ mutants of *R. palustris* and other purple nonsulfur bacteria, like
64 *Rhodospirillum rubrum*, have slower growth rates than parental strains with an intact

Calvin cycle (5–8). Recently it was shown that this slower growth was largely due to phosphoribulokinase (PRK) activity (7, 8), the enzyme directly upstream of Rubisco in the Calvin cycle (Fig. 1). A *Rs. rubrum* Δ Rubisco mutant, with remaining PRK activity, accumulated the Rubisco substrate, ribulose 1,5-bisphosphate (RuBP) (8). Genetically disrupting PRK in a Δ Rubisco mutant of either *R. palustris* or *Rs. rubrum* restored the growth rate to parental levels (7, 8). Eliminating the activity of another Calvin cycle enzyme upstream of PRK (fructose bisphosphatase) also alleviated the growth defect in a *Rs. rubrum* Δ Rubisco mutant and restored wild-type RuBP levels (8). These results suggest that RuBP has a toxic effect on growth.

Since the H₂ production rate is largely dependent on the growth rate we hypothesize herein that PRK activity also limits the H₂ production rate in a Δ Rubisco mutant of *R. palustris* (Fig. 1, Model 2), either in concert with Model 1 or separately from it. To test this hypothesis, we looked at the effects of PRK activity on the H₂ yield, the H₂ production rate, and the growth rate. In support of this hypothesis, disrupting PRK activity resulted in proportional increases to both the H₂ yield and production rate. We propose that PRK is a favorable target over Rubisco to improve H₂ production while avoiding negative physiological side effects.

2. MATERIALS AND METHODS

2.1. Strains and growth conditions. All experiments were performed with a NifA⁺-derivative (5) of *R. palustris* CGA009 (9) with constitutively active nitrogenase (Table 1). *R. palustris* was grown anaerobically in 16-mL volumes of a chemically defined medium with NH₄⁺ (PM) (10) in sealed 27-mL tubes under an argon headspace at 30°C in front of a 60 W light bulb. Disodium succinate, sodium acetate, or sodium butyrate were supplied to a final concentration of 40 mM carbon (i.e., 10 mM succinate, 20 mM acetate, and 10 mM butyrate). When appropriate, kanamycin and gentamicin were each

used at a final concentration of 50 µg/mL for *R. palustris*. Antibiotics were used for starter cultures and for experiments with plasmid-bearing strains but were omitted from other experiments. *Escherichia coli* was grown aerobically in LB broth with gentamicin (15 µg/mL) when appropriate.

2.2. *R. palustris* strain construction. All strains and plasmids used in this study are listed in Table 1 and Table 2 respectively. pBBPgdh empty vector (5) and pBBPcbbP expressing *R. palustris* cbbP (7) were introduced into *R. palustris* via conjugation with *E. coli* S17 (11). Briefly, *E. coli* S17 plasmid donor and *R. palustris* recipient were grown to mid-exponential phase in their respective media, washed and resuspended in PM, and then mixed at a 2:1 v/v ratio of *R. palustris* : *E. coli*. The mixed cells were then spotted to a nutrient agar plate and incubated upright at 30°C overnight. The plate was then inverted and incubated at 30°C for 24 h. The spot was then streaked to PM agar with 50 µg/mL gentamicin. Presence of the correct plasmid in *R. palustris* colonies was confirmed using PCR.

2.3. Analytical techniques. Cell density was measured by optical density at 660 nm (OD₆₆₀) with a Genesys 20 visible spectrophotometer (Thermo-Fisher, Pittsburgh, PA). Specific growth rates were determined using at least three measurements in exponential phase between 0.05 and 0.8 OD₆₆₀. H₂ was sampled upon culture inoculation, periodically throughout exponential growth and in early stationary phase. As before (5), we found H₂ production to be linearly correlated with cell density during exponential growth. H₂ was quantified by sampling culture headspace with a gas-tight syringe and injecting into a Shimadzu GC-2014 gas chromatograph with a thermal conductivity detector as described (12). Organic acids were quantified from culture supernatants sampled at the start of experiments and early in stationary phase when the carbon source was exhausted using a Shimadzu 20A high-performance liquid chromatograph with a UV detector (13). Total protein measurements for calculating specific H₂

production rates were assayed using a Pierce BCA Protein Assay Kit as described (5). OD₆₆₀ values were converted into protein concentrations using standard curves that we established for every strain on every carbon source used. H₂ yields were calculated from the change in H₂ divided by the change in substrate consumed between the start of the experiment and at a time point in stationary early phase when the carbon source was exhausted and H₂ production had stopped. Specific H₂ production rates were determined by linear regression analysis of H₂ versus cell density during exponential phase, then multiplying the slope ($\mu\text{mol H}_2 \times \text{mg protein}^{-1}$) by the growth rate (h^{-1}) according to the Monod model as described (14). Substrate oxidation states were determined as described (6, 15). Biological replication is indicated in each figure legend.

3. RESULTS

It was previously observed that an *R. palustris* NifA* Δ Rubisco mutant exhibited a higher H₂ yield than the NifA* parent but the H₂ production rate remained unchanged (5). It was originally thought that the nitrogenase reaction rate was unable to fully compensate for the loss of the Calvin cycle as an electron sink, thereby limiting both the H₂ production rate and the growth rate (Fig. 1, Model 1) (5). More recently it was shown that eliminating PRK activity, the enzyme upstream of Rubisco (Fig. 1), restored the growth rate of the NifA* Δ Rubisco mutant to near-parental levels as long as electron balance could be maintained via H₂ production (7). Thus, an alternative model exists wherein PRK activity impedes the H₂ production rate (Fig. 1, Model 2).

To identify contributions of each model towards the inhibition of the H₂ production rate we first quantified growth rates, H₂ yields, and production rates for the NifA* parent strain and three Calvin cycle mutants, lacking either or both Rubisco and PRK, during phototrophic growth. To potentially highlight a possible rate-limiting effect of electron carrier oxidation (Fig. 1, Model 1) we collected this data during growth on three

substrates that have different oxidation states (+/-) and are known to impact the H₂ yield by *R. palustris* (6): succinate (+0.5), acetate (0), and butyrate (-1). Representative growth curves for these batch cultures are shown in Figure 2.

The general trends for each measured parameter were similar across all substrates, though differing in magnitude (Fig. 3). Calvin cycle mutations resulted in a higher H₂ yield regardless of whether Rubisco, PRK, or both activities were disrupted (Fig. 3A). The NifA*ΔRubisco mutant had a lower growth rate than the parent strain for each substrate tested whereas mutants lacking PRK had similar growth rates to the parent strain (Fig. 3B). Finally, the highest H₂ production rates were observed whenever PRK activity was disrupted (Fig. 3C).

These results indicate that PRK activity has a negative effect on growth and H₂ production rates, regardless of the substrate oxidation state. The largest increases in the H₂ production rate relative to the NifA* strain were observed in the NifA*ΔPRK and NifA*ΔRubiscoΔPRK mutants during growth on butyrate (Fig. 3C), the most electron rich substrate supporting the slowest growth rate (Fig. 3B). This trend suggests that PRK activity was the primary rate-inhibiting factor (Model 2), rather than limiting electron carrier oxidation (Model 1).

However, the two models are not mutually exclusive. While the data in Figure 3 points to an inhibiting role of PRK activity it does not rule out an additional contribution of limiting electron carrier oxidation to the phenotype. Furthermore, the two models could be connected, as NADH has been shown to stimulate PRK activity in purple nonsulfur bacteria (16).

To gage the relative impacts of each model on the H₂ production rate we considered how much the rate could be expected to increase upon the loss of the Calvin cycle. Using Figure 1 as a guide, we assumed that there is a fixed electron demand from biosynthesis and that electron balance is maintained by a flexible distribution of

electrons between H₂ production and the Calvin cycle, in accordance with previous results (6). If nitrogenase cannot fully compensate for the elimination of electron flow to the Calvin cycle (e.g., rate-limiting nitrogenase activity; Fig. 1, Model 1) then central metabolism would slow to match the combined oxidation rates of nitrogenase and biosynthesis. Thus, the H₂ yield would increase in the absence of Rubisco but the rate would still be limited by nitrogenase activity. However, if the nitrogenase reaction rate can fully compensate for the elimination of electron flow to the Calvin cycle (exclusively Model 2 with expansion of nitrogenase activity) then it leaves PRK activity as the sole inhibitory factor. In this case, elimination of PRK should relieve the inhibition and the H₂ production rate should scale proportionately to the yield.

To address whether H₂ yields and rates increase proportionately with the loss of PRK activity we transformed the data from Figure 3 to compare fold increases in H₂ yield versus rate in the NifA* Calvin cycle mutants over the NifA* parent values (Fig. 4). For the NifA*ΔRubisco mutant, the fold increase in the H₂ yield was significantly more than that for H₂ production rate during growth on every carbon source (Fig. 4). The discrepancy between the fold increase in H₂ yield and rate for NifA*ΔRubisco mutant does not widen when more reduced substrates are provided (Fig. 4). However, for both the NifA*ΔPRK and NifA*ΔRubiscoΔPRK mutants, the fold-increase in the H₂ yield and production rate were proportional on all carbon sources (Fig. 4). These proportional increases, regardless of the substrate oxidation state, suggest that inhibition of the H₂ production rate in the NifA*ΔRubisco mutant was due entirely to PRK activity (Fig. 1; Model 2).

To verify that the ΔPRK mutant trends were not due to an unanticipated consequence of the *cbbP* deletion construct and to examine whether additional PRK expression inhibits growth and H₂ production rates we expressed the native *cbbP* gene from a constitutive expression vector. Since we observed similar trends on all substrates

using vector-less strains (Fig. 3) we limited this current analysis to growth on succinate. Expressing PRK from a plasmid had no effect on the growth rate, H₂ yield, or production rate in the NifA* parent nor the NifA*ΔRubisco mutant (Fig. 5). Expressing PRK partially complemented the NifA*ΔPRK H₂ yield and fully complemented H₂ production rate (Fig 5A and 5C). A positive effect on the growth rate was observed when expressing PRK in the NifA*ΔPRK mutant, as was observed previously (7). Expressing PRK had no effect on the NifA*ΔRubisco H₂ yield as was expected because the ΔRubisco mutation still left the Calvin cycle disrupted (Fig. 5A). However, expressing *cbbP* had a detrimental effect on the NifA*ΔRubiscoΔPRK growth rate and H₂ production rate, partially complementing the ΔPRK mutation in this strain (Fig 5B and 5C). Thus, the inhibitory effects we observed were due to PRK activity rather than an artifact of the genetic constructs. However, expressing PRK from our plasmid did not have an additional inhibitory effect on strains having a chromosomal *cbbP* gene.

4. DISCUSSION

We have shown that PRK activity, in the absence of Rubisco, negatively affects growth, and in turn, the H₂ production rate. The inhibitory effect of PRK activity is unknown but likely involves RuBP accumulation. A *Rs. rubrum* ΔRubisco mutant grew at less than a third of the wild-type growth rate and exhibited RuBP levels that were 100-fold higher than the wild-type (8). Expression of spinach PRK in *E. coli*, which does not have the Calvin cycle, also inhibited growth (17). Rubisco and PRK are the only enzymes known to catalyze reactions involving RuBP as a substrate or product (18). Thus, it seems likely that in the absence of Rubisco, PRK can generate RuBP levels that adversely affect growth, perhaps by competing against a preferred substrate in an enzyme active site or by acting as an allosteric enzyme inhibitor.

An alternative explanation for our results is that PRK activity inhibits nitrogenase, which would directly impede H₂ production and indirectly impede growth by limiting the rate of electron carrier oxidation. However, this hypothesis is unlikely since PRK activity had a negative effect on *E. coli* growth (17), and *E. coli* does not have nitrogenase. Thus, the inhibitory effect of PRK activity is likely associated with a common feature between purple non-sulfur bacteria and *E. coli*.

Expressing PRK from a plasmid partially or fully complemented the effects of Δ PRK mutations on growth rate, H₂ yield, and H₂ production rate (Fig. 5). These results indicate that functional PRK was expressed from our plasmid. It is therefore curious that expressing PRK from a plasmid did not negatively affect the growth rate or H₂ production rate of strains encoding PRK in the genome (Fig. 5). For the NifA* parent, it is possible that Rubisco can compensate for increased PRK activity or that the substrate for PRK was limiting, such that increasing the PRK level had little effect on the reaction rate. For the NifA* Δ Rubisco mutant it is possible that there is an upper limit to the toxic effects of RuBP accumulation or that high RuBP levels feed back to inhibit PRK activity, as has been shown for PRK from *Synechococcus* (19).

Rubisco is often considered to be synonymous to the Calvin cycle, yet it is only one of four enzymes that are unique to the pathway. When disrupting the Calvin cycle, either to increase H₂ production or to understand its role in cell physiology, researchers tend to target Rubisco (5, 6, 20–23). From this current study and others (7, 8), it is apparent that PRK activity in the absence of Rubisco can have pleiotropic effects. PRK is also unique to the Calvin cycle and disrupting its activity is sufficient to eliminate Calvin cycle flux (7, 8, 24, 25). The substrate for PRK, ribulose-5-phosphate, can be processed by common pentose phosphate pathway enzymes, likely explaining the lack of negative effects associated with PRK mutants. Thus to avoid pleiotropic effects of PRK activity and to reap the benefits of proportional increases to both H₂ yield and

production rate, we propose that PRK is a more suitable target to disrupt Calvin cycle flux than Rubisco.

5. CONCLUSIONS

We have shown that the H₂ production rate in the *R. palustris* NifA* Δ Rubisco mutant is hindered by PRK activity (Model 2) rather than by a diminished capacity for electron carrier oxidation (Model 1). Proportional increases in both the H₂ yield and production rate in PRK mutants show that nitrogenase reaction rate can fully compensate for the loss of electron carrier oxidation by the Calvin cycle. PRK is therefore an attractive target to increase both the H₂ yield and production rate.

ABBREVIATIONS

PRK: phosphoribulokinase

Rubisco: ribulose-1,5-bisphosphate carboxylase

RuBP: ribulose-1,5-bisphosphate

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REFERENCES

1. Nath K, Das D. 2004. Improvement of fermentative hydrogen production: various approaches. *Appl. Microbiol. Biotechnol.* 65:520–9.
2. McKinlay JB, Harwood CS. 2010. Photobiological production of hydrogen gas as a biofuel. *Curr. Opin. Biotechnol.* 21:244–251.
3. Turner J, Sverdrup G, Mann MK, Maness P-C, Kroposki B, Ghirardi M, Evans RJ, Blake D. 2008. Renewable hydrogen production. *Int. J. Energy Res.* 32:379–407.
4. Rey FE, Heiniger EK, Harwood CS. 2007. Redirection of metabolism for biological hydrogen production. *Appl. Environ. Microbiol.* 73:1665–1671.
5. McKinlay JB, Harwood CS. 2010. Carbon dioxide fixation as a central redox cofactor recycling mechanism in bacteria. *Proc. Natl. Acad. Sci. U S A* 107:11669–11675.
6. McKinlay JB, Harwood CS. 2011. Calvin cycle flux, pathway constraints, and substrate oxidation state together determine the H₂ biofuel yield in photoheterotrophic bacteria. *mBio* 2:10.1128/mBio.00323–10.
7. Gordon GC, McKinlay JB. 2014. Calvin cycle mutants of photoheterotrophic purple non-sulfur bacteria fail to grow due to an electron imbalance rather than toxic metabolite accumulation. *J. Bacteriol.* 196:1231–1237.
8. Wang D, Zhang Y, Pohlmann EL, Li J, Roberts GP. 2011. The poor growth of *Rhodospirillum rubrum* mutants lacking RubisCO is due to the accumulation of ribulose-1,5-bisphosphate. *J. Bacteriol.* 193:3293–3303.

- 294 9. Larimer FW, Chain P, Hauser L, Lamerdin J, Malfatti S, Do L, Land ML, Pelletier
295 DA, Beatty JT, Lang AS, Tabita FR, Gibson JL, Hanson TE, Bobst C, Torres JL,
296 Peres C, Harrison FH, Gibson J, Harwood CS. 2004. Complete genome
297 sequence of the metabolically versatile photosynthetic bacterium
298 *Rhodopseudomonas palustris*. Nat. Biotechnol. 22:55–61.
- 299 10. Kim M, Harwood CS. 1991. Regulation of benzoate-CoA ligase in
300 *Rhodopseudomonas palustris*. FEMS Microbiol Lett 83:199–203.
- 301 11. Simon R, Priefer U, Pühler A. 1983. A broad host range mobilization system for in
302 vivo genetic engineering: transposon mutagenesis in gram negative bacteria.
303 Bio/Technology 1:784–791.
- 304 12. Huang JJ, Heiniger EK, McKinlay JB, Harwood CS. 2010. Production of hydrogen
305 gas from light and the inorganic electron donor thiosulfate by *Rhodopseudomonas*
306 *palustris*. Appl. Environ. Microbiol. 76:7717–7722.
- 307 13. McKinlay JB, Zeikus JG, Vieille C. 2005. Insights into *Actinobacillus succinogenes*
308 fermentative metabolism in a chemically defined growth medium. Appl. Environ.
309 Microbiol. 71:6651–6656.
- 310 14. McKinlay JB, Shachar-Hill Y, Zeikus JG, Vieille C. 2007. Determining
311 *Actinobacillus succinogenes* metabolic pathways and fluxes by NMR and GC-MS
312 analyses of ¹³C-labeled metabolic product isotopomers. Metab. Eng. 9:177–192.
- 313 15. Gottschalk G. 1986. Bacterial Metabolism. Springer New York, New York, NY.
- 314 16. Tabita FR. 1980. Pyridine nucleotide control and subunit structure of
315 phosphoribulokinase from photosynthetic bacteria. J. Bacteriol. 143:1275–80.
- 316 17. Hudson G, Morell M, Arvidsson Y, Andrews T. 1992. Synthesis of spinach
317 phosphoribulokinase and ribulose 1,5-bisphosphate in *Escherichia coli*. Aust. J.
318 Plant Physiol. 19:213.
- 319 18. Caspi R, Altman T, Billington R, Dreher K, Foerster H, Fulcher CA, Holland TA,
320 Keseler IM, Kothari A, Kubo A, Krummenacker M, Latendresse M, Mueller LA,

321 Ong Q, Paley S, Subhraveti P, Weaver DS, Weerasinghe D, Zhang P, Karp PD.
322 2014. The MetaCyc database of metabolic pathways and enzymes and the
323 BioCyc collection of Pathway/Genome Databases. *Nucleic Acids Res.* 42:D459–
324 71.

325 19. Wadano A, Nishikawa K, Hirahashi T, Satoh R, Iwaki T. Reaction mechanism of
326 phosphoribulokinase from a cyanobacterium, *Synechococcus* PCC7942.
327 *Photosynth. Res.* 56:27–33.

328 20. Wang D, Zhang Y, Welch E, Li J, Roberts GP. 2010. Elimination of Rubisco alters
329 the regulation of nitrogenase activity and increases hydrogen production in
330 *Rhodospirillum rubrum*. *Int. J. Hydrogen Energy* 35:7377–7385.

331 21. Hemschemeier A, Fouchard S, Cournac L, Peltier G, Happe T. 2008. Hydrogen
332 production by *Chlamydomonas reinhardtii*: an elaborate interplay of electron
333 sources and sinks. *Planta* 227:397–407.

334 22. Marín-Navarro J, Esquivel MG, Moreno J. 2010. Hydrogen production by
335 *Chlamydomonas reinhardtii* revisited: Rubisco as a biotechnological target. *World*
336 *J. Microbiol. Biotechnol.* 26:1785–1793.

337 23. Farmer RM, Laguna R, Panescu J, McCoy A, Logsdon B, Zianni M, Moskvina O V,
338 Gomelsky M, Tabita FR. 2014. Altered residues in key proteins influence the
339 expression and activity of the nitrogenase complex in an adaptive CO₂ fixation-
340 deficient mutant strain of *Rhodobacter sphaeroides*. *Microbiology* 160:198–208.

341 24. Gharibi H, Atikol U, Öztürk Y, Gökçe A, Peksel B, Gürkan M, Özgür E, Gündüz U,
342 Eroğlu İ, Yücel M. 2012. Hydrogen production properties of *Rhodobacter*
343 *capsulatus* with genetically modified redox balancing pathways. *Int. J. Hydrogen*
344 *Energy* 37:2014–2020.

345 25. Hallenbeck PL, Lerchen R, Hessler P, Kaplan S. 1990. Phosphoribulokinase
346 activity and regulation of CO₂ fixation critical for photosynthetic growth of
347 *Rhodobacter sphaeroides*. *J. Bacteriol.* 172:1749–61.

Figure Legends

Fig 1. Two models can explain the relatively low specific H₂ production rate in Rubisco mutants despite a higher H₂ yield. An *R. palustris* NifA* strain consumes organic acids and generates reduced electron carriers (e.g., NADH). H₂ production via nitrogenase, biosynthetic reactions, and the Calvin cycle replenish oxidized electron carrier pools to maintain metabolic flow. There are no other major electron sinks (5, 6). In **Model 1**, the rate of NADH oxidation by nitrogenase does not increase to compensate for the loss of Rubisco activity (red Δ). A slower rate of NADH oxidation in turn leads to slower central metabolism, growth, and H₂ production. In **Model 2**, loss of Rubisco activity leads to an accumulation of RuBP. RuBP negatively affects central metabolism and in turn results in slower growth and H₂ production, even though the nitrogenase rate is capable of increasing (light blue region around nitrogenase arrow). The two models are not mutually exclusive and were tested with the consideration that both could contribute to the low H₂ production rate exhibited by ΔRubisco mutants. Solid arrows represent specific metabolic rates (e.g., μmol x mg protein⁻¹ x h⁻¹) for electron flow (blue) or carbon flow (black). Dashed arrows indicate negative feedback.

Fig 2. Growth curves for H₂-producing NifA* Calvin cycle mutants and the NifA* parent. Batch cultures were grown phototrophically with either succinate (A), acetate (B), or butyrate (C), which differ in their oxidation state. The oxidation states are: succinate, +0.5; acetate, 0; butyrate, -1. Representative values from single biological replicates are shown. Similar trends were observed for at least four biological replicates.

Fig 3. The negative effects of PRK activity on the growth rate and H₂ production rate in the absence of Rubisco are independent of the substrate oxidation state. H₂ yields (**A**), growth rates (**B**), and specific H₂ production rates (**C**) were determined for the NifA* parent (Black), the NifA*ΔRubisco mutant (light gray), the NifA*ΔPRK mutant (white) and the NifA*ΔRubiscoΔPRK mutant (dark gray) during phototrophic growth with succinate (Suc), acetate (Ace), or butyrate (But), which differ in oxidation state. Sampling times are described in the methods. Different letters indicate significant differences with $p < 0.01$ using one-way ANOVA with Tukey's posttest. Statistical comparisons were performed within each carbon source. Error bars indicate standard deviation (n = 4-11).

Fig 4. Disruption of PRK activity results in a proportional increase in both H₂ yield and specific H₂ production rate. Fold increases are relative to the corresponding values from the NifA* parent strain for growth on succinate (**A**), acetate (**B**), and butyrate (**C**). All data was transformed from that shown in Figures 3A and 3C. NifA*ΔRubisco, light gray; NifA*ΔPRK, white; NifA*ΔRubiscoΔPRK, dark gray. Different letters indicate significant differences with $p < 0.01$ using one-way ANOVA with Tukey's posttest. Statistical comparisons were performed within each carbon source. Error bars indicated standard deviation (n = 4-11).

Fig 5. Expressing PRK from a plasmid partially complements the ΔPRK mutation but does not have an additive effect on strains already encoding PRK. H₂ yields (**A**), growth rates (**B**), and specific H₂ production rates (**C**), were determined for the NifA* parent and Calvin cycle mutants with either an empty vector control (pBBPgdh; black) or with the PRK gene expressed from the same vector under a constitutive promoter (pBBPcbbP; gray). All strains were grown phototrophically with 10mM succinate.

397 Sampling times are described in the methods. Error bars indicated standard deviation.
398 Different letters indicate significant differences with $p < 0.01$ using two-way ANOVA
399 correcting for multiple comparisons using the Sidak method. ($n = 4-6$).

Figure 1

Figure 2

A H_2 yield **B** Growth rate **C** Sp. H_2 production rate

A**Succinate** **B****Acetate** **C****Butyrate**