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Calvin Cycle Mutants of Photoheterotrophic Purple Nonsulfur Bacteria Fail To Grow Due to an Electron Imbalance Rather than Toxic Metabolite Accumulation

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Purple nonsulfur bacteria grow photoheterotrophically by using light for energy and organic compounds for carbon and electrons. Disrupting the activity of the CO_2-fixing Calvin cycle enzyme, ribulose 1,5-bisphosphate carboxylase (RubisCO), prevents photoheterotrophic growth unless an electron acceptor is provided or if cells can dispose of electrons as H_2. Such observations led to the long-standing model wherein the Calvin cycle is necessary during photoheterotrophic growth to maintain a pool of oxidized electron carriers. This model was recently challenged with an alternative model wherein disrupting RubisCO activity prevents photoheterotrophic growth due to the accumulation of toxic ribulose-1,5-bisphosphate (RuBP) (D. Wang, Y. Zhang, E. L. Pohlmann, J. Li, and G. P. Roberts, J. Bacteriol. 193:3293-3303, 2011, http://dx.doi.org/10.1128/JB.00265-11). Here, we confirm that RuBP accumulation can impede the growth of *Rhodospirillum rubrum* (*Rs. rubrum*) and *Rhodopseudomonas palustris* (*Rp. palustris*) RubisCO-deficient (ΔRubisCO) mutants under conditions where electron carrier oxidation is coupled to H_2 production. However, we also demonstrate that *Rs. rubrum* and *Rp. palustris* Calvin cycle phosphoribulokinase mutants that cannot produce RuBP cannot grow photoheterotrophically on succinate unless an electron acceptor is provided or H_2 production is permitted. Thus, the Calvin cycle is still needed to oxidize electron carriers even in the absence of toxic RuBP. Surprisingly, Calvin cycle mutants of *Rs. rubrum*, but not of *Rp. palustris*, grew photoheterotrophically on malate without electron acceptors or H_2 production. The mechanism by which *Rs. rubrum* grows under these conditions remains to be elucidated.

Purple nonsulfur bacteria (PNSB) are renowned for their ability to employ versatile metabolic modules to thrive under different growth conditions. PNSB can grow photoautotrophically using light for energy, inorganic compounds other than water (e.g., thiosulfate, Fe^{2+}) for electrons, and CO_2 for carbon. The Calvin cycle is well-known for permitting autotrophic growth by converting CO_2 into organic precursors for biosynthesis (Fig. 1). In this pathway phosphoribulokinase (PRK) expends ATP to generate ribulose 1,5-bisphosphate (RuBP). RuBP is then combined with CO_2 via ribulose 1,5-bisphosphate carboxylase (RubisCO), resulting in two molecules of 3-phosphoglycerate. CO_2 fixation generates relatively oxidized metabolites that accept electrons from NAD(P)H via glyceraldehyde-3-phosphate dehydrogenase.

PNSB can also grow photoheterotrophically using light for energy and organic compounds for carbon and electrons. Unlike the process in a respiring heterotroph, reducing power from oxidative pathways [e.g., NAD(P)H] is not used to reduce a terminal electron acceptor and generate ATP by oxidative phosphorylation. Rather, ATP generation is largely decoupled from the oxidative pathways of central metabolism as photoheterotrophs repeatedly energize electrons and shuttle them through a H^+-pumping electron transfer chain to generate ATP by cyclic photophosphorylation (Fig. 1). Nevertheless, photoheterotrophs generate ample reducing power that must be oxidized to replenish pools of oxidized electron carriers [e.g., $NAD(P)^+$] and maintain metabolic flow. CO_2 fixation was first hypothesized to fulfill this essential role of maintaining oxidized electron carriers during photoheterotrophic growth in 1933 by Muller (1), prior to the elucidation of the Calvin cycle itself (2) (Fig. 1, model 1). Muller devised this hypothesis to explain why there was net CO_2 fixation when PNSB grew photoheterotrophically on compounds, like butyrate, that are more electron rich than the average carbon in biomass (1). Supporting this hypothesis, it was later shown that PNSB could be grown photoheterotrophically on butyrate without added CO_2 if an electron acceptor like dimethyl sulfoxide (DMSO) was provided (3) or if PNSB were allowed to dispose of electrons as H_2 (4). More compelling, deleting genes encoding RubisCO (i.e., *cbbM* and, in some cases, *cbbLS*) in model PNSB, including *Rhodobacter sphaeroides* (*Rb. sphaeroides*), *Rhodobacter capsulatus* (*Rb. capsulatus*), and *Rhodopseudomonas palustris* (*Rp. palustris*), prevented photoheterotrophic growth, even with relatively oxidized substrates, unless an alternative electron acceptor was provided (5–7), or if electrons were disposed of as H_2 (8), or if an alternative reductive CO_2-fixing pathway was available (9). ^{13}C-labeling experiments with *Rp. palustris* have since shown that the Calvin cycle is one of the most active pathways during photoheterotrophic growth, and it oxidizes 40 to 60% of the reducing power even during growth with relatively oxidized substrates (8, 10). Thus, there is a long history of evidence to support a model wherein the Calvin cycle plays an essential role in oxidizing excess reducing power generated during photoheterotrophic growth (Fig. 1, model 1).

This electron-balancing role of the Calvin cycle was recently called into question. Wang et al. showed that the deletion of the

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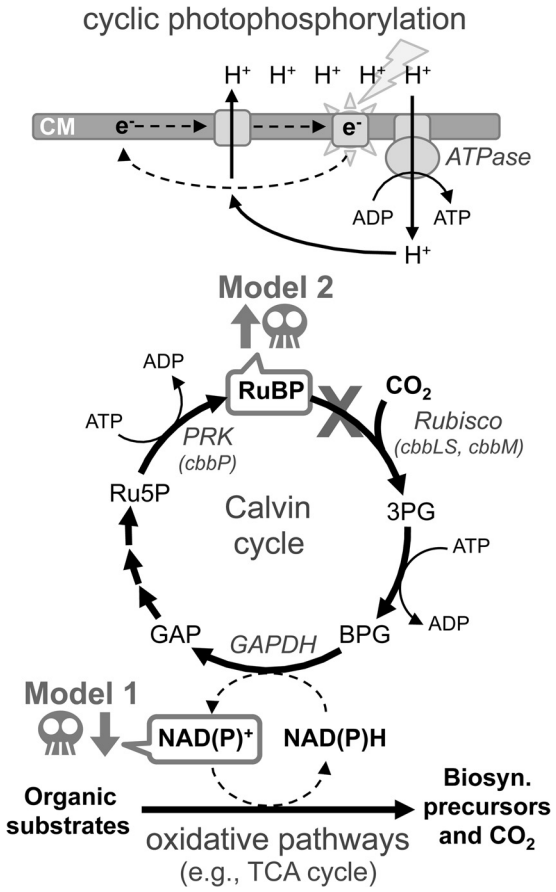

FIG 1 Two models to explain why disrupting RubisCO activity prevents photoheterotrophic growth in PNSB. In both models, ATP generation is decoupled from central metabolism as electrons are repeatedly energized and cycled through a H^+-pumping electron transfer chain (cyclic photophosphorylation; top). Oxidative pathways convert organic substrates into biosynthetic precursors and CO_2 and reduce electron carriers (bottom). The Calvin cycle generates biosynthetic precursors, fixes CO_2, and oxidizes electron carriers (middle). In model 1, disrupting RubisCO activity (gray X) prevents Calvin cycle flux, leading to a depletion of oxidized electron carriers that halts all metabolic activity. In model 2, disrupting RubisCO activity leads to a lethal accumulation of toxic RuBP, produced by PRK. BPG, 1,3-bisphosphoglycerate; CM, cytoplasmic membrane; GAP, glyceraldehyde-3-phosphate; GAPDH, GAP dehydrogenase; PRK, phosphoribulokinase; R5P, ribulose-5-phosphate; RuBP, ribulose-1,5-bisphosphate; RubisCO, ribulose-1,5-bisphosphate carboxylase; TCA, tricarboxylic acid.

RubisCO gene, *cbbM*, in *Rhodospirillum rubrum* (*Rs. rubrum*) resulted in poor photoheterotrophic growth on malate but, oddly, did not prevent growth (11). Disrupting RubisCO activity resulted in the accumulation of RuBP, the product of the preceding Calvin cycle enzyme, PRK (Fig. 1). A strong inverse correlation between RuBP levels and growth rate suggested that RuBP was toxic (11). When the gene encoding PRK, *cbbP*, was deleted in a RubisCO-deficient (ΔRubisCO) mutant to prevent RuBP production, a wild-type growth rate was restored (11). Wang et al. proposed that the previously observed inability of various PNSB ΔRubisCO mutants to grow photoheterotrophically was due to the accumulation of toxic RuBP rather than an electron imbalance (Fig. 1, model 2). In advocacy of this alternative hypothesis, we note that the addition of electron acceptors or permitting the disposal of electrons as H_2 has been shown to result in lower Calvin cycle transcript levels in multiple PNSB (5, 8, 10, 12). Thus, simply providing electron acceptors and permitting H_2 production could repress Calvin cycle activity and prevent toxic RuBP accumulation.

Even so, we were skeptical of this alternative model for several reasons. First, ^{13}C metabolic flux and flux balance analyses agree that disrupting Calvin cycle activity should lead to a lethal depletion of oxidized electron carriers or, in other terms, a lethal excess of reducing power (8, 10, 13, 14). Electron balance must be maintained to support metabolic flow and cell viability. Second, deleting the gene encoding the RuBP-producing PRK enzyme, *cbbP*, was previously shown to prevent photoheterotrophic growth in *Rb. sphaeroides* (12) and *Rb. capsulatus* (15). Adding the electron acceptor DMSO or allowing the cells to produce H_2 restored growth, supporting the original model since these ΔPRK Calvin cycle mutants cannot produce RuBP (12, 15). Third, the experiments by Wang et al. were performed in a medium that contained glutamate as the sole nitrogen source (11), which is known to induce H_2 production via the enzyme, nitrogenase (16). When H_2 production was repressed by using NH_4^+ as the nitrogen source, the same *Rs. rubrum* ΔRubisCO mutant displayed questionable photoheterotrophic growth (achieved 10% of the wild-type final OD) (17). Furthermore, another *Rs. rubrum* ΔRubisCO mutant previously shown to grow photoheterotrophically with NH_4^+ (18) was later shown to produce H_2 due to the acquisition of a *nifA** mutation that bypassed the repression of nitrogenase by NH_4^+ (17). We suspected that photoheterotrophic growth of *Rs. rubrum* observed by Wang et al. (11) was only possible due to the disposal of electrons as H_2.

The purpose of this study was to reexamine the impact of electron imbalance versus RuBP accumulation on PNSB photoheterotrophic growth. To distinguish between phenotypes that might be unique to *Rs. rubrum* versus those that might be more broadly applicable to PNSB, we performed experiments on ΔPRK and ΔRubisCO mutants of *Rs. rubrum* and *Rp. palustris*. Furthermore, we employed two different carbon sources that have different numbers of available electrons, malate (12 e^-) and succinate (14 e^-), but are both expected to enter metabolism and be processed by the tricarboxylic acid (TCA) cycle (10). We confirmed the observations of Wang et al. (11) that ΔRubisCO mutations can impede growth rates, likely through the accumulation of toxic RuBP. However, we also confirmed the original model wherein an alternative means of electron disposal is required for any photoheterotrophic growth in the absence of the Calvin cycle. Surprisingly, *Rs. rubrum* appears to have an alternative mechanism to H_2 production that permits photoheterotrophic growth of Calvin cycle mutants with malate but not with succinate.

MATERIALS AND METHODS

Strains and growth conditions. All strains are summarized in Table 1. *Rs. rubrum* strains were derived from strain UR2 (19) and were provided by Gary Roberts, University of Wisconsin—Madison. *Rs. rubrum* genotypes were verified by PCR using primers that were either flanking or internal to the mutated region. *Rs. rubrum* strains were streaked to supplemented malate-ammonium (SMN) (19) agar plates from 10% DMSO frozen stocks and grown aerobically in darkness at 30°C. Five-milliliter aerobic SMN cultures were inoculated from single colonies and incubated at 30°C with shaking. Aerobic starter cultures were used to inoculate 10 ml of anaerobic defined medium in 28-ml anaerobic test tubes. The defined medium was based on a previously described recipe (20) and contained (per liter) 0.9 g of K_2HPO_4, 0.6 g of KH_2PO_4, 0.1 g of $MgSO_4$, 0.075 g of

TABLE 1 Strains used in this study

Strain[a]	Phenotype	Relevant genotype	Reference or source
E. coli			
S17		*thi pro hdsR hdsM*$^+$ *recA* RP4-2 (Tc::Mu Km::Tn*7*)	27
NEB10β		F$^-$ λ$^-$ *recA1* Δ(*lacZYA-argF*)*U169 hsdR17 thi-1 gyrA96 supE44 endA1 relA1* ϕ80*lacZ*ΔM15	New England Biolabs
Rp. palustris			
CGA009	Wild type	*hupV* mutant	21, 22
CGA4007	ΔPRK	Δ*cbbP*::Km *hupV* mutant	This study
CGA676	NifA*	*nifA** *hupV* mutant	8
CGA678	NifA* ΔRubisCO II	*nifA** Δ*cbbM hupV* mutant	8
CGA4010	NifA* ΔPRK	*nifA** Δ*cbbP*::Km^r *hupV* mutant	This study
CGA4009	NifA* ΔRubisCO	*nifA** Δ*cbbLS* Δ*cbbM hupV* mutant	This study
CGA4011	NifA* ΔRubisCO ΔPRK	*nifA** Δ*cbbLS* Δ*cbbM* Δ*cbbP*::Km^r *hupV* mutant	This study
Rs. rubrum			
UR2	Wild type		19
UR2565	ΔPRK	Δ*cbbP*::Km^r	11
UR5251	ΔRubisCO	Δ*cbbM*::Gm^r	17
UR2557	ΔRubisCO ΔPRK	Δ*cbbM*::Gm^r Δ*cbbP*::Km^r	11

[a] *Rp. palustris* encodes both RubisCO type I (*cbbLS*) and type II (*cbbM*). CGA678 (NifA* ΔRubisCO II) still has RubisCO activity due to *cbbLS*. Any *Rp. palustris* strain designated ΔRubisCO (e.g., CGA4009 and CGA4011) lacks both *cbbM* and *cbbLS* and does not have RubisCO activity.

TABLE 2 Plasmids used in this study

Plasmid	Description	Reference or source
pBBPgdh	Gm^r; mobilizable broad-host-range cloning vector with a constitutive *Rp. palustris* promoter	8
pJQ200SK	Gm^r, *sacB*; mobilizable *Rp. palustris* suicide vector	26
pUC19	Ap^r; High-copy-number cloning vector	25
pGEM	High-copy-number cloning vector for direct insertion of PCR products	Promega
pPS858_Km2	Ap^r Km^r; FRT[a]	8
pGEM*cbbP*	Ap^r; *cbbP* and ribosomal binding site cloned into pGEM	
pBBP*cbbP*	Gm^r; derived from pBBPgdh; complementation vector for Δ*cbbP*::Km^r	This study
pUCΔ*cbbLS*	Ap^r; in-frame Δ*cbbLS* cloned into XbaI/BamHI sites of pUC19	This study
pGEMΔ*cbbP*	Ap^r; in-frame Δ*cbbP* cloned into pGEM	This study
pGEMΔ*cbbP*::Km^r	Ap^r, Km^r; in-frame Δ*cbbP*::Km^r in pGEM	This study
pJQΔ*cbbLS*	Gm^r; in-frame Δ*cbbLS* cloned into pJQ200SK	This study
pJQΔ*cbbP*::Km^r	Gm^r; in-frame Δ*cbbP*::Km^r cloned into pJQ200SK	This study

[a] FRT, Flp recognition target.

$CaCl_2 \cdot 2H_2O$, 0.012 g of $FeSO_4 \cdot 7H_2O$, 0.02 g of EDTA, 0.015 g of biotin, 1 ml of trace elements (20), and either 5.9 mM sodium glutamate to permit H_2 production or 7.5 mM $(NH_4)_2SO_4$ to prevent H_2 production. DMSO was added to a final concentration of 60 mM where indicated in the text and figure legends. Culture medium was made anaerobic by bubbling medium with Ar gas and then sealing tubes with rubber stoppers (Geo-Microbial Technologies, Ochelata, OK) and aluminum crimps. Cultures were incubated at 30°C in front of a 60-W light bulb. *Rp. palustris* strains were derived from the type strain CGA009 (21), which is defective for uptake hydrogenase activity (22). *Rp. palustris* was grown in a similar manner to *Rs. rubrum* except in a defined photosynthetic medium which always contained $(NH_4)_2SO_4$ as the nitrogen source (23). H_2 production was accomplished for *Rp. palustris* using a *nifA** background (Table 1) that allows for H_2 production in the presence of NH_4^+ (8). Disodium salts of fumarate, malate, or succinate were used as carbon sources for all defined media at a final concentration of 10 mM. *Rs. rubrum* growth experiments in medium with succinate and NH_4^+ were inoculated from starter cultures that were grown photoheterotrophically with malate and NH_4^+. *Escherichia coli* was grown on LB agar or in LB broth. Gentamicin and kanamycin were used at 100 μg/ml each for *Rp. palustris*, at 10 μg/ml each for *Rs. rubrum*, and at 10 μg/ml and 30 μg/ml, respectively, for *E. coli*. Antibiotics were used on plates and in starter cultures but were omitted during experiments in which growth rates were compared (including for strains carrying plasmids).

***Rp. palustris* strain construction.** In-frame deletions of *cbbLS*, encoding type I RubisCO, and *cbbP*, encoding PRK, were constructed using plasmids and primers listed in Tables 2 and 3, respectively, as described previously (22). Briefly, regions ~1 kb upstream and downstream of the gene to be deleted were amplified by PCR. The two PCR products were combined in frame by overlap extension PCR (24) and cloned into either pGEM (Promega, Madison, WI) or pUC19 (25) using the restriction sites indicated in Table 3 and maintained in *E. coli* NEB10β (New England BioLabs, Ipswich, MA). Constructs were confirmed by sequencing to ensure that no point mutations were introduced. The Km^r cassette from pPS858_Km2 was amplified and inserted at a KpnI site within the Δ*cbbP* construct. Deletion constructs were then subcloned into the *Rp. palustris* suicide vector pJQ200SK (26), maintained in *E. coli* S17 (27), and transferred to *Rp. palustris* by conjugation. Counterselection on agar plates supplemented with 10% sucrose and screening for sensitivity to gentamicin were used to obtain recombinant *Rp. palustris* strains. Genotypes were confirmed by PCR. To complement the Δ*cbbP*::Km^r mutation in the *Rp. palustris* ΔPRK strain, CGA4007, the *cbbP* gene and the native ribosomal binding site were amplified using primers listed in Table 3, cloned into pGEM, sequenced, and then subcloned into the *Rp. palustris* constitutive expression vector pBBPgdh (8) in *E. coli* NEB10β. The vector was then moved into *E. coli* S17 and transferred into CGA4007 by conjugation.

Analytical techniques. Cell density was assayed by optical density at 660 nm (OD_{660}) using a Genesys 20 visible spectrophotometer (ThermoFisher, Pittsburgh, PA). Specific growth rates were determined using measurements with values that were <0.8 OD_{660}, where a linear relationship between cell density and OD_{660} was maintained. H_2 was sampled from culture headspace using a gas-tight syringe and analyzed using a Shimadzu GC-2014 gas chromatograph as described previously (28). Polyhydroxybutyrate was hydrolyzed from dry cells and extracted as crotonic acid by boiling cells in 1 ml of pure sulfuric acid in screw-cap glass test tubes. Extracts were diluted with 4 ml of water, centrifuged, filtered, and diluted 10-fold with water, and then crotonic acid was quantified by high-performance liquid chromatography (HPLC) (Shimadzu) as described previously (29). Culture supernatants were analyzed for formate using the same HPLC parameters.

TABLE 3 Primers used in this study

Primer	Sequence (5′–3′)[a]	Description (restriction site)
JBM47	AAAGCAAGCCGCTCTAGACATCAACG	Δ*cbbLS* upstream primer (XbaI)
JBM48	GTTCATGTCGTCCTCCTTGAAAGCC	Δ*cbbLS* in-frame deletion reverse
JBM49	CAAGGAGGACGACATGAACGGCTGATCGTGGACGCGACAGC	Δ*cbbLS* in-frame deletion forward
JBM50	GTCGCGCGAATTGTGGATCCAACGT	Δ*cbbLS* downstream primer (BamHI)
JBM128	GCTGCAGGATGTGCCGTACGC	Δ*cbbP* upstream primer (PstI)
JBM129	GTTTGCGGTCGATCAGGTACCGATGGAGATGATCGGATGCTTAC	Δ*cbbP* in-frame deletion reverse (KpnI)
JBM130	CCGATCATCTCCATCGGTACCTGATCGACCGCAAACGAAGCATG	Δ*cbbP* in-frame deletion forward (KpnI)
JBM131	CTTCTAGACCTCGTCGGCGCC	Δ*cbbP* downstream primer (XbaI)
GG010	GGATCCACGTCGTCTCTCCCGGT	*cbbP* complementation forward (BamHI)
GG011	TCTAGACGTGCGGGAACGTTCAAA	*cbbP* complementation reverse (XbaI)
JBM114	AGGTACCAATTCCGCGAACCCCAGA	Km^r from pPS858_Km2 forward (KpnI)
JBM115	TGGTACCAATTCCGCTAGCTTCACGCT	Km^r from pPS858_Km2 reverse (KpnI)

[a] Restriction sites are underlined.

RESULTS AND DISCUSSION

Preventing RuBP accumulation in ΔRubisCO mutants improves growth rates under conditions permitting H_2 production. Previously, Wang et al. proposed that PNSB RubisCO mutants fail to grow due to the toxic accumulation of RuBP (11). This served as an alternative hypothesis to the long-standing model that disrupting the Calvin cycle prevents photoheterotrophic growth by preventing the oxidation of reduced electron carriers (Fig. 1). We were concerned that the growth conditions used by Wang et al. (11) permitted the disposal of excess electrons as H_2 and therefore were unsuitable to rule out the original model. To confirm that H_2 was produced and to verify the growth trends observed by Wang et al. (11), we grew the same *Rs. rubrum* strains from the Wang et al. study (UR2, UR2565, UR5251, and UR2557) (Table 1) with malate and glutamate, the latter being a nitrogen source that permits H_2 production via nitrogenase. We observed over 1 mmol of H_2 production from all strains during the growth phase (Fig. 2A). As was observed previously, the *Rs. rubrum* ΔRubisCO mutant, UR5251, grew more slowly than the wild-type strain, UR2, at 45% of the wild-type growth rate (Fig. 2B). The ΔPRK mutant grew at the same rate as the wild-type strain (Fig. 2B). Deleting the gene encoding PRK, *cbbP*, in a ΔRubisCO background restored the growth rate to the wild-type level (Fig. 2B). Interestingly, the ΔRubisCO mutant growth rate was more variable than that of other strains (Fig. 2B), perhaps due to selective pressure for variants that produce less RuBP.

We also performed the same experiment with *Rp. palustris* using a *nifA** genetic background, succinate as the carbon source, and NH_4^+ as the nitrogen source. NifA* strains produce H_2 in the presence of NH_4^+ (8). Similar to what was observed for *Rs. rubrum*, the *Rp. palustris* NifA* ΔRubisCO strain exhibited a severely impaired growth rate (56% that of the NifA* parent), whereas the NifA* ΔPRK strain had a growth rate that was 83% that of the parent (Fig. 3). Deleting the gene encoding PRK, *cbbP*, in the NifA* ΔRubisCO mutant improved the growth rate of the ΔRubisCO mutant to a similar level observed in the NifA* ΔPRK strain (91% that of the NifA* parent) (Fig. 3). Thus, our results support the notion that RuBP accumulation can negatively impact photoheterotrophic growth rates in other PNSB. However, since these experiments permitted the disposal of excess electrons as H_2, they did not rule out the original model wherein electron imbalance prevents photoheterotrophic growth of Calvin cycle mutants.

***Rs. rubrum* Calvin cycle mutants are capable of photoheterotrophic growth with malate or fumarate but not with succinate.** Several studies have demonstrated that PNSB Calvin cycle mutants, including ΔPRK mutants of *Rb. sphaeroides* and *Rb. capsulatus*, cannot grow photoheterotrophically with compounds such as malate, succinate, and acetate unless electron acceptors are added or H_2 production is permitted (6–8, 12, 15). To verify that Calvin cycle PRK activity is essential for photoheterotrophic growth, we made an *Rp. palustris* ΔPRK mutant and tested it for

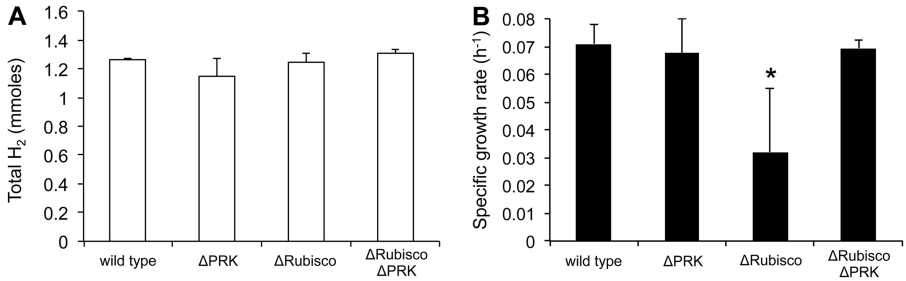

FIG 2 Preventing RuBP accumulation using a ΔPRK mutation improved the growth rate of an *Rs. rubrum* ΔRubisCO mutant under conditions permitting H_2 production. *Rs. rubrum* strains were grown phototrophically in minimal medium with malate and glutamate. (A) H_2 levels measured at the onset of stationary phase (i.e., when the highest OD_{660} value was observed). Values are averages from three biological replicates, with error bars representing standard deviations. (B) Average specific growth rates from three to five biological replicates are shown, with error bars representing standard deviations. The asterisk indicates that a value was significantly different from that of the wild-type ($P < 0.05$; two-tailed *t* test, equal variance). Wild type, UR2; ΔPRK, UR2565 (Δ*cbbP*::Km^r); ΔRubisCO, UR5251 (Δ*cbbM*::Gm^r); ΔRubisCO ΔPRK, UR2557 (Δ*cbbM*::Gm^r Δ*cbbP*::Km^r).

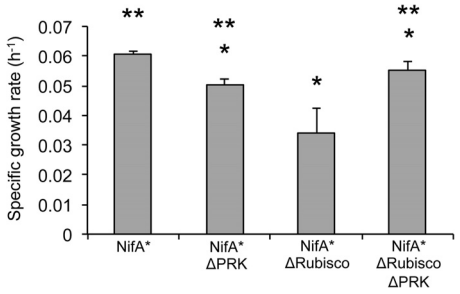

FIG 3 Preventing RuBP accumulation using ΔPRK mutations improved the growth rate of a H_2-producing *Rp. palustris* NifA* ΔRubisCO mutant. Average specific growth rates from three biological replicates are shown, with error bars representing standard deviations. *Rp. palustris* strains were grown phototrophically in minimal medium with succinate and NH_4^+. All strains produced H_2 during growth due to a *nifA** mutation. The asterisk indicates that a value was significantly different from that of the NifA* parent ($P < 0.05$; two-tailed *t* test, equal variance); double asterisks indicate that a value was significantly different from that of the NifA* ΔRubisCO strain ($P < 0.05$; two-tailed *t* test, equal variance). NifA*, CGA676 (*nifA**); NifA* ΔPRK, CGA4010 (*nifA** Δ*cbbP*::Km^r); NifA* ΔRubisCO, CGA4009 (*nifA** Δ*cbbLS* Δ*cbbM*); NifA* ΔRubisCO ΔPRK, CGA4011 (*nifA** Δ*cbbLS* Δ*cbbM* Δ*cbbP*::Km^r).

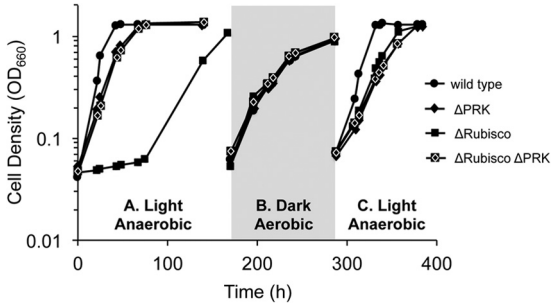

FIG 5 *Rs. rubrum* Calvin cycle mutants grew photoheterotrophically on malate with NH_4^+ without producing H_2. *Rs. rubrum* strains were grown aerobically in rich SMN medium and then transferred to anaerobic phototrophic minimal medium with malate and NH_4^+ (graph A). Cultures were then transferred to aerobic, rich SMN medium and allowed to grow (graph B) before being transferred to the same conditions as for graph A (graph C). Representative data from single biological replicates are shown. Similar trends were observed for at least three biological replicates for each trend except that the initial ΔRubisCO strain lag phase ended at different times. No culture produced more than 2.5 μmol of H_2.

growth under conditions that repress H_2 production via nitrogenase. This *Rp. palustris* ΔPRK mutant was incapable of photoheterotrophic growth with malate and NH_4^+ unless *cbbP*, the gene encoding PRK, was expressed in *trans* (Fig. 4, ΔPRK pBBP*cbbP*) or if H_2 production was permitted (Fig. 4, NifA* ΔPRK). The same trends were observed when succinate was used in place of malate (data not shown).

Based on observations that PRK is critical for photoheterotrophic growth with NH_4^+ in *Rp. palustris* (Fig. 4), *Rb. sphaeroides* (12), and *Rb. capsulatus* (15), we expected that *Rs. rubrum* Calvin cycle mutants would also not grow with NH_4^+. However, we observed growth of the *Rs. rubrum* Calvin cycle mutants to approximately the same final OD as the wild-type strain under phototrophic conditions with malate and NH_4^+ (Fig. 5). We confirmed that growth was not due to contaminating bacteria

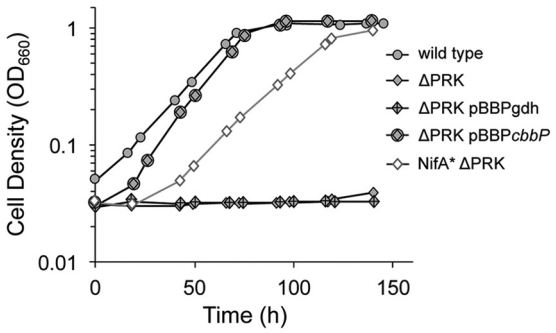

FIG 4 A ΔPRK mutant of *Rp. palustris* that cannot produce RuBP was incapable of photoheterotrophic growth unless H_2 production was permitted. *Rp. palustris* strains were grown in a minimal medium with malate and NH_4^+. Only the NifA* strain can produce H_2 under these growth conditions. Representative values from single biological replicates are shown. Similar trends were observed for at least three biological replicates. Wild type, CGA009; ΔPRK, CGA4007 (Δ*cbbP*::Km^r); ΔPRK pBBPgdh, CGA4007 (Δ*cbbP*::Km^r) with empty vector (Table 2); ΔPRK pBBP*cbbP*, CGA4007 (Δ*cbbP*::Km^r) with complementation vector (Table 2); NifA* ΔPRK, CGA4010 (*nifA** Δ*cbbP*::Km^r).

through PCR amplification of genes encoding RubisCO and PRK both before and after growth experiments. The ΔRubisCO mutant, UR5251, showed an initial lag phase under phototrophic conditions with malate and NH_4^+ (Fig. 5). However, this lag phase was not the result of an adaptation to anaerobic conditions as it was not observed again after cultures were transferred to rich aerobic medium and then back into anaerobic phototrophic conditions with malate and NH_4^+ (Fig. 5). The lag phase was also not observed again when the ΔRubisCO mutant was transferred directly from anaerobic phototrophic conditions with malate and NH_4^+ to identical conditions (data not shown). Thus, there may have been selective pressure for suppressor mutations that limit the production of toxic RuBP by the ΔRubisCO mutant. Such a mutation could explain the difference between our observations and those previously where the ΔRubisCO mutant, UR5251, only grew to ~10% of the final OD of the wild-type when supplied with malate and NH_4^+ (17).

Even though *Rs. rubrum* Calvin cycle mutants unexpectedly grew with malate and NH_4^+, they did not grow with succinate and NH_4^+ unless DMSO was provided as an electron acceptor (Fig. 6). For these experiments, medium with succinate and NH_4^+ was inoculated with strains that were previously grown phototrophically with malate and NH_4^+ (Fig. 5). The DMSO requirement for growth with succinate but not with malate suggests that *Rs. rubrum* Calvin cycle mutants have an additional mechanism to maintain electron balance when they are grown on malate but not on succinate. It is possible that this mechanism cannot oxidize the extra reducing power produced during the metabolism of succinate since succinate has two more available electrons than malate. In support of this notion, all *Rs. rubrum* Calvin cycle mutants grew phototrophically with fumarate and NH_4^+ (data not shown). Fumarate has the same number of available electrons as malate and is also expected to be processed by the TCA cycle (10).

Several mechanisms are possible that could allow *Rs. rubrum* Calvin cycle mutants to grow photoheterotrophically with malate and NH_4^+, some of which we can rule out while others we can only speculate about. Mutations in *nifA* that allow for constitutive H_2 production (17, 30, 31) can be ruled out as we did not detect H_2 in any of the *Rs. rubrum* Calvin cycle mutant cultures grown with

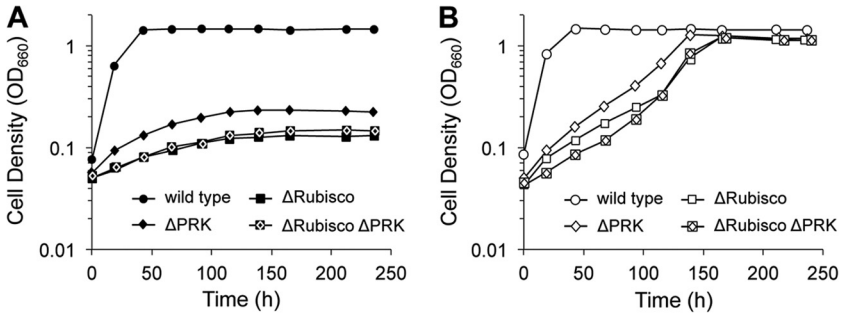

FIG 6 *Rs. rubrum* Calvin cycle mutants could not grow photoheterotrophically with succinate and NH_4^+ (A, no DMSO) unless DMSO was provided (B) as an electron acceptor. Cultures with succinate and NH_4^+ were inoculated from cultures grown phototrophically with malate and NH_4^+ but without DMSO (Fig. 5). Representative data from single biological replicates are shown. Similar trends were observed for at least three biological replicates.

malate and NH_4^+. Production of polyhydroxybutyrate storage polymer as an electron sink can also be ruled out as we did not detect any polyhydroxybutyrate in any *Rs. rubrum* strain grown with malate and NH_4^+. A flux balance model predicted that an *Rs. rubrum* Calvin cycle mutant could grow photoheterotrophically with malate by producing formate but that formate production would not permit growth with succinate because there would still be excess reducing power (13). However, we did not detect formate in the supernatants of any *Rs. rubrum* culture.

A mechanism we can currently only speculate about is the use of an alternative CO_2-fixing pathway in *Rs. rubrum* that could substitute for the Calvin cycle. In support of such a mechanism, an *Rs. rubrum* ΔRubisCO strain was previously reported to grow with CO_2 as the sole carbon source and with thiosulfate as an electron donor though the CO_2-fixing pathway responsible was not identified (32). One possibility is the ethylmalonyl-coenzyme A (CoA) pathway, which permitted phototrophic growth of *Rb. sphaeroides* ΔRubisCO mutants with acetate and NH_4^+ (9). However, the ethylmalonyl-CoA pathway is unlikely to permit photoheterotrophic growth of PNSB Calvin cycle mutants with malate since excessive reducing power would be made en route to the start of the pathway (9, 13). Indeed, the *Rb. sphaeroides* RubisCO mutant that grew with acetate and NH_4^+ did not grow with malate and NH_4^+ (9). Another possibility for a CO_2-fixing pathway is the reverse TCA cycle. A flux balance model predicted that the reverse TCA cycle could substitute for the Calvin cycle in maintaining electron balance in *Rs. rubrum* (13). Consistent with this possibility, fumarate reductase activity was previously detected in *Rs. rubrum* under anaerobic conditions (33). However, it is not clear whether reverse TCA cycle flux could explain growth with malate and fumarate but not with succinate.

The mechanism that permits *Rs. rubrum* Calvin cycle mutants to grow phototrophically on malate without producing H_2 remains to be elucidated. Even so, the facts that *Rs. rubrum* ΔPRK mutants, which cannot produce RuBP, still require an electron acceptor to grow on succinate (Fig. 6) and that similar observations have been made for ΔPRK mutants of *R. palustris* (Fig. 4) and other PNSB (12, 15) with succinate and malate indicate that RuBP accumulation is not the reason why Calvin cycle mutants fail to grow photoheterotrophically. Rather, our data support the long-standing hypothesis that the Calvin cycle is required during photoheterotrophic growth to oxidize NAD(P)H in the absence of other electron-accepting processes.

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