Calvin Cycle Flux, Pathway Constraints, and Substrate Oxidation State Together Determine the H₂ Biofuel Yield in Photoheterotrophic Bacteria

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ABSTRACT Hydrogen gas (H₂) is a possible future transportation fuel that can be produced by anoxygenic phototrophic bacteria via nitrogenase. The electrons for H₂ are usually derived from organic compounds. Thus, one would expect more H₂ to be produced when anoxygenic phototrophs are supplied with increasingly reduced (electron-rich) organic compounds. However, the H₂ yield does not always differ according to the substrate oxidation state. To understand other factors that influence the H₂ yield, we determined metabolic fluxes in Rhodopseudomonas palustris grown on ¹³C-labeled fumarate, succinate, acetate, and butyrate (in order from most oxidized to most reduced). The flux maps revealed that the H₂ yield was influenced by two main factors in addition to substrate oxidation state. The first factor was the route that a substrate took to biosynthetic precursors. For example, succinate took a different route to acetyl-coenzyme A (CoA) than acetate. As a result, R. palustris generated similar amounts of reducing equivalents and similar amounts of H₂ from both succinate and acetate, even though succinate is more oxidized than acetate. The second factor affecting the H₂ yield was the amount of Calvin cycle flux competing for electrons. When nitrogenase was active, electrons were diverted away from the Calvin cycle towards H₂, but to various extents, depending on the substrate. When Calvin cycle flux was blocked, the H₂ yield increased during growth on all substrates. In general, this increase in H₂ yield could be predicted from the initial Calvin cycle flux.

IMPORTANCE Photoheterotrophic bacteria, like Rhodopseudomonas palustris, obtain energy from light and carbon from organic compounds during anaerobic growth. Cells can naturally produce the biofuel H₂ as a way of disposing of excess electrons. Unexpectedly, feeding cells organic compounds with more electrons does not necessarily result in more H₂. Despite repeated observations over the last 40 years, the reasons for this discrepancy have remained unclear. In this paper, we identified two metabolic factors that influence the H₂ yield, (i) the route taken to make biosynthetic precursors and (ii) the amount of CO₂-fixing Calvin cycle flux that competes against H₂ production for electrons. We show that the H₂ yield can be improved on all substrates by using a strain that is incapable of Calvin cycle flux. We also contributed quantitative knowledge to the long-standing question of why photoheterotrophs must produce H₂ or fix CO₂ even on relatively oxidized substrates.

H₂ydrogen gas (H₂) is a promising transportation fuel that can be used in hydrogen fuel cells to generate an electric current with water as the only waste product. Anoxygenic phototrophic bacteria, including purple nonsulfur bacteria (PNSB), produce H₂ via nitrogenase (1). H₂ production is an obligate product of the nitrogenase reaction, which is better known for converting N₂ gas to NH₃. In fact, nitrogenase will produce H₂ as the sole product in the absence of N₂. To invoke H₂ production, PNSB are grown under conditions that induce nitrogenase activity, such as by supplying N₂, or in some cases glutamate, as the sole nitrogen source (2–5). Also, several PNSB mutants have been identified that produce H₂ when grown with NH₄⁺ as a nitrogen source, a condition that normally represses nitrogenase synthesis (4, 6–8). These mutants typically have activating mutations in nifA, encoding the master transcriptional activator of nitrogenase, and are termed NifA⁺ strains (4, 6, 7).

The preferred mode of growth for PNSB is photoheterotrophy, where light provides energy by photophosphorylation and organic compounds are used for carbon. In a recent study, we found that Rhodopseudomonas palustris cells grown with ¹³C-labeled acetate incorporated most of the acetate into cell material but that only half of the reducing equivalents that were generated during acetate oxidation were used in biosynthetic reactions. The bacteria were required to oxidize the other half of the reduced carriers of reducing equivalents (e.g., NADH, NADPH, and ferredoxins, here collectively referred to as electron carriers) by some other means. In the case of acetate, cells accomplished this by carrying out CO₂ fixation via the Calvin cycle or by producing H₂ (7). Others have shown that the Calvin cycle is essential during photoheterotrophic growth on other substrates, even substrates that are substantially more oxidized than biomass (9, 10). PNSB mutants lacking the CO₂-fixing enzyme of the Calvin cycle, ribulose
1,5-bisphosphate carboxylase (RuBisCO), were unable to grow on malate, succinate, or acetate unless cells were grown under nitrogen-fixing conditions to allow H$_2$ production (7, 11) or unless the electron acceptor dimethyl sulfoxide was provided (9, 10).

Given the important role for H$_2$ production in oxidizing electron carriers, one would expect PNSB to produce more H$_2$ from more-reduced substrates and less H$_2$ from more-oxidized substrates. However, it has long been known that H$_2$ yields from PNSB do not always differ accordingly with the substrate oxidation state. In 1977, Hillmer and Gest reported that *Rhodobacter capsulatus* produced about twice as much H$_2$ from pyruvate as from glucose, a more reduced substrate (2). Similar results have been reported for other PNSB (3, 5). One factor that certainly affects H$_2$ yields from different substrates is the amounts of storage products, such as polyhydroxybutyrate, and excreted organic acids produced (5, 12). H$_2$ yields from *Rhodobacter sphaeroides* also appeared to correlate with the substrate free energy (5), a surprising result given that H$_2$ production is not expected to be limited by energy during photosynthetic growth.

To identify factors other than substrate oxidation state that influence H$_2$ production, we performed $^{13}$C metabolic flux analysis with *R. palustris* provided with organic compounds with a range of oxidation states. We determined metabolic fluxes for the wild type (WT) and a NifA* strain grown anaerobically in light in mineral medium containing NH$_4$ as the nitrogen source. The wild-type strain does not produce H$_2$ under these conditions, whereas the NifA* strain expresses nitrogenase and produces H$_2$ constitutively. This comparison allowed us to determine the contribution of the Calvin cycle to the growth obtained when nitrogenase is not present compared to the growth obtained when nitrogenase is active and competing against the Calvin cycle for electrons by producing H$_2$. Our results illustrate how the biochemical constraints of a metabolic network can affect the H$_2$ yield when meeting demands for biosynthetic precursors. Our results also show that Calvin cycle activity decreases to different extents, depending on the organic substrate supplied, thereby affecting the H$_2$ yield by competing for electrons.

**RESULTS**

**H$_2$ yields and characteristics of growth on different substrates.** Previous reports with various PNSB indicated that H$_2$ yields do not always differ according to the substrate oxidation state (2, 3, 5). To confirm this, we determined the H$_2$ yields from *R. palustris* grown on unlabeled substrates with various oxidation states that were also available in $^{13}$C-labeled forms (Table 1). To achieve H$_2$ production, we used a NifA* strain that synthesizes active nitrogenase in mineral medium containing NH$_4$ as the nitrogen source. The wild-type strain does not synthesize nitrogenase and produces no H$_2$ when grown in this medium. Fumarate, succinate, and acetate all gave similar H$_2$ yields despite differing in oxidation state (Table 1). The most H$_2$ was produced during growth on the most reduced substrate, butyrate.

We accounted for nearly all of the consumed carbon and electrons in biomass, CO$_2$, H$_2$, and excreted organic acids (Table 2). As expected (13), growth on butyrate, a compound more reduced than biomass, required NaHCO$_3$ unless H$_2$ was produced (Table 2). In all cases, more CO$_2$ was produced by the NifA* strain than on the wild type, consistent with electrons being shifted away from CO$_2$ fixation towards H$_2$ production. This is reflected in lower biomass yields for the NifA* strain (Table 2), whereas the NifA* strain expresses nitrogenase and produces H$_2$ constitutively. This comparison allowed us to determine the contribution of the Calvin cycle to the growth obtained when nitrogenase is not present compared to the growth obtained when nitrogenase is active and competing against the Calvin cycle for electrons by producing H$_2$. Our results illustrate how the biochemical constraints of a metabolic network can affect the H$_2$ yield when meeting demands for biosynthetic precursors. Our results also show that Calvin cycle activity decreases to different extents, depending on the organic substrate supplied, thereby affecting the H$_2$ yield by competing for electrons.

**TABLE 1 Oxidation states of *R. palustris* biomass and growth substrates and the H$_2$ yield from each substrate**

| Compound | Formula | Oxidation state$^a$ | H$_2$ yield (mol H$_2$/100 mol organic C consumed)$^b$ |
|----------|---------|---------------------|--------------------------------------------------|
| Fumarate | C$_4$H$_6$O$_4$ | +1 | 18 ± 3$^d$ |
| Succinate | C$_4$H$_6$O$_4$ | +0.5 | 23 ± 1 |
| Acetate | C$_3$H$_4$O$_2$ | 0 | 21 ± 3 |
| Biomass$^c$ | CH$_2$N$_{0.18}$O$_{0.38}$ | −0.5 | |
| Butyrate | C$_7$H$_{10}$O$_2$ | −1 | 41 ± 10 |

$^a$ Based on the elemental composition of *R. palustris* 42OL (25).

$^b$ Values were determined for each carbon atom as described previously (7, 26) and then averaged by dividing the sum by the number of carbon atoms.

$^c$ NifA* cultures were grown in minimal medium with NH$_4$ as the nitrogen source. Values are averages from 3 to 5 biological replicates ± standard deviations (SD) based on samples taken during early exponential growth. Values are normalized for organic C consumed to account for the different carbon contents between acetate and the other substrates.

$^d$ Calculated by grouping fumarate and malate as a single metabolite [i.e., $d$H$_2$/d(fumarate + malate)] × 100/4 (carbon atoms). The H$_2$ yield from fumarate consumed alone would give a value of 12 ± 2.

Whereas acetate was converted entirely into CO$_2$ and biomass, growth on all other substrates resulted in organic acid excretion (Table 2). A relatively large proportion of fumarate was excreted as malate (growth on malate also resulted in fumarate excretion), a relatively large proportion of butyrate was excreted as acetate, and a small proportion of succinate was excreted as fumarate. For all substrates, excreted organic acids were eventually consumed. Since fumarate and malate have the same oxidation state, we grouped them as one metabolite to account for their simultaneous consumption and to better reflect the effects of intermediary metabolic fluxes on H$_2$ yield. If we were to show the H$_2$ yield as a proportion of fumarate consumed alone, without accounting for its consumption, the H$_2$ yield would be deceptively low. We did not group butyrate and acetate, since these compounds have different oxidation states and thus acetate excretion informs us about fluxes that reduce electron carriers.

*R. palustris* showed typical exponential growth curves on all substrates except for fumarate. Growth on fumarate was biphasic, with rapid growth (i.e., 5.8 ± 0.3 h for the wild type and 6.4 ± 0.5 h for the NifA* strain) before a cell density of about 0.06 units of optical density at 660 nm (OD$_{660}$) was reached, followed by a lower growth rate (Table 2) that lasted for the remainder of exponential growth. $^{13}$C metabolic flux analysis is based on equations that describe a metabolic steady state (or a pseudometabolic steady state in batch cultures). Therefore, our flux maps for biphasic fumarate-grown cultures may not be quantitatively accurate. However, the majority of the labeling patterns (~85%) accumulated during the second exponential growth phase. Thus, the statements about fluxes on fumarate relative to other substrates should be qualitatively accurate.

The Calvin cycle is important for maintaining electron balance on all substrates. After confirming that cells convert the compounds provided entirely into biomass, CO$_2$, H$_2$, and organic acids, we determined metabolic fluxes in wild-type and NifA* cells by use of $^{13}$C-labeled fumarate, succinate, and butyrate and $^{13}$C-labeled butyrate with unlabeled NaHCO$_3$. We determined the metabolic fluxes using measurements of organic acids and CO$_2$, the *R. palustris* biomass composition (7), and the $^{13}$C-labeling patterns obtained from proteinaceous amino acids (see Tables S2 to S4 in the supplemental material). The labeling patterns that
were informative for determining specific fluxes for [1,4-\(^{13}\)C]fumarate and [1,4-\(^{13}\)C]succinate essentially the same as those described for [1-\(^{13}\)C]acetate (7). For example, fully labeled serine was a pattern uniquely generated by the Calvin cycle as it assimilated \(^{13}\)CO\(_2\) originating from the \(^{13}\)C-carboxyl groups of the organic substrate. Conversely, metabolism of [2,4-\(^{13}\)C]butyrate resulted in the liberation of unlabeled carboxyl groups, such that the informative labeling patterns were the inverses of those obtained when the carboxyl groups were labeled (e.g., the Calvin cycle uniquely generated fully unlabeled serine).

The central metabolic flux maps obtained for the wild type and the NifA* strain grown on the different substrates and previously reported flux maps for cells grown on acetate (7) are shown in Fig. 1 (values and confidence intervals, including those for biosynthetic fluxes, are in Tables S5 to S8 in the supplemental material).

% C recovery % electron recovery

Table 2: Conversion of substrates to biomass, CO\(_2\), organic acids, and H\(_2\) by R. palustris during exponential growth

| Substrate       | WT     | NifA*   | WT     | NifA*   | WT     | NifA*   | WT     | NifA*   | WT     | NifA* |
|-----------------|--------|---------|--------|---------|--------|---------|--------|---------|--------|-------|
| Fumarate        | 10.6 ± 1.0| 13.2 ± 0.8| 62 ± 3| 47 ± 2| 28 ± 1| 29 ± 1| 12 ± 2| 30 ± 3| 18 ± 3| 101 ± 5| 106 ± 2| 104 ± 6| 109 ± 3|
| Succinate       | 6.5 ± 0.4| 8.2 ± 0.5| 82 ± 3| 67 ± 3| 15 ± 1| 27 ± 1| 0.1 ± 0.0| 0.1 ± 0.0| 23 ± 1| 97 ± 4| 94 ± 3| 106 ± 4| 99 ± 3|
| Acetate         | 8.4 ± 0.6| 9.4 ± 0.6| 88 ± 8| 79 ± 4| 6 ± 1| 17 ± 2| 2 ± 0| 21 ± 3| 93 ± 8| 96 ± 5| 98 ± 9| 99 ± 5|
| Butyrate-HCO\(_3\) | 8.6 ± 0.4| 10.7 ± 1.0| 83 ± 6| 84 ± 6| 18 ± 4| 11 ± 3| 23 ± 5| 23 ± 5| 11 ± 3| 94 ± 5| 97 ± 5| 97 ± 9| 99 ± 8|
| Butyrate        | No growth| 32.4 ± 7.6| 67 ± 12| 6 ± 1| 24 ± 2| 41 ± 10| 97 ± 14| 96 ± 15|

a Unlabeled cultures were grown in minimal medium with NH\(_4\) as the nitrogen source. Values are averages from 3 to 5 biological replicates ± DSD based on samples taken during early exponential growth.

b Values are normalized for organic C consumed to account for acetate having two carbon atoms, whereas the other substrates have four. Negative signs indicate that there was a net consumption of CO\(_2\), which was made possible by the NaHCO\(_3\) supplement.

c Molecules of biomass were determined from the R. palustris 420L elemental composition (25): CH\(_{1.8}\)N\(_{0.18}\)O\(_{0.38}\) (mole weight, 22.426 g/mol).

d Maltose was excreted during growth on fumarate, fumarate was excreted during growth on succinate, and acetate was excreted during growth on butyrate.

e The percentage of organic carbon and electrons consumed that were observed in products. The sum of the values in biomass, CO\(_2\), and organic acids would equal 100 for full carbon recovery. Electron recovery was based on available hydrogen as described previously (7, 26).

f Growth rates during the second growth phase on fumarate.

g Calculated by grouping fumarate and malate as a single metabolite [i.e., \(\Delta H_2/d\text{fumarate} + \text{malate} \times 100/4\text{carbon atoms}\)]. The H\(_2\) yield from fumarate consumed alone would give a value of 12 ± 2.

h The acetate data were previously published (7).
When acetate is taken up, it is converted directly to acetyl-CoA. However, succinate is processed through the TCA cycle and decarboxylated twice to produce acetyl-CoA. As a consequence, growth on succinate involved a relatively high forward flux through pyruvate dehydrogenase and/or pyruvate ferredoxin oxidoreductase (PDH/POR), which reduces electron carriers. The high forward PDH/POR flux during growth on succinate contributed to a total flux through reactions that reduce electron carriers that was similar to that observed during growth on acetate. A second consequence of the route from succinate to acetyl-CoA is the loss of carbon as CO₂. The net CO₂ yield was three times higher during growth on succinate than during that on acetate. This high CO₂ yield from succinate resulted in less carbon for biosynthesis. Therefore, biosynthetic reactions during growth on succinate do not require as many reducing equivalents as those during growth on acetate. To counter the extra electron carrier reduction by PDH/POR and the lack of electron carrier oxidation by biosynthesis, the Calvin cycle refixed about half of the CO₂ produced from succinate oxidation.

FIG 1 Central metabolic fluxes in non-H₂-producing wild-type cells and H₂-producing NifA* cells. The metabolic network is based on the R. palustris genome sequence and simplified by grouping reactions that do not affect labeling patterns. The Calvin cycle is highlighted in green, the TCA cycle in blue, and the glyoxylate shunt in red. Underlined metabolites indicate starting points for biosynthetic reactions that are not shown. The complete network was described previously (7) and is described in Table S1 in the supplemental material. Net flux magnitude, as mole percentage of the substrate uptake flux, is indicated by arrow thickness. The value for acetate uptake was normalized to 100, whereas the values for 4-carbon substrates were normalized to 50 to account for the different carbon contents. Net flux direction is indicated by an enlarged arrowhead for those fluxes assumed to be bidirectional. Wild-type flux distributions are shown along the top for fumarate (A), succinate (B), acetate (C), and butyrate with NaHCO₃ (D). NifA* flux distributions during H₂ production are shown along the bottom for fumarate (E), succinate (F), acetate (G), butyrate with NaHCO₃ (H), and butyrate with NaCl (I). Flux distributions for acetate were previously reported (7). All fluxes are based on samples taken during early exponential growth. Reactions: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDH/POR, pyruvate dehydrogenase/pyruvate ferredoxin oxidoreductase; RuBisCO, ribulose 1,5-bisphosphate carboxylase. Metabolites: 3PG, 3-phosphoglycerate; AcCoA, acetyl coenzyme A; αKG, α-ketoglutarate; Cit/Itc, citrate/isocitrate; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; Fum/Mal, fumarate/malate; Fr, ferredoxin; G6P, glucose-6-phosphate; GAP, glyceraldehyde 3-phosphate; Glx, glyoxylate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate; QOH, quinol; R5P, pentose phosphates; R1,5P, ribulose 1,5-bisphosphate; S7P, sedoheptulose 7-phosphate; Succ, succinate; XH, unknown reduced electron carrier.
locations, such as succinate and fumarate, result in Calvin cycle fluxes that more closely reflect the substrate oxidation state. Fumarate was processed similarly to succinate to reach biosynthetic precursors, including a relatively large flux through PDH/POR (Fig. 1). The similar metabolic flux distributions are reflected in the similar levels of substrate converted to CO₂ (Table 3). However, since fumarate is not processed by succinate dehydrogenase (Fig. 1) (Suc → Fum/Mal), there was less total reduction of electron carriers than during growth on succinate (Fig. 2A). Correspondingly, the wild-type Calvin cycle (GAPDH) flux that recycles oxidized electron carriers was about half of that obtained during growth on succinate (Fig. 1 and 2A). The lower RuBisCO flux recaptured only 21% of the CO₂, resulting in 1.8-fold-higher net CO₂ yield during growth on fumarate than during that on succinate (Fig. 1 and Table 3).

Similarly, acetate and butyrate are both processed through acetyl-CoA and the glyoxylate shunt. However, degradation of butyrate to two acetyl-CoA molecules through -oxidation results in two reduced electron carriers (Fig. 1). As a result, the total flux that reduces electron carriers was nearly 1.5 times higher during growth of wild-type R. palustris on butyrate with NaHCO₃ than during that on acetate (Fig. 2A). To compensate, GAPDH flux was 1.8-fold higher during growth on butyrate with NaHCO₃ than during that on acetate (Fig. 1). As observed during growth on acetate (7), growth on butyrate was associated with a small amount of electron carrier oxidation by a proposed POR flux from

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**TABLE 3** CO₂ produced by various metabolic reactions and refixed by RuBisCO⁺

| Substrate            | % of substrate converted to CO₂ (relative to amt of substrate consumed) | % of CO₂ refixed by Calvin cycle (relative to amt of substrate converted to CO₂) | Net CO₂ yield (% relative to amt of substrate consumed) |
|----------------------|-------------------------------------------------------------------------|---------------------------------------------------------------------------------|--------------------------------------------------------|
|                      | WT | NifA⁺ | WT | NifA⁺ | WT | NifA⁺ |
| Fumarate             | 40 ± 4 | 44 ± 4 | 21 ± 9 | 6 ± 1 | 32 ± 2 | 42 ± 2 |
| Succinate            | 37 ± 3 | 40 ± 2 | 49 ± 7 | 30 ± 5 | 19 ± 2 | 28 ± 2 |
| Acetate              | 22 ± 2 | 23 ± 1 | 68 ± 11 | 13 ± 3 | 6 ± 1 | 18 ± 1 |
| Butyrate-HCO₃⁻       | 16 ± 1 | 15 ± 3 | 180 ± 16 | 149 ± 36 | -16 ± 1/ | -10 ± 3/ |
| Butyrate             | 23 ± 3 | 23 ± 3 | 76 ± 17 | 6 ± 1 | |

* Average values with 90% confidence intervals were derived from the fluxes shown in Fig. 1. Minor variations between CO₂ yields in Tables 2 and 3 are due to changes made by the fitting algorithm to find the most likely set of fluxes to explain all of the data.

+ All values were calculated by grouping malate and fumarate as a single pool. This grouping results in different CO₂ yields between Tables 2 and 3, because the CO₂ yields in Table 2 were normalized to fumarate alone so that the amount of malate produced could also be reported. If fumarate and malate were grouped in Table 2, the CO₂ yields would be the same as those reported in Table 3.

- The acetate data were previously published (7).

- Wild-type cells do not grow without the NaHCO₃ supplement.

- One hundred percent of the butyrate converted to CO₂ was refixed along with CO₂ from the NaHCO₃ supplement.

- The negative values indicate that there was a net uptake of CO₂ from the NaHCO₃.
maps look very similar for non-H$_2$-producing (wild-type) and
Grows under these conditions. As can be seen in Fig. 1, the flux
for. Unlike what was observed on acetate, increased TCA cycle
and 43% of the H$_2$ production during growth on fumarate, succi-
(7), the decrease in Calvin cycle flux made nearly 90% of the elec-
tron carrier that is reduced, which in turn affects the amount of
electron sink (Fig. 2B). For example, the NifA$^+$ CO$_2$-fixing
RuBisCO flux was still 75% of the wild-type value during growth
on butyrate with NaHCO$_3$ (Fig. 2). The importance of CO$_2$ fixa-
tion during growth on butyrate was further exemplified by our
analysis of cells grown in the absence of NaHCO$_3$. Without
NaHCO$_3$, the NifA$^+$ strain grew about 3 times more slowly on
butyrate (Table 2) and relied heavily on the Calvin cycle to oxidize
electron carriers, using CO$_2$ released endogenously from butyrate
itself (Table 3).

Calvin cycle fluxes decrease in response to H$_2$ production.
Using the NifA$^+$ strain, we examined the effect of H$_2$ production
on metabolic fluxes for the four different substrates. We also ob-
tained a flux map for cells grown on butyrate without added
NaHCO$_3$, since unlike wild-type R. palustris, the NifA$^+$ strain
grows under these conditions. As can be seen in Fig. 1, the flux
maps look very similar for non-H$_2$-producing (wild-type) and
H$_2$-producing (NifA$^+$) cells except for the Calvin cycle fluxes,
which are lower in H$_2$-producing cells. During growth on acetate
(7), the decrease in Calvin cycle flux made nearly 90% of the elec-
trons for the H$_2$ produced available, with the rest being accounted
for by decreased use of reducing equivalents for biosynthesis
(compare Fig. 2A and B). There is less need for reducing equiva-

tents generated by the oxidation of each substrate were used for
fixing CO$_2$ into biomass, as
indicated by the lower biomass yields for the NifA$^+$ strain
(Table 2). Similar to what was observed on acetate, the decrease in
Calvin cycle flux during growth of the NifA$^+$ strain on butyrate
with NaHCO$_3$ was enough to account for all of the H$_2$ produced
(Fig. 2). For the other substrates, the decrease in Calvin cycle flux
did not make a large contribution, accounting for 29%, 64%,
and 43% of the H$_2$ production during growth on fumarate, succi-
nate, and butyrate, respectively. Nevertheless, in each case the de-
crease in Calvin cycle GAPDH flux was the largest single contribu-
tor of electrons for H$_2$ production. This cannot be said with
absolute certainty for growth on fumarate, as 36% of the electrons
needed to explain the amount of H$_2$ observed were unaccounted
for. Unlike what was observed on acetate, increased TCA cycle
fluxes contributed some electrons for H$_2$ production during
growth on fumarate, succinate, and especially butyrate without
NaHCO$_3$ (Fig. 2). This is not evident from a visual inspection of
the flux maps in Fig. 1, because several reactions contribute to the
overall TCA cycle flux.

H$_2$ production accounted for one-third of electron carrier oxida-
tion in the NifA$^+$ strain during growth on acetate, compensat-
ning for the low Calvin cycle flux (Fig. 2B) (7). However, on other
carbon sources, like succinate and butyrate, the Calvin cycle still
oxidized a large proportion of the reduced electron carriers, even
when nitrogenase was induced and available to form H$_2$ as an
electron sink (Fig. 2B). For example, the NifA$^+$ CO$_2$-fixing
RuBisCO flux was still 75% of the wild-type value during growth
on butyrate with NaHCO$_3$ (Fig. 2). The importance of CO$_2$ fixa-
tion during growth on butyrate was further exemplified by our
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butyrate (Table 2) and relied heavily on the Calvin cycle to oxidize
electron carriers, using CO$_2$ released endogenously from butyrate
itself (Table 3).

As we observed with acetate (7), the decrease in Calvin cycle flux
was accompanied by a decrease in the transcription of Calvin
cycle genes during growth on the other substrates (Fig. 3). In par-
allel with the flux data, decreases in Calvin cycle gene expression
were generally not as drastic as those observed during growth on
acetate, especially for $cbbM$ (RuBisCO type II) and $cbbP$ (phos-
phoribulokinase).

H$_2$ yields increase when Calvin cycle flux is blocked by
mutation. Since a significant percentage of the reducing equiva-

teurs generated by the oxidation of each substrate were used for
CO$_2$ fixation, even during H$_2$ production (Fig. 2), the Calvin cycle
represents an attractive, nonessential target to mutate to force
more electrons towards H$_2$. To test this, we grew the NifA$^+$ strain
and a NifA$^+$ $\Delta$RuBisCO strain (CGA679; NifA$^+$ strain with both
sets of RuBisCO genes deleted) side by side on the four substrates
and compared the H$_2$ yields. For each substrate, the H$_2$ yield from
the NifA$^+$ $\Delta$RuBisCO strain was higher than that from the NifA$^+$
parent (Fig. 4). When RuBisCO type I was expressed from a plas-
mid vector in the NifA$^+$ $\Delta$RuBisCO strain, the H$_2$ yields were
similar to or lower than those of the parent. Introduction of an
eempty vector into this strain has no effect on H$_2$ production. The
NifA$^+$ parent still relied heavily on the Calvin cycle during growth
on succinate and butyrate (Fig. 2B). Thus, we expected a larger
increase in the H$_2$ yields for the $\Delta$RuBisCO strain on succinate and

![FIG 3](mbio.asm.org March/April 2011 Volume 2 Issue 2 e00323-10)
butyrate than on acetate. Indeed, the \( H_2 \) yields on succinate and butyrate increased about 2-fold, compared to 1.3-fold during growth on acetate. In general, our data indicate that the Calvin cycle flux of a NifA* strain can be used to predict the \( H_2 \) yield of a NifA* ΔRuBisCO strain (Fig. 4). However, the increase in the \( H_2 \) yield for fumarate was unexpectedly high, as the parent NifA* strain was estimated to have low Calvin cycle flux (Fig. 2B). As we observed previously (7), during growth on acetate, the NifA* ΔRuBisCO strain grew more slowly than the NifA* parent on all substrates (the NifA* ΔRuBisCO strain growth rate ranged from 0.6 [fumarate] to 0.9 [acetate] times that of the parent growth rate).

DISCUSSION

Balancing electrons is a challenge for a PNSB like \( R. \) palustris growing phototrophically because energy is obtained by cycling electrons in cyclic photophosphorylation and not by transferring them to a terminal electron acceptor. During this mode of growth, reducing equivalents that are generated during the oxidation of an organic carbon source, but which cannot be put towards biosynthesis, can be used to fix \( CO_2 \) via the Calvin cycle or released acid excretion does not affect the final \( H_2 \) yield, since excreted compounds were eventually consumed. Changes to polyhydroxybutyrate and other biomass components (in addition to the potential effects of biphasic growth on labeling patterns) could help explain why we were unable to account for 36% of the electrons in \( H_2 \) produced from fumarate by the NifA* strain. We assumed that the biomass composition on fumarate was the same as that observed for growth on succinate and acetate (7). It was also suggested that the free energy of a substrate can influence the \( H_2 \) yield from PNSB (5). However, our results (and data from others) do not show the same correlation (see Fig. S2 in the supplemental material). Rather, there appears to be a large variability in \( H_2 \) yields among PNSB (Fig. S2), barring any influence from the different experimental procedures used. In this paper, we identified two metabolic factors that help explain variable \( H_2 \) yields among different PNSB: (i) the route taken to generate biosynthetic precursors and (ii) the amount of competing Calvin cycle flux.

In some cases, the biochemical constraints of a metabolic network affect the overall need for electron carrier oxidation, and thereby the amount of \( CO_2 \) fixed or \( H_2 \) produced, by dictating the route that must be taken towards biosynthetic precursors. Specifically, the route succinate took to generate acetyl-CoA produced a level of reducing equivalents similar to that produced by growth on acetate (Fig. 4). This led to Calvin cycle fluxes and \( H_2 \) yields that were unexpectedly high with succinate relative to those with acetate. This effect is expected to be more pronounced when bacteria that have different metabolic inventories are compared. For example, \( R. \) sphaeroides assimilates \( CO_2 \) using the reductive ethylmalonyl-CoA pathway (14), unlike \( R. \) palustris, which uses the oxidative glyoxylate shunt. It was recently shown that the ethylmalonyl-CoA pathway oxidizes enough electron carriers during acetate assimilation such that the Calvin cycle and \( H_2 \) production were dispensable for phototrophic growth (15). Given the obligate nature of this reductive pathway in \( R. \) sphaeroides for growth on acetate, one would expect that \( H_2 \) yields would be lower than those of a bacterium using the glyoxylate shunt.

To produce \( H_2 \), the NifA* strain shifted electrons away from \( CO_2 \) fixation to \( H_2 \) production, such that the necessary electron carrier oxidation was shared by the two activities. However, the Calvin cycle flux decreased to different extents, depending on the substrate (Fig. 1 and 2), and thereby affected the \( H_2 \) yield. We verified this by showing that a NifA* ΔRuBisCO strain that is incapable of Calvin cycle flux had higher \( H_2 \) yields than the NifA* parent (Fig. 4). Preventing Calvin cycle flux in a \( R. \) rubrum NifA* mutant was also recently shown to improve the \( H_2 \) yield (11). It is not clear why there was a greater decrease in Calvin cycle flux in response to \( H_2 \) production during growth on acetate than during growth on other substrates. One possibility is that the higher levels of \( CO_2 \) produced during growth on fumarate and succinate and the addition of NaHCO\(_3\) to the butyrate cultures allowed for greater participation of RuBisCO type II, which has a low affinity for \( CO_2 \).

There are other factors that can also affect the \( H_2 \) yield. It is well documented that excretion of organic acids or synthesis of electron-rich polymers such as polyhydroxybutyrate can influence the \( H_2 \) yield (5, 12). Under our growth conditions, organic acid excretion does not affect the final \( H_2 \) yield, since excreted compounds were eventually consumed. Changes to polyhydroxybutyrate and other biomass components (in addition to the potential effects of biphasic growth on labeling patterns) could help explain why we were unable to account for 36% of the electrons in \( H_2 \) produced from fumarate by the NifA* strain. We assumed that the biomass composition on fumarate was the same as that observed for growth on succinate and acetate (7). It was also suggested that the free energy of a substrate can influence the \( H_2 \) yield from PNSB (5). However, our results (and data from others) do not show the same correlation (see Fig. S2 in the supplemental material). Rather, there appears to be a large variability in \( H_2 \) yields among PNSB (Fig. S2), barring any influence from the different experimental procedures used. In this paper, we identified two metabolic factors that help explain variable \( H_2 \) yields among different PNSB: (i) the route taken to generate biosynthetic precursors and (ii) the amount of competing Calvin cycle flux.

**FIG 4** The \( H_2 \) yield increases when Calvin cycle flux is blocked by mutation. Hydrogen yields from a NifA* ΔRuBisCO strain that is incapable of Calvin cycle flux due to the deletion of all genes encoding RuBisCO enzymes (CGA679; black bars) were higher than those from the NifA* parent (CGA676; white bars). Including pBBPgdh as a vector control (dark gray bars) does not affect \( H_2 \) yields, but expressing RuBisCO type I from pBBPebhLSX in the ΔRuBisCO strain results in \( H_2 \) yields similar to those from the NifA* parent (light gray bars). Averages from 3 to 10 biological replicates are shown with 90% confidence intervals. Hydrogen yields from acetate were reported previously (7). The shaded boxes show the \( H_2 \) yield ranges, based on 90% confidence intervals, expected if all electrons associated with Calvin cycle flux in the NifA* parent were diverted to \( H_2 \) production.
TABLE 4 Strains and plasmids used

| Strain or plasmid | Genotype or phenotype | Reference |
|------------------|-----------------------|-----------|
| **R. palustris strains** | | |
| CGA009 | Wild-type strain; spontaneous Cm\(^t\) derivative of CGA001 | 17 |
| CGA676 | NifA\(^t\); produces H\(_2\) in the presence of NH\(_4\)\(^+\) | 7 |
| CGA669 | \(\Delta\)bblS::Km\(^t\) \(\Delta\)cbbM mutant of CGA009 | 7 |
| CGA679 | \(\Delta\)bblS::Km\(^t\) \(\Delta\)cbbM mutant of CGA676 | 7 |
| **Plasmids** | | |
| pBBPgdh | Gmr\(^t\); pBBR1MCS-5 with RPA0944 promoter between Knpl and Xhol sites | 7 |
| pBBPcbbLSX | Gmr\(^t\); cbbLSX with native ribosomal binding site cloned into pBBPgdh | 7 |

**MATERIALS AND METHODS**

**Chemicals, bacteria, and culture conditions.** [1,4-\(^13\)C]fumaric acid and [1,4-\(^13\)C]succinic acid were purchased from Cambridge Isotope Laboratories (Andover, MA) and were neutralized with NaOH prior to use. Sodium [2,4,4-\(^13\)C]butyrate was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from either Sigma-Aldrich or Fisher Scientific (Pittsburg, PA). All experiments were conducted on *R. palustris* wild-type strain CGA009 or its derivatives and are listed in Table 4. CGA009 is defective in uptake hydrogenase activity (16). CGA669 is a NifA\(^t\) strain derived from CGA009 that constitutively produces H\(_2\) via a NifA\(^t\) strain derived from CGA009 that constitutively produces H\(_2\) via.

**Fisher Scientific (Pittsburg, PA). All experiments were conducted on *R. palustris* wild-type strain CGA009 or its derivatives and are listed in Table 4. CGA009 is defective in uptake hydrogenase activity (16). CGA669 is a NifA\(^t\) strain derived from CGA009 that constitutively produces H\(_2\) via a NifA\(^t\) strain derived from CGA009 that constitutively produces H\(_2\) via.

**Analytical techniques.** Organic acids were quantified using a Varian high-performance liquid chromatograph (HPLC) with a UV detector at 210 nm as described previously (18). H\(_2\) was quantified using a gas chromatograph-mass spectrometry (GC-MS) as described previously (21). Mass isotopomer distributions were corrected for natural abundances of all atoms except for the carbon atoms in amino acid backbones using previously described software (22). Corrected amino acid mass isotopomer distributions and extracellular flux measurements (i.e., organic acid excretion, CO\(_2\) production, and biosynthetic fluxes based on the *R. palustris* biomass composition [7]) were used with a metabolic model (see Table S1 in the supplemental material) based on the *R. palustris* genome sequence (7) to solve intermediary metabolic fluxes using previously described software (23). Confidence intervals (90\%) for individual fluxes were determined as described previously (24). No redox constraints were used in determining metabolic flux distributions. In our metabolic model, fumarate and malate were treated as a single pool to account for simultaneous production and consumption of fumarate and malate (see Results). Since fumarate and malate have the same oxidation state, grouping these two metabolites did not affect our electron balance calculations. The *R. palustris* biomass compositions were based on data collected for CGA009 and CGA676 grown on either succinate or acetate in the presence of NH\(_4\)\(^+\) (7) and were assumed to be similar for growth on fumarate and butyrate for this study.

**Electron balance and H\(_2\) yield calculations.** Electron balance was assessed by two different methods. The first method used extracellular measurements to determine the sum of the electrons in the H\(_2\), organic acids, and biomass produced as a fraction of electrons in substrate consumed. *R. palustris* biomass was assumed to have 4.5 electrons per mole of carbon and was determined using a standard curve relating OD\(_{560}\) to dry cell weight and an assumed biomass composition of CH\(_{1.8}\)N\(_{0.18}\)O\(_{0.38}\) (25) as described previously (7). The second method used intermediary flux values obtained from \(^1\)C-labeling experiments to determine the sum of fluxes through reactions that oxidize electron carriers as a fraction of the sum of fluxes that reduce electron carriers. For this calculation, we did not distinguish between different types of electron carriers, as we assumed that *R. palustris* uses transhydrogenase to transfer electrons between different carriers (7).

**qRT-PCR analysis.** RNA was purified from cultures in early exponential growth (0.3 to 0.5 OD\(_{560}\) units) using an RNAsen mini kit (Qiagen, Valencia, CA), and genes encoding the large subunit of Rubisco type I (cbbL), Rubisco type II (cbbM), and phosphoribulokinase (cbbP) were used to assess Calvin cycle gene expression by quantitative reverse transcription-PCR (qRT-PCR) as described previously (7). Further details are provided in Fig. S1 in the supplemental material.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00323-10/-/DCSupplemental.

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