Chapter 7

Systems Biology of Photobiological Hydrogen Production by Purple Non-sulfur Bacteria

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Summary

Photosynthetic purple non-sulfur bacteria (PNSB) can naturally convert electrons from organic compounds, protons from water, and energy from light into H_2 gas, via the enzyme nitrogenase. In 2004, the first PNSB genome sequence was reported, that of *Rhodopseudomonas palustris* strain CGA009. The CGA009 genome sequence revealed natural attributes that favored H_2 accumulation and revealed further potential for enhancing H_2 production. Since

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then, the genomes of several more *Rp. palustris* species and other PNSB have been sequenced. Comparing these genomes has led to new ideas for improving the substrate range, rate, and photosynthetic efficiency of H_2 production. Furthermore, systems biology or 'omics' approaches, including transcriptomics, proteomics, and fluxomics have been applied. Many of these systems level approaches have focused on the regulation and activity of nitrogenase – the enzyme responsible for H_2 production. Guided by these approaches, metabolic engineering has targeted metabolic pathways that compete with H_2 production for electrons, leading to strains with higher H_2 yields and potentially linking the survival of these strains to the production of H_2 biofuel. A systems level examination of PNSB is now turning to characterize largely unexplored but potentially crucial aspects involved in H_2 production including non-coding small RNAs and post-translational modifications. Systems biology approaches are also being designed to eliminate experimenter bias and highlight genes of unknown function that contribute to H_2 production, ideally providing clues to their function and their place in bacterial physiology. This chapter describes the contributions of systems biology to our understanding and application of H_2 production by PNSB, focusing on *Rhodopseudomonas palustris* and referencing examples from other PNSB.

I. Introduction

A. Background on Purple Non-sulfur Bacterial Physiology

Purple non-sulfur bacteria (PNSB) are α- and β-proteobacteria that have long fascinated researchers with their metabolic versatility. PNSB employ different metabolic modules to thrive in different environments. In the dark, PNSB grow using aerobic respiration or anaerobic respiration. The anaerobic electron acceptors that PNSB use vary between species and strains, but can include electron acceptors of denitrification (e.g, NO_3^- and N_2O), dimethylsulfoxide, and trimethylammonium oxide (McEwan et al. 1985; Ferguson et al. 1987; McEwan 1994). However, PNSB are most commonly grown and studied under photosynthetic conditions. Photosynthesis by PNSB is anoxygenic. Thus, PNSB are unlike oxygenic cyanobacteria, plants, and algae that obtain electrons from water and produce O_2 as a waste product. PNSB use a single photosystem that resembles photosystem II but it is incapable of oxidizing water and thus no O_2 is produced. Instead, PNSB use organic compounds as both a source of carbon and electrons during photosynthetic growth – a photoheterotrophic lifestyle (Fig. 7.1a). Alternatively, PNSB can use inorganic electron donors other than water (e.g., H_2, thiosulfate, or Fe^{2+}) as an electron source and CO_2 as a carbon source – a photoautotrophic lifestyle (Fig. 7.1b). Electrons pulled from the electron donor are energized by the photosystem using light and channeled through a H^+-pumping electron transfer chain. The resulting proton motive force can be used to make ATP via ATP synthase or to power other energy-requiring processes (e.g., solute uptake via H^+-symport). The electrons can be donated to $NADP^+$ to generate NADPH for biosynthesis through reverse electron transfer (a process that utilizes the proton motive force). Alternatively, the electrons can be repeatedly energized and cycled through the electron transfer chain. This cycling allows for the continuous maintenance of the proton motive force and ATP pools in a process called cyclic photophosphorylation. Cyclic photophosphorylation is particularly advantageous under starvation conditions as cycling a few electrons can generate usable energy for cell maintenance and repair.

Abbreviations: αKG – α-ketoglutarate or 2-oxoglutarate; GOGAT – Glutamine 2-oxoglutarate aminotransferase; PNSB – Purple nonsulfur bacterium/bacteria; Rubisco – Ribulose 1,5 bisphosphate carboxylase

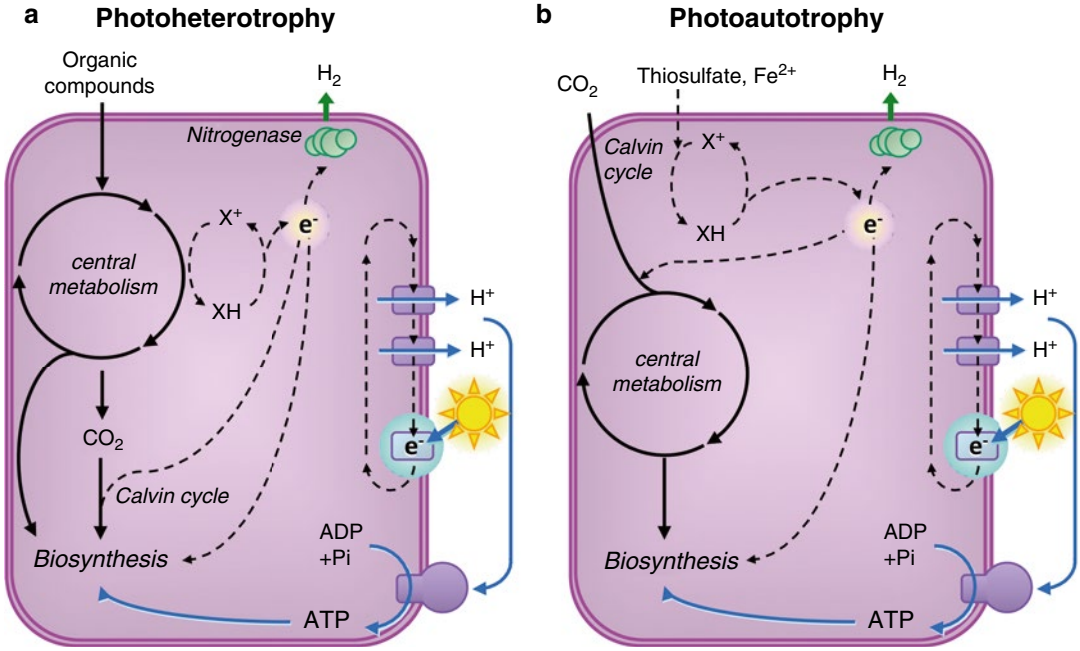

Fig. 7.1. PNSB like *Rp. palustris* can grow using light for energy and either organic or inorganic carbon and electron sources. (**a**) During photoheterotrophic growth, organic compounds serve as carbon and energy sources. Excess reductant can be oxidized through CO_2 fixation via the Calvin cycle or through H_2 production via nitrogenase. This oxidation of excess reductant is an essential process required for growth. (**b**) During photoautotrophic growth, CO_2 serves as the carbon source and is reduced to organic biosynthetic intermediates by the Calvin cycle. Inorganic compounds other than water, such as thiosulfate, serve as the electron source. Under certain conditions, some electrons can be channeled to H_2 production, resulting in simultaneous fixation of CO_2 greenhouse gas and production of H_2 biofuel (Figure modified from McKinlay and Harwood 2010a).

During photoheterotrophic growth, PNSB can use a wide variety of organic acids and alcohols but the ability to utilize sugar is less common trait observed in PNSB, though some PNSB like *Rhodobacter sphaeroides* are routinely grown with sugars (Fuhrer et al. 2005; Kontur et al. 2011). *Rp. palustris* is relatively unique among PNSB for its ability to degrade aromatic compounds (e.g., p-coumarate), released during the degradation of lignin by certain fungi (Harwood 2009). Additionally, *Rp. palustris* can degrade some chlorinated aromatic compounds. As a result, much of the earlier research on *Rp. palustris* was devoted to understanding the biochemistry of its aromatic compound degrading pathways which are potentially useful in bioremediating sites contaminated with chlorinated and aromatic pollutants (Harwood 2009). During photoautotrophic growth, PNSB obtain electrons from inorganic electron donors such as H_2 (most if not all PNSB), thiosulfate (i.e., *Rp. palustris* (van Niel 1944; Rolls and Lindstrom 1967)), and in some cases Fe^{2+} (i.e., *Rp. palustris* TIE-1 (Jiao et al. 2005) and *Rhodobacter* sp. SW2 (Croal et al. 2007)). When using inorganic electron donors, PNSB employ the Calvin cycle to utilize CO_2 as a carbon source. As will be described in detail later on, CO_2 fixation can actually be essential during photosynthetic growth on organic compounds, as it allows the cell to deal with the excess electrons that are invariably generated during growth on organic compounds. PNSB can also obtain nitrogen from atmospheric N_2 using the enzyme, nitrogenase. As described below, it is nitrogenase that is most often exploited by researchers when using PNSB to produce H_2 gas.

B. Hydrogen Gas Production by PNSB

As PNSB primarily consume compounds other than sugars, most research on H_2 production by PNSB has focused on using fermented agricultural waste as a feedstock. In this situation, the cellulose in agricultural material has been broken down into sugars and fermented mainly into organic acids. It is these organic acids (e.g., acetate and butyrate) that serve as the carbon and electron source for PNSB growth and H_2 production. Thus, it is envisioned that PNSB could couple H_2 production to waste-water remediation.

The two classes of enzymes known to produce H_2 are hydrogenase and nitrogenase. Though there are examples of PNSB producing H_2 via hydrogenase (e.g. *Rhodospirillum rubrum* (Fox et al. 1996a) and *Rp. palustris* BisB18 (Oda et al. 2008)), H_2 is more commonly produced via nitrogenase. Nitrogenase is better known for producing NH_4^+ from atmospheric N_2. However, H_2 is an obligate product of the nitrogenase reaction (Eq. 7.1). Even in the presence of 50 ATM of N_2, nitrogenase will continue to produce H_2 at a 1:2 ratio with NH_4^+ (Simpson and Burris 1984). In an atmosphere devoid of N_2, nitrogenase behaves like a hydrogenase, producing H_2 as the sole product (Eq. 7.2). An argument that is sometimes made against using nitrogenase to produce H_2 is that its turnover rate is an order of magnitude lower than Ni-hydrogenase and three orders of magnitude lower than Fe-hydrogenase (McKinlay and Harwood 2010b). However, this comparison is based on in vitro rates. In vivo rates of specific H_2 production show a much narrower gap (McKinlay and Harwood 2010b), perhaps because bacteria that use nitrogenase tend to produce large amounts of the enzyme to compensate for its slow rate.

$$\text{Mo-nitrogenase: } N_2 + 8H^+ + 8e^- + 16\,\text{ATP} \rightarrow 2\,NH_3 + H_2 + 16\,\text{ADP} \tag{7.1}$$

$$\text{Mo-nitrogenase in absence of } N_2\text{: } 8H^+ + 8e^- + 16\,\text{ATP} \rightarrow 4H_2 + 16\,\text{ADP} \tag{7.2}$$

$$\text{Hydrogenase: } 2H^+ + 2e^- \longleftrightarrow H_2 \tag{7.3}$$

Unlike hydrogenase, which simply requires an electron donor (Eq. 7.3), nitrogenase also requires ATP (Eqs. 7.1 and 7.2). This ATP requirement allows nitrogenase to generate very high levels of H_2 without the reaction slowing and eventually running in reverse, as is the case with hydrogenase. The high ATP requirement is not a barrier to H_2 production for PNSB, since they can produce ample ATP from recycled electrons via cyclic photophosphorylation, provided that they are illuminated. However, a significant hurdle in producing H_2 via nitrogenase is the repressive effects of NH_4^+. The repression of nitrogenase in response to NH_4^+ and strategies to bypass this regulation are described in Sect. III.A.

II. Purple Non-sulfur Bacteria in the Light of Genomics and Systems Biology

A. Rhodopseudomonas palustris *CGA009 – The First Purple Genome Sequence*

Rp. palustris CGA009 was the first PNSB genome to be published in 2004 (Larimer et al. 2004). Prior to that time most work on *Rp. palustris* had focused on the biochemistry of anaerobic pathways for the degradation of aromatic compounds and the biophysics of its photosynthetic apparatus. The genome sequence of *Rp. palustris* CGA009 revealed several features that made it naturally suited for H_2 production such as (i) an inactive

uptake hydrogenase, (ii) multiple nitrogenase isozymes, (iii) multiple pathways for consumption of aromatic compounds, and (iv) pathways for the utilization of inorganic electron donors. This section will describe these key features and the insights made into *Rp. palustris* physiology and H_2 production from comparative genomics, functional genomics, and targeted biochemical and mutational analyses.

1. An Inactive Uptake Hydrogenase

N_2 fixation by nitrogenase is electron-intensive – each enzymatic cycle requiring six electrons to make two NH_4^+ and another two electrons for the obligate production of H_2. From the perspective of maximizing cell growth, the production of H_2 is 'wasteful' as it would be beneficial to instead use the electrons in H_2 to fix more N_2. Indeed, N_2-fixing prokaryotes tend to encode a Ni-containing uptake hydrogenase to recapture H_2 electrons. Eliminating uptake hydrogenase is often the first step in increasing H_2 yields in PNSB but this was unnecessary in *Rp. palustris* CGA009. The CGA009 genome sequence revealed that *Rp. palustris* had an uptake hydrogenase but curiously CGA009 was incapable of growing photoautotrophically with H_2 as an electron donor (Rey et al. 2006). Upon closer examination, a 4-nucleotide deletion was noticed in *hupV*, which encodes a subunit of a hydrogen sensor protein needed to activate transcription of the hydrogenase gene cluster. Without uptake hydrogenase activity, H_2 produced via nitrogenase could escape the cell and accumulate in the sealed growth container (Rey et al. 2006). When the mutated gene was replaced with a 'repaired' sequence, *Rp. palustris* was able to grow photoautotrophically on H_2 and accumulated less H_2 when grown under N_2-fixing conditions (Rey et al. 2006). This repaired strain, CGA010, is sometimes referred to as the wild-type strain though it is derived from CGA009.

A microarray analysis was used to compare CGA009 with CGA010 grown under N_2-fixing conditions where the uptake hydrogenase is expected to capture H_2 from nitrogenase (Rey et al. 2006). The comparison confirmed that *hupV* is required for the expression of the hydrogenase gene cluster. Curiously, five other genes were differentially expressed between the two strains. Two genes encoding a putative dicarboxylic acid transporter, a predicted formate transporter, and a glutamine synthetase were all upregulated 2–8-fold in CGA010 relative to the *hupV*-defective CGA009, suggesting that HupV is involved in activating transcription of these genes under N_2-fixing conditions. It was speculated that the dicarboxylic acid transporter and glutamine synthetase could allow *Rp. palustris* to better assimilate oxidized organic acids and N_2 gas in the presence of H_2. In support of this hypothesis, CGA010 had slightly higher growth rates than CGA009 (Rey et al. 2006). The fifth gene encoded a hypothetical protein and showed 47-fold lower expression in CGA010 relative to CGA009, suggesting that HupV is involved in strong repression of this gene. As of yet, no phenotype has been associated with the differential expression of these genes and it is worth noting that CGA010 is indistinguishable from CGA009 if Ni^{2+} is not added to the growth medium. In other words, CGA010 cannot consume H_2 if Ni^{2+} levels are insufficient to support synthesis of Ni-containing uptake hydrogenase.

2. An Arsenal of H_2 Producing Nitrogenases

Perhaps the biggest surprise from the CGA009 genome sequence was the presence of genes encoding all three nitrogenase isozymes. Most N_2 fixing prokaryotes encode Mo-nitrogenase, which has a Fe-Mo cluster in the active site. However, there are two 'alternative' nitrogenases, V-nitrogenase and Fe-nitrogenase, named for the metals used in place of Mo in the active site. Many bacteria encode one alternative nitrogenase in addition to Mo-nitrogenase but prior to *Rp. palustris* CGA009, the only organisms known to harbor all three were non-photosynthetic *Azotobacter*

vinelandii (Joerger et al. 1989) and *Methanosarcina acetivorans* (Galagan et al. 2002). *Rp. palustris* CGA009 remains the only example of a photosynthetic microbe that encodes all three nitrogenase isozymes.

Alternative nitrogenases are advantageous for H_2 production because they are better producers of H_2 than NH_4^+ (Eqs. 7.4, 7.5, and 7.6). Furthermore, in vivo rates of H_2 production are comparable between strains expressing individual forms of each nitrogenase (Table 7.1). The alternative nitrogenases were shown to be expressed in *Rp. palustris* in response to genetic mutations rendering Mo-nitrogenase non-functional (Oda et al. 2005), similar to what was found for the expression of Fe-nitrogenase in *Rs. rubrum* (Lehman and Roberts 1991). This observation suggests that the alternative nitrogenases are expressed in response to severe nitrogen starvation (e.g., when Mo availability limits Mo-nitrogenase function). Microarray analysis of *Rp. palustris* cells expressing either of the two alternative nitrogenases show upregulation of genes involved in acquiring diverse forms of nitrogen, supporting the hypothesis that the alternative nitrogenases are part of a general response to nitrogen starvation. In other bacteria like *Rhodobacter capsulatus* the presence of Mo represses alternative nitrogenase gene expression (Masepohl et al. 2002) but this does not hold true for *Rp. palustris* (Oda et al. 2005) or *Rs. rubrum* (Lehman and Roberts 1991).

Table 7.1. Strains that use alternative nitrogenases grow more slowly but have higher specific productivities.

Nitrogenase expressed	Growth rate (h^{-1})[a]	H_2 production (μmol (mg protein)$^{-1}$)[a]	Specific H_2 productivity (μmol (mg protein)$^{-1}$ h^{-1})[b]
Mo-only	0.048	30	1.44
V-only	0.036	51	1.84
Fe-only	0.028	140	3.92

[a]Data taken from Oda et al. (2005)

[b]Specific H_2 productivities were calculated according to the Monod model where the specific rate of product formation is equal to the amount of product produced per unit biomass multiplied by the growth rate. This equation assumes a constant ratio between H_2 and biomass during the period in which the growth rate was measured

$$\text{Mo-nitrogenase: } N_2 + 8H^+ + 8e^- + 16\,\text{ATP} \rightarrow 2NH_3 + H_2 + 16\,\text{ADP} \quad (7.4)$$

$$\text{V-nitrogenase: } N_2 + 12H^+ + 12e^- + 24\,\text{ATP} \rightarrow 2NH_3 + 3H_2 + 24\,\text{ADP} \quad (7.5)$$

$$\text{Fe-nitrogenase: } N_2 + 24H^+ + 24e^- + 48\,\text{ATP} \rightarrow 2NH_3 + 9H_2 + 48\,\text{ADP} \quad (7.6)$$

As mentioned earlier, Mo-nitrogenase can produce H_2 as the sole product in the absence of N_2. In such a situation, all nitrogenase isozymes are equally matched in terms of electrons devoted to H_2 production. Thus, alternative nitrogenases are most advantageous under conditions where N_2 is plentiful, thereby allowing the electron balance to be shifted towards H_2 production while still assimilating enough nitrogen for growth. There is still much to be learned in regards to the regulation of the alternative nitrogenases. Through understanding how the enzymes are regulated it may be possible to express all three isozymes at once, increasing the copy number of these relatively slow enzymes and therefore the rate of H_2 production.

3. Finding the Route from Lignin Monomers to H_2

Rp. palustris is distinguished from most other PNSB by its ability to grow phototrophically on aromatic compounds. Many of these aromatic compounds are lignin monomers released during lignin degradation by fungi. The CGA009 genome sequence suggested two distinct routes for the degradation of the lignin monomer *p*-coumarate: a β-oxidation route and a non-β-oxidation route. Microarray and quantitative

^{15}N-proteomic analyses were used to identify which routes were used by comparing transcript and protein levels during growth on succinate versus *p*-coumarate (Pan et al. 2008). The agreement between the transcriptional and proteomic data sets pointed to the non-β-oxidation route for *p*-coumarate degradation and putative genes were identified for every step in the pathway (Pan et al. 2008). This approach greatly narrows the targets for identifying and characterizing lignin monomer-degrading enzymes through genetic and biochemical approaches, more so than basing predictions on a genome sequence alone. For example, the CGA009 genome was predicted to have a single β-oxidation pathway for degrading fatty acids encoded by *pimFABCDE*. Elimination of this gene cluster resulted in slower growth on several straight chain fatty acids and on benzoate – a common intermediate for many aromatic compound degradation pathways that itself is degraded by β-oxidation after ring cleavage (Harrison and Harwood 2005). However, even without this gene cluster, growth on these compounds was not eliminated and growth was unimpaired on some fatty acids like 8-carbon caprylate (Harrison and Harwood 2005). Thus, other pathways must exist to degrade long chain fatty acids and transcriptomic and proteomic approaches could help identify them.

4. Removing Greenhouse Gases While Producing Biofuels Through the Use of Inorganic Feedstocks

The genome sequence also revealed genes for utilizing some inorganic electron donors. A carbon monoxide dehydrogenase was found that could be used to convert CO (e.g., from syngas) into H_2 (Larimer et al. 2004). Thus far, the functionality of the *Rp. palustris* CO dehydrogenase has not been tested but *Rs. rubrum* has a CO dehydrogenase that has been intensively characterized (Bonam et al. 1989; Kerby et al. 1995; Shelver et al. 1995; Spangler et al. 1998; Munk et al. 2011). Although not mentioned in the original annotation, *Rp. palustris* CGA009 also encodes genes for the utilization of ferrous iron (RPA0746-4). Attempts to grow CGA009 on Fe^{2+} were unsuccessful, but closely related *Rp. palustris* TIE-1 grows photoautotrophically on Fe^{2+} using the homologous *pio* operon (Jiao et al. 2005; Jiao and Newman 2007). The CGA009 genome annotation also pointed to a *sox* operon, encoding a thiosulfate oxidizing complex (Larimer et al. 2004). In 1944, Van Neil demonstrated that *Rp. palustris* could use CO_2 as the sole carbon source and inorganic thiosulfate as the electron donor, setting it apart from purple non-sulfur bacteria like *Rb. capsulatus, Rb. sphaeroides*, *Rp. gelatinosa* (van Niel 1944), and *Rs. rubrum* (Rolls and Lindstrom 1967). Recently, it was shown that *Rp. palustris* can grow autotrophically on CO_2 and thiosulfate while fixing N_2/producing H_2 (Huang et al. 2010). Thus, when grown on inorganic electron donors like thiosulfate, PNSB can produce H_2 biofuel while removing CO_2 greenhouse gas – a claim usually reserved for processes using cyanobacteria and algae.

B. Comparative Rp. palustris *Genomics*

As of 2012, the genome sequences for seven *Rp. palustris* strains were available – more than any other PNSB species (genomes sequences were available for five *Rb. sphaeroides* strains, two *Rs. rubrum* strains, and single strains of several other PNSB species). Furthermore there are genome sequences available for 14 *Bradyrhizobium* species, which are more closely related to *Rp. palustris* than most PNSB are. In BLAST alignments performed in 2008, less than 1 % of the genes in a given *Rp. palustris* genome had top hits to *Rb. sphaeroides* or *Rs. rubrum* but about 80 % of the genes had a top hit to a *Bradyrhizobium* or *Nitrobacter* (excluding comparisons with other *Rp. palustris* genomes) (Oda et al. 2008). A specialized visual tool for comparing the first six *Rp. palustris* genomes to be sequenced is publically

available (http://public.tableausoftware.com/views/rhodo_palustris/uniquepfam2strain) (Simmons et al. 2011) as well as more general web-based resources for comparative genomics such as Integrated Microbial Genomes (http://img.jgi.doe.gov/). Comparative genomic analysis between *Rp. palustris* CGA009, closely related strain TIE-1, and four strains isolated from the Netherlands: BisA53, BisB5, BisB18, HA2 (with the two BisB strains isolated from the same half-gram of sediment) have been described (Oda et al. 2008; Simmons et al. 2011). About half of the genes were shared by all five strains while 10–18 % of the genes in each genome were strain specific. Surprisingly, the two strains isolated from the same half-gram of sediment shared fewer orthologs than any other comparison.

Overall, the genome of each strain suggested a specialization to a specific environment. Some of these specializations present potentially beneficial features for H_2 production. CGA009 was the only strain found to encode three nitrogenase isozymes (more recently sequenced strains, TIE-1 and DX-1, are missing the V-nitrogenase). CGA009 is thus specialized for H_2 production in diverse environments, with the potential to activate all three enzymes through genetic engineering. The V-nitrogenase in CGA009 was likely acquired by horizontal gene transfer. One comparative genomic study highlighted the presence of an aquaporin in CGA009 and BisB5 that could impart an advantage under freezing conditions, whereas the other strains lacking the aquaporin could have an advantage in an environment with high sugar concentrations (Simmons et al. 2011). Strain BisB18 showed some capacity for fermentative metabolism, encoding pyruvate formate-lyase and formate hydrogen-lyase. *Rs. rubrum* also encodes a fermentative hydrogenase that allows it to produce H_2 from CO in the dark (Fox et al. 1996b). This fermentative capacity raises the possibility of producing hydrogen both via nitrogenase and hydrogenase, which has been shown to result in a twofold higher H_2 yield when the *Rs. rubrum* hydrogenase was expressed in *Rb. sphaeroides* (Kim et al. 2008). BisB18 also has the ability to grow on methanol. Strain BisB5 encoded a larger repertoire of enzymes for degrading aromatic compounds under anaerobic conditions, perhaps making it ideally suited for using lignin monomers as a renewable feedstock for H_2 production. Strain BisA53 was able to absorb light at additional wavelengths not absorbed by the other strains, giving it the potential to have a higher efficiency in converting light energy into chemical H_2 energy by harnessing more of the light spectrum. As mentioned above, TIE-1 can potentially use Fe^{2+} as an electron source for H_2 production while fixing CO_2. CGA009 was unable to use Fe^{2+} as an electron donor despite encoding the necessary *pio* operon. BisB18 and BisA53 also encode the *pio* operon but growth on Fe^{2+} has not been tested. Finally, strain DX-1 is reported to interact with electrodes in a microbial fuel cell allowing for electricity generation (Xing et al. 2008) which can also be converted to H_2 via electrolysis (Cheng and Logan 2007).

The genome sequences of various PNSB genomes have revealed an impressive inventory of metabolic and physiological attributes that allow for the production of H_2 under a wide range of conditions. However, this metabolic versatility also introduces a challenge to identify and harness attributes that would enhance H_2 production while distinguishing them from those attributes that would work against H_2 production.

III. Deciphering and Engineering the Metabolic and Regulatory Mechanisms Involved in H_2 Production

A. Regulation of Nitrogenase in Response to NH_4^+

Although PNSB can photosynthetically generate ample ATP to run nitrogenase, the enzyme is subject to negative feedback by NH_4^+ (and other nitrogen compounds) at multiple levels. Nitrogenase is a complicated enzyme. It requires over 20 accessory genes

for its proper assembly and the individual subunits of the active enzyme must associate and disassociate eight times in one catalytic cycle to convert one N_2 into 2 NH_4^+, expending 16 ATP in the process. Thus, any microbe has good reason not to synthesize the enzyme if it can obtain NH_4^+ from the environment.

The inhibition of nitrogenase in response to NH_4^+ in PNSB has been most intensively studied in *Rs. rubrum* (Munk et al. 2011) (the PNSB in which nitrogenase was first discovered by Howard Gest (Gest 1999)) and *Rb. capsulatus* (Masepohl et al. 2002). Nitrogenase regulation has also been examined in other PNSB with seemingly subtle but sometimes important differences. Nitrogenase regulation is closely tied to the intracellular levels of α-ketoglutarate (αKG) and glutamine, which respectively signal nitrogen starvation and abundant NH_4^+. Both of these signal metabolites serve as substrates for the enzyme that sets the stage for most of the aminotransferase reactions in the cell: glutamine 2-oxoglutarate aminotransferase or GOGAT (note: 2-oxoglutarate is another name for αKG). As depicted in Fig. 7.2 the enzyme transfers an amino group from glutamine to αKG, producing two molecules of glutamate. Glutamate then serves as the amino donor for the synthesis of nearly all amino acids. If NH_4^+ is abundant, glutamine synthetase provides ample glutamine to move the GOGAT reaction forward (Fig. 7.2). If NH_4^+ is low, then the GOGAT reaction stalls, waiting for glutamine substrate. As a result, αKG accumulates and triggers a nitrogen starvation response including the synthesis of nitrogenase. The ratio of αKG to glutamine is first sensed by the uridylyltransferase, GlnD. GlnD then transmits the nitrogen status through the uridylylation state of small trimeric signal transduction proteins called PII proteins. PII proteins are uridylylated by GlnD when αKG is abundant and de-uridylylated by GlnD when glutamine is abundant. The PII uridylylation state determines how they will interact with downstream regulatory proteins involved in nitrogen metabolism.

In general for PNSB, the PII proteins are involved in nitrogenase regulation at three levels (Fig. 7.3): (i) transcriptional regulation involving the two-component regulatory system NtrBC, (ii) transcriptional regulation involving the σ^{54}-enhancer-binding protein NifA, and (iii) post-translational covalent modification of nitrogenase by DraT and DraG. When NH_4^+ is scarce and αKG accumulates, the uridylylated PII proteins cannot interact with NtrB. NtrB is then free to phosphorylate NtrC. Phosphorylated NtrC then activates the transcription of a regulon

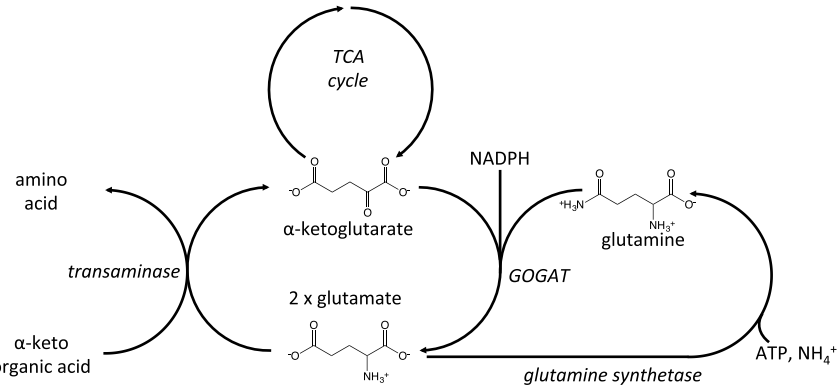

Fig. 7.2. The ammonium assimilation cycle. The nitrogen status of the cell (abundant ammonium or nitrogen starvation) is signaled through the levels of the two substrates for the glutamine 2-oxoglutarate aminotransferase (*GOGAT*) reaction: αKG and glutamine. The reaction produces two glutamate. Glutamate serves as an amino donor for the synthesis of nearly all amino acids via transaminase reactions. If NH_4^+ is scarce, glutamine cannot be synthesized via glutamine synthetase and αKG accumulates, signaling nitrogen starvation and nitrogenase is expressed.

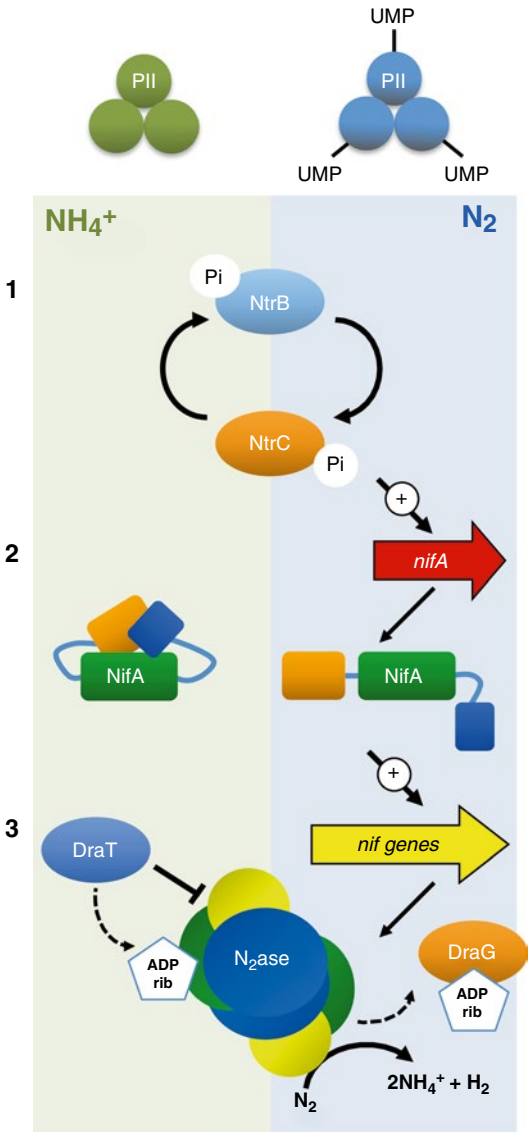

Fig. 7.3. Nitrogenase is regulated at three levels. (*1*) In the presence of NH_4^+ PII proteins respond to high glutamine levels and prevent phosphorylation of NtrC by NtrB. During nitrogen starvation, high α-ketoglutarate levels lead to the uridylylation of PII proteins and allow NtrB to phosphorylate NtrC. NtrC then promotes the transcription of genes involved in nitrogen fixation, including *nifA*. (*2*) PII proteins respond to the nitrogen status of the cell and either allow or prevent NifA from activating the transcription of nitrogenase-encoding genes. (*3*) Nitrogenase enzyme activity can be switched off if glutamine levels rise. PII proteins interact with DraT, which halts nitrogenase activity by adding ADP-ribosyl groups to nitrogenase. If α-ketoglutarate levels rise, PII proteins are uridylylated and DraG removes the ADP-ribosyl groups to allow nitrogenase activity to continue (Reprinted with permission from the American Society for Microbiology (Microbe, January 2006, p. 20–24)).

involved in nitrogen starvation, including *nifA*, which encodes the master transcriptional activator of nitrogenase. PII proteins are also generally thought to interact with NifA and during nitrogen-starvation cause NifA to bind to the enhancer of the nitrogenase operon, leading to the expression of nitrogenase and its many accessory proteins. Once nitrogenase is expressed and active there is a post-translational mechanism to switch off its activity in response to ammonium. If NH_4^+ becomes available, deuridylylated PII proteins interact with DraT, which adds an ADP-ribosyl group to nitrogenase, preventing its activity. If NH_4^+ becomes scarce before the inactivated nitrogenase is degraded, DraG can reactivate nitrogenase by removing the ADP-ribosyl groups.

The above regulatory network presented a major hurdle for photobiological production of H_2. In the lab, environmental conditions can be easily modified to induce nitrogenase expression. For example, providing glutamate as the nitrogen source and omitting N_2 gas (e.g., by growing cultures under argon) is a common method used to induce nitrogenase activity and maximize its hydrogenase activity. This technique carries over from the serendipitous discovery of nitrogenase in PNSB where Howard Gest observed H_2 production because he used a growth medium with glutamate as the sole nitrogen source (Gest 1999). However, most PNSB-based strategies for H_2 production envision using agricultural or industrial waste as a feedstock. These wastes invariably contain nitrogen compounds at concentrations that can repress nitrogenase and therefore H_2 production (Adessi et al. 2012). Overcoming this complicated and multilayered nitrogenase regulatory network appeared to be a monumental task. However, in *Rp. palustris* all that was necessary was a single nucleotide change.

B. Bypassing the Repression of Nitrogenase in Response to NH_4^+

The repression of nitrogenase in response to NH_4^+ is entirely due to regulatory mechanisms. The repression is not a direct chemical or thermodynamic effect of NH_4^+. To bypass

this regulatory network in *Rp. palustris* the Harwood lab applied a strong selective pressure for spontaneous mutations that would require *Rp. palustris* to produce H_2 to grow (Rey et al. 2007). Since the 1930s it has been known that PNSB require an electron acceptor to grow photosynthetically on organic compounds that contain more electrons per carbon than the average carbon in cellular biomass (Muller 1933). The cell must dispose of these excess electrons in order to maintain a pool of oxidized electron carrier molecules (e.g., NAD^+) required by crucial metabolic reactions. CO_2 is the traditional electron acceptor used in most experiments but the production of H_2 also suffices for eliminating excess electrons (McKinlay and Harwood 2011). The Harwood lab used this knowledge to select for *Rp. palustris* strains that constitutively produce H_2 by incubating cells in growth medium with NH_4^+ and an electron rich carbon source but without CO_2. After several months under constant illumination, some cultures suddenly grew and produced H_2 in the presence of NH_4^+ (Rey et al. 2007). Sequencing genes involved in nitrogenase regulation revealed that each mutant had a single nucleotide change in *nifA* – the gene encoding the master transcriptional activator of nitrogenase. A single nucleotide was confirmed to be all that was necessary for constitutive H_2 production by introducing the mutated gene into a wild-type genetic background. These mutants that produce H_2 constitutively are called NifA* strains. Since then a NifA* strain containing a 48-nucleotide deletion in *nifA* was constructed and has a more stable phenotype than the original spontaneous NifA* strains (McKinlay and Harwood 2010a).

It is remarkable that a single nucleotide change could bypass the entire nitrogenase regulatory network. As it turns out, this is a feature that may be unique to *Rp. palustris*. In *Rs. rubrum* a similar *nifA* mutation is required but the DraT activity must also be disrupted to prevent post-translational repression (Zou et al. 2008). Microarray and genetic approaches have been used to determine why nitrogenase is not switched off in *Rp. palustris* NifA* strains. In *Rp. palustris*, NtrBC activates the expression of the PII protein, GlnK2 (one of three PII proteins encoded in the CGA009 genome) which in turn controls DraT2 (one of two DraT proteins encoded in the genome) (Heiniger et al. 2012). When *Rp. palustris* is growing by N_2 fixation, NtrC is phosphorylated and GlnK2 is expressed. Under these conditions, the introduction of NH_4^+ causes GlnD to remove the uridylyl groups from GlnK2, and GlnK2 can activate DraT2 to switch off nitrogenase. However, when NifA* cells are grown with NH_4^+ (i.e., prolonged exposure to NH_4^+), NtrC is not phosphorylated and GlnK2 levels are low. Thus, there is insufficient GlnK2 to activate the switch off mechanism in NifA* strains grown with NH_4^+. When NifA* cells are grown with N_2 and then exposed to NH_4^+, a switch off response occurs but it is not nearly strong enough to prevent H_2 production. One reason for this low switch off activity appears to be insufficient DraT2 to completely switch off nitrogenase in NifA* strains. Comparisons of NifA* strains grown with NH_4^+ to wild-type cells grown with N_2 show that nitrogenase activity is about threefold higher in NifA* strains while DraT2 levels are similar. Indeed, expressing both *glnK2* and *draT2* from a plasmid in the NifA* strain resulted in H_2 production levels at 22 % that of the NifA* strain with an empty vector (Heiniger et al. 2012). Knocking out *draT2* resulted in a 1.3-fold increase in H_2 production indicating that NifA* strains were still subject to a low level of switch-off activity when grown with NH_4^+ (Heiniger et al. 2012).

Microarray analysis of NifA* strains has also been useful in defining the NifA regulon. When *Rp. palustris* is switched from growth on NH_4^+ to N_2, over 200 genes are differentially expressed – about 4 % of the genome (Oda et al. 2005). However, microarray comparisons between the NifA* strain and the wild type, both grown with NH_4^+, show that only 18 genes outside of the nitrogenase gene cluster increase their expression levels (Rey et al. 2007). Thus, it appears that most of the genes involved in N_2 fixation are not essential for the functioning of nitrogenase but are more likely part of a broad response to

nitrogen starvation. In contrast, the small regulon revealed by the NifA* strains potentially points to genes that are involved in nitrogenase function, and therefore H_2 production. These genes encode proteins that could form novel electron transfer chains delivering electrons to nitrogenase or in iron scavenging and storage to meet the high iron demands of a large pool of functional nitrogenase (Rey et al. 2007). Proteomic analysis of *Rp. palustris* also pointed to the importance of iron acquisition for nitrogenase activity with the detection of 14 different TonB-dependent iron transporters (VerBerkmoes et al. 2006). Other genes upregulated in the NifA* strains included those encoding light harvesting complex II proteins, perhaps to meet the energetic demands of nitrogenase, and hypothetical proteins of unknown function (Rey et al. 2007).

C. Identifying and Eliminating Pathways That Compete with H_2 Production

In addition to the increased expression levels of 18 genes in NifA* strains, microarray comparisons also indicated that several genes had lower transcript levels in the NifA* strains compared to wild-type (Rey et al. 2007). Among these genes were those encoding the CO_2-fixing Calvin cycle (Fig. 7.4).

Similar decreases in Calvin cycle gene expression were observed during N_2 fixation/H_2 production in microarray analyses of *Rb. sphaeroides* (Kontur et al. 2011). The Calvin cycle is best known for its role in allowing autotrophic organisms like plants, algae, and some bacteria to grow on CO_2 as the sole carbon source at the expense of ATP and reductant. In PNSB, the Calvin cycle also functions to maintain electron balance during photosynthetic growth on organic compounds (photoheterotrophic growth; Fig. 7.1a). When PNSB grow photoheterotrophically, the organic substrates are oxidized, resulting in reduction of electron carriers such as $NAD(P)^+$ to NAD(P)H. In respiring organisms, this reductant would be oxidized by H^+-pumping electron transfer chains, intimately associated with the formation

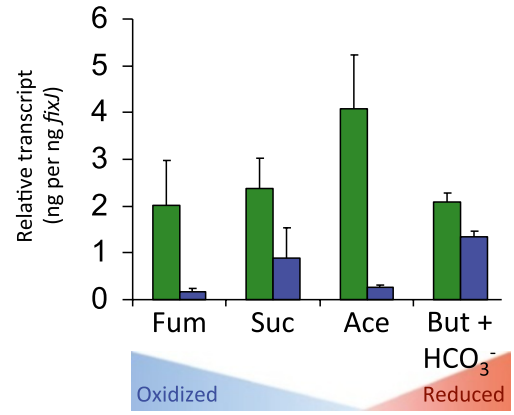

Fig. 7.4. Calvin cycle gene expression levels are lower during H_2 production. Examples for Type I Rubisco transcript levels (*cbbL*) determined by RT-qPCR analysis are shown for wild-type *Rp. palustris* (*green*) and a H_2-producing NifA* strain (*blue*) grown in the presence of NH_4^+ on substrates having different electron contents (McKinlay and Harwood 2011). Similar trends were observed for other Calvin cycle genes in other NifA* strains and in wild-type during N_2 fixation by microarray analysis (McKinlay and Harwood 2010a) (Figure reproduced and amended with permission from the American Society for Microbiology under a Creative Commons Attribution Non-commercial Share Alike license (McKinlay and Harwood 2011)).

of ATP. In photoheterotrophic PNSB, ATP is formed by cyclic photophosphorylation, without the need for a terminal electron acceptor. Even so, the reduced electron carriers must be oxidized to maintain metabolic flow and avoid cell death. By fixing CO_2 via ribulose 1,5 bisphosphate carboxylase (Rubisco), the Calvin cycle eventually forms glyceraldehyde-3-phosphate that can accept electrons from NAD(P)H. The CO_2 'electron acceptor' is ultimately incorporated into biomass. Elimination of Calvin cycle genes encoding Rubisco and phosphoribuokinase can disable PNSB from growing on organic carbons sources. However, growth of such Calvin cycle mutants can be rescued by the addition of electron acceptors or by allowing the cells to rid themselves of excess electrons through H_2 production (Hallenbeck et al. 1990a, b; Falcone and Tabita 1991; McKinlay and Harwood 2010a). In fact, uncharacterized constitutive H_2-producing strains of *Rb. sphaeroides* were obtained

through the long-term incubation of Rubisco mutants (Joshi and Tabita 1996) – a similar strategy to what the Harwood lab used to obtain NifA* strains of *Rp. palustris* (Rey et al. 2007). An electron balancing activity such as the Calvin cycle or H_2 production is required for photoheterotrophic growth even on organic compounds that have less electrons per carbon than the average carbon in cellular biomass. In the absence of added CO_2, *Rp. palustris* relies on a Rubisco type I enzyme to scavenge CO_2 released by oxidative metabolic pathways rather than the Rubisco type II enzyme it encodes, as indicated by proteomic analysis and followed up with biochemical and mutational approaches (VerBerkmoes et al. 2006; Joshi et al. 2009). These results are consistent with Rubisco type I having a higher affinity for CO_2 than the type II enzyme (Tabita 1988).

Recent observations with *Rs. rubrum* have led to the argument that preventing Rubisco activity results in an accumulation of ribulose-1,5-bisphosphate and it is the toxic effect of this compound rather than an inability to maintain electron balance that disrupts growth (Wang et al. 2011). As observed in other PNSB, a Rubisco mutant of *Rs. rubrum* had severe growth defects (Wang et al. 2010, 2011). However, knocking out phosphoribulokinase, the enzyme that produces the ribulose 1,5-bisphosphate substrate for Rubisco, restored normal growth (Wang et al. 2011). Though toxic accumulation of ribulose-1,5-bisphosphate could disrupt growth it does not rule out the fact that electrons must be balanced to obey conservation of mass. We have since confirmed the observations made with *Rs. rubrum* and suggest that it has alternative mechanisms to maintain electron balance since phosphoribulokinase mutants of other PNSB including *Rp. palustris* (G.C. Gordon and J.B. McKinlay, unpublished), *Rb. sphaeroides* (Hallenbeck et al. 1990a), and *Rb. capsulatus* (Öztürk et al. 2012) do not grow or show severe growth defects under photoheterotrophic conditions with NH_4^+.

Importantly, the Calvin cycle and H_2 production are both vital mechanisms by which PNSB deal with excess electrons during photoheterotrophic growth. Thus, because of their common roles they can potentially compete for reductant. Even though the Calvin cycle is down-regulated when nitrogenase is active (Fig. 7.4), the Calvin cycle could still consume electrons that could otherwise be used to produce H_2. Rarely do genomic transcript levels correlate with metabolic activity in a quantitative manner. To determine the effect of the Calvin cycle on H_2 production ^{13}C-metabolic flux analysis or 'fluxomics' was performed. This approach provides a quantitative view of the in vivo flow of carbon (and associated electrons and ATP) through a metabolic network. Metabolic flux distributions with and without H_2 production (i.e., NifA* vs wild-type *Rp. palustris*) were compared on four different carbon sources having different oxidation states – fumarate, succinate, acetate, and butyrate (McKinlay and Harwood 2011).

In the absence of H_2 production, and in the absence of added CO_2 or bicarbonate, the Calvin cycle fixes a significant amount of the CO_2 released by other metabolic reactions as the organic carbon source is oxidized (i.e., ranging from 20 % on fumarate to 70 % on acetate). When H_2 is produced, the Calvin cycle flux always decreased (Fig. 7.5a), supporting the microarray observations (Fig. 7.4). However, the Calvin cycle flux magnitude depended on the carbon source used. For example, during growth on acetate, H_2 production resulted in a Calvin cycle flux that was ~20 % of that in the absence of H_2 production (Fig. 7.5a). However, during growth on succinate, H_2 production only resulted in a decrease of Calvin cycle flux to 60 % of the level observed during the absence of H_2 production. Thus, depending on the growth conditions the Calvin cycle can divert a considerable portion of available electrons away from H_2 production. Calvin cycle flux was prevented by deleting the genes encoding Rubisco enzymes resulting in increased H_2 yields that were proportional in magnitude to the Calvin cycle fluxes observed in the parental NifA* strain (Fig. 7.5b). On some carbon sources such as succinate and

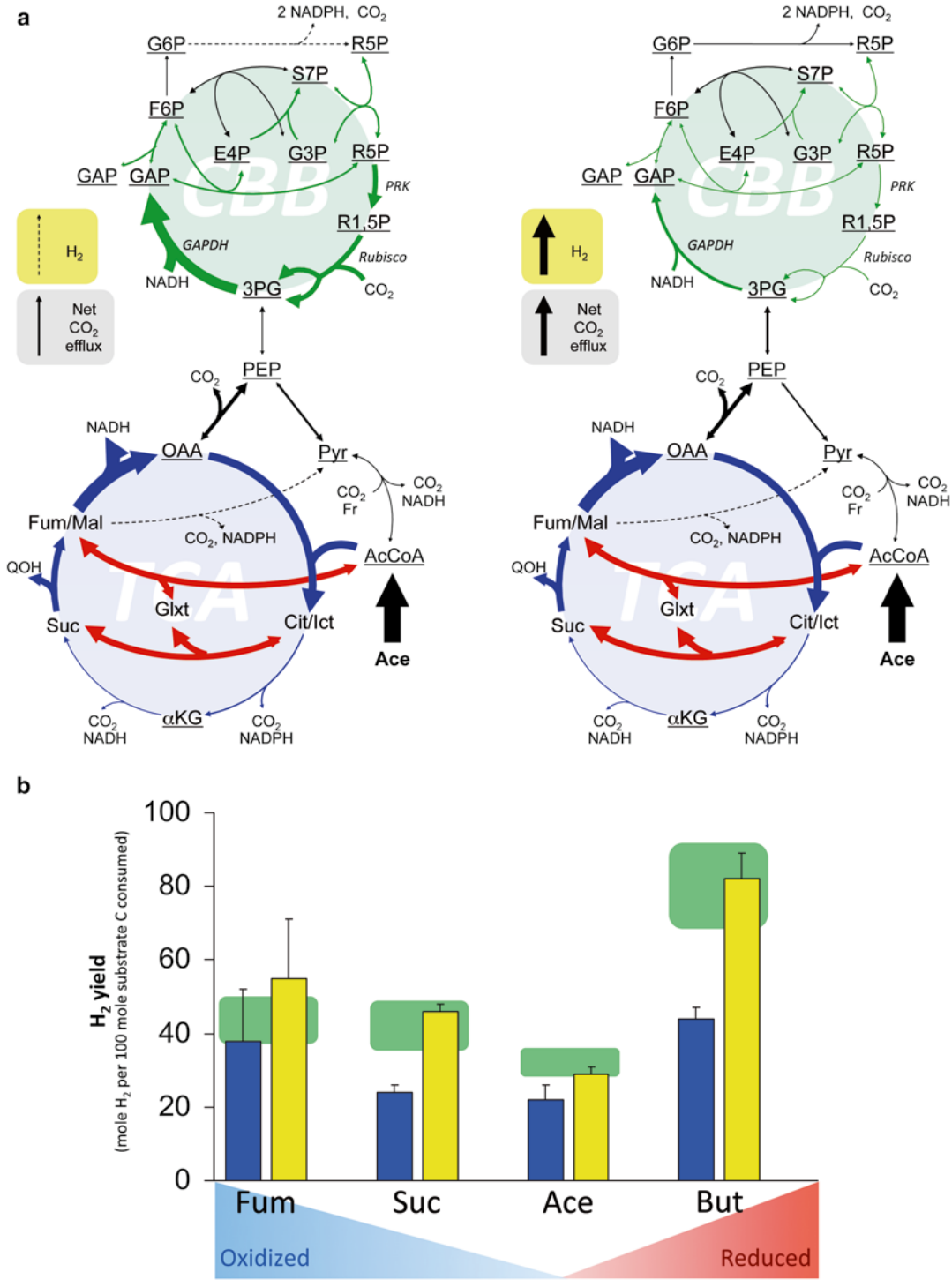

Fig. 7.5. **The Calvin cycle competes with H_2 production for electrons.** (**a**) A comparison of metabolic fluxes between wild-type *Rp. palustris* (*left*) and an H_2-producing NifA* strain (*right*) from ^{13}C-metabolic flux analyses (^{13}C-labeling experiments) show that there is less metabolic flow through the Calvin cycle (*green arrows*) when H_2 is being produced. *Arrow thickness* is proportional to the flux through corresponding pathway or reaction. *Dotted arrows* signify fluxes that are less than 5 molar percent of the acetate uptake rate. (**b**) When Calvin cycle flux is prevented by deleting the genes encoding Rubisco the H_2 yields from the Calvin cycle NifA* mutant (*yellow*) are higher than the NifA* parent strain (*blue*). The *green boxes* show the 90 % confidence intervals for H_2 yields that were predicted to result from eliminating Calvin cycle flux based on flux maps obtained for the four different carbon sources (Figures reproduced and amended with permission from the American Society for Microbiology under a Creative Commons Attribution Non-commercial Share Alike license (McKinlay and Harwood 2011)).

butyrate, preventing Calvin cycle activity resulted in a twofold increase in the H_2-yield (Fig. 7.5b) (McKinlay and Harwood 2011). This study illustrated how functional genomics and systems biology approaches like fluxomics can be used to identify targets for improving product formation. Several *in silico* flux balance models of PNSB have also pointed to the importance of the Calvin cycle (and other reductive pathways including sulfide production and an alternative CO_2-fixing ethylmalonyl-CoA pathway found in some PNSB) in maintaining electron balance (Klamt et al. 2002; Hädicke et al. 2011; Imam et al. 2011; Rizk et al. 2011). Although these models require more assumptions than those that incorporate experimental data from ^{13}C-labeling experiments, they can identify potentially good targets for metabolic engineering. Like other 'omics' approaches, these models are most effective when resulting hypotheses are tested through follow up biochemical or mutational experiments and the resulting information used to refine the models.

An important benefit of producing H_2 in the absence of the Calvin cycle is that formation of the desired product (i.e., H_2) is required for cell viability by maintaining redox balance. This is a rare scenario for engineered biofuel-producing microbes. Typically, the production of the desired product is at odds with cell growth. Thus, H_2-producing PNSB strains that lack Calvin cycle activity may be unique examples of biofuel-producing microbes with a stable phenotype. However, one must always consider that if a more efficient way to maintain electron balance exists, it will likely evolve. For example, a *Rb. sphaeroides* Calvin cycle mutant was reported to have evolved the ability to reduce sulfate to sulfide as an electron balancing mechanism instead of producing H_2 (Rizk et al. 2011). It is not clear whether this is indeed a more efficient means of dealing with excess electrons than producing H_2. Accumulation of internal polyhydroxybutyrate is also another potential alternative electron balancing mechanism (De Philippis et al. 1992; Imam et al. 2011). Knocking out this pathway in some PNSB has resulted in higher H_2 yields (Yilmaz et al. 2010).

IV. Future Directions for a System-Level Understanding of Photobiological H_2 Production

Systems biology approaches such as microarrays and ^{13}C-metabolic flux analysis have provided insights into how PNSB produce H_2 and have guided the successful engineering of PNSB to improve H_2 production. In the process, these approaches have also provided fundamental knowledge about the physiology of PNSB and the regulatory mechanisms that govern their diverse metabolic modules. With advances in systems biology technologies we can expect further complementary insights into the fundamental and applied aspects of PNSB. As systems level experimental and computational approaches grow in popularity and garner more attention we must not lose sight of the importance of rigorous biochemical and genetic approaches, particularly in verifying the trends and testing the predictions that emerge from systems level approaches.

A. RNAseq Analysis of Coding and Non-coding RNAs

New sequencing technologies have made it cost-effective to sequence and count cDNA copies of transcripts in a process called RNAseq. A major challenge in RNAseq is to focus the sequencing power on non-ribosomal RNA molecules. Recently, a method called not-so-random (NSR) priming was developed, which uses a random selection of hexamer primers to convert RNA into cDNA, but excludes those sequences predicted to bind rRNA (Armour et al. 2009). This approach makes use of only 1 μg of RNA. Combining NSR random hexamer primers with short 5′ barcode tags (e.g., so that multiple samples can be read per lane in an Illumina sequencer) also allows for directional sequencing and the

identification of antisense RNA molecules. This new method is being applied to *Rp. palustris* with results now starting to reach publication (Hirakawa et al. 2011).

Thus far, RNAseq has revealed an antisense transcript involved in the quorum sensing response of *Rp. palustris* CGA009. *Rp. palustris* has an unusual quorum sensing system involving production of a homoserine lactone with an aromatic side chain instead of a typical acyl side chain (Schaefer et al. 2008). Interestingly this aromatic p-coumaryl homoserine lactone signal is only made when aromatic compounds like p-coumarate are provided (*Rp. palustris* cannot synthesize p-coumarate itself). The RNAseq analysis revealed a small transcript that is anti-sense to *rpaR* which encodes the quorum sensing receptor/regulatory protein (Hirakawa et al. 2011). A follow up study confirmed the antisense activity of this transcript as it repressed the expression of *rpaR* and therefore production of the quorum sensing signal (Hirakawa et al. 2012).

Aside from the antisense transcript, this single use of RNAseq on *Rp. palustris* revealed several intergenic regions that are differentially expressed and could encode small RNA molecules (sRNA) (Hirakawa et al. 2011). A computational approach was also used to predict sRNA coding regions, four of which were confirmed to exist using RNA gel blots (Madhugiri et al. 2012). Noncoding RNAs are receiving increasing attention for their regulatory roles in diverse prokaryotes (Gottesman 2005). sRNAs add an additional layer of complexity that must be understood to successfully engineer a microbial system. sRNA and other functional RNAs including aptamers and riboswitches also offer the potential for new tools to redirect metabolic flux towards desired products like H_2 (Win et al. 2009).

B. Proteomic Analysis of Post-translational Regulatory Mechanisms

Though traditional transcriptomics and proteomics have made invaluable contributions to our understanding and application of microbial systems, these functional genomic approaches often do not explain phenotypic trends, such as metabolic flux. For example, a study that compared multiple omics with various *E. coli* mutants found that it was an exception, rather than the rule, for transcriptomics, proteomics, and metabolomics data sets to correlate with metabolic fluxes (Ishii et al. 2007). Metabolic fluxes are often determined by thermodynamics, kinetic parameters, and through post-translational modifications of the enzymes.

Mass spectrometry has been used to examine post-translational modifications of specific proteins in *Rp. palustris.* For example, focusing on the regulation of nitrogenase (and therefore H_2 production) the uridylylation state of the three *Rp. palustris* PII proteins, GlnK1, GlnK2, and GlnB were examined in the presence and absence of ammonium (Connelly et al. 2006). In the future, mass spectrometry-based proteomic analyses will likely target post-translational modifications on a systems scale. For example, Crosby et al. recently used such a proteomic approach to identify N-lysine acetylated proteins in *Rp. palustris* (Crosby et al. 2012). N-lysine acylation (i.e., modification of lysine residues with acetyl from acetyl-CoA, or in some cases, propionyl, butyryl, succinyl, or malonyl groups) can be used to activate or deactivate activity of a wide variety of proteins in both prokaryotes and eukaryotes with deacylation having the opposite effect (Albaugh et al. 2011; Kim and Yang 2011). In *Rp. palustris*, biochemical and mutational analyses had previously revealed that N-lysine acetylation inhibits activity of three enzymes involved in degradation of lignin monomers such as benzoate and cyclohexanecarboxylate, and deacetylation reactivates these enzymes (Crosby et al. 2010). The proteomics analysis originally identified hundreds of potential targets for acylation. However, after stringent parameters were applied to the screen, requiring that acetylated proteins be identified by two different proteomics software packages and that proteins show elevated levels of acylation in a mutant strain lacking two

known deacetylases, 14 candidate acetylation targets were identified. Biochemical assays then verified that nine of these proteins were actual targets for acetylation by two known *Rp. palustris* acetyltransferases (Crosby et al. 2012). One of the candidate proteins (that was not a target for the known acetyltransferases) was glyceraldehyde-3-phosphate dehydrogenase – a key enzyme of the Calvin cycle and other metabolic pathways. The importance of the acetylation of this enzyme and others identified in the study for *Rp. palustris* physiology and H_2 production have not yet been reported.

C. Global Identification and Characterization of Ligand-Binding Proteins

Part of the metabolic versatility of PNSB involves their ability to use a wide range of organic and inorganic substrates. *Rp. palustris* was the focus of a recent study that attempted to characterize all of the solute-binding proteins associated with transporters in the cell (Giuliani et al. 2011). 107 genes encoding candidate *Rp. palustris* solute binding proteins were expressed in *E. coli*, 75 of the resulting proteins were screened and ligands were ultimately identified for 45 proteins. The proteins were assayed for their ability to bind compounds in a fluorescence-based thermal shift assay using a library of small molecules including metals, aromatic compounds, amino acids, fatty acids, and other compounds. Related to H_2 production, this approach identified a vanadate transporter that is likely important for supplying vanadium required by V-nitrogenase. This gene product was previously incorrectly annotated as a phosphate transporter. Two solute binding proteins were found to bind a wide range of fatty acids, and not coincidentally, were encoded near the *pimFABCDE* gene cluster encoding enzymes for the β-oxidation of a broad range of fatty acids (Harrison and Harwood 2005; Giuliani et al. 2011). The screen also identified six gene products capable of binding aromatic compounds. These proteins are therefore potentially important for H_2 production from lignin monomers. Four of these proteins were further studied to determine their thermodynamic and structural properties using isothermal calorimetry and small/wide angle X-ray scattering (Pietri et al. 2012). The data was used to guide the development of structural models that may prove useful for identifying aromatic compound-binding proteins based on sequences in other genomes (Pietri et al. 2012).

A surprising result to emerge from the screen was that only 11 gene products bound ligands that were predicted from their original annotation (Giuliani et al. 2011). This study is therefore invaluable for refining our knowledge of the function of poorly annotated gene products – knowledge that can ideally be extended to other genomes.

D. The Physiology of Non-growing Cells – Approaching the Maximum Theoretical H_2 Yield

Although advances have been made in understanding and engineering *Rp. palustris* to produce H_2, most work has focused on producing H_2 using growing cells – in other words, cells that are dedicating electrons to biosynthesis that could otherwise be used to produce H_2. Even the NifA* Calvin cycle mutants only use 20–36 % of the consumed electrons for H_2 production and the rest are almost exclusively used for biosynthesis (McKinlay and Harwood 2011). Certainly biosynthesis must occur to have biocatalysts to produce H_2, and the cells themselves may even have value as fertilizer or animal feed (Honda et al. 2006). However, once cell numbers accumulate, there are great advantages to using them to produce H_2 in a non-growing state. First, when growth is prevented by nitrogen starvation, wild-type CGA009 will divert about 50 % of the electrons from acetate to H_2 and up to 75 % to H_2 from an inorganic substrate like thiosulfate (Huang et al. 2010). Second, *Rp. palustris* is ideally suited for long-term use in a non-growing state. As long as *Rp. palustris* is illuminated, it can repeatedly energize and recycle electrons through an electron transfer chain, maintaining a proton motive force and ATP

pools to repair itself. This ability to maintain itself may explain why no classical stress proteins were detected in a proteomics analysis of stationary phase (carbon-starved) *Rp. palustris* cells (VerBerkmoes et al. 2006). *Rp. palustris* cells have been immobilized in artificial latex biofilms and maintained in a non-growing state for months at a time. These biological solar panels remained metabolically active when transferred between batches of fresh medium losing little activity after the first 6 days maintaining H_2 productivity at 2.08 ± 0.08 mmol H_2 m^{-2} h^{-1} for over 5 months (Gosse et al. 2010). It is worth noting that the mode of starvation can greatly influence H_2 yields. Unlike the favorable effects of nitrogen starvation on H_2 production, sulfur starvation did not result in high H_2 yields but rather favored polyhydroxybutyrate synthesis (Melnicki et al. 2008).

Techniques such as RNAseq and recent advances in ^{13}C-metabolic flux analysis procedures (Rühl et al. 2012) have facilitated our ability to examine non-growing cells. Such approaches are expected to reveal novel targets for improving H_2 yields by non-growing cells and to lead to a better general understanding of the physiology of bacteria under stressful starvation conditions.

E. Non-biased Interpretation and Utilization of Systems Biology Data

Though the immense volumes of data from systems biology is undeniably useful, is also introduces a challenge in effectively interpreting the data and applying it to a given problem. For example, when comparing transcriptomic datasets in which gene expression levels vary for hundreds of genes it is impractical to follow up on each and every variation. Thus, experimenters tend to focus on well-annotated and characterized gene products than genes of unknown function. As a result, some potentially important aspects of microbial physiology are overlooked and metabolic engineering efforts are heavily weighted on hypotheses that can be biased by a researcher's background knowledge. However, there are approaches to identify targets on a systems scale without introducing experimenter bias. For example, screening transposon mutant libraries can link a gene of unknown function to a phenotype. Such approaches have turned up useful targets for metabolic engineering *E. coli* for lycopene production (Alper et al. 2005). Integrating systems biology approaches such as genomic variation with transcriptomic data can also highlight key drivers of complex functions through predictions of causal relationships (Schadt et al. 2005). This approach led to the identification of three genes previously not known to be involved in obesity in mice, which were validated through analyses of mice with mutations in these genes (Schadt et al. 2005). It may be possible to use similar approaches to identify key, and potentially unexpected, drivers of H_2 production in PNSB. Identifying targets that an experimenter would not otherwise predict is not only useful for improving product formation but can lead to a functional characterization of gene products with previously unknown function and define their role in the greater context of microbial physiology.

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