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Metabolic Engineering and Modeling of Metabolic Pathways to Improve Hydrogen Production by Photosynthetic Bacteria

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Project description and rational

Rising energy demands and the imperative to reduce carbon dioxide (CO_2) emissions are driving research on biofuels development. Hydrogen gas (H_2) is one of the most promising biofuels and is seen as a future energy carrier by virtue of the fact that 1) it is renewable, 2) does not evolve the "greenhouse gas" CO_2 in combustion, 3) liberates large amounts of energy per unit weight in combustion (having about 3 times the energy content of gasoline), and 4) is easily converted to electricity by fuel cells. Among the various bioenergy strategies, environmental groups and others say that the concept of the direct manufacture of alternative fuels, such as H_2, by photosynthetic organisms is the only biofuel alternative without significant negative criticism [1]. Biological H_2 production by photosynthetic microorganisms requires the use of a simple solar reactor such as a transparent closed box, with low energy requirements, and is considered as an attractive system to develop as a biocatalyst for H_2 production [2]. Various purple bacteria including *Rhodopseudomonas palustris*, can utilize organic substrates as electron donors to produce H_2 at the expense of solar energy. Because of the elimination of energy cost used for H_2O oxidation and the prevention of the production of O_2 that inhibits the H_2-producing enzymes, the efficiency of light energy conversion to H_2 by anoxygenic photosynthetic bacteria is in principle much higher than that by green algae or cyanobacteria, and is regarded as one of the most promising cultures for biological H_2 production [3]. Here implemented a simple and relatively straightforward strategy for hydrogen production by photosynthetic microorganisms using sunlight, sulfur- or iron-based inorganic substrates, and CO_2 as the feedstock. Carefully selected microorganisms with bioengineered beneficial traits act as the biocatalysts of the process designed to both enhance the system efficiency of CO_2 fixation and the net hydrogen production rate. Additionally we applied metabolic engineering approaches guided by computational modeling for the chosen model microorganisms to enable efficient hydrogen production.

RESULTS

I. Biological H_2 production based on inorganic substrates

Biological H_2 production by anoxygenic photosynthetic microorganisms has attracted considerable attention as an efficient way of converting biomass to H_2. But some of the important issues still remain unaddressed: 1) Although specific H_2 productivities by photosynthetic bacteria have been achieved growing on organic substrates, H_2 production based on inorganic electron donor coupled with CO_2 fixation has yet to be explored. The ability for using inorganic substrates coupling with primary production by photosynthesis for H_2 production is of great significance, because it eliminates the requirement of organic substrate and therefore greatly reduces greenhouse gas (CO_2) emission commonly associated with its production. 2) It is known that two enzyme systems including nitrogenase and hydrogenase are responsible for H_2 production. Although their enzymatic activities in H_2 production has been examined individually, but the functional interplay between these two enzyme systems during H_2 production is unclear in any microorganism.

R. palustris has an extremely versatile metabolism, and can grow phototrophically on organic substrates as well as inorganic substrates [4]. The energetics of this process is plausible, however, as shown in **Fig. 1**. Considering the redox potential of the electron donors that purple bacteria can use, iron couple [Fe^{2+}/Fe^{3+}] and reduced sulfur species such as [HSO_3^-/SO_4^{2-}] at neutral pH have values ranging from 0 -250 mV [5], which are low enough to donate electrons to the reaction center of purple bacteria (E0', +450 mV) and therefore provide reducing power to sustain cellular growth [6]. However, compared to the H_2/H^+ couple (E0', -410 mV) [7], both iron and sulfur have a higher redox potential, suggesting a reverse electron flow is needed for the production of H_2 when growing on these electron donors. Here we examined H_2 production H_2 production of

R. palustris and the Hup mutant lacking the H_2 uptake hydrogenase under carbon fixing conditions with either thiosulfate or acetate as the electron donor (**Fig. 1**). H_2 production was monitored using gas chromatography. The results showed that inorganic-substrate based H_2 production system by purple bacteria is a viable pathway, alleviating the current requirement of organic substrates for biological H_2 production and therefore reduces CO_2 emission commonly associated with it the production of the organic substrates.

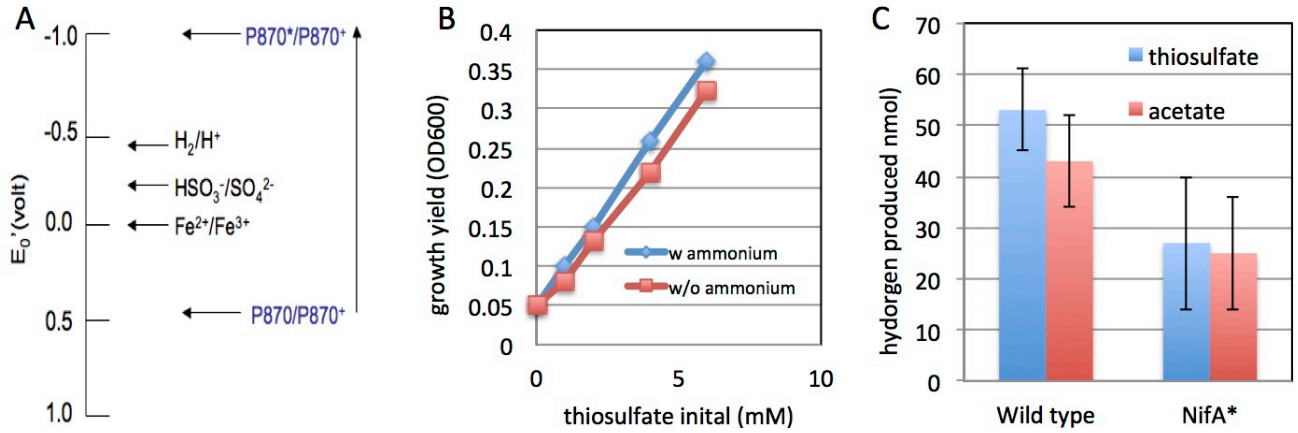

Fig. 1 (A) Comparison of redox potential of some inorganic electron donor in relation to Photosystem I in purple bacteria. (B) *R. palustris* growth yields with thiosulfate as the sole electron donor in photoautotrophic growth. Different initial thiosulfate concentration was provided and growth yield was recorded as the final optical density reached after 5 to 7 days. With initial concentration of zero thiosulfate, the growth yield corresponds to the size of the inoculum. (C) H_2 production by *R. palustris* in a cell suspension assay with thiosulfate or acetate as the electron donor. When using thiosulfate as the electron donor, bicarbonate (5 mM) was provided as the sole carbon source. H_2 yield was measured after 24 h incubation.

II. Understanding the functions of nitrogenases and hydrogenases for H_2 production

Purple nonsulfur bacterium *R. palustris* has promise for eventual use in a biological process for H_2 production because it can access both organic and inorganic compounds, as well as lignin monomers as sources of electrons [8]. H_2 production by purple bacteria can be achieved through two enzyme systems including hydrogenase and nitrogenase (**Fig. 2A**). Although light dependent H_2 evolution is *generally* coupled with nitrogenase activity (N_2 fixation), light dependent H_2 evolution by growing cultures of purple bacteria under nitrogenase-repressed conditions has been reported, suggesting the use of hydrogenase for H_2 production [9]. However, it is not clear how the electrons are being "shared" between nitrogenase and hydrogenase enzymes, and which system is more efficient in H_2 production under certain conditions. The question is complicated by the fact that multiple isozymes for both hydrogenase and nitrogenase are present in *R. palustris*. A striking feature of *R. palustris* is that it expresses three distinct functional nitrogenases, whereas most nitrogen-fixing bacteria encode only a molybdenum nitrogenase [10]. The presence of these alternative nitrogenases has been proposed to serve as a route for N_2 fixation in situations where molybdenum is limited in the environment, but the individual function of these alternative nitrogenases with regard to H_2 production has not been investigated. Mutant strains of *R. palustris* expressing a single nitrogenase isozyme were either constructed by overlap extension PCR as previously described [11] or obtained from other laboratories, H_2 production under N_2 fixing conditions was measured and compared with those of expressing a single isozyme, a combination of two isozymes, as well as wide type strain expressing all three nitrogenases (**Fig. 2B, C**). Beside three nitrogenases, there are two known hydrogenases present in *R. palustris*, including a H_2 uptake hydrogenase (Hup) and a formate-dependent hydrogenase [12]. The expression and activation of Hup is associated with H_2 uptake, when H_2 is supplied as an electron donor for photoautotrophic growth. The formate-dependent hydrogenase couples H_2 production when formate is supplied as a growth substrate. In order to examine the interplay of hydrogenases and nitrogenases, ongoing effort focuses on testing H_2 productivity as

well as hydrogenase and nitrogenase expression profile with different combinations of the expressed hydrogenase and nitrogenase isozymes, and determine which combination results in the most efficient H_2 production. The results from these experiments will provide clearer understanding of the interplay between nitrogenases and hydrogenases in relation to their roles in H_2 production, and lay the groundwork for future bioengineering of these enzyme pathways to improve H_2 production.

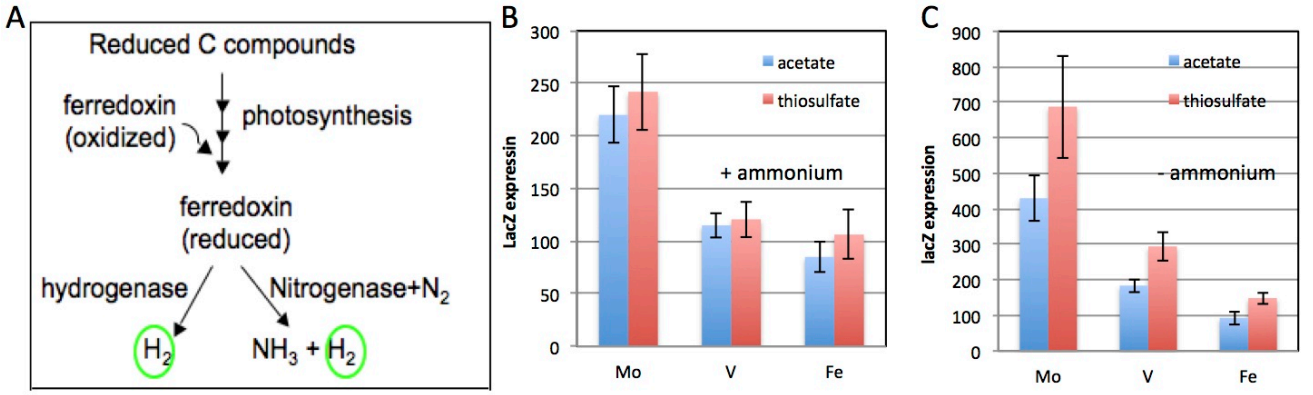

Fig. 2 (A) Hydrogen production pathways through hydrogenase and nitrogenase in photosynthetic bacteria. (B, C) Expression levels of molybdenum-Fe nitrogenase (Mo), vanadium nitrogenase (V), and iron nitrogenase (Fe) in *R. palustris* measured by beta-galactosidase activity when grown with acetate or thiosulfate as the electron donor, in the presence or absence of a fixed nitrogen source ammonium, respectively. The absence of ammonia induced the expression level of all 3 nitrogenases; the expression levels in thiosulfate are significantly higher than those with acetate, especially for the Mo-containing nitrogenase, highlighting its importance for hydrogen production.

III. Integration of a cellulose-based syntrophic co-culture for H_2 production

For the established H_2 producing co-culture systems based on cellulosic materials, although certain H_2 productivity has been achieved in bioreactors with microorganisms with different syntrophic relationship, the basic knowledge concerning the metabolic interactions between the organisms in the bioreactor is not understood, which hinders strategic system optimization for improving H_2 producing efficiency. _Development of cellulose-based infrastructure for various biofuel productions has been growing rapidly in the past decade. Bioreactors containing two or more microorganisms have been designed for integrated biological H_2 production through coalition of different metabolic processes [2, 13]. In a established model co-culture system [2, 13], dark fermentation by *Clostridia* decomposes cellulose into small organic acids, CO_2 and H_2 (**Fig. 3**). *R. palustris,* who cannot use cellulose directly as a growth substrate, further degrades the secondary metabolites (small organic acids) produced by *Clostridia,* for further H_2 production. The integration of two metabolically distinct, yet compatible, microorganisms allows us to combine cellulose hydrolysis, fermentation of breakdown sugars, and conversion of H_2 in one single step.

In an artificial co-culture containing *Clostridium cellulolyticum* and *R. palustris* with cellulose as the sole carbon source, we examined cell growth kinetics, cellulose consumption, H_2 production, and carbon transfer from *C. cellulolyticum* to *R. palustris*. When cultured alone, *C. cellulolyticum* degraded only 73% of the supplied cellulose. However, in co-culture *C. cellulolyticum* degraded 100% of the total cellulose added (5.5 g/L) and at twice the rate of *C. cellulolyticum* monocultures. Concurrently, the total H_2 production by the co-culture was 1.6-times higher than that by the *C. cellulolyticum* monoculture. Co-culturing also resulted in a 2-fold increase in the growth rate of *C. cellulolyticum* and a 2.6-fold increase in final cell density. The major metabolites present in the co-culture medium include lactate, acetate and ethanol, with acetate serving as the primary metabolite transferring carbon from *C. cellulolyticum* to *R. palustris*. Our results suggest that the stimulation of bacterial growth and cellulose consumption under the co-culture conditions is likely caused by

R. palustris' removal of inhibitory metabolic byproducts (i.e., pyruvate) generated during cellulose metabolism by *C. cellulolyticum*.

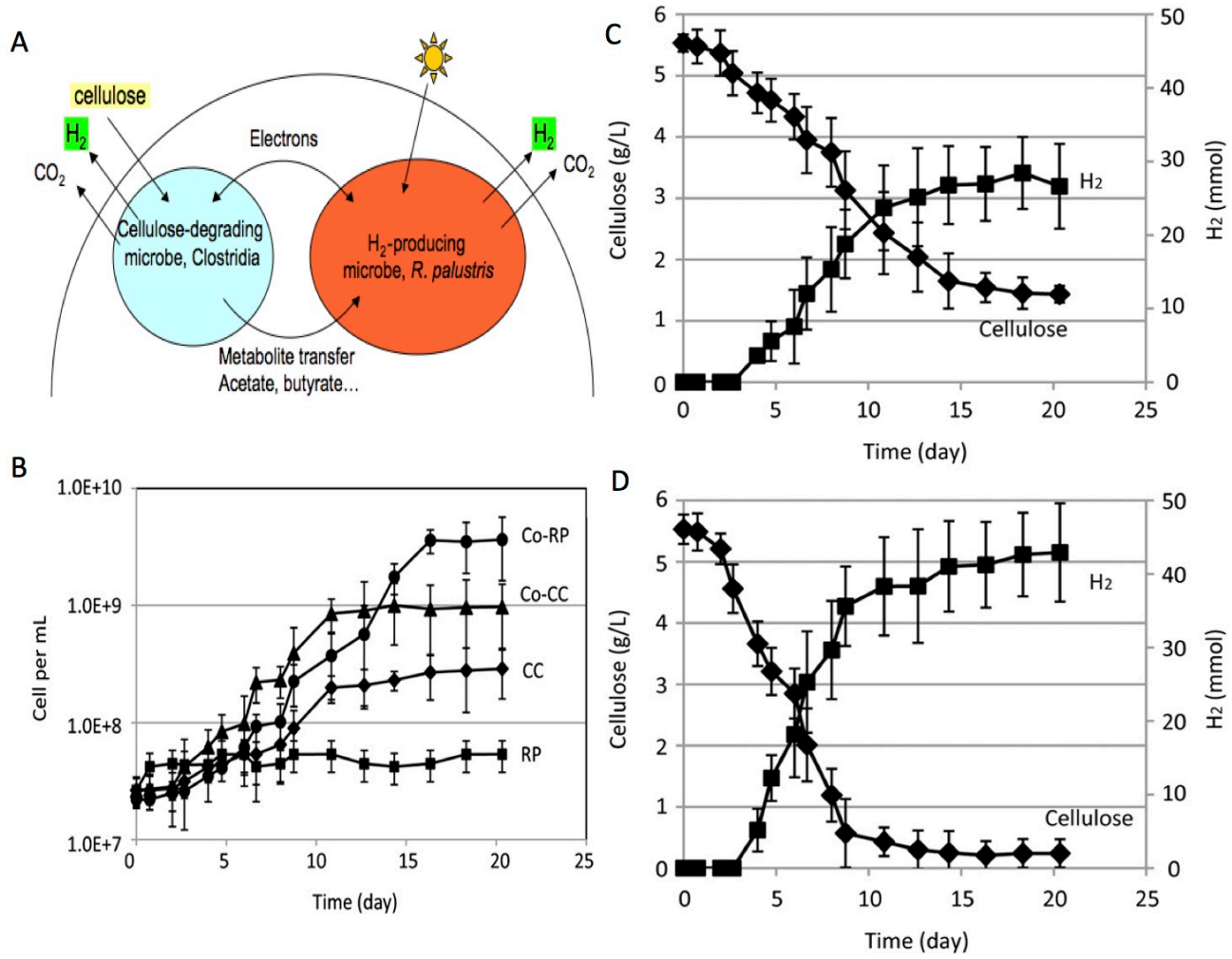

Fig. 3 (A) Possible interaction in a synthrophic co-culture containing cellulose-degrading Clostridia and H_2-producing *R. palustris*. (B) Comparison of cell growth when *C. cellulolyticum* and *R. palustris* were cultured individually on cellulose versus co-cultured together. The Y-axis is in logarithmic scale. (CC, diamonds) *C. cellulolyticum* in monoculture; (RP, squares), *R. palustris* in monoculture, (Co-CC, triangles) *C. cellulolyticum* in co-culture; (Co-RP, circles) *R. palustris* in coculture. Error bars represent standard deviations from triplicate cultures. (C, D) Measurements of residual cellulose concentration in the medium and H_2 produced over time when *C. cellulolyticum* was cultured alone on cellulose (C) versus cocultured with *R. palustris* (D). Error bars represent standard deviations from triplicate cultures.

IV. Flux balance analysis for H_2 production by *R. palustris*

In order to study system-wide carbon and energy flow within this co-culture, we have developed genome-scale reconstruction of metabolism in RP and use Flux Balance Analysis (FBA) [14] to study the metabolic capabilities of these organisms under different genetic and environmental conditions. We have used this model to examine the modes of metabolism most conducive to H_2 production. The model's predicted behaviors have been compared with experimental observations to ensure accuracy. As expected, our *in silico* analysis showed the RP does not require light to grow in nutritionally rich environments. Furthermore, we tested its ability to grow on organic acid byproducts of CC metabolism (acetate, ethanol, lactate and pyruvate). Consistent with results of fluxomic analysis, our results indicate that H_2 production is closely linked with

production of CO_2. Carbon fixation results in reduced production of H_2. Consumption of carbon sources like ethanol and acetate that are more reduced than the cellular biomass results in greater production of H_2 in comparison to more oxidized compounds like pyruvate. Additionally, our *in silico* analyses indicate that RP has a very robust mechanism for autotrophic growth and is not dependent on Calvin cycle for photoheterotrophic growth. This result has not been experimentally verified and is in disagreement with observed essentiality of Rubisco for photoheterotrophic growth in other purple- non-sulfur bacteria. Re-oxidation of reduced cofactors is energetically expensive and although light-dependent energy producing mechanisms are not essential for limited growth of RP, growth at the optimal level requires this mode of energy production. As a matter of fact our latest analyses indicate that given RP uses cyclic photophosphorylation, the amount of light energy converted to ATP is a significant determinant in whether the organism uses carbon-fixation or H_2 production as the means by which to oxidize reduced cofactors. Increased efficiency in capturing light and producing ATP increases the metabolic capacity of the system to use H_2 production as a mode of oxidation. Overall, since H_2 production is inversely linked to carbon fixation/conservation, increased H_2 yield adversely effects cellular growth.

We have previously shown that addition of new constraints to FBA models drastically improves the predictive capability of these models [15]. In order to verify the results of our FBA, we used the results of the gene expression microarray studies detailed above to eliminate pathways whose constituent enzymes have not been expressed. Similar work has previously been shown to improve the predictions of metabolic behavior and intracellular fluxes [16]. Additionally, we have used the results gene-expression analyses with our GX-FBA [17] method to gain added insight into metabolism of RP as it adapts to different environments and transitions between different stages of its lifecycle.

One significant challenge encountered during the course of FBA model development was the unique structure of RP's Lipid A. The lipid A base of lipopolysaccharides (LPS) in RP has been shown to be composed of 2,3-diamino-2,3-dideoxyglucose [18]; however, the metabolic pathway for production of this compound are unknown. We used microarray analysis results to examine the expression of those genes that are associated with the usual pathways of LPS production. We wanted to know if the pathway used in most bacteria for generation of typical glucosamine-based lipid A were active in RP. The results of our analyses showed that most of these genes were prominently expressed in RP. Given this information, we used a number of *in silico* methods such as LLNL's AS2TS [19] protein structure modeling tool and tools for identification of catalytic sites [20] and protein function predictions (CatSId) [21] to see if any of these enzyme could catalyze catalysis of a diamino-glucose. Our analysis of proteins in RP, could not find any enzyme that could catalyze the required chemical reaction. The results of all these analyses are included in a manuscript that is in the final stages of preparation and should be published in the near future.

DISSEMINATION

DOE OBER biofuel SFA project is providing support for additional research in both the experimental and modeling aspects of physiological studies on hydrogen production by *R. palustris* when co-culturing with cellulose degrading bacteria.

ADC/DUSA (required)
Enter the name of the ADC who reviewed your text in this section or the name of the applicable Designate Unclassified Subject Area (DUSA):

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