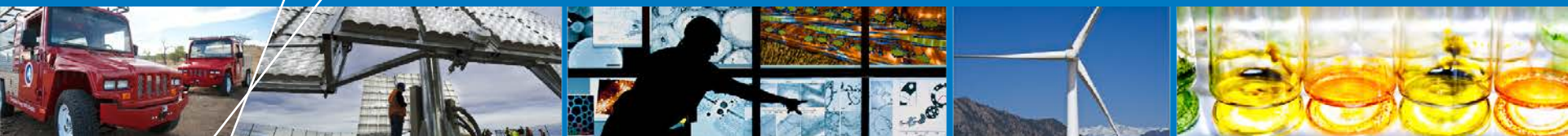


Biological Systems for Hydrogen Photoproduction



**2012 Annual Merit Review and
Peer Evaluation Meeting**

**Maria L. Ghirardi, PI
National Renewable Energy Laboratory**

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PD037

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Overview

Timeline

- Project start date: FY00
- Project end date: 9/30/2012*
- Percent complete: 80%

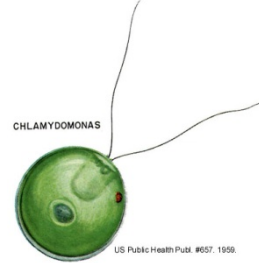
*Project continuation and direction determined annually by DOE

Budget

- Total project funding: \$9,951K
- Funding received in FY11: \$750K
- Planned funding for FY12: \$600K

Barriers

- Barriers addressed:
 - Rate of H₂ production (AH)
 - Continuity of H₂ production (AI)
 - Engineering issues (AJ)
- Targets (see next page)
 - light conversion efficiency
 - rates of production
 - duration of production



Partners

- Dr. Sergey Kosourov, Institute of Basic Biological Problems, RAS, Pushchino, Russia
- Dr. Eric Johnson, Johns Hopkins University

Relevance/Objectives

- **General goal:** Develop photobiological systems for large-scale, low cost and efficient H₂ production from water (barriers AH, AI and AJ).



Characteristics	Units	2003	2006	2013 Target	2018 Target
Utilization efficiency of incident solar energy	%	10	15	15	20
Efficiency of incident light energy conversion of water to hydrogen	%	0.1	0.1	2	5
Duration of continuous H ₂ photoproduction	Time units	NA	NA	30 min	4 h
O ₂ tolerance (half-life in air)	Time units	1 sec	1 sec	10 min	2 h

- **Specific tasks:**

Task 1: Address the O₂ sensitivity of hydrogenases that prevent continuity of H₂ photoproduction under aerobic, high solar-to-hydrogen (STH) light conversion efficiency conditions.

Task 2: Utilize a limited STH H₂-producing method (sulfur deprivation) as a platform to address or test other factors limiting commercial algal H₂ photoproduction, including low rates due to biochemical and engineering mechanisms.

Approach/Milestones – Task 1

Task 1: Address the O₂ sensitivity of hydrogenase by (a) using targeted random mutagenesis to generate O₂-tolerant hydrogenases; and (b) introducing the gene encoding for a more O₂-tolerant hydrogenase from *Clostridium acetobutylicum* into the photosynthetic alga *Chlamydomonas reinhardtii*; measure its linkage to water oxidation and *in vivo* O₂ tolerance.

Task 1	Milestone	Due date	Status
3.3.2	Go/NoGo: Assess progress in the random mutagenesis approach to evaluate whether to further pursue this approach in FY12.	12/31/11	Completed: NoGo
3.3.5	Demonstrate expression of an active Ca1 in a <i>C. reinhardtii</i> hydrogenase-less background and characterize O ₂ -sensitivity of light-driven H ₂ production.	9/30/12	80% completed



Dr. Paul King, NREL



Dr. Kath Ratcliff, NREL



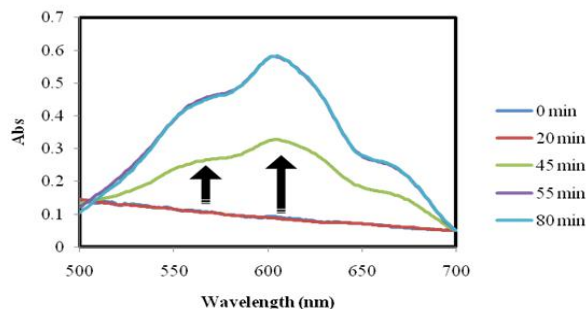
Dr. David Mulder, NREL

Accomplishments and Progress – Task 1

Task 1 – Random mutagenesis – completion of milestone 3.3.2 – NoGo.

We re-evaluated the effects of O₂ on the H₂-production activity of the more O₂-tolerant *Clostridium acetobutylicum* Ca1 hydrogenase and concluded that (a) a targeted random mutagenesis effort would require more funding than currently available through the FCT Program; (b) we need to first validate that higher in vitro O₂ tolerance translates into higher *in vivo* O₂ tolerance. We decided, instead, to focus on the expression of Ca1 in *Chlamydomonas reinhardtii* (see next slide).

Progress before the Go/NoGo milestone included:



(a) Development of a chemochromic high-throughput assay for H₂ production in micro-wells, based on methyl viologen reduction by H₂.

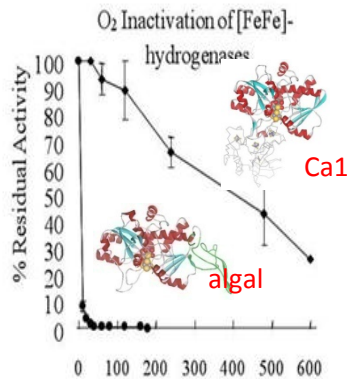
Strain	Ca1 whole cell activity (nmol
	H ₂ /min/mL)
Rosetta-2 (DE3)	1625
MC4100 (DE3)	1194
MW1001 (DE3)	173

Background control rate: 16 nmol H₂/min/mL

(b) Selection of a *E. coli* strain lacking native hydrogenase activity and expressing Ca1 at high levels.

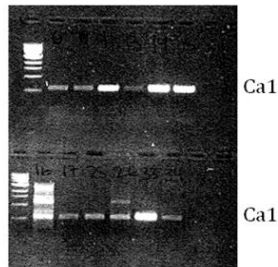
Accomplishments and Progress – Task 1

Task 1 – Expression of Ca1 in *C. reinhardtii* and measurement of *in vivo* O₂ tolerance – towards completion of milestone 3.3.5.

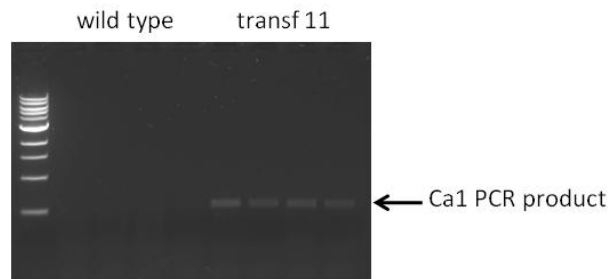


Justification: the Ca1 hydrogenase is 2 orders of magnitude more O₂-tolerant than the algal hydrogenase *in vitro*.

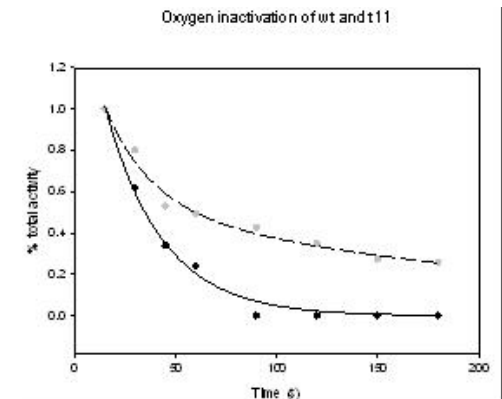
Previous work: we successfully expressed Ca1 in a wild-type strain of *C. reinhardtii* that contained native hydrogenase activity; O₂-inactivation kinetics were biphasic, suggesting the presence of a more O₂-tolerant enzyme in combination with the native hydrogenases.



PCR (genomic DNA)



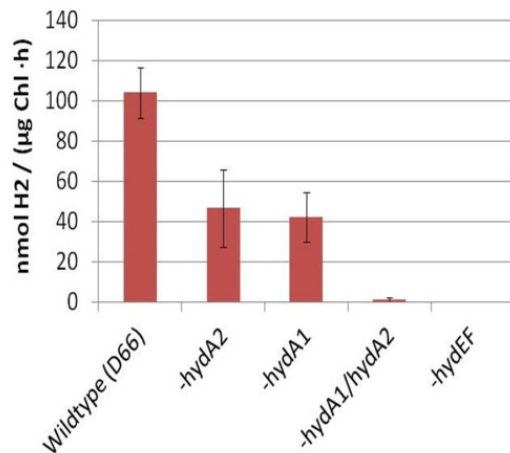
RT-PCR (mRNA)



Accomplishments and Progress – Task 1

Task 1 (cont.) – Expression of Ca1 in *C. reinhardtii* and measurement of *in vivo* O₂ tolerance – towards completion of milestone 3.3.5.

Current Progress: A double hydrogenase knock-out mutant was isolated under BES funding and served as a host for expression of Ca1 behind the *psaD* promoter.



Meuser et al., Bioch. Biophys.
Res. Comm. 417, 704

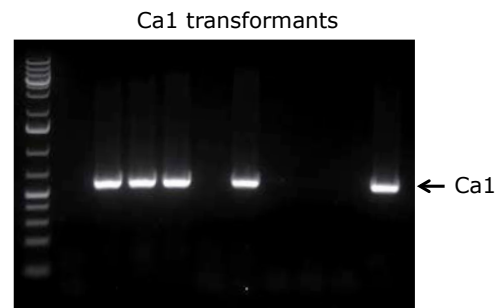
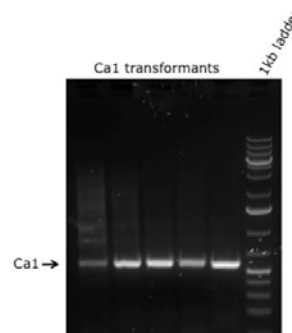
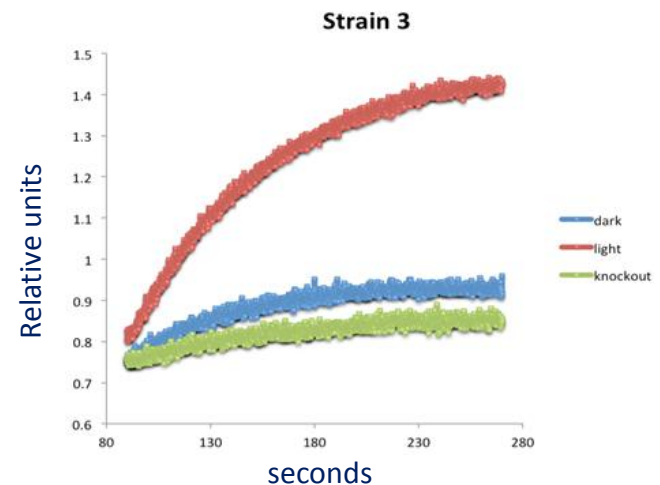


Figure 1. PCR of genomic DNA from various Ca1 transformants
PCR (genomic DNA)



RT-PCR (mRNA)

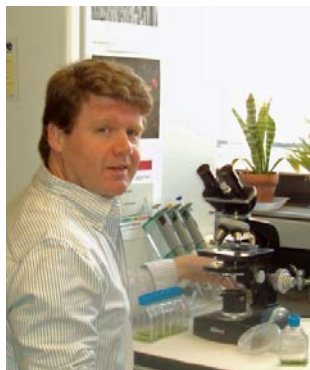
Light-dependent H₂
production was
detected!



Approach/Milestones – Task 2

Task 2: Utilize the limited STH sulfur-deprivation method to test (a) the rates of H_2 production by inducible ATP synthase mutants that are not limited by the non-dissipation of a proton gradient; and (b) the long-term performance of immobilized algal cultures.

Task 2	Milestone	Due date	Status
3.3.3	Physiologically characterize mutant strains with a defective AtpE gene under the regulation of an inducible promoter	3/30/12	Completed
3.3.4 (CSP Agreement milestone 51536)	Demonstrate continuous operation of the sulfur-deprivation process for a total of 2 months upon addition of phosphate/sulfate to alginate-immobilized cultures	9/30/12	50% completed



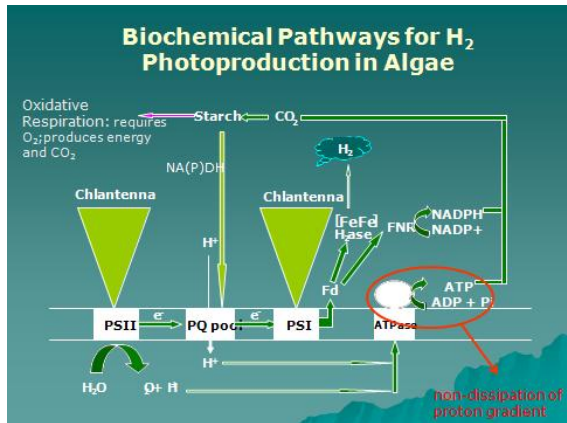
Dr. Eric Johnson
JHU



Dr. Sergey Kosourov
RAS

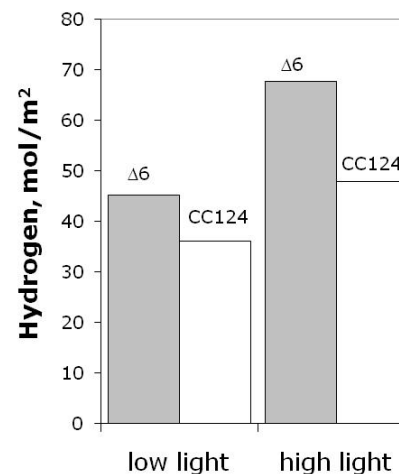
Accomplishments and Progress – Task 2

Task 2 — Generate, physiologically characterize and test the rate of inducible ATP synthase mutants that are defective in maintaining the proton gradient – completion of milestone 3.3.3.



Justification: electron transport from water to ferredoxin is accompanied by the formation of a proton gradient that drives the production of ATP (essential for CO₂ fixation). During H₂ photoproduction, ATP demand drops, impeding the efficient dissipation of the proton gradient and impairing electron transport (and thus of H₂ production).

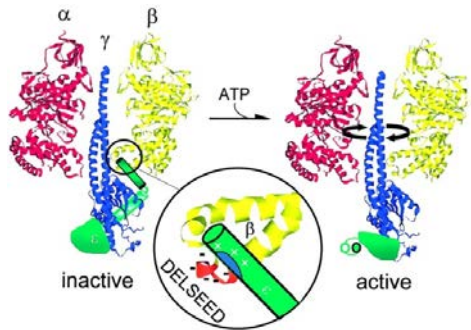
Previous results: *C. reinhardtii* mutants defective in one of the ATP synthase subunits, AtpE ($\Delta 6$, dark bars), grow more slowly but produce H₂ at higher rates than the corresponding wild-type (cc124, light bars) strains, particularly at high light intensities.



Accomplishments and Progress – Task 2

Task 2 (cont.) – Generate, physiologically characterize and test the H₂-production rate of inducible ATP synthase mutants that are defective in maintaining the proton gradient – milestone 3.3.3 completed.

Current progress:



(a) Generated six mutants in the C-terminal of the ATP synthase subunit ϵ ; four showed similar rates of H₂ production as their parental strain but two, EJ13 and EJ17, were unable to grow constitutively.

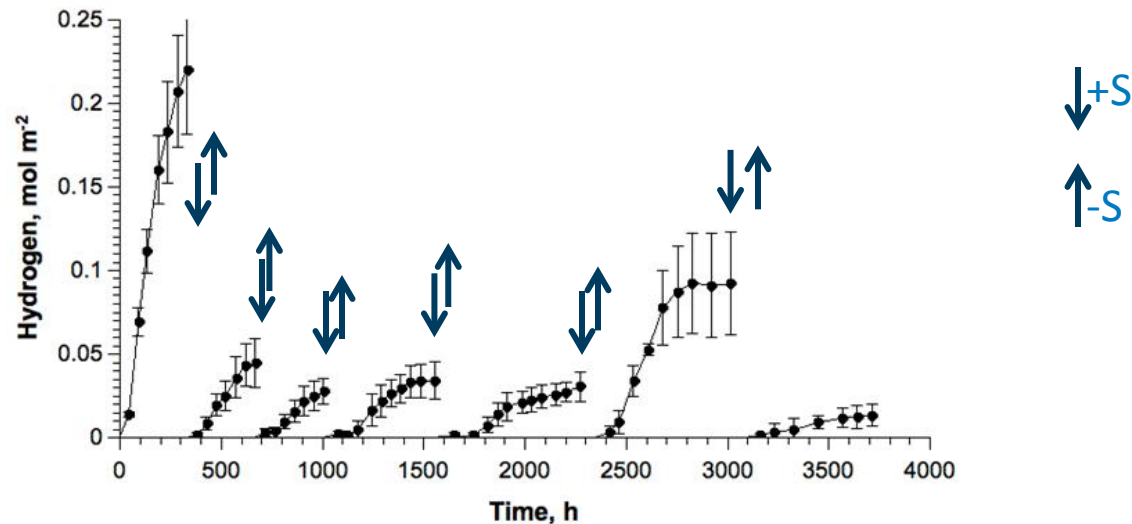
(b) The defective EJ17 gene was transformed behind the PsbD promoter into strain Ind41_15D. The transformed gene was shown to be stably incorporated into the chloroplast genome and to allow growth of the transformant under photo or heterotrophic conditions in the presence of spectinomycin - **completion of milestone 3.3.3.**

(c) The inducibility of the Ind41_15D/psbD system under selective pressure was positively demonstrated by using the expression of an orange fluorescent protein as a marker.

Accomplishments and Progress – Task 2

Task 2 – Demonstrate continuous H_2 production for 2 months by sulfur-deprived, alginate-immobilized algae – towards completion of milestone 3.3.4.

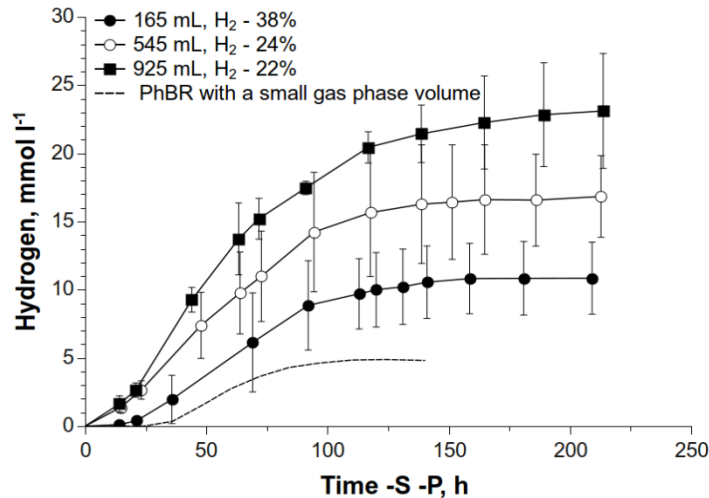
Previous results: alginate-immobilized algae photoproduce H_2 at higher specific rates and light conversion efficiencies than cultures in suspension upon sulfur deprivation, and they show higher tolerance to aerobic environments; cycles of +S/-S resulted in prolongation of H_2 production for an additional 6 cycles of about 500 hours each.



Current results: preliminary results by using continuous flow of S+ medium resulted in low rates of H_2 production; the experiment is being repeated under different operational conditions.

Accomplishments and Progress – Task 2

Task 2 – Demonstrate the effect of headspace volume on the H_2 photoproduction yield.



Current results: In suspension cultures, a 4x increase (from ~0.5 to ~2) in the ratio of gas/liquid volume results in a **2x increase in the total yield of H_2 gas**. Similar results were shown with immobilized cultures.

Remarkably, **565 ml of H_2 gas per liter of the suspension culture is the highest yield ever reported for a wild-type strain in a time period of less than 180 hours**. A control PhBR with a historically small gas phase volume of ~5 – 10 ml only produced up to 120 ml L⁻¹ of H_2 gas.

Collaborations

Partners (subcontractors):

- Dr. Sergey Kosourov, Russian Academy of Sciences – applies sulfur deprivation to sulfur-immobilized *C. reinhardtii* cultures and tests their H₂-production capabilities (Task 2).
- Dr. Eric Johnson, Johns Hopkins University – generates ATP synthase mutants, develops transformation protocols and transforms *Chlamydomonas reinhardtii*; tests physiological properties of transformants (Task 2).

Proposed Future Work

2012

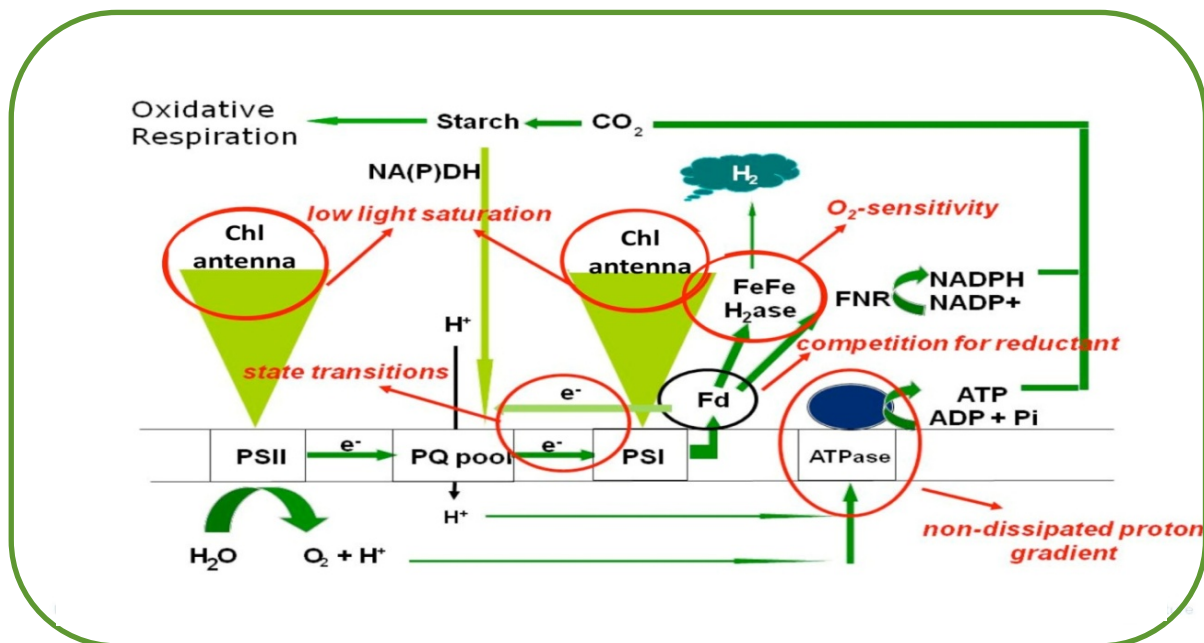
Task 1 – Complete milestone 3.3.5: Demonstrate expression of an active Ca1 in a *C. reinhardtii* hydrogenase-less background and characterize O₂ sensitivity of light-driven H₂ production.

Task 2 – Complete milestone 3.3.4: Demonstrate continuous operation of the sulfur-deprivation process for a total of 2 months upon addition of phosphate/sulfate to alginate-immobilized cultures.

Work beyond FY12: Start to genetically express mutated ATP synthase and truncated antenna regulatory genes into a strain expressing the Ca1 hydrogenase. Develop photobioreactor systems for cyclic or continuous H₂ production based on current optimization of gas space composition (not shown), alginate immobilization, and others.

Summary Slide

Relevance: Photobiological water splitting coupled to hydrogenase-mediated H_2 production has the potential to convert *about 10% of incident solar energy into H_2* . Various barriers have been identified as currently limiting green algal H_2 production, including the *O_2 sensitivity of the hydrogenase enzyme*, and *down-regulation of photosynthesis due to non-dissipation of the proton gradient*.



Summary Slide (cont.)

Approach: NREL is expressing a more O₂-tolerant bacterial hydrogenase in green algae and generating ATP synthase mutants that prevent down-regulation of photosynthesis with JHU. NREL and RAS use the low STH sulfur-deprivation process to test candidates that have been generated to address various barriers, as well as optimizing photobioreactor conditions for efficient and sustainable production of H₂ gas.

Technical Accomplishments and Progress:

1. Successfully expressed a bacterial hydrogenase in a *Chlamydomonas* strain lacking native hydrogenase activity and detected photoproduction of H₂.
2. Generated inducible ATP synthase mutants, tested for growth, and are testing their phenotype with respect to H₂ photoproduction.
3. Continues to optimize long-term H₂ photoproduction by alginate-immobilized *Chlamydomonas*.

Collaborations: Drs. Sergey Kosourov (RAS) and Eric Johnson (JHU).

Proposed Future Research: For 2012: complete milestones 3.3.4 (demonstrate continuous H₂ photoproduction by addition of sulfate) and 3.3.5 (measure the *in vivo* O₂ tolerance of mutants expressing Ca1); measure the inducible H₂ photoproduction capability of the ATP synthase mutant. After 2012: genetically combine useful phenotypes into a single organism expressing Ca1.