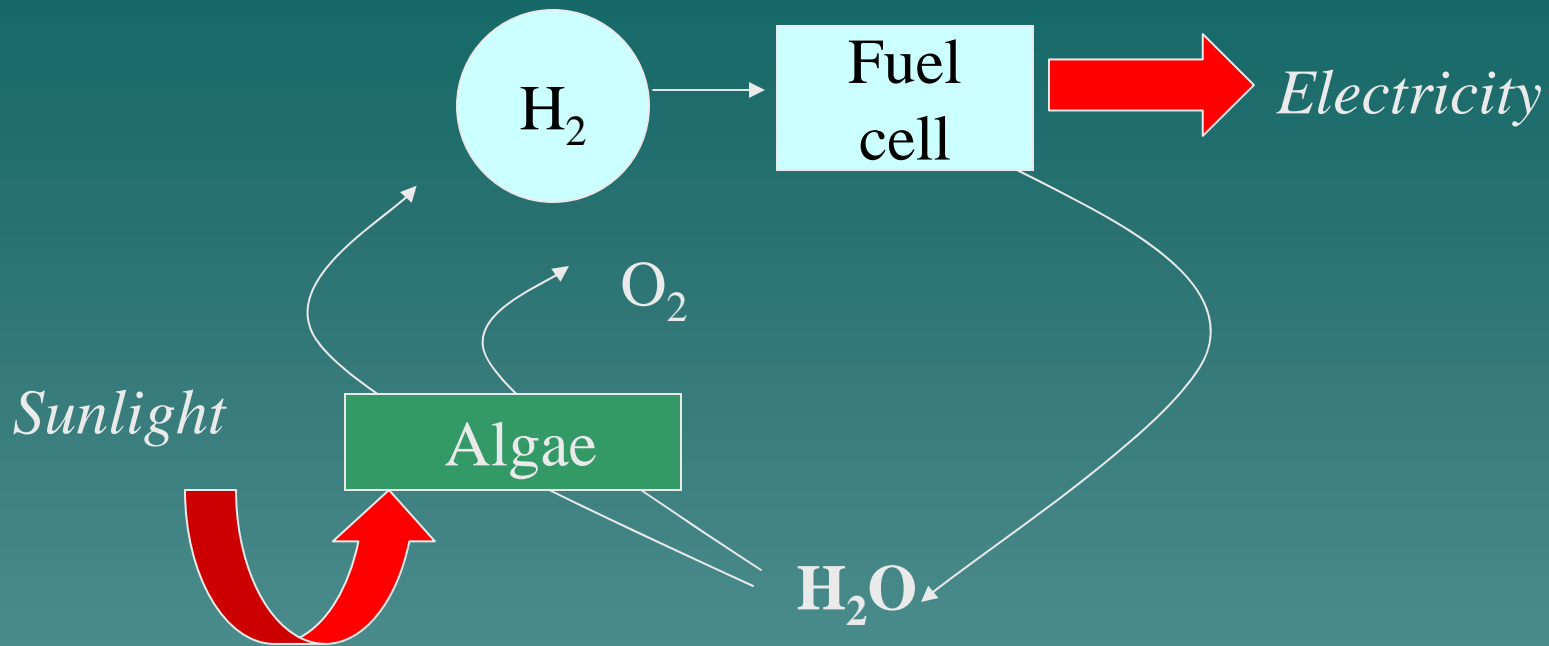


# Algal Biohydrogen: Opportunities and Challenges

**Maria L. Ghirardi**  
**Group Manager/Principal Scientist**

**National Renewable Energy  
Laboratory, Golden CO 80401**

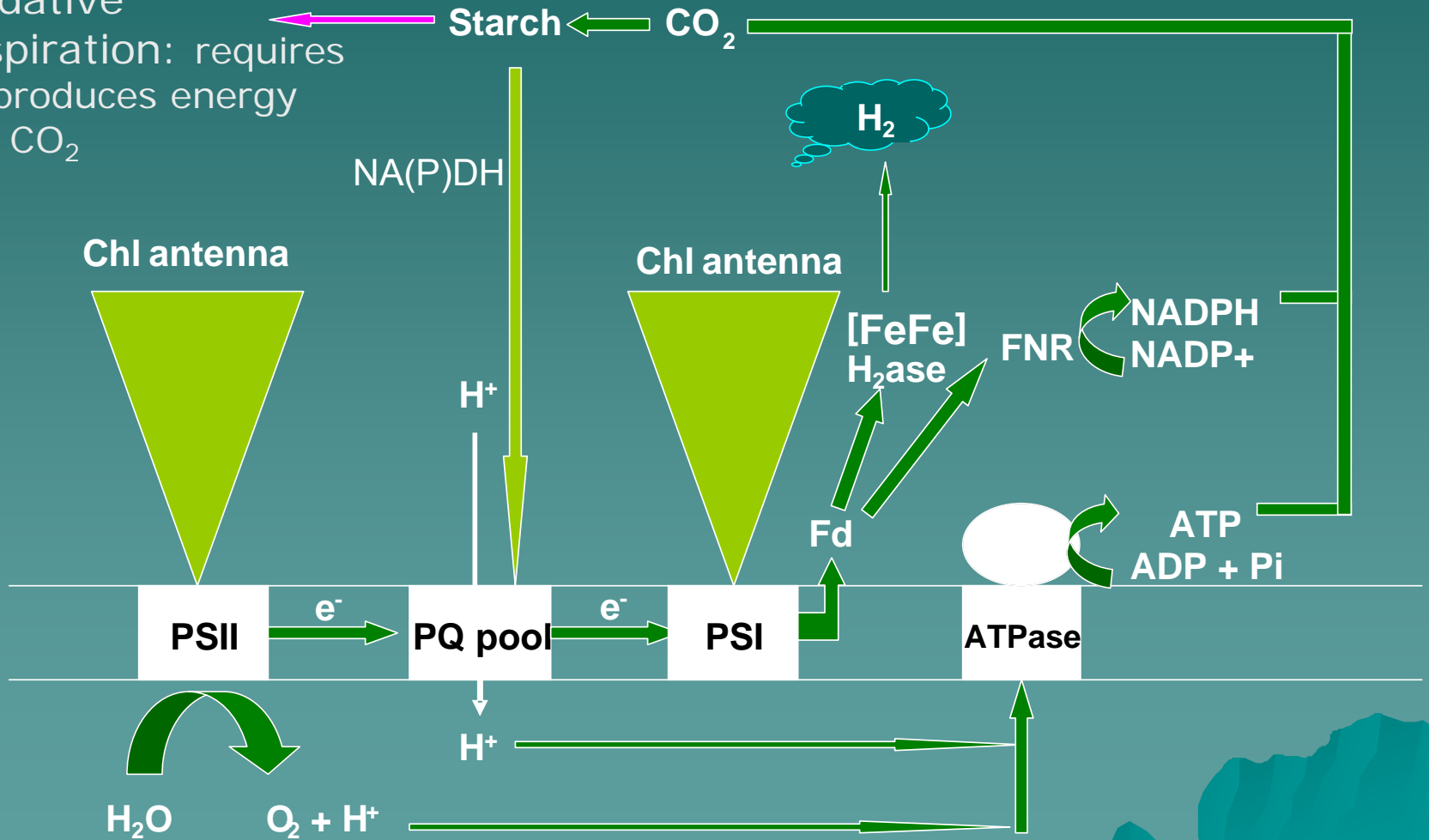
# Vision



- Maximum conversion efficiency of about 10-13% of incident sunlight (needs to be corrected for yearly sunlight intensities);
- Land area of about 100 x 100 square kilometers (or 4,500 square miles) is required to provide enough energy to fully supply the U.S. transportation needs (263 million vehicles, 60 mi/gge); this equals about 0.12% of the U.S. surface area.
- Estimated cost of photobiologically-produced  $H_2$  could be as low as \$3/kg (or gge).

# Biochemical Pathways for H<sub>2</sub> Photoproduction in Algae

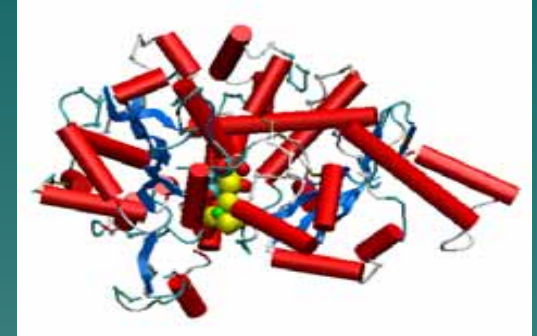
Oxidative  
Respiration: requires  
O<sub>2</sub>; produces energy  
and CO<sub>2</sub>



# Biochemical Issues to be Solved

1. The algal hydrogenase is extremely sensitive to  $O_2$  inactivation; other factors that regulate its expression are not known;
2.  $H_2$  production competes with  $CO_2$  fixation and other metabolic pathways for reductant;
3. Photosynthetic electron transport is down-regulated in the absence of ATP consumption (due to lack of  $CO_2$  fixation, which induces cyclic electron transport);
4. The large light-harvesting antennae of the photosystems prevents high light conversion efficiencies at sunlight.

# Issue 1. O<sub>2</sub> Sensitivity of the Two Algal Hydrogenases

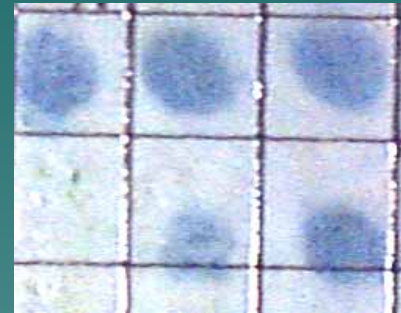
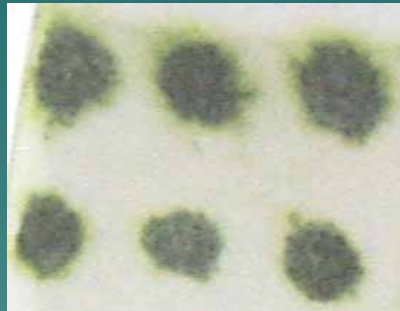


- ◆ The algal hydrogenase genes are not transcribed in the presence of oxygen;
- ◆ The enzymes required for hydrogenase maturation are not expressed under oxygenic conditions;
- ◆ The algal hydrogenases are inactivated by oxygen and are quickly degraded under oxygenic conditions.

# Regulation of Hydrogenase Gene Transcription by Redox Potential

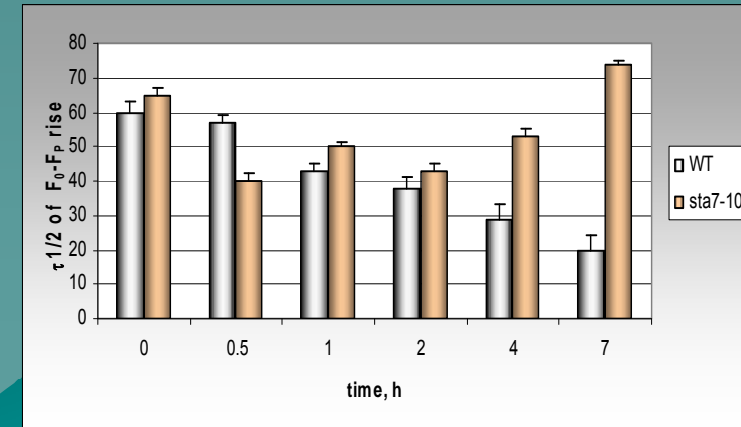
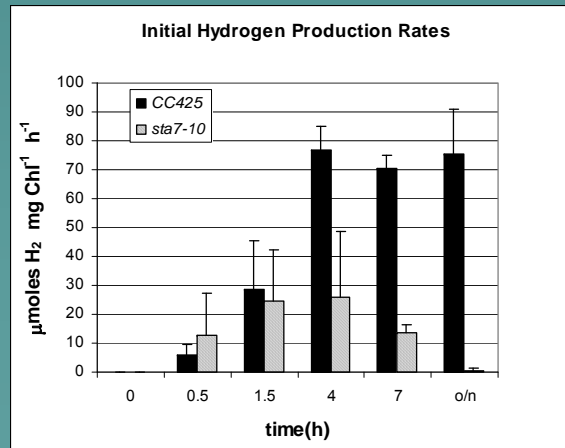
(M. Posewitz and S. Smolinski)

Colonies on TAP plates



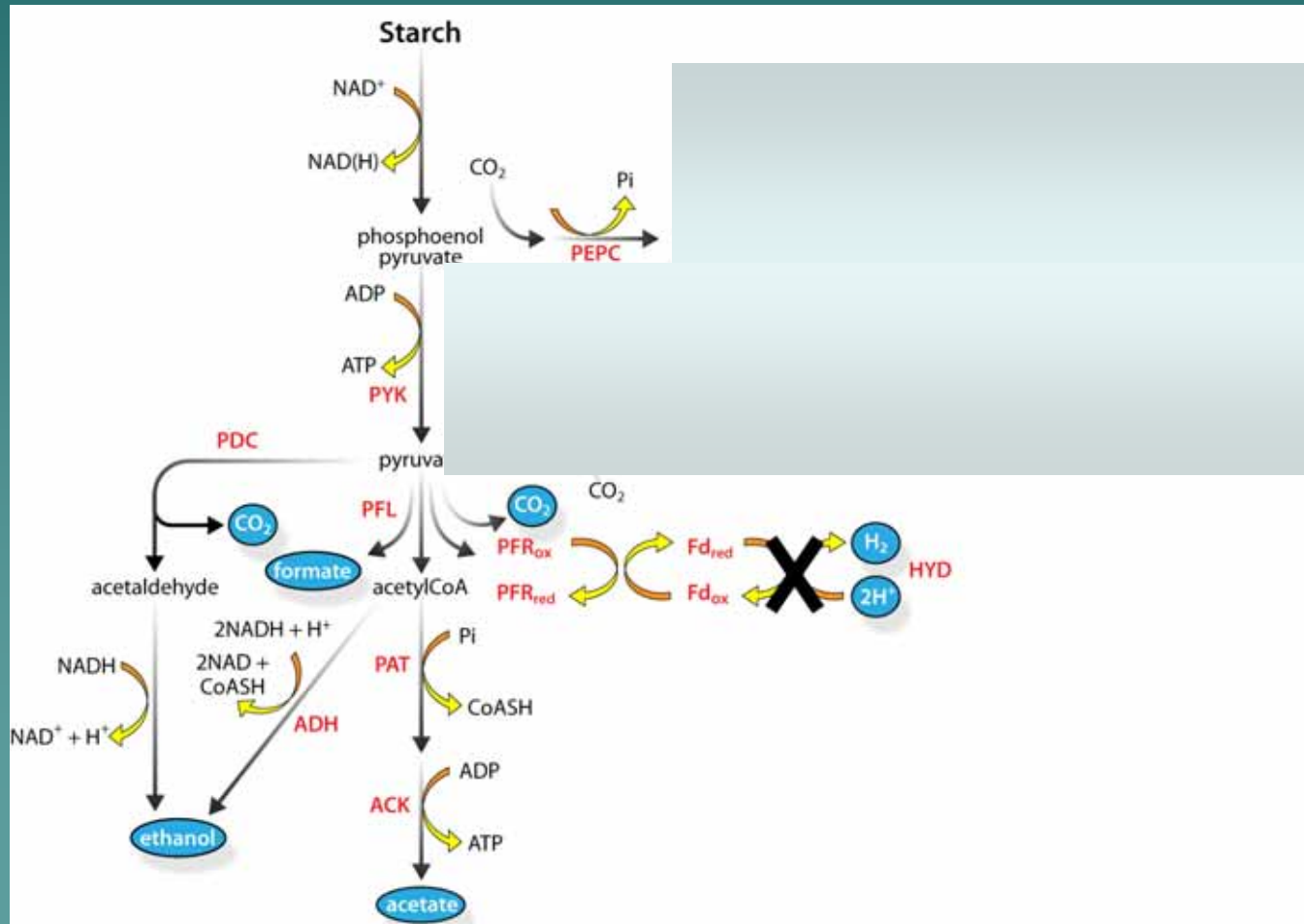
Chemochromic sensor

**Sta7 mutant** - non-functional isoamylase gene; does not accumulate starch; transient transcription of the hydrogenase genes and transient induction of hydrogenase activity upon anaerobiosis: correlation between H<sub>2</sub>-production and PQ pool redox state.



# O<sub>2</sub> Regulation of Metabolic Pathways under Anaerobic Conditions

(A. Dubini, F. Mus, M. Posewitz, M. Seibert, A. Grossman)



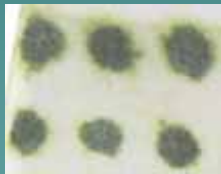
# Transcriptional Regulation and Assembly of the Hydrogenase Catalytic Site

(M. Posewitz, P. King)

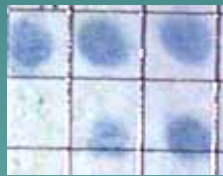
## Insertional Mutagenesis



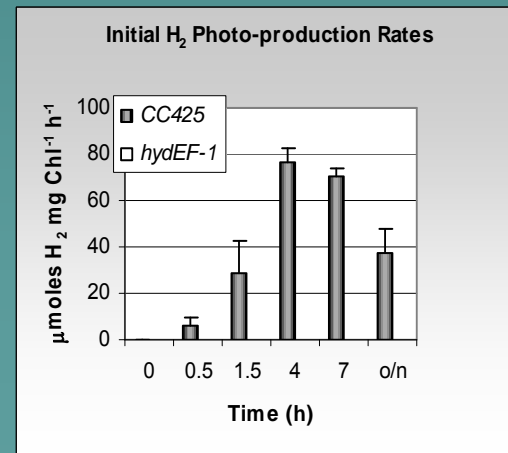
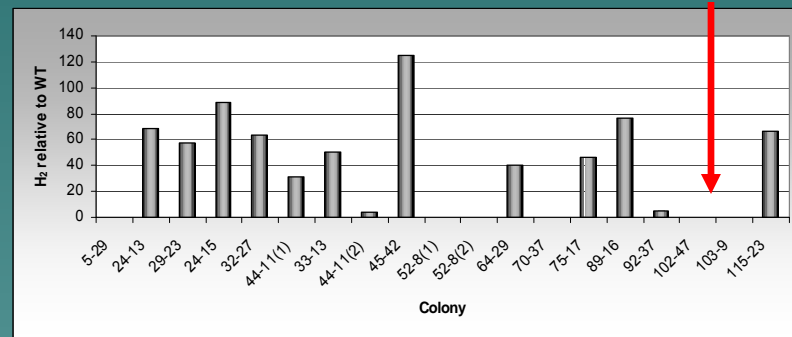
Chemochromic Screen (overnight anaerobiosis) for H<sub>2</sub>-photoproduction



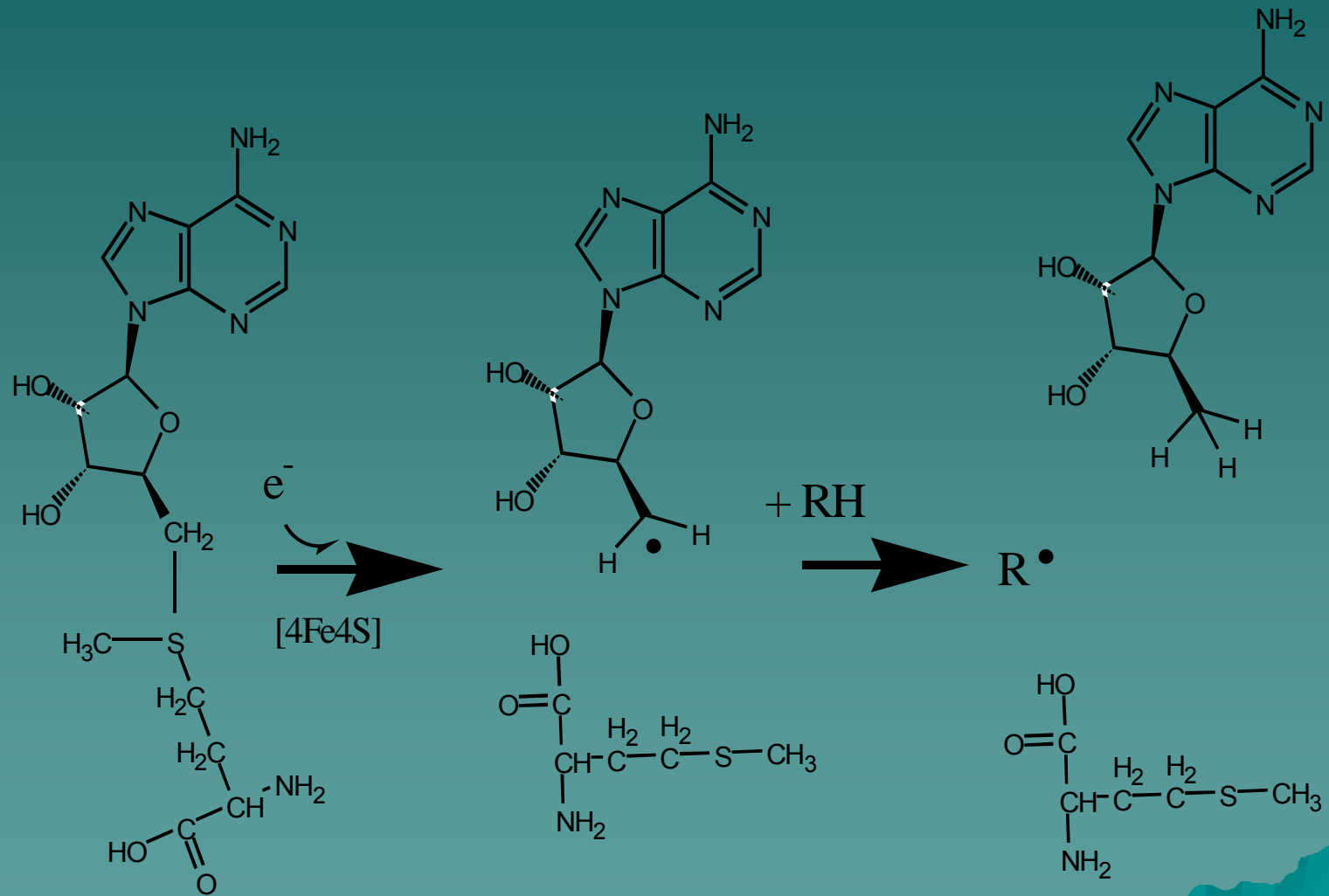
Colonies on TAP plates



Chemochromic sensor



# SAM Cleavage into the Ado Radical



# Recent results from other groups

- HYDE and HYDG have been shown to have SAM activity when expressed in vitro (Fontecave et al.).
- HYDF has been shown to have GTPase activity in vitro (Fontecave et al.) and to insert a sulfur into tyrosine, converting it into p-cresol (Fontecave and Fontecilla-Camps).

Suggested role for each of the assembly proteins:

**HydE** – S insertion into the apoprotein

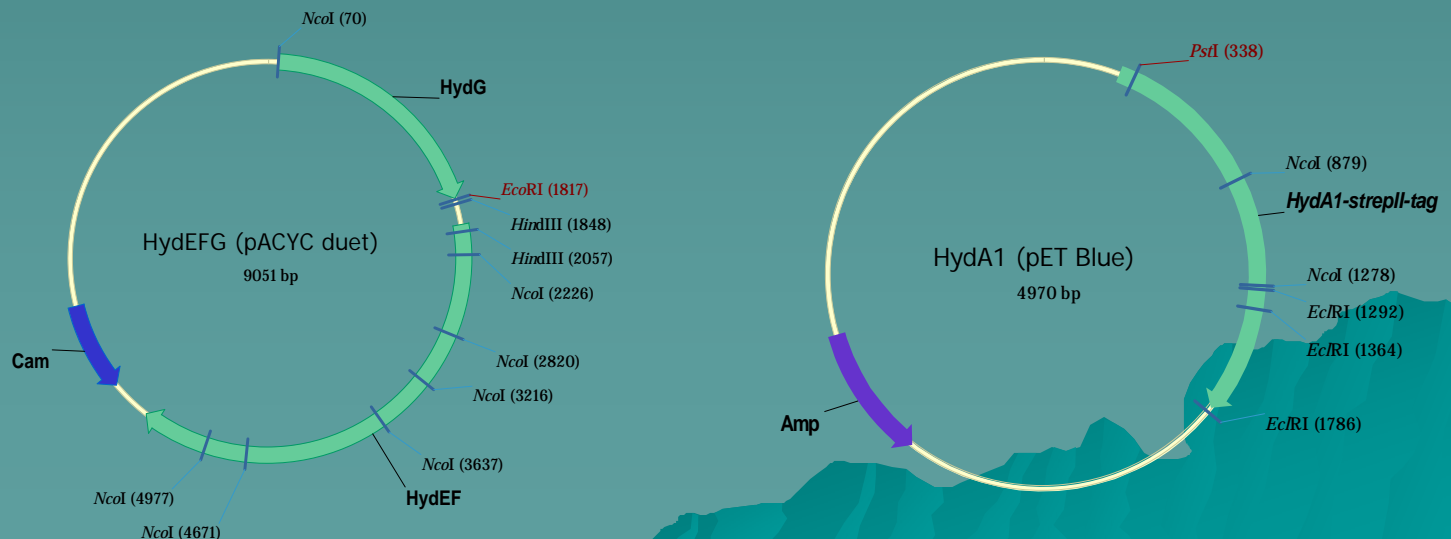
**HydF** – scaffold for assembly of the H-cluster

**HydG** – S insertion into the apoprotein or synthesis of CO and CN<sup>-</sup> ligands

# O<sub>2</sub>-regulation of the H-Cluster Assembly

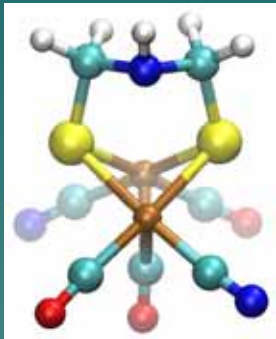
(P. King, M. Posewitz)

- Identified assembly genes (HydE, HydF and HydG) and used them to produce active, tagged [Fe]-hydrogenase by co-expression in *E. coli*.
- The tagged enzyme was partially purified and its activity measured with reduced MV as the electron donor.
- Accelerates our ability to generate and test candidate O<sub>2</sub>-resistant proteins.

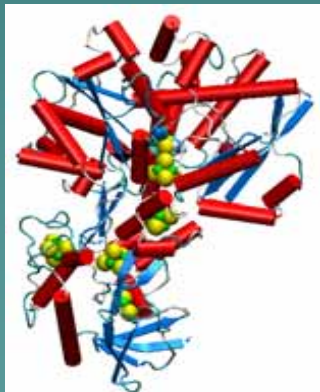


# Inactivation of [FeFe]-Hydrogenase by O<sub>2</sub>

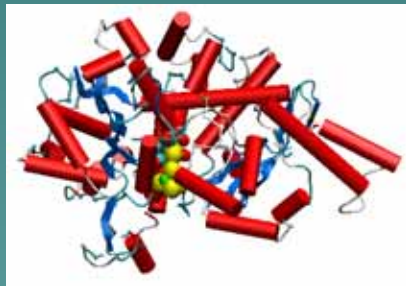
(D. Sverdruzic and P. King)



The catalytic center consists of a 4Fe4S center coupled by a cysteine residue to a unique 2Fe2S center.

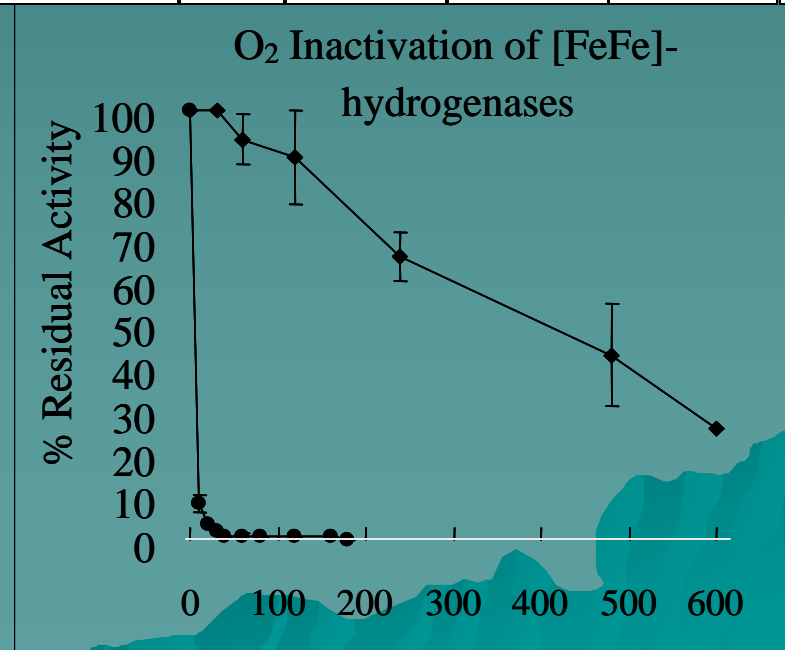


*Clostridium acetobutylicum*  
HydA



*C. reinhardtii* HYDA2

Organism	Protein Name	Subunit Composition	Activity (nMol H <sub>2</sub> /ml/min)	Specific Activity (nMol H <sub>2</sub> /mg/min)	Half-life after exposure to air
<i>Chlamydomonas reinhardtii</i>	HydA1	Monomeric	42	212	< 1 sec
<i>Chlamydomonas reinhardtii</i>	HydA2	Monomeric	12	708	< 1 sec
<i>Clostridium acetobutylicum</i>	CaI	Monomeric	28	2894	415±115
	CaII	Monomeric	3	682	
<i>Clostridium pasteurianum</i>	CpI	Monomeric	20	ND	120-300
<i>Shewanella oneidensis</i>	HydAB	Dimeric	14	850	



# Approaches for Solving Issue #1

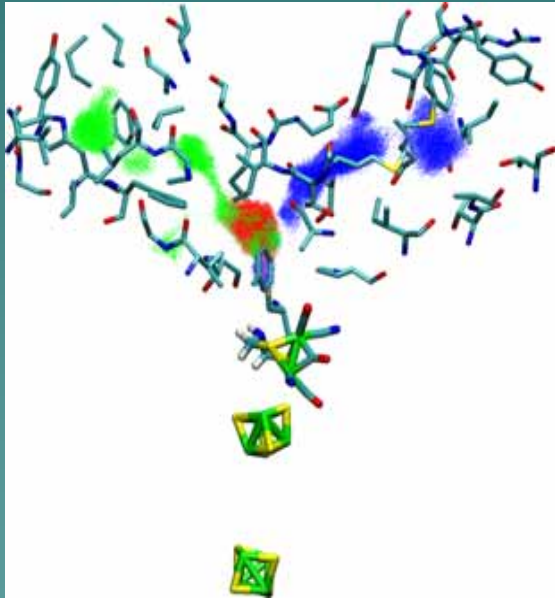
- ◆ Engineer a hydrogenase that is more tolerant to  $O_2$  inactivation;
- ◆ Transform a more  $O_2$ -tolerant hydrogenase into the alga;
- ◆ Induce culture anaerobiosis by partially inactivating photosynthetic  $O_2$  evolution or by increasing the rates of respiratory  $O_2$  consumption.

# Engineering a more O<sub>2</sub>-tolerant [FeFe]-hydrogenase

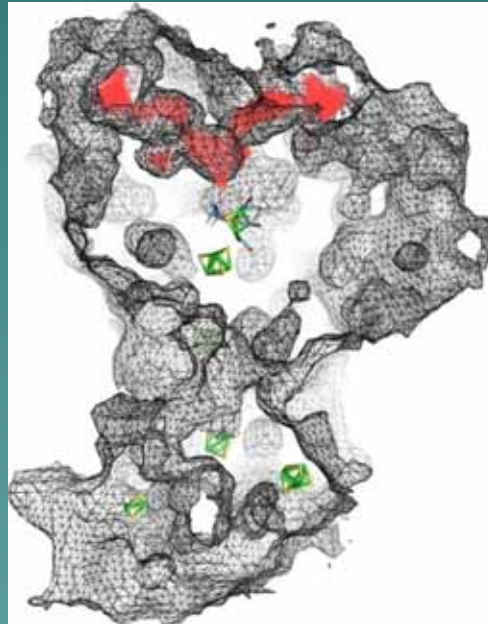
Hypothesis:  $O_2$  sensitivity is governed by  $O_2$  access to the catalytic site

# Computational Simulations

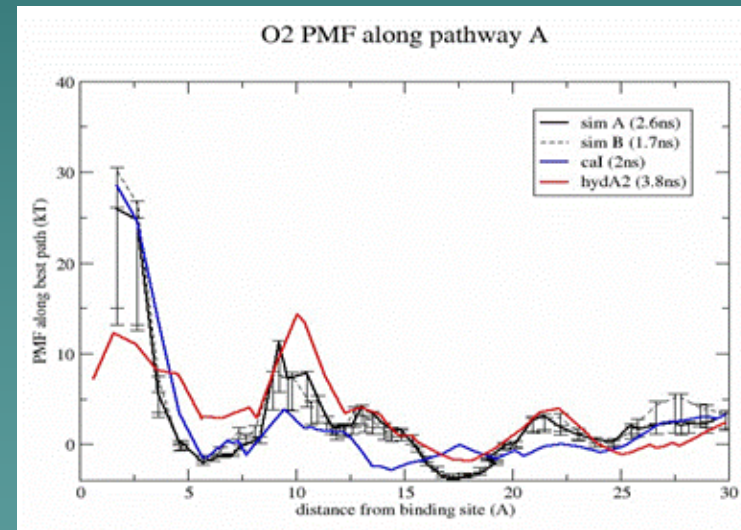
(J. Cohen, P. King, K. Schulten)



$O_2$  pathways



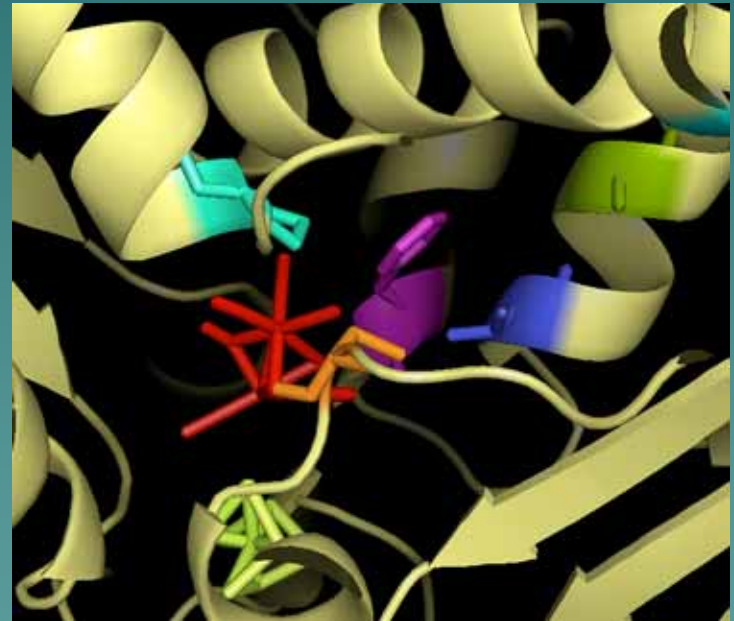
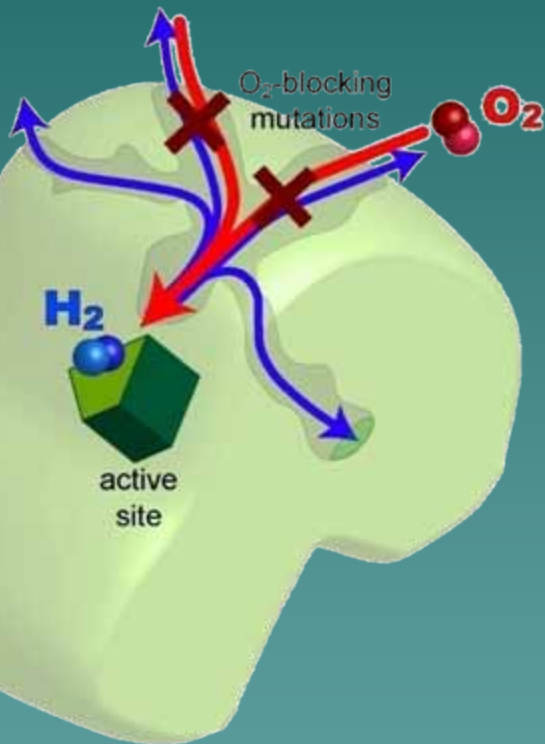
$O_2$  cavities



Energy barriers to  $O_2$  diffusion

# Molecular Engineering O<sub>2</sub> Tolerance into the Algal Hydrogenase

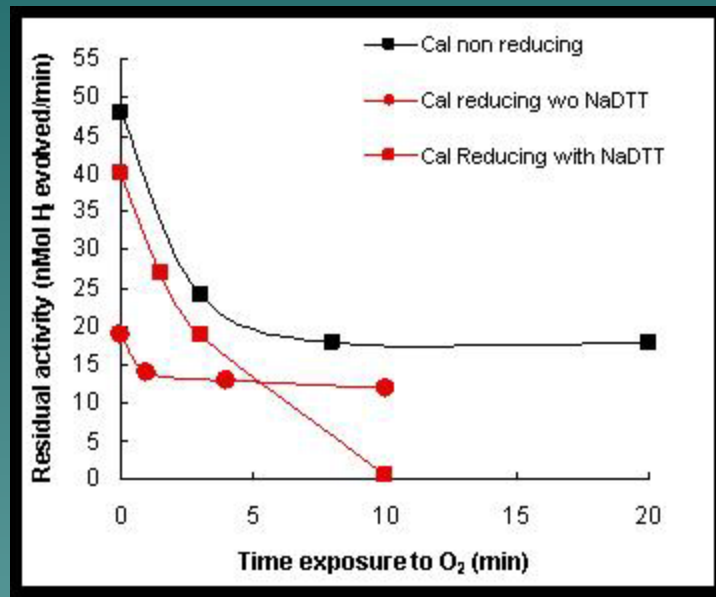
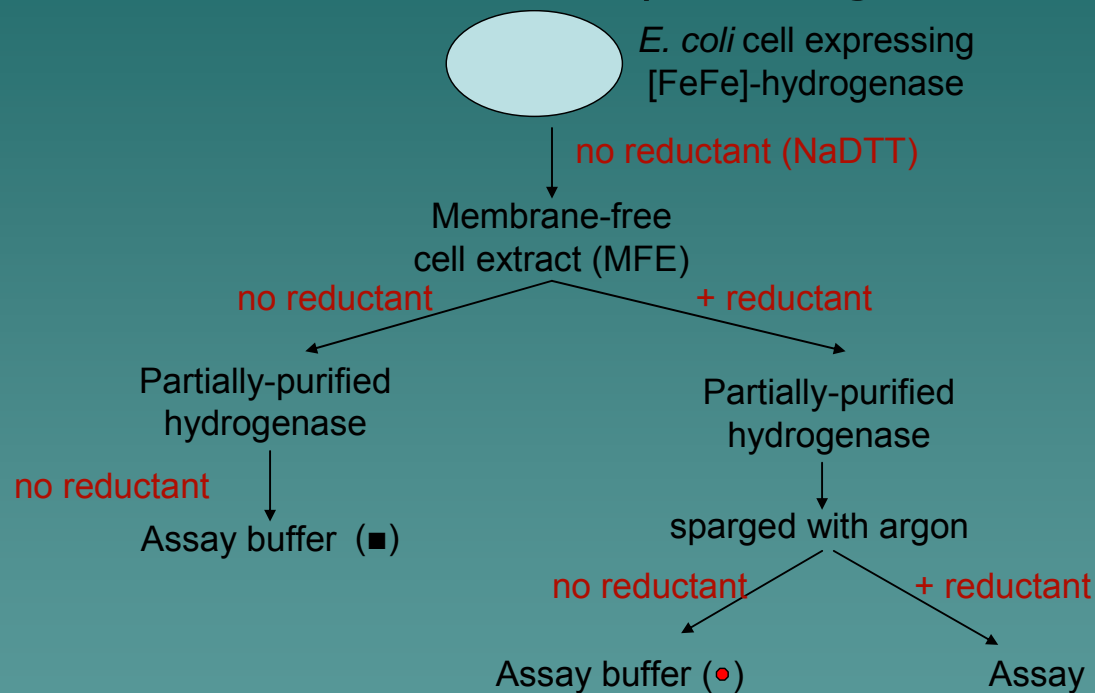
(C. English and P. King)



We focused our engineering efforts in the area the high energy barrier. We substituted local amino acid residues for larger ones, sterically hindering the access of O<sub>2</sub> to the catalytic site.

# O<sub>2</sub> Tolerance Varies According to the Redox State of the Enzyme

(C. English, P. King)

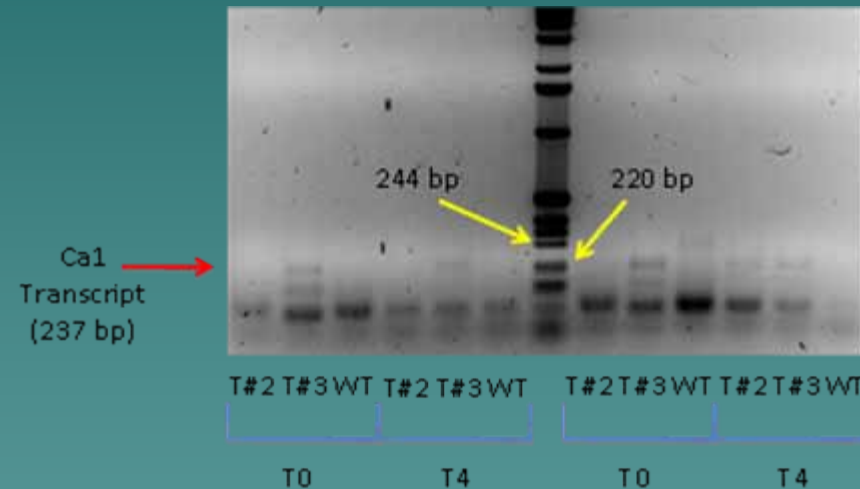
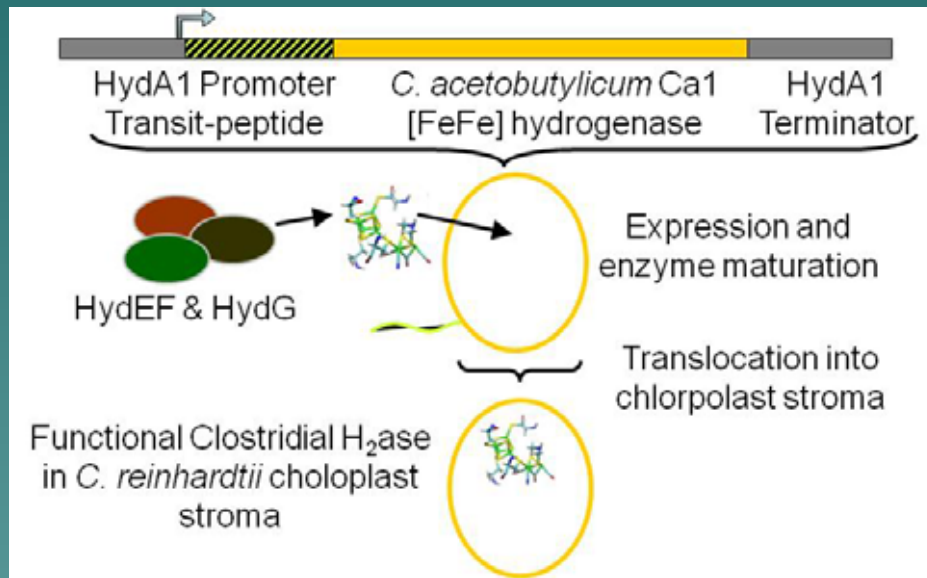


**Conclusion:** lack of (■) or short exposure to reductant (●) results in enzymes that are partially insensitive to O<sub>2</sub> inactivation, while enzymes exposed to reductant throughout the purification process are totally sensitive to O<sub>2</sub> (●). The inactive state of the enzyme could be due to the presence of a CO ligand at the H-cluster, as suggested by others, which, upon illumination or extensive argon sparging, would be removed to yield the O<sub>2</sub>-tolerant form of the enzyme.

Expressing a more O<sub>2</sub>-Tolerant  
Hydrogenase in  
*Chlamydomonas*

# Expression of more O<sub>2</sub>-tolerant hydrogenases in algae

(C. English, P. King)



The *Clostridium acetobutylicum* hydrogenase gene (*Ca1*) has been successfully incorporated into the *Chlamydomonas reinhardtii* genome, and that it is correctly transcribed upon anaerobic induction.

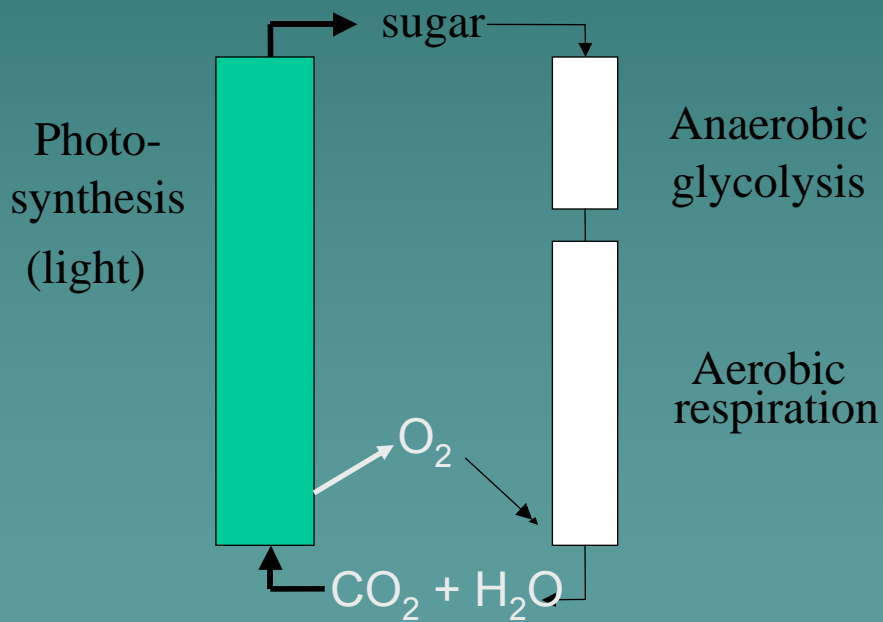
DNA (gene) → RNA (transcript) → protein (enzyme) → activity (H<sub>2</sub> production)

Induce Culture Anaerobiosis by  
Partially Inactivating  
Photosynthetic O<sub>2</sub> Evolution

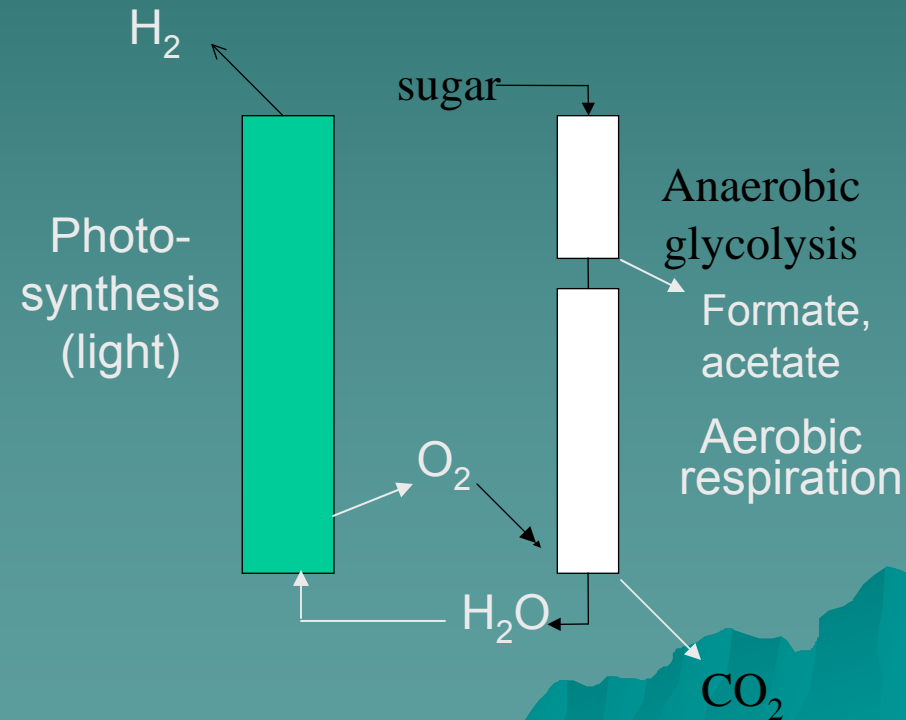
# Effects of Sulfur Deprivation

(Wykoff et al., 1998; Melis et al., 2000)

Sulfur replete

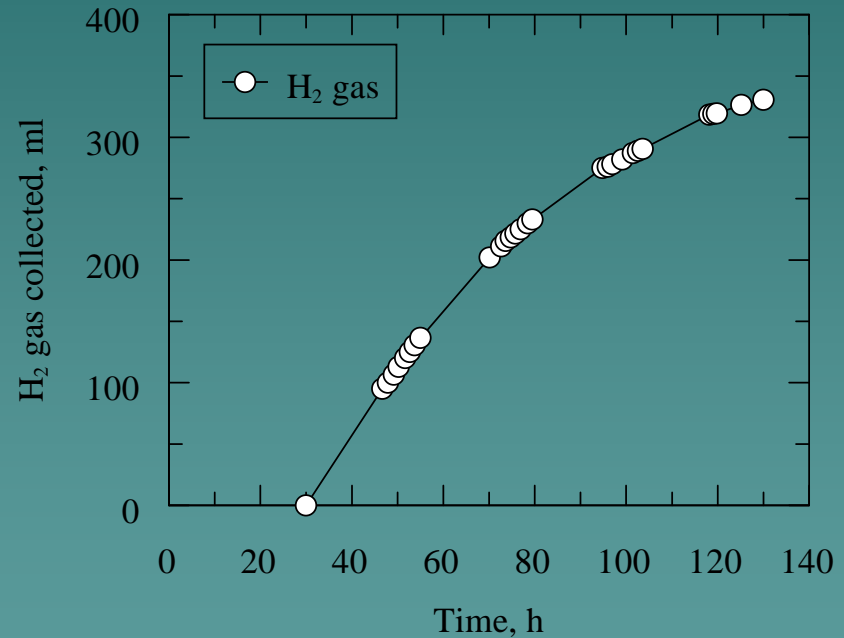
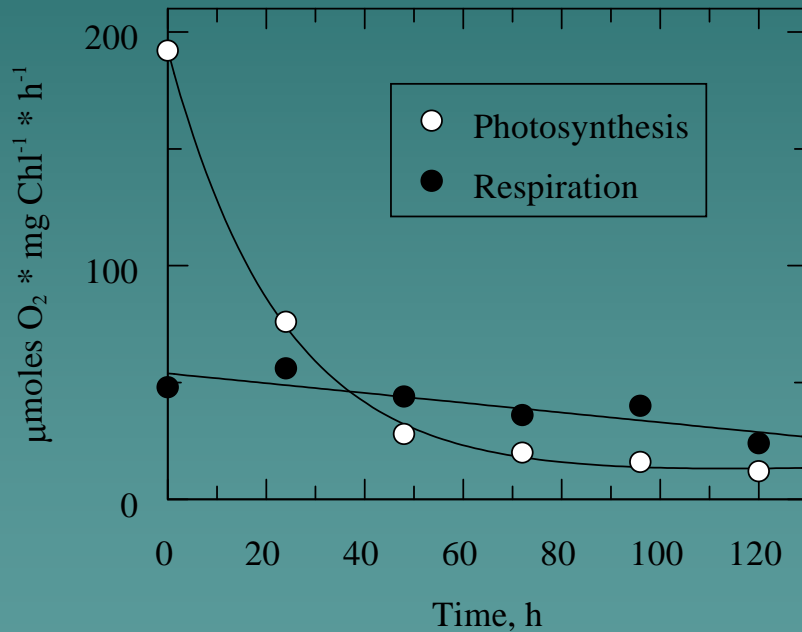


Sulfur depleted



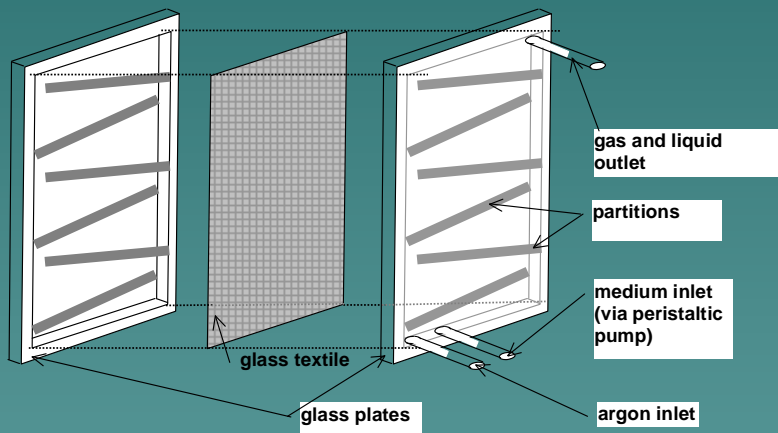
# Temporal Separation of O<sub>2</sub> Evolution and H<sub>2</sub> Production

(Forestier and U.C. Berkeley)

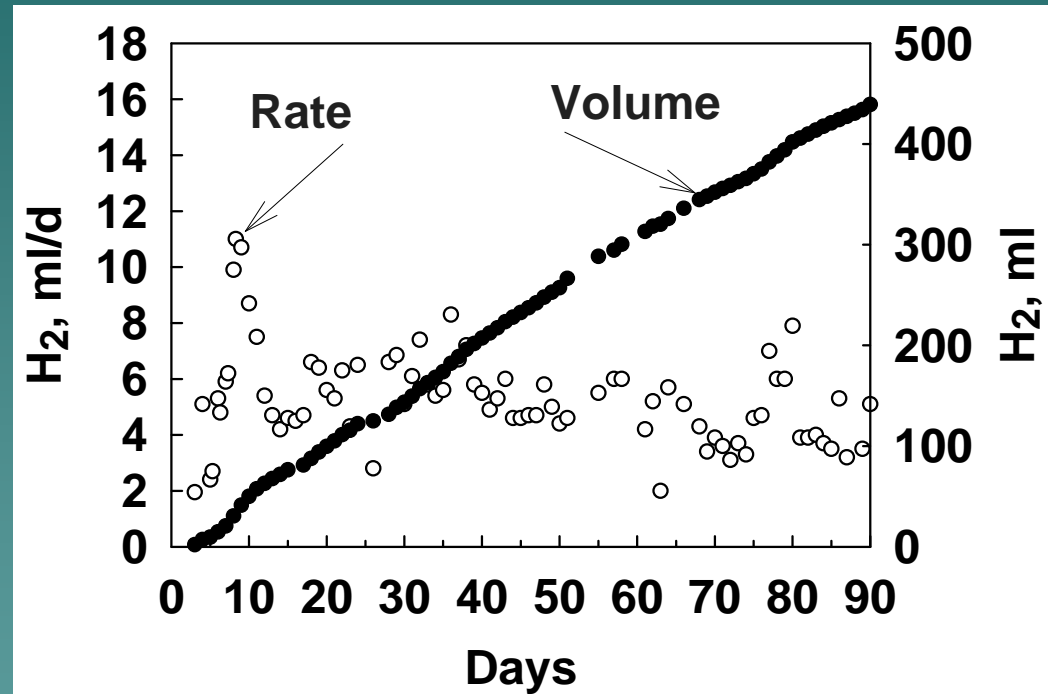


Cycles of +S (2 days) and -S (5 days) can be repeated up to about 3 times without significant decrease in yields

# Photobioreactor with Immobilized *C. reinhardtii* in Chemostat Mode (Fedorov and Pushchino)



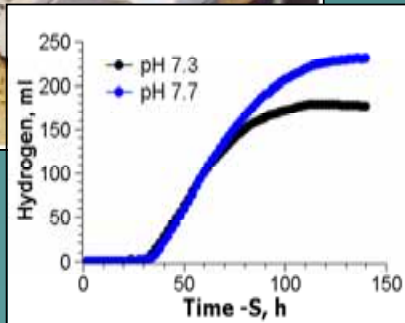
Cells were grown on the glass textile



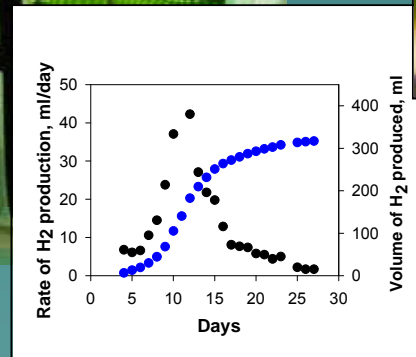
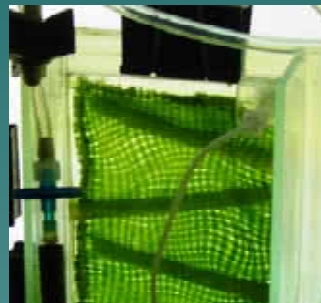
H<sub>2</sub> production was observed for 3 months at about the average rate as seen in suspensions; continuous input of 10-20  $\mu$ M sulfate in the flow medium was used.

# Sustained H<sub>2</sub>-production by sulfur-deprived, immobilized algae

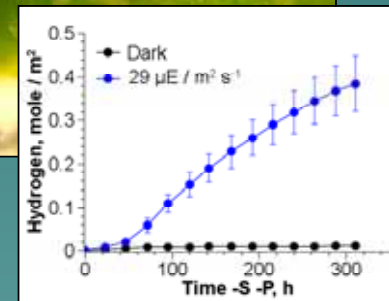
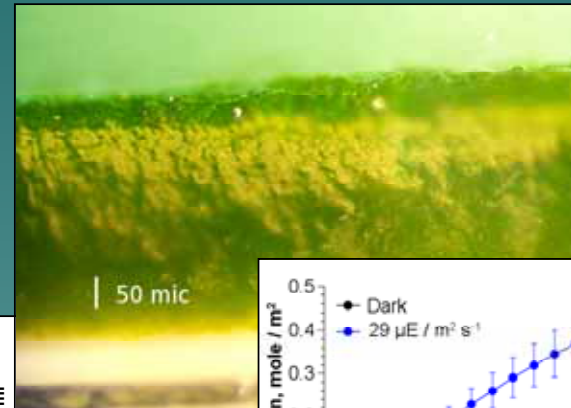
Cell suspensions



Immobilized onto glass fibers



Immobilized into alginate films



Higher cell density, longer duration  
Non-degradable matrix

High cell density  
Long duration  
Degradable matrix

Low cell-density, short duration

Maximum light conversion efficiency of 1 % under low intensity

# Other Approaches for Inducing Culture Anaerobiosis

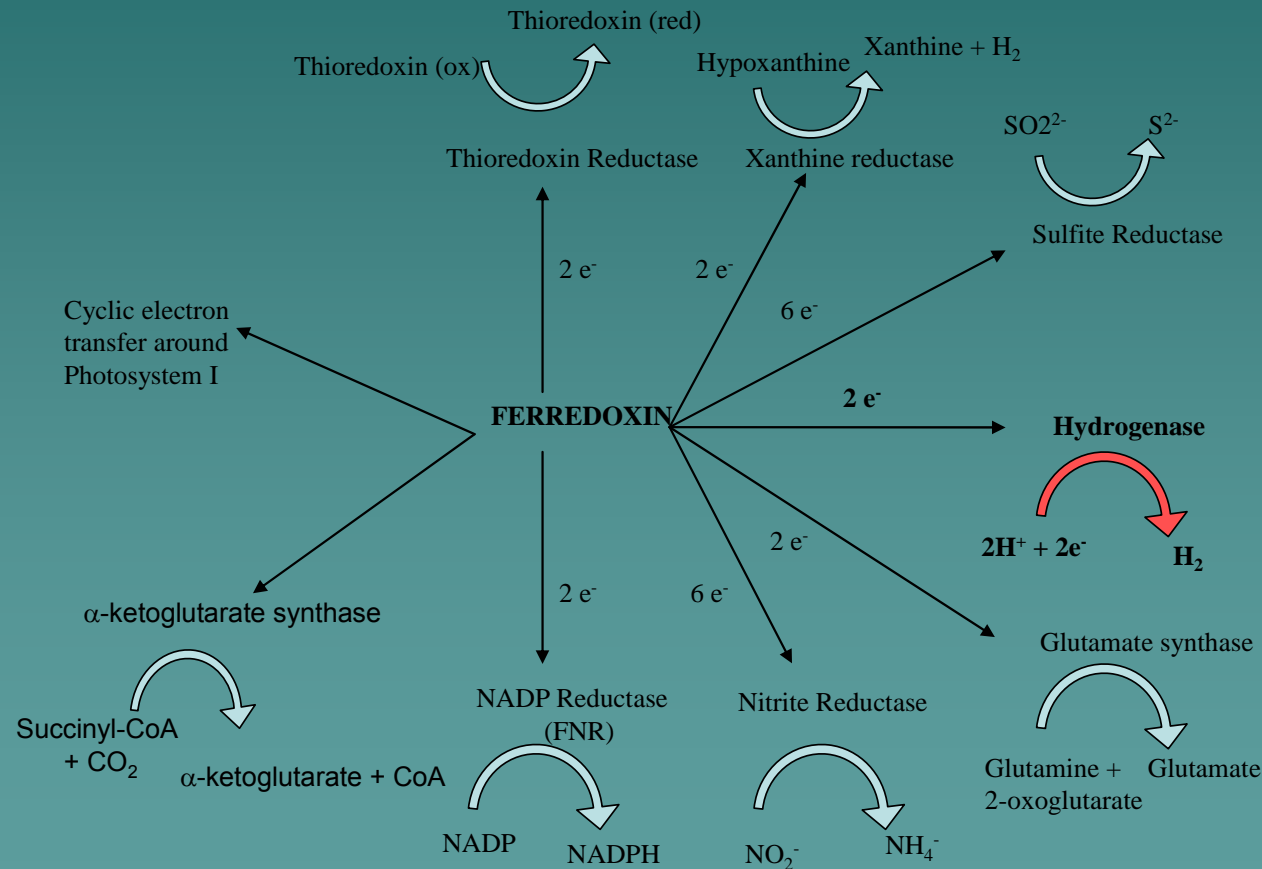
- ◆ Increase the photosynthesis/respiration ratio using sulfate permease mutants (Melis et al.)
- ◆ Regulate the expression of PSII reaction center protein (psbD) by expressing it behind the cyt c6 promoter that responds to addition of copper to the medium (Surzycki et al.)
- ◆ Generate PSII mutants with higher violaxanthin to zeaxanthin activity (Torzillo et al.)

# Biochemical Issues to be Solved

1. The algal hydrogenase is extremely sensitive to  $O_2$  inactivation; other factors that regulate its expression are not known;
2.  $H_2$  production competes with  $CO_2$  fixation and other metabolic pathways for reductant;
3. Photosynthetic electron transport is down-regulated in the absence of ATP consumption (due to lack of  $CO_2$  fixation);
4. The large light-harvesting antennae of the photosystems prevents high light conversion efficiencies at sunlight.

# Issue 2. H<sub>2</sub> production competes with the CO<sub>2</sub> fixation pathway

(A. Dubini)



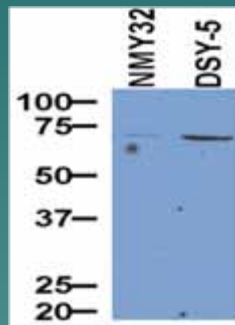
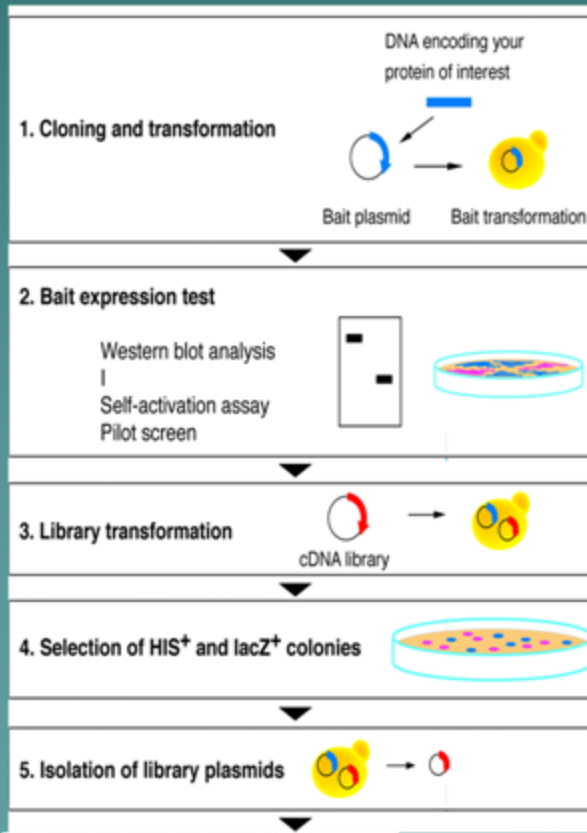
The genome of *C. reinhardtii* has six ferredoxin homologs, besides the petF gene whose gene product is the electron acceptor for PSI. Two of those genes, FDX2 and FDX5 are upregulated during anaerobiosis.

# The yeast two-hybrid assay using HYD2 as bait

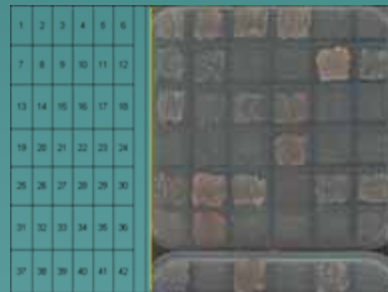
Western blot of fused protein

Summary of results

Yeast two-hybrid protocol



Growth in adenine-



Galactosidase assay



Prey #	Growth on SD-his	lacZ positive	Growth on SD-ade	Summary +++	This interaction is ...
1	+	++	+	4	strong
2	+	++	+	4	strong
3	+	-	+	2	weak
4	+	-	+	2	weak
5	+	-	-	1	weak
6	+	-	(+)	1	weak
7	+	-	+	2	weak
8	+	-	+	2	weak
9	+	-	-	1	weak
10	+	-	-	1	weak
11	+	-	++	3	intermediate
12	+	++	+	4	strong
13	+	++	++	5	strong
14	+	-	+	2	weak
15	+	+	+	3	intermediate
16	+	-	+	2	weak
17	+	-	-	1	weak
18	+	-	-	1	weak
19	+	-	-	1	weak
20	+	-	-	1	weak
21	+	+	(+)	2	weak
22	+	-	+	2	weak
23	+	++	-	3	intermediate
24	+	-	(+)	1	weak
25	+	-	+	2	weak
26	+	-	++	3	intermediate
27	+	-	+	2	weak
28	+	-	-	1	weak
29	+	-	+	2	weak
30	+	-	+	2	weak
31	+	-	+	2	weak
32	+	-	+	2	weak
33	+	-	-	1	weak
34	+	-	(+)	1	weak
36	+	-	-	1	weak
36	+	-	(+)	1	weak
37	+	+	+	3	intermediate
38	+	-	-	1	weak
39	+	-	+	2	weak
40	+	-	-	1	weak
41	+	-	+	2	weak
42	+	+	-	2	weak

# Recent results from other groups

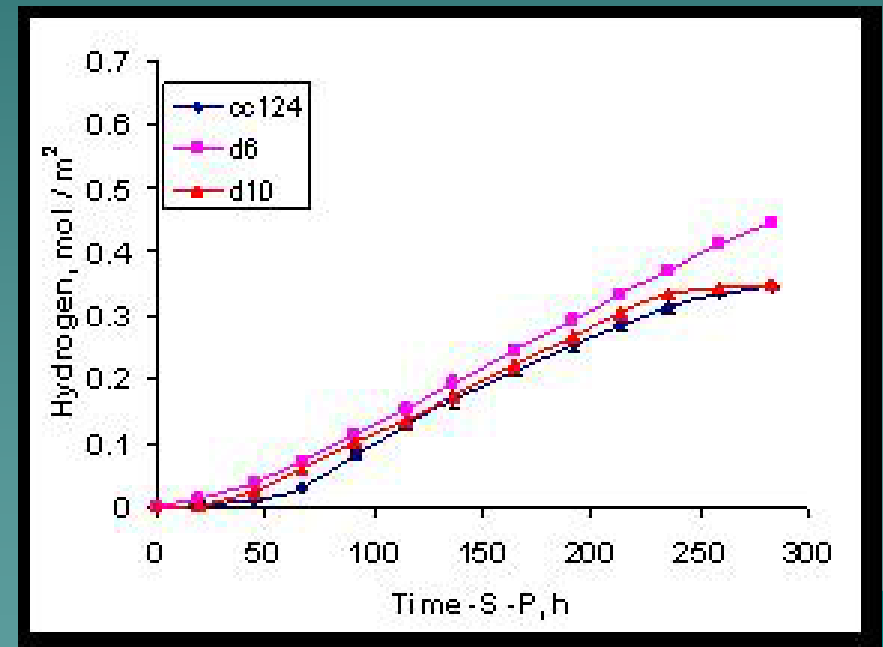
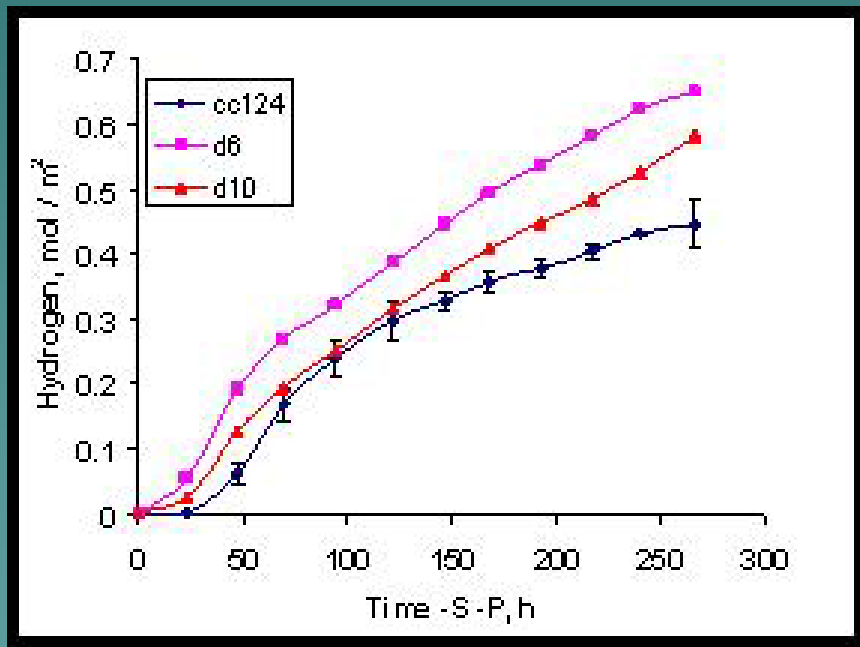
- ◆ Happe et al. reported that FDX5 was upregulated under sulfur deprivation; when overexpressed *in vitro*, however, it was unable to reduce HYDA1 or interact with FNR.
- ◆ Merchant et al. identified interactions between FDX2 and nitrite reductase.

# Issue 3. H<sub>2</sub> production is down-regulated by non-dissipation of the proton gradient

(Kosourov and Johns Hopkins Institute)

High light

Low light



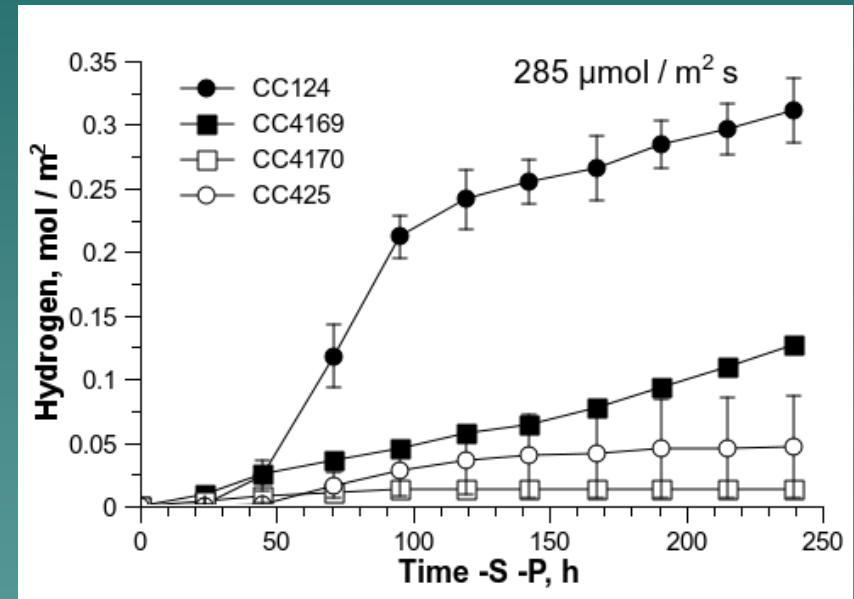
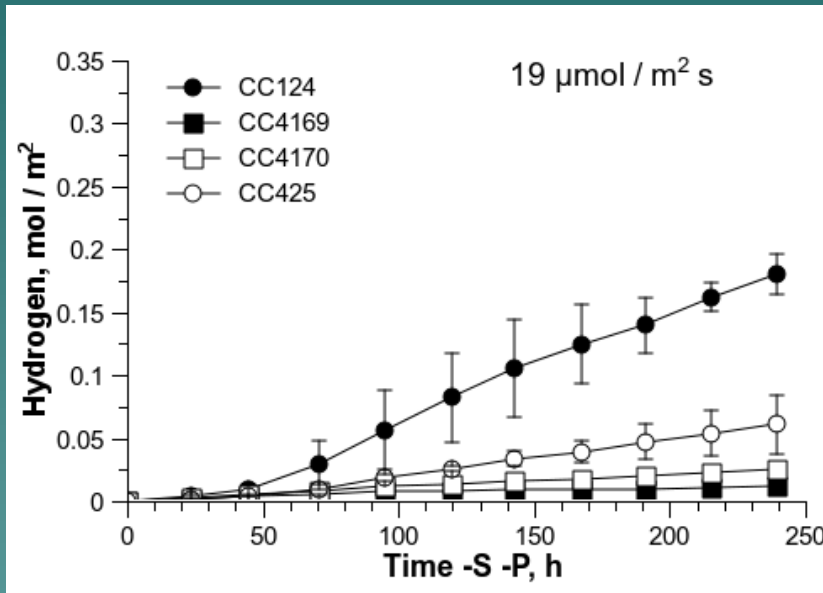
Leaky ATPase mutants (where the proton gradient is uncoupled from ATP synthesis) show higher rates and yields of H<sub>2</sub> under sulfur deprivation

# Recent results from other groups

- ◆ Many groups demonstrated that the addition of proton uncouplers can temporarily increase the rates of H<sub>2</sub> production by *C. reinhardtii*.
- ◆ Kruse and Hankamer showed that the *stm6* multi-phenotype mutant (that includes an inability to transition from linear to cyclic electron transfer), produces H<sub>2</sub> at higher rates and for longer periods of time than its parental strain.

# Issue 4. Large antennae saturate photosynthesis at low light intensity

(Kosourov and U.C. Berkeley)



Mutants with 2.5 smaller antennae show about 2X higher H<sub>2</sub> production yields at high light intensity

# Members of the Team and Collaborators

**NREL Team:** Kate Brown, Chris Chang, Alexandra Dubini, Christine English, Maria L. Ghirardi, Kwiseon Kim, Paul King, Hai Long, Murthy Narayana, Michael Seibert, Sharon Smolinski, Venkat Subramanian, Drazenka Svedruzic

## **Collaborators:**

Matthew Posewitz, Colorado School of Mines

Klaus Schulten and Jordi Cohen, Beckman Institute of the University of Illinois

Anatoly Tsygankov and Sergey Kosourov, Russian Academy of Sciences, Pushchino

Eric Johnson, Johns Hopkins Institute

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