Investigating the link between fermentation and H_2 production in *Chlamydomonas reinhardtii*

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H_2 Production Pathways in *C. reinhardtii*

When cultures of the green alga, *C. reinhardtii,* are deprived of sulphur, the rate of photosynthetic oxygen evolution drops below the rate of respiration triggering anaerobic H_2 production [1]. Electrons are fed to the hydrogenase from both PSII dependent and independent pathways, the latter proposed to be controlled by the activity of a type II NADH dehydrogenase [2,3], or a pyruvate:ferredoxin oxidoreductase [4] with reductant coming from the catabolism of starch and pyruvate [1]. We aim to investigate the fermentative pathways believed to compete with H_2 production [4] by artificial microRNA (amiRNA) knockdown [5], with a view to increasing yields. We describe here a survey of the predicted fermentative enzymes in *C. reinhardtii* and the construction and characterisation of a knock-down mutant of pyruvate formate lyase (PFL1) which is predicted to be involved in the anaerobic production of formate, and pyruvate decarboxylase 3 (PDC3) involved in ethanol formation [4].

Predicted Enzymes Involved in Pyruvate Metabolism

Enzyme		ID	Phytozome† ID	Predicted location*	Predicted size (kDa)	IUBMB
Mitochondrial pyruvate	E1α	PDC1	Au9.Cre07.g337650	C / M / C	43.7	EC 1.2.4.1
dehydrogenase complex	E1β	PDH1	Au9.Cre01.g063700	M / - / M	44.7	EC 1.2.4.1
	E2	DLA1	Au9.Cre29.g778200	C / M / M	64.3	EC 2.3.1.12
	E3	DLD1	Au9.Cre31.g780600	- / M/ M	52.5	EC 1.8.1.4
Chloroplast pyruvate	E1α	PDC2	Au9.Cre02.g099850	- / C / -	43.5	EC 1.2.4.1
dehydrogenase complex	E1β	PDH2	Au9.Cre03.g194200	M / C / M	36.3	EC 1.2.4.1
	E2	DLA2	Au9.Cre03.g158900	C / C / -	43.1	EC 2.3.1.12
Pyruvate decarboxylase		PDC3	Au9.Cre03.g165700	- / - / -	61.9	EC 4.1.1.1
Dual function alcohol/ acetaldehyde dehydrogenase		ADH1	Au9.Cre20.g758200	C / C / M	102.2	EC 1.1.1.1
Pyruvate ferredoxin oxidoreductase		PFOR	Au9.Cre11.g473950	- / - / -	128.3	EC 1.2.7.1
Pyruvate formate lyase		PFL1	Au9.Cre01.g044800	C / C / C	91.1	EC 2.3.1.54
D-Lactate dehydrogenase (2-hydroxyacid-dehydrogenase)		dLDH	Au9.Cre07.g324550	C / C / C	45.6	EC 1.1.1.28
[Fe]-hydrogenase		HYD1	Au9.Cre03.g199800	C / M / C	53.1	EC 1.12.7.2
[Fe]-hydrogenase		HYD2	Au9.Cre09.g396600	C / M / C	53.7	EC 1.12.7.2

Table 1 Bioinformatic analysis of enzymes with predicted involvement in pyruvate metabolism †JGI v4 Phytozome (http://www.phytozome.net/). *predicted location determined using TargetP/ ChloroP/ Predotar; chloroplast, C; mitochondria, M. Only enzymes for which ESTs are available are shown

Expression of Enzymes Involved in Pyruvate Metabolism Under Varying Physiological Conditions

Antibodies were raised against expressed sequence tags of putative fermentative enzymes (Table 1) and used to investigate changes in expression under different physiological conditions (Figure 1). All enzymes were expressed under sulphur-depleted conditions (TAP-S) which promote production of hydrogen.

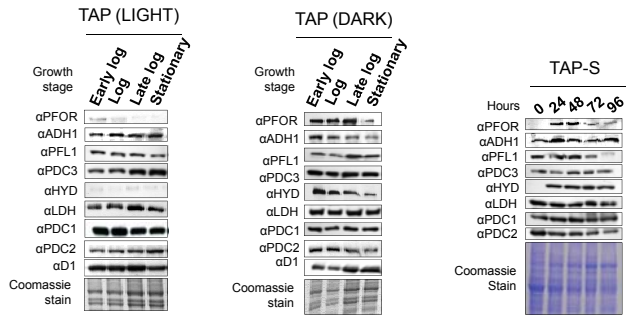

Figure 1 Immunoblot analysis of fermentative enzyme expression under different conditions. 10µl of cells at a concentration equivalent to OD_{750} = 10 were loaded.

Partitioning of Pyruvate Metabolism

Localisation of fermentative enzymes was deduced by immunoblot analysis of purified *C. reinhardtii* organelle fractions (Figure 2A). These data, plus earlier genomic [6], biochemical [4,7,8] and proteomic [9,10] results were used to construct a model of fermentative metabolism (Figure 2B)

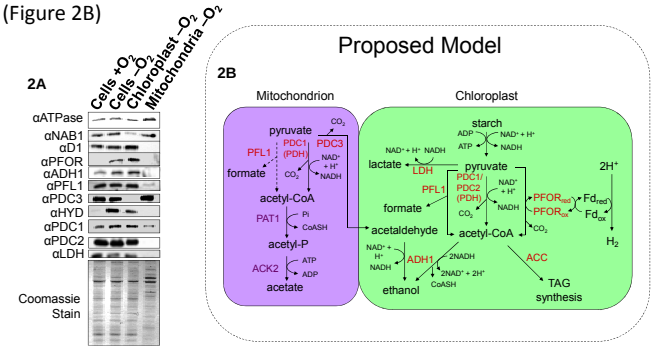

Figure 2A Sub-cellular localisation of putative *C. reinhartii* fermentative enzymes by immunoblot, ~10µg protein loaded

Figure 2B Putative model of fermentative metabolism based on immunoblot analysis and previously reported experimental data. Note presence of some of the enzymes in the cytoplasm cannot be ruled out (e.g. PDC3 and ADH1).

Effect of Pyruvate Formate Lyase (PFL1) and Pyruvate Decarboxylase (PDC3) Knockdown On H_2 Production

Artificial microRNA knockdowns [3] of PFL1 (strains PFL1-KD1 and PFL1-KD2) and PDC3 (strain PDC3-KD) were created in *C. reinhardtii* CC-124. The H_2 production ability was assessed during sulphur deprivation using 325ml bioreactors by water displacement, and gas analysed by gas chromotography.

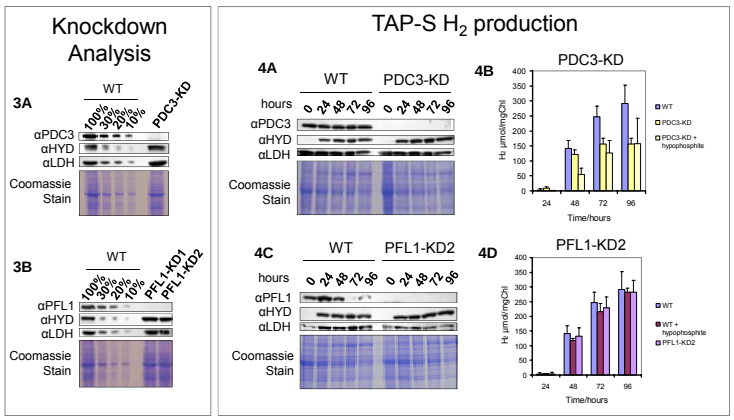

Figure 3 Immunoblot analysis of knockdown cell lines (A) PDC3 (B) PFL1

Figure 4 Analysis of fermentative enzyme expression in PDC3 and PFL1 knockdown cell lines (A and C) and hydrogen production (B and D) during sulphur depletion, with and without PFL1 inhibitor hypophosphite; error bars were calculated from three independent experiments

Conclusions

1.The fermentative enzymes analysed here are constitutively expressed in *C. reinhardtii*, with the strict requirement for anaerobic conditions limited to HYD and PFOR (Figure 1).

2. Fermentative metabolism in *C. reinhardtii* comes from the interaction of chloroplast and mitochondrial enzymatic pathways (Figure 2).

3. Artificial microRNA is useful tool for reverse genetic analysis of metabolic processes in *C. reinhardtii* (Figure 3).

4. It is likely that silencing of multiple pathways will be required to increase H_2 production in *C. reinhardtii* strain CC-124 (Figure 4).

Acknowledgements

We would like to thank Dr. Attila Molnar (University of Cambridge) for providing amiRNA vector pChlamiRNA3, and his assistance in the design of artificial micro RNAs. This work was funded by the EPSRC

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