THE D-LACTATE DEHYDROGENASE GENE OF *CHLAMYDOMONAS REINHARDTII*: Isolation of Gene Product, Transcript Knock-Down, and Transcriptional Regulation

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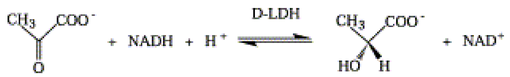
Background

• ***Chlamydomonas reinhardtii*** **is a single cell green alga which can use photosynthesis and respiration to provide energy.**

• **When *Chlamydomonas* cells are grown under anaerobic conditions, they generate energy through anaerobic metabolism.**

• **Lactate Dehydrogenase (LDH) catalyzes the interconversion of pyruvate to lactate and is essential for anaerobic metabolism.**



• **While L-lactate and D-lactate are stereoisomers, the D-LDH and L-LDH enzymes are structurally distinct and have evolved independently.**

• ***Chlamydomonas*** **cells have several different LDH enzymes, but the predominant one involved in anaerobic metabolism is a D-LDH.**

• **The *Chlamydomonas* D-LDH coding sequence was isolated by reverse transcriptase PCR from total cell RNA using gene specific primers. This sequence was ligated into plasmid pGEX 6P-1 and introduced into the protease deficient *E. coli* strain BL-21. The recombinant plasmid encodes a glutathione S-transferase (GST)/D-LDH fusion protein in which the two protein sequences are separated by a PreScission protease cleavage site.**

Experimental Goals

• **Isolate recombinantly expressed *Chlamydomonas* D-LDH protein from BL-21 cells**

GST pull-down experiments followed by PreScission protease cleavage will be used to purify the protein.

The isolated D-LDH protein will be used for structural analyses and for the generation of protein-specific antisera.

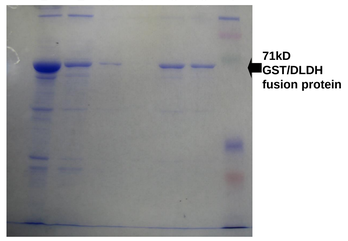
• **Knock-down the expression of the D-LDH protein using an RNAi approach to test the hypothesis that the D-LDH gene is essential for growth under anaerobic conditions**

D-LDH sequences will be cloned into an effective RNAi vector and transformed into *Chlamydomonas* cells.

D-LDH mRNA levels will be measured by qRT-PCR to confirm knock-down.

D-LDH knock-down strains will be tested for their ability to grow under anaerobic conditions.

SDS-PAGE of GST/D-LDH Fusion Protein Purification



1: total cell lysate, 2: flow through, 3: wash 1, 4: wash 2, 5: eluate 1, 6: eluate 2

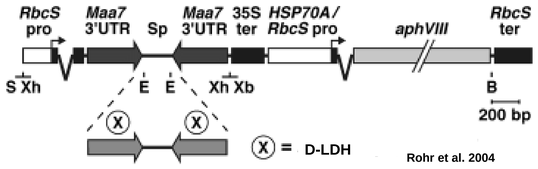
Recombinantly expressed GST/D-LDH fusion protein was successfully isolated from *E. coli*

D-LDH Knock-Down Strategy

• **D-LDH sequences will be cloned in sense and anti-sense orientation into the *Maa7* inverted repeat transgene**

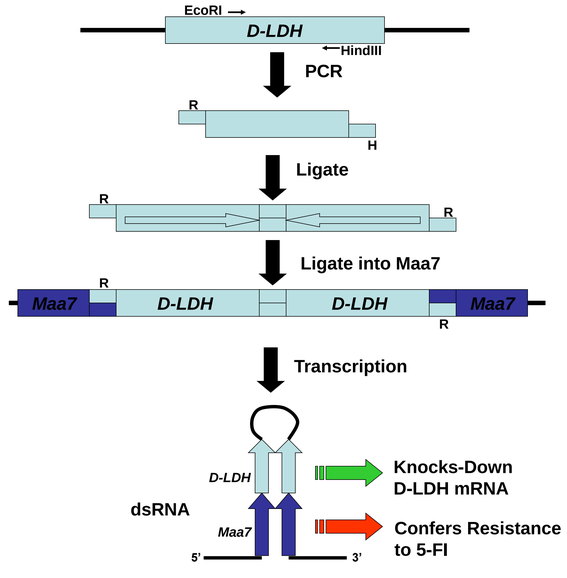
• **The tandem inverted repeat of the aminoglycoside 3'-phosphotransferase gene (*Maa7*) confers 5-FI resistance**

• ***aphVIII*** **confers resistance to paromycin**



Rohr et al. 2004

Cloning Strategy



Background (Part 2)

• **D-LDH catalyzes the interconversion of pyruvate to lactate and is essential for anaerobic metabolism.**

• **Hypothesis 1: Wild type *Chlamydomonas* cells grown under anaerobic conditions will up-regulate the production of D-LDH mRNA compared to cells grown in aerobic conditions.**

• **Sulfur deprivation of *Chlamydomonas* cells causes inhibition of photosynthesis. As a result, the rate of O_2 production by photosynthesis drops below that of O_2 consumption by respiration. *Chlamydomonas* cells grown in a sealed chamber in medium without sulfur generate an anaerobic state after 48 hours.**

• **Hypothesis 2: *Chlamydomonas* cells grown in medium without sulfur will up-regulate D-LDH mRNA compared to cells grown in an open chamber.**

qRT-PCR

• **Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) currently is the most sensitive technique for measuring mRNA levels.**

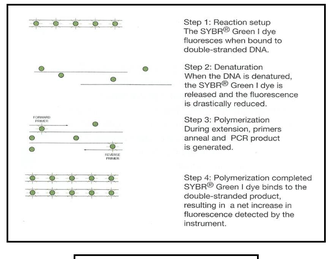
• **SYBR Green binds dsDNA and emits fluorescence upon excitation.**

• **The addition of SYBR Green to a PCR reaction allows for the quantification of DNA product after each reaction cycle. The number of cycles of replication needed to cross a threshold is measured and recorded – this number is the CT value.**

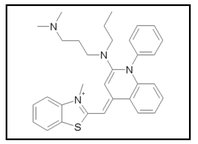
• **The CT values for a transcript of interest (D-LDH) are normalized with respect to house-keeping gene transcripts (β-tubulin and RUBISCO) for each sample – these are the ΔCT values.**

• **A high ΔCT value indicates a low amount of starting mRNA since it takes more cycles to reach the threshold, while a low ΔCT value indicates a higher amount of starting mRNA since it takes less cycles to reach the threshold.**

qRT-PCR with SYBR Green

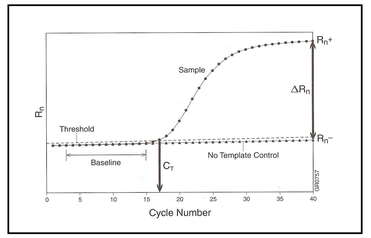


Structure of SYBR Green



From: Applied Biosystems Absolute Quantification (2004)

Representative qRT-PCR Amplification Plot



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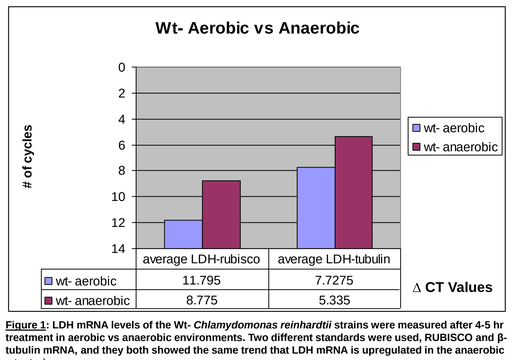

Figure 1: LDH mRNA levels of the Wt- *Chlamydomonas reinhardtii* strains were measured after 4-5 hr treatment in aerobic vs anaerobic environments. Two different standards were used, RUBISCO and β-tubulin mRNA, and they both showed the same trend that LDH mRNA is upregulated in the anaerobic wt- strain.

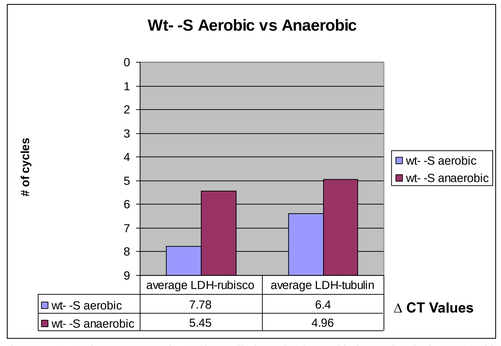

Figure 2: Wt- strains were grown in –Sulfur media for 48 hrs in aerobic (open chamber) vs anaerobic (closed chamber) conditions. The same trend that was seen when growing the strains with normal media was seen; LDH mRNA is upregulated under anaerobic conditions compared to aerobic conditions.

Conclusions

• **D-LDH mRNA is up-regulated 3 – 8 fold in anaerobic conditions compared to aerobic conditions.**

• **Preliminary results suggest that D-LDH mRNA levels are *constitutively* up-regulated in mitochondrial mutants with defective electron transfer.**

• **Future studies will involve measuring D-LDH protein levels by immunoblot analysis to determine if an increase in D-LDH mRNA levels corresponds to an increase in D-LDH protein levels.**

• **The *cis* (D-LDH promoter) and *trans*-acting factors (proteins that bind to these sequences) will be characterized to understand the basis of D-LDH transcriptional regulation.**