Genetic Engineering of *Escherichia coli* to Enhance Biological Hydrogen Production from Biomass-derived sugars

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Research Goals and Objectives

Specific Aims:

- Genetically engineer an *E. coli* strain that will increase H_2 production by increasing the protein components of formate hydrogenase lyase enzyme complex (FHL).

- Determine the increase in formate dehydrogenase levels (FDH-H), H_2 production, and FHL activity in the engineered strains.

Bacterial Hydrogenases – Catalyst for Biohydrogen production

- Bacteria can reduce protons using several electron donors (formate, ferredoxins) to catalytically produce H_2 – via enzymes known as Hydrogenases

- Two major classes of Hydrogenases – (1) Ni-Fe (which includes Ni-Fe-Se) and (2) Fe only

- One of the best studied (model) hydrogenase systems is Hydrogenase 3 from *E. coli*, part of a multi-enzyme membrane associated complex called the formate hydrogenlyase (FHL)

Formate Hydrogenlyase enzyme complex

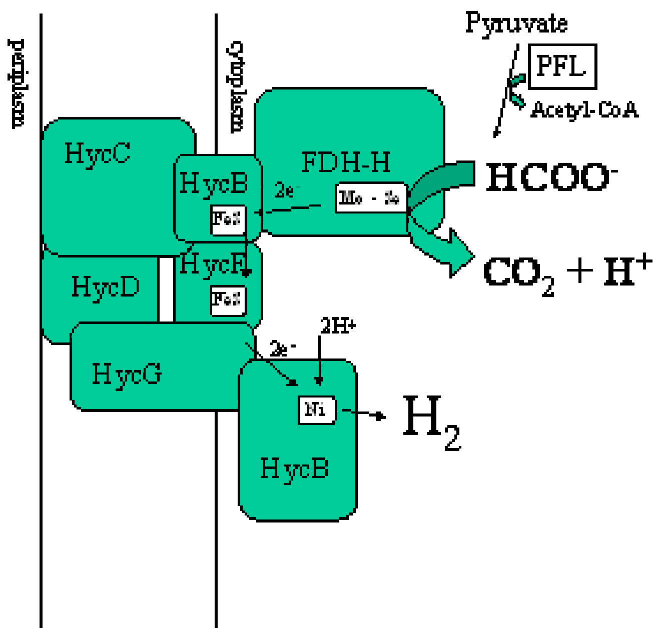


Figure 1. Schematic of FHL complex of *E. coli*

FHL complex consists of a selenoenzyme formate dehydrogenase (FDH-H), electron carrier proteins (Hyc) and hydrogenase 3 (Hyc). Formate (HCOOH) is the electron donor and also the intermediate metabolite that induces expression of the genes encoding FHL complex.

FhlA is a transcriptional activator of the genes encoding all of these components. Previous work identified a mutant FhlA protein (FhlA165) which resulted in unregulated and elevated expression of genes encoding FHL components. In cells containing FhlA165 one would expect higher levels of FHL complex, and thus higher flux of carbon through the FHL system (resulting in higher Hydrogen-Glucose ratio)

Selenoenzyme Biosynthesis

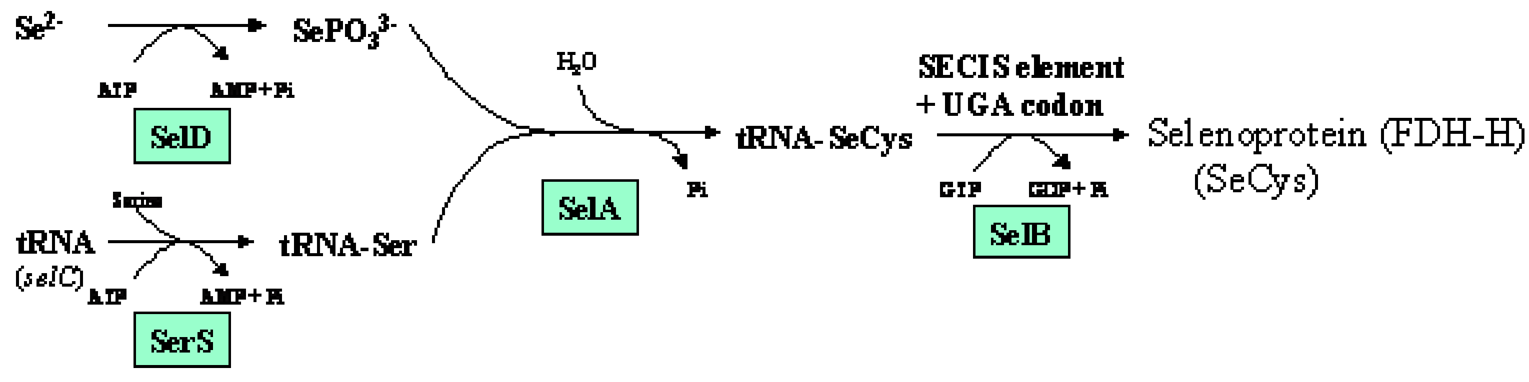
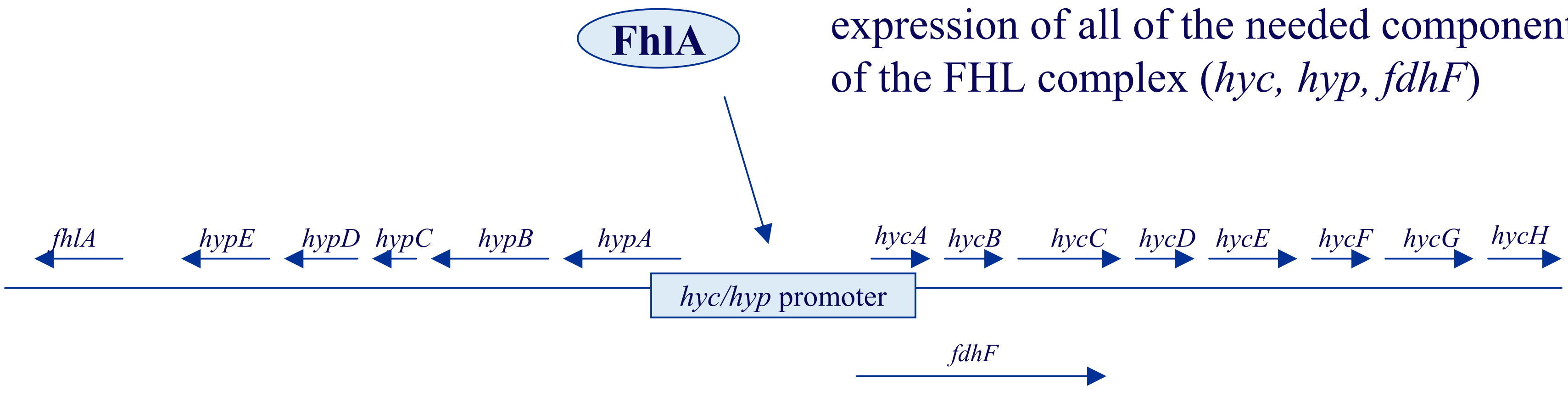


Figure 2. Overall pathway for insertion of selenium into selenoenzymes in *E. coli*

Selenoprotein synthesis could be limiting to FHL complex formation since the components of the selenocysteine insertion is only in low abundance in the cell. Hypothesis to be tested relates to the need for these components for optimal FHL complex formation, and thus optimal engineering of *E. coli* to produce hydrogen.

Regulation of Formate Hydrogenlyase

FhlA is the global regulator that controls expression of all of the needed components of the FHL complex (*hyc, hyp, fdhF*)



hyc **– Hydrogenase 3 and electron carrier proteins**
hyp **– Hydrogenase maturation (Ni, CO, CN insertion)**
fdhF **– formate dehydrogenase (selenoenzyme)**

FhlA165 is a **mutant** version of FhlA which activates expression of *hyp*, *hyc* and *fdhF* genes to a higher level (about 3-fold higher expression of the mRNA levels [1])

[1] Self et al (2000) FEMS Microbiol Lett. 2000 Mar 1;184(1):47-52.

Hypothesis

- Increasing the level of *selABC* genes (needed for selenoprotein synthesis) will increase the cell's ability to make active FHL complex by increasing the level of FDH-H (selenoenzyme)
- Combining a plasmid carrying FhlA165 (highly active mutant FhlA activator) with pSUABC (*selABC*+) will enable optimal expression and activity of FHL activity
- This combination should provide the highest possible hydrogen production potential of the model organism *E. coli*

Relevance to Current State-of-the-Art

- Biological production of hydrogen represents potential use of biomass as a source of hydrogen
- Harnessing reducing potential in this biomass can be achieved in a more efficient manner using defined biological catalysts (versus mixed culture systems)
- Limitations of biohydrogen production – still a serious issue in biohydrogen production, and one that is directly addressed in this study

Relevance to NASA

- Hydrogen is and will be used as a fuel for launch and for long term exploration of space (e.g. to supply energy for fuel cells on moon and Mars bases)
- Development of these technologies benefits not only space program, but energy research in general
- Potential renewable sources of hydrogen can be achieved in long term space exploration from biomass-derived hydrogen

Budget, Schedule and Deliverables

- Budget: Currently on budget ($95,000 total cost)

  Total expenditures - $74,000

  Remaining expenses and salary costs - $12,750

  (Indirect costs - $8,270)

- Schedule and Deliverables:

  One deliverable: Two plasmid system to increase FHL activity (and thus hydrogen production) in an *E. coli* model. Genetic Engineering is complete; Testing of hydrogen production in process (nearly complete), and thus on schedule with respect to studies proposed for this fiscal year.

Anticipated Technology End Use

- Engineered *Escherichia coli* strains can be used to couple the fermentation of biomass to production of hydrogen
- Currently strains are being developed with enhanced capabilities to utilize cellulosic biomass at the University of Florida (collaborators), and these strains can be tested for their potential use to increase hydrogen production using available feedstocks
- Optimization of hydrogen production needed prior to testing in biomass fermentation systems – to start with an optimal catalyst

Accomplishments and Results

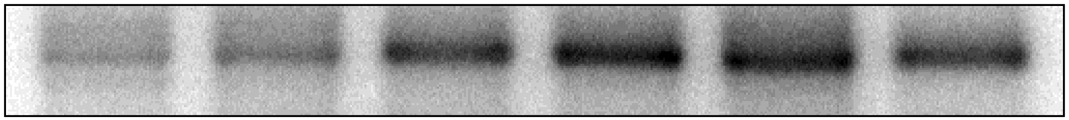
- **Genetic Engineering:**

  pDG1: Plasmid constructed which carries *fhlA165* under its own promoter in medium copy (20-30 copies per cell)

  pDG2: Plasmid constructed which carries *fhlA165* under its own promoter in high copy (50 or more copies per cell)

  Both are compatible with pSUABC, which carries *selABC* genes in low copy (pACYC184 derivative)

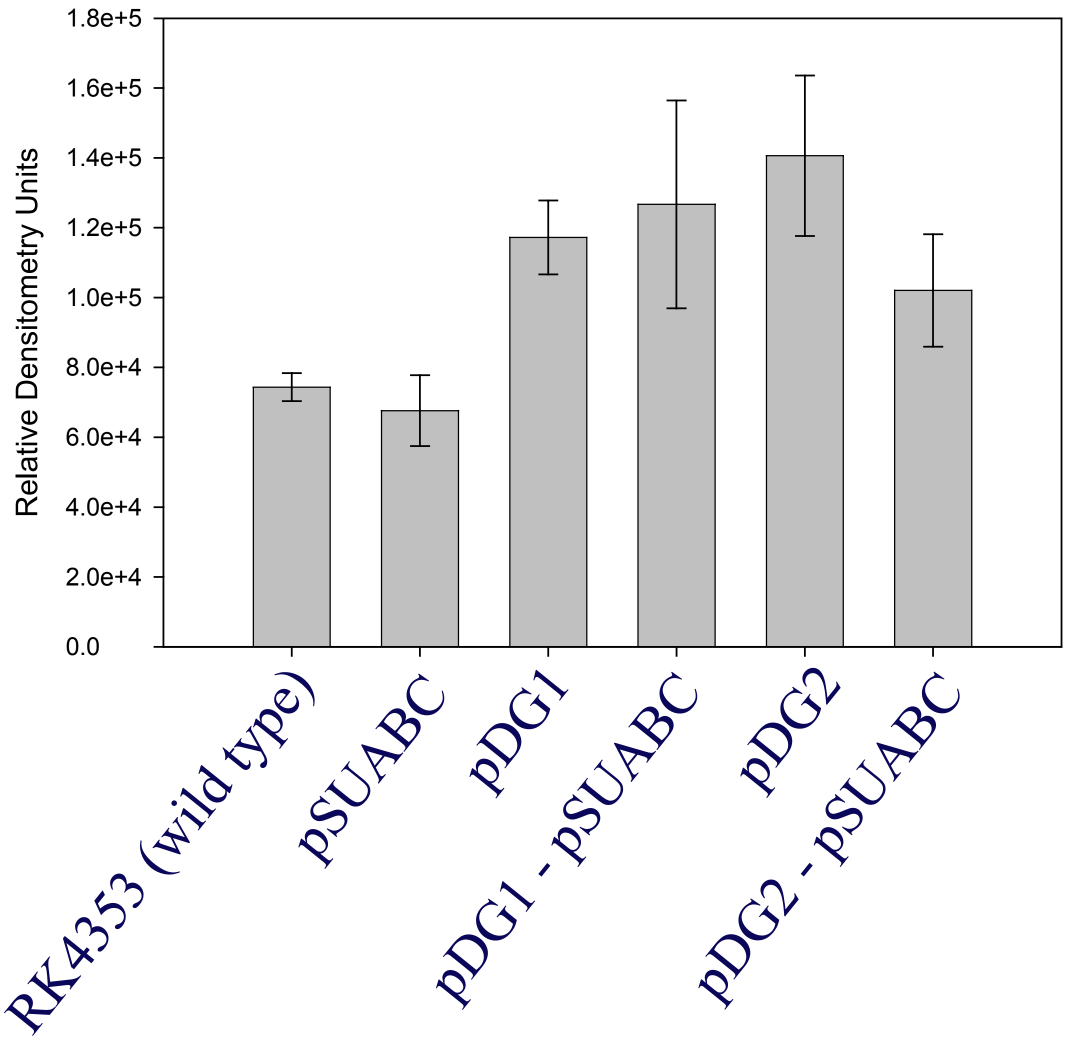
- **Selenoenzyme FDH analysis:**



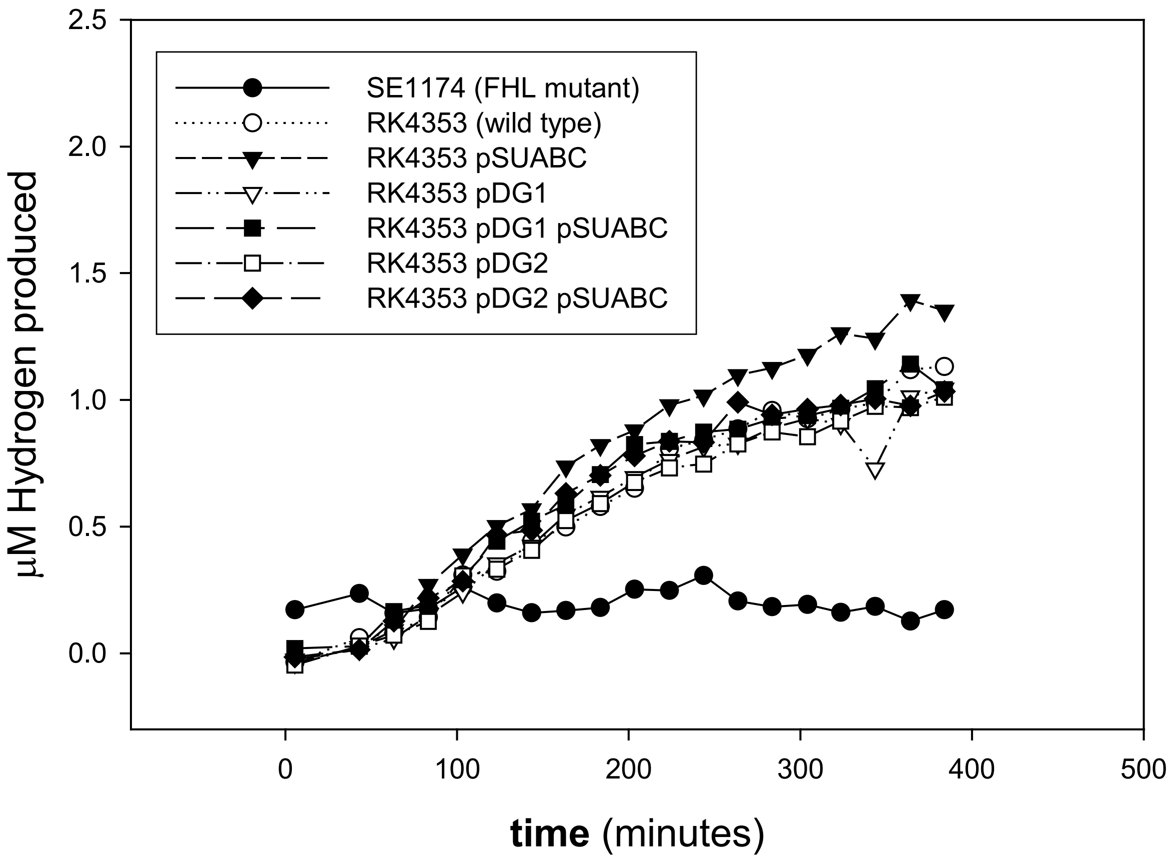
SDS-PAGE
^{75}Se-labeled proteins

- Presence of FhlA165 mutant activator results in significantly higher levels of selenoenzyme formate dehydrogenase (FDH)

- Presence of pSUABC (*selABC*+) has little effect on FDH levels (not expected)

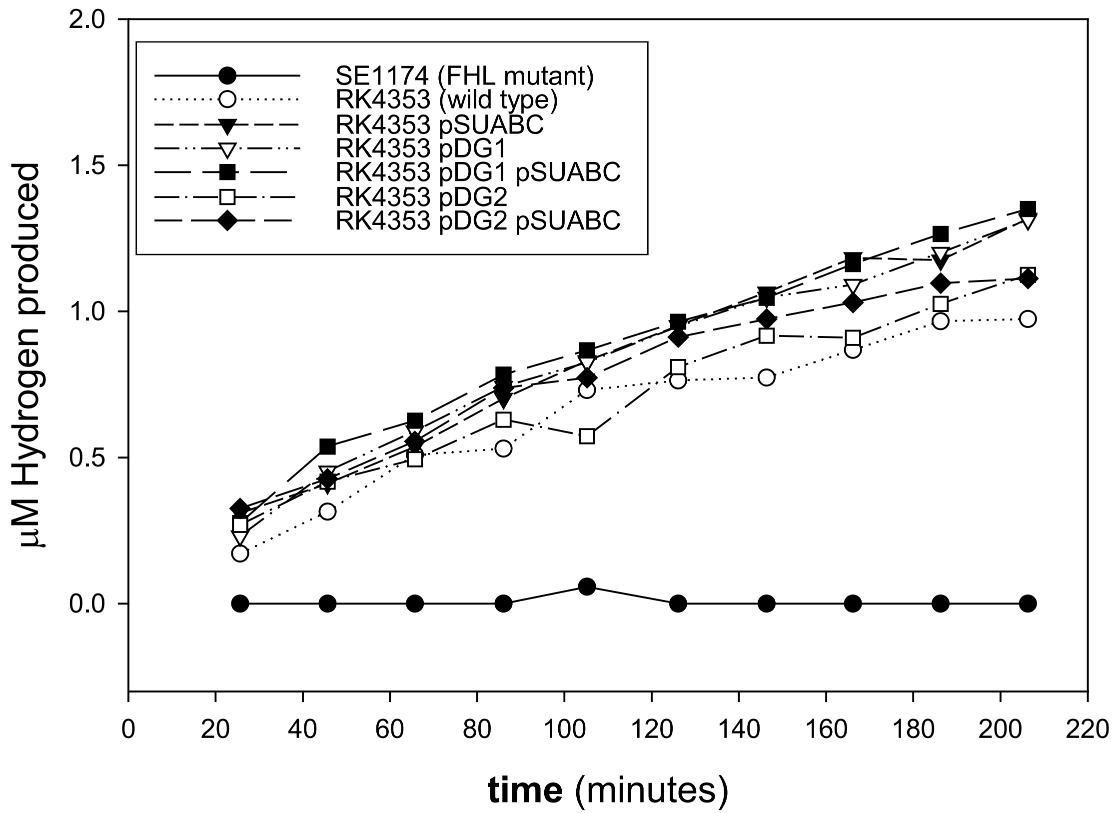

1.8e+5
1.6e+5
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
Relative Densitometry Units
RK4353 (wild type)
pSUABC
pDG1
pDG1 - pSUABC
pDG2
pDG2 - pSUABC

- **FHL activity analysis – room temperature**



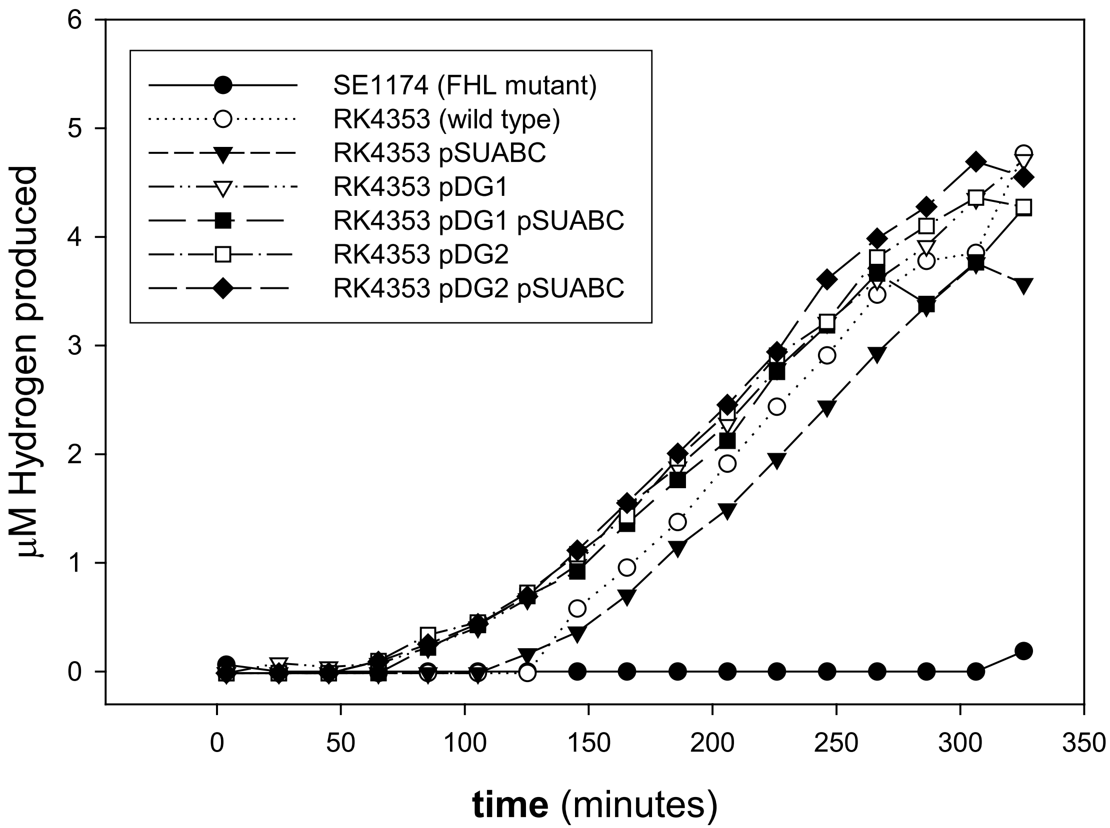
- Only pSUABC containing strain produced more active FHL complex in standard FHL activity assay
- Contradictory to results of labeled selenoenzyme analysis

• FHL activity analysis – 37 degrees



• When carried out at 37 degrees, FHL activities match well with selenoprotein labeling, and indicate a signficant increase in hydrogen production versus wild type strain

Fermentation conditions (more applicable to industrial setting)



- FhlA165 expressing strains produce more hydrogen in the initial growth period
- Correlates better to analysis of FDH enzyme levels by selenium radioisotope

Summary of Results to date

- FhlA165 expression (mid-level or high level) results in increases in the level of the selenoenzyme formate dehydrogenase (electron donor for hydrogenase 3 isoenzyme)
- Presence of *selABC* in multiple copies (via pSUABC) has little effect on FDH levels, but somehow enhances the level of hydrogen produced by FHL complex in standard assay
- Presence of FhlA165 results in significantly higher production of hydrogen in a batch fermentation growth experiment – more applicable to industrial or applied biohydrogen fermentation

Future Plans

1.) Continue optimizing expression of FHL and applying two plasmid system to engineered *E. coli* strains (e.g. KO11) that utilize cellulosic biomass as a carbon source

2.) In addition, we are investigating a simpler and (potentially) more efficient enzyme catalyst – Fe-hydrogenase from *Clostridium sp. (C. perfringens* and *C. acetobutylicum*)

3.) Goal: Achieve high-level expression of active ferredoxin/Fe-hydrogenase from *C. perfringens* in *E. coli.* Currently both *hydA* and *fer* genes already cloned into pDuet expression vector and are being tested for Hyd activity in the presence of accessory genes (*hydEFG*) expressed from separate plasmid (pPMD25 – cloned by K. T. Shanmugam's laboratory)

Future Plans (continued)

4.) In collaboration with K. T. Shanmugam (UF) we are expressing ferredoxin and Fe-hydrogenase (*C. acetobutylicum*) in *E. coli* to purify and demonstrate direct oxidation of NADH coupled to hydrogen formation using a cloned NADH dehydrogenase that can reduce ferredoxin

Acknowledgements:

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