Emergence of Renewable Biohydrogen: Cell Line Engineering of *Thermotoga maritima*.

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Abstract: The anaerobic hyperthermophilic bacterium, *Thermotoga maritima (Tma),* ferments carbohydrates to form maximum possible molecular hydrogen (H_2) as one of its by-products which makes it a candidate for consolidated bioprocessing. To investigate the metabolic carbon flux associated with hydrogen synthesis, a genetic system in *Tma* was developed. The first genetic marker, a drug resistant, allele of the gyrase subunit GyrB (*gyrB*) was selected because of the dominance when placed in trans to a drug resistant copy and higher temperature stability of antibiotic, novobiocin, used to isolate mutants. Based on DNA sequence analysis of a collection of independent spontaneous novobiocin resistance mutations in *gyrB*, an artificial *gyrB* allele was constructed possessing a G to A transition mutation at nt 401 and synonymous codon changes at third codon positions (nt399,405). Recombinants arising from transformation with a plasmid encoded codon optimized *gyrB* allele were recovered on drug plates combined with discriminatory allele specific PCR screening promoting amplification of only the recombinants allele precluding the wild type template. To apply the end product metabolic engineering in *Tma*, the genetic system was used to inactivate the *Tma* L- lactate dehydrogenase (*ldh)* by cloning a terminally-truncated internal fragment of *ldh* in a suicide plasmid together with the *groESp*::*kan* marker. In resultant *ldh* knockout mutant the presence of four predicted amplicons, including a unique fusion joints between 5' and 3' ends of the disrupted *ldh* joint along with the selectable genetic markers for *Tma* and Eco (*amp*), were confirmed by PCR. The impact of *ldh* inactivation on H_2 formation was then studied in batch culture using gas chromatography. Loss of lactate dehdyrogenase resulted in an apparent increase in H_2 relative to the wild type of over 30% under normal growth conditions. Presumably this shift reflects a metabolic adaptation to maintain redox homeostasis in response to the loss of the ability to excrete excess reductant in the form of an organic acid.

2 Figure 1 DNA Sequence of WT *gyr*B and novobiocin resistant *gyrB* mutant

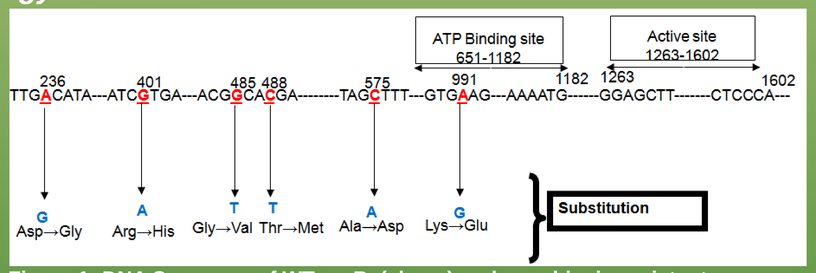

Figure 1: DNA Sequence of WT *gyr*B (above) and novobiocin resistant *gyr*B mutant (below)

3 Figure 2. Homologous recombination at *gyrB* locus.

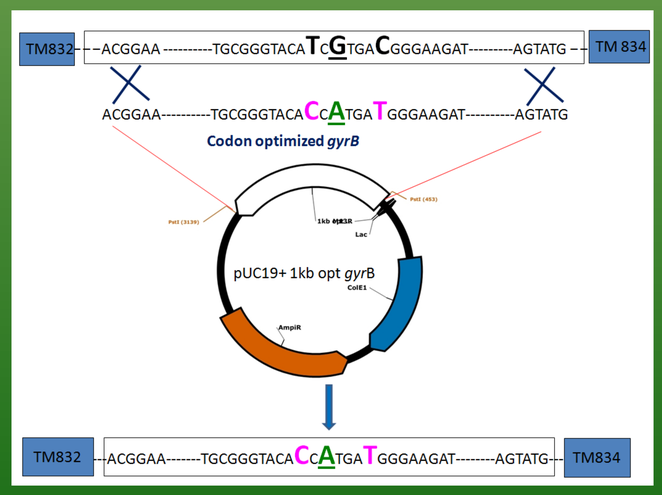

Figure 2: A schematic of crossover events through homologous recombination in *T. maritima* at *gyrB* locus with a codon-tagged *gyr*B allele.

4 Figure 3. Allele Specific PCR amplification of codon optimized *gyrB*

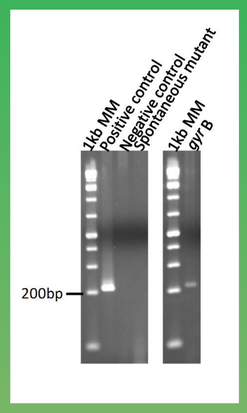

Figure 3:A gel picture representing the presence of codon optimized *gyrB* genes *in T. maritima*

5 Figure 4. Promoter fusion construct

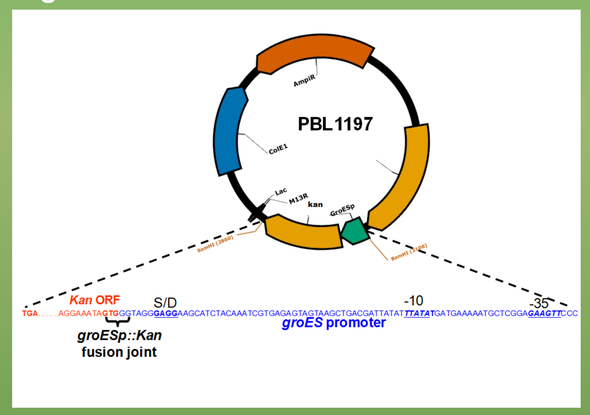

Figure 4: Schematic of promoter (*groESp*) fusion with thermostable kanamycin nucleotidyl transferase

6 Figure 5. Carbon metabolism in *T. maritima* and redox homeostasis

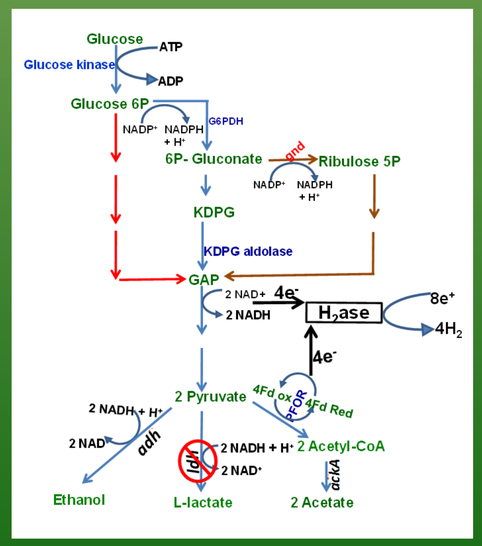

Figure 5: Schematic of carbon flow and disruption of lactate dehydrogenase *(ldh)*

7 Figure 6. Disruption of L- *lactate dehydrogenase* of *T. maritima*

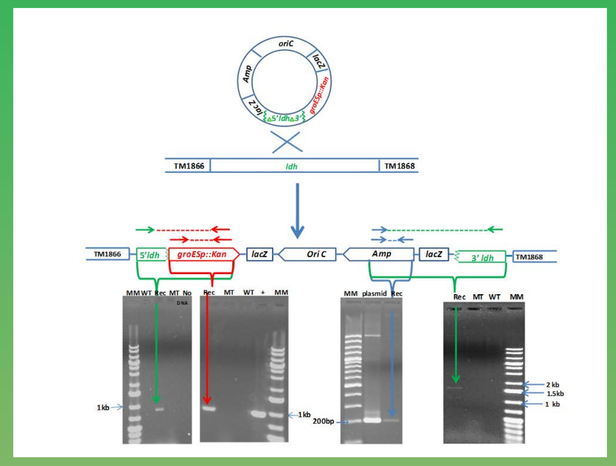

Figure 6:Targeted disruption of lactate dehydrogenase *(ldh)* via homologous recombination. PCR detection of four amplicons including 5' and 3' *ldh*-marker joints, *Tma* marker (*groESp*::*kan*) and *Eco* marker (amp)

8 Figure 7. Hydrogen synthesis in *ldh* mutant

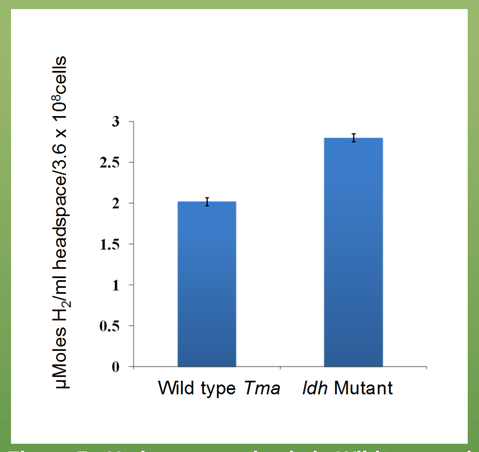

Figure 7: Hydrogen synthesis in Wild type and *ldh* mutant

9 Conclusion

The *gyrB* genetic marker was used to perform allele replacement in *T. maritima* through homologous recombination. Selection of *ldh* (L-lactate dehydrogenase) was based on the predicted importance of this enzyme in redox homeostasis and H_2 synthesis. Further use of the *Tma* genetic system to conduct metabolic engineering will aid in an improved understanding of Hydrogen Biogenesis.

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