Pathways and Regulatory Network of Hydrogen Production from Cellulose by *Clostridium thermocellum*

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Clostridium thermocellum, a thermophilic, ethanogenic, and cellulolytic anaerobe, produces a complex extracellular cellulolytic organelle called the cellulosome. The cellulosome contains various depolymerizing enzymes that are arrayed on a protein scaffold and effectively degrades complex cellulosic substrates. During cellulose fermentation, the bacterium evolves hydrogen at a high rate. Analysis of its genome sequence reveals the existence of at least three putative hydrogenases (CtHydA1, CtHydA2 and CtHydA3) central to hydrogen metabolism. Furthermore, at least 20 genes are potentially related to hydrogen metabolism. The bacterium is thus remarkably versatile in employing various enzymes, some of which are potentially novel, for hydrogen metabolism. The versatility indicates the significance of this biological process. Yet little is known concerning the pathway for hydrogen production and the underlying regulatory mechanism/network that control these hydrogenase and the related genes as well as cellulolytic process and other metabolic pathways in the organism.

Due to the novelty of CtHydA3, it was selected for initial study. Genes encoding CtHydA3 (Ct_3003), a ferredoxin-like protein (Ct_3004), and three FeFe-hydrogenase maturation proteins (CtHydE, CtHydF, and CtHydG) have been cloned into three plasmids and co-transformed into *E. coli* strain Rosetta (DE3) and BL21 (DE3) for heterologous expression. In addition, a 6X His-tag sequence was fused to either the C- or N-terminus of CtHydA3. Protein immunoblots confirmed the expression of both the C- and N-terminus His-tagged CtHydA3 (73 kDa band) in *E. coli* Rosetta, but not in *E. coli* BL21, likely due to differences in codon usage between *C. thermocellum* and *E. coli*. The recombinant protein was affinity-purified with *in vitro* activity linking to reduced methyl viologen. However, the low yield of the purified protein prevented its biochemical characterization. The *E. coli* Rosetta strain also displayed a high background hydrogenase activity. We have since initiated transformation using an *E. coli* FTD strain (DE3) lacking all background hydrogenase activity with the intent to determine cofactors requirements and directionality (H_2 production or H_2 uptake) of CtHydA3 in *E. coli* cell-free extract. The outcome will reveal the physiological functions of CtHydA3 during cellulose metabolism.

To identify transcription factors controlling metabolic pathways, we developed an affinity purification method by immobilizing promoter DNA sequences to a solid support. DNA-binding proteins from the *C. thermocellum* cell lysate, obtained by growing on cellobiose or crystalline cellulose and eluted from the affinity columns, were identified by the MALDI-TOF or LC-MS-MS techniques. We thus identified a REX-like protein that regulates the expression of a hydrogenase gene and many other genes, indicating that it is a global regulator. It is likely the first global regulator experimentally verified in this bacterium. We also carried out the proteomic analysis and identified the cellulosomal proteins and non-cellulosomal glycosyl hydrolases that are up- or down-regulated when the cells were grown on the cellulose substrate vs. cellobiose.

The studies will provide important insights into the pathway and regulatory mechanism/network controlling hydrogen metabolism and cellulolysis as well as other pertinent metabolic pathways in this very intriguing cellulolytic and thermophilic bacterium, which catalyzes the rate-limiting cellulose-degradation reaction in a single-step process of biomass conversion (or CBP, Consolidated Bioprocessing). Detailed understanding of the pathway and regulatory

mechanism/network will ultimately provide rationales for engineering, alternating, or deregulating the organism for biomass conversion to liquid and hydrogen fuels.