Report on Site-Directed Mutagenesis and Protein Expression of TmHydB Variants.

Thermotoga maritima [FeFe] hydrogenase (*TmHydABC*) is a key enzyme involved in hydrogen production in nature, functioning to catalyze the oxidation of NADH and ferredoxin and production of hydrogen gas as a waste product. Understanding the structural and functional aspects of *Tm*HydABC could provide insights into its biocatalytic activity, enabling its potential use in biohydrogen production and other biotechnological applications. Furthermore, the enzyme uses electron bifurcation, a complicated mechanism of energy coupling in nature. The understanding of electron bifurcation will have far-reaching consequences for understanding microbial metabolism and developing new energy conversion technologies. *Tm*HydABC is composed of three subunits (HydA, B, and C), where the HydA subunit contains the hydrogenase active site, the HydB subunit contains the sites of NADH and ferredoxin oxidation, and HydC contains a single [2Fe-2S] cluster, whose function is still unclear. The aim of this project was to explore the effects of specific site-directed mutations in the *Tm*HydB subunit on its function, using molecular biology techniques such as polymerase chain reaction (PCR), transformation, and protein expression.

PCR amplification was used to introduce specific point mutations into the *hydB* gene. The primers designed for these reactions were tailored to replace key residues, such as cysteines, with alanine, serine, and glycine in order to assess their roles in *Tm*HydB function. After amplification, the PCR products were treated with **DpnI** to degrade the methylated parental DNA template, leaving only the newly synthesized, mutated DNA. The presence of PCR products was confirmed by gel electrophoresis, and bands of the expected size were excised for gel extraction to purify the mutant *hydB* genes. The purified PCR products were then ligated and transformed into chemically competent E. coli TOP10 cells. After thawing, the cells were transformed with the ligation product, incubated on ice, heat-shocked to facilitate plasmid uptake, recovered in LB medium, and plated on LB-agar with ampicillin to select for successful transformants. The plates were incubated overnight at 37°C, and colony formation was observed the following day.

Single colonies from each plate were picked and used to inoculate 10 mL of LB broth with 100 µg/mL ampicillin and incubated overnight at 37°C in a shaking incubator. Cells were then harvested, plasmid DNA was prepared, and sent for sequencing. BL21(DE3)*ΔiscR* cells were transformed with the purified, sequenced mutant plasmid DNA to express the *Tm*HydB protein. This strain is highly suitable for recombinant protein expression, particularly iron-sulfur proteins, due to the iscR deletion, which upregulates iron-sulfur cluster synthesis. Antibiotic selection included both 50 µg/mL kanamycin and 100 µg/mL ampicillin. Plates with these transformed cells were incubated overnight to allow colony formation.

Single colonies of BL21(DE3)*ΔiscR* transformants were picked for overnight cultures, which were in turn used for 500 mL cultures in LB + 10 g/L K2HPO4, 10 g/L Na2HPO4, 0.5% glucose, 2 mM ferric ammonium citrate, 50 µg/mL kanamycin, and 100 µg/mL ampicillin. These cultures were grown for approximately 3 hours in a shaking incubator at 37°C to reach mid-log phase (optical density of 0.5). Then the cultures were transferred to bottles, 2 mM L-cysteine was added, expression was induced with 200 µg/L anhydrotetracycline, and the bottles were sealed and incubated at 25°C overnight. The cells were harvested by

centrifugation at 4000 rpm for 10 minutes at 4°C, and the pellets were resuspended in 25 mL of 100 mM Tris-HCl, 150 mM NaCl, pH 8 (lysis buffer). Cells were broken using an Emulsiflex, followed by centrifugation at 20,000 rpm for 30 min at 4°C. The supernatant was applied to a 10 mL streptactin affinity column, washed with 3 column volumes of lysis buffer, and protein eluted with 10 mL of lysis buffer containing 50 mM biotin. HydB was concentrated using centrifugal concentrators (30 kDa molecular weight cut-off) and protein was stored at -80°C.

Both TOP10 and BL21(DE3)*ΔiscR* cultures exhibited normal growth patterns in LB broth, reaching mid-log phase after approximately 3 hours of incubation. This observation indicated that the mutations introduced into the *TmHydB* gene did not negatively affect cell growth or protein expression at this stage. Sequencing of the plasmid DNA confirmed the successful introduction of the desired point mutations into the *TmHydB* gene. All eight mutants (C560A, C560S, C555A, H555C, C565A, C565S, C468A, and G468S) were verified by comparing the sequences to the wild-type gene. The alignment showed the specific mutations at the expected sites with no other alterations to the coding sequence. The sequencing data confirmed that each mutation was introduced correctly, allowing further studies into how these changes impact the activity and stability of *TmHydB*. The mutations targeted conserved cysteine residues, which are likely involved in coordinating metal ions or forming disulfide bonds critical for enzyme function. The impact of these mutations on hydrogenase activity will be assessed in subsequent experiments using biochemical assays.

The results obtained so far indicate that the site-directed mutagenesis and transformation procedures were successful, with all intended mutations introduced into the *hydB* gene. The colonies grown on selective media and the confirmation by sequencing suggest that the mutations did not disrupt the ability of E. coli to replicate the plasmid or express the protein. Future steps will involve expressing the mutated *Tm*HydB proteins in BL21(DE3)*ΔiscR* cells under controlled conditions and purifying the proteins for activity assays. These assays will determine whether the introduced mutations affect the enzyme's catalytic activity, stability, or interaction with metal cofactors. The successful transformation, colony growth, and verification of site-directed mutations in *Tm*HydB set the stage for further functional analysis of these mutants. These experiments will contribute to a deeper understanding of the role of specific cysteine residues and other key amino acids in the catalytic mechanism of *Tm*HydB. Through this work, we aim to elucidate how alterations to the enzyme's active site affect its hydrogen metabolism, which could have important implications for the development of biohydrogen production technologies.