Dynamic Remodeling of Protein Acetylation in Fuel-Producing *E. coli* with Different Carbon Sources

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Project Goals: The goal of this project is to determine how protein acetylation affects metabolism in engineered microorganisms. Lysine acetylation is a common post-translational modification that eukaryotes, archaea, and bacteria employ to regulate protein activity. Multiple studies have recently shown that lysine acetylation predominantly targets metabolic enzymes – in fact, most metabolic enzymes are subject to lysine acetylation. We hypothesize that bacteria employ lysine acetylation as a global mechanism to regulate metabolism in response to their energy and redox status. Our previous work suggests that lysine acetylation may be an attractive and innovative target for metabolic engineering. We are investigating how lysine acetylation affects fuel production in engineered microorganisms. The significance of this work is that it will address a fundamental gap in our understanding of bacterial metabolism and identify new approaches for overcoming the problems associated with the production of advanced biofuels.

Recently, we published a study reporting that glucose-regulated lysine acetylation (Kac) was predominant in central metabolic pathways and overlapped with acetyl phosphate-regulated acetylation sites (1). We proposed that acetyl phosphate-dependent acetylation across hundreds of proteins is a response to carbon flux that can regulate central metabolism. Here, we extend our investigations to examine the effect on both protein expression levels and the alterations in acetylation status across the *E. coli* proteome when it is provided with alternative carbon sources beyond glucose.

For these experiments, *E. coli* K-12 wild-type strain BW25113 was grown in M9 media, a minimal growth media supplemented with either 0.4% or 4% glucose (Glc) or xylose (Xyl). Four independent biological replicates were performed for each of the four conditions and a total protein fraction was subjected to label-free quantitation both before and after immuno-affinity enrichment of peptides containing acetyllysine modifications. Anti-acetyllysine Western blot analysis revealed a proteome that became more acetylated with higher concentrations of glucose or xylose, respectively. To obtain a more precise understanding of glucose- and xylose-induced changes in protein acetylation, as well as in protein expression, we examined protein fractions that had undergone trypsin digestion both before and after antibody-based affinity-enrichment of acetyllysine-containing peptides by quantitative mass spectrometry. To monitor changes in protein acetylation and expression, we used a novel label-free and data-independent acquisition (DIA or SWATH) approach on a SCIEX TripleTOF 6600 LC/MS system that we have modified for this purpose (2). Overall, we confidently identified 3840 unique acetylation sites from 978 acetylated proteins across all growth conditions: 1608 Kac sites at 0.4% Xyl; 2824 Kac sites at 4% Xyl; 1489 Kac sites at 0.4% Glc, and 2949 Kac sites at 4% Glc. Both the number of identified sites and the extent of acetylation increased when the concentration of glucose or xylose was increased from 0.4% to 4%. Using our label-free quantitative approach with Skyline 3.5, an open source software project, we were able to identify hundreds of acetylation sites in over 150 proteins that showed robust and statistically significant acetylation increases when cells

were grown with the larger amount of sugar. These acetylated proteins were involved in glycolysis/gluconeogenesis, pyruvate metabolism, TCA cycle etc. In addition, performing a functional annotation analysis for these acetylated proteins showed a statistically relevant enrichment for the ontology term "generation of precursor and metabolites and energy". Interestingly, the acetylation levels and relative changes with increased sugar concentrations appear very similar between glucose and xylose. We also found, as expected, significant changes in the expression levels of proteins relevant in carbohydrate metabolism and sugar transport, as well as key metabolic proteins, including acetyl-coenzyme A synthetase (ACS), and 2-methylcitrate synthase. For example, ACS showed a ~10 fold reduction in protein levels at high concentrations (4%) of either glucose or xylose compared to those observed in the lower sugar (0.4%) supplementation experiments.

Lastly, we have begun an independent assessment of site occupancy or stoichiometry of lysine acetylation using a novel SWATH acquisition method. In this modified approach, ion intensities of both MS1 precursors and MS2 fragments are used to determine site occupancy, thus increasing the confidence and accuracy of these determinations. Such determinations are typically prone to error and over-estimation given the large dynamic range of observed acetylation stoichiometries (~20% to less than 0.01%). Our modified and optimized approach was based on a recently reported method by Baeza and colleagues (3) that depends on the accurate determination of isotopic forms that are generated both *in vivo* (normal endogenous isotopic abundances) and *in vitro*, the latter by quantitative per-acetylation with stable isotope labeled acetic anhydride at the protein level. Our revised method is based on measuring abundances of otherwise identical specific fragments ion pairs in a data-independent acquisition that encompass the same site of lysine acetylation and whose relative ion intensities represent the extent to which they originated from endogenous versus exogenous labeling. In these *E. coli* experiments, most of the lysine-acetylation stoichiometries measured were low (<5%). However, there were some 'hot spots' detected at specific sites showing higher site occupancy. These site occupancy levels reveal information in addition to the relative fold-change of acetylation levels of individual Kac sites under different sugar supplement conditions, and will provide additional mechanistic assessments of which pathways may be most functionally affected by acetylation.

References

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