Final report: The Systems Biology of Protein Acetylation in Fuel-Producing Microorganisms

ABSTRACT

Our goal 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. Still, we know very little about lysine acetylation, particularly in the case of bacteria. 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 propose to investigate 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.

RESULTS (number refers to associated publication)

1. Acetylation results from acetate fermentation

Lysine acetylation is thought to provide a mechanism for regulating metabolism in diverse bacteria. Indeed, many studies have shown that the majority of enzymes involved in central metabolism are acetylated and that acetylation can alter enzyme activity. However, the details regarding this regulatory mechanism are still unclear, specifically with regards to the signals that induce lysine acetylation. To better understand this global regulatory mechanism, we profiled changes in lysine acetylation during growth of *Escherichia coli* on the hexose glucose or the pentose xylose at both high and low sugar concentrations using label-free mass spectrometry. The goal was to see whether lysine acetylation differed during growth on these two different sugars. No significant differences, however, were observed. Rather, the initial sugar concentration was the principal factor governing changes in lysine acetylation, with higher sugar concentrations causing more acetylation (**Figure 1**). These results suggest that acetylation does not target specific metabolic pathways but rather simply targets accessible lysines, which may or may not alter enzyme activity. They further suggest that lysine acetylation principally results from conditions that favor accumulation of acetyl phosphate, the principal acetate donor in *E. coli.*

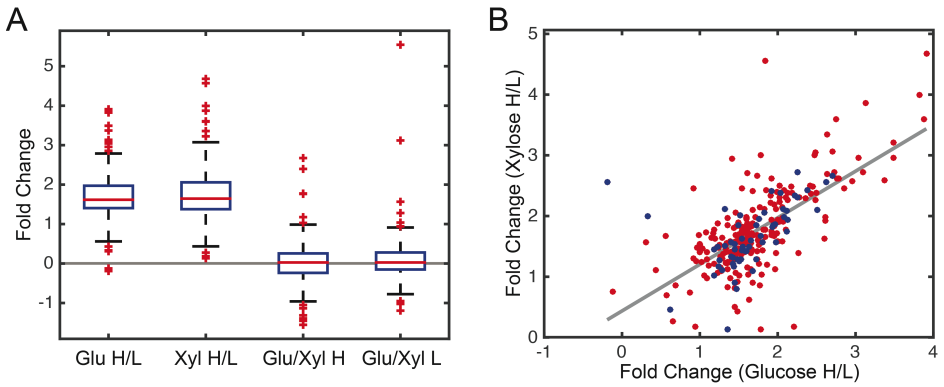

Figure 1. Relative changes in lysine acetylation under the four growth conditions. A. Box plot showing relative change in acetylation for the four different growth conditions. B. Comparison of

differentially acetylated lysines during growth on xylose versus glucose. The blue dots denote lysines on the metabolic enzymes depicted in **Figure 2**. Abbreviations: Glu H/L (4% glucose versus 0.4% glucose); Xyl H/L (4% xylose versus 0.4% xylose); Glu/Xyl H (4% glucose versus 4% xylose); and Glu/Xyl L (0.4% glucose versus 0.4% xylose).

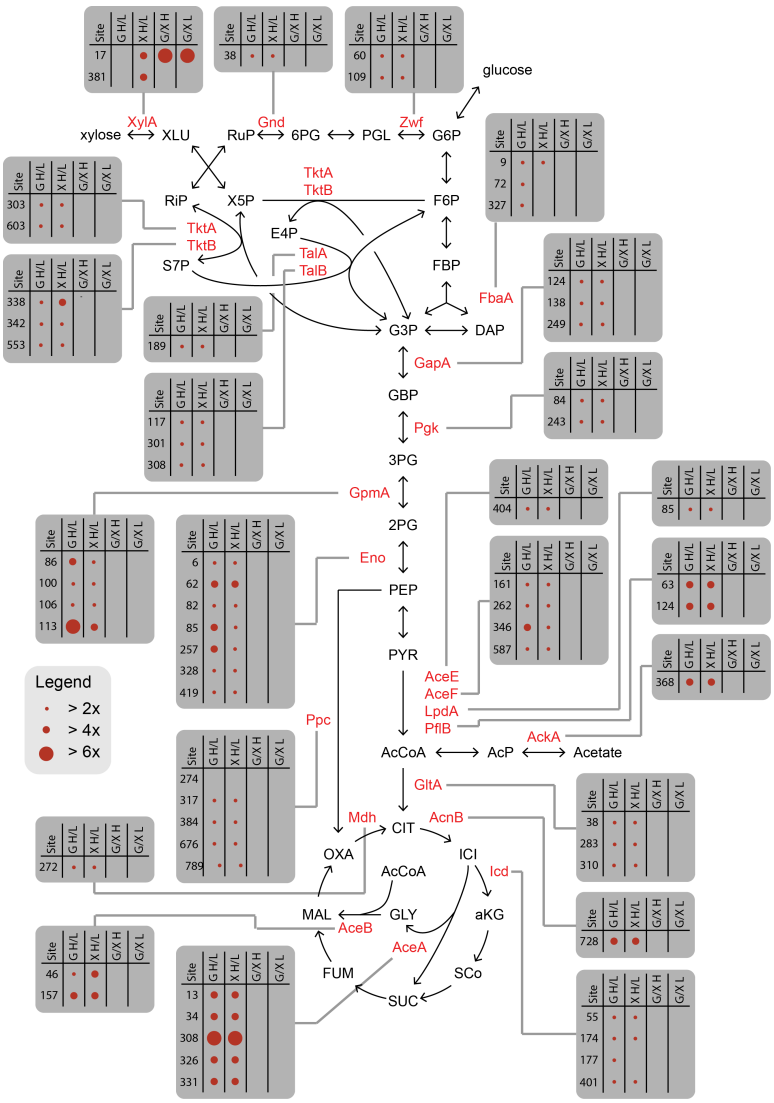

Figure 2. Enzymes in central metabolism exhibiting changes in lysine acetylation under the four growth conditions. Specific lysines are shown in gray boxes. Abbreviations: XLU: xyluose; RuP: ribulose 5-phosphate; 6PG: gluconate 6-phosphate; PGL: phosphogluconolactone; G6P: glucose 6-phopshate; RiP: ribulose 5-phosphate; X5P: xylulose 5-phosphate; F6P: fructose 6-phosphate; S7P: sedoheptulose 7-phosphate; E4P: erythrose 5-phosphate; FBP: fructose 1,6-bisphosphate; G3P: glyceraldehyde 3-phosphate; DHAP: dihydroxyacetone phosphate; 3PG; GBP: 1,3-bisphosphoglycerate; 3PG: 3-phosphoglycerate; 2PG: 2-phosphoglycerate; PEP: phosphoenolpyruvate; PYR: pyruvate; AcCoA: acetyl-CoA: AcP: acetyl-phosphate; CIT: citrate; ICI: isocitrate; aKG: α-ketoglutarate; SCo: succinyl-CoA; SUC: succinate; FUM: fumarate; MAL: malate; OXA: oxaloacetate; Glu H/L (4% glucose versus 0.4% glucose); Xyl H/L (4% xylose versus 0.4% xylose); Glu/Xyl H (4% glucose versus 4% xylose); and Glu/Xyl L (0.4% glucose versus 0.4% xylose).

The significance of this work is that it demonstrates that lysine acetylation is not determined by the carbon source but rather results from the accumulation of acetyl-CoA within the cell (which in turn also causes acetate to be excreted from the cell). These results were actually surprising to us because we initially hypothesized that the carbon source would affect acetylation patterns. However, in retrospect, it makes sense given the central role of acetate fermentation in inducing lysine acetylation.

2. pH affects acetate fermentation.

When *E. coli* is grown aerobically on sugars, it will produce acetate. Acetate production is thought to occur when the carbon flux through central metabolism exceeds the capacity of the tricarboxylic acid (TCA) cycle. To deal with this bottleneck, the cell diverts this excess flux to a fermentation product (e.g. acetate). This strategy enables cells to produce both energy and anabolic precursors at high rates despite limitations in their metabolic capacity.

In many organisms, overflow metabolism can be reversed: once the cells consume the available sugar, they will then consume the overflow metabolite in order to generate energy and provide anabolic precursors for additional growth. For example, during batch growth on glucose, *E. coli* consumes the acetate produced from overflow metabolism. Indeed, *E. coli* can grow on acetate as its sole carbon source.

We found that the pH of the growth medium affects the ability of *E. coli* to consume acetate – when the initial pH is less than 6, the cells are unable to re-consume the acetate. (**Figure 3**). To determine the mechanism, we characterized a set of metabolic mutants and found that those defective in the TCA cycle or glyoxylate shunt exhibited reduced rates of acetate consumption. We further found that expression of the genes in these pathways was reduced during growth in acidic media. Expression of the genes involved in the AckA-Pta pathway, which provides the principal route for both acetate production and consumption, were also inhibited in acidic media but only after glucose was depleted, which correlates with the acetate consumption phase. Based on these results, we conclude that growth in acidic environments inhibits the expression of the acetate catabolism genes, which in turn prevents acetate consumption.

While this work is not directly related to lysine acetylation, it nonetheless has uncovered an important facet to acetate fermentation. These results also demonstrate how acetate alters cell metabolism; they also may be useful for the design of aerobic fermentation processes.

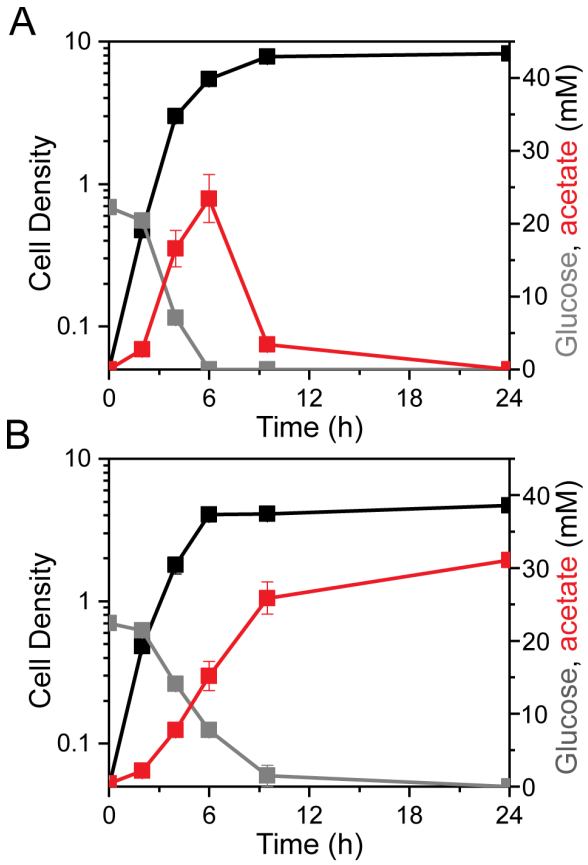

Figure 3. Growth of *E. coli* on glucose when initial pH is 7 (A) or 6 (B).

3. Lysine acetylation is highly conserved.

We investigated whether lysine acetylation is found on different enzymes and whether this modification is conserved throughout bacterial evolution by performing a comprehensive phyloproteomic analysis involving 48 phylogenetically distant bacteria. We found that acetylation occurs in conserved lysine residues located in catalytic pockets of enzymes from the glycolytic pathway and the TCA cycle (**Figure 4**). Two-thirds of glycolytic and tricarboxylic acid (TCA) cycle enzymes are acetylated at these critical sites. Our data suggest that acetylation may play a direct role in metabolic regulation by switching off enzyme activity.

To test this hypothesis, we cloned and purified enolase from *E. coli* and *B. subtilis*. This enzyme has two catalytically active lysines known to be acetylated. We found that acetylating these enzymes inhibits the activity (**Figure 5**). These results demonstrate the regulatory effect of lysine acetylation is conserved across phylogenetically distinct bacteria.

This significance of this work was to demonstrate that what is true for *E. coli* is also true for other bacteria. In these regards, our results from this project, which principally derive from *E. coli*, also apply to other bacteria as well.

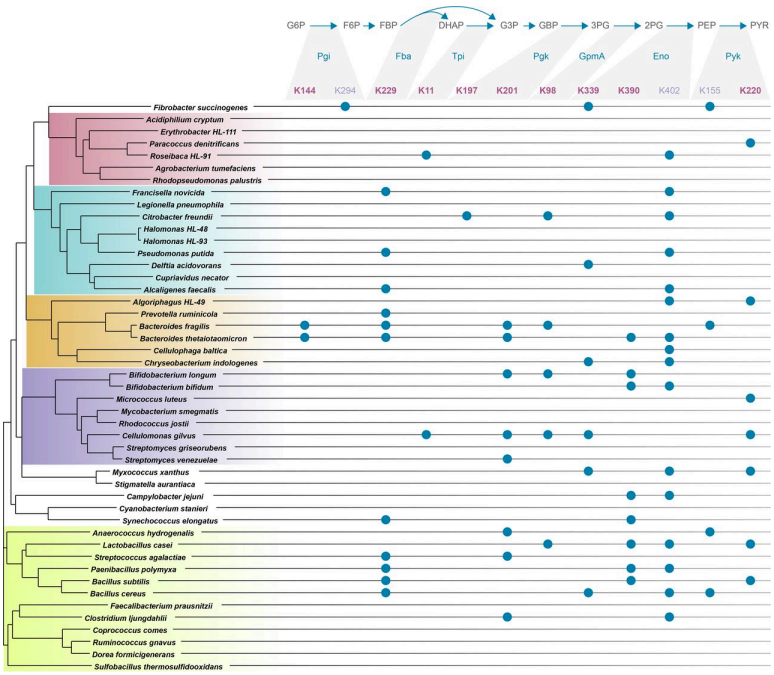

Figure 4. Acetylation of substrate/cofactor-binding lysine residues across taxa. The phyloproteomic data identify acetylated lysines from 48 organisms. Enzymes from glycolysis are listed along with universally conserved lysine residues, numbered according to the numbering system for *Bacillus subtilis* (except for GpmA from *L. casei* gene S6CBB3). Catalytically essential sites as described in the text are shown in boldface type. Nonboldfaced sites are listed if they are universally conserved across bacteria but are not known to be involved in substrate/cofactor binding. Observed acetylations are indicated with solid blue circles. The phylogenetic tree of organisms is based on RplB sequence alignment with the major taxonomic groups colored: Alphaproteobacteria, Gamma- or Betaproteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes. Protein name abbreviations: Pgi, phosphoglucose isomerase; Fba, fructose-bisphosphate aldolase; Tpi, triose-phosphate isomerase; Pgk, phosphoglycerate kinase; GmpA, 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase; Eno, enolase; Pyk, pyruvate kinase.

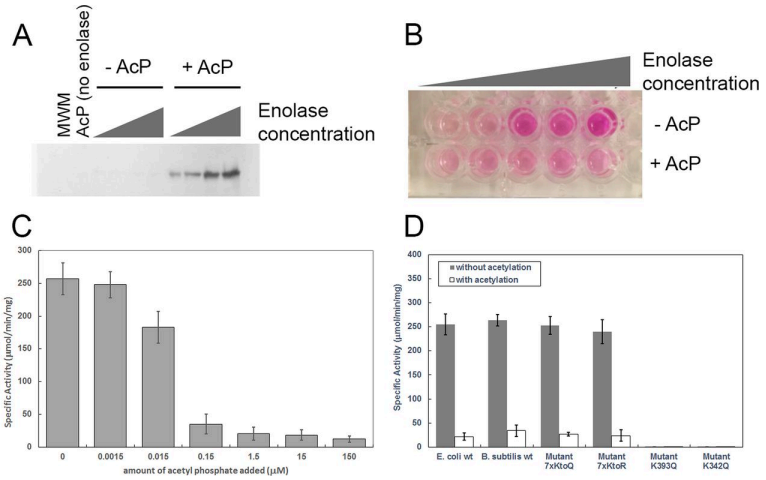

Figure 5. Enolase activity regulation by acetylation. (A) Western blot analysis of enolase treated with acetylphosphate (AcP). From right to left, serial 10 dilutions of enolase incubated in the presence (+) or absence (-) of 15 M acetylphosphate. MWM, molecular weight markers. (B)

Colorimetric assay of serial 10 dilutions of enolase incubated in the presence or absence of 15 M acetylphosphate. (C) Enzymatic activity assay for *E. coli* enolase with and without addition of acetyl phosphate. Acetyl phosphate was added at concentrations ranging from 1.5 nM to 150 M. Acetylation of *E. coli* enolase by acetyl phosphate resulted in a dose-dependent inhibition of *E. coli* enolase activity. (D) Enzymatic activity assay showing that acetylation inhibits enolase catalytic activity. The assay was performed in triplicate. Enolase activity was measured in the presence or absence of 1.5 M acetylphosphate. Along with wild-type *E. coli* and *B. subtilis* enzymes, four *E. coli* mutant strains were tested. In the 7xKtoQ mutant and 7xKtoR mutant, the seven lysine residues that are not conserved or at the active site were replaced with either glutamine (K_iQ) or arginine (K_iR) (see Materials and Methods). In the K393Q mutant and K342Q mutant, either active site lysine 393 or active site lysine 342 were mutated to glutamine. wt, wild type.

4. Magnesium tunes lysine acetylation.

Previously, our lab and others have found that *E. coli* grown in carbon excess leads to high acetylation levels due to the production of acetate. We found that carbon is directed into biomass rather than acetate when magnesium, the limiting nutrient in our experiments, is in excess. In particular, we found that *E. coli* grown in typtone broth buffered to pH7 (TB7) supplemented with 0.4% glucose grew to an OD_{600} value of ~3 (**Figure 6, left**). When we further supplemented the growth medium with 1 mM magnesium sulfate ($MgSO_4$), *E. coli* had an extended exponential phase and reached an OD_{600} 2-3 times greater. Six additional carbon sources showed the same magnesium-induced biomass increase. We also found that cells grown in media where typtone was replaced with either casamino acids or peptone exhibited the same effect. Even the common laboratory medium LB was found to benefit from magnesium supplementation when provided with excess carbon. More significantly, magnesium-induced biomass increase was accompanied by a significant reduction in acetylation as measured by Western blot analysis (**Figure 6, right**).

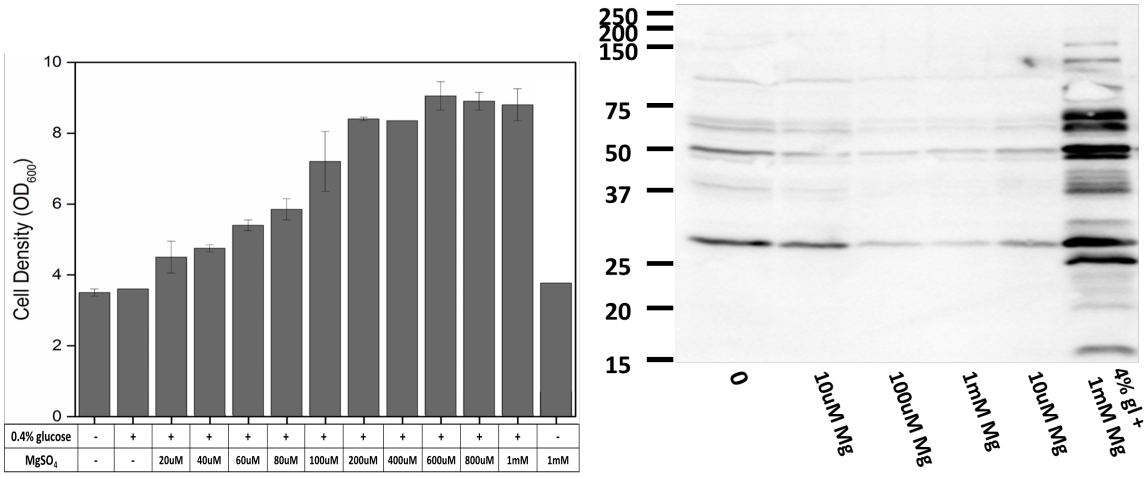

Figure 6. Left. When wild type *E. coli* is grown in tryptone broth buffered to pH 7 (TB7) supplemented with 0.4% glucose, stationary phase cell density reaches an OD_{600} of about 3. However, increasing concentrations of magnesium support cell density up to an OD_{600} of 9. Magnesium does not permit increased biomass if glucose is not present. **Right.** When these stationary phase cell lysates were analyzed by anti-acetyllysine western blot, increasing magnesium concentration reduced acetylation. However, even in the presence of additional

magnesium, increasing glucose concentration 10-fold restored carbon excess and thus restored and even increased acetylation.

These results demonstrate that magnesium indirectly affects protein acetylation by determining whether carbon flux is diverted to biomass or acetate. The mechanism is likely related to ribosome abundance, because magnesium is known to increase the stability of the ribosomes. Collectively, these results provide a deeper understanding of how different media formation influence bacterial metabolism and physiology, and demonstrate how *E. coli* regulates its metabolism accordingly.

5. Acetylation of CRP

The cyclic AMP receptor protein (CRP) is a global regulator of metabolism in *E. coli*. It directly regulates over 280 promoters. It is also acetylated at lysine 100. In this work, acetylation of lysine 100 was shown to reduce the ability of the regulator to activate transcription from class II but not class 1 promoters. Briefly, CRP-dependent promoters can be classified into two groups depending on where CRP binds relative to the transcription start site. Interestingly, acetylation was also found to increase steady-state CRP levels, suggesting that it could increase transcription from some class I promoters. Based on these results, we propose that K100 acetylation is a mechanism by which the cell could potentially turn down transcription from a subset of Class II CRP-dependent promoters, whilst simultaneously turning up transcription from a subset of Class I promoters. This mechanism would be most relevant when the cell ferments or consumes high concentrations of acetate.

6. Stoichiometry of lysine acetylation.

Although many studies have clearly shown that lysine acyl modifications undergo large fold-changes under different conditions, precise measures of acylation site occupancy, or stoichiometry, are scarce. To address the challenges inherent in accurate quantification of lysine acylation stoichiometry, we have developed a method that applies a variation of the stable isotope labeling method, followed by SWATH acquisition that collects both precursor and multiple fragment ion abundances (**Figure 7**). We benchmarked this new acylation occupancy workflow with experiments using (acetylated) bovine serum albumin (BSA), and then we investigated protein lysates from *E. coli* under different growth conditions where we had previously observed large fold-changes in acetylation levels, but where data on the site occupancy of these changes was not known. We also developed a tractable method for data analysis using a combination of Skyline and custom scripts written in-house. The combination of our SWATH fragment ion quantification and our custom software allow us to determine site-specific acylation stoichiometry from peptides containing multiple lysines. We extended the method to allow determination of lysine succinylation stoichiometry. Our results indicate that using SWATH fragment ion quantification improves accuracy and precision compared to results from only precursor quantification. We find the majority of both lysine acetylation and succinylation site occupancies in *E. coli* are relatively low (<1%–5%), but some sites have higher stoichiometry (>5%).

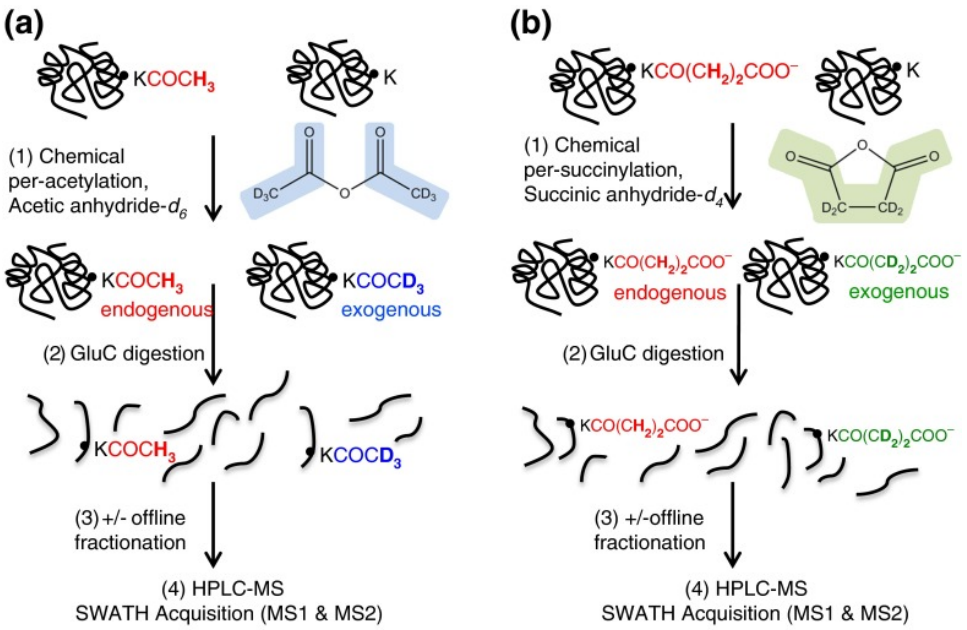

Figure 7. Stoichiometry workflow. **(a)** First, protein lysates were incubated three times with acetic anhydride-*d6* to acetylate unmodified lysine residues. Second, samples were digested with endoproteinase Glu-C, followed by optional offline fractionation of the proteolytic peptides by basic reversed-phase chromatography. Finally, peptides were analyzed by LC-MS in SWATH acquisition mode. **(b)** To determine succinylation stoichiometry the same workflow was used as described in **(a)**, except the heavy acylation reagent was changed to using succinic anhydride-*d4*.

7. Protein acetylation dynamics.

In *E. coli*, acetylation of proteins at lysines depends largely on a non-enzymatic acetyl phosphate-dependent mechanism. To assess the functional significance of this post-translational modification, we first grew wild-type cells in buffered tryptone broth with glucose and monitored acetylation over time by immunochemistry. Most acetylation occurred in stationary phase and paralleled glucose consumption and acetate excretion, which began upon entry into stationary phase.

During growth, both the number of identified sites and the extent of acetylation increased with considerable variation among lysines from the same protein. As glucose-regulated lysine acetylation was predominant in central metabolic pathways and overlapped with acetyl phosphate-regulated acetylation sites, we deleted the major carbon regulator CRP and observed a dramatic loss of acetylation that could be restored by deleting the enzyme that degrades acetyl phosphate. We propose that acetyl phosphate-dependent acetylation is a response to carbon flux that could regulate central metabolism.

In summary, this study provides new insights into the important role of glucose regulation for *E. coli* protein acetylation in a time-dependent fashion. It reveals a mechanism for regulating this acetylation and its potential to quickly modify proteins with impacts on entire biological pathways or networks. This study also provides experimental foundation for future studies that could investigate the dependence of acetylation on glucose levels, the precise mechanism(s) used by cells to delay glucose consumption until they transition to stationary phase, the mechanism used by the cAMP-CRP complex to regulate acetyl phosphate concentrations, and the effects exerted by

acetylation on protein function.

PUBLICATIONS RESULTING FROM THIS PROJECT

1. B. Schilling, N. Basisty, D. G. Christensen, D. Sorensen, J. S. Orr, A. J. Wolfe, C. V. Rao. Global lysine acetylation in *Escherichia coli* results from growth conditions that favor acetate fermentation. bioRxiv. 2018. doi: 10.1101/457929.

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