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Understanding the Regulation of Metabolic Enzyme Acetylation in E. Coli

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UNDERSTANDING THE REGULATION OF METABOLIC ENZYME ACETYLATION IN *E. coli*

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For Daysha and Ravi,
My never ending sources of inspiration

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LIST OF ABBREVIATIONS

KAT	Lysine acetyltransferase
KDAC	Lysine deacetylase
GNAT	GCN5-related N-Acetyltransferase
cAMP	3'-5'-cyclic adenosine monophosphate
CRP	cAMP receptor protein, catabolite repressor protein
WT	Wild type
VC	Vector control
TB	Tryptone broth
TB7	Tryptone broth pH 7
ARs	Activating regions, AR1, AR2, AR3
RNAP	RNA polymerase
Acetyl-CoA	Acetyl-Coenzyme A
CoA	Coenzyme A
PTM	Post-translational modification
NTD	N-terminal domain
CTD	C-terminal domain
PAGE	Polyacrylamide gel electrophoresis
PVDF	Polyvinylidene fluoride
PBST	Phosphate Buffered Saline with Tween 20

ABSTRACT

Global protein acetylation is a newly discovered phenomenon in bacteria. Of the more than 250 acetylations reported in *E. coli*, many are of metabolic enzymes [1-3]. Thus, acetylation could represent a novel posttranslational mechanism of metabolic control. Yet, almost nothing is known about the regulation of these acetylations or of their metabolic outcomes. Here, we report that the cAMP receptor protein (CRP) regulates protein acetylation in *E. coli* and provide evidence that protein acetylation modulates the flux of carbon through central metabolism. When we grew cells in mixed amino acids supplemented with glucose and cAMP, global protein acetylation increased in a CRP-dependent manner and several of the acetylated proteins were central metabolic enzymes. Much of this CRP-mediated acetylation required activation region 1 (AR1), a surface patch that allows CRP to interact with RNA polymerase. A second surface patch (AR2) also was involved, albeit to a lesser degree. These results raise the possibility that CRP might regulate the transcription of a protein acetyltransferase. Indeed, a recent report suggested that CRP might regulate transcription of the protein acetyltransferase YfiQ (also known as Pat) by a mechanism that would require AR2 [4]. We further obtained bioinformatic evidence that supports the hypothesis that CRP also could regulate *yfiQ* transcription in an AR1-dependent manner. Since CRP regulates metabolism, we asked if YfiQ could influence metabolism. Using Phenotype MicroArray™ analysis [5], we found that a *yfiQ* null mutant exhibits a distinctive defect during growth on gluconeogenic

carbon sources and a distinct advantage during growth on a carbon source that bypasses the need for gluconeogenesis. *In vitro* acetylation assays identified four substrates of YfiQ. Three YfiQ substrates were the strictly irreversible glycolytic enzymes PfkA, PfkB, and LpdA. The fourth was CRP itself. We thus hypothesize that CRP activates *yfiQ* transcription, whose protein product acetylates a subset of metabolic enzymes, altering their function and shifting the balance between glycolysis and gluconeogenesis. We further propose that YfiQ acetylates CRP. Efforts to determine how this acetylation affects the CRP-dependent transcriptome are underway.

CHAPTER I

LITERATURE REVIEW

N_{ε}-Lysine Acetylation

The post-translation modification (PTM) of proteins by acetylation can occur in two forms: 1) N_{α}-acetylation, where an acetyl group from the acetyl donor (acetyl-CoA) is transferred to the amino terminus of a protein, or 2) N_{ε}-acetylation, where an acetyl group is transferred to the ε-amino group of a lysine residue. N_{α}-acetylation is a non-reversible PTM that is rare in bacteria, In contrast, N_{ε}-lysine protein acetylation is a reversible PTM that can function to alter protein structure, function, stability, localization, and protein-protein interactions [6, 7]. In *E. coli*, over 250 proteins are reported to be post-translationally modified by N_{ε}-lysine acetylation [1, 2]. The acetylated proteins fall into many different functional classes, including some involved in transcription, translation, and metabolism, and some in sensing redox states and heat-shock stress. Currently, there are only 5 bacterial proteins where the effects of N_{ε}-lysine acetylation have been studied in any detail: the signaling protein CheY, the central metabolic enzyme Acs, the response regulator RcsB, the α subunit of RNA polymerase (RNAP), and the exoribonuclease RNase R [8-17].

CheY is the response regulator of the chemotaxis system, and upon phosphorylation, binds to the flagellar switch FliM, leading to an increase in the likelihood of clockwise flagellar rotation [18]. Acetylation of CheY is thought to occur by three mechanisms: 1) spontaneous acetylation, where CheY is able to acetylate itself using acetyl-CoA as an acetyl donor (this will be explained in detail in the next section) [12], 2) acetylation by the central metabolic enzyme Acs using acetate as the acetyl donor [11, 19], or 3) acetylation by a yet undiscovered lysine acetyltransferase (KAT) [13, 20]. Acetylation of CheY is hypothesized to affect protein-protein interactions between CheY and the rest of the chemotaxis machinery, thereby affecting the ability of *E. coli* to respond to chemotatic signals [10].

The central metabolic enzyme, acetyl-CoA synthetase (Acs), is responsible for converting acetate, adenosine triphosphate (ATP) and coenzyme A (CoA) into acetyl-CoA, pyrophosphate, and adenosine monophosphate (AMP) [21]. Acetylation regulates ACS enzymatic activity. Acetylation of an active site lysine of Acs by the KATs Pat in *S. enterica* and AcuA in *Bacillus subtilis* inhibits Acs activity, but the mechanism of inhibition remains unknown [16, 22]. Deacetylation of Acs results in restored catalytic activity, and is regulated by the sirtuin CobB in *S. enterica*, and the sirtuin SrtN and the class I KDAC AcuC in *B. subtilis* [16, 22-24].

The response regulator RcsB is part of the Rcs two component system involved in regulating complex cellular processes such as flagellar synthesis, cell division, and capsule biosynthesis. Posttranslational modification by phosphorylation of RcsB occurs on a conserved aspartate residue (D56), which serves as the acceptor of the phosphorelay

signal from its cognate sensor kinase RcsC via the histidine phosphotransferase RcsD [25], leading to either repression or activation of RcsB-dependent promoters. *In vitro* studies into the effects and regulation of RcsB acetylation have shown that: 1) RcsB acetylation occurs on lysine 180 (K180) [17], 2) acetylation of K180 affects RcsB activity, most likely by affecting the ability of RcsB to bind to the DNA, and 3) the KAT Pat and the sirtuin CobB of *S. enterica* and their homologs in *Escherichia coli* are sufficient to regulate K180 acetylation and deacetylation, respectively [17]. Data from the Wolfe lab furthers these findings. *In vivo* analysis of RcsB acetylation in *E. coli* indicates that: 1) CobB is required for RcsB-dependent *rprA* exponential phase transcription, 2) YfiQ, the *E. coli* homolog of Pat, activates *rprA* transcription, but 3) deletion of *yfiQ* has no discernible affect on RcsB acetylation, 4) *in vivo* acetylation occurs on multiple RcsB lysines, but rarely on K180, and 5) some of these acetylations are sensitive to the presence of the sirtuin KDAC CobB and some are not (Hu *et al.,* in preparation). These findings suggest the possibility that acetylation of K180 may not affect transcription at the *rprA* promoter and suggest a novel role for acetylation of RcsB in *E. coli*.

Acetylation of RNA polymerase core subunits was identified in 2 different global acetylome studies [1, 2]. More recently, the Wolfe lab has identified acetylation of RNAP on three of these core subunits (β, β', α). Approximately 30 acetylated lysines were detected in *E. coli* cells grown in an amino acid-based medium (TB) supplemented with glucose, but not when the glucose was omitted [14]. Acetylation of K298 of the carboxy-terminal domain (CTD) of α occurred in a glucose- and YfiQ-dependent manner, while

glucose-induced transcription of the stress responsive *cpxP* promoter required K298 and was sensitive to nicotinamide, an inhibitor of CobB [14].

Acetylation of K291, another α-CTD lysine, is detected when an *ackA* mutant is grown in TB supplemented with glucose [26]. The acetylation of K291 is hypothesized to inhibit glucose-induced *cpxP* transcription, as mutation of K291 to K291A alleviates inhibition, while mutation to the acetylation mimic K291Q resulted in a decrease in glucose-induced *cpxP* transcription even in wild-type cells [26]. YfiQ is not involved in this behavior, suggesting that either K291 can undergo spontaneous acetylation, or is acetylated by a yet unknown KAT [26]. These results show that acetylation of the α-CTD can have differential transcriptional effects dependent upon environmental and genetic conditions. These manuscripts reporting the effects of acetylation on RcsB and RNAP are the first to suggest that acetylation can affect transcription *in vivo.*

The exoribonuclease RNase R is known to degrade structured RNAs in *E. coli*, and RNase R activity is regulated, in part, by regulation of RNase R stability [27]. Stability of RNase R protein is highest in stationary phase cells, with RNase R in exponential phase cells having a half life of approximately 10 minutes [28]. RNase R stability is regulated by two components of the trans-translation system: tmRNA and SmpB, which both bind to the C-terminal region of RNase R, resulting in instability of RNase R in exponential phase cells [27]. Comparison of RNase R protein from exponential and stationary phase cells revealed that RNase R was acetylated on K544 in exponential, but not in stationary, phase cells. This acetylation is regulated by the KAT YfiQ, and leads to tighter binding of tmRNA and SmpB, thus increasing RNase R instability [8, 9].

Although much is known about the affects and regulation of CheY, Acs, RcsB, the α subunit of RNAP, and RNase R acetylation, there are still more than 245 proteins in *E. coli* reported to be acetylated, whose regulation and effects of protein acetylation remain unknown. Interestingly, of all the acetylated proteins identified in *E. coli*, many are enzymes involved in carbon metabolism [1, 2]. In the closely related bacteria, *Salmonella enterica*, lysine acetylation is thought to regulate not only the activity of certain central metabolic enzymes, but is also predicted to affect carbon flux through central metabolism [3]. While one group investigated the regulation of acetylation of the *S. enterica* central metabolic enzymes GapA, AceE, and AceK, the full effects of metabolic enzyme acetylation in *S. enterica* are not known. However, the evidence suggests that metabolic enzyme acetylation has the potential to regulate carbon flux between glycolysis and gluconeogenesis [3].

Regulation of N_ε- Lysine Acetylation

Currently, there are two proposed mechanisms by which N_ε-lysine acetylation is thought to occur: 1) enzymatic regulation of N_ε-lysine acetylation and 2) spontaneous acetylation of lysines.

Enzymatic regulation of N_ε-lysine acetylation is controlled by two groups of proteins: lysine acetyltransferases (KATs) and lysine deacetylases (KDACs). Enzymes that acetylate lysines are known as lysine acetyltransferases (KATs). 5 groups of proteins have been shown to exhibit KAT activity (reviewed in Hu *et al.,* 2011). Of these, the GCN5-like acetyltransferase (GNAT) family is the most widely distributed with over

10,000 members identified across all three domains of life [29]. KATs are intimately tied to central metabolism because they use the key central metabolite acetyl coenzyme A (acetyl-CoA) as the acetyl donor [30]. In *E. coli*, there are 23 known and putative KATs [31].

YfiQ (also known as Pat in *S. enterica,* and Pka and PatZ in *E. coli*) is the only well-studied bacterial KAT, and is known to acetylate the central metabolic enzyme Acs both *in vivo* and *in vitro* [15], the response regulator RcsB *in vitro* (Hu *et al.,* in preparation) [17], the exoribonuclease RNase R *in vitro* in *E. coli* [8] , and is sufficient to acetylate the enzymes GapA, AceE, and AceK *in vitro* in *S. enterica* [3]. YfiQ is a large, 98 kD, multi-domain protein, where the N-terminal region of YfiQ has a conserved CoA binding domain, and the C-terminus has a conserved GNAT RimL-acetyltransferase domain **(Figure 1)** [32]. RimL is a known ribosomal protein acetyltransferase, and has a conserved GNAT domain [33].

Biochemical characterization of *S. enterica* Pat indicates several key properties: 1) isothermal titration calorimetry experiments indicate that Pat has 2 binding sites for acetyl-CoA, 2) Pat has high affinity for acetyl-CoA, with a dissociation constant (K_d) of 0.29 ± 0.03 μM for the first site, and 2.38 ± 0.03 μM for the second site, and 3) Pat oligomerizes from a monomer to a tetramer in the presence of acetyl-CoA [32]. It is not known if the binding of acetyl-CoA to Pat induces a change in conformation that leads to tetramer formation. Furthermore, the mechanism of substrate recognition by Pat has not been determined, but there are likely three possibilities: 1) sequence-specific recognition of a lysine substrate [1], 2) structural specificity of the area surrounding the lysine, or 3) a

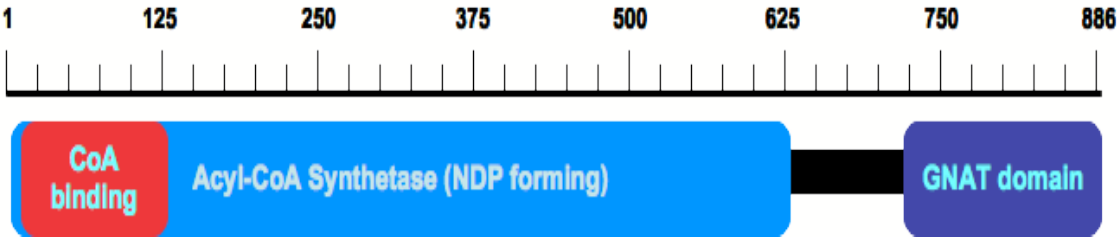

Figure 1. Domain structure of *E. coli* YfiQ. Identification of domains was based on Conserved domain database [34] search. The CoA binding domain belongs to the NADP-Rossman superfamily of CoA-binding proteins. The Acyl-CoA Synthetase (NDP-forming) domain is generally believed to catalyze the conversion of acetyl-CoA and ADP to acetate, ATP, and CoA. The C-terminal GNAT domain catalyzes the N-acetyltransferase reaction, and is homologous to the RimL GNAT domain

combination of the two. Although no evidence exists to suggest the possibility of structural specificity regulating substrate specificity, a combination of both structural and sequence-specificity is a likely mechanism for substrate recognition, similar to the proposed mechanism utilized by histone acetyltransferases [35].

In bacteria, the regulation of KAT activity remains unknown. However, it is likely that KAT activity is tied intimately to the levels of the acetyl donor, acetyl-CoA [30], which fluctuate in response to periods of carbon starvation and carbon excess. It is not known whether KAT activity changes with nutrient conditions; however, published work indicates increases in metabolic enzyme acetylation when *S. enterica* is grown on glucose as the sole carbon source, but not citrate [3]. Furthermore, the Wolfe lab has shown that growth of *E. coli* in TB supplemented with 0.4 % glucose results in an increase in protein acetylation relative to growth in TB alone [14]. These results indicate that protein acetylation can change in response to altered nutrient conditions. However, it remains to be seen if KAT activity does indeed change in response to fluctuations in acetyl-CoA levels.

Although the regulation of KAT activity is still unclear, the regulation of KAT expression is being uncovered. In one report, expression of YfiQ was shown to change during growth, with detectable YfiQ protein levels during exponential growth decreasing over time to undetectable levels in late exponential and stationary phase cells [9]. Additionally, there is a report that suggests that the central carbon regulator in enteric bacteria, cAMP receptor protein (CRP) could regulate expression of at least one known and two putative KATs: YfiQ, YedL, and YjhQ, respectively [4, 36, 37], although it is not

known how this regulation occurs. Nevertheless, this relationship between CRP and KATs is exciting, because CRP activity also is intimately tied to carbon metabolism via availability of its allosteric effector, cAMP. The highest CRP activity occurs during times of nutrient limitation, while the lowest activity occurs during carbon excess [5] (e.g. exposure to glucose). This relationship between cAMP and KATs may be a conserved mechanism across bacteria, by which cells can regulate KAT expression and/or activity, as it has recently been shown that cAMP regulates KAT activity in *Mycobacterium tuberculosis* [38, 39].

Spontaneous acetylation of a bacterial protein was first identified for the metabolic enzyme Acs [11]. The chemotaxis response regulator CheY also has been shown to spontaneously acetylate [12]. Spontaneous acetylation of CheY and Acs was identified using *in vitro* acetylation experiments where either CheY or Acs were incubated with either radiolabeled C^{14}-acetyl-CoA or radiolabeled C^{14}-sodium acetate and ATP, respectively [11, 12]. The mechanisms by which spontaneous acetylation is regulated are being elucidated. Two possibilities exist: a catalytic event called autoacetylation and chemical acetylation that does not involve catalysis. Autoacetylation by acetyltransferases has been shown to occur as an intramolecular reaction; i.e., the acetyltransferase is capable of catalyzing the transfer of the acetyl group from the donor to itself. This is the case for the eukaryotic p300 histone acetyltransferase [40]. Autoacetylation also can be an intermolecular process. For example, the P/CAF histone acetyltransferase autoacetylates by a mechanism in which one P/CAF protein acetylates another P/CAF protein *in trans* [41].

Chemical acetylation is seen when the addition of acetyl-CoA, acetic anhydride, or sodium acetate and ATP, induces protein acetylation *in vitro*, as seen with CheY and Acs, respectively [11, 12, 42]. It is not understood how proteins can undergo chemical acetylation without a KAT being present. Evidence suggests that acetyl-CoA, acetic anhydride, and sodium acetate can act as acetyl-donors *in vitro*. Whether this is the case *in vivo* remains to be seen.

Lysine deacetylases (KDACs) deacetylate acetylated lysines. There are two major families of KDACs, grouped into four classes, for each of which there are putative bacterial homologues [43, 44]. The only studied KDAC in bacteria, however, is the sirtuin CobB (a member of class III KDACs). CobB is a NAD^+-dependent deacetylase **(Figure 2)**, which is reported to deacetylate the following bacterial proteins *in vitro:* RcsB, GapA, AceE, and AceK, and *in vivo:* CheY and Acs [3, 16, 17, 20]. The dependence on NAD^+ to deacetylate certain acetylated proteins and the current knowledge of the CobB-regulated metabolic substrates indicates that CobB activity may also respond to changes in cellular metabolic status, but the research to definitively show this has not been done [45].

Structural research into the mechanism of substrate recognition by CobB has determined that substrate-specific binding by CobB involves both the zinc-binding domain and a region distal to acetyl-lysine-binding site **(Figure 2)** [46]. Both sites are hypothesized to play a role in determining substrate-specificity. Among the sirtuin class, the zinc domain is variable, while the acetyl-lysine binding site is conserved [46]. Thus, additional work must be done to elucidate how CobB achieves substrate-specificity.

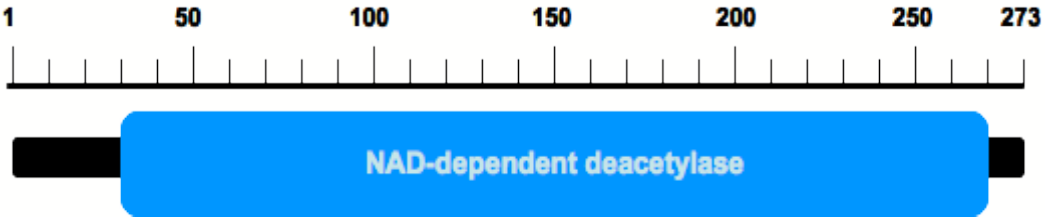

Figure 2. Domain structure of *E. coli* CobB. Identification of domains was based on Conserved domain database [34] search. The NAD-dependent deacetylase domain belongs to the silent information regulator 2 (Sir2) superfamily of proteins, which catalyzes NAD^+-dependent deacetylation.

Studies into the regulation of CobB expression have yielded little information: CRP, which is hypothesized to regulate expression of 2 putative (*yjhQ, yedL*) and 1 known KAT (*yfiQ*), has not been implicated in regulating expression of *cobB*. Thus far, there are no reports of studies researching the regulation of *cobB* expression in E. coli.

Overview of Glycolysis and Gluconeogenesis

Central metabolism of carbon is defined by the utilization of three pathways for carbon catabolism: Glycolysis (also referred to as the Embden-Meyerhof-Parnas or EMP pathway), the pentose-phosphate-pathway, and the tricarboxylic acid (TCA) cycle [47]. Glycolysis functions in opposition to a carbon anabolism pathway known as gluconeogenesis. Glycolysis and gluconeogenesis are universal pathways that describe the catabolism and anabolism, respectively, of the six-carbon monosaccharide glucose.

Glycolysis is an anaerobic pathway, in that oxygen is not required for the catabolism of glucose, and this pathway is responsible for metabolizing 1 molecule of glucose to 2 molecules of pyruvate while producing 2 molecules of ATP [48-50]. Pyruvate can be further catabolized by anaerobic or aerobic means using fermentative pathways or the tricarboxylic acid cycle, respectively. Under aerobic conditions, pyruvate is oxidized to CO_2. In anaerobic condition pyruvate is fermented into partially oxidized intermediates, e.g. lactate, acetate or ethanol. The central function of the glycolytic pathway is to produce energy [48-51].

The glycolytic pathway consists of ten reactions **(Figure 3)**, and is divided into 3 stages: 1) stage 1 or the investment stage, where 2 molecules of ATP are utilized for each

molecule of glucose taken into the cell with the end product fructose 1,6-bisphosphate; 2) stage 2, where 1 molecule of fructose 1,6-bisphosphate is catabolized into two three-carbon molecules, and 3) stage 3 or the harvesting stage wherein the cell gains four molecules of ATP and 2 molecules of NADH from one molecule of glucose [52]. The ten different reactions of glycolysis are sequentially as follows. 1) In *E. coli,* phosphorylation of exogenous glucose to glucose-6-phospate is catalyzed by the phosphotransferase system (PTS) concomitant with glucose uptake [53]. For intracellular glucose, phosphorylation is catalyzed by glucokinase (*glk*) [53]. In eukaryotes, this reaction is catalyzed by the enzyme hexokinase. 2) Reversible isomerization of glucose-6-phosphate to fructose-6-phosphate catalyzed by phosphoglucomutase (*pgm)* [54, 55]. 3) The phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate, coupled to the hydrolysis of ATP to ADP and Pi and catalyzed by the unidirectional enzyme phosphofructokinase (*pfkA, pfkB)* [56]. 4) The catabolism of one molecule of fructose-1,6-bisphophate into one molecule of dihydroxyacetone phosphate (DHAP) and one molecule of glyceraldehyde-3-phosphate (GAP), catalyzed by fructose bisphosphate aldolase (*fbaA, fbaB*) [57]. 5) The interconversion of the isomers DHAP to GAP, catalyzed by the enzyme triose-phsophate isomerase (*tpiA)*. 6) Dehydrogenation of GAP to 1,3-bisphosphoglycerate by the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH, *gapA*) [58]. 7) The generation of ATP and the production of 3-phosphoglycerate from 1,3-bisphosphoglycerate catalyzed by phosphoglycerate kinase (*pgk*) [59]. 8) The shift of phosphate from 3-phosphoglycerate to 2-phosphoglycerate catalyzed by phosphoglycerate mutase (*gpmA*) [59]. 9) Dehydration of 2-phosphoglycerate to

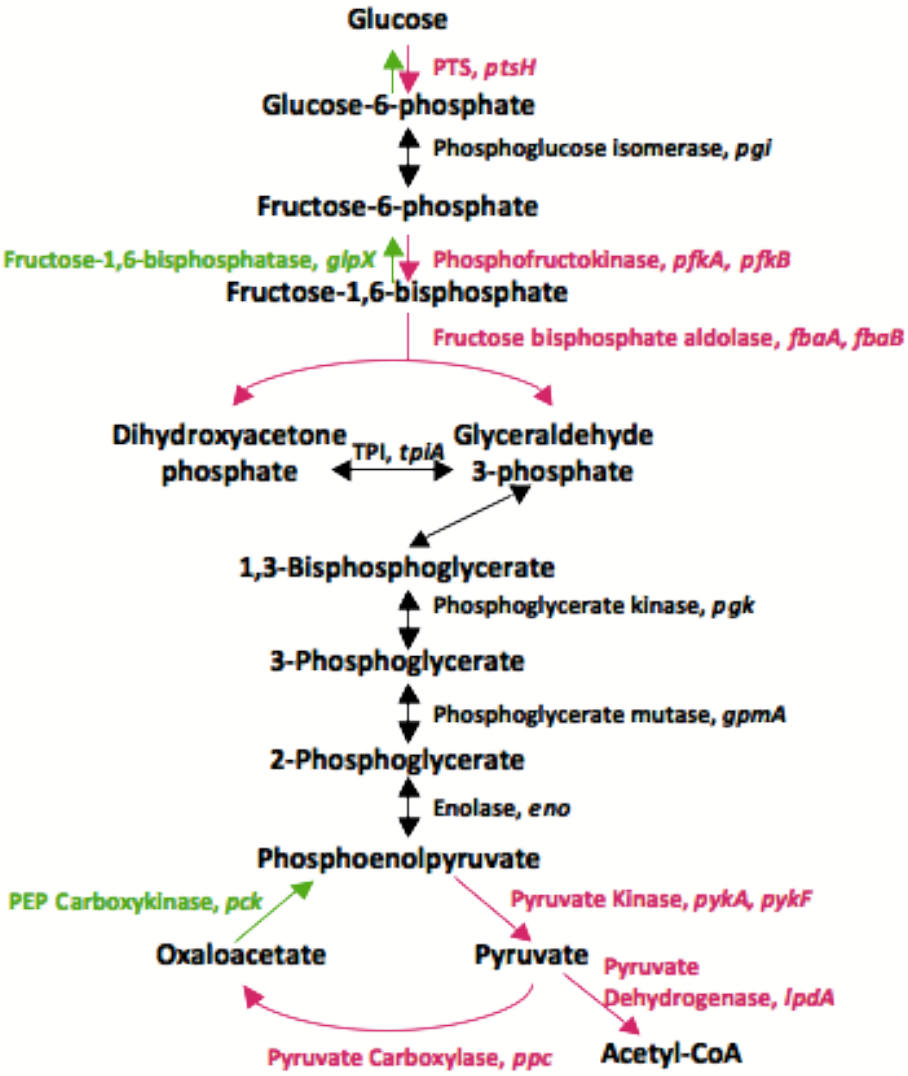

Figure 3. Glycolysis and Gluconeogenesis [60]. Irreversible glycolytic enzymes are shown in unidirectional pink arrows, and irreversible gluconeogenic enzymes are in unidirectional green arrows. Reversible glycolytic and gluconeogenic enzymatic reactions are denoted with bidirectional arrows in black. Enzyme name and gene notations are given for each enzyme.

phosphoenolpyruvate catalyzed by enolase (*eno*) [61]. 10) The irreversible action of pyruvate kinase (*pykA, pykF*) to form pyruvate and to synthesize ATP [62]. Thus, the net result of glycolysis is to catabolize 1 glucose molecule into 2 pyruvate molecules, 2 ATP molecules, and 2 NADH molecules.

Gluconeogenesis works in opposition to glycolysis to produce glucose from non-carbohydrate precursors, such as pyruvate, lactate, glycerol, amino acids and TCA cycle intermediates **(Figure 3)**. There are four steps of glycolysis that are irreversible and thus need bypass reactions for gluconeogenesis: 1) the conversion of glucose to glucose-6-phosphate catalyzed by glucokinase (Glk) [53], 2) the synthesis of fructose-1,6-bisphosphate from fructose-6-phosphate, catalyzed by PfkA and PfkB [63], 3) the conversion of phosphoenolpyruvate to pyruvate by 2 pyruvate kinase isoenzymes (PykA and PykF) [64, 65], and 4) the synthesis of phosphoenolpyruvate from oxaloacetate, catalyzed by phosphoenolypyruvate carboxykinase (Pck) [66]. To bypass these irreversible steps of glycolysis, gluconeogenic carbon sources utilize the following enzymes: 1) the conversion of fructose-1,6-bisphosphate from fructose-6-phosphate is catalyzed by four isoenzymes (Fbp, GlpX, YbhA, YggF) [67-70], and 2) phosphoenolpyruvate synthetase (PpsA) catalyzes the conversion of pyruvate to phosphoenolpyruvate [71]. The rest of the glycolytic reactions can work in reverse, and thus these enzymes work in both the glycolytic and gluconeogenic pathways. Gluconeogenic carbon sources, once converted into pyruvate or PEP through other cellular metabolic processes, are fed into the anabolic pathway leading to the production of glucose and other glycolytic intermediates. The

conversion of pyruvate to glucose during gluconeogenesis requires the utilization of 2 molecules of ATP and 2 molecules of NADH.

Regulation of Carbon Flux Between Glycolysis and Gluconeogenesis

Since glycolysis and gluconeogenesis are interconnected pathways, the flux of carbon between the two pathways must be controlled; if both pathways were active at the same time, a futile cycle would occur resulting in the net hydrolysis per reaction cycle of 4 NTPs (2 ATPs and 2GTPs) [52]. Cells utilize a combination of three regulatory mechanisms to regulate flux of carbon between these two seemingly opposing pathways: 1) controlling the concentration of enzymes responsible for the rate-limiting steps of glycolysis and/or gluconeogenesis, 2) allosteric regulation of enzymatic activity, and 3) covalent modification of enzymes by post-translational modification (PTM) [72]. Thus, the *in vivo* capacity of an enzyme to affect metabolic flux is a function of the enzyme's abundance and its kinetic properties [72, 73].

Regulating the concentration of metabolic enzymes

Regulating the concentration of metabolic enzymes is most commonly accomplished by controlling their expression [72]. In *E. coli*, there are seven global transcriptional regulators (ArcA, CRP, Fis, Fnr, IHF, Lrp, and NarL) [74]. Of these, three regulate expression of genes involved in central metabolism: 1) the cAMP-receptor protein (CRP) [75], 2) the two component signal transduction pathway ArcB/ArcA, which regulates expression of genes in response to redox conditions [76, 77], and 3) the anoxic

responsive regulator FNR [76, 78, 79]. There are also two additional regulators of central carbon metabolism: the cAMP-independent catabolite repressor-activator Cra [75, 80], and the global repressor Mlc, which regulates expression of parts of the glucose phosphotransferase system (PTS) [81]. Although expression of metabolic enzymes is tightly regulated by CRP, ArcB/ArcA, FNR, Cra, Mlc, or a combination of these regulators, research suggests that central metabolic flux is not regulated by controlling the expression of metabolic enzymes alone as deletions of each of these regulators does not greatly affect metabolic flux [82]. Perrenoud and Sauer systematically deleted ArcA, ArcB, Cra, Crp, Cya, Fnr, and Mlc, and tested the affects of each deletion on aerobic glucose catabolism using metabolic flux analyses. Only deletion of *arcA* showed any effect on metabolic flux, with TCA activity increasing over 60% when compared to the WT strain [82]. Deletion of *cra* and *crp* resulted in a growth defect but no significant change in metabolic flux when compared to WT [82]. These results indicate that gene expression alone does not regulate carbon flux through central metabolism.

Although deletion of CRP shows little effect on carbon flux [82], CRP is thought to play an important role in regulating the flux of carbon, by regulating expression of several of the irreversible glycolytic and gluconeogenic enzymes: GlpX, Glk, YggF, PpsA, and Pck [83]. These enzymes dictate the entry and exit from the central glycolysis and gluconeogenic pathways, leading to the hypothesis that CRP may regulate the switch between glycolysis and gluconeogenesis [83].

Allosteric regulation of metabolic enzyme activity

Allosteric regulation of enzyme activity plays an important role in regulating carbon flux through glycolysis and gluconeogenesis. This type of regulation exclusively modulates the kinetic activity of enzymes via the non-covalent binding of inhibitors or activators to non-catalytic sites (allosteric sites) [72]. Inhibition of enzymatic activity can occur using many different allosteric regulators: 1) malate and aspartate inhibit Ppc activity [84-87], 2) ATP and phosphoenol pyruvate (PEP) inhibit Pck activity [66], 3) ADP, AMP malate, oxaloacetate, and PEP inhibit Pps activity [88], and 4) both GTP and succinyl coenzyme A inhibit PykA activity [89]. Activation of enzymatic activity occurs via a number of different allosteric activators: 1) calcium allosterically activates Pck activity [90], 2) fructose-1,6-diphosphate activates PykA [89], and 3) both NMP and ribose-5-phosphate activate PykF activity [91]. These are just a few of the factors that are known to inhibit or activate glycolytic and gluconeogenic enzyme activity; there are many more known and unknown factors that are capable of allosterically regulating enzymatic activity. Thus, allosteric regulation of enzymatic activity plays a critical role in regulating carbon flux through central metabolism [73].

Affects of non-acetylation PTMs on metabolic enzyme activity

Of the three layers of metabolic enzyme regulation, post-translational modifications of enzymes has the ability to affect both enzyme abundance and kinetic activity [72]. There are many different types of PTMs that are known to affect metabolic enzyme activity, with phosphorylation of enzymes being the most well-understood and

studied PTM of metabolic enzymes. The phosphorylation of tyrosine, serine, and threonine residues on enzymes serves as an important control mechanism for enzyme activity and abundance [92, 93]. The first identified and studied phosphorylation of a bacterial metabolic enzyme was that of isocitrate dehydrogenase (ICDH) from *E. coli*. ICDH, a TCA cycle enzyme, catalyzes the conversion of isocitrate into α-ketoglutarate [93, 94]. Phosphorylation of ICDH disrupts the binding of the ICDH cofactor NADPH, which leads to an almost complete loss in enzyme activity [95-97].

Besides phosphorylation, other PTMs, such as lysine succinylation, and pupylation via the conjugation with the ubiquitin-like protein (PUP), are hypothesized to regulate metabolic flux [72]. In *E. coli*, lysine succinylation has been identified on three metabolic enzymes: ICDH, serine hydroxymethyltransferase, and GAPDH [98]. Succinylation of these proteins is hypothesized to occur using the succinyl donor succinyl-CoA, with succinylation postulated to alter protein structure and function [98]. The full effects of lysine succinylation on metabolic enzyme activity and carbon flux, however, are not known.

Pupylation of proteins occurs when a small protein, Pup, is transferred to the ε-amino group of a target lysine [99]. Pupylation is considered the bacterial analog of ubiquitin modification in eukaryotes. When bacterial proteins become pupylated, they are targeted for degradation using archaeal-type mycobacterial proteosome machinery [100-102]. In bacteria, pupylation has thus far only been identified and studied in *Mycobacterium tuberculosis,* with over 40% of the identified pupylations occurring on

metabolic enzymes [103]. To date, there are no studies on the effects and regulation of metabolic enzyme pupylation.

Affects of acetylation on metabolic enzyme activity and carbon flux

Acetylation of a bacterial metabolic enzyme was first detected on *S. enterica* acetyl-CoA synthetase (Acs) [16]. K609 was found to be reversibly acetylated by the KAT Pat and the sirtuin CobB. This acetylation blocks Acs catalytic activity [16]. This regulation of Acs acetylation extends to a wide variety of bacteria [15, 22, 39]. It also extends to eukaryotes, as the two isoforms of eukaryotic Acs, AceS1 (cytoplasm) and AceS2 (mitochondria), are acetylated on K661, the residue that corresponds to *S. enterica* K609. Furthermore *S. enterica* Pat and eukaryotic SIRT1 can regulate AceS acetylation and deacetylation, respectively [104, 105]. Acetylation of Acs, whether bacterial or eukaryotic, was shown to inhibit its activity both *in vivo* and *in vitro* [15, 22, 39].

Since the discovery of Acs acetylation, proteomic studies in both *E. coli* and *S. enterica* have provided evidence for acetylation of many additional metabolic enzymes [1-3]. Of these, many are involved in glycolysis, the TCA cycle, and amino acid and lipid metabolism. Similarly, metabolic enzyme acetylation was found to be a highly prevalent modification in mammalian liver cells [106-109] and in liver mitochondria [108], suggesting the possibility that acetylation may be an ancient mechanism by which cells regulate metabolism [60].

The diversity of acetylated metabolic enzymes suggests that acetylation may perhaps serve as a switch that can regulate enzymatic activity [1-3]. In addition to Acs,

there are only two other glycolytic and gluconeogenic enzymes where studies to understand the effects and regulation of enzyme acetylation have been done: GAPDH, and PEPCK. Phosphoenolpyruvate carboxykinase (PEPCK) is a key enzyme that dictates the switch between glycolysis and gluconeogenesis, and catalyzes the conversion of oxaloacetate to phosphoenolpyruvate. PEPCK acetylation has been studied in yeast, and that research reveals that PEPCK acetylation and deacetylation is regulated by the nucleosome KAT of the H4 complex, and the KDAC Sirtuin 2, respectively [110]. PEPCK acetylation increases under high nutrient conditions, when the cells are fed glucose, and decrease in a low-nutrient condition, when cells are solely fed amino acids [109]. Acetylation of PEPCK affects its activity by regulating the stability, and thereby the abundance, of the protein. Under high nutrient conditions, PEPCK is acetylated and thus unstable. Stability returns when nutrients become depleted and PEPCK becomes deacetylated [109]. It appears, therefore, that PEPCK, and perhaps other metabolic enzymes, can respond to changes in environmental conditions, perhaps through the actions of its cognate KAT and KDAC.

Acetylation and deacetylation of GAPDH (also known as GapA) was reported to be regulated by *S. enterica* Pat and CobB, respectively [3], and it is hypothesized that acetylation of GAPDH affects flux between glycolysis and gluconeogenesis. Although Wang *et. al.* convincingly show in their study that GAPDH can get acetylated, contrary to popular opinion [60], the study did not fully elucidate the *in toto* affects of GAPDH acetylation on metabolic flux. This is because metabolic flux analysis only reveals the pathways affected by mutation of either the KAT or KDAC thought to regulate

acetylation [3], or of a transcription factor hypothesized to regulate metabolism [82, 111, 112]. At best, there is correlative evidence in *S. enterica* that metabolic enzyme acetylation regulates flux, as deletion of the sirtuin *cobB* resulted in a 47% increase, and deletion of the KAT *pat* resulted in a 40.7% decrease in the glycolysis/gluconeogenesis flux ratio, respectively, when compared to the WT strain [3]. Due to the lack of knowledge obtained using the singular approach of Wang *et al.,* researchers have proposed that future studies should incorporate traditional flux analyses with omics data from transcriptional analyses so that instead of assessing individual regulatory events, genome-scale fluxes can be estimated through stoichiometric modeling [72], as was performed in yeast [113, 114].

Thus, the full effects of metabolic enzyme acetylation on glycolytic/gluconeogenic flux and central metabolism are not known, and much work remains. Our lab aims to further this research by trying to elucidate the mechanisms of the regulation of metabolic enzyme acetylation, and to start to elucidate the effects of acetylation on carbon flux through central metabolism.

cAMP Receptor Protein, CRP

The discovery of the cAMP receptor protein, CRP, occurred when researchers tried to identify the components required to regulate β-galactosidase transcription in *E. coli*. Adenosine 3', 5'-cyclic phosphate (cyclic AMP) was known to regulate β-galactosidase (*lacZ*) transcription [115-117], as both biochemical and genetic studies indicated that cAMP stimulated *lacZ* transcription [118, 119], cell-free extracts of *E. coli* were able to produce β-galactosidase *in vitro* when the extracts were supplemented with

lac operon-containing DNA[120], and the addition of cAMP to this system increased β-galactosidase synthesis [121]. Purification and characterization of a cAMP binding protein then followed [122], and this protein, CRP, now serves as a model for transcriptional regulation in bacteria.

CRP is now known as one of the seven global transcription factors of *E. coli* [123], regulating expression of over 200 genes or roughly 5% of all *E. coli* genes [124]. CRP is activated upon binding of the second messenger cAMP, in response to the advent of carbon starvation. When bound by cAMP, the CRP dimer binds its DNA sites with high affinity and, as such, can either activate or repress transcription depending on promoter context.

CRP regulates transcription by using three surface-exposed patches known as activating regions that interact with RNA polymerase (RNAP) to control gene expression from various promoters **(Figure 4a)** [123]. Transcription activation by CRP at the simplest promoters occurs when CRP binds to a consensus site [125], centered at about 60, 70 or 80 (Class I) or at about 40 (Class II) base pairs upstream of the transcription start site (+1) [126]. At Class I promoters, activating region 1 (AR1) interacts with the C-terminal domain of the α subunit of RNAP (α-CTD) **(Figure 4b)** [126, 127]. At Class II promoters, the α-NTD (N-terminal domain of the α subunit of RNAP) interacts with the activating region 2 (AR2) **(Figure 4c)**. At some Class II promoters, the AR1/α-CTD interaction also participates in transcription initiation [126, 128-131]. At other Class II promoters, an interaction between AR3 and the σ subunit of RNAP participates. Of the three activating regions, AR2 & AR3 contain charged residues that, when disrupted, result in altered

interactions between CRP and RNAP, leading to a change in gene expression from CRP-activated promoters. We will discuss the AR2 and AR3 interactions with RNAP in detail, and thus, we will focus on CRP-dependent transcription from Class II promoters.

Interactions between Activating Region 2 and RNA polymerase

At Class II CRP-dependent promoters, CRP interacts with RNAP polymerase in three different places: AR1 makes contact with the α-CTD of RNAP, AR2 makes contact with the α-NTD of RNAP, and AR3 can make contact with domain 4 of the σ^{70} subunit of RNAP **(Figure 4c)** [128]. Detailed and elegant work by several researchers has elucidated the different mechanistic consequences of the AR1 and AR2 interactions on CRP-dependent transcriptional activation. At Class II CRP-dependent promoters, the α-CTD acts as an inhibitory determinant, and the AR1-α-CTD interaction overcomes the inhibitory effect of the α-CTD, resulting in an increase in binding between RNAP and the DNA [132, 133]. Thus, the AR1-α-CTD interaction allows the formation of the transcription closed complex (TCC) [126].

The AR2-α-NTD interaction increases the rate of isomerization from the TCC to the transcription open complex (TOC), by stabilizing the transition state between the TCC and the TOC [128]. AR2 is a surface exposed patch of CRP consisting of the following residues: Histidine-19, Histidine-21, Glutamate-96, and Lysine-101 [128, 132]. These residues interact with a string of four negatively charged residues on the α-NTD: Glutamate-162, Glutamate-163, Aspartate-164, and Glutamate-165 [128].

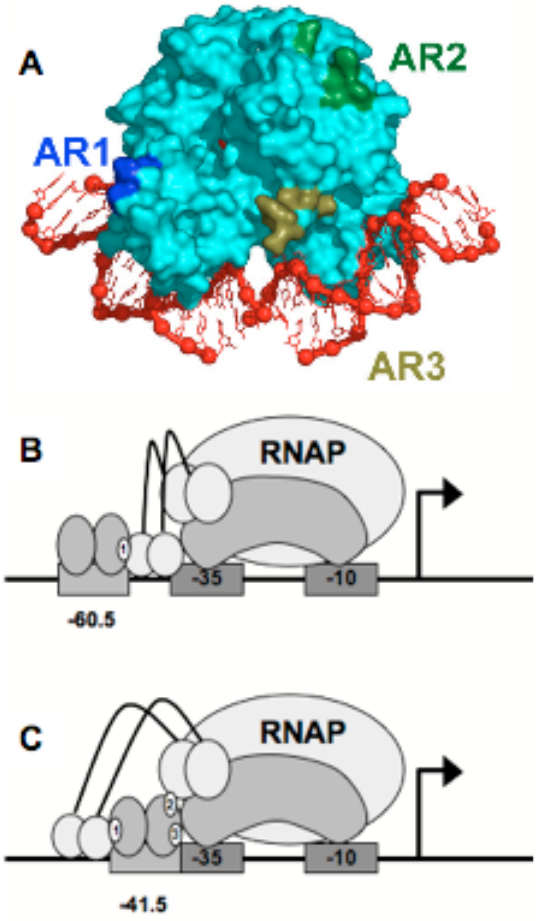

Figure 4. Regulation of transcription by cAMP receptor protein (CRP). A) Structure of the CRP-DNA complex showing AR1 in blue, AR2 green, and AR3 in olive green. B) Transcription activation can occur by a Class I mechanism, where CRP binds to an upstream site centered near positions –61, -71, -81, -91 and interacts through Activation Region 1 (AR1, 1) with an α-CTD C). Activation can also occur via a Class II mechanism, where CRP binds a site near the –35 hexamer and interacts with RNAP via two surfaces: activating region (AR1, 1) and Activation Region 2 (AR2, 2). Figures adapted from Wolfe (2009), and Lawson *et al.* (2004).

This interaction between AR2 of CRP and residues 162-165 of the α-NTD appears to be electrostatic in nature, with the distance between the two proteins dictating the strength of the interaction [128]. This indicates that both charge and size of the amino acid residues on both CRP and RNAP are required to maintain the needed interactions. Thus, mutation of any of the residues required for participating in this interaction leads to a disruption of the electrostatic interaction, and consequently, loss of transcription from CRP-dependent Class II promoters [128].

Specifically, mutation of the charged residue K101 residue in AR2, to the uncharged and smaller residue alanine (K101A), or charge reversal to a glutamate (K101E) results in loss of transcription from Class II promoters [134]. These results indicate that the positive charge of K101 is likely required for Class II transcriptional activation [134]. Interestingly, K101 was one of two residues of CRP whose acetylation was identified using mass spectrometry of whole cell lysates of *E. coli* [1, 2]. While the regulation and effects of K101 acetylation are not known, acetylation of K101 would be predicted to neutralize the charge of the K101 [135, 136] and, as previous studies have shown, the charge of K101 is required to maintain the electrostatic interactions between AR2 of CRP and the αNTD of RNAP [128, 132]. We therefore hypothesize that acetylation of K101 leads to a disruption in the electrostatic interactions between AR2 and the αNTD, resulting in loss of transcription from Class II promoters.

Interactions between Activating Region 3 and RNA polymerase

AR3 of CRP is a complex region of CRP, containing both an activating determinant consisting of the negatively charged residues Aspartate-53, Glutamate-54, Aspartate-55, and Glutamate-58, and an inhibitory determinant consisting of the positively charged residue Lysine-52 [130, 131, 137-139]. The residues of AR3 interact with a positively charged patch of residues on domain 4 of σ^{70} subunit of RNAP, consisting of residues Arginine-596, Arginine-599, Lysine-593, and Lysine-597 [130]. Specifically, the side chains of K593, K597, and R599 are thought to form a cluster that interacts with residues K52-D55 and E58 of AR3 of CRP **(Figure 5)**. R596 of domain 4 of σ^{70} is thought to be separate from the cluster, and clashes with the inhibitory residue K52 of AR3.

Thus, AR3 residues 53-55 and 58 increase the rate of isomerization from the TCC to the TOC at CRP-dependent Class II promoters, while K52 plays an inhibitory role at these promoters [130, 131]. The positively charged K52 interacts with a positively charged surface of RNAP, sterically preventing tight interactions between the two proteins. Mutation of the charged K52 residue on AR3 to an uncharged asparagine (N52) results in a tighter interaction between CRP & RNAP, because the negatively charged residues on CRP can interact with the positively charged residues on RNAP [130, 131]. Thus, neutralization of the charged K52 permits a tighter interaction between the two proteins. AR3, therefore, consists of activating and inhibiting determinants that balance each other, so that at Class II promoters, the dominant interactions between CRP and RNAP occur via AR1 and AR2 [130, 131].

Proteomics studies identified acetylation of CRP at two locations, K101 (as described above) and K52 [1, 2]. Acetylation of K52 would be predicted to neutralize its charge [135, 136] and thus could play a physiologically relevant role similar to that of the K52N mutation - by neutralizing K52, acetylation would permit a tighter RNAP-CRP interaction, and lead to a change in transcription from Class II promoters. Thus, CRP acetylations of K52, or K101, or both K52 and K101, have the potential to impact gene expression from a wide variety of CRP-dependent Class II promoters, and further the mechanistic understanding of how CRP regulates Class II-dependent transcription.

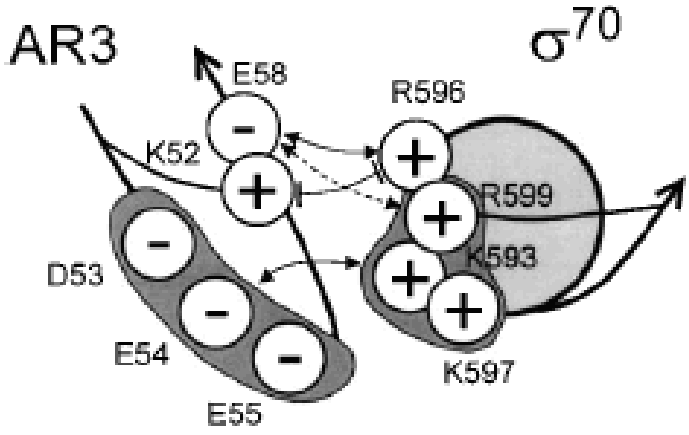

Figure 5. Proposed interactions between AR3 and the σ^{70} subunit of RNAP that may regulate transcription from a Class II CRP-dependent promoter. AR3 lies within an exposed β-turn, with the large arrowhead depicting the direction of the C-terminus of the peptide chain. AR3 residues D53, E54, and E55 form a negatively charged patch, and residues K52 and E58 are adjacent to each other. σ^{70} residues are located on an α-helix, with the large arrowhead depicting the direction of the C-terminus of the peptide chain. σ^{70} residues K593, K597, and R599 form a positively charged cluster. Interactions between AR3 and σ^{70} are indicated as follows: productive (double headed arrows), non-productive (line with perpendicular ends), and weak interactions (dotted line). Figure adapted from Rhodius & Busby (2000).

Summary

Lysine acetylation is a post-translational modification that affects proteins in many different ways, and thus has a significant impact on many different aspects of cellular physiology. In bacteria, lysine acetylation is a relatively newly discovered phenomenon, and much remains to be elucidated concerning the regulation of acetylation, the effects of acetylation on various cellular processes, and the evolutionary costs and reasoning for the conservation of acetylation across the three domains of life.

We wish to understand the regulation and effects of metabolic enzyme acetylation in the enterobacterium *E. coli*. It is known that metabolic enzyme acetylation occurs in *E. coli*, but much remains unknown concerning both the mechanisms that regulate acetylation and the downstream effects of acetylation on enzyme function and carbon flux through central metabolism. Furthermore, we aim to understand the regulation and effects of acetylation of the transcriptional regulator CRP, as this transcription factor responds to and regulates carbon metabolism and has been implicated in potentially regulating acetylation in *E. coli*.

The goal of my thesis project is to start the process of elucidating the factors that regulate metabolic enzyme acetylation, and attempt to understand how metabolic enzyme acetylation affects metabolism. There is research in eukaryotes, and preliminary research in *S. enterica,* suggesting that acetylation serves to regulate carbon flux through central metabolism, but significant work needs to be done to fully understand the effects of metabolic enzyme acetylation on central metabolism.

Our lab has shown that acetylation of RNAP and RcsB can affect gene expression from at least two promoters, *cpxP* and *rprA*, respectively. Acetylation of CRP is predicted to affect expression of at least some of the Class II-CRP-dependent-genes, and we hope to elucidate some of the effects of CRP acetylation on Class II-gene expression.

CHAPTER II

MATERIALS AND METHODS

Bacterial strains and plasmids

All bacterial strains used in this study are listed in **Table 1**. Derivatives were constructed by generalized transduction with P1kc, as described previously [140]. Plasmids used in this study are listed in **Table 2.** Oligonucleotide primers used in this study are listed in **Table 3.**

Culture conditions

For strain construction, cells were grown in Luria-Bertani (LB) containing 1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract, and 0.5% (wt/vol) sodium chloride. LB plates also contained 1.5% agar. For Western immunoblot analysis, cells were grown in buffered TB that contained 1% (wt/vol) tryptone buffered at pH 7.0 with 100 mM potassium phosphate (TB7). Cell growth was monitored spectrophotometrically (DU640; Beckman Instruments, Fullerton, CA, USA) by determining the optical density at 600 nm (OD_{600}). Kanamycin (40 μg ml^{-1}), ampicillin (100 μg ml^{-1}), and chloramphenicol (25 μg ml^{-1}) were added as needed.

Western immunoblot analysis

To observe the differences in protein acetylation profiles, 10 ml cell cultures were grown at 37^{o}C with agitation at 250 rpm in TB7 for 7.5 hours (to allow entry into stationary phase). Cells were harvested and pelleted by centrifugation and pellets resuspended with Tris-EDTA pH 8.0. Samples were lysed by sonication, and the proteins separated by SDS-12% polyacrylamide gel electrophoresis (PAGE), transferred to PVDF membranes, and subjected to Western immunoblot analysis. Two polyclonal anti-acetyllysine antibodies were used: Cell Signaling Technology at a 1:200 dilution, and ImmuneChem at a 1:500 dilution. Primary incubation was carried at 4^{o}C with shaking, followed by 4 washes for 5 minutes each in phosphate buffer with 1% Tween (PBST). Secondary goat anti-rabbit Immunoglobulin G antibody at a 1:1000 dilution conjugated to horseradish peroxidase was incubated for 2 hours at room temperature with shaking, followed by 3 washes for 5 minutes each in PBST. Enhance chemiluminescence Western immunoblotting reagents (LumiGlo, Cell Signaling Technology) were used for visualization, according to manufacturer's instructions. When needed, OneMinute Western Blot Stripping Buffer (GM Biosciences) was used to strip membranes, according to manufacturer's instructions.

Phenotype MicroArray^{TM} assays

Metabolic Phenotype MicroArray^{TM} assays were performed as previously described [5, 141]. Briefly, bacteria were resuspended from LB plates into 10 ml of IF-0a GN/GP Base IF (Biolog Inc.) to an 85% transmittance, and PM media was prepared

according to manufacturer's instructions. PM1 and PM2 microplates (Biolog Inc.) were inoculated with 100 μl of PM media containing the bacterial suspension and incubated at 37^{o}C for 48 hours in the Omnilog Incubator (Biolog). OD600 measurements were taken at 15 minute intervals (OmniLog PM DC 1.30.01), and OmniLog PM software was used for data analysis and kinetic and parametric plot generation. Parametric plot generations of three independent experiments comparing mutant and wild type strains was used to generate average plot height for data comparisons using a significance threshold >20, as per manufacturer's instructions.

Growth curve analyses

To determine the effects of mutation of genes encoding putative and known KATs on carbon metabolism, we grew cells in 25 ml of TB7 supplemented with and without the following carbon sources: 0.4% glucose (w/v), 0.8% lactate (w/v), 15 mM acetate, 0.6% succinate (w/v), 0.8% glycerol (v/v), under different oxygenation conditions: either high levels of oxygenation or aeration, corresponding to 250 rpm, medium levels of oxygenation, 100 rpm, or static cultures for low levels of oxygenation, or microaerophilic conditions. Cultures were grown for 8-10 hours, with OD600 readings taken every 30 minutes.

Expression and purification of proteins

Chloramphenicol resistant ASKA plasmids [142] for YfiQ, RcsB (our positive control, see Hu *et al.,* in preparation), and our metabolic enzymes were purified from the

ASKA collection and transformed into kanamycin resistant BL21 Magic cells [143]. Protein was expressed in the presence of 34 μg/ml chloramphenicol and 35 μg/ml kanamycin and purified as described (Kuhn *et al.*, 2012, in preparation). Since the ASKA clones did not contain a cleavable polyhistidine affinity tag, we cloned the *yfiQ, rcsB, pfkA, pfkB,* and *lpdA genes* into the pMCSG7 vector [143, 144], which contains a tobacco etch virus cleavage site after the N-terminal polyhistidine tag. All assays were performed using the protein produced from ASKA clones because we did not obtain soluble expression of the pMCSG7 clones and therefore could not remove the tag.

In vitro acetylation assay

Purified YfiQ and potential substrates were incubated in Tris-HCl buffer pH 8 with the following components: 50 mM Tris-HCl pH 8.0, 10% glycerol (v/v), 0.1 mM EDTA, 1 mM DTT, and 10 mM sodium butyrate. Each reaction mixture was prepared just prior to use in a total reaction volume of 100 μl and reactions were incubated for 2 hours at 37^{o}C in a water bath. 2x SDS loading buffer was added to stop the reaction, and samples were heated for 5 minutes at 100^{o}C to denature proteins. Proteins were then separated by 12% SDS-PAGE. To compare the acetylation state of proteins before and after incubation with YfiQ, anti-acetyllysine Western immunoblot analysis was performed using the method described above.

Ellman's assay

Biochemical assays were performed as described previously (Kuhn *et al.,* 2012, in preparation) with some modifications. First, reactions were performed in the presence of

0.15 mM NaCl at 25°C for 1 hour to prevent protein precipitation during the reaction. Additionally, after stopping the reactions with guanidine HCl samples were transferred to Nanosep 10K MWCO centrifugation devices (Pall Life Sciences, VWR) and centrifuged at 13,000 rpm for 10 minutes to separate CoA and the protein. The Ellman's reagent was then added to the flow-through and monitored as described (Kuhn *et al.,* 2012, in preparation). High concentrations of YfiQ (100 μg) were necessary to detect activity (see note in cloning section of methods), most likely due to the polyhistidine tag competing with binding of AcCoA.

Promoter activity assays

To monitor promoter activity from *Pacs205-lacZ* [145], or *PCC-41.5-lacZ* [146], cells were grown in TB7 or TB7 supplemented with various carbon sources for 8 hours. Every 60 minutes, 50 μl aliquots were harvested and 50 μl of All-in-One β-galactosidase reagent (Pierce Biochemical) was added at the end of the 8-hour experiment. β-Galactosidase activity was then determined quantitatively using a microtiter format, as described previously [147].

Orbitrap-mass spectrometry and protein identification.

Either whole cell lysates from complemented CRP strains (AJW 2313), or vector control (AJW 4524) strains were run on an 12% SDS-PAGE gel, and gel slices were excised and subjected to tryptic digestion, as described previously [148]. Tryptic peptides were separated and measured online by ESI-mass spectrometry using a nanoACQUITY UPLC™ system (Waters, Milford, MA) coupled to an LTQ Orbitrap™ XL mass

spectrometer (Thermo Fisher Scientific, Waltham, MA). A trap column (Symmetry® C18, 5 µm, 180 µm inner diameter x 20mm, Waters) was used for desalting. Elution was performed onto an analytical column (BEH130 C18, 1,7 µm, 100 µm inner diameter x 100 mm, Waters) by a binary gradient of buffers A (0.1% (v/v) acetic acid) and B (99.9 % (v/v) acetonitrile, 0.1% (v/v) acetic acid) over a period of 80 min with a flow rate of 400 nl/min. The Orbitrap XL was operated in data-dependent MS/MS mode using the lockmass option for real time recalibration. Proteins were identified by searching all MS/MS spectra in "dta" format against an *E. coli* database (extracted from the Uniprot-KB database: http://www.uniprot.org/uniprot/?query=Escherichia+coli+K12&sort=score), using **Sorcerer™-SEQUEST®** (Sequest v. 2.7 rev. 11, Thermo Electron including Scaffold_3_00_05, Proteome Software Inc., Portland, OR).

The Sequest search was carried out considering the following parameters: a parent ion mass tolerance - 10 ppm, fragment ion mass tolerances of 1.00 Da. Up to two tryptic miscleavages were allowed. Methionine oxidation (+15.99492 Da), cysteine carbamidomethylation (+57,021465 Da) and lysine acetylation (+42.010571 Da) were set as variable modifications. Proteins were identified by at least two peptides applying a stringent SEQUEST filter. Sequest identifications required at least ΔCn scores of greater than 0.10 and XCorr scores of greater than 1.9, 2.2, 3.3 and 3.8 for singly, doubly, triply and quadruply charged peptides. Acetylated peptides that passed these filter criteria were examined manually and accepted only when b- or y- ions confirmed the acetylation site

TABLE 1. Bacterial strains used in this study.

Strain	Relevant characteristics	Source/Reference
AJW678	*thi-1 thr-1(Am) leuB6 metF159(Am) rpsL136 lacX74*	[149]
AJW2179	AJW678 λ*pacs205*	[145, 150]
AJW2198	AJW2179 Δ*crp::kan*	[145, 150]
AJW2313	AJW2198 pDCRP	[129, 150]
AJW2314	AJW2198 pDCRP/K101E	[129, 150]
AJW2315	AJW2198 pDCRP/K52N	[129, 150]
AJW2316	AJW2198 pDCRP/K101E/K52N	[129, 150]
AJW3660	AJW2198 pDCRP/H159L	[129, 150]
AJW3661	AJW2198 pDCRP/H159L/K52N	[129, 150]
AJW3763	AJW2316 pCA24N	[142]
AJW3764	AJW2316 pCoaA	[142]
AJW3897	AJW2198 pCA24N	[142]
AJW3898	AJW2198 pCRP (pCA24N)	[142]
AJW4333	AJW2179 Δ*yjhQ::kan*	P1:JW4269→AJW2179 [151]
AJW4344	AJW2179 Δ*yfiQ::kan*	P1:JW2568→AJW2179 [151]
AJW4355	AJW4333 pDCRP	[129, 150]
AJW4366	AJW4344 pDCRP	[129, 150]
AJW4456	AJW678 Δ*crp::kan*	P1:JW5702→AJW2179 [148]
AJW4524	AJW2198 pBR322/pDU9	[129, 150]
AJW4525	AJW2179 pBR322	[129, 150]
AJW4526	AJW2179 pDCRP	[129, 150]
AJW4527	AJW4333 pBR322	[129, 150]
AJW4786	AJW4456 pRW50 CC-41.5	[152]
AJW4787	AJW4456, pDCRP	[129, 150, 152]
AJW4788	AJW4786 pDCRP/K101E	[129, 150, 152]
AJW4870	AJW4786 pDCRP/K52N	[129, 150, 152]
AJW4871	AJW4786 pDCRP/K101E/K52N	[129, 150, 152]
AJW4872	AJW4786 pDCRP/H159L	[129, 150, 152]
AJW4873	AJW4786 pDCRP/H159L/K52N	[129, 150, 152]
AJW4956	AJW4786 pDCRP/K101A	[129, 150]
AJW4957	AJW4786 pDCRP/K101Q	[129, 150]
AJW4958	AJW4786 pDCRP/K101R	[129, 150]

TABLE 2. Plasmids used in this study

Plasmid	Relevant Characteristics	Source/Reference
pBR322	Control plasmid (Amp^R)	[129, 150]
pDCRP	Plasmid expressing CRP (Amp^R)	[129, 150]
pDCRP/K101E	Plasmid expressing K101E (AR2) variant of CRP (Amp^R)	[129, 150]
pDCRP/K52N	Plasmid expressing K52N (AR3) variant of CRP (Amp^R)	[129, 150]
pDCRP/K101E/K52N	Plasmid expressing K101E/K52N (AR2,3) variant of CRP (Amp^R)	[129, 150]
pDCRP/H159L	Plasmid expressing H159L (AR1) variant of CRP (Amp^R)	[129, 150]
pDCRP/H159L/K52N	Plasmid expressing H159L/K52N (AR1,3) variant of CRP of CRP (Amp^R)	[129, 150]
pCA24N	Control plasmid (Cm^R)	[142]
pCA24N-*coaA*	Plasmid expressing 6xHis-CoaA under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*crp*	Plasmid expressing 6xHis-Crp under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*tpiA*	Plasmid expressing 6xHis-TpiA under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*fbaA*	Plasmid expressing 6xHis-FbaA under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*maeB*	Plasmid expressing 6xHis-MaeB under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*gpmA*	Plasmid expressing 6xHis-GpmA under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*yjhQ*	Plasmid expressing 6xHis-YjhQ under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*lpdA*	Plasmid expressing 6xHis-LpdA under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*pykF*	Plasmid expressing 6xHis-PykF under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*pgk*	Plasmid expressing 6xHis-Pgk under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*mdh*	Plasmid expressing 6xHis-Mdh under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*pck*	Plasmid expressing 6xHis-Pck under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*ppc*	Plasmid expressing 6xHis-Ppc under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*pykA*	Plasmid expressing 6xHis-PykA under the control of an IPTG-inducible promoter (Cm^R)	[142]

TABLE 2 (cont). Plasmids used in this study

Plasmid	Relevant Characteristics	Source/Reference
pCA24N-*gapA*	Plasmid expressing 6xHis-GapA under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*pfkA*	Plasmid expressing 6xHis-PfkA under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*pfkB*	Plasmid expressing 6xHis-PfkB under the control of an IPTG-inducible promoter (Cm^R)	[142]
pCA24N-*ptsH*	Plasmid expressing 6xHis-PtsH under the control of an IPTG-inducible promoter (Cm^R)	[142]
pPRW50 CC-41.5	Plasmid expressing *lacZ* under the control of the semi-synthetic CC-41.5 promoter (Tet^R)	[152]
pDCRP/K101R	Plasmid expressing K101R variant of CRP (Amp^R)	[129, 150]
pDCRP/K101A	Plasmid expressing K101A variant of CRP (Amp^R)	[129, 150]
pDCRP/K101Q	Plasmid expressing K101Q variant of CRP (Amp^R)	[129, 150]

TABLE 3. Oligonucleotide primers used in this study

Target gene	Sequence from 5' to 3'
crp	
F:	AACAGACCCCAGTCTCGAATCGTT
R:	TACGTTCCTGGCCCTCTTCAAACA
Purpose: Primer set binds at 5' and 3' ends of CRP. Used to verify *crp* deletions.	
yjhQ	
F:	TCAATGGCCAGCCCTATCGAATCA
R:	TTGCATCATCCAGCAGGCTTTGTG
Purpose: Primers bind upstream of and in *yjhQ* gene to verify deletion.	
lacZ	GGCGATTAAGTTGGGTAACG
Purpose: Sequence promoters of pRW50	
6XHis	CATTAAAGAGGAGAAATTAACTATGAGAGG
Purpose: Sequence and verify insertions into pCA24N from ASKA collection.	
***crp* – K101R**	
F:	GAAGTGGCTGAAATTTCGTACAGAAAATTTCGCCAATTGATTCAG
R:	CTGAATCAATTGGCGAAATTTTCTGTACGAAATTTCAGCCACTTC
Purpose: Used to construct site directed mutant of K101 to K101R.	
***crp* – K101A**	
F:	GTGAAGTGGCTGAAATTTCGTACGCAAAATTTCGCCAATTGATTCAGG
R:	CCTGAATCAATTGGCGAAATTTTGCGTACGAAATTTCAGCCACTTCAC
Purpose: Used to construct site directed mutant of K101 to K101A.	
***crp* – K101Q**	
F:	GTGAAGTGGCTGAAATTTCGTACCAGAAATTTGGCCAATTGATTCAGGT
R:	ACCTGAATCAATTGGCGAAATTTCTGGTACGAAATTTCAGCCACTTCAC
Purpose: Used to construct site directed mutant of K101 to K101Q.	

CHAPTER III

RESULTS: CRP=DEPENDENT REGULATION OF PROTEIN ACETYLATION IN *E. coli.*

In eukaryotes, protein acetylation regulates many cellular functions, including gene expression, protein degradation, and enzymatic activity [153]. In bacteria, the effects of protein acetylation are not understood. Recent reports, however, indicate that protein acetylation has the potential to regulate many aspects of cellular physiology [3, 13, 154]. Although it is now clear that bacterial protein acetylation occurs [1-3], the following remain unknown: 1) the mechanism by which the cells regulate the majority of protein acetylations, and 2) if these acetylation events affect cellular physiology. In this chapter, I aim to elucidate both the regulation and the effect of lysine acetylation of several proteins in *E. coli.*

Two types of enzymes regulate protein acetylation: lysine acetyltransferases (KATs) and lysine deacetylases (KDACs). Lysine acetyltransferases catalyze protein acetylation, and these enzymes have been implicated in a number of acetylation events in bacteria [3, 30]. The cAMP receptor protein (CRP), the major carbon regulator of enteric bacteria, e.g. *E. coli*, is reported to regulate expression of at least three KATs: 2 putative (YedL and YjhQ) and one known (YfiQ) [4, 37]. Because it can regulate transcription of

genes that encode KATs, we hypothesized that CRP regulates protein acetylation in *E. coli*. If so, these acetylation events should impact central metabolism and other CRP-regulated cellular processes. This chapter describes experiments to test this hypothesis and, if it is correct, to identify the requirements for CRP-dependent acetylation.

Characterization of the Nutrient Requirements for CRP-Dependent Protein Acetylation

To determine if CRP regulates protein acetylation in *E. coli*, we constructed two strains: a Δ*crp* mutant (strain AJW2198, **Table 1**) transformed with a plasmid (pCRP, **Table 2**) that expresses wild-type CRP to generate the complemented strain (AJW2313, **Table 1**) and the same Δ*crp* mutant transformed with pDU9 (**Table 2**) to generate the vector control (VC) strain (AJW4524, **Table 1**). We grew the resultant transformants under four conditions: tryptone broth buffered at pH 7 (TB7), TB7 supplemented with 3 mM cAMP, TB7 supplemented with 0.4% glucose, and TB7 supplemented with both 3 mM cAMP and 0.4% glucose. We chose these conditions based in part, on the previously reported observation that supplementation of TB7 with 0.4% glucose intensifies the acetylome, as assessed by anti-acetyllysine Western immunoblot analysis [14]. To the glucose-supplemented TB7, we also added 3 mM cAMP. The exogenous cAMP is critical because: 1) the transport of exogenous glucose into the cell represses cAMP production [155], 2) cAMP must bind to CRP to promote its DNA-binding activity [155], and, 3) 3 mM cAMP has been reported to be sufficient to overcome the effects of glucose [156, 157]. Following 7.5 hours incubation at 37°C, we harvested cells, prepared whole cell

lysates and analyzed changes in the acetylome using anti-acetyllysine Western immunoblot analysis.

When grown in TB7, both the complemented (AJW2313) and non-complemented VC (AJW4524) cells showed very little protein acetylation **(Figure 6, lane 1** and data not shown**),** similar to previous findings [14]. When grown in TB7 supplemented with 3 mM cAMP, complemented cells (lane 2), there was little detectable difference in lysine acetylation relative to growth in TB7 **(Figure 6)**. In contrast, supplementation of TB7 with 0.4% glucose intensified the acetylome (lane 3) **(Figure 6)**, as reported previously[14]. Supplementation with both cAMP and glucose, however, resulted in a much more intense acetylome (lane 4) **(Figure 6)**.

To determine if CRP regulates this glucose- and cAMP-dependent acetylome, we compared the acetylation profiles of the complemented strain (Figure 1, lane 4) and its VC *(Δcrp)* **(Figure 6)**. If CRP regulates protein acetylation, we reasoned that the VC would exhibit decreased protein acetylation relative to the complemented strain. Indeed, most of the glucose- and cAMP-dependent acetylome was decreased or undetectable in the VC, supporting the hypothesis that CRP is required for much of the glucose- and cAMP-dependent protein acetylation **(Figure 6)**. CRP also appeared to be required for some glucose-dependent, but cAMP-independent, acetylations, as the acetylation signal of some bands present in the glucose-dependent acetylome **(Figure 6, lane 3)**, was not detected in the cAMP-dependent acetylome **(Figure 6, lane 2)**. Finally, some protein acetylation did not require CRP, as the acetylation signal of at least one band did not

change with the status of *crp*. We conclude that the *E. coli* acetylome varies relative to both growth conditions and *crp* status.

Glucose is a catabolite-repressing carbon source [155]. To determine if the CRP-dependent acetylome responds specifically to catabolite repression, we tested diverse carbon sources, chosen on the basis of their catabolite repression abilities: glucose and pyruvate are strong catabolite repressors, sorbitol and glycerol are moderate catabolite repressors, while succinate, lactate, and acetate are poor catabolite repressors. We added each carbon source to TB7 supplemented with or without 3 mM cAMP, grew the complemented strain and its VC for 7.5 hours at 37°C, harvested cells, prepared cell lysates, and assessed changes in global lysine acetylation by anti-acetyllysine Western immunoblot analysis.

Like glucose (**Figure 7a**, lane 1), sorbitol (**Figure 7a,** lanes 3 and 4), pyruvate (**Figure 7a,** lanes 6 and 7), and succinate (**Figure 7a,** lanes 15 and 16) supported robust protein acetylation only in the presence of cAMP. In contrast, acetate, glycerol and lactate supported robust protein acetylation in the absence of cAMP (**Figure 7a,** lanes 9, 12, and 18, respectively). When cAMP was present, glycerol (**Figure 7a,** lane 13) and lactate (**Figure 7a,** lane 18), but not acetate (**Figure 7a,** lane 9), supported additional acetylation. Like glucose (**Figure 7a,** lane 2), other carbon source-induced protein acetylation depended on CRP (**Figure 7a,** lanes 5, 8, 11, 14, 17 and 20). We conclude that CRP-dependent protein acetylation during growth in TB7 requires some additional carbon source and that this behavior is not a direct consequence of catabolite repression,

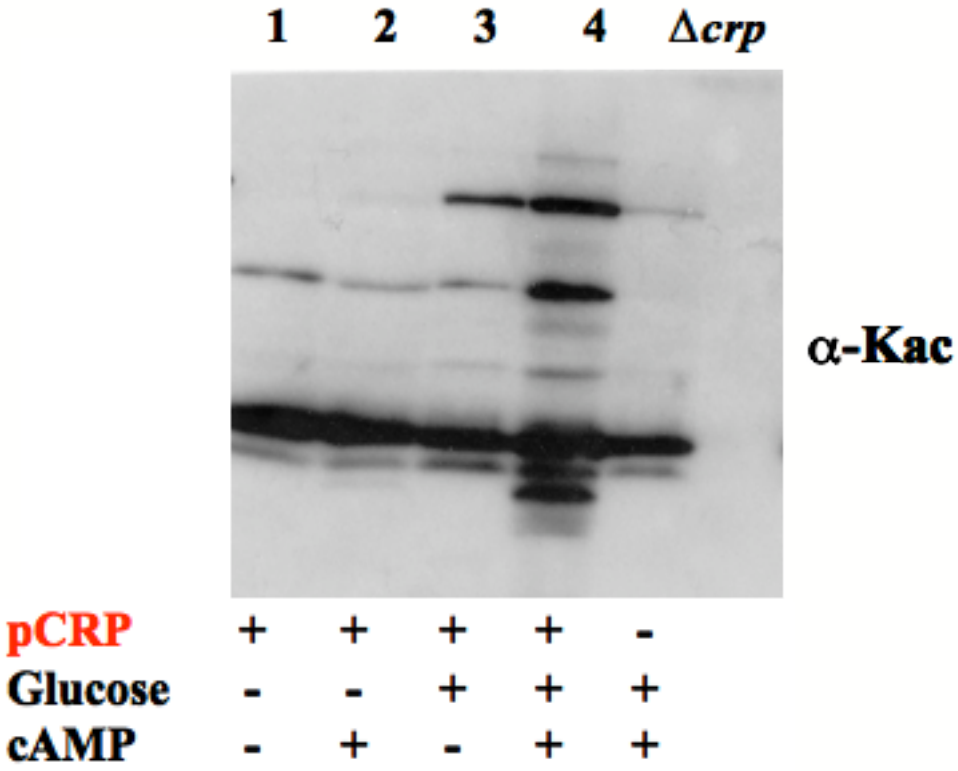

Figure 6. CRP regulates protein acetylation in *E. coli.* Western immunoblot analyses of whole cell lysates from cells grown at 37°C with aeration in TB buffered at pH7 (TB7) supplemented with either 0.4% glucose, 3 mM cAMP, or both. Δ*crp* VC cells (rightmost lane) or Δ*crp* cells complemented with pCRP (lanes 1-4).

but that catabolite repression must be overcome to permit binding of CRP to its DNA sites.

Since TB7 is a mixed amino acid-based medium, we asked if the CRP-dependent acetylome requires growth on amino acids. To test the role of amino acids, we grew complemented cells in MOPS minimal media supplemented with or without casamino acids. Both glycerol and glucose supported an acetylome similar to that observed in TB7 but only in the presence of casamino acids **(Figure 7b)**. The addition of casamino acids alone resulted in little acetylation, similar to TB7 alone **(Figure 6, lane 1)**. Thus, we conclude that CRP-dependent protein acetylation requires both amino acids and an additional carbon source.

Taken together, these findings support the hypothesis that the central carbon regulator CRP regulates protein acetylation in *E. coli* under certain environmental conditions, and suggests a novel link between central metabolism and the regulation of protein acetylation.

Determination if Lysine Acetylation involves the Activating Regions of CRP

Since we had shown that CRP could regulate protein acetylation in *E. coli*, we aimed to elucidate the mechanism underlying this regulation. CRP is a global transcription factor that regulates gene expression of over 200 genes in *E. coli* [123, 124, 131, 158]. Much of this regulation depends on CRP's activation regions (ARs), surface-exposed patches that permit CRP to make contact with RNA polymerase (RNAP) **(Figure 4A)** [158]. AR1 and AR2 are required for CRP-activated transcription from Class I and Class II

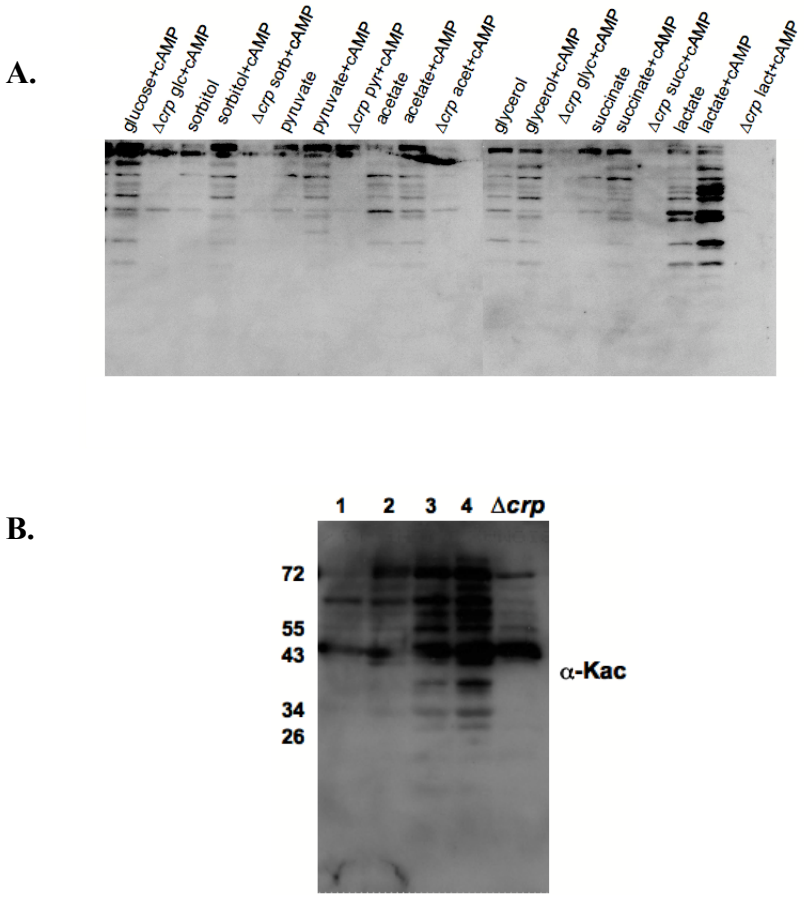

Figure 7: CRP regulates protein acetylation in *E. coli* in multiple carbon sources. Western immunoblot analyses of whole cell lysates of **A)** Δ*crp* or Δ*crp* cells complemented with pCRP grown at 37^{o}C with aeration in TB buffered at pH7 (TB7) or in TB7 supplemented with various carbon sources or both the indicated carbon source and 3 mM cAMP, or **B)** Δ*crp* cells complemented with pCRP grown at 37^{o}C with aeration in MOPS casamino acids (lane 1), MOPS casamino acids supplemented with 3 mM cAMP (lane 2), MOPS casamino acids supplemented with 0.4% glucose (lane 3), MOPS casamino acids supplemented with both 0.4% glucose and 3 mM cAMP (lane 4). Lane 5 represents Δ*crp* cells grown in MOPS casamino acids supplemented with both 0.4% glucose and 3 mM cAMP (same treatment as lane 4).

promoters, respectively **(Figure 4B, 4C)** [123]. At some Class II promoters, AR1 also contributes to CRP-dependent transcription [126, 128-131].

To elucidate the mechanism by which CRP regulates protein acetylation, we asked if these ARs are required for the CRP-dependent acetylation profile. We introduced well-characterized plasmid-borne AR mutants **(Table 2)** into the Δ*crp* mutant strain **(AJW 2198, Table 1)**, grew the resultant transformants in the 4 growth conditions described previously, and used Western immunoblot analysis to evaluate glucose- and cAMP-dependent acetylation. If CRP-dependent acetylation depends on either AR, then disruption of that AR should result in decreased protein acetylation relative that exhibited by cells that express intact WT CRP.

Indeed, transformants expressing the AR1 mutant (strain AJW3660, **Table 1**) and grown in TB7 supplemented with glucose and cAMP exhibited a significant decrease in the acetylome relative to transformants that expressed WT CRP (strain AJW2313) **(Figure 8)**. In contrast, transformants expressing the AR2 mutant (strain AJW2314, **Table 1**) exhibited a more moderate decrease in acetylome intensity relative to cells expressing WT CRP **(Figure 8)**. We conclude that glucose- and cAMP-dependent acetylation requires AR1 and involves AR2.

Since Class I promoters require AR1 and Class II promoters require AR2, we conclude that multiple CRP-dependent mechanisms regulate glucose- and cAMP-dependent protein acetylation. These mechanisms could regulate protein acetylation by: 1) regulating expression of proteins that are post-translationally modified, 2) regulating expression of acetylation-regulating enzymes (e.g. KATs and KDACs), or 3) a

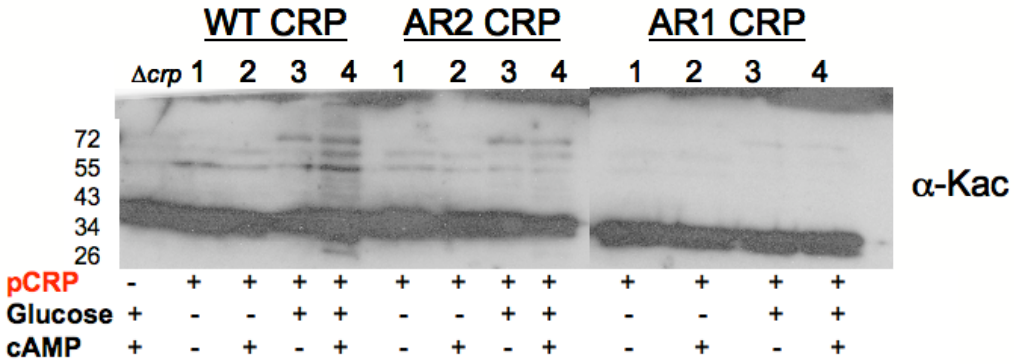

Figure 8. Both activating regions of CRP regulate CRP-dependent acetylation. Western immunoblot analyses of cell lysates from Δ*crp* cells (leftmost lane marked with Δ*crp*), or Δ*crp* cells complemented with WT *crp* allele (pCRP), KE101 mutant allele (AR2) or the HL159 mutant allele (AR1) grown at 37°C with aeration in TB buffered at pH7 (TB7) supplemented with either 0.4% glucose, 3 mM cAMP, or both.

combination of the two, where CRP regulates expression of the proteins that are substrates for acetylation, as well as the enzymes that regulate acetylation.

Determination if CRP regulates KAT expression in *E. coli*

Since we obtained evidence that CRP can regulate protein acetylation *in vivo*, and that this acetylation requires the presence of CRP's activating regions, we next sought to determine if CRP regulates protein acetylation by regulating expression of one or more of the enzymes that regulate lysine acetylation: i.e., KDACs or KATs. A search through previously published global transcriptional analyses revealed evidence that CRP can regulate transcription of the genes that encode two putative KATs, YedL, and YjhQ [37]. A recent report also suggests that CRP may regulate transcription of *yfiQ*, which encodes the first KAT identified in enteric bacteria [4].

Next, we perused the promoters responsible for expression of these three genes, seeking DNA stretches with similarity to the CRP consensus sequence (TGTGA n6 TCACA). Since none of these promoter regions had been studied previously, the transcription start sites were not known. We therefore first sought putative CRP binding sites in the promoter regions that drive transcription of *yfiQ, yjhQ* and *yedL.* Knowing that CRP tends to bind to sites centered about 40 nucleotides (Class II) or 60 nucleotides (Class I) upstream of the transcription start site (+1), we next scanned for a putative RNAP binding site [126], which contains the -10 hexamer (consensus TATAAT) and often a -35 hexamer (TTGACA) positioned 17±1 nucleotides upstream. Utilizing this method,

we identified putative CRP binding sites in front two of the KAT genes previously identified as putatively regulated by CRP: *yjhQ,* and *yfiQ* **(Figure 9)**.

yjhQ, a putative KAT gene, lies in an operon downstream of *yjhX*, a gene of unknown function. The *yjhX* promoter is thought to drive *yjhQ* transcription [159]. Overexpression of CRP in a strain deleted for *yjhQ* (strain AJW4355, **Table 1**) resulted in decreased protein acetylation compared to a strain in which *yjhQ* was intact (strain AJW4526, **Table 1**), indicating that YjhQ regulates protein acetylation (data not shown). We used the previously described bioinformatics approach to determine if CRP could regulate the putative KAT YjhQ. Indeed, upstream of the *yjhX* open reading frame, we identified sequences with good similarity to the consensus sequences for the CRP binding site (gGgGA n6 TCAtc), the -35 hexamer (ccGACc) and the -10 hexamer (TATcAa). If these sequences indeed recruit CRP and RNAP, then the DNA site for CRP would be centered at -61.5 relative to the putative +1 **(Figure 9A)**. This would be a Class I promoter, requiring AR1 for its activity. Efforts to determine whether these sequences constitute a bonafide CRP-dependent Class I promoter are planned.

A recent report provides evidence that supports the hypothesis that CRP regulates transcription of *yfiQ*[4]. These authors identified a putative CRP site centered at -41.5 base pairs upstream of the putative +1. We verified the existence of this sequence **(Figure 9B)**. However, since this would be a Class II promoter requiring AR2 for its activity and we had found that that CRP-dependent protein acetylation did not require AR2, we sought other putative CRP binding sites. Because *yfiQ* lies in a putative operon with *yfiP* [4, 159], a gene of unknown function, we analyzed the sequence upstream of the

A.


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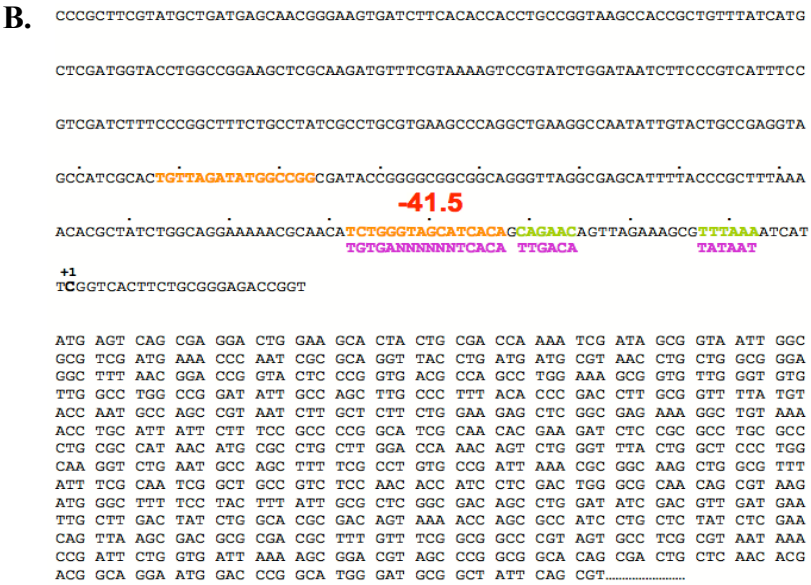
AAGCCGATGAGGCCATTTCAGGCGTACTTCAACATGCATTCGTCAGTGTCATCTGCCTTTTCCAGTAAATGGGTT
GGCATTCAATTTCCAAAGTACATCACACTTTTTCTACTGAAACATATCCCTCCTATAACTACGTGTTAATATTCG
CTCCGTAAATACCTTTCAACTCAACCCGAGGCTTTGATGCTGGAGAATGTAATCATCCGATAATCAGTTCCCCGA
TGTGANNNNNNTCACA
+1
CCTTTTCAGGCCGGACTGATTATCAATGCGCCGAAATCGAATGCGGACACCGCGGTGTGTTTGCACGTTTTGCAC
TTGACA
TATAAT
ATGATGATTAAATAGGTTCTTCAGT
ATG AAT TTA TCC CGT CAG GAA CAA CAT ACC TTA CAC GTT CTC GCT AAA GGT AGA CGT
ATT GCG CAC GTC CGC GAT TCT TCA GGC CGC GTC ACT TCC GTT GAA TGC TAC AGC CGC
GAA GGG CTG TTG CTG ACC GAC TGC ACG CTC GCC GTC TTC AAA AAA CTC AAA ACC AAA
AAA CTT ATC AAG TCC GTC AAT GGC CAG CCC TAT CGA ATC AAC ACC ACC GAG CTG AAT
AAA GTT CGC GCT CAG CTC GAT AAT CGC TAA

B.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GCCATCGCACTGTTAGATATGGCCGGCGATACCGGGGCGGCGGCAGGGTTAGGCGAGCATTTTACCCGCTTTAAA
-41.5
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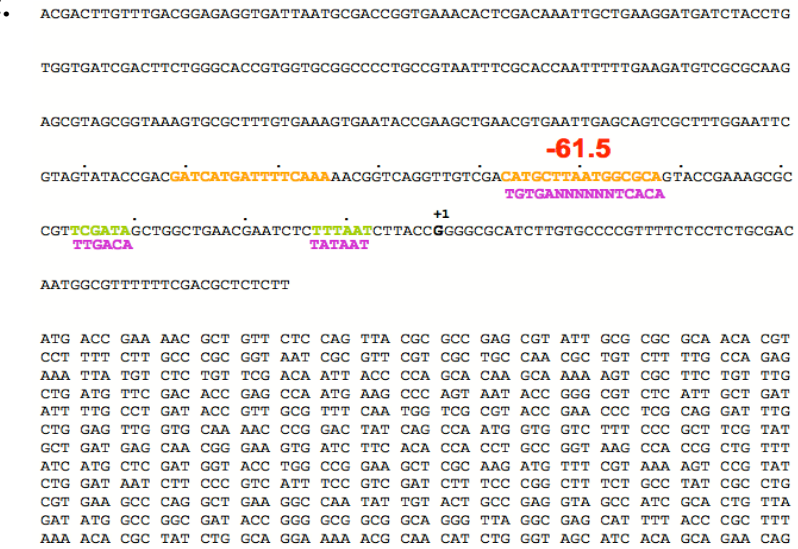
TGTGANNNNNNTCACA TTGACA
TATAAT
+1
TCGGTCACTTCTGCGGGAGACCGGT
ATG AGT CAG CGA GGA CTG GAA GCA CTA CTG CGA CCA AAA TCG ATA GCG GTA ATT GGC
GCG TCG ATG AAA CCC AAT CGC GCA GGT TAC CTG ATG ATG CGT AAC CTG CTG GCG GGA
GGC TTT AAC GGA CCG GTA CTC CCG GTG ACG CCA GCC TGG AAA GCG GTG TTG GGT GTG
TTG GCC TGG CCG GAT ATT GCC AGC TTG CCC TTT ACA CCC GAC CTT GCG GTT TTA TGT
ACC AAT GCC AGC CGT AAT CTT GCT CTT CTG GAA GAG CTC GGC GAG AAA GGC TGT AAA
ACC TGC ATT ATT CTT TCC GCC CCG GCA TCG CAA CAC GAA GAT CTC CGC GCC TGC GCC
CTG CGC CAT AAC ATG CGC CTG CTT GGA CCA AAC AGT CTG GGT TTA CTG GCT CCC TGG
CAA GGT CTG AAT GCC AGC TTT TCG CCT GTG CCG ATT AAA CGC GGC AAG CTG GCG TTT
ATT TCG CAA TCG GCT GCC GTC TCC AAC ACC ATC CTC GAC TGG GCG CAA CAG CGT AAG
ATG GGC TTT TCC TAC TTT ATT GCG CTC GGC GAC AGC CTG GAT ATC GAC GTT GAT GAA
TTG CTT GAC TAT CTG GCA CGC GAC AGT AAA ACC AGC GCC ATC CTG CTC TAT CTC GAA
CAG TTA AGC GAC GCG CGA CGC TTT GTT TCG GCG GCC CGT AGT GCC TCG CGT AAT AAA
CCG ATT CTG GTG ATT AAA AGC GGA CGT AGC CCG GCG GCA CAG CGA CTG CTC AAC ACG
ACG GCA GGA ATG GAC CCG GCA TGG GAT GCG GCT ATT CAG CGT……

C.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AGCGTAGCGGTAAAGTGCGCTTTGTGAAAGTGAATACCGAAGCTGAACGTGAATTGAGCAGTCGCTTTGGAATTC
-61.5
GTAGTATACCGACGATCATGATTTTCAAAAACGGTCAGGTTGTCGACATGCTTAATGGCGCAGTACCGAAAGCGC
TGTGANNNNNNTCACA
+1
CGTTCGATAGCTGGCTGAACGAATCTCTTTAATCTTACCGGGGCGCATCTTGTGCCCCGTTTTCTCCTCTGCGAC
TTGACA
TATAAT
AATGGCGTTTTTTCGACGCTCTCTT
ATG ACC GAA AAC GCT GTT CTC CAG TTA CGC GCC GAG CGT ATT GCG CGC GCA ACA CGT
CCT TTT CTT GCC CGC GGT AAT CGC GTT CGT CGC TGC CAA CGC TGT CTT TTG CCA GAG
AAA TTA TGT CTC TGT TCG ACA ATT ACC CCA GCA CAA GCA AAA AGT CGC TTC TGT TTG
CTG ATG TTC GAC ACC GAG CCA ATG AAG CCC AGT AAT ACC GGG CGT CTC ATT GCT GAT
ATT TTG CCT GAT ACC GTT GCG TTT CAA TGG TCG CGT ACC GAA CCC TCG CAG GAT TTG
CTG GAG TTG GTG CAA AAC CCG GAC TAT CAG CCA ATG GTG GTC TTT CCC GCT TCG TAT
GCT GAT GAG CAA CGG GAA GTG ATC TTC ACA CCA CCT GCC GGT AAG CCA CCG CTG TTT
ATC ATG CTC GAT GGT ACC TGG CCG GAA GCT CGC AAG ATG TTT CGT AAA AGT CCG TAT
CTG GAT AAT CTT CCC GTC ATT TCC GTC GAT CTT TCC CGG CTT TCT GCC TAT CGC CTG
CGT GAA GCC CAG GCT GAA GGC CAA TAT TGT ACT GCC GAG GTA GCC ATC GCA CTG TTA
GAT ATG GCC GGC GAT ACC GGG GCG GCG GCA GGG TTA GGC GAG CAT TTT ACC CGC TTT
AAA ACA CGC TAT CTG GCA GGA AAA ACG CAA CAT CTG GGT AGC ATC ACA GCA GAA CAG
TTA GAA AGC GTT TAA

Figure 9. Bioinformatic analyses of the *yjhQ, yfiP,* and *yfiQ* promoter regions. The transcription start site is marked with a +1, and the dots above the sequences are 10 base pairs apart. The putative CRP binding sites are shown in orange, with the CRP consensus sequence below in pink. The putative -10 and -35 hexamers are in green, with their respective consensus sequences below in pink. **A)** The *yjhX* promoter (which drives *yjhPQ* transcription) is shown. A putative CRP binding site centered at -41.5 and a second putative site centered at -140.5 are shown. **B)** The *yfiQ* promoter. A close-to-consensus CRP site centered at -41.5 is shown, along with a second putative, lower consensus CRP binding site centered at -145.5. **C)** The *yfiP* promoter (which drives transcription of both *yfiP* & *yfiQ* transcription) is shown. A putative CRP site centered at -61.5 is shown, along with a second putative, lower consensus CRP binding site centered at -95.5.

yfiP open reading and identified a putative CRP binding site centered at -61.5 base pairs upstream of the putative +1 **(Figure 9C)**. Thus, CRP has the potential to regulate *yfiQ* expression by two mechanisms: regulation of *yfiPQ* transcription in an AR1-dependent manner, and regulation of *yfiQ* transcription in an AR2-dependent manner.

Using the same bioinformatics approach, we found no evidence that CRP regulates transcription of *cobB,* which encodes the only KDAC identified in *E. coli*. While it remains to be determined *in vivo* if CRP does indeed regulate expression of either *yfiQ* or *yjhQ*, we have bioinformatic evidence that CRP could regulate protein acetylation in *E. coli* by regulating the genes that encode a putative (YjhQ) and a known KAT (YfiQ).

Identification of the Targets of CRP/Glucose/cAMP-Dependent Protein Acetylation

To determine if CRP has the potential to regulate protein acetylation in *E. coli*, we used anti-acetyllysine Western immunoblot analyses to identify any lysine acetylations that may be regulated by CRP **(Figure 6)**. Using this approach, we determined that CRP does indeed regulate protein acetylation, but were unable to identify the targets of CRP-dependent protein acetylation.

To identify CRP-dependent acetylations, we utilized a mass spectrometry approach. We grew the complemented strain (strain AJW2313) and its VC (strain AJW4524) in TB7 supplemented with glucose and cAMP, separated the proteins by SDS-PAGE, and sent the samples to our collaborator Haike Antelmann, who used linear trap quadrupole (LTQ) Orbitrap mass spectrometry (MS) to detect acetylated peptides.

Dr. Antelmann and her group mapped 18 acetylation sites to 16 different proteins from whole cell protein preparations of the complemented strain **(Table 4)**.

In contrast, from the VC, they mapped only three acetylations **(Table 4)**; each of these three acetylations was also detected in the complemented strain. Of the acetylated proteins detected solely in the complemented strain, we identified proteins involved in at least seven different cellular functions. The most prominently represented function was central metabolism. Other acetylated proteins function in translation, transcription, and regulation of redox state **(Table 4)**.

Although these data do not provide direct evidence that CRP regulates acetylation of these proteins, the data supports the model that CRP contributes to the acetylation of multiple proteins, including several metabolic enzymes.

Table 4: Glucose- and cAMP-dependent protein acetylations detected in WT cells but not in *crp* mutant cells**.** Mass spectrometry was used to identify acetylated peptides in Δ*crp* VC and Δ*crp* pDCRP cells grown in TB7 supplemented with 0.4% glucose and 3 mM cAMP. There are six functional classes of proteins acetylated in a CRP-dependent manner, as assessed by mass spectrometry. The proteins listed in red were found acetylated in both the Δ*crp* VC and Δ*crp* pDCRP cells.

Protein	Molecular Weight	K-Acetyl-Peptide sequence	K-Ac Sites	Cellular Mechanism
Udp	27 kDa	TQQEIPNAETMKQTESHAVK	K235	Metabolism
DeoC	28 kDa	FGASSLLASLLKALGHGDGK	K246	Metabolism
PflB	85 kDa	KSGVLTGLPDAYGR	K162	Metabolism
LpdA	51 kDa	VINQLTGGLAGMAKGR	K106	Metabolism
GapA	36 kDa	AVGKVLPELNGK	K227	Metabolism
GapA	36 kDa	GASQNIIPSSTGAAKAVGK	K213	Metabolism
Pgk	41 kDa	KDDETLSK	K120	Metabolism
Pgk	41 kDa	GEKKDDETLSK	K119	Metabolism
GmpA	29 kDa	HYGALQGLNKAETAEK	K100	Metabolism
Adk	24 kDa	KDDQEETVR	K157	Metabolism
PtsH	9 kDa	PAAQFVKEAK	K24	Metabolism
Eda	22 kDa	TSAESILTTGPVVPVIVVKK	K24	Metabolism
PyrB	34 kDa	KGETLADTISVISTYVDAIVMR	K85	Metabolism
GroL	57 kDa	VAAVKAPGFGDR	K277	Protein folding
TufA	43 kDa	AIDKPFLLPIEDVFSISGR	K209	Translation
RpsF	16 kDa	LHKAHYVLMNVEAPQEVIDELETTFR	K56	Translation
FusA	78 kDa	IAFVNKMDR	K143	Translation
Crp	24 kDa	YPSKSTLIHQGEK	K27	Transcription
HupB	9 kDa	NPQTGKEITIAAAK	K67	DNA binding
SodA	23 kDa	AEFEKAAASR	K119	ROS
DegQ	47 kDa	IATTEPGTKVK	K342	Protease

Summary

To date, very little is known about the regulation and effects of protein acetylation in bacteria. While our lab and a few others have studied the regulation and consequences of lysine acetylation for a few proteins (i.e. RcsB, RNAP, Acs, CheY and RNase R), the majority of protein acetylations in *E. coli* remain uncharacterized.

The work described in this chapter aimed to understand CRP-dependent regulation of protein acetylation. We hypothesized that CRP helps to regulate protein acetylation in *E. coli* on the basis of published reports that: 1) CRP itself is acetylated, and 2) that CRP can regulate transcription of two putative (*yedL* and *yjhQ*) and one known KATs (*yfiQ*). Using anti-acetyllysine Western immunoblot analyses, we determined that CRP can regulate protein acetylation *in vivo*, and that CRP-dependent protein acetylation was only detected when cells were grown in amino acid rich media supplemented with excess carbon. We next assessed the mechanism by which CRP regulates protein acetylation and determined that the activating regions of CRP are both involved, with AR1 required for the response, and AR2 involved. We hypothesized that CRP may regulate protein acetylation by controlling expression of a KAT or a KDAC, the enzymes that regulate acetylation. Indeed, we identified putative CRP binding sites in front of the promoters that transcribe the genes that encode two KATs, YjhQ and YfiQ, suggesting that CRP regulates protein acetylation by regulating the expression of one or more KATs. To identify CRP-dependent acetylations, we used mass spectrometry and found several proteins whose acetylations seem to be affected by CRP. These data support the hypothesis that CRP regulates protein acetylation in *E. coli*, and that CRP

likely regulates acetylation by regulating expression of one or more KATs. These findings suggest a novel link between the central metabolism and protein acetylation, where carbon transport and metabolism controls the concentration of cAMP, which facilitates the binding of CRP to its DNA sites, including those that control transcription of two KATs. The resultant expression of these KATs could affect protein acetylation with potential implications in regulating multiple cellular processes.

CHAPTER IV

RESULTS: EVIDENCE THAT KATs MAY REGULATE CENTRAL METABOLISM

KATs are enzymes that use the central metabolic intermediate acetyl-CoA as an acetyl donor to acetylate their protein target. The use of acetyl-CoA as the acetyl donor provides the cell with the unique ability to sense and respond to changes in central metabolic flux [3, 30]. In the last chapter, we showed previously published and nascent evidence that together support the hypothesis that CRP regulates transcription of the genes that encode two KATs: YjhQ and YfiQ [4, 37]. Wang *et al.* recently reported that the *Salmonella enterica* KAT Pat (the homolog of YfiQ) somehow senses changes in metabolic flux and responds by acetylating a number of key metabolic enzymes. This would appear to lead to a change in metabolic activity, as deletion of *pat* resulted in a growth defect when the cells were grown in minimal media supplemented with glucose [3]. These and other results implicate acetylation as a key posttranslational regulatory mechanism of bacterial central metabolism [1, 2]. We therefore considered the possibility that one of the functions of *E. coli* KATs may be to acetylate metabolic enzymes. We further predicted that this acetylation would affect the flux of carbon through central metabolism [160, 161]. In this chapter, we will describe analyses designed to determine if YfiQ and/or YjhQ have the potential to regulate central metabolism.

The Effects of Mutation of *yfiQ* on Central Metabolism

To test the hypothesis that YfiQ regulates central metabolism, we used Phenotype MicroArray™ (Biolog, Hayward, CA), an experimental technique that allows for high-throughput analyses of cellular phenotypes [162]. The Phenotype MicroArray™ is a broad-based phenotypic assay of gene function. It utilizes microtiter plates containing a different lyophilized carbon source in each of 90 wells. By inoculating each well with a strain, one can determine the ability of that cell to grow and respire on each of the 90 carbon sources. After inoculation, cells are then grown in the microtiter plates at 37°C under microaerophilic conditions while the OMNILOG reader records growth and tetrazolium reduction readings at 15-minute intervals during the course of the 37-hour growth period. We utilized this method to test if deletion of *yfiQ* affected the ability of *E. coli* to utilize carbon.

Of the 90 different carbon sources tested, the Δ*yfiQ* mutant grew and respired significantly worse than its WT parent on 20 **(Table 5).** Of these 20, many require gluconeogenesis to support growth and respiration. These include compounds that are metabolized to glycolytic intermediates (e.g., D-galactitol, D-galactarate, 5-keto-D-gluconic acid, dihydroxyacetone and D, L-α-glycerol phosphate). They also include TCA intermediates and associated compounds (e.g., D- and L-malic acid, succinic acid, fumaric acid, and L-aspartic acid). Growth on each of these compounds requires gluconeogenesis, whose end product is glucose-6-phosphate [67-71]. Thus, it was telling that the Δ*yfiQ* mutant grew and respired better than its WT parent on maltotriose, which is metabolized directly to glucose-6-phosphate. Taken together, these data are consistent

with the hypothesis that the Δ*yfiQ* mutant is defective for gluconeogenesis. The observation that the mutant grew and respired worse than its WT parent on gluconeogenic carbon sources and better than its parent on maltotriose suggests the possibility that YfiQ regulates the balance between gluconeogenesis and glycolysis, perhaps by acetylating one or more central metabolic enzymes.

The Phenotype MicroArray™ experiment is performed under microaerophilic conditions. To determine if the YfiQ-dependent growth defect was influenced by aeration, we grew the Δ*yfiQ* mutant and its WT parent in TB7 supplemented with glucose with either high levels of shaking (250 rpm), medium levels of shaking (100 rpm) or no shaking (static cultures). The Δ*yfiQ* mutant did not exhibit any dramatic growth differences in TB7 supplemented with glucose when aeration was altered, suggesting that the YfiQ-dependent growth defect seen with the Phenotype MicroArray™ assay is not subject to differences in aeration **(Figure 10)**. These results indicate that YfiQ is active regardless of the differences in aeration, though the mechanism of the YfiQ-dependent growth defect remains to be elucidated.

The KAT YfiQ is often thought to function in close opposition to the only known KDAC in *E. coli* CobB [3, 17]. In *S. enterica,* for example, CobB has been found to deacetylate many of the substrates of YfiQ [3, 17], and deletion of Δ*cobB* results in some growth phenotypes that are in opposition to the growth phenotypes exhibited by the Δ*yfiQ* mutant [3]. Since YfiQ is often linked to CobB in *S. enterica* [3], we subjected an isogenic *cobB* mutant to analysis by Phenotype MicroArray™, with the expectation that if YfiQ and CobB of *E. coli* work as a binary system, then deletion of *cobB* should result

Table 5. Phenotypes gained and lost by the Δ*yfiQ* mutant as shown by Biolog metabolome analysis. Carbon sources denoted with an (*) indicate gluconeogenic carbon sources.

Test Metabolite	Growth Difference[a]	Mode of Action/Metabolic Pathway
Phenotype Gained by Δ*yfiQ*		
Maltotriose	61.41	C-Source/Glycolysis, glucose
Phenotypes Lost by Δ*yfiQ*		
Dulcitol (D-galactitol)	-67.09	C-Source/Galactose
Mucic Acid (D-galactarate)	-66.9	C-Source/Galactose
* L-Aspartic Acid	-61.9	C-Source/Amino acid, TCA
* L-Malic Acid	-54.62	C-Source/TCA
5-Keto-D-Gluconic Acid	-51.52	C-Source
D,L-a-Glycerol Phosphate	-41.61	C-Source/Gluconeogenesis
* Succinic Acid	-40.29	C-Source/TCA
* Dihydroxy Acetone	-38.24	C-Source
* D,L-Malic Acid	-37.24	C-Source/TCA
* D-Alanine	-36.88	C-Source/Amino acid, TCA
* Beta-D-Allose	-34.19	C-Source
D-Saccharic Acid	-33.11	C-Source
* Fumaric Acid	-32.88	C-Source/TCA
a-Methyl-D-Galactoside	-30.11	C-Source/Galactose
D-Sorbitol	-29.37	C-Source
D-Galactonic Acid-g-Lactone	-27.66	C-Source/Galactose
* D-Malic Acid	-26.39	C-Source/TCA
* m-Tartaric Acid	-24.04	C-Source/TCA
D-Arabinose	-22.08	C-Source
* Bromosuccinic Acid	-21.26	C-Source/TCA

a. Growth differences are determined by measuring the difference in average height of the kinetic plots in arbitrary units.

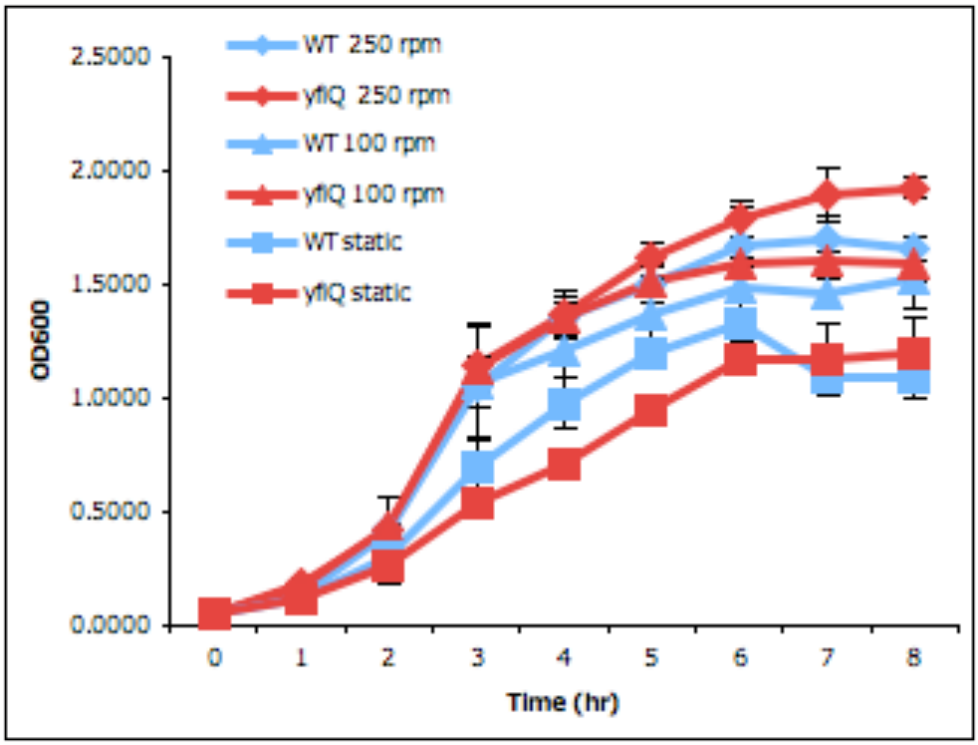

Figure 10. Growth curves of Δ*yfiQ* mutant and its WT parent grown at 37°C with TB7 supplemented with 0.4% Glucose, under different aeration conditions. The blue lines represent the WT, and the red lines represent the Δ*yfiQ* mutant in each condition. Diamonds represent cells grown in high levels of aeration (250 rpm), triangles represent cells grown in moderate levels of aeration (100 rpm), and squares represent cells grown in static cultures.

Table 6. Phenotypes gained and lost by the Δ*cobB* mutant as shown by Biolog metabolome analysis.

Test Metabolite	Growth Difference[a]	Mode of Action/Metabolic Pathway
Phenotypes Gained by Δ*cobB*		
a-D-Lactose	31.26	C-Source/Glycolosis
D-Melibiose	28.53	C-Source/Glycolosis
Phenotypes Lost by Δ*cobB*		
D-Tagatose	-19.89	C-Source

a. Growth differences are determined by measuring the difference in average height of the kinetic plots in arbitrary units

in phenotypes that are similar but opposite to those obtained with the Δ*yfiQ* mutant **(Table 5)**. Surprisingly, the Δ*cobB* mutant grew and respired as well as its WT parent on almost all the 90 tested carbon sources. Only two carbon sources yielded significantly different results: the Δ*cobB* mutant grew and respired better than the WT parent on α-D-lactose and D-melibiose, which are both disaccharides composed of glucose and galactose, indicating perhaps an altered ability of the Δ*cobB* mutant to catabolize these two disaccharides (**Table 6).** It is not clear whether the Δ*cobB* mutant is altered in its ability to either transport these disaccharides, split them into their component monosaccharides, or catabolize these monosaccharides, as the Δ*cobB* mutant grew similarly to its WT parent in both glucose and galactose. The data obtained by the Δ*cobB* mutant Phenotype MicroArray™ argues against a direct functional linkage, in *E. coli*, of the KDAC CobB and the KAT YfiQ. The lack of direct functional linkage also suggests the possibility that one or more presently unidentified KDACs regulate metabolism by counterbalancing the action of YfiQ.

Preliminary Metabolic Analyses of the Putative KAT YjhQ

In the last chapter, we provided evidence suggesting that the general carbon regulator CRP might regulate expression of the putative KAT YjhQ. We therefore asked if YjhQ has the potential to regulate carbon metabolism. We reasoned that loss of an enzyme that coordinated carbon metabolism would cause a growth defect. To test this hypothesis, we grew the Δ*yjhQ* mutant and its WT parent at 37°C in TB7 supplemented with diverse carbon sources. Relative to its WT parent, the Δ*yjhQ* mutant grew at a more

rapid rate in the presence of 0.6% succinate or 0.4% glucose, but not in their absence and not when TB7 was supplemented with 0.8% lactate or 15 mM acetate **(Figure 11)**.

To extend our analysis to a much wider array of carbon sources, we used Phenotype MicroArray™ (Biolog, Hayward, CA). If YjhQ plays a major role in coordinating metabolism, we anticipated that the Δ*yjhQ* mutant would exhibit a defect in the utilization of multiple carbon sources. Of the 90 different carbon sources tested, however, only 4 supported poor growth and respiration by the Δ*yjhQ* mutant relative to its WT parent: L-arabinose, D-xylose, dihydroxy acetone, and D-tagatose **(Table 7)**. Given the growth defects observed in the original experiment, we were a bit surprised by the general lack of phenotypes exhibited by the Δ*yjhQ* mutant in the Phenotype MicroArray™. We therefore compared experimental conditions. Whereas the earlier experiments were performed in large volume cultures that were shaken, the Phenotype MicroArray™ experiment was performed in small volumes that were not shaken. Thus, the relative lack of mutant phenotypes in the Phenotype MicroArray™ experiment could be due to decreased oxygenation. We examined this hypothesis by comparing growth curves of the Δ*yjhQ* mutant under several different aeration conditions, either with no aeration (static), moderate levels of aeration (100 rpm), or high levels of aeration (250 rpm). The Δ*yjhQ* mutant grew more rapidly than its WT parent only when aerated (250 and 100 rpm) **(Figure 12)**, indicating that the lack of growth phenotypes exhibited by the Δ*yjhQ* mutant in the Phenotype MicroArray™ experiments is likely due to the low levels of aeration. Thus, we propose that YjhQ regulates central metabolism under highly oxygenated conditions, perhaps by acetylating central metabolic enzymes.

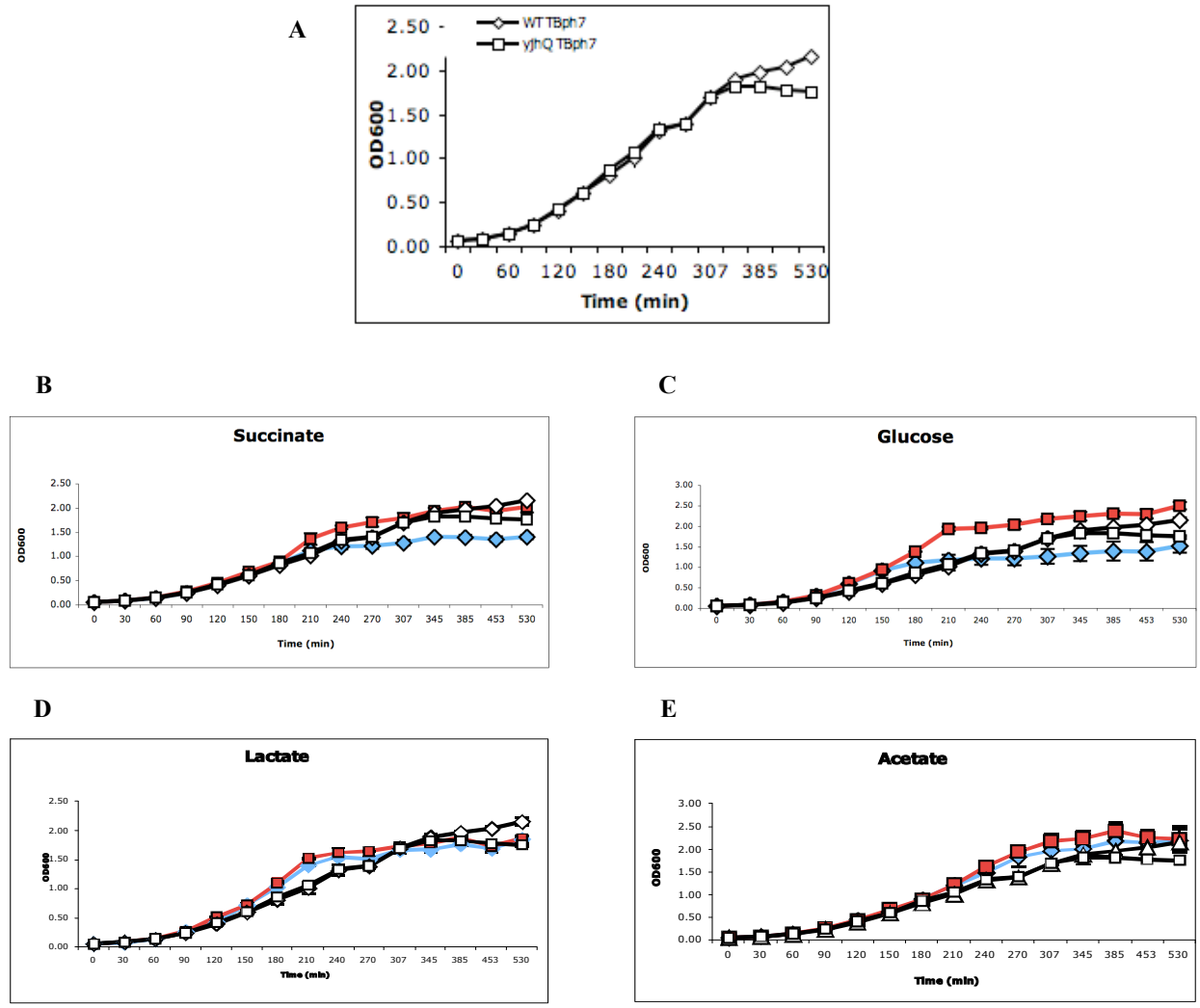

Figure 11. Growth curves of Δ*yjhQ* and its WT parent grown at 37°C with aeration in TB7 (A) or supplemented with 0.6% Succinate (B), 0.4% Glucose (C), 0.8% Lactate (D), or 15 mM Acetate (E). For comparison, the no supplement growth curves from Panel A (black symbols) are shown in Panels B-E. In each panel, the Δ*yjhQ* mutant is represented by squares. The black lines represent growth in unsupplemented TB7; the red lines represent growth in TB7 supplemented with a given carbon source. The WT is represented by diamonds. The black lines represent growth in unsupplemented TB7; the blue lines represent TB7 supplemented with a given carbon source.

Table 7. Phenotypes lost by the Δ*yjhQ* mutant as shown by Biolog metabolome analysis.

There were no significant phenotypes gained by the Δ*yjhQ* mutant.

Test Metabolite	Growth Difference[a]	Mode of Action/Metabolic Pathway
Phenotypes lost by Δ*yjhQ*		
Dihydroxy Acetone	-45.77	C-Source
D-Tagatose	-23.46	C-Source
D-Xylose	-22.57	C-Source
L-Arabinose	-20.19	C-Source/Glycolysis

a. Growth differences are determined by measuring the difference in average height of the kinetic plots in arbitrary units.

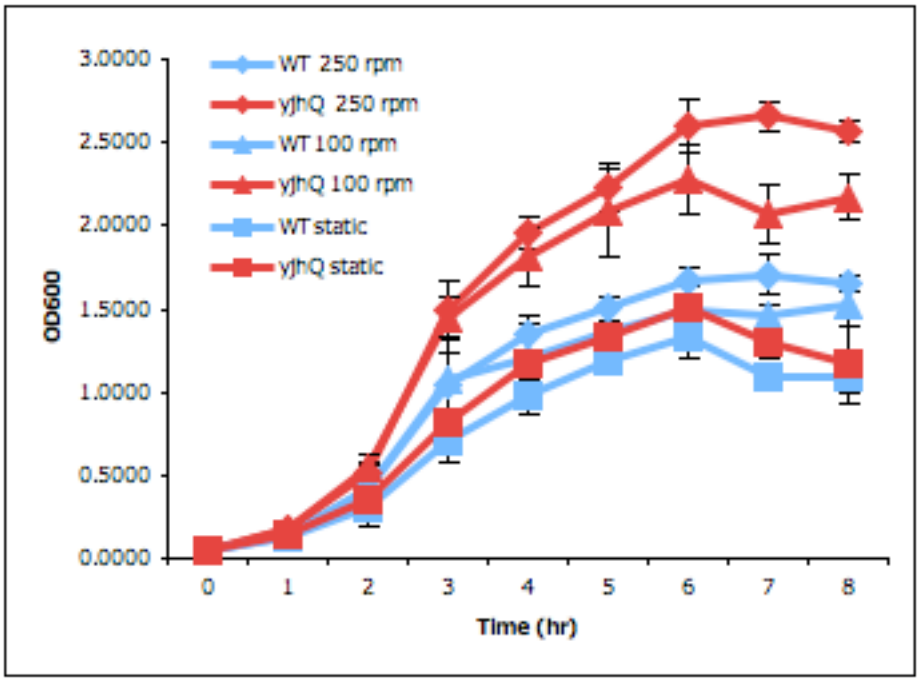

Figure 12. Growth curves of Δ*yjhQ* mutant and its WT parent grown at 37°C with TB7 supplemented with 0.4% glucose, under different aeration conditions. The blue lines represent the WT, and the red lines represent the Δ*yjhQ* mutant in each condition. Diamonds represent cells grown in high levels of aeration (250 rpm), triangles represent cells grown in moderate levels of aeration (100 rpm), and squares represent cells grown in static cultures.

In Vitro **Acetylation Analyses of YfiQ and Various Metabolic Enzymes**

On the basis of mass spectrometric and Phenotype MicroArray^{TM} analysis of the Δ*yfiQ* mutant, we hypothesized that the KAT YfiQ acetylates a subset of metabolic enzymes and that this acetylation alters the balance between glycolysis and gluconeogenesis, favoring the latter. To test this hypothesis, we purified irreversible strictly glycolytic, irreversible strictly gluconeogenic, and reversible bifunctional glycolytic/gluconeogenic enzymes (**Table 8)**, used the purified enzymes as putative substrates for YfiQ, tested the ability of YfiQ to acetylate the purified metabolic enzymes using *in vitro* acetylation experiments, and monitored metabolic enzyme acetylation status by anti-acetyllysine Western immunoblot analysis. As a positive control, we used the known YfiQ substrate RcsB (Hu *et al.,* in preparation) [17].

When our collaborators (Wayne Anderson's laboratory at Northwestern University) purified these metabolic enzymes, they discovered that some of the proteins purified in two peaks, representing different oligomeric states (**Table 8)**. Using size exclusion chromatography, they determined that PykA, PykF, LpdA, and Pgk each purified as two differently sized oligomers. Currently, we do not know the oligomeric forms of these proteins, and this is something the Anderson lab aims to determine in future work.

To monitor acetylation status of the purified proteins, we subjected each form of each enzyme to anti-acetyllysine Western immunoblot analysis. Some of enzymes that purified in a single state (e.g. fructosebisphosphatase aldolase (Fba) and phosphoenol carboxykinase (Pck)) were acetylated *in vivo*, while others (e.g. triosephosphate

Table 8. Summary of *in vitro* acetylation reactions with YfiQ and various substrates. Reactions were run at 37°C for 2 hours, and changes in acetylation status were determined using both Western immunoblot analysis and Ellman's assays (Kuhn *et al.,* in preparation; Hu *et al.,* in preparation). The table lists the following results: substrate acetylation alone (Kac *in vivo*), Substrate + acetyl-CoA, and Substrate + acetyl-CoA + YfiQ. Y indicates acetylation, N indicates no evidence of substrate acetylation under the conditions tested. Certain substrates purified in two different oligomeric states (PykA, PykF, LpdA, and Pgk), denoted as (1) or (2), indicating acetylation of either oligomeric state 1 or state 2.

Enzyme	Type	Gene	MW (kDa)	Kac (*in vivo*)	Acetylate with Acetyl-CoA	Acetylated by YfiQ
Phosphofructokinase	Glycolytic	PfkA PfkB	PfkA: 34.8, PfkB: 32.5	Y (PfkA, PfkB)	Y	**Y**
Triosephophate isomerase	Glycolytic	TpiA	26.9	N	N	N
Pyruvate kinase	Glycolytic	PykA PykF	PykA: 51.4 PykF: 50.7	Y (PykF -1, 2-N)	Y (PykF -1, 2-N)	N
Pyruvate dehydrogenase	Glycolytic	LpdA	50.7	1-N, 2-Y	Y	**Y**
Fructosebisphosphotase aldolase	Gluconeogenic	FbaA	39.1	Y	N	N
Phosphoenol pyruvate carboxykinase	Gluconeogenic	Pck	59.6	Y	Y	N
Malate dehydrogenase	Gluconeogenic	Mdh	32.3	Y	N	N
Phosphoenolpyruvate carboxylase	Gluconeogenic	Ppc	99	N	N	N
Malate dehydrogenase (oaa-reducing)	Gluconeogenic	MaeB	82.4	N	N	N
Glyceraldehyde phosphate dehydrogenase	Glycolytic, Gluconeogenic	GapA	35.5	N	N	N
Phosphoglycerate kinase	Glycolytic, Gluconeogenic	Pgk	41.1	1-Y, 2-N	Y (both oligomers)	N
Phosphoglycerate mutase	Glycolytic, Gluconeogenic	Gpm A	28.6	Y	N	N

isomerase (TpiA) and phosphoenolpyruvate carboxylase (Ppc)) were not (**Table 8)**. For those that purified in two forms, the acetylation status of both oligomers were either similar (e.g. lipoamide dehydrogenase (LpdA) and phosphofructokinase B (PfkB) or not ((e.g. phosphoglycerate kinase (Pgk) and pyruvate kinase F (PykF)) **(Table 8)**. For example, Pgk oligomer 1 was acetylated, while Pgk oligomer 2 was not (data not shown). The same was true for PykF. These observations suggest that the oligomer state can dictate *in vivo* acetylation status and perhaps suggest a potential mechanism for the regulation of acetylation.

To determine if YfiQ is sufficient to acetylate any of the purified metabolic enzymes *in vitro*, we first asked if YfiQ is acetylated *in vivo* or if it can autoacetylate *in vitro,* similar to what is known about eukaryotic KATs [29, 40]. We reasoned that such a finding would help us understand the mechanism by which YfiQ acetylates its target substrates. Indeed, purified *E. coli* YfiQ was acetylated, albeit at low levels **(Figure 13)** (Hu *et al.,* in preparation). We next asked if addition of acetyl-CoA to YfiQ increased YfiQ acetylation, and determined that YfiQ has the potential to autoacetylate **(Figure 13)**. These findings were supported by mass spectrometry, which detected ten acetylated lysine residues on YfiQ **(Figure 13)** (Hu *et al.,* in preparation). We also observed a 40 kD protein whose acetylation seemed to require both YfiQ and acetyl-CoA **(Figure 13)**. Mass spectrometry identified this protein as the translation factor EF-Tu (data not shown) and detected both acetylation of residue K177 in the purified protein and further acetylation of K264 after incubation with Acetyl-CoA (Hu *et al.,* in preparation). These data confirm reports that EF-Tu is acetylated *in vivo* [1, 2], and shows that YfiQ may

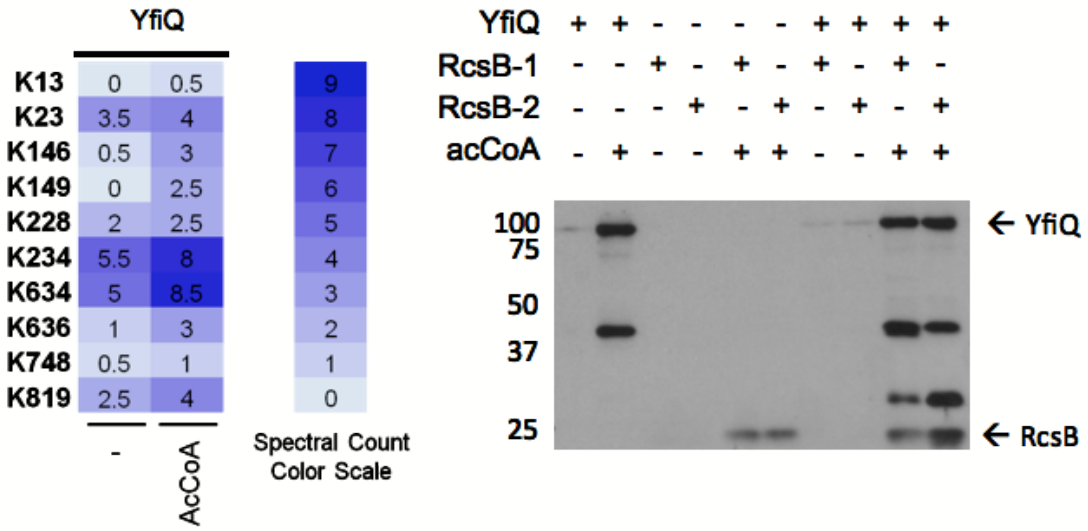

Figure 13. YfiQ can acetylate in the presence of acetyl-CoA, and can acetylate the response regulator RcsB. Data taken from Hu *et al.,* in preparation. *In vitro* acetylation reactions were run at 37^{o}C for 2 hours, and changes in acetylation status were determined using Western immunoblot (right) and mass spectrometry (MS) analysis (left). MS analysis identified changes in the acetylation status of various lysines on YfiQ. Peptide peak intensity or spectral count analysis shows semi-quantative analyses for changes in YfiQ acetylation.

be sufficient to acetylate EF-Tu *in vitro*. Alternatively, EF-Tu could have the potential to react with acetyl-CoA independently of YfiQ, either by autoacetylation or by chemical acetylation. Thus, in determining if YfiQ is post-translationally modified, we also determined that the translation factor EF-Tu co-purifies with YfiQ, and that YfiQ and acetyl-CoA or acetyl-CoA alone can induce its acetylation.

To test our hypothesis that YfiQ acetylates a subset of central metabolic enzymes, we incubated each purified protein with acetyl-CoA alone and with both acetyl-CoA and YfiQ, and performed anti-acetyllysine Western immunoblot analysis to establish acetylation status **(Figure 14)** or Ellman's assay **(Figure 15)** (Kuhn *et al.,* in preparation) to monitor CoA evolution. When incubated with acetyl-CoA alone, several proteins (e.g. RcsB, PfkA, GapA, and both oligomers of LpdA) became more acetylated **(Figure 14, Table 8)** and evolved more CoA **(Figure 15)**. This suggests the potential for autoacetylation, chemical acetylation or contamination with some unidentified acetyltransferase. When incubated with both acetyl-CoA and purified YfiQ, only four proteins (RcsB, both oligomers of LpdA, PfkA and PfkB) became substantially more acetylated **(Figure 14)** and evolved significantly more CoA **(Figure 15)** relative to incubation with acetyl-CoA alone.

Interestingly, all three of the YfiQ-acetylated enzymes, LpdA, PfkA, and PfkB are irreversible glycolytic enzymes. Each regulates critical steps of glycolysis: the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate (by PfkA and PfkB), and the conversion of pyruvate to acetyl-CoA (by LpdA, which is a subunit of the pyruvate dehydrogenase complex). Thus, we hypothesize that one or more of these YfiQ-

dependent acetylations results in the metabolic defect observed with the Phenotype MicroArray™ **(Table 5).** We predict that acetylation of PfkA, PfkB and LpdA by YfiQ inhibits the activity of these enzymes such that, when *yfiQ* is absent, glycolysis is favored over gluconeogenesis. This would lead to a defect in growth on gluconeogenic carbon sources, similar to the results obtained with the Δ*yfiQ* mutant in the Phenotype MicroArray™. Thus, YfiQ has the potential to regulate central metabolism by balancing carbon flux between glycolysis and gluconeogenesis. Future studies will examine if deletion of *yfiQ* does indeed alter carbon flux through central metabolism, and if YfiQ is necessary to acetylate these metabolic enzymes *in vivo*.

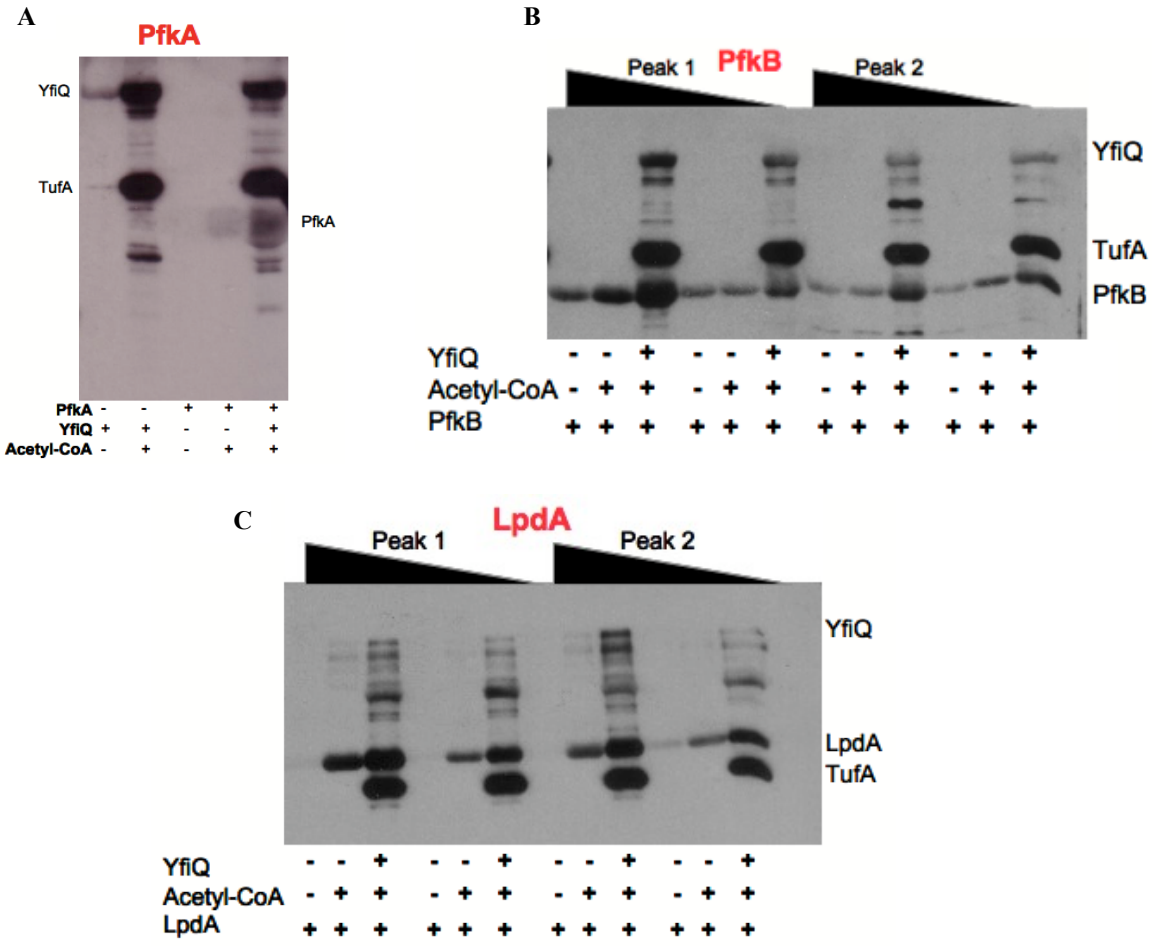

Figure 14. Anti-acetyllysine Western immunoblot analyses of *in vitro* acetylation reactions of YfiQ toward metabolic enzyme substrates. Reactions were run for 2 hours at 37^{o}C with YfiQ and various substrates. A) Acetylation reactions of PfkA, with and without YfiQ and acetyl-CoA. B) Acetylation reactions of PfkB peak 1 or peak 2 (which represent different oligomeric states), at various concentrations with and without YfiQ and acetyl-CoA. C) Acetylation reactions of LpdA peak 1 or peak 2, at various concentrations with and without YfiQ and acetyl-CoA. TufA is a contaminant of YfiQ purifications (Hu *et al.,* in preparation).

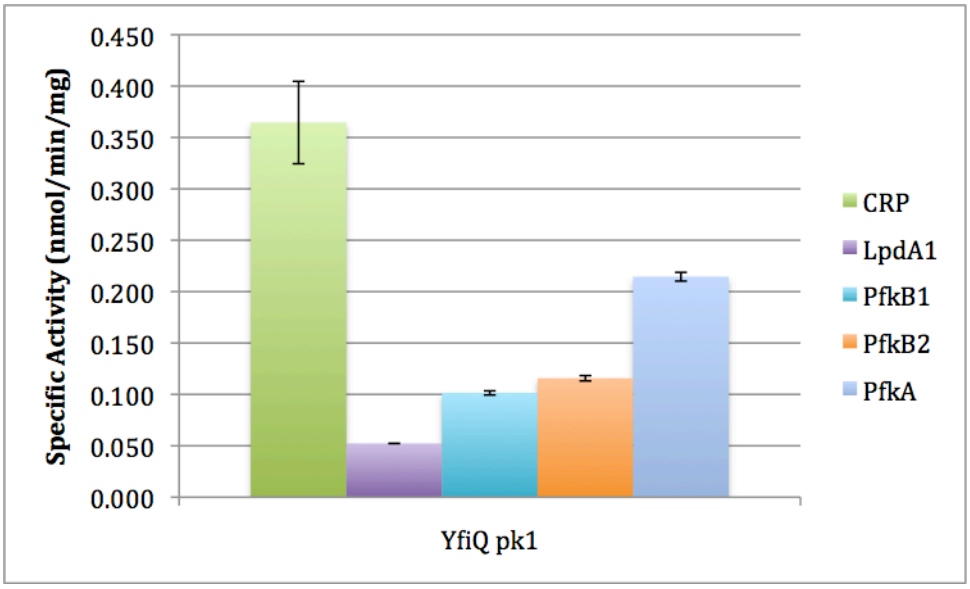

Figure 15. Ellman's assay measuring activity of YfiQ toward metabolic enzyme substrates. The enzyme activity of YfiQ was determined using a discontinuous kinetic assay with 82 μg of YfiQ (peak 1), 3 mM substrate and 0.5 mM acetyl-CoA at 37°C for two hours. Ellman's reagent was used to detect the production of CoA at A_{415nm}.

Summary

In this chapter, we asked if any of the putative CRP-dependent KATs identified in Chapter 3 have the potential to regulate central metabolism. Previously, we identified two KATs whose expression may be regulated by CRP: the known KAT YfiQ, and the putative KAT YjhQ. We hypothesized that the function of these KATs was to acetylate metabolic enzymes and alter metabolic homeostasis. We based this hypothesis on our preliminary mass spectrometry results, which identified several metabolic enzymes whose acetylation was detected when *crp* was present, but were not detected when *crp* was absent, indicating perhaps that CRP can regulate metabolic enzyme acetylation in *E. coli*.

We therefore asked if mutation of either KAT affected the ability of the cells to grow and respire carbon. Relative to its WT parent, the Δ*yfiQ* mutant grew and respired poorly on many gluconeogenic carbon sources. In contrast, the mutant grew and respired better than its WT parent on maltotriose, which when catabolized directly produces glucose-6-phosphate, the end product of gluconeogenesis. Since the mutant grew and respired better on maltotriose, we hypothesized that YfiQ controls the balance between glycolysis and gluconeogenesis. In contrast, mutation of *yjhQ* resulted in a growth defect in TB7 supplemented with glucose or succinate, and this growth defect required high levels of aeration. It is not clear from our experiments if YjhQ functions to regulate metabolism. We also tested if mutation of the only known KDAC, CobB, affected carbon utilization, and determined that mutation of *cobB* had little effect on the ability of the cells to grow and respire on all but two of the carbon sources tested. These results

indicate that there may be other deacetylases in *E. coli* that function to oppose YfiQ activity and regulate central metabolism. Indeed, data from our lab suggests the existence of novel deacetylases in *E. coli* (Hu and Wolfe, unpublished data).

Using *in vitro* acetylation analyses with purified YfiQ and potential substrates, we determined that YfiQ is sufficient to acetylate three metabolic enzymes: PfkA, PfkB, and LpdA, which are all irreversible glycolytic enzymes. Some of the proteins tested exhibited acetylation when the proteins were incubated with acetyl-CoA alone *in vitro*. This would suggest the potential for acetylation of proteins without the presence of an acetyltransferase, though the mechanism remains unclear. We also discovered that YfiQ along with some of the other purified proteins (PfkA, LpdA, RcsB, GapA), purified into different peaks, which represent different unidentified oligomeric states of the proteins. In some cases, both peaks exhibited the same acetylation profile (LpdA, PfkB); in other cases (RcsB), the peaks showed varying acetylation patterns across the different *in vitro* conditions. These results indcate that oligomeric states many dictate protein actylation. Taken together, our *in vivo* and *in vitro* findings support the hypothesis that acetylation of these enzymes by YfiQ has the potential to inhibit enzymatic activity, thereby altering the balance between gluconeogenesis and glycolysis, favoring the latter.

Future studies should utilize metabolic flux analyses to determine if deletion of *yfiQ* does indeed alter carbon flux through central metabolism in *E. coli*, as has already been shown in *S. enterica* [3], and whether mutation of the YfiQ-acetylated lysine on the metabolic enzymes does indeed alter enzymatic activity. Additionally, it remains to be determined if YjhQ is sufficient to acetylate proteins.

CHAPTER V

RESULTS: CRP ACETYLATION AND ITS IMPACT ON GENE EXPRESSION

The first report, in 2008, of a posttranslational modification of the global transcriptional regulator CRP used mass spectrometry to identify acetylation of K52 of CRP [2]. Shortly thereafter, K101 of CRP also was identified as acetylated [1], indicating that CRP could become acetylated on multiple lysines *in vivo*. In our previous chapters, we aimed to determine if 1) CRP can regulate protein acetylation and 2) if the putative CRP-dependent KATs have the potential to regulate central metabolism. In this chapter, we aim to: 1) identify the KAT(s) and KDAC(s) that regulate CRP acetylation, 2) identify any additional sites of acetylation on CRP and, 3) determine the effects of CRP acetylation on CRP-dependent transcriptional activation.

Preliminary Analyses of the Regulation of CRP Acetylation

Although it is known that CRP can become acetylated *in vivo*, it is not known how this acetylation is regulated. Thus, we tested whether the KAT YfiQ could regulate CRP acetylation. Previously, we and others had determined that CRP may regulate YfiQ transcription [4], and we had used *in vitro* acetylation analyses to determine that YfiQ is sufficient to induce acetylation of three metabolic enzymes: PfkA, PfkB, and LpdA. Since CRP is thought to regulate YfiQ transcription, we also asked if YfiQ could

acetylate its putative transcriptional regulator, CRP. To test if YfiQ can induce CRP acetylation, we used the same *in vitro* acetylation analyses described previously (Materials and Methods and Chapter 4).

Purified CRP showed little to no acetylation when assessed by anti-acetyllysine Western immunoblot analysis **(Figure 16)**. When acetyl-CoA was incubated with CRP, the acetylation state of CRP increased, indicating that CRP may have the potential to autoacetylate or to chemically acetylate. However, when YfiQ was added to acetyl-CoA and CRP, the acetylation state of CRP increased significantly **(Figures 15, 16)**. These results were confirmed by an independent, quantitative approach using the Ellman's assay to detect CoA evolution **(Figure 15)**. Currently, we do not know the sites of YfiQ-dependent acetylation, but mass spectrometry to identify the YfiQ-dependent lysine acetylations on CRP is planned.

We conclude that the KAT YfiQ is sufficient to induce CRP acetylation. Since CRP controls the transcription of many genes, CRP acetylation has the potential to alter global gene expression. Because CRP likely regulates transcription of *yfiQ* and YfiQ acetylates CRP, there may be an auto-regulatory loop between CRP and YfiQ that might play a homeostatic role **(Figure 22)**.

Summary of the Identified Sites of CRP Acetylation

Acetylation of CRP was first identified in 2008 by mass spectrometry analysis, where acetylation of K52 was identified in stationary but not exponential phase Luria broth (LB) cultures [2]. Acetylation of K101 was identified a year later in exponential

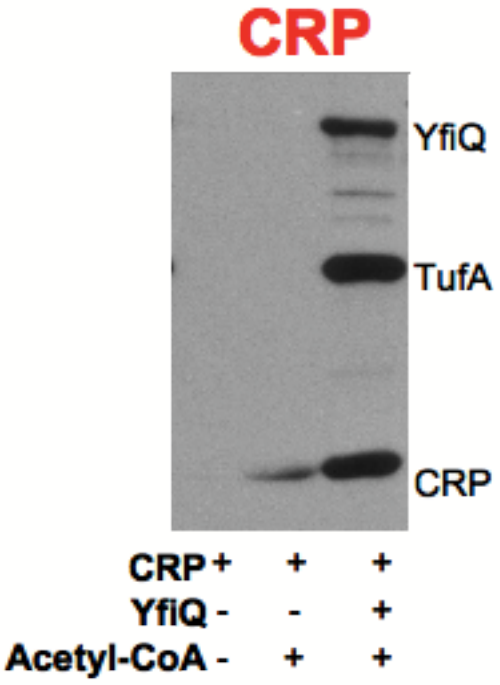

Figure 16: YfiQ acetylates the global regulator CRP. Anti-acetyllysine Western immunoblot analysis of in vitro acetylation assays with YfiQ and CRP. In vitro acetylation reactions were run for 2 hours at 37^{o}C, with either YfiQ, CRP, or acetyl-CoA added to each reaction.

phase LB cultures [1]. In Chapter 3, we described a combination of approaches that helped us determine that CRP has the potential to regulate protein acetylation. During that study, we used mass spectrometry analysis to compare the acetylation status of proteins between Δ*crp* mutant cells either complemented with CRP (AJW 2313) or carrying the vector control plasmid (AJW 4524), each grown in TB7 supplemented with 0.4% glucose and 3 mM cAMP. We identified 20 proteins whose acetylations were present in the complemented cells but not identified in the vector control cells. One of these proteins was CRP: we identified three novel acetylation sites on CRP: K27, K36, K153. We also identified acetylation of K101, whose acetylation was previously shown to occur during exponential phase growth in LB media [1]. We did not observe acetylation of K52, which was previously reported [2]. Therefore, of the 15 lysines in CRP, 5 have been detected as acetylated, indicating that CRP can be acetylated on multiple lysines under different growth conditions **(Figure 17)**. Of the 5 sites of CRP acetylation, K52 and K101 are located on RNA polymerase interaction surfaces, and thus are of great interest. The effects of acetylation of these two sites will be explored in further detail.

Preliminary Analyses of the Affects of K52 Acetylation on CRP-Dependent Transcriptional Activation

K52 of CRP is located in a patch of residues of CRP known as Activating Region 3 (AR3). AR3 is thought to play an inhibitory role in the interaction between CRP and RNAP [130] as the positively charged K52 forms a salt bridge with negatively charged amino acid, masking the negatively charged AR3 and preventing interaction with a

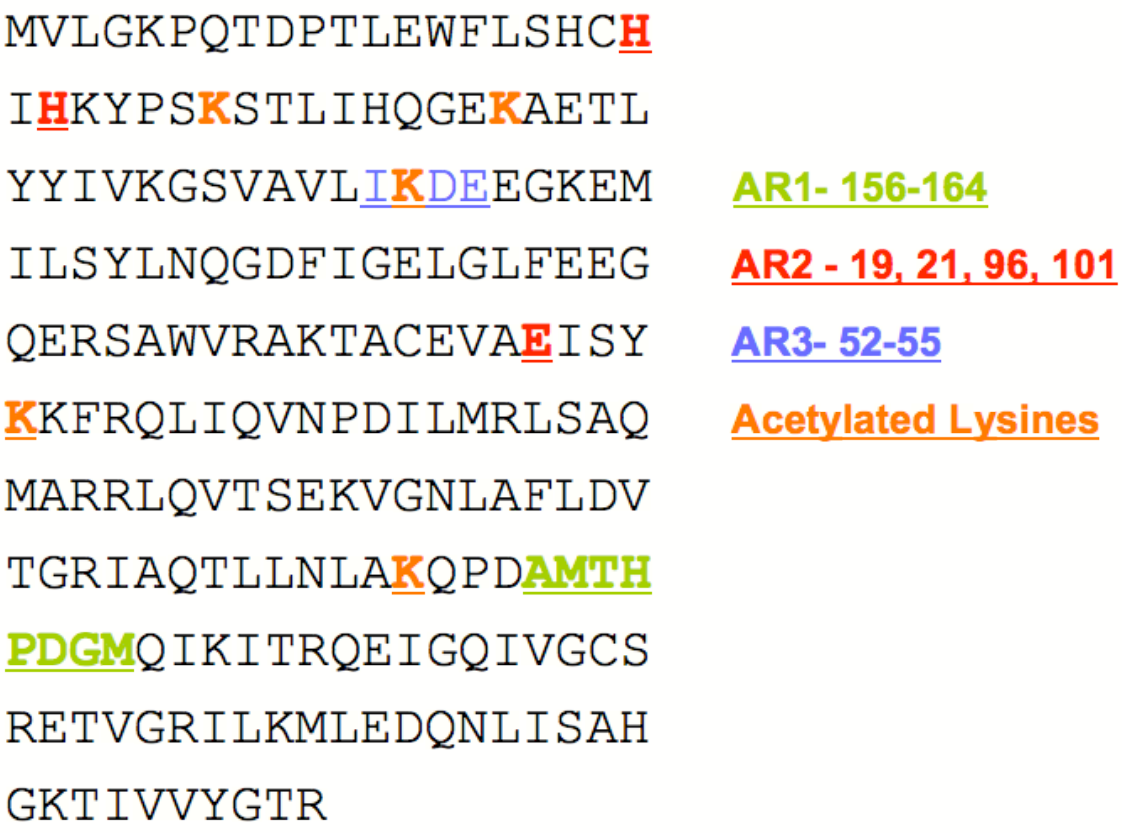

Figure 17. Locations of the five acetylated lysines on CRP. K101 and K52 are the two acetylations that are located directly within activating regions. Acetylated lysines are shown in orange. Activating region residues are underlined and colored (green AR1, red AR2, blue AR3). Notice that the AR2 residues (2 histidine, 1 glutamate and 1 lysine) are distantly located in the primary sequence.

positively charged surface of RNAP **(Figure 5)**. Previous research has shown that mutation of the positively charged K52 residue to an uncharged asparagine (N52) results in a tighter interaction between CRP & RNAP, presumably because it permits the negatively charged residues on CRP to interact with the positively charged residues on RNAP [130]. Thus, neutralization of the charged K52 permits a tighter interaction between the two proteins. Acetylation of K52 would neutralize the charge of the lysine. Since K52 acetylation had been reported [2], we hypothesized that K52 acetylation could play a physiologically relevant role similar to that of the K52N mutation; by neutralizing the charge of K52, acetylation would permit a tighter RNAP-CRP interaction, and lead to a change in gene expression.

We chose to test this hypothesis in the context of the *acs205* promoter (*Pacs205)* for the following reasons: 1) *acs* encodes acetyl-CoA synthetase (Acs), an enzyme that synthesizes acetyl-CoA from acetate [21]; 2) reversible acetylation regulates Acs enzyme activity [21]; 3) CRP is required to activate *acs* transcription [150]; and 4) the CRP K52N mutant actively inhibits transcription from *Pacs205*, a truncated form of the *acs* promoter that is primarily regulated by CRP [150]. We used TB7 supplemented with glucose and cAMP as a stimulus because the addition of glucose to TB7 leads to an increase in the acetylation of proteins [14]. Using *Pacs205,* we tested the following predictions: 1) The addition of glucose and cAMP will result in a decrease in CRP-dependent transcription, 2) mutation of K52 will alter the CRP-dependent transcriptional response to glucose and cAMP, and 3) manipulation of the acetyl-CoA:CoA ratio will alter the activity of CRP, and result in a change in transcription from *Pacs205*.

To monitor the effect of glucose and cAMP addition on CRP activity, we introduced an *Pacs205-lacZ* promoter fusion into the CRP complemented strain that had been deleted for the *lac* operon and grew the cells under four different conditions: TB7, TB7 supplemented with 3 mM cAMP, TB7 supplemented with 0.4% glucose, and TB7 supplemented with both glucose and cAMP, as described previously. Indeed, as predicted for a catabolite-repressible promoter, growth in the presence of glucose caused a 7-fold decrease in *acs* transcription relative to growth in the absence of glucose **(Figure 18)**. In contrast, addition of both cAMP and glucose increased *acs* transcription by about 2.5-fold relative to addition of glucose alone **(Figure 18)**, which returned transcription to the unsupplemented level. Thus, cAMP can antagonize the effect of glucose, as predicted by their metabolic relationship [156, 157]. This allowed us to test the roles of K52 and acetyl-CoA in CRP-dependent *acs* transcription.

We used the well-characterized CRP K52N mutant [129, 150] to test the role of K52 in cAMP-glucose-induced CRP-dependent *acs* transcription. If the cAMP-glucose-induced increase in *acs* transcription involves K52 acetylation, then the K52N substitution should result in decreased transcription. Indeed, the K52N mutant (AJW2315) exhibited an approximately 2-fold decrease in transcription under conditions of high acetyl-CoA:CoA ratio (glucose plus cAMP) relative to transcription in unsupplemented TB7 **(Figure 19)**. However, the sole addition of cAMP failed to induce transcription of the K52N mutant, indicating that the K52N mutant is unresponsive to cAMP addition.

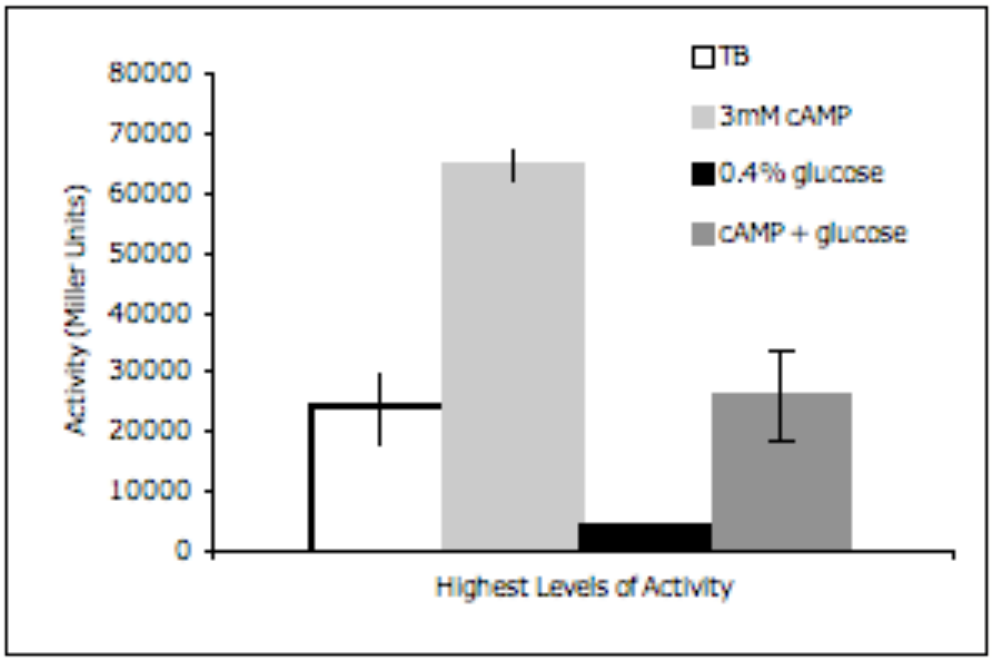

Figure 18. CRP responds to the addition of glucose and cAMP. Cells expressing *pacs205-lacZ* reporter fusion were transformed with the plasmid pDCRP (WT CRP). The transformants were grown at 37°C with shaking in TB7 (white bar), TB7 supplemented with 3 mM cAMP (light grey bar), TB7 supplemented with 0.4% glucose (black bar), or TB7 supplemented with 3 mM cAMP and 0.4% glucose (dark grey bar). Cells were harvested at regular intervals and OD_{600} and β-galactosidase activity were measured. The highest levels of activity across an 8-hour time course are shown. The mean of three independent cultures is shown, and the error bars indicate standard deviations.

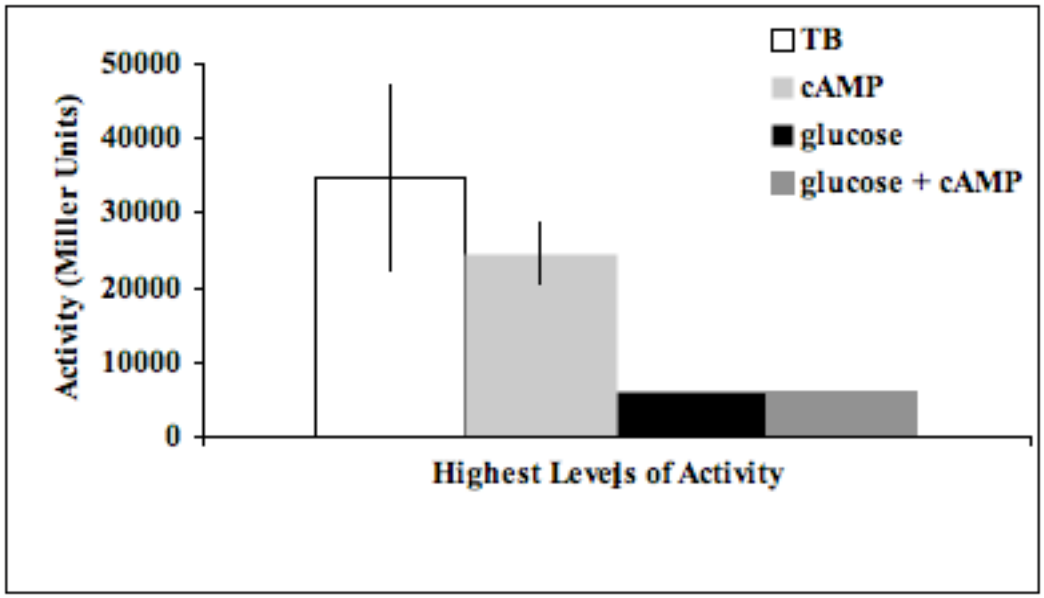

Figure 19. The K52N mutant of CRP does not respond to the addition of glucose and cAMP. Δ*crp* mutant cells expressing the *pacs205-lacZ* reporter fusion were transformed with the plasmid pDCRP/K52N, expressing the K52N AR3 mutant of CRP. The transformants were grown at 37^{o}C with shaking in TB7 (white bar), TB7 supplemented with 3 mM cAMP (light grey bar), TB7 supplemented with 0.4% glucose (black bar), or TB7 supplemented with 3 mM cAMP and 0.4% glucose (dark grey bar). Cells were harvested at regular intervals and OD_{600} and β-galactosidase activity were measured. The highest levels of activity across an 8-hour time course are shown. The mean of three independent cultures is shown, and the error bars indicate standard deviations.

Since the K52N mutant can activate transcription in TB7 alone, this indicates that the mutant can bind to the DNA, but the K52N mutant does not respond to cAMP, which we know activates DNA binding of CRP. If the K52N mutant is able to bind the DNA, but is unable to activate transcription in the presence of cAMP alone, or glucose and cAMP together, this suggests that glucose must have an additional effect on CRP independent of K52.

If glucose-cAMP-induced *acs* transcription results from an increase in the acetyl-CoA:CoA ratio, and if the acetyl-CoA:CoA ratio leads to high levels of acetylation of CRP, then decreasing that ratio should eliminate the response to glucose. To achieve this reduction, we increased the CoA pool by overexpressing pantothenate kinase (PK), an enzyme that catalyzes the first step in CoA synthesis. Overexpression of PK leads to a 3-fold increase in CoA concentration [163], and this resulted in a decrease in the glucose-cAMP-induced *acs* transcription mediated by wild-type CRP (AJW3762) **(Figure 20A)**, but had no effect on transcription by the K52N mutant (AJW3764) **(Figure 20B)**. These data support the hypothesis that glucose-cAMP-induced *acs* transcription is sensitive to the acetyl-CoA:CoA ratio and are consistent with the hypothesis that acetylation of a lysine other than K52 of CRP, a lysine either on CRP or RNAP, is responsible for regulating the *acs* transcription response to glucose and cAMP.

Our preliminary studies into the effects of K52 acetylation indicate that: 1) CRP-dependent *acs* transcription responds to the addition of glucose and cAMP, which we predict leads to an increase in the acetyl-CoA:CoA ratio [14, 163], and the acetylation of CRP, 2) that this activity correlates with the acetyl-CoA:CoA ratio, 3) that K52 is

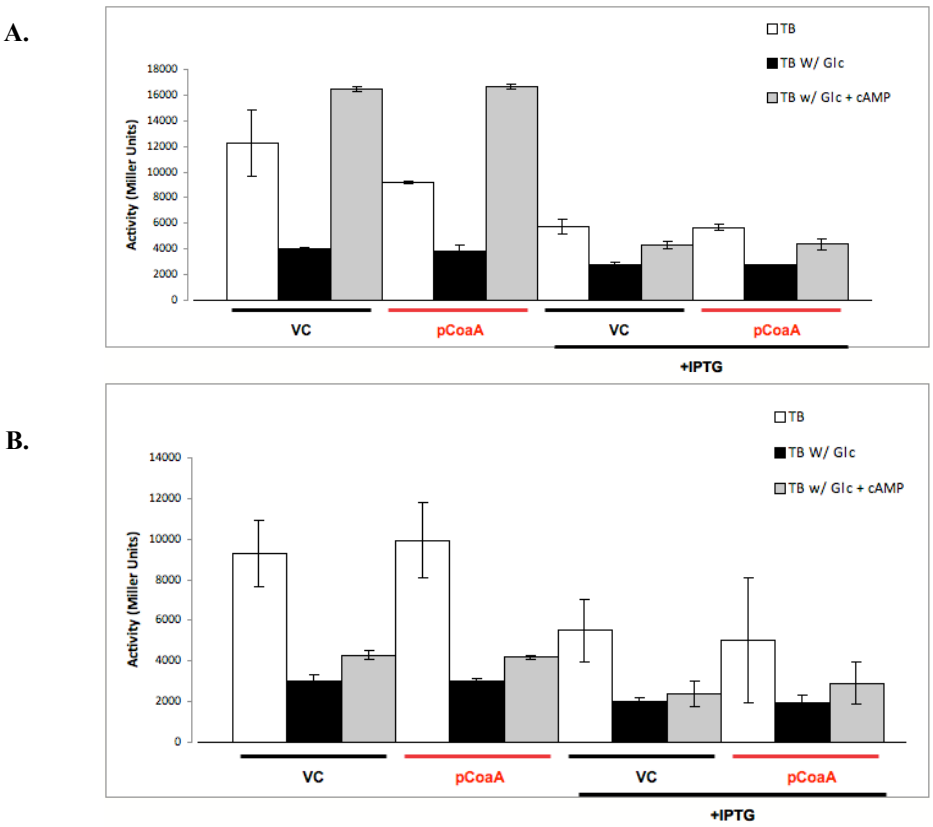

Figure 20. Only WT CRP is sensitive to manipulations of Acetyl-CoA and CoA levels. Δ*crp* mutant cells expressing *pacs205-lacZ* reporter fusion and carrying either WT CRP **(Panel A)**, or pDCRP/K52N **(Panel B)** plasmids, were transformed with the vector pCA24N plasmid (black lines), or a pCA24N derivative containing the *coaA* ORF (red lines). The transformants were grown at 37^{o}C with shaking in TB7 (white bar), TB7 supplemented with 0.4% glucose (black bar), or TB7 supplemented with 3 mM cAMP and 0.4% glucose (dark grey bar). 50 μM IPTG was used to induce *coaA* expression. Cells were harvested at regular intervals and OD_{600} and β-galactosidase activity were measured. The highest levels of activity across an 8-hour time course are shown. The mean of three independent cultures is shown, and the error bars indicate standard deviations.

important to regulate *acs* transcription, but the glucose-cAMP response seems to be independent of K52, and 4) that there must be another glucose-cAMP specific acetylation of a lysine either on CRP, RNAP or both CRP and RNAP. These results are consistent with the hypothesis that glucose and cAMP impact the acetylation status of CRP & that this PTM influences CRP function. However, we have yet to determine: 1) how CRP acetylation is regulated and 2) how CRP acetylation affects interaction of CRP with RNA polymerase.

Preliminary Analyses of the Affects of K101 Acetylation on CRP-Dependent Transcriptional Activation

The transcription data we acquired with the K52N mutant argues against acetylation of K52 as the primary actor in the glucose-cAMP-dependent transcriptional response. These results suggest that there may be another lysine involved in regulating CRP-dependent transcription in the presence of glucose and cAMP. Indeed, published reports identified another acetylation of CRP on K101 [1], a lysine found within a surface exposed patch of CRP called activating region 2 (AR2). K101 of AR2 is known to make direct contact with a negatively charged residue on surface of the N-terminal domain of the α subunit of RNAP (α-NTD). This contact is required for transcription from Class II promoters [128] **(Figure 4)**. At CRP-dependent Class II promoters, CRP binds at a site near the -35 hexamer and interacts with the α-NTD of RNAP using the AR1 and AR2 surfaces [126].

Disruption of the charge on either CRP or the α-NTD results in a loss of transcription from Class II promoters, indicating that these charge-charge interactions are required for transcriptional activation [128]. Acetylation of a lysine neutralizes its charge, which in the case of K101 would result in loss of a positive charge of the AR2 surface. Indeed, both reversal of charge (K101E) and loss of charge (K101A) result in complete loss of transcription from Class II promoters [128], indicating that K101 is critical for Class II-CRP-dependent transcription. Based on the previously published results, we hypothesized that the acetylation of K101 would disrupt the charge-charge interaction between AR2 of CRP and the α-NTD of RNAP, thereby affecting transcription from Class II promoters.

To test the hypothesis that K101 acetylation affects CRP-dependent Class II transcription, we have constructed acetylated lysine mimics of K101, similar to those used in the well-studied field of histone acetylation [164]. In this approach, the lysine is converted either to a glutamine (Q) to mimic an acetylated lysine, or converted to an arginine (R) to mimic an unacetylated lysine **(Strains listed in Table 1)**. We used the well-characterized semi-synthetic *CC-41.5-lacZ* promoter [165, 166] as the readout for CRP activity at a Class II promoter. If acetylation of K101 affects the charge-charge interaction between CRP and the α-NTD of RNAP, then the K101Q mutation should disrupt this interaction and diminish transcription, while the K101R mutation should behave more like WT, since arginine carries the same charge properties as an unacetylated lysine.

Preliminary experiments suggest that the charge of K101 is indeed important in regulating CC-41.5 transcription, but it is unclear how mutation of K101 to K101Q affects transcription from this promoter. The K101E mutant which is known to disrupt transcription from the CC-41.5 promoter, failed to do so in our experiment, rendering the results of the K101Q, R, and A mutants unreadable. We however can say that mutation of K101 to K101Q, R, and A does not affect DNA binding of these CRP variants, as all of the K101 variants have levels of transcription that were comparable to WT **(Figure 21)**. Surprisingly, none of the mutants exhibited any significant changes in transcription when compared to transcription with WT CRP.

These experiments were performed in unsupplemented TB7, and future studies must investigate transcriptional activation of WT CRP and the K101 variants when cells are grown in TB7 supplemented with 0.4% glucose and 3 mM cAMP; a growth condition shown to induce K101 acetylation (see Summary of the Identified Sites of CRP Acetylation section above). Thus, it is possible then that we were unable to detect differences in transcription due to the lack of induction of CRP acetylation. Future studies must also determine which KATs and KDAC, if any, regulate K101 acetylation, and determine if K101 acetylation affects the interaction between CRP and RNA polymerase.

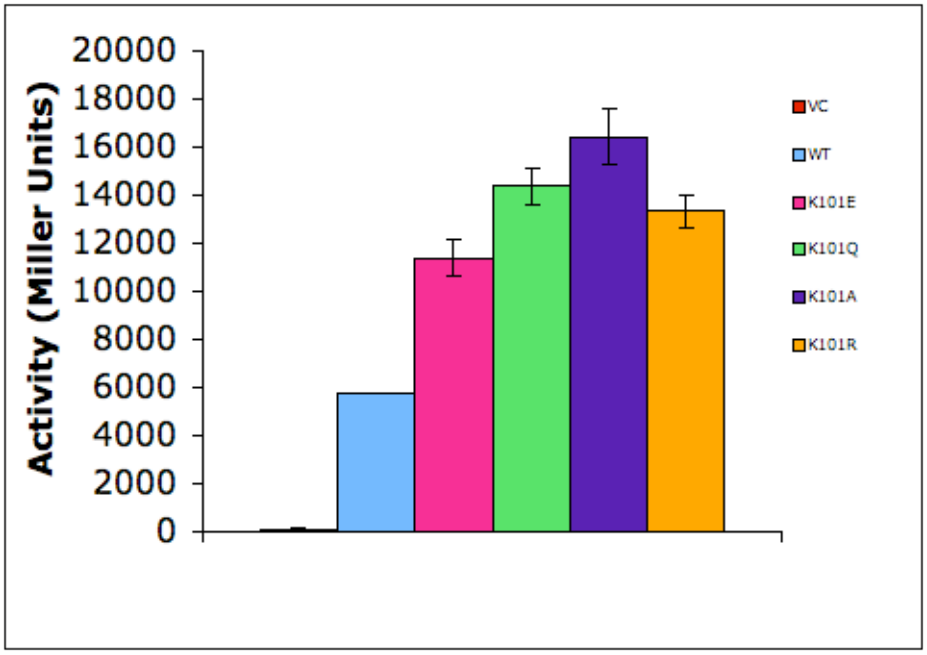

Figure 21. Mutation of K101 and the response from the CC-41.5 promoter. Cells expressing *pCC-41.5-lacZ* reporter fusion were transformed with pBR322 vector control (red bar) pDCRP (blue bar), pDCRP/K101E (pink bar), pDCRP/K101Q (green bar), pDCRP/K101A (purple bar), or pDCRP/K101R (orange bar) plasmids. The transformants were grown at 37°C with shaking. Cells were harvested at regular intervals and OD_{600} and β-galactosidase activity were measured. The highest levels of activity across an 8-hour time course are shown. The mean of three independent cultures is shown, and the error bars indicate standard deviations.

Summary

In this chapter, we assessed the regulation and transcriptional effects of CRP acetylation. Previously, two lysines on CRP had been identified as acetylated: K52 and K101 [1, 2]. Our studies confirmed acetylation of K101 and identified 3 new sites of acetylation: K27, K36, and K153. The putative CRP-dependent KAT, YfiQ was sufficient to induce CRP acetylation, although the lysines whose acetylations are YfiQ-dependent remain to be identified.

We hypothesized that the functions of two of the acetylations, K52 and K101, were to neutralize the charge and thereby alter interactions between CRP and RNA polymerase, affecting transcription from various CRP-dependent promoters. We predicted that acetylation of K101 would result in a decrease in interactions between CRP and RNAP, and acetylation of K52 would result in the opposite – increased interactions between CRP and RNAP. Preliminary evidence using transcriptional assays suggests that the charges of both these lysines are required for transcription from the two promoters assayed in this study, and acetylation of these lysines is likely to have an affect on CRP-dependent transcription. Although we have yet to identify a clear effect of these acetylations on CRP-dependent transcription, we have strong preliminary evidence that supports the merit of our hypothesis that acetylation of CRP affects CRP-dependent transcriptional activation. This hypothesis will be extensively tested in future studies.

CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

The discovery of large-scale protein lysine acetylation in bacteria has spurred a new field: the study of the regulation and effects of bacterial protein acetylation. To date, however, the acetylation of only five proteins in *E. coli* have been studied in any depth: CheY, Acs, RNAP, RcsB, and RNase R [8-17].

Of the more than 200 *E. coli* proteins with detectable acetylations, many are central metabolic enzymes [1, 2]. Some evidence exists that suggests that acetylation can affect metabolic enzyme function in bacteria [3, 15, 22]. On this basis, acetylation is predicted to affect carbon flux through central metabolism [3]. However, much remains to be discovered.

Preliminary evidence suggests that CRP may regulate protein acetylation in *E. coli*. One study has shown that CRP can regulate transcription of two putative KATs *in vitro* [37]. Another reports that cAMP, the allosteric effector of CRP, can regulate expression *yfiQ*, which encodes the only studied KAT in *E. coli* [4].

The goal in this study was to determine if CRP actually regulates protein acetylation in *E. coli*, and to gain insight into the mechanism(s) by which this regulation might occur. We also aimed to understand the regulation of metabolic enzyme acetylation and some of the downstream effects of that acetylation. We hypothesized that CRP would

regulate protein acetylation, that one or more KAT might regulate acetylation of central metabolic enzymes and that acetylation of at least one central metabolic enzyme would impact carbon flux.

Using both *in vivo* and *in vitro* approaches, we determined that CRP does indeed regulate protein acetylation in *E. coli*, most likely by regulating the expression of one or more KATs. We also determined that at least one of these putative-CRP dependent KATs, YfiQ, is sufficient to acetylate several metabolic enzymes. One is lipoamide dehydrogenase (LpdA), a subunit of both the pyruvate dehydrogenase complex (PDHC) and α-ketoglutarate dehydrogenase, which synthesize acetyl-CoA and succinyl-CoA, respectively. The others are both phosphofructokinases PfkA and PfkB, which convert fructose-6-phosphate to fructose-1,6-bisphosphate. All three are irreversible glycolytic enzymes. Since, deletion of *yfiQ* resulted in a growth defect on over 20 mostly gluconeogenic carbon sources, we hypothesized that CRP- and YfiQ-dependent protein acetylation of certain strictly glycolytic enzymes shifts central metabolic equilibrium towards gluconeogenesis.

We also began to study the downstream effects of CRP acetylation. Although no conclusive evidence was obtained, the preliminary results support the hypothesis that acetylation of certain lysines on the surface of CRP has the potential to impact CRP-dependent Class II promoter activity.

The implications of this work and future directions for this study will be elaborated in the following sections.

Nutritional requirements for CRP-dependent protein acetylation

To test the role of CRP in controlling protein acetylation, we first determined the nutritional requirements for CRP-regulated acetylation, finding that acetylation required growth on both amino acids and carbon sources that feed into central metabolism. Growth on amino acids or carbon sources alone did not stimulate CRP-dependent acetylation **(Figures 6 and 7)**. Previous research from our lab had shown that the addition of glucose to TB7 induces protein acetylation [14]. The work presented in the thesis verifies that finding and furthers it by identifying additional carbon sources that can induce protein acetylation **(Figure 7)**.

This protein acetylation does not require catabolite repression, as growth on either catabolite repressing (e.g. glucose) or non-catabolite repressing (e.g. acetate) carbon sources produced similar acetylation patterns **(Figure 7)**. These results also provide additional evidence for the hypothesis that the critical element is the acetyl-donor, acetyl-CoA [14, 167]. Transcriptional evidence from our lab suggests that it is the acetyl-CoA:CoA ratio that is important, as decreasing acetyl-CoA or increasing CoA concentrations have a similar effect [14]. Further work must be done to fully understand the growth requirements for acetylation. For example, what is the role of the amino acids? Which amino acids are critical? Does acetyl-CoA concentration or the acetyl-CoA:CoA ratio affect the activity of KATs or KDACs? Indeed, a recent publication reported that acetyl-CoA activates, while CoA inhibits the activity of certain eukaryotic KDACs [168].

CRP-dependent regulation of protein acetylation in *E. coli*

No definitive studies exist showing that CRP can regulate protein acetylation in *E. coli*. While evidence exists to support the hypothesis, it is correlative at best. For example, one study shows that CRP is sufficient to activate *in vitro* transcription of two putative KATs: *yjhQ* and *yedL* [37]. Another study reports that exogenous cAMP stimulates transcription from a promoter that drives transcription of *yfiQ,* which encodes a known KAT [4]. Taken together, these two studies raise the possibility that CRP may regulate protein acetylation.

We have extended these studies, using anti-acetyllysine Western immunoblot analysis to identify the nutritional requirements for CRP-dependent protein acetylation *in vivo*, to show that CRP regulates protein acetylation in *E. coli*, and to demonstrate that both CRP activating regions regulate CRP-, glucose-, and cAMP-dependent acetylation: while activating region 1 is required, activating region 2 contributes **(Figure 8)**. The identification of sequences that resemble the consensus CRP binding site located upstream of *yfiQ* and *yjhQ* **(Figure 9)** strengthens the hypothesis that CRP could regulate protein acetylation in *E. coli* by regulating expression of KATs.

While all the available evidence supports the hypothesis that CRP regulates protein acetylation in *E. coli*, much remains to be determined. For example, there is no actual evidence that CRP binds to the identified sequences. Also, while it is clear that cAMP regulates *yfiQ* transcription, no solid evidence shows that this effect requires CRP. Furthermore, no *in vivo* evidence exists to demonstrate that either cAMP or CRP regulates *yjhQ.* Thus, future studies should determine if CRP controls the transcription of

any of the other known or putative KATs. If not, future studies should determine how CRP influences protein acetylation *in vivo* and elucidate the mechanisms by which KAT transcription is regulated.

This putative link between cAMP via CRP and KAT regulation is exciting. Evidence for a similar system exists in other bacteria. Work done in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* indicates that cAMP regulates protein acetylation in these bacteria by directly regulating KAT activity [38, 169]. MSMEG_5458 and its homolog in *M. tuberculosis* are composed of a cAMP-binding domain fused to a KAT domain that can acetylate Acs. Thus, in response to cAMP, these KATs acetylate Acs [38, 169]. Could we be looking at a similar but less hard-wired relationship, where cAMP binds the transcription factor CRP, to activate transcription of KAT-encoding genes? If so, it is intriguing that YfiQ acetylates Acs, whose gene is transcribed in a strictly cAMP- and CRP-dependent manner.

Metabolic Enzyme Acetylation in *E. coli*

Acetylation of acetyl-CoA synthetase (Acs) was the first reported and best-studied acetylation of a bacterial metabolic enzyme [15, 16]. Since these studies, however, proteomics studies using mass spectrometry have identified many additional acetylations of metabolic enzymes [1-3]. Indeed, acetylation occurs on most of the central metabolic enzymes of *E. coli* and *S. enterica*. Acetylation was detected on all of the enzymes involved in glycolysis, except two (glucokinase and phosphofructokinase). Acetylation also was detected on two gluconeogenic enzymes (phosphoenolpyruvate carboxykinase

and aldolase). Finally, acetylation was detected on several TCA cycle enzymes (citrate synthase, isocitrate lyase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase) [1-3]. Although the YfiQ homolog of *S. enterica* (Pat) was shown to acetylate GapA, Acs, AceE, and AceK [3], it is not known if these events occur in *E. coli* and, if so, whether these acetylations affect carbon flux.

Our work furthers these studies and provides preliminary insights into how acetylation might affect carbon flow through central metabolism. We used mass spectrometry analysis to identify several central metabolic enzymes whose acetylation was detected when CRP was expressed, but were not detected when CRP was absent **(Table 4)**. While non-quantitative, these data are supportive of the hypothesis that CRP regulates acetylation of central metabolic enzymes.

These data and others [3], combined with observations supporting the hypothesis that CRP regulates expression of YfiQ, led us to hypothesize that YfiQ may acetylate central metabolic enzymes in *E. coli*. If YfiQ does acetylate metabolic enzymes, and if enzyme acetylation affects activity, and therefore carbon flux through central metabolism, then deletion of *yfiQ* should affect the ability of *E. coli* to utilize and grow on different carbon sources. Indeed, deletion of *yfiQ* did affect the ability of *E. coli* to grow and respire on more than 20 different carbon sources **(Table 5)**. However, it is not clear where the defect in metabolism lies. Many of the carbon sources that supported poorer growth and respiration of the *yfiQ* mutant than its WT parent require the gluconeogenic pathway. In contrast, the only carbon source that permitted better growth and respiration of the *yfiQ* mutant than its WT parent was maltotriose, which is directly

catabolized into glucose-6-phosphate and thus does not require gluconeogenesis. That the *yfiQ* mutant actually fared better than its parent on maltotriose suggests that the WT sacrifices some growth to build glucose-6-phosphate and that YfiQ plays a role in establishing the proper balance between glycolysis and gluconeogenesis.

Based on our *in vivo* mass spectrometry data, the PM array data with the *yfiQ* mutant, and the potential CRP-dependent regulation of *yfiQ* transcription, we hypothesized that YfiQ acetylates metabolic enzymes in *E. coli*, and that such acetylation would alter metabolic flux. We therefore determined if YfiQ (along with its substrate acetyl-CoA) is sufficient to acetylate central metabolic enzymes using *in vitro* acetylation reactions, and determined that YfiQ can specifically acetylate three enzymes: lipoamide dehydrogenase (LpdA), and the two phosphofructokinases (PfkA, PfkB). All other enzymes tested showed no changes in acetylation status when YfiQ was added to the *in vitro* reaction mixture. The finding that YfiQ acetylated only three irreversible glycolytic enzymes warrants further investigation into how YfiQ-dependent acetylation of these enzymes affects their activity, and whether acetylation of LpdA, PfkA, and/or PfkB affects the equilibrium between glycolysis and gluconeogenesis. The combination of the PM array data with the acetylation data suggests that acetylations of LpdA, PfkA, and PfkB function to inhibit the activity of these enzymes, such that when *yfiQ* is absent, glycolysis is favored over gluconeogenesis.

These results differ from the findings of Wang *et al.*, who showed that deletion of *pat* from *S. enterica* leads to a 40.7% decrease in the glycolysis/gluconeogenesis flux ratio when compared to WT [3]. Thus, when *pat* is absent, gluconeogenesis is favored

over glycolysis. This would imply that Pat-dependent acetylation of metabolic enzymes activates glycolysis, perhaps by acetylating the reversible glycolytic/gluconeogenic enzyme GapA and favoring its glycolytic activity [3]. We found the opposite in *E. coli*; deletion of *yfiQ* appeared to disfavor gluconeogenesis and reversible GapA did not appear to be specifically acetylated by YfiQ. Instead, the YfiQ-dependent acetylations were on strictly glycolytic enzymes. Could Pat and YfiQ, which contain 92% amino acid similarity, function differently in *S. enterica* and *E. coli*, respectively?

During the course of the *in vitro* experiments, we discovered that six of the fourteen enzymes tested acetylated *in vitro* in the presence of acetyl-CoA alone **(Table 8)**. It is possible that the purification preparations of some metabolic enzymes are contaminated with a co-purifying acetyltransferase. It is not clear whether enzyme-independent acetylation occurs *in vivo*, and the physiological affects of this type of acetylation are not known. Future studies might elucidate whether non-enzymatic acetylation of proteins occurs *in vivo*, and determine the factors that dictate if a protein can undergo spontaneous acetylation.

We also discovered that YfiQ in *E. coli* can acetylate itself in the presence of acetyl-CoA **(Figure 13)**. We do not know if this acetylation of YfiQ affects YfiQ structure, substrate recognition, or stability, and thus function of YfiQ. Data from *S. enterica* suggests that Pat also undergoes acetylation in the presence of acetyl-CoA, but studies into the thermodynamics and kinetics of Pat activity did not assess if Pat acetylation affected KAT activity [17, 32]. Thus, future work must take into account the acetylation of YfiQ/Pat and determine if the acetylation affects YfiQ function, similar to

the work that has been done to elucidate the effects of acetylation of KATs on KAT activity in eukaryotes [40, 41].

Affects and Regulation of CRP Acetylation

The acetylation of CRP on two lysine residues deemed critical for transcriptional regulation from Class II-CRP dependent promoters was recently discovered [1, 2]. Currently, however, there are no studies into the regulation and affects of CRP acetylation. Acetylation of K101 and K52 have the potential to impact transcription by disrupting the protein-protein interactions between CRP and RNAP that are necessary for transcription from Class II promoters. Currently, there are only two other bacterial transcription factors where acetylation has been studied: RNAP and RcsB (Hu *et al.,* in preparation) [14, 17], and these studies support the hypothesis that acetylation can disrupt interactions between RNAP, transcription factors, and their DNA sites.

In our work, we sought to understand the regulation and affects of CRP acetylation, and determined that: 1) the KAT YfiQ is sufficient to acetylate CRP, 2) CRP acetylation also seems to occur in the presence of acetyl-CoA alone, 3) CRP can become acetylated on 5 of its 15 lysines, and 4) acetylation of CRP might alter CRP-dependent transcriptional activation.

The finding that YfiQ is sufficient to acetylate CRP **(Figure 16)** is exciting as it suggests the possibility of a feedback loop in which CRP regulates YfiQ expression and YfiQ acetylates CRP, altering its activity **(Figure 22)**. Studies to test this hypothesis should be performed.

We currently do not know which lysine acetylations require both YfiQ and acetyl-CoA and which acetylations require only acetyl-CoA. To identify the locations of these acetylations, one would perform mass spectrometry analysis on *in vitro* acetylated CRP. Ultimately, one would want to delineate the impact of these two different types of CRP acetylation on CRP-dependent transcription.

Previous studies had detected acetylation of K52 and K101. Our preliminary mass spectrometry studies of whole cell lysates from WT and complemented CRP cells revealed 3 additional sites of CRP acetylation: K27, K36, and K153. These lysines have not been implicated in regulating transcription or in affecting cAMP binding to CRP. Thus we chose to focus our efforts on the two lysines that are known to affect transcription: K52, and K101. However, it is possible that acetylation of K27, K36, and K153 or K101 and K52 could affect CRP activity by altering the structure of CRP, which would have the potential to disrupt CRP binding: 1) its allosteric affector cAMP, 2) consensus sequence on the DNA, or 3) both cAMP and the DNA. Additionally, acetylation of CRP could affect stability of the protein, thereby affecting CRP-dependent gene expression. These possibilities need to be tested in future studies of CRP acetylation.

The finding that CRP undergoes acetylation on multiple residues **(Figure 17)** indicates perhaps another regulatory mechanism to regulate CRP activity, the first being cAMP binding to CRP. Fluctuations in metabolism, leading to changes in the levels of acetyl-CoA, are also thought to affect the acetylation status of proteins in bacteria [30, 161, 170]. Thus, both cAMP levels and potentially the levels of acetylation are intimately tied to

the fluxes in central metabolism [30, 161, 170], indicating that CRP activity may respond in multiple ways to changes in metabolic conditions **(Figure 22)**. These multiple layers of CRP regulation are necessary since CRP controls expression of around 5% of genes in *E. coli*, and rampant activation of CRP would affect cell growth and viability.

Thus, the study of the regulation and affects of CRP acetylation is crucial to our understanding of the response of *E. coli* to changes in environmental conditions. Our work has given credence to the hypothesis that acetylation of CRP affects CRP-dependent transcriptional activation.

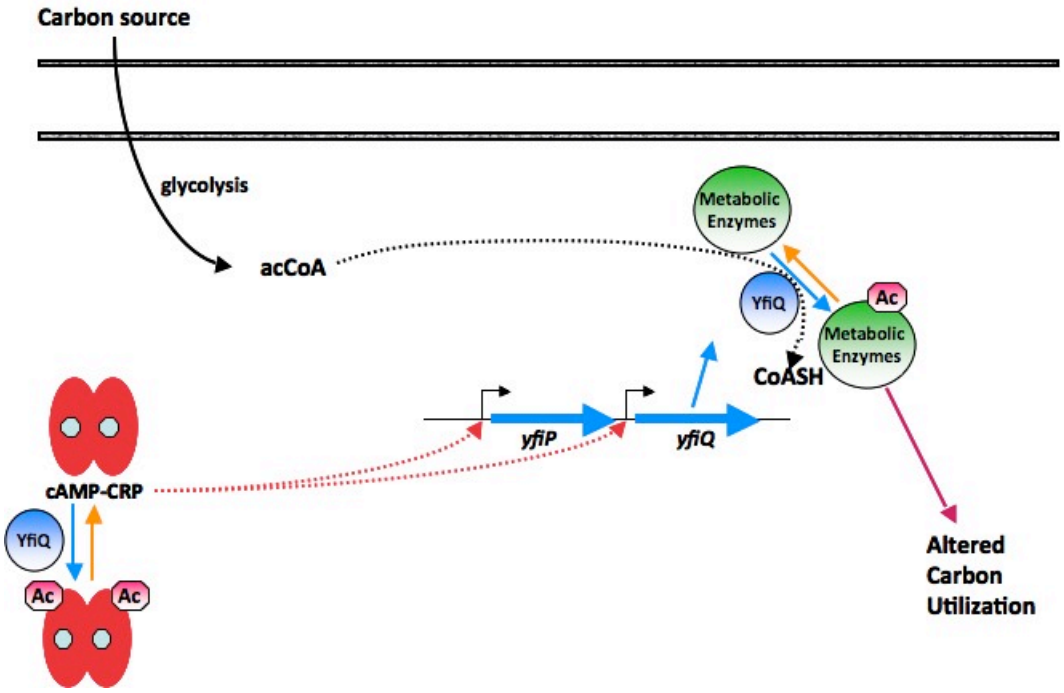

Figure 22. Proposed Model. Based on our work and others [4], we propose a model in which the central carbon regulator in *E. coli*, cAMP receptor protein (CRP) activates transcription from promoters located upstream of *yfiP* and *yfiQ*. In this model, the GNAT YfiQ acetylates various substrates in *E. coli*, including the metabolic enzymes phosphofructokinase (PfkA/PfkB) and lipoamide dehydrogenase (LpdA), the latter a subunit of pyruvate dehydrogenase. We predict that YfiQ-dependent acetylation of these metabolic enzymes alters their function, shifting the equilibrium between glycolysis and gluconeogenesis. We also propose that YfiQ acetylates CRP, altering its function & forming an autoregulatory loop.

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VITA

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After leaving the Army, Arti then returned to Grand Rapids to finish her undergraduate studies at Grand Valley State University (GVSU), in Allendale, Michigan, graduating with a Bachelors of Science in Biology with Honors in 2008. Her undergraduate research work on the discovery of novel antimicrobials led to the filing of a U.S. Patent (US 2011/0054034 A1, Methods of Using Carboxylic Amides as Antimicrobial Agents. W. Schroeder Jr., A. J. Walker, R. P. Smart, R. M. Morgan). While studying at GVSU, Arti was awarded two summer research grants (Summer Student Scholars, and the Salksi Award), and was chosen by the Department of Biology faculty to receive the Outstanding Biology Graduate Award.

Upon finishing her undergraduate coursework, Arti continued to pursue her passion for science and entered the Graduate Program in the Department of Microbiology and Immunology at Loyola University of Chicago in August of 2008. Under the mentorship of Dr. Alan Wolfe, Arti studied the regulation and affects of protein acetylation in *E. coli*, discovering novel inputs into the regulation of protein acetylation, and performed preliminary studies suggesting the potential involvement of acetylation in regulating central metabolism in *E. coli.*

During her time at Loyola, Arti was voted by the graduate student to serve as the GSC-LUMC student representative, acting as a liaison between the graduate students and the graduate faculty. Arti received Academic Honors as a Military Veteran from Loyola, and the National Science Foundation awarded Arti Honorable Mention for the Graduate Student Fellowship program. She also received two awards for poster presentations: the American Society of Microbiology Student Travel Grant (2011), and Molecular Genetics of Bacteria and Phages Poster Finalist (2011).