The Effect of Glucose on Transcription at CpxR- and OmpR-Regulated Promoters

Andrew Charles Cosgrove
Loyola University Chicago

Follow this and additional works at: https://ecommons.luc.edu/luc_theses

Part of the Microbiology Commons

Recommended Citation
https://ecommons.luc.edu/luc_theses/716

This Thesis is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Master's Theses by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.

This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 2012 Andrew Charles Cosgrove
LOYOLA UNIVERSITY CHICAGO

THE EFFECT OF GLUCOSE
ON TRANSCRIPTION AT
CPXR AND OMPR-REGULATED PROMOTERS

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN MICROBIOLOGY AND IMMUNOLOGY

BY

ANDREW CHARLES COSGROVE
CHICAGO, IL
MAY 2012
ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor Dr. Alan Wolfe for his time and guidance in my graduate career. I would also like to thank my committee members Dr. Chris Weithoff and Dr. Karen Visick for not only their time as committee members but for valuable technical advice.

I would also like to thank my fellow classmates as well as members of the Forest Preserve United Football Club for providing me with fun and unique ways to discuss science.

Finally, I would like to thank my parents and grandparents, whom without their never ending support and advice, this thesis would not have been possible.
For my grandparents Robert (1930-2009) and Nancy (1929-2011) Larson for providing me with the gift of education
TABLE OF CONTENTS

ACKNOWLEDGMENTS iii

LIST OF TABLES vii

LIST OF FIGURES viii

LIST OF ABBREVIATIONS x

ABSTRACT xii

CHAPTER ONE: INTRODUCTION 1

CHAPTER TWO: LITERATURE REVIEW 7
    Phosphorylation 7
    Glycosylation 10
    Acetylation 11

CHAPTER THREE: MATERIALS AND EXPERIMENTAL METHODS 15
    Bacterial strains 15
    Chemical and biological reagents 15
    Bacterial media and growth conditions 15
    Antibiotics 16
    Generalized P1 transduction 16
    Transformation 17
    Luminescence readings 17
    Fluorescence readings 18

CHAPTER FOUR: RESULTS 21
    Response of other CpxR-regulated promoters to exogenous glucose 21
    Screen of CpxR-regulated promoters 26
    Categories of CpxR-regulated promoters in response to glucose 27
    Response of CpxR-regulated promoters to nicotinamide 29
    Summary 30

CHAPTER FIVE: RESULTS 32
    Validating the fluorescent dual-reporter strain 33
    $ompC$ transcription varies between strain backgrounds 39
Glucose-regulated *ompC* and *ompF* transcription requires the response regulator OmpR

EnvZ is required for glucose-regulated transcription at *ompC*, but not at *ompF*

Glucose-regulated *ompC* and/or *ompF* transcription does not involve acetylation of the RNAP-OmpR complex

Glucose-regulated *ompC* and/or *ompF* transcription does not depend on the acetyltransferase YfiQ

Glucose-regulated *ompC* and *ompF* transcription is not sensitive to the deacetylase CobB

Glucose-regulated *ompC* and/or *ompF* transcription does not require acetylation of certain lysine residues on RNAP

Glucose-regulated *ompC* and/or *ompF* transcription is not sensitive to the AcCoA:CoA ratio

Summary

CHAPTER SIX

The effect of glucose on CpxR-regulated promoters

The effect of glucose on the *ompC* and *ompF* promoters

The effect of NAM on the *ompC* and *ompF* promoters

Differences in strain backgrounds

Development of luminescence and fluorescent techniques

Significance

REFERENCES

VITA
LIST OF TABLES

Table 1. Strains, phage, and plasmids used in this study 19
Table 2. Glucose-regulated behavior of CpxA*-regulated promoters: a summary 23
LIST OF FIGURES

Figure 1. Schematic describing reversible Nε-lysine acetylation 2

Figure 2. Schematic showing conventional cpxP activation through the CpxAR two-component system 3

Figure 3. Schematic showing CpxA-independent cpxP activation 5

Figure 4. Representative measurements associated with the cpxP promoter 25

Figure 5. Response to glucose by several CpxR-regulated promoters in WT, ΔcpxA and cpxR1 strains 28

Figure 6. Response to NAM by the ompC and ompF promoters in a cpxR1 strain 31

Figure 7. Schematic of the fluorescent dual-reporter strain 34

Figure 8. Response to osmolarity and glucose by the ompC and ompF promoters in the WT strain 38

Figure 9. Response to NAM by the ompC and ompF promoters in a WT strain 40

Figure 10. Response to glucose and NAM by the ompC promoter in two different background strains 41

Figure 11. Response to glucose by the ompC and ompF luminescence reporter plasmids in the fluorescent dual-reporter strain 43

Figure 12. Response to glucose by the ompC and ompF promoters in ΔcobB and ΔompR strains 45

Figure 13. Response to glucose by the ompC and ompF promoters in WT and ΔenvZ strains 47

Figure 14. Response to glucose by the ompC and ompF promoters in ΔcobB and ΔyfiQ strains 50

Figure 15. Response to glucose by the ompC and ompF promoters in strains expressing lysine-to-alanine mutants of the α-CTD of RNAP 52
Figure 16. Response to glucose by the *ompC* and *ompF* promoters 
in PHB- and CoaA-expression strains
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-CTD</td>
<td>carboxyl terminal domain of the α-subunit of RNA polymerase</td>
</tr>
<tr>
<td>AcCoA</td>
<td>acetyl-coenzyme A</td>
</tr>
<tr>
<td>AcP</td>
<td>acetyl phosphate</td>
</tr>
<tr>
<td>ACS</td>
<td>acetyl-CoA synthetase</td>
</tr>
<tr>
<td>amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>cam</td>
<td>chloramphenicol</td>
</tr>
<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>HK</td>
<td>histidine kinase</td>
</tr>
<tr>
<td>kan</td>
<td>kanamycin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µL</td>
<td>microliter</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
</tbody>
</table>
nm  nanometer
NAM  nicotinamide
OD600  optical density measured at 600nm
P-CpxR  phosphorylated CpxR
P-OmpR  phosphorylated OmpR
PTM  post-translational modification
RR  response regulator
RPM  revolutions per minute
RNAP  RNA polymerase
spc  spectinomycin
tet  tetracycline
TSS  transformation and storage solution
TB  tryptone broth
TB7  tryptone broth buffered to pH 7.0
TBT  tryptone broth for transduction
TCS  two-component system
vol  volume
wt  weight
YFP  yellow fluorescent protein
ABSTRACT

While reversible acetylation of proteins has been well studied in eukaryotes and is now recognized in bacteria, global protein acetylation in bacteria is a recently appreciated phenomenon. Protein acetylation is known to affect almost every aspect of cellular physiology in eukaryotes and there is proteomic evidence that this may also hold true in bacteria. In eukaryotes, lysines are acetylated by acetyltransferases that use acetyl-CoA as the acetyl group source, and de-acetylated by deacetylases. In bacteria, this reversible process uses enzymes homologous to those used by eukaryotes.

Our lab has recently found that acetylation of RNA polymerase (RNAP) can activate transcription at the CpxR-dependent promoter cpxP independent of activation by its cognate sensor kinase CpxA [1]. The RNAP acetylation is detected when cells grow in mixed amino acids (e.g. tryptone broth (TB)) supplemented with a glycolytic carbon source such as 0.4% glucose. In E. coli, this growth condition results in a global increase in protein acetylation [1]. CpxA-independent activation of cpxP in response to glucose requires AcCoA as the acetyl group donor, the acetyltransferase YfiQ, and K298 on the α-CTD of RNAP, which is acetylated in a YfiQ-dependent manner. This activation is reversed by the deacetylase CobB. These data support the hypothesis that glucose-induced CpxA-independent activation of cpxP transcription involves acetylation of K298 [1]. This effect of acetylation on cpxP transcription was discovered using a cpxP-lacZ-promoter fusion inserted in single copy into the chromosome [1, 2] and confirmed using a
cpxP-lux reporter fusion carried by a low-copy plasmid (Lima, Patel and Wolfe, unpublished data).

I performed a screen of CpxR-regulated promoters each carried on the same low copy plasmid [3], testing each promoter’s response to glucose. These plasmids were originally characterized in a constitutive kinase mutant of CpxA known as CpxA*[4, 5]. These mutants accumulate high levels of P-CpxR and the response to CpxA* depends entirely on CpxR phosphorylation [3-5]. This screen identified two categories of CpxR-regulated promoters: (1) those that are activated by CpxA* and (2) those that are repressed by CpxA* [3]. When I investigated the response to glucose by these promoters, I found that the response to glucose did not necessarily correlate with the response to CpxA*. This suggests that glucose can control CpxR-regulated promoters by a mechanism distinct from the conventional phosphorylation cascade.

To generalize glucose-induced control of CpxR-regulated promoters, we further characterized the glucose response of the CpxR-regulated and OmpR-dependent promoters ompC and ompF. The EnvZ-OmpR two-component system is one of the most extensively studied two-component systems in bacteria. Like CpxA, EnvZ is a sensor kinase that responds to changes in the extracellular environment, most prominently osmolarity and pH. Also like CpxA, it can act either as a net kinase or a net phosphatase for its cognate response regulator, OmpR. While the role of phosphorylation in the regulation of OmpR-dependent promoters is well known, the role of acetylation in this pathway has not been studied. The cpxP promoter responds similarly to both glucose and the CobB inhibitor, NAM, through an acetylation mechanism. When we saw that ompC and ompF responded similarly to glucose and NAM, we had compelling evidence that
suggested that acetylation might influence OmpR-dependent transcription. If glucose was affecting \textit{ompC} and \textit{ompF} transcription through an acetylation-dependent mechanism, then we hypothesized that it might be through a mechanism similar to the way glucose affects transcription at the \textit{cpxP} promoter.

If glucose affected transcription at the \textit{ompC} and \textit{ompF} promoters using the mechanism used at the \textit{cpxP} promoter, then we proposed that it would require AcCoA as an acetyl group donor, the acetyltransferase YfiQ, K298 on the $\alpha$-CTD of RNAP and be sensitive to CobB. We investigated the role of each of these components in \textit{ompC} and \textit{ompF} transcription and found that glucose does indeed affect transcription at these promoters, but it does so using a mechanism distinct from glucose-induced \textit{cpxP} transcription. Therefore, the role of acetylation at the \textit{ompC} and \textit{ompF} promoters remains to be determined.
CHAPTER ONE

INTRODUCTION

Although N\textsubscript{ε}-lysine acetylation of bacterial proteins has been known for some time, the observation that it is a global phenomenon is a recent discovery (for reviews, see [6, 7]). Like many regulatory processes, N\textsubscript{ε}-lysine acetylation is a reversible process: an acetyltransferase transfers an acetyl group from acetyl-CoA (AcCoA) to the epsilon nitrogen of a lysine within a target protein, while a deacetylase removes that acetyl group (Figure 1). In contrast to bacterial protein phosphorylation, bacterial N\textsubscript{ε}-lysine acetylation remains mostly uncharacterized. Yet, it has been suggested that acetylation might be as prevalent as phosphorylation [6, 8] and thus merits deeper investigation.

Lysine acetylation can affect protein stability, location, and/or function [6, 9, 10]. Indeed, the Wolfe lab recently reported that multiple lysines on multiple subunits of RNA polymerase (RNAP) can become acetylated and that at least one of these acetylations can alter the ability of RNAP to activate transcription from the CpxR-dependent cpxP promoter [1].

The conventional view of cpxP activation (Figure 2), and that of other CpxR-regulated promoters, is as follows: (1) in response to an extracytoplasmic stimulus, CpxA
Figure 1. Schematic describing reversible Nε-lysine acetylation [11]. KATs catalyze the reversible acetylation of lysines using the acetyl group from acetyl-CoA as the acetyl donor. Lysines are deacetylated by KDACs. Lys, lysine. KAT, lysine acetyl transferase. KDAC, lysine deacetylase.
Figure 2. Schematic showing conventional $cpxP$ activation through the CpxAR two-component system [1]. For details, see text. P, phosphoryl group. H, histidine. RNAP, RNA polymerase. NTD, N-terminal domain of the $\alpha$-subunit of RNAP. CTD, C-terminal domain of the $\alpha$-subunit of RNAP.
autophosphorylates on a conserved histidine residue; (2) phosphorylated CpxA donates this phosphoryl group to a conserved aspartate on its cognate response regulator CpxR; and (3) phosphorylated CpxR (P-CpxR) and RNAP cooperate to activate cpxP transcription.

_cpxP_ can also be activated in the absence of CpxA. It also can be activated in the absence of the extracytoplasmic stimulus, a condition that shifts CpxA from a net kinase to a net P-CpxR phosphatase [2]. This CpxA-independent activation requires that cells growing in a medium composed of mixed amino acids (e.g. tryptone) also be exposed to glucose or certain other carbon sources [1]. The current model for this CpxA-independent activation is as follows (Figure 3): (1) glucose is metabolized to AcCoA, raising the AcCoA:CoA ratio; (2) some of the AcCoA is converted to acetyl phosphate (AcP) causing its concentration to rise; (3) using AcCoA as the acetyl donor, the acetyltransferase YfiQ acetylates lysine 298 (K298) of the α-CTD of RNAP; (4) this acetylation is reversed by the NAD⁺-dependent (and thus nicotinamide (NAM) inhibited) deacetylase CobB; (5) using AcP as the phosphoryl donor, CpxR autophosphorylates on aspartate 51 (D51); and (6) together, both post-translational modifications activate _cpxP_ transcription. Thus, this CpxA-independent activation requires exposure to glucose (or other carbon sources that increase the AcCoA:CoA ratio), the acetyltransferase YfiQ, P-CpxR, and K298 of the α-CTD. It also is sensitive to NAM and CobB [1](Lima _et al._, submitted).
Figure 3. Schematic showing CpxA-independent cpxP activation [1]. For details see text. P, phosphoryl group. Ac, acetyl group. Glc, glucose. Pyr, pyruvate. AcCoA, acetyl-CoA. AcP, acetyl-phosphate. NAM, nicotinamide. RNAP, RNA polymerase. NTD, N-terminal domain of the α-subunit of RNAP. CTD, C-terminal domain of the α-subunit of RNAP.
This model for glucose-induced acetylation-dependent activation was generated by the exclusive study of the *cpxP* promoter; however, it is reasonable to believe that this may be a more global regulatory mechanism. In this thesis, I tested this hypothesis.

Upon characterization of a subset of CpxR-regulated promoters, I chose *ompC* and *ompF* for further study, specifically to determine if these two promoters were regulated by acetylation of RNAP in response to exogenous glucose in a manner analogous to the *cpxP* promoter. Results from this study definitively show that while glucose does regulate *ompC* and *ompF* transcription, it utilizes a mechanism that is distinct from the one that operates at the *cpxP* promoter.
CHAPTER TWO
LITERATURE REVIEW

Post-translational modification has long been thought to be restricted to eukaryotes; however, it is becoming increasingly clear that post-translational modification in bacteria may rival that of eukaryotes. For example, a recent whole proteome analysis of the Gram-negative bacterium *Shewanella oneidensis* revealed 390 distinct post-translational modifications (PTMs) on 1673 proteins [12]. This is in accordance with a previously published report suggesting that there are up to 300 PTMs in eukaryotes [13]. While many of these PTMs have no known function, several PTMs are known to have physiological function. These include phosphorylation, glycosylation, and acetylation.

**Phosphorylation**

Phosphorylation is one of the most prevalent PTMs in both eukaryotes and bacteria. Phosphorylation can occur on tyrosine, serine, threonine, histidine and aspartate residues. Protein phosphorylation on serine, threonine and tyrosine residues in eukaryotes was discovered in the 1950s, but protein phosphorylation was not discovered in bacteria until the late 1970s. Despite the initial discovery that bacteria can phosphorylate on serine and threonine residues [14, 15], it was long thought that serine/threonine and tyrosine phosphorylations were primarily restricted to eukaryotes, while bacteria utilized
aspartate/histidine phosphorylation [16]. However, in 1991, the first eukaryotic-like bacterial serine/threonine kinase, Pkn1 was characterized in *Myxococcus xanthus* [17]. Since then, more than 30 serine/threonine kinases in 13 different bacterial species have been discovered and characterized. 13 phosphatases have also been identified and characterized [18]. The physiological roles of these kinases and phosphatases range from central and secondary metabolism to cell division and cell wall synthesis to virulence, while PknA and PknB of *Mycobacterium tuberculosis* are essential, most bacterial serine/threonine kinases are non-essential [19-21].

Tyrosine kinases in bacteria have proven to be more difficult to find and characterize. Indeed, only two proteins in bacteria containing structural homology to eukaryotic tyrosine kinases have been shown to actually exhibit tyrosine kinase activity [22, 23]. Recently, this deficiency in tyrosine kinases in bacteria was changed with the discovery of a new class of tyrosine kinases found exclusively in bacteria [16]. First discovered and characterized in *Acinetobacter johnsonii* [24], BY-kinases play important roles in diverse cellular processes, including capsule and exopolysaccharide formation [25-27], antibiotic resistance [28], stress response [29] and DNA metabolism [30]. Like eukaryotic tyrosine kinases, BY-kinases phosphorylate tyrosine residues in an ATP-dependent manner. However, they do not share sequence similarity to eukaryotic tyrosine kinases [31]. This lack of structural similarity to eukaryotic tyrosine kinases has inhibited further characterization of many putative bacterial tyrosine kinases [32].

In contrast to the relatively uncharacterized serine/threonine and tyrosine phosphorylation in bacteria, histidine phosphorylation and aspartate phosphorylation has
been very well characterized, especially as it relates to two-component signal transduction systems (TCS). A prototypical TCS consists of two proteins: a sensor histidine kinase (HK) and a response regulator (RR). In such a system, stimulation of the HK causes it to autophosphorylate on a conserved histidine residue, using ATP as the phosphoryl donor. This phosphorylated histidine subsequently acts as the phosphoryl group donor for autophosphorylation on a conserved aspartate by the RR. The RR can then function as an effector, often as a transcription factor that binds to DNA and regulates transcription to regulate cellular physiology [33].

To date, TCS have been identified in nearly every sequenced bacterial genome. Some genomes encode more than 200 HKs and RRs [33]. For example, the *Escherichia coli* genome encodes 30 HKs and 32 RRs for a total of 62 TCS components [34], while the *Streptococcus pneumoniae* genome encodes 13 HKs and 14 RRs for a total of 27 TCS components [35] and *Myxococcus xanthus* encodes 272 TCS genes [36]. TCS can aide bacteria in responding to a wide range of changing environmental conditions and stimuli. For example, in *E. coli*, TCS have been implicated in many responses, including phosphate regulation, potassium transport, osmotic regulation, respiratory control, virulence, catabolite repression, capsule synthesis, nitrate regulation, and chemotaxis [34]. A novel mechanism of regulating the output of a TCS pathway forms the basis of this thesis [1].
**Glycosylation**

Another post-translational modification long thought to be restricted to eukaryotes is protein glycosylation, of which there are two types: N-glycosylation and O-glycosylation. N-glycosylation was first identified in the Gram-negative bacteria *Campylobacter jejuni* [37]. To date, this remains the only bacterial species in which the N-glycosylation pathway has been well characterized [38], in part by fully reconstituting the *C. jejuni* N-glycosylation in *E. coli* [39, 40]. *C. jejuni* contains the genes responsible for N-linked glycosylation in its *pgl* gene cluster that, along with other necessary enzymes, encodes 5 putative glucosyltransferases. The enzymes in the *pgl* gene cluster of *C. jejuni* show significant homology to enzymes involved in LPS and capsule biosynthesis [41] and the delineation of this gene cluster has allowed investigators to recreate the complete synthesis of a lipid-linked *C. jejuni* heptasaccharide from precursors and subsequent transfer to a target protein *in vitro* [42]. Deletion of the *pglB* and *pglE* genes in *C. jejuni* reduces bacterial adherence to host cells and colonization, suggesting an important role for N-glycosylation in pathogenesis [41, 43]. Indeed, over 65 *C. jejuni* proteins have been shown definitively to be N-glycosylated and up to 150 are predicted to be N-glycosylated [44]. While the *pgl* cluster in *C. jejuni* is the only well-characterized N-glycosylation system in bacteria, it clearly exhibits the potential to be a global regulator of bacterial physiology. The high number of glycosylated proteins in *C. jejuni*, combined with the ability of the *pgl* system to function in *E. coli* [39, 40], suggests that N-glycosylation could be a widely functional PTM in many other bacterial species.
In addition to N-glycosylation, bacteria also possess O-glycosylation abilities. O-glycosylation in bacteria is most predominantly studied and characterized in flagella assembly and in some cases may even be necessary for its assembly [45]. In *C. jejuni*, for example, the FlaA flagellar protein is glycosylated by O-linkage on up to 19 different sites [46-48]. The FlaB protein is also O-glycosylated [49]. This glycosylation is essential for flagellum synthesis, as mutations in the genes responsible for sugar synthesis causes cells to accumulate intracellular flagellin of a reduced mass, which renders *C. jejuni* non-motile [50].

**Acetylation**

Acetylation is yet another post-translational modification long thought to be restricted to eukaryotes. In 1992, evidence was first presented that the chemotaxis response regulator CheY could be acetylated [51, 52]. In 2002, it was reported that the bacterial sirtuin CobB mediated reversible N\(_{\varepsilon}\)-lysine acetylation of acetyl-CoA synthetase (ACS) in *Salmonella enterica* [53]. Since then, over 200 proteins have been detected as acetylated in *E. coli* and *S. enterica* [6, 54-56]. It has even been suggested that protein acetylation could be as common as phosphorylation [8].

While protein acetylation in bacteria is a newly appreciated phenomenon, it is apparent that many central metabolic enzymes can become acetylated and it is predicted that acetylation plays a major role in their function and regulation [6, 54-56]. It has been proposed that the cell can use their acetylation status as a measure of the metabolic status of the cell and adjust protein activity and stability as needed [6].
Several prominent examples of acetylation controlling protein function in bacteria exist. Acetylation serves as a simple on-off switch for the enzymatic activity of the central metabolic enzyme ACS [6]. ACS is inhibited by acetylation at its active site [53, 57] and reactivated by deacetylation [53]. The inactivation of ACS is catalyzed by the acetylation of a lysine residue in the ACS active site by the acetyltransferase YfiQ [53, 57]. Although it is not known how the acetylation inactivates ACS, it is known that ACS must be deacetylated to become reactivated [53]. In S. enterica, the deacetylation is catalyzed by the sirtuin CobB [53, 58].

Acetylation also affects the chemotaxis response regulator CheY. CheY is acetylated on 6 lysines at the carboxy-terminal surface and 5 of these lysines interact with other proteins [59, 60]. Several possibilities exist for the role of acetylation at these residues. It is thought that acetylation could simply inhibit protein-protein interactions [61]. It is also known that CheY becomes activated by phosphorylation (reviewed in [62]); however, evidence also exists for a phosphorylation-independent, acetylation-dependent chemotactic response in E. coli, possibly through CheY acetylation [52, 60].

Two CheY acetylation mechanisms have been reported: (1) autoacetylation using AcCoA as the acetyl group donor [63] and (2) acetylation by ACS using acetate as the acetyl donor [51, 64]. A third mechanism has been proposed with CheY being acetylated by an unknown acetyltransferase [59, 60]. Two deacetylation mechanisms also have been reported: (1) deacetylation mediated by ACS [51, 64] and (2) deacetylation by the sirtuin CobB [59, 60]. While the predominant method for acetylation is unknown, it is thought that CobB-mediated deacetylation is predominantly utilized in vivo.
More recently, it was reported that RcsB can be acetylated *in vitro* and that this acetylation may control its function [65]. Like CheY, RcsB is a response regulator. It controls cell division and regulates both capsule and flagellum synthesis (reviewed in [66, 67]). RcsB is acetylated by YfiQ and this acetylation is opposed by the sirtuin CobB. The acetylated lysine (K180) is located on the predicted DNA binding helix-turn-helix motif [68]. Upon acetylation of (K180), RcsB loses its affinity for its DNA site. Thus, acetylation of RcsB is predicted to regulate gene expression [65].

This model for RcsB acetylation appears to be too simple. Linda Hu in the Wolfe lab has found that RcsB can be acetylated *in vivo* on up to 7 different residues. More often than not, K180 is not acetylated, suggesting that acetylation of this residue plays a minor role *in vivo*. To monitor the effect that acetylation has on RcsB function, Linda has evaluated transcription from the *rprA* promoter, which encodes a small RNA that regulates the stress responsive sigma factor σS [69]. Using this approach, she has obtained evidence to support the hypothesis that RcsB becomes acetylated by at least one acetyltransferase other than YfiQ and that it is deacetylated by both CobB and at least one additional deacetylase. Furthermore, she has evidence that at least one acetylated lysine other than K180 is critical for RcsB function. Taken together, these data suggest that RcsB regulation by acetylation is more complicated than the published model.

A final example of acetylation serves as the functional basis for this thesis. The conventional wisdom is that *cpxP* transcription is activated because CpxR is phosphorylated by the CpxA sensor kinase in response to an extracytoplasmic stress [70-72]. When this happens, P-CpxR combines with RNAP to initiate *cpxP* transcription.
However, it is now apparent that \textit{cpxP} can be activated in a CpxR-dependent, but CpxA-independent manner when cells grown in amino acid-based medium are supplemented with glucose, pyruvate or acetate [2, 73]. The underlying mechanism requires both phosphorylation of CpxR by acetyl phosphate (Lima \textit{et al.}, submitted) and acetylation of the C-terminal domain of the \(\alpha\) subunit (\(\alpha\)-CTD) of RNAP by the acetyltransferase YfiQ. Furthermore, this acetylation is opposed by the deacetylase CobB [1].
CHAPTER THREE
MATERIALS AND EXPERIMENTAL METHODS

Bacterial strains

Bacterial strains used in this study are listed in Table 1. Unless otherwise noted, all strains are from our laboratory collection and were created using standard techniques.

Chemicals and biological reagents

All chemicals and biological reagents used in this study were purchased from Sigma Chemical Company or Fisher Scientific.

Bacterial media and growth conditions

All *E. coli* strains were grown in tryptone broth (TB) supplemented with buffering salts (TB7) unless otherwise noted. TB7 contains 10% (wt/vol) tryptone, 6.15% (vol/vol) 1M K₂HPO₄ and 3.85% (vol/vol) 1 M KH₂PO₄ in water. When noted, Luria broth (LB) was utilized. LB contains 10% (wt/vol) tryptone, 5% (wt/vol) yeast extract and 5% (wt/vol) NaCl in water.

Glucose was added to cultures from a stock concentration of 20% glucose (wt/vol) in water to a final concentration of 0.4% (wt/vol). NAM was made monthly and added from a stock concentration of 2 M in water to a final concentration of 50 mM. Sucrose was added to cultures from a stock concentration of 50% sucrose (wt/vol) in
water to a final concentration of 20% (wt/vol). Sodium citrate was added to cultures from a stock concentration of 1 M in water to a final concentration of 0.4% (wt/vol).

**Antibiotics**

Antibiotic concentrations used were prepared as a stock solution 1000 times the working concentration. Working concentrations are as follows: ampicillin, 100 µg/mL; kanamycin, 40 µg/mL; spectinomycin 100 µg/mL; tetracycline, 15 µg/mL and chloramphenicol, 25 µg/mL. Stock solutions of ampicillin, kanamycin and spectinomycin were dissolved in water and filter sterilized. Stock solutions of tetracycline and chloramphenicol were dissolved in 50% ethanol and 100% ethanol, respectively, and filter sterilized. All antibiotics were stored at -20°C.

**Generalized P1 transduction**

A single colony was inoculated and grown at 37°C with aeration overnight in TB7. In the morning, 50-100 µL of overnight culture was added to 5 mL of TBT. TBT contains 0.2% (wt/vol) glucose from a 20% (wt/vol) stock solution, 10 mM CaCl₂, 10 mM MgSO₄, and 0.4 mM FeCl₃ in TB. The overnight culture was grown in TBT until it reached an OD600 of 0.5-1.0. 1 mL of cells were infected with 50-100 µL of phage lysate and incubated unaerated at 37°C for 30 minutes. Next, 200 µL of 1M sodium citrate (pH 5.5) were added to prevent a second round of phage absorption. The cells were spun in a centrifuge at 13000 RPM for 1 minute to concentrate. The supernatant liquid was decanted and the pellet resuspended in 500 µL LB and 200 µL 1M sodium citrate. The
cells were then incubated unaerated at 37°C for 70 minutes to give them time to develop antibiotic resistance. Finally, the cells were spun at 13000 RPM for 1 minute, the supernatant was decanted and the pellet was resuspended in 50 µL 1 M sodium citrate. The entire cell suspension was plated onto LB agar plates with appropriate antibiotics.

**Transformation**

Transformations were performed in TSS. TSS contains 10% (wt/vol) poly(ethylene glycol), 5% (vol/vol) DMSO and 50 mM MgSO₄ or MgCl₂ in LB. Cells were grown overnight in TB and 1mL of culture per transformation was pelleted by spinning for 1 minute at 13000 RPM. The supernatant liquid was decanted, the cells were resuspended in 100 µL TSS, and the plasmid was added. Cells were then incubated at 4°C for 30 minutes. Next, 0.9 mL of LB was added and the cells were grown for 1-1.5 hours at 37°C with aeration. Finally, the cells were centrifuged, the supernatant was decanted and the cells were resuspended in 250 µL LB before plating.

**Luminescence readings**

Luminescence readings were detected on a TD-20/20 luminometer (Turner Designs) with a detector wavelength of 300-650 nm with peak detection at 420 nm. 500 µL of culture was added to 28 mm scintillation vials, swirled 4 times to aerate and placed in the luminometer to be read at 37°C for 6 seconds.
Fluorescence readings

Fluorescence readings were taken on POLARstar Omega (BMG Labtech) fluorescent plate reader. At each time point, 200 µL of culture was added to one well of a BD Falcon (BD Biosciences) 96-well black/clear plate. Samples were stored at 4°C in the dark until assayed. YFP fluorescence was measured using an excitation wavelength of 585 nm and an emission wavelength filter of 520 ± 5 nm. CFP fluorescence was measured using an excitation wavelength of 430 nm and an emission wavelength filter of 520 ± 5 nm.
Table 1. Strains, phage, and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Characteristic</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAD282</td>
<td>$F^{-}$ araD139 $\Delta$(argF-lac) U169 rpsL150 (Str$^r$) relA1 flbB5301 deoC1 ptsF25 rbsR $\lambda$ [cpxP'-lacZ+]</td>
<td>[74]</td>
</tr>
<tr>
<td>PAD292</td>
<td>PAD282 cpxR1::spc (spectinomycin insertion in cpxR with a polar effect on cpxA)</td>
<td>[74]</td>
</tr>
<tr>
<td>PAD348</td>
<td>PAD282 cpxA::cam</td>
<td>[74]</td>
</tr>
<tr>
<td>MDG131</td>
<td>MC41000\Phi(ompF+-yfp+)\Phi(ompC+-yfp+)</td>
<td>[75]</td>
</tr>
<tr>
<td>MDG133</td>
<td>MDG131ompR101 zhf::Tn10 (non-polar 57bp in-frame deletion in ompR)</td>
<td>[75]</td>
</tr>
<tr>
<td>MDG135</td>
<td>MDG131 envZ::Kan</td>
<td>[75]</td>
</tr>
<tr>
<td>AJW 4791</td>
<td>MDG131 ackA::tet</td>
<td>P1:AJW2620 --&gt; MDG131&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AJW4790</td>
<td>MDG131 yfiQ::kan</td>
<td>P1:JW2568 --&gt; MDG131&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AJW4789</td>
<td>MDG131 cobB::kan</td>
<td>P1:JW1106 --&gt; MDG131&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AJW2620</td>
<td>ackA::tet</td>
<td>Wolfe Lab Collection</td>
</tr>
<tr>
<td>JW1106</td>
<td>cobB::kan</td>
<td>[76]</td>
</tr>
<tr>
<td>JW2568</td>
<td>yfiQ::kan</td>
<td>[76]</td>
</tr>
</tbody>
</table>

Plasmid

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJW1</td>
<td>Low-copy-number plasmid expressing luxCDABE under control of the cpxP promoter (kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>[3]</td>
</tr>
<tr>
<td>pNLP11</td>
<td>Low-copy-number plasmid expressing luxCDABE under control of the degP promoter (kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>[3]</td>
</tr>
<tr>
<td>pNLP19</td>
<td>Low-copy-number plasmid expressing luxCDABE under control of the rpoErseABC promoter (kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>[3]</td>
</tr>
<tr>
<td>pNLP20</td>
<td>Low-copy-number plasmid expressing luxCDABE under control of the ompC promoter (kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>[3]</td>
</tr>
<tr>
<td>pNLP43</td>
<td>Low-copy-number plasmid expressing luxCDABE under control of the ompF promoter (kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>[3]</td>
</tr>
<tr>
<td>pNLP56</td>
<td>Low-copy-number plasmid expressing luxCDABE under control of the csgDEFG promoter (kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>[3]</td>
</tr>
<tr>
<td>pNLP63</td>
<td>Low-copy-number plasmid expressing luxCDABE under control of the ybaJ promoter (kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>[3]</td>
</tr>
<tr>
<td>pREII-rpoA</td>
<td>Plasmid expressing wild-type rpoA or alanine substitution derivatives (amp&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>[77]</td>
</tr>
<tr>
<td>pCA24n</td>
<td>Vector. Expresses chloramphenicol acetyltransferase (cam&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>[78]</td>
</tr>
<tr>
<td>pCA24n-coaA</td>
<td>Plasmid expressing 6 x His-CoaA under the control of an IPTG-inducible promoter</td>
<td>[78]</td>
</tr>
</tbody>
</table>
pTAC85 | Vector. Contains an ampicillin resistance cassette (amp<sup>R</sup>) | [79]
---|---|---
pPHB3 | pTAC85 expressing *phbCAB* under the control of an IPTG-inducible promoter | [1]

*This study.*
CHAPTER FOUR

RESULTS

DETERMINE IF OTHER CPXR-REGULATED PROMOTERS RESPOND TO EXOGENOUS GLUCOSE AND/OR NICOTINAMIDE

Previously, the Wolfe lab showed that the addition of glucose to a buffered amino-acid medium (TB7) (1) alters the cellular acetylation profile, including that of RNA polymerase [1], and (2) activates transcription from the CpxR-regulated promoter \( cpxP \) independently of the cognate sensor kinase CpxA [1, 2]. Since CpxR regulates many other genes [5, 70, 71, 80-83], we hypothesized that the addition of 0.4% glucose or 50 mM NAM to TB7 would activate transcription from other CpxR-regulated promoters in a manner that depends on CpxR and protein acetylation, but not CpxA.

Response of other CpxR-regulated promoters to exogenous glucose.

To test the role of CpxR and P-CpxR in glucose-induced transcription, we monitored the transcriptional response to glucose of a subset of CpxR-regulated promoters in a reference strain and its isogenic null mutants of \( cpxA \) and \( cpxR1 \). The mutant \( cpxR1 \) allele has a polar effect on the downstream gene \( cpxA \); thus, this strain expresses neither CpxR nor CpxA [70].

To measure transcription of CpxR-regulated genes, we used \( lux \) promoter fusions constructed on a low copy number plasmid by Price and Raivio [3]. According to these authors’ analysis, this set of CpxR-regulated promoters fell into two distinct classes:
those that are activated in the presence of the cpxA* allele and those that are repressed (Table 2). cpxA* encodes a CpxA mutant protein that functions constitutively as a net kinase [4, 5] and thus accumulates P-CpxR, which activates CpxA*-activated promoters and represses CpxA*-repressed promoters. CpxA*-dependent behavior strictly depends on CpxR [3, 73].

By transformation with this library of plasmids, we introduced each promoter-lux fusion into a Δlac reference strain (WT, strain PAD282; Table 1) that contains a cpxP promoter-lacZ fusion inserted in single copy at the λ attachment site of the chromosome. Similarly, we introduced these fusions into the isogenic cpxA null and cpxR1 mutants. The mutant cpxR1 allele has a polar effect on the downstream gene cpxA; thus, this strain expresses neither CpxR nor CpxA [70].

We know the response of the cpxP promoter to all of the tested combinations of strains and culture conditions [1, 2]; therefore, it served as an internal control. All experiments in which the well-characterized cpxP promoter did not behave as previously reported were eliminated from analysis and repeated.

Three independent colonies per strain were inoculated overnight in TB7 supplemented with kanamycin to retain the reporter plasmids. In the morning, cultures were measured at OD600 to estimate cell number and diluted appropriately to an initial OD600 of 0.1 into 25 mL of the same medium (control condition) or the same medium supplemented with 0.4% glucose (experimental condition). We grew the bacteria at 37°C for 8 hours with shaking at 225 rpm, while taking growth and cpxP-lacZ measurements every hour. Luminescence was measured at hour 3 (i.e. during exponential growth phase)
Table 2. Glucose-regulated behavior of CpxA*-regulated promoters: a summary.a

<table>
<thead>
<tr>
<th>Promoter</th>
<th>CpxA* b</th>
<th>WT w/ 0.4% glucose</th>
<th>ΔcpxA w/ 0.4% glucose</th>
<th>cpxRI c w/ 0.4% glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpxP</td>
<td>Activates</td>
<td>Activates</td>
<td>Activates</td>
<td>NR</td>
</tr>
<tr>
<td>ybaJ</td>
<td>Activates</td>
<td>Inhibits</td>
<td>Inhibits</td>
<td>Inhibits</td>
</tr>
<tr>
<td>degP</td>
<td>Activates</td>
<td>Activates</td>
<td>Activates</td>
<td>NR</td>
</tr>
<tr>
<td>ompC</td>
<td>Activates</td>
<td>Activates</td>
<td>Activates</td>
<td>Activates</td>
</tr>
<tr>
<td>ycfS</td>
<td>Activates</td>
<td>NR</td>
<td>Activates</td>
<td>NR</td>
</tr>
<tr>
<td>csgDEFG</td>
<td>Inhibits</td>
<td>Inhibits</td>
<td>Inhibits</td>
<td>Inhibits</td>
</tr>
<tr>
<td>ompF</td>
<td>Inhibits</td>
<td>Inhibits</td>
<td>Inhibits</td>
<td>Inhibits</td>
</tr>
<tr>
<td>rpoErseABC</td>
<td>Inhibits</td>
<td>Activates</td>
<td>Activates</td>
<td>Activates</td>
</tr>
</tbody>
</table>

aActivation or inhibition represents a greater than 2-fold change in transcription compared to the no glucose control. NR, no response.

bCpxA* is a constitutively active kinase mutant that depends on CpxR for activity.

cThe mutant cpxRI allele has a polar affect on the downstream cpxA gene, making cpxRI mutants deficient in both cpxR and cpxA.
and then hours 6, 7 and 8 (i.e. during the transition to and during early stationary phase). For optimal luminescence activity, luminescence was measured for 6 seconds at 37°C immediately after aeration. All luminescence and β-galactosidase activities were normalized to growth as measured by absorbance at OD600. All experiments were performed at least twice.

To test the validity of our system, we first compared the growth of the mutant strains to that of their WT parent. In this initial experiment (the only one that was not repeated), the three strains exhibited similar growth characteristics in TB7, but somewhat different behaviors in TB7 supplemented with glucose (Figure 4A). However, this was not representative of most experiments. A more representative growth curve (Figure 5A) shows no major difference in the growth patterns of the three strains. This growth curve also clearly demonstrates that exogenous glucose tends to enhance growth in a Cpx-independent manner. To validate the plasmid-borne promoter-luminescence fusions, we next compared transcription from the cpxP-lacZ and cpxP-lux fusions in the same strains, monitoring the activities of both β-galactosidase (Figure 4B) and luminescence (Figure 4C). Regardless of reporter and as reported previously [1, 2], (1) exposure to exogenous glucose enhanced cpxP transcription in a WT strain (Figure 4B and Figure 4C inset, squares), (2) glucose-induced transcription was further enhanced in the cpxA-null mutant (Figures 4B and 4C, diamonds), and (3) cpxP transcription required CpxR (Figures 4B and 4C, triangles). We conclude that the lux and lacZ fusions yield similar results and thus the plasmid-borne lux fusions could be used to monitor CpxR-regulated promoter activity.
Figure 4. Representative measurements associated with the cpxP promoter. **A.** Growth curve as measured by absorbance at OD600 over time. **B.** β-galactosidase activity for the cpxP promoter over time normalized to cell growth as measured by OD600. **C.** Luciferase activity for the cpxP promoter over time normalized to cell growth as measured by OD600. In all panels, strains are WT (□, ■ ΔcpxA (○, ●) and ΔcpxR1 (Δ, ▲) and are from the same experiment with cells grown at 37°C with shaking in TB7 (open symbols) or in TB7 supplemented with 0.4% glucose (closed symbols).
Screen of CpxR-regulated promoters.

Given that the plasmid-borne lux reporter provided a valid readout of glucose-induced cpxP transcription (Figure 4C), we proceeded to test the glucose response by other CpxR-regulated promoters. For each experiment, after confirming that the cpxP-lacZ reporter responded properly (i.e., as in Fig. 4B, data not shown), we analyzed the luminescence activities of the WT and mutant transformants in the presence or absence of exogenous glucose.

For any given promoter (Table 2), transcriptional behavior in the CpxA* mutant strain (Column 1) did not necessarily correlate with its response to glucose (Column 2). For example, cpxP and other CpxA*-activated promoters (degP and ompC) were strongly activated by glucose, while the CpxA*-activated ybaJ promoter was repressed. Similarly, the CpxA*-repressed promoters csgDEFG and ompF were repressed by glucose, while the CpxA*-repressed rpoErseABC promoter was activated by glucose. As observed with the cpxP promoter, all glucose-regulated behavior, whether it was activation or repression, was independent of CpxA. In most but not all cases, glucose-regulated behavior was enhanced. Indeed, the CpxA*-induced promoter ycfS responded to glucose in the absence of CpxA even though it did not in the presence of CpxA (Column 3). Finally, glucose-regulated behavior depended on CpxR in only three of the CpxA*-regulated promoters (Column 4, cpxP, degP and ycfS). These results provide evidence that glucose-regulated behavior is mechanistically different than regulation through the conventional CpxAR pathway. We found that these promoters fell into three distinct categories.
Categories of CpxR-regulated promoters in response to glucose.

CpxA-repressed, CpxR-dependent, Glucose-activated promoters. Like the $cpxP$ promoter (Figure 4B and 4C), the CpxA*-activated $degP$ and $ycfS$ promoters responded to glucose in a CpxR-dependent and CpxA-repressed manner (Figure 5B and 5C). CpxA-dependent repression of glucose-induced activation was particularly strong at the $ycfS$ promoter, which did not respond to glucose unless CpxA was absent (Figure 5C).

CpxR-independent, Glucose-activated promoters. Glucose induced transcription from the $ompC$ and $rpoErseABC$ promoters. Glucose activation of the CpxA*-activated $ompC$ promoter was strictly independent of both CpxA and CpxR (Figure 5D), while glucose-induced activation of the CpxA*-repressed $rpoErseABC$ promoter depended weakly on CpxR and moderately on CpxA (Figure 5E): the $cpxR1$ mutant exhibited as robust a response to glucose as did its WT parent, albeit more slowly. In contrast, the $cpxA$ mutant responded more weakly than its parent. This is an unusual result, as CpxA appeared to influence the response to glucose by the $rpoErseABC$ promoter in a CpxR-independent manner.

Cpx-independent, Glucose-repressed promoters. In contrast, glucose repressed transcription at the CpxA*-repressed $ompF$ and $csgDEFG$ promoters (Figures 5G and 5H) and the CpxA*-activated $ybaJ$ promoter (Figure 5F). In all three cases, this glucose effect was independent of both CpxA and CpxR (Figures 5F, 5G and 5H inset).
Figure 5. Response to glucose by several CpxR-regulated promoters in WT, ΔcpxA and cpxR1 strains. A. Representative growth curve for all experiments in WT (■) ΔcpxA (♦) and cpxR1 (▲) strains as measured by absorbance at OD600 over time. Luminescence measurements for CpxR-regulated promoter transcription over time normalized to cell growth at OD600 in WT (□, ■) ΔcpxA (◊, ♦) and cpxR1 (△, ▲) strains. Open symbols represent growth in TB7 at 37°C with shaking and closed symbols represent growth in TB7 with 0.4% glucose at 37°C with shaking. This growth curve was generated during the experiment measuring ompF transcription in panel G. Graphs represent readings taken in triplicate for the following promoters B. degP C. yctS D. ompC E. rpoErseABC F. ybaJ G. ompF H. csgDEFG
Repression by CpxA and CpxR at the *ompF* promoter and by CpxR at the *csgDEFG* promoter was apparent, as cells lacking *cpxR* (Figure 5H) or *cpxA* and *cpxR* (Figure 5G) exhibited more glucose-independent transcription from these two promoters than did their WT parent.

**Response of CpxR-regulated promoters to nicotinamide**

The addition of NAM activates *cpxP* transcription even in the absence of exogenous glucose [1]. Since NAM inhibits CobB, the only known deacetylase in *E. coli*, NAM presumably leads to increased acetylation and, as such, activates transcription via RNAP acetylation in a manner similar to that induced by glucose.

After observing the response of CpxR-regulated promoters to glucose, we chose the *ompC* and *ompF* promoters for further study. We chose these two promoters because (1) they exhibited opposing, Cpx-independent responses to exogenous glucose and (2) they are primarily regulated by a different two-component system: the EnvZ-OmpR pathway. We reasoned that glucose-regulated acetylation-dependent activation might be applicable to other two-component systems and that demonstration of such a phenomenon would generalize acetylation-dependent activation more than further study of CpxR-dependent promoters.

To determine if the *ompC* and *ompF* responses to glucose were due to changes in acetylation, we monitored the transcriptional response to NAM. NAM is a pharmacological inhibitor of the only known deacetylase in *E. coli*, CobB. The Wolfe lab recently reported that exposure to NAM activates *cpxP* transcription and that NAM
increases the cellular acetylation profile in a manner similar to that caused by glucose [1].

If this OmpR-dependent pair of promoters responds to NAM as they respond to glucose, then we hypothesized that acetylation might regulate transcription at these promoters and, if so, that acetylation might be a global method of gene regulation.

Since \textit{ompC} and \textit{ompF} are activated in a Cpx-independent manner, we tested the effect of NAM on \textit{ompC} and \textit{ompF} transcription in a \textit{cpxR} mutant strain and found that exposure of this strain to 50 mM NAM activated \textit{ompC} and repressed \textit{ompF} transcription, similar to glucose-induced behavior (compare \textbf{Figures 6A & 6B to Figures 5D & 5F}).

\textbf{Summary}

We have observed that glucose can regulate transcription from a number of CpxR-regulated promoters and that their response to glucose does not necessarily correlate with their response to P-CpxR as a result of the \textit{cpxA}\textsuperscript{*} allele. Of these, the primarily OmpR-regulated \textit{ompC} and \textit{ompF} promoters are of particular interest. We showed that \textit{ompC} and \textit{ompF} are activated and repressed, respectively, in the presence of both glucose and NAM and that this effect occurs independently of the CpxAR two-component system. Based on these observations, we chose to further characterize glucose-dependent regulation of \textit{ompC} and \textit{ompF} transcription.
Figure 6. Response to NAM by the *ompC* and *ompF* promoters in a *cpxR1* strain. Luminescence measurements for OmpR-regulated promoter-*lux* fusions over time normalized to cell growth at OD600 in a *cpxR1* strain. Open symbols represent growth in TB7 at 37°C with shaking and closed symbols represent growth in TB7 supplemented with 50 mM NAM at 37°C with shaking. Graphs represent readings taken in triplicate for the following promoters A. *ompC* B. *ompF*. 
CHAPTER FIVE

RESULTS

DETERMINE WHETHER GLUCOSE-REGULATED ompC AND ompF TRANSCRIPTION IS REGULATED BY BOTH OmpR PHOSPHORYLATION AND ACETYLATION OF SOME COMPONENT OF THE OmpR-RNAP COMPLEX.

Glucose-induced transcription of the cpxP promoter depends on both phosphorylation of the response regulator CpxR (Lima et al., submitted) and acetylation of RNAP [1]. Here, we determined whether the same is true for glucose-regulated ompC and/or ompF transcription.

For ompC and ompF transcription, it is reported that OmpR is the primary regulator; without OmpR, little or no transcription occurs [84, 85]. To become active, OmpR must become phosphorylated [86-89]. Two phosphoryl donors have been reported: EnvZ and AcP [90, 91]. EnvZ is the cognate sensor kinase/phosphatase, while AcP is a central metabolite [92]. AcP can function as an EnvZ-independent phosphoryl group donor to OmpR [91].

We first determined the role phosphorylation plays in the glucose effect on these promoters. Because glucose-induced transcription of the cpxP promoter requires the acetyltransferase YfiQ, is opposed by the deacetylase CobB, and responds to the AcCoA:CoA ratio [1], we then determined whether YfiQ, CobB and the AcCoA:CoA ratio influence glucose-regulated ompC and/or ompF transcription.
Validating the fluorescent dual-reporter strain

On the basis of our findings in the luminescence-reporter system, we chose to further investigate the *ompC* and *ompF* promoters. Our lab possesses the fluorescence-based dual-reporter strain MDG131 and several isogenic derivatives (Table 1; a generous gift of Mark Goulian, University of Pennsylvania). Constructed in the same MC4100 background used by Lima *et al.* to investigate glucose-induced *cpxP* transcription [1, 2], this strain contains 2 transcriptional fusions: (1) the CFP gene fused to *ompC* and (2) the YFP gene fused to *ompF* (Figure 7). These fusions were constructed by integrating a promotorless *cfp* with a ribosome-binding site into the chromosome after the *ompC* translational stop codon, but before the transcriptional termination signal. In the same strain, a promotorless *yfp* with a ribosome-binding site was integrated after the *ompF* translational stop codon, but before the transcriptional termination signal. The result is 2 bicistronic operons, in which each native promoter drives transcription of its native gene and its respective reporter. Finally, the presence of both reporters in the same strain permits the use of one reporter as an internal control for the other [75].
Figure 7. Schematic of the fluorescent dual-reporter strain. This strain, constructed in the MC4100 background, contains transcriptional fusions of \textit{yfp} and \textit{cfp} to \textit{ompF} and \textit{ompC}, respectively. The arrow at +1 represents the transcriptional start site. S-D represents the Shine-Dalgarno sequence, a ribosomal binding site.
This system has many advantages over the luminescence-based reporter system used in Aim 1. (1) The enzymatic-based luminescence reporter can vary in its response depending on many factors, including aeration. This can cause quite a bit of variation in data and can lead to false interpretation. (2) The plasmid-based luminescence system only permits monitoring of one promoter at a time. Thus, the reporters are in different strains and therefore are measured in independent environments. (3) The fluorescence-based dual-reporter system is not enzymatic and therefore limits the potential for human error. (4) The fluorescent reporters are inserted in the chromosome downstream of the native \textit{omp} promoter and gene in contrast to the luminescence-based system, which is carried on a plasmid. By being in the context of the native promoter, we can be more confident that the response is physiologically relevant. (5) By having both the \textit{ompC} and \textit{ompF} promoters in the same strain, comparing the two can serve as an internal control independent of growth, total cell mass and other factors that can affect protein content in the cells. (6) The fluorescence-based dual-reporter system has been well established [75].

To further investigate glucose-regulated \textit{ompC} and \textit{ompF} transcription, I therefore chose to use the fluorescence-based dual-reporter strains.

To validate use of the dual reporter strain, we first needed to replicate previously reported data. EnvZ is known to detect changes in osmolarity [93] and this strain was developed to specifically evaluate transcriptional changes in response to osmolarity. These previous studies were performed under culture conditions that differ quite dramatically from those that we have used to evaluate the glucose-induced response. Thus, validation of this strain will serve two purposes: (1) to replicate the response to
osmolarity under our culture conditions and (2) to identify the effect that our base culture conditions have on the behavior of the \textit{ompC} and \textit{ompF} promoters.

The dual-reporter strain (MDG131) was originally tested in a minimal medium supplemented with sucrose as the osmolyte [75]. While \textit{E.coli} cells do not metabolize sucrose, the cells metabolize glucose, producing acetic acid and other acidic byproducts. To avoid acidifying the media, therefore, we grew cells in a buffered media. We used TB7 because glucose-induced \textit{cpxP} transcription depends on an amino-acid based medium such as TB.

We measured \textit{ompC} and \textit{ompF} fluorescence in the dual-reporter strain under 5 conditions: (1) unbuffered TB, (2) unbuffered TB with 20% sucrose, (3) buffered TB (TB7), (4) TB7 with 0.4% glucose, and (5) TB7 with 0.4% citrate (an osmolyte that \textit{E. coli} cannot metabolize).

We inoculated 3 independent colonies per strain overnight in TB7. In the morning, we measured culture density at OD600 and diluted appropriately to an initial OD of 0.1 into 25 mL of the same medium (control condition) or the same medium supplemented with 0.4% glucose (experimental condition). We then incubated at 37°C for 6 hours with shaking at 225 rpm, taking samples for growth and fluorescence measurements every 45 minutes. Samples were stored in the dark at 4°C until assayed. YFP fluorescence was measured using an excitation wavelength of 585 nm and an emission wavelength filter of 520 ± 5 nm. CFP fluorescence was measured using an excitation wavelength of 430 nm and an emission wavelength filter of 520 ± 5 nm. All
fluorescence readings were normalized to growth as measured by absorbance at OD600. All experiments were repeated at least once.

Relative to osmolarity, we were able to replicate the previously reported results [75]. Addition of sucrose to unbuffered TB increased *ompC* transcription and repressed *ompF* transcription, as previously reported (Figures 8A and B, compare white squares (TB) to black squares (TB + sucrose)). Adding salts to buffer the TB also increased osmolarity and exerted the same effect on transcription as sucrose, i.e. it increased *ompC* transcription and repressed *ompF* transcription (Figures 8A and B, compare white diamonds to white and black squares). The addition of glucose to TB7 further repressed *ompF* transcription (Figure 8B, compare black to white diamonds) and caused a slight decrease in *ompC* transcription (Figure 8A, compare black to white diamonds). These effects were not the result of a further increase in osmolarity, as adding an equivalent amount of citrate to TB7 had little effect on *ompC* and *ompF* transcription. Citrate was added at a concentration of 0.4% (wt/vol) as a sodium citrate salt. An equivalent amount of sodium citrate (Na$_3$C$_6$H$_5$O$_7$) contains 4 osmoles compared to 1 osmole for glucose (C$_6$H$_12$O$_6$); therefore, 0.4% citrate is predicted to have a larger effect on osmolarity than does 0.4% glucose. We conclude that the glucose effect is independent of osmolarity (Figures 8A and B, compare gray and white diamonds).

We previously observed that the *cpxP*, *ompC* and *ompF* promoters each responded to NAM in a manner similar to glucose, but with an intermediate effect ([1] and Figure 6A and B). Adding NAM to the fluorescent reporter strain weakly increased *ompC* transcription, while moderately repressing *ompF* transcription. Thus, these
Figure 8. Response to osmolarity and glucose by the *ompC* and *ompF* promoters in the WT strain. Fluorescence activity for the OmpR-regulated promoter transcription over time normalized to cell growth at OD600. Open symbols represent growth in TB (□) or TB7 (◊) at 37°C with shaking and closed symbols represent growth in TB with 20% sucrose (■) or TB7 with 0.4% glucose (♦) or 0.4% citrate (♦) at 37°C with shaking. Graphs represent readings taken in triplicate for the following promoters A. *ompC* B. *ompF*
promoters responded to NAM in both genetic backgrounds (compare Figure 9A and B to Figure 6A and B).

We obtained conflicting results in regards to the response of *ompC* to glucose in the two different strain backgrounds. When we performed the initial screen of CpxR-regulated promoters, we only measured luminescence during stationary growth phase. Subsequent experiments show that the initial strain (i.e., the strain that harbors the *cpxP-lacZ* reporter) does indeed activate *ompC* in response to glucose throughout the growth curve. In contrast, the fluorescent dual-reporter strain represses *ompC* in response to glucose throughout growth. However, NAM activated *ompC* transcription in both strains (Figure 10A and B).

In conclusion, we have shown that the double reporter strain behaves as published, that glucose-regulated behaviors are not due to the further increase in osmolarity, and that these promoters can be used for further investigation into glucose-regulated behavior. However, with respect to glucose-regulated *ompC* transcription, we obtained conflicting results relative to the luminescence-based reporter. Whereas NAM increased activity from *ompC* in both strains and glucose increased activity from the *ompC-lux* fusion, glucose slightly diminished activity from the *ompC-cfp* fusion.

**ompC transcription varies between strain backgrounds**

In our original screen of CpxR-regulated promoters, which utilized the plasmid-based *lux* reporter fusions, we saw strong *ompC* transcriptional activation and strong *ompF* transcriptional repression in response to exogenous glucose. When we decided to
Figure 9. Response to NAM by the \textit{ompC} and \textit{ompF} promoters in a WT strain.
Fluorescence activity for OmpR-regulated promoters over time normalized to cell
growth at OD600 in a WT strain. Open symbols represent growth in TB7 at 37°C with
shaking, dark symbols represent growth in TB7 supplemented with 0.4% glucose and
gray symbols represent growth in TB7 supplemented with 50 mM NAM at 37°C with
shaking. Graphs represent readings taken in triplicate for the following promoters \textbf{A.}
\textit{ompC} \textbf{B.} \textit{ompF}.
Figure 10. Response to glucose and NAM by the *ompC* promoter in two different background strains. Luminescence and fluorescence activity for OmpR-regulated promoters over time normalized to cell growth at OD600 in a *cpxR1* and WT strains. Open symbols represent growth in TB7 at 37°C with shaking, dark symbols represent growth in TB7 supplemented with 0.4% glucose and gray symbols represent growth in TB7 supplemented with 50 mM NAM at 37°C with shaking. Graphs represent readings taken in triplicate for the following strains A. PAD292 B. MDG131.
pursue investigation of these promoters, we began to use a fluorescent dual-reporter strain which was constructed in the same MC4100 background used in the lux reporter screen. Although the strain backgrounds were the same, the behavior of the ompC promoter was different in each strain: whereas the ompC-lux fusion responded robustly to glucose, the ompC-fluorescent reporter did not.

We envisioned two potential causes for the discrepancy in ompC transcription: (1) the activation was an artifact of the luminescence assay, which is sensitive to many factors, including aeration, or (2) the MC4100 backgrounds were different.

To determine the reason for the conflicting results, we transformed the WT dual-reporter strain and its isogenic envZ (MDG135) and ompR (MDG133) mutants with the ompC- and ompF-lux reporter plasmids. We measured both luminescence and fluorescence as previously described with the fluorescence readings serving as a control.

In the fluorescent dual-reporter strain, ompC was not activated in response to exogenous glucose when measured using either the luminescence or fluorescence reporters (Figure 8A and 11A). In contrast, glucose repressed ompF regardless of the reporter (Figure 8B and 11B). We conclude that the difference in ompC behavior results from some unknown polymorphism in the two background strains and not from differences in the assays. This experiment gave further evidence that the fluorescent dual-reporter strain provides valid results and could be used for subsequent experiments, at least with respect to the ompF reporter.
Figure 11. Response to glucose by the *ompC* and *ompF* luciferase reporter plasmids in the fluorescent dual-reporter strain. Luminescence measurements for OmpR-dependent promoter-*lux* fusions over time normalized to cell growth at OD600 in WT (□, ■), Δ*envZ* (◇, ♦) and Δ*ompR* (△, ▲) strains. Open symbols represent growth in TB7 at 37°C with shaking and closed symbols represent growth in TB7 supplemented with 0.4% glucose at 37°C with shaking. Graphs represent readings taken in triplicate for the following promoters A. *ompC* B. *ompF*.
Glucose-regulated *ompC* and *ompF* transcription requires the response regulator OmpR.

OmpR is the major response regulator required for *ompC* and *ompF* transcription under all reported conditions [84, 85]. Thus, we tested the hypothesis that glucose-regulated *ompC* and *ompF* transcription also requires OmpR.

To test the role of OmpR, we compared *ompC* and *ompF* transcription in the wild-type double reporter strain MDG131 to that of an isogenic *ompR* mutant strain MDG133 (Table 1; both generous gifts of Mark Goulian, U. Penn). The *ompR* mutation is a non-polar 57 bp in-frame deletion in *ompR* that does not disrupt expression of the downstream *envZ* gene. Growth, harvesting and monitoring were performed as previously described.

As expected, the *ompR* null mutant exhibited levels of *ompC* and *ompF* transcription significantly below that of the *cobB* null control strain. In this particular experiment, a *cobB* null mutant was used as a control since it behaves similarly to its WT parent (MDG131) (see Figure 14 for explanation). Despite the low levels of transcription, glucose further reduces *ompC* and *ompF* transcription (Figure 12A and 12B). Interestingly, glucose reduced WT *ompF* transcription (Figure 8B and 12B) down to the levels of the *ompR* null mutant (Figure 12B), suggesting that glucose essentially shuts off *ompF* transcription. While OmpR is the major regulator of *ompC* and *ompF* transcription, other regulators have been reported, including CpxR [94], Lrp [95] and CadC [96]. It is possible that glucose has an effect on the low levels of transcription induced by these regulators.
Figure 12. Response to glucose by the *ompC* and *ompF* promoters in Δ*cobB* and Δ*ompR* strains. Fluorescence activity for the OmpR-regulated promoter transcription over time normalized to cell growth at OD600 in Δ*cobB* (□, ■) and Δ*ompR* (△, ▲) strains. Open symbols represent growth in TB7 at 37°C with shaking and closed symbols represent growth in TB7 with 0.4% glucose at 37°C with shaking. Graphs represent readings taken in triplicate for the following promoters. **A. ompC** **B. ompF.**
EnvZ is required for glucose-regulated transcription at \textit{ompC}, but not at \textit{ompF}.

The EnvZ-OmpR two-component system regulates \textit{ompC} and \textit{ompF} transcription in response to changes in osmolarity. In high osmolarity, \textit{ompC} is activated while \textit{ompF} is repressed; in low osmolarity, the opposite occurs. These behaviors require EnvZ, which senses changes in osmolarity and a variety of other stimuli [93, 97, 98]. We tested the hypothesis that EnvZ also detects glucose and thus plays an essential role in glucose-regulated transcription of \textit{ompC} and/or \textit{ompF}.

To test the role of EnvZ, we compared \textit{ompC} and \textit{ompF} transcription in the wild-type double reporter strain MDG131 to that of the isogenic \textit{envZ} mutant strain MDG135 (Table 1; a generous gift of Mark Goulian, U Penn). In this \textit{envZ} mutant allele, a \textit{kan} resistance cassette disrupts the \textit{envZ} gene. Growth, harvesting and monitoring were performed as previously described.

Relative to its WT parent, the \textit{envZ} null strain exhibited reduced \textit{ompC} transcription and glucose appeared to exert no effect (Figure 13A). In contrast, exogenous glucose reduced \textit{ompF} transcription in both WT and \textit{envZ} null strains (Figure 13B). These results suggest that EnvZ plays a prominent role in \textit{ompC} transcription. Without EnvZ, transcription levels are much lower than in a WT strain. Additionally, EnvZ must be required for the glucose effect at the \textit{ompC} promoter, as there is no effect on transcription when glucose is added and EnvZ is absent. In contrast, the \textit{ompF} promoter responded similarly whether it was present in the WT parent or the \textit{envZ} null mutant. Thus, glucose causes repression of \textit{ompF} regardless of the presence of EnvZ.
Figure 13. Response to glucose by the *ompC* and *ompF* promoters in WT and Δ*envZ* strains. Fluorescence activity for the OmpR-regulated promoter transcription over time normalized to cell growth at OD600 in WT (○, ■) and Δ*envZ* (◊, ♦) strains. Open symbols represent growth in TB7 at 37°C with shaking and closed symbols represent growth in TB7 with 0.4% glucose at 37°C with shaking. Graphs represent readings taken in triplicate for the following promoters A. *ompC* B. *ompF*
Glucose-regulated *ompC* and/or *ompF* transcription does not involve acetylation of the RNAP-OmpR complex.

Glucose-induced *cpxP* transcription requires the acetyltransferase YfiQ [1], presumably because it acetylates certain lysines on the surface of the α-CTD of RNAP. This acetylation is opposed by the deacetylase CobB and requires AcCoA as the acetyl group donor [1]. We tested the hypotheses that YfiQ, CobB and AcCoA are required and/or involved in glucose-regulated *ompC* and *ompF* transcription.

**Glucose-regulated *ompC* and/or *ompF* transcription does not depend on the acetyltransferase YfiQ.**

We recently reported that glucose-induced *cpxP* transcription requires both the acetyltransferase YfiQ and K298 of the α-CTD of RNAP, and that YfiQ is required for K298 acetylation [1]. We hypothesized that YfiQ is required for glucose-regulated *ompC* and/or *ompF* transcription.

To test the role of YfiQ, we used P1 generalized transduction to move a *yfiQ* mutant allele from a donor strain into the WT double-reporter strain (MDG131). This allele and all subsequent mutant alleles are present in the Keio collection of gene deletions [76]. All Keio collection gene deletions lack the entire gene and are marked with kanamycin (*kan*) resistance and all Keio mutant alleles were verified by colony PCR prior to use. We compared glucose-regulated *ompC* and *ompF* transcription from the resultant *yfiQ* mutant double-reporter strain to that of its parent. All other aspects of the experiment were performed as previously described.
ompC and ompF transcription in the yfiQ null mutant resembled that of its WT parent in both the presence and absence of glucose (Figures 14A and 14B). We conclude that YfiQ does not play a role in the response to glucose by the ompC or ompF promoters.

**GLUCOSE-REGULATED ompC AND ompF TRANSCRIPTION IS NOT SENSITIVE TO THE DEACETYLASE CobB.**

Inhibition of the deacetylase CobB by exposure to NAM caused CpxA-independent cpxP transcription, while overexpression of the deacetylase CobB antagonized glucose-induced cpxP transcription [1]. Since exposure to NAM influenced ompC and ompF transcription (Aim 1), we tested the hypothesis that CobB is involved in glucose-regulated ompC and ompF transcription.

To test the role of CobB, we constructed a cobB null mutant of the WT double-reporter strain (MDG131), as previously described. We compared glucose-regulated ompC and ompF transcription in the WT and cobB null double-reporter strains as previously described.

ompC and ompF transcription in the cobB null mutant resembled that of its WT parent in both the presence and absence of glucose (Figures 14A and 14B). We conclude that CobB does not play a role in the response to glucose by the ompC or ompF promoters.
Figure 14. Response to glucose by the *ompC* and *ompF* promoters in ∆*cobB* and ∆*yfiQ* strains. Fluorescence activity for the OmpR-regulated promoter transcription over time normalized to cell growth at OD600 in ∆*cobB* (○, ■) and ∆*yfiQ* (◊, ♦) strains. Open symbols represent growth in TB7 at 37°C with shaking and closed symbols represent growth in TB7 with 0.4% glucose at 37°C with shaking. Graphs represent readings taken in triplicate for the following promoters. A. *ompC* B. *ompF*. 
**Glucose-regulated ompC and/or ompF transcription does not require acetylation of certain lysine residues on RNAP.**

Certain lysine residues of the α-CTD of RNAP are acetylated when glucose is added to the growth media. Of these, K298 is essential for glucose-induced cpxP transcription [1]. We tested the hypothesis that at least one of these lysines is essential for glucose-regulated ompC and/or ompF transcription.

We possess a plasmid-borne library consisting of alanine mutant alleles of every non-alanine residue of the α-CTD (a generous gift of Richard Gourse, University of Wisconsin-Madison). We used transformation to introduce 3 lysine-to-alanine mutant alleles into the WT double-reporter strain and determined if any mutant allele inhibits glucose-regulated ompC and/or ompF transcription, as previously described.

Each of the lysine-to-alanine mutants exhibited behavior to that of their WT parent (Figure 15A and 15B). Thus, we conclude that the lysine residues on the α-CTD of RNAP, including K298, play no role in the response to glucose by the ompC or ompF promoters. We further conclude that these behaviors do not operate in the same manner as glucose-induced cpxP transcriptional activation. If acetylation is playing a role it may be on another part of the OmpR-RNAP complex.
Figure 15. Response to glucose by the \textit{ompC} and \textit{ompF} promoters in strains expressing lysine-to-alanine mutants of the $\alpha$-CTD of RNAP. Fluorescence activity for OmpR-regulated transcription over time normalized to cell growth at OD600 in WT (□, ■) K291A (◊, ◦) K297A (△, ▲), and K298A (○, ●) strains. Open symbols represent growth in TB7 at 37°C with shaking and closed symbols represent growth in TB7 with 0.4% glucose at 37°C with shaking. Graphs represent readings taken in triplicate for the following promoters. A. \textit{ompC} B. \textit{ompF}. 
GLUCOSE-REGULATED *ompC* AND/OR *ompF* TRANSCRIPTION IS NOT SENSITIVE TO THE AcCoA:CoA RATIO.

AcCoA is the acetyl donor for acetylation [6]. The ability to generate AcCoA from pyruvate is required for glucose-induced *cpxP* transcription and this transcription is sensitive to the AcCoA:CoA ratio [1]. It has been previously shown that the carbon:nitrogen balance in the cell can affect *ompC* and *ompF* transcription [99, 100] and that the AcCoA:CoA ratio can affect *cpxP* transcription [1]. Addition of glucose may affect the AcCoA:CoA ratio, leading to glucose-regulated *ompC* and *ompF* transcription. To test the hypothesis that AcCoA is involved in glucose-regulated *ompC* and/or *ompF* transcription, we manipulated the AcCoA:CoA ratio.

To decrease AcCoA levels in the WT double-reporter strain, we used two different IPTG-inducible plasmid systems. One system involves overexpressing CoaA, which encodes pantothenate kinase. Pantothenate kinase catalyzes the first step in CoA synthesis. Overexpression of CoaA will increase CoA levels in the cell, effectively reducing the AcCoA:CoA ratio [101, 102]. The other system involves overexpression of the genes *phbCAB*. This method synthesizes the biopolymer polyhydroxybutyrate (PHB), using acetyl groups from AcCoA as the substrate [103, 104]. We used transformation to introduce each of these plasmids and their vector controls into the WT double-reporter strain. For each transformant, we compared *ompC* and *ompF* transcription in the presence or absence of glucose and in the presence or absence of IPTG. All other aspects of the experiment were performed as previously described.

*ompC* and *ompF* transcription were not substantially affected by either type of AcCoA:CoA ratio manipulation (Figures 16A and 16B, compare squares and triangles).
to diamonds and circles). This result suggests that the glucose effect does not depend on AcCoA:CoA ratio and that acetylation may not play a role at all in glucose-regulated *ompC* and *ompF* transcription.

**Summary**

We have shown that glucose while regulates *ompC* and *ompF* transcription, it appears to do so through an acetylation-independent mechanism. Our model of CpxA-independent *cpxP* activation requires exogenous glucose, the acetyltransferase YfiQ, AcCoA, acetylation at K298 of RNAP and is sensitive to the deacetylase CobB. Glucose-regulated transcription of *ompC* and *ompF* appears to be independent of YfiQ, CobB and AcCoA suggesting that glucose is regulating *ompC* and *ompF* by a different mechanism. K291 and K298 on the α-CTD of RNAP may play a role in *ompF* transcription.
Figure 16. Response to glucose by the *ompC* and *ompF* promoters in PHB- and CoaA-expression strains. Fluorescence activity for the OmpR-regulated transcription over time normalized to cell growth at OD600 in vector control (□, ■, ▲), PHB expression (◊, ●) and CoaA expression (○, ●) strains. Plasmid expression was induced by addition of 5 µM IPTG. Open symbols represent growth in TB7 at 37°C with shaking and closed symbols represent growth in TB7 with 0.4% glucose at 37°C with shaking. Graphs represent readings taken in triplicate for the following promoters. A. *ompC* B. *ompF*. 
CHAPTER SIX
DISCUSSION

The effect of glucose on CpxR-regulated promoters

This thesis provides evidence that exogenous glucose regulates transcription from a diverse set of CpxR-regulated promoters. The mechanism can be acetylation-dependent, as occurs at the \textit{cpxP} promoter or by a distinct mechanism, as suggested by the \textit{ompC} and \textit{ompF} data. Alternatively, it may be acetylation-independent.

Lima \textit{et al.} previously reported that exogenous glucose can activate the CpxA*-activated \textit{cpxP} promoter in a CpxA-independent matter [1]. This study showed that exogenous glucose also can repress certain CpxA*-activated promoters. It also showed that glucose can either activate or repress CpxA*-repressed promoters.

While screening the CpxR-regulated promoters for their response to exogenous glucose, we found several classes of responses. Some coincided with their response to the constitutively activate \textit{cpxA*} allele, while others did not. For example, the CpxA*-activated promoters \textit{cpxP}, \textit{degP} and \textit{ompC} also were activated in response to glucose (Figure 4B, 4C, 5B, 5D). The CpxA*-activated \textit{ycfS} promoter also responded to glucose but only in a \textit{cpxA} null strain (Figure 5C). Similarly, the CpxA*-repressed promoters \textit{csgDEFG} and \textit{ompF} also were repressed in response to glucose (Figure 5G and 5H). Not all promoters responded to glucose as they did to CpxA*. For example, the CpxA*-
activated promoter ybaJ was repressed in response to glucose (Figure 5F) and the CpxA*-repressed promoter rpoErseABC was activated in response to glucose (Figure 5E). The existence of promoters that respond differently to CpxA* and glucose strongly supports the hypothesis that some CpxR-regulated promoters have an additional level of control.

While we chose ompC and ompF for further study (see below), several other promoters could warrant further investigation. As described above, the ybaJ and rpoErseABC promoters exhibit opposing responses to glucose relative to the cpxA* allele. Whereas the CpxA*-repressed ybaJ promoter was inhibited by glucose, the CpxA*-inhibited rpoErseABC promoter was activated by glucose. These observations make it obvious that CpxR-regulated promoters can be activated by at least two mechanisms: phosphorylation via CpxA and the mechanism that responds to glucose. I recommend these promoters for further investigation because they are the only tested promoters to exhibit opposing responses to glucose and the cpxA* allele. Interestingly, the rpoErseABC promoter can be regulated by both CpxR-dependent and σE-dependent mechanisms, like the degP promoter.

Our lab has previously investigated the degP promoter, which is controlled by both CpxR and the essential alternative sigma factor σE. Bozena Zemaitaitis and Alan Wolfe previously showed that glucose-induced degP transcription acts on σE-dependent transcription (Zemaitaitis and Wolfe, unpublished data). In this study, my co-workers found that CpxR represses glucose-induced degP transcription, a result that conflicts with my observation that glucose-induced degP transcription depends on CpxR. The previous
study, however, was performed in a different genetic background. While this discrepancy must be resolved, it is clear that glucose can enhance $\sigma^E$-dependent transcription. Since I also observed that CpxR represses the glucose response from *rpoErseABC* (Figure 5E), it is possible that glucose activates *degP* and *rpoErseABC* through the same mechanism.

The previous investigation into glucose-induced *degP* transcription comes as a result of studies of the strictly $\sigma^E$-dependent *rpoHP3* promoter [105, 106]. Since CpxR repressed the glucose response, Zemaitaitis and Wolfe reasoned that glucose must act on *degP* transcription via the $\sigma^E$-dependent mechanism. Because it is regulated solely by $\sigma^E$, Zemaitaitis and Wolfe chose *rpoHP3* for further investigation. However, glucose-induced *degP* transcription appears to parallel that observed with *rpoHP3*. For example, they found that the *rpoHP3* promoter’s response to glucose was enhanced by deletion of RseA, a $\sigma^E$ inhibitor. Thus, the glucose response does not require RseA. In contrast, the glucose effect required ppGpp and DksA, presumably to destabilize RNAP from rapidly transcribed promoters (e.g. those that transcribe ribosomal RNA) and thus makes RNAP available to initiate $\sigma^E$-dependent transcription.

While exogenous glucose activates *rpoHP3*, exogenous pyruvate does not. This is surprising because glucose is metabolized into pyruvate and because both glucose and pyruvate activate transcription from the *cpxP* promoter [1, 2]. Intriguingly, Zemaitaitis and Wolfe found that succinate and fumarate could also activate *rpoHP3* transcription. Apparently, this activation depends on the conversion of these TCA cycle intermediates to aspartate, as exogenous aspartate also activated *rpoHP3* transcription. A methodical genetic analysis showed that glucose, succinate and aspartate-induced *rpoHP3*
transcription requires the *de novo* synthesis of nucleotide mono- or diphosphates (AMP, ADP, GMP, GDP, UMP, UDP, CMP or CDP), but not nucleotide triphosphates. The role of the nucleoside mono- or diphosphates remains unknown.

While *degP* is activated by both P-CpxR and glucose and *rpoErseABC* is repressed by P-CpxR but activated by glucose (Table 2), we believe that the glucose response is being controlled by $\sigma^E$ at both of these promoters. It would be interesting to determine why *degP* and *rpoErseABC* exhibit opposing responses to P-CpxR but similar responses to exogenous glucose and to determine the mechanism for $\sigma^E$-dependent activation in response to exogenous glucose.

Another interesting promoter is *ycfS*, which behaves most similarly to *cpxP*. It is induced by CpxA* and by glucose, and it is inhibited by CpxA. The study of the *ycfS* promoter could prove problematic, as it exhibits very low levels of transcription. Additionally, the gene function of *ycfS* remains uncharacterized and any interesting results from studies of this promoter could be difficult to publish without any physiological relevance.

As demonstrated by my study of the *ompC* and *ompF* promoters, glucose must regulate transcription through multiple mechanisms: (1) glucose can regulate transcription through acetylation combined with the conventional phosphorylation-dependent mechanism, like *cpxP* regulation, (2) glucose can utilize a mechanism that is independent from phosphorylation by either inhibiting the phosphorylation-dependent mechanism or by a completely phosphorylation-independent mechanism and (3) finally, glucose could be regulating transcription by an acetylation and phosphorylation-
independent mechanism that may rely on other PTMs. Although glucose can clearly regulate CpxR-regulated promoters through multiple mechanisms, it also appears to play a role in regulating $\sigma^E$-dependent promoters suggesting that glucose may be a more global regulator of transcription and the glucose effect is not be restricted to just the CpxR-regulated promoters that we tested.

**The effect of glucose on the ompC and ompF promoters**

We initially chose the *ompC* and *ompF* promoters for further study due to their opposing responses to glucose in a CpxAR-independent manner and previous reports that they are primarily regulated by the well-characterized EnvZ-OmpR two-component system [84, 85]. Additionally, the *ompC* and *ompF* promoters were activated and repressed, respectively, in response to exogenous NAM and glucose (Figure 5C, 5G, Figure 6, Figure 8, Figure 9, Figure 11), like *cpxP*, which provided intriguing evidence that glucose might regulate these promoters through acetylation.

If acetylation was the cause of the glucose-effect, then we inferred that it would operate in a manner analogous to that of glucose-induced *cpxP* transcription. If it were to operate in the same manner, we expected it to utilize the same components of glucose-induced *cpxP* transcription, namely YfiQ, AcCoA, K298 of RNAP and to be sensitive to a CobB deletion. However, it is clear that glucose does not regulate *ompC* and *ompF* transcription through the same acetylation-dependent mechanism that activates *cpxP* transcription. Yet, glucose and NAM both regulate the transcription of these promoters
and while it is beyond the scope of this thesis to further investigate the mechanism, a multitude of mechanistic possibilities exist.

The promoter architecture and binding of OmpR to the *ompC* and *ompF* promoters varies significantly from CpxR binding to the *cpxP* promoter. Like CpxP, OmpR binds to its promoter region when phosphorylated [86]. A model has been proposed where, when low levels of P-OmpR are present, it binds to the higher affinity binding sites at the *ompF* promoter and initiates transcription. As higher levels of P-OmpR accumulate, it begins to bind to the lower affinity binding sites at the *ompC* and *ompF* promoters, activating *ompC* transcription and turning off *ompF* transcription [107].

The more complicated promoter architecture at the *ompC* and *ompF* promoters may play a role in the mechanism by which glucose regulates their transcription, which must be through a mechanism that is distinct from the one that permits glucose-induced activation at the *cpxP* promoter.

NAM inhibits the deacetylase CobB, which causes a global increase in acetylation that activates *cpxP* transcription through the same mechanism as glucose [1]. Since NAM activated and repressed *ompC* and *ompF* transcription, respectively, it is still possible that acetylation plays a role in regulating these OmpR-dependent promoters. One possible mechanism could be through acetylation of OmpR, a response regulator like CheY and RcsB, which are known to be acetylated. Like RcsB, OmpR has a lysine that is predicted to interact with DNA [108]. Acetylation of this lysine would be predicted to alter the affinity of OmpR for DNA. This possibility warrants investigation.
The effect of NAM on the *ompC* and *ompF* promoters

Like glucose, exogenous NAM caused an increase in *ompC* transcription while repressing *ompF* transcription (Figure 6 and 9). NAM inhibits the NAD$^+$-dependent class of deacetylases known as sirtuins [109, 110]. The only known deacetylase in *E. coli*, CobB, is a sirtuin. By inhibiting CobB, NAM increases the global acetylation profile of *E. coli* and activates *cpxP* in an acetylation-dependent manner [1]. Since the addition of NAM to the culture media gave results similar to the addition of glucose, we reasoned that both NAM and glucose regulated *ompC* and *ompF* transcription through an acetylation mechanism. Yet, glucose does not appear to be regulating *ompC* and *ompF* transcription via acetylation, at least not of K291, K297, K298 of the α-subunit of RNAP. While it is still possible that NAM regulates *ompC* and *ompF* transcription via an acetylation-dependent mechanism, it is also possible that NAM and glucose act through the regulation of intracellular NAD$^+$ levels or thus inhibiting NAD$^+$-dependent processes. Metabolism of glucose via glycolysis drives down the NAD$^+$ concentration because glyceraldehyde dehydrogenase uses NAD$^+$ as a co-factor. Another possibility is that another NAD$^+$-dependent deacetylase exists in *E. coli* but has yet to be identified. If so, this deacetylase would be the founding member of a novel family because, with the exception of *cobB*, the *E. coli* chromosome does not encode a sirtuin homolog.

Differences in strain backgrounds

We initially characterized the *ompC* and *ompF* responses in the MC4100 *E. coli* strain background. We utilized the exact same strain used to characterize the *cpxP*
response to exogenous glucose [1, 2]. When we chose to further pursue the \textit{ompC} and \textit{ompF} promoters, we utilized a fluorescent dual-reporter strain constructed by the Goulian lab in the same MC4100 background [75]. While we managed to replicate their previously published results concerning the transcription of \textit{ompC} and \textit{ompF} in response to osmolarity (Figure 8 and [75]), the response to glucose by the \textit{ompC} promoter in the fluorescent dual reporter strain was different from that observed with strain used to monitor the \textit{ompC-lux} fusion (Figure 10 and compare Figure 5C and 5G to Figure 8). Intriguingly, \textit{ompC} was activated by NAM, regardless of strain background (Figure 6, Figure 9 and Figure 10).

The MC4100 background strain used by both labs was constructed over 35 years ago at Harvard Medical School [111]. Since then, it has been used in laboratories worldwide to study \textit{E. coli}. It is not unreasonable to consider the possibility that as this strain has been passed around that it may have acquired mutations. Differences in how strains are maintained by different labs and the source of MC4100 received by those labs has almost certainly contributed to differences amongst supposedly identical strains. We propose that the conflicting results regarding \textit{ompC} transcription between our MC4100 strain and the MC4100 strain received from the Goulian lab arose from an unknown mutation in one of the strains. Since \textit{ompC} responded similarly to NAM in both strains, the mutation may only affect how \textit{ompC} responds to glucose. Our lab now has the ability to perform deep sequencing and we propose to sequence both MC4100 strains to determine the genetic differences that may have caused the discrepancy in the \textit{ompC} response to glucose.
Development of luminescence and fluorescent techniques

One of the biggest obstacles to overcome in acquiring these data was in developing techniques to achieve consistent results. Our lab had previously worked with luminescence, so the techniques to acquire luminescence data were in place but required significant modification before the data could become reproducible. Additionally, the techniques for acquiring fluorescence data had to be developed with no previous protocol in place.

We originally acquired luminescence readings using the default setting on the luminometer of a 3 second delay followed by a 15 second read. Upon consulting with Karen Visick, a faculty member in the department with extensive luminescence experience, we were told to adjust our readings to 6 seconds with no delay. Additionally, since aeration affects luminescence, we began to take luminescence readings immediately after taking a sample from the growing culture while still in the 37°C warm room. While this change did not affect the overall conclusions, it gave more consistent readings between samples.

When we decided to use the fluorescent dual-reporter strain to further investigate *ompC* and *ompF* transcription, our lab had never used fluorescence as a measurement of gene activity, so protocols were not in place to measure fluorescence in this strain. We consulted another faculty member in the department, Chris Wiethoff, whose lab has had previous experience measuring fluorescence from bacterial cells. He recommended lysing the cells using a solution of 1% Triton X-100 and a protease inhibitor since his lab had
problems with background fluorescence and light refracting off the cells. The published protocol used when the strain was developed called for cooling the samples without lysis and adding chloramphenicol to inhibit protein synthesis [75]. We compared fluorescence measurements from cells stored at 4°C with and without chloramphenicol and found that adding chloramphenicol had no effect on fluorescence measurements. We also compared measurements from lysed cells and intact cells and found that we measured similar fluorescence readings regardless of cell lysis. Furthermore, we acquired more consistent data points when we did not lyse the cells compared to when we lysed them. Since lysing the cells requires more steps and therefore increases the chance for human error, we chose to measure the cells without any other modifications to them.

**Significance**

In conclusion, we have shown that glucose can regulate transcription at many different CpxR-regulated promoters. At some of these promoters, however, CpxR is not required for glucose to affect transcription. Thus, the response to glucose is not restricted to CpxR-regulated promoters. The underlying mechanism for some of these responses may be due to acetylation, while others appear to be acetylation-independent. Most, if not all, of these mechanisms appear to be novel and thus warrant further investigation.

We chose to pursue the mechanism by which glucose regulates transcription at the OmpR-dependent *ompC* and *ompF* promoters. While most of the data suggests that acetylation may not play a role in glucose-regulated transcription at these promoters, the
results of the NAM experiments still suggest a potential role for acetylation. If so, it is clear that the mechanism is different from the one that regulates cpxP transcription.
REFERENCES


VITA

Andrew Cosgrove was raised in Commerce, Michigan, a suburb of Detroit. Before attending Loyola University Chicago, he attended the University of Michigan, where he earned a Bachelor of Science in Cellular and Molecular Biology in 2007. After graduation, he moved to Boston where he spent two years running a flow cytometry core facility at the Ragon Institute, a joint HIV/AIDS research venture between Massachusetts General Hospital, Harvard Medical School and Massachusetts Institute of Technology (MIT).

In August of 2009, Andrew began his graduate studies at Loyola University of Chicago. He spent time in several different labs before finding a home studying the role of acetylation in *E. coli* in the lab of Dr. Alan Wolfe. His research focused on the regulation of transcription in response to exogenous glucose.

After graduation, Andrew plans to continue to pursue a research career in the pharmaceutical or biotechnology industries, preferably in a location near mountains and snow.