CHAPTER ONE

General Overview

Significance and Pathogenesis:

Clostridium perfringens is a Gram-positive, pathogenic, anaerobic, spore-forming rod. Due to its ability to produce thirteen different toxins, *C. perfringens* is the causative pathogen in tissue infections such as gas gangrene (clostridial myonecrosis) (21), and a variety of intestinal diseases including enteritis necroticans (26), infectious diarrhea, and antibiotic-associated diarrhea (34). *C. perfringens* is also the third leading cause of bacterial food poisoning in the United States behind *Campylobacter jejuni* and *Salmonella* (43). Clostridial food poisoning develops after the ingestion of food containing a large number of vegetative cells (~10^9cells). The low pH of the stomach kills the majority of the *Clostridium* but triggers the initiation of sporulation in the surviving cells as they move to the small intestine where they continue the sporulation process (24). At the end of the sporulation process, the cells lyse to release their endospores, concomitantly releasing *Clostridium perfringens* enterotoxin (CPE), a toxin produced during the sporulation process (26). It has been established that non-sporulating cells do not transcribe the *cpe* gene (6, 45, 76), although it has been determined that *C. perfringens* can sporulate and not produce CPE (44). CPE first comes into contact with the intestinal epithelial cell membranes at the site of a CPE receptor protein and then complexes with a 70-kDa host protein forming a pore that eventually leads to cell death due to an influx of ions and water. As the epithelial cells die, the claudins of the tight junctions are exposed. CPE is also capable of binding and degrading these exposed claudins, breaking down the tight junctions between cells (42). Cell death

of these intestinal epithelial cells and the breakdown of the tight junctions results in the major symptoms associated with *C. perfringens* food poisoning, diarrhea and intestinal cramping (32, 33, 73).

C. perfringens was first recognized as a possible cause of food poisoning in 1943 in England and in 1945 in the United States (26). Since that time, *C. perfringens* has become recognized as a significant source of food poisoning. In the United States, the CDC estimates there are approximately fourteen million cases of food poisoning annually, from various sources. Of this total number, 250,000 cases are singly attributed to *C. perfringens*, resulting from more than 600 major outbreaks with a fatality rate of 1% (43).

Symptoms of *C. perfringens* food poisoning include diarrhea, abdominal pain and occasionally nausea and fever (62). However, the disease is self-limiting and runs its course in under twenty-four hours (27). Annually, the economic costs of *C. perfringens* food poisoning, in the U.S. alone, ranges in the millions of dollars (69).

C. perfringens is identified also as a cause in the non-food related illnesses of antibiotic-associated diarrhea and infectious diarrhea. Infectious diarrhea caused by *C. perfringens* is found primarily in the institutionalized elderly (39). Clostridia related infectious diarrhea targeted mainly women (72%) and in people over the age of sixty (86%). The symptoms of *C. perfringens* infectious diarrhea include all of the symptoms of *C. perfringens* food poisoning with the addition of blood in the stools and the ability to last more than seven days (27).

C. perfringens is now recognized as a major cause of antibiotic-associated diarrhea, an illness typically attributed to *Clostridium difficile* (65, 74). Abrahao *et al*

showed that the detection rates of *C. difficile* and *C. perfringens* are actually very similar in cases of antibiotic associated diarrhea when properly tested (1, 2).

The Phosphotransferase System:

The phosphotransferase system (PTS) is a well-documented mechanism of active sugar transport found in both Gram-positive and Gram-negative bacteria, composed of a network of sugar transporters and phosphate shuttling proteins (66). The PTS pathway is also able to couple this sugar transport to the subsequent phosphorylation of the sugar using phosphoenol pyruvate (PEP) as the phosphate donor. Sugar phosphorylation by PTS transport allows the sugar to immediately be introduced into the glycolytic pathway as a sugar phosphate. Only specific sugars known as PTS sugars, including glucose, fructose, mannitol, and mannose are taken up by the PTS system in this manner (58). In addition to sugar transport/phosphorylation, the PTS systems of both Gram-positive and Gram-negative bacteria are able to sense the nutritional state of the cell via pathways recognizing the phosphorylation state of the PTS proteins used in phosphate transfer. The mechanisms and components behind PTS transport and nutritional sensing in Gram-positive and Gram-negative bacteria, however, differ in many aspects (66, 67).

C. perfringens is capable of utilizing a broad variety of sugars (15); however, it has been shown that in the presence of the PTS sugar glucose, *C. perfringens* is subject to a repressive effect on the uptake and metabolism of non-PTS sugars and exhibits varied effects on several aspects of its physiology (71, 75). The genes and operons required for the utilization of poorer carbon sources, for example, are generally expressed only if the corresponding non-PTS sugar is present and more preferable sugars are not present. This ability to globally control gene expression based on the presence or absence of a PTS

sugar is known as carbon catabolite repression (CCR) and has been demonstrated in multiple species of bacteria (67). Examples of cell functions known to be regulated by CCR include acetate and acetoin utilization in *Bacillus subtilis* (16, 70), amylase production in *Clostridium acetobutylicum* (7), and maltose utilization in *Listeria monocytogenes* (4). The sporulation process is also known to be suppressed by CCR in both *C. perfringens* and *Bacillus subtilis* (63, 71).

Carbon Catabolite Repression:

Carbon catabolite repression in *B. subtilis* (68) and in many other Gram-positive low G+C subfamily members, including *C. perfringens,* is mediated by the transcriptional regulator Catabolite Control Protein A (CcpA) (4, 38, 41, 67). CcpA has the ability to bind to cis-elements called catabolite responsive elements (CRE), which are operators found near the promoters of genes controlled through CCR (70). CcpA belongs to the LacI/GalR family of repressors, which are capable of binding DNA at operator sites based on the presence or absence of low molecular weight effectors (72). (The mechanism of CcpA mediated catabolite repression is diagramed in Figure 1.) The effector responsible for CcpA binding the CRE-site was experimentally shown to be HPr-serine 46-Phosphate (36).

In the enteric Gram-negative PTS system, HPr (histidine-containing phosphocarrier protein) is the protein responsible for transferring a phosphate from the E1 protein to the E2 protein, which then transfers the phosphate to the incoming sugar. In the event that this transfer cannot take place the phosphorylated EII protein activates adenylate cyclase, which increases cAMP levels. cAMP then acts as a cofactor for the CRP (catabolite responsive protein), which binds DNA, affecting transcription rates (52).

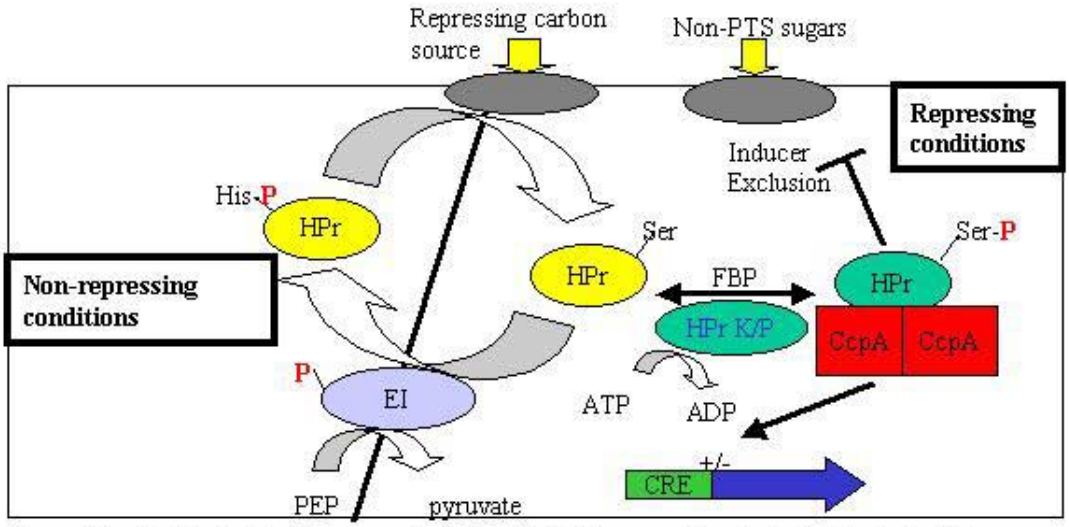

Figure 1. Model of catabolite repression in low G+C Gram-positive bacteria. The left of the diagram represents the system under non-repressing conditions while the right area represents CCR in the presence of a repressing carbon source. As repressing carbon sources are taken in through PTS transport, HPr will transfer a phosphate from the Histidine-15 position. HPr Kinase/Phosphatase responds to the high levels of fructose-1, 6-bisphosphate present from increased glycolysis and phosphorylates HPr on the regulatory Serine-46. This form of HPr inhibits the uptake of non-PTS sugars (inducer exclusion) and interacts with catabolite control protein A (CcpA) to bind DNA at CRE regions located near the promoters of catabolically responsive genes.

In low G+C Gram-positive bacteria, there is a vastly different cAMP-independent mechanism of carbon catabolite repression. In this system, HPr-histidine-phosphate, the form of HPr phosphorylated via PEP through EI, is required for PTS transport of several carbon sources. The transport of a sugar through the PTS will accept the phosphate donated from histidine-phosphorylated HPr, leaving HPr. In the presence of high amounts of glucose (repressing conditions), the high level of the glycolytic intermediate fructose 1, 6- bisphosphate stimulates the ATP-dependent HPr kinase/phosphatase to phosphorylate HPr on the serine-46 (20). Two molecules of seryl-phosphorylated HPr interact with a CcpA dimer allowing it to bind DNA at the CRE regions. The ratio of HPr-His-phosphate (no PTS sugar present) and HPr-Ser-phosphate (PTS sugars are present) determines whether or not a particular carbon source is utilized or not by interacting with CcpA to repress or activate selected genes. This controlled system of nutritional sensing and gene regulation allows for the sequential utilization of carbon sources that allow the growth of the cell to be maximized for a given environment (67).

Past research into understanding the role of CcpA in catabolite repression was accomplished by mutating the *ccpA* gene and testing phenotypes under different environmental stimuli. In this manner, it was demonstrated that CcpA repressed transcription of multiple genes and operons in *B. subtilis* (12,9)*, B. megaterium* (37) and several other Gram positive low G+C bacteria (4, 22, 37) and is responsible for transcription of the *las* operon, containing the genes for the glycolytic enzymes phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase in *Lactococcus lactis* (41), and the *ackA* gene, which is involved in acetate excretion in *B. subtilis* (70). Many of the single substitution mutations of CcpA were affected in their ability to bind the co-

repressor HPr-serine phosphate (38), lost the ability to dimerize, or were affected in the binding cleft for DNA binding. These mutants were characterized by constitutive binding of CRE-sites, or by an inability to recognize and bind the operator (37). In *C. perfringens*, a *ccpA*$^-$ mutant has been shown to exhibit altered sugar utilization as well as a lack of sporulation ability (71).

CHAPTER TWO

Characteristics of *C. perfringens* HPr K/P and HPr

Abstract:

Carbon catabolite repression in many low G+C Gram-positive bacteria is mediated through the bifunctional enzyme HPr kinase/phosphatase. This enzyme has been shown to phosphorylate HPr on the regulatory serine-46 residue producing HPr-Ser-P, a cofactor that increases the affinity of CcpA for a cis-element sequence known as a Catabolite Responsive Element or CRE-site, found near the promoters of regulated genes. We have cloned the genes encoding the proteins HPr and HPr kinase/phosphatase from the chromosome of *C. perfringens* strain SM101 into an expression vector and overproduced the recombinant protein in *E. coli* for better characterizations of the protein interactions. We were able to demonstrate the phosphorylation of HPr by HPr kinase/phosphatase through phosphorylation assays designed to also determine the concentration of FBP necessary for optimal phosphorylation activity. HPr kinase/phosphatase was unable to phosphorylate an HPr-Ala-46 substitution mutant, providing evidence that in *C. perfringens* the serine-46residue is involved in the accepting the phosphate from ATP via HPr kinase/phosphatase.

Introduction:

HPr kinase/phosphatase is the bifunctional enzyme responsible for phosphorylating and dephosphorylating the serine-46 residue of the histidine-containing protein (HPr) of the CCR pathway of low G+C Gram-positive bacteria. This enzyme has been of particular interest to researchers since 1983 because of its integral role in this regulatory pathway, as well as its absence in eukaryotes, making it a prime antibiotic

target (8, 53, 54, 56). Early studies in *B. subtilis* and the lactic acid bacteria elucidated the mechanism of the novel enzyme through various biochemical assays designed to characterize the conditions necessary for either the kinase or the phosphatase activity of the bifunctional enzyme (13, 40). From these studies, it was determined that the kinase/phosphatase was able to respond to the nutritional state of the cell and accordingly adjust kinase/phosphatase activity (8, 55). The next problem came in determining if the enzyme independently adjusted its activity in response to intracellular stimuli, or whether it was part of a network where its activity was modulated by other proteins that actively sensed the carbon situation. Radioactive kinase and dephosphorylation assays were employed to measure the rate of phosphorylation/dephosphorylation of HPr by an HPr K/P obtained from *Bacillus subtilis*. These assays were performed under varying concentrations of several intermediates of glycolysis as well as several high-energy phosphate containing molecules. It was found through this method that fructose-1,6-bisphosphate (indicative of high glycolytic activity) was able to stimulate kinase activity of the enzyme and inorganic phosphate (indicative of low ATP) activated phosphatase activity (8, 54). In addition to its ability to phosphorylate HPr on Serine-46, HPr K/P is able to phosphorylate a second protein, called Crh (catabolite repression HPr), a protein found in *B. subtilis*, on a similar Serine-46 residue (12). HPr-Ser-46-P and Crh-Ser-46-P have both been shown to function as allosteric effectors for CcpA in *B. subtilis*, increasing the transcriptional regulator's affinity for CRE-sites found near the promoters of regulated genes (12, 61).

Methods and Materials:

Cloning of genes encoding CCR proteins of *C. perfringens* into pBAD30 vector:

The genes encoding HPr (11kDa), HPr K/P (33kDa), and CcpA (36kDa) of *C. perfringens* strain SM101 were identified through homology searches of the recently sequenced *C. perfringens* strain SM101 genome using sequences identified in *C. perfringens* strain 13 as the CCR protein encoding genes *hpr*, *ptsK*, and *ccpA*. Once identified, primers were engineered to amplify these three genes, which contained restriction sites for simplified cloning (*SacI* and *SalI*) and also encoding a 6X-Histidine tag on the C- terminus of the translated gene product. After the PCR reactions, the amplified genes with the newly encoded 6xHis tag and restriction sites were then purified by agarose gel electrophoresis (0.8% agarose in 1X TAE buffer), excised, and cleaned using the Qiagen Gel Extraction Kit (Qiagen 2002). The purified DNA fragments were then digested with *SacI* and *SphI* (New England Biolabs Beverly, MA) for two hours at 37˚ C, separated through gel electrophoresis, excised, and cleaned as described above. The digested fragments were then individually ligated into *SacI* and *SphI* digested pBAD30 at 15˚ C, using two units of New England Biolabs T4 Ligase (19). The resultant ligation was dialyzed on 0.025 μm pore filters against water and electroporated into electrocompetent *Escherichia coli* strain DH10B (Gibco BRL/Invitrogen Carlsbad, CA) in a BTX BCM399 cell electroporator set to 2500 V (BTX Holliston, MA). Electroporated cells were then allowed to recover for 45 minutes in SOC (20 g Bacto tryptone, 5 g Bacto yeast extract, 2 ml of 5 M NaCl, 2.5 ml of 1 M KCl, 10 ml of 1 M $MgCl_2$, 10 ml of 1 M $MgSO_4$, 20 ml of 1 M glucose, adjusted to 1 litre with dH_2O) (59). The recovered electroporation culture was then centrifuged at 14,000 x g and plated on

LB plates containing ampicillin at a concentration of 100 μg/ml (selecting for the pBAD30 resistance marker). The pBAD30 plus insert plasmids obtained from ampicillin resistant colonies were then checked for proper inserts by restriction digestion with *SacI* and *SphI* and agarose gel electrophoresis. Clones shown to contain a proper sized insert were then sequenced using the BigDye Terminator system (Applied Biosciences, Foster City, CA) at the Virginia Bioinformatics Institute core facility (Blacksburg, VA) to ensure the correct insert sequence and orientation. Plasmids containing the desired insert in the correct translation frame were then obtained through alkaline lysis plasmid preparation (Qiagen Miniprep) and electroporated into *E. coli* strain BL-21 RIL Codonplus for use in overproduction of the encoded protein (Stratagene). A map of these expression vectors is included as Figure 2. All plasmids and strains used in these protocols are listed in Table 1.

PCR Mutagenesis of *hpr* gene to encode HPr-Ala-46:

To determine experimentally that the serine residue at position 46 in the HPr protein of *C. perfringens* was the residue phosphorylated by HPr K/P, PCR mutagenesis was undertaken to change the sequence encoding the serine residue to a sequence encoding an alanine residue. Four primers were designed for this purpose: OWH14, OWH22, OWH23, and OWH24. Two of these primers are designed to flank the HPr encoding sequence (OWH14, OWH24) and two of which will cover and introduce the desired mutation into the amplified product (OWH22, OWH23) (see Figure 3) (59). Two PCR reactions are performed first to obtain the two halves of the total DNA region, and a second round of reactions using the outside flanking primers and first round products to

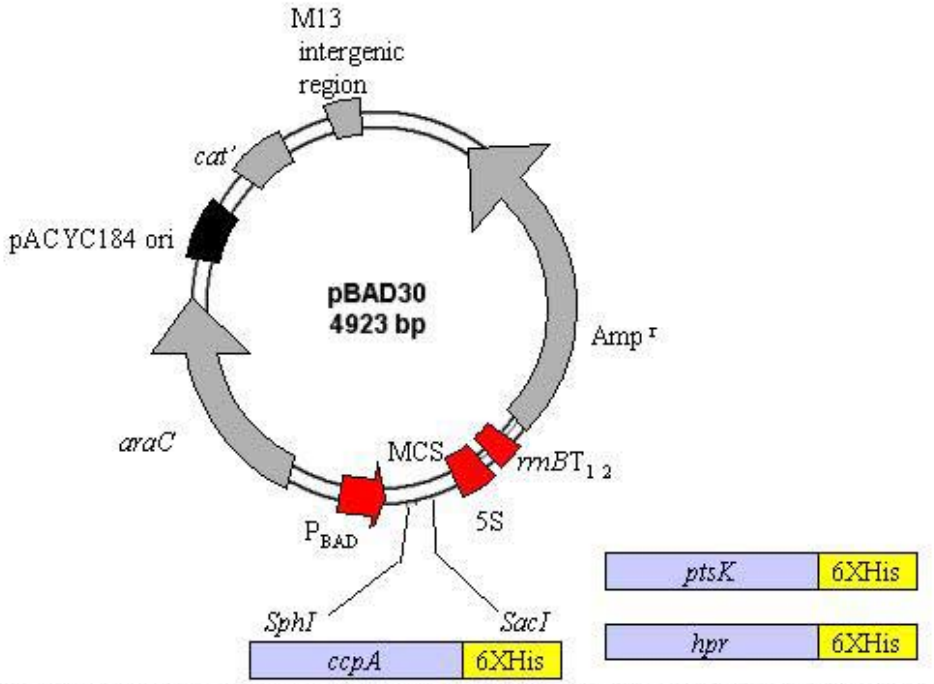

Figure 2. Construction of pBAD30 based expression vectors for expression of *C. perfringens* strain SM101 CCR proteins. Abbreviations: *rrnBT*$_{1,2}$- parts of the 5S rRNA and the strong *rrnB* terminators, P_{BAD}- arabinose inducible promoter, pACYC184 ori- low copy number *E. coli* origin, *araC*- encodes arabinose repressor protein, *cat'*-truncated chloramphenicol resistance gene, Amp ^{r}- ampicillin resistance gene, MCS- multiple cloning site.

Table 1. Bacterial strains and plasmids used in the expression and characterization of components of *C. perfringens* CCR

Strain/Plasmid	Relevant Characteristics	Reference/Source
E. coli		
DH10B	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) F80d *lacZ* ΔM15 *lacX74 deoR recA1 araD139 (ara, leu*)7697 *galU galK λ-rpsL endA1 nupG*	Gibco/BRL Corp.
BL21 Codonplus (DE3)- RIL	*argU* (AGA, AGG), *ileY* (AUA), *leuW* (CUA)	Stratagene
C. perfringens		
SM101	*hpr, ptsK, ccpA*	Zhao and Melville, 1998
Plasmids		
pBAD30	L-arabinose inducible promoter, MCS, *rrnB* T_1 T_2 transcription terminators, Ampicillin resistance, low copy number pACYC184 *E. coli* origin	Guzman et al, 1995
pBAD30+HPr	*hpr* (261bp) of *C. perfringens* strain SM101 amplified to also encode a C-terminal 6X-His tag cloned into 5' *SacI* and 3' *SphI* sites of the MCS of pBAD30	This Study
pBAD30+K/P	*ptsK* (897bp) of *C. perfringens* strain SM101 amplified to also encode a C-terminal 6X-His tag cloned into 5' *SacI* and 3' *SphI* sites of the MCS of pBAD30	This Study
pBAD30+CcpA	*ccpA* (999bp) of *C. perfringens* strain SM101 amplified to also encode a C-terminal 6X-His tag cloned into 5' *SacI* and 3' *SphI* sites of the MCS of pBAD30	This Study
pBAD30+HPr-Ala	*hpr* (261) of *C. perfringens* strain SM101 containing an alanine codon substitution at the 46-serine position amplified to also encode a C-terminal 6X-His tag cloned into 5'*SacI* and 3'*SphI* sites of the MCS of pBAD30	This Study

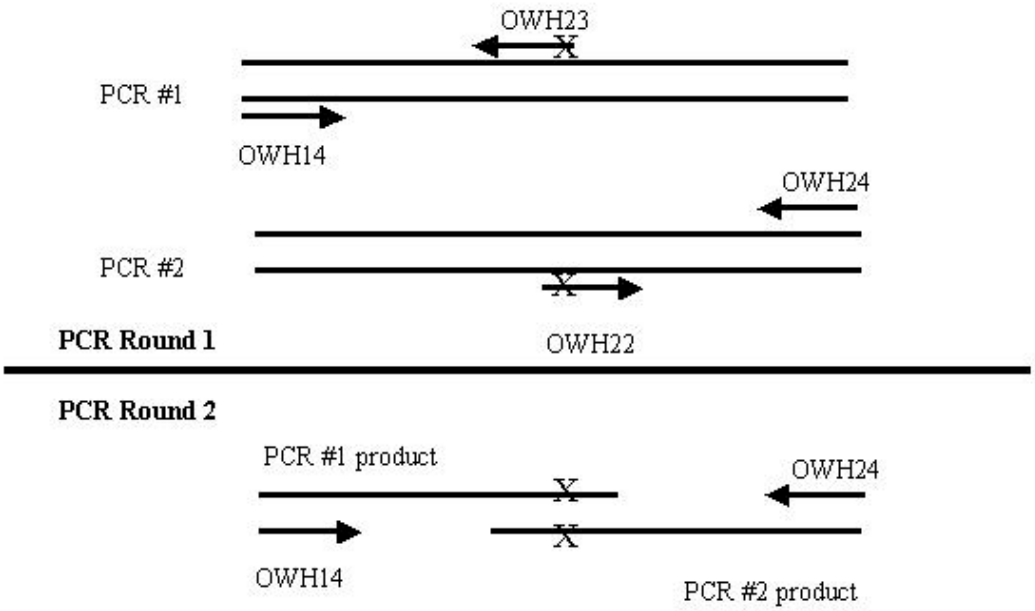

Figure 3. PCR mutagenesis procedure performed to change the codon encoding residue 46-serine to an alanine residue in the *hpr* gene amplified from *C. perfringens* strain SM01. The newly mutated gene was then ligated into the pBAD30 expression vector and electroporated into *E. coli* strain BL-21 for expression of the HPr-46-Ala protein.

obtain a fragment of the same size as the *Clostridium perfringens* strain SM101 wild type *hpr*, but containing the desired mutation. After agarose electrophoresis, the newly amplified fragment was then digested with *SacI* and *SphI* and ligated into pBAD30 (also digested with *SacI* and *SphI*). The resulting ligation was electroporated into *E. coli* strain DH10B (as described above) and plated on LB plates containing ampicillin 100 μg/ml. Positive colonies were regrown, then plasmid DNA was purified through alkaline lysis, and checked through restriction digest for correct insert size. Positive restriction digests, however, looked identical for those of pBAD30+ HPr so further analysis was necessary to determine if the mutation occurred as planned. Sequencing at the VBI core facility (Blacksburg VA), using the BigDye Terminator Kit, determined that the AGC codon of wild-type *C. perfringens* had indeed been mutated to GCT, encoding alanine in this plasmid. The primers used for the amplification of the genes encoding the CCR pathway proteins of *C. perfringens* and in the PCR mutagenesis protocol to mutagenize HPr are included as Table 2.

Protein expression: Induction Curve:

Vector pBAD30 is a protein expression vector designed to provide a stringent mechanism of control of genes that may encode proteins toxic to the host cell (19). To control the transcription of genes inserted into pBAD vectors, the *araBAD* promoter, is placed upstream of the multiple cloning site. The *E. coli araBAD* promoter is bound by a dimer of the repressor AraC until a sufficient level of L-arabinose is present to bind to the AraC proteins, releasing the DNA loop that was preventing transcription of P_{araBAD}. The

Table 2. Primers used in amplifying the *hpr, ptsK*, and *ccpA* genes from *C. perfringens* strain SM101 and in the PCR mutagenesis procedure to mutagenize the *hpr* gene

Primer Name	Primer Sequence (5'-3')	Reference
OWH14	CAAATAGAGCTCAGGAGGTGTTTATAATGGTAACTAAAG AAGTTATAG	This Study
OWH15	CCTTGCATGCTCATTAATGATGATGATGATGATGCTCTTCTAAA TTTTCGATTAACTTAGCTATTTCTTCAGC	This Study
OWH16	TAAGAAGAGCTCAGGAGGTTTTATATGGGTGTTACAATAGAG	This Study
OWH17	GTTTATACGCATGCTCATTAATGATGATGATGATGATGAATTTT ACTCTCTTTTTCAATTATATCAAC	This Study
OWH18	GGCAATAAGTTAGAGCTCAGGAGGTGAGTTAGATGGCTGCTTC	This Study
OWH19	GCATTTTTTATGGCATGCTCATTAATGATGATGATGATGATGCT TACAGCTATCTCTCTCTATTAAATC	This Study
OWH20	ATTTTAGGATCCGATGATTTAGTAAAATAATTTAGTAAAATTATA	This Study
OWH21	CT TCACTGCAGTTAAAAATCCCCCTCATATTTC	This Study
OWH22	GGAAAAAAAGCTAATGTTAAGGCTTTAATCGGAGTTTTATC	This Study
OWH23	GATAAAACTCCGATTAAAGCCTTAACATTAGCTTTTTTTCC	This Study
OWH24	AAGCCTGCATGCTCATTAATGATGATGATGATGATGCTCTTC	This Study
OWH25	CATTTAGGATCCGAATTTAGTATATTTCTGCA ATTTGG TCA	This Study
OWH26	CATATTCTGCAGGAATTAATATTTAATTTTATTACAATTATTAATA AGAAAACGT	This Study

AraC proteins then bind to a secondary site, where, with the aid of CRP-cAMP, it is able to strongly activate transcription. To gauge the responsive expression in *E. coli* strain BL21 RIL Codonplus (Stratagene) containing pBAD30+HPr, HPr-Ala-46, HPr K/P, or CcpA, an induction curve was set up to determine the concentration of L-arabinose that would produce the highest yield of protein upon induction. Single colonies that contained the pBAD30 plus insert plasmid were grown in 5 mls of Terrific Broth (per liter:12 g Bacto tryptone, 24 g Bacto yeast extract, 4 ml glycerol, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4) plus 100 μg/ml ampicillin overnight and reinnoculated 1:60 into 5 ml fresh Terrific broth plus antibiotic. These cultures were grown to an $O.D._{600}$ of 0.6 and then induced to overexpress the protein of interest by the addition of L-arabinose to a final concentration of 0.2%, 0.02%, or 0.002%. 1 ml alliquots were pelleted and frozen at 0, 2, and 4 hours for the induced cultures. Pellets were washed in 500 μl TE and then resuspended in 50 μl of milliQ water. 6 μl of 10X sample buffer (10 mM Tris HCl, 1 mM EDTA pH 8, 2.5% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue) was then added to the cell suspensions and the samples were boiled for 5 minutes. The samples were cooled on ice for 5 minutes and briefly centrifuged to collect condensation and pellet insoluble material. The now denatured proteins were then electrophoresed on an 8%-25% SDS-PAGE PhastGel (Amersham Pharmacia Upsala, Sweden), stained with PhastGel Blue R dye, and examined to determine the optimal concentration and length of induction with L-arabinose.

Protein Overexpression and Purification:

E. coli colonies shown through plasmid purification and restriction digest to contain pBAD30 (19) plus the HPr, HPr-Ala-46, HPr K/P, or CcpA encoding inserts were inoculated to 10 ml of Terrific Broth containing 100 μg/ml ampicillin and grown overnight at 37˚C and reinnoculated into 250 ml of fresh Terrific Broth plus 100 μg/ml ampicillin. To retrieve the overexpressed protein, the protocol outlined in the Qiagen Ni-NTA Spin Handbook (Qiagen, February 2003) was followed. This culture was grown with vigorous shaking to an $O.D_{600}$ of 0.6 and then L-arabinose was added to a concentration of 0.02% (determined earlier as optimal concentration for induction). Four hours after induction the cells were pelleted at 4,000 x g in a JA-14 rotor, washed with 10 mls TE, centrifuged again at 4,000 x g, and frozen at -20˚C. These pellets were then thawed and resuspended in 1 ml of lysis buffer (50 mM NaH_2P0_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) supplemented with 1 mg/ml lysozyme. After incubating on ice for 30 minutes the cells were sonicated and centrifuged at 10,000 x g for 30 minutes at 4˚C. Qiagen Ni-NTA spin columns were equilibrated with 600 μl of lysis buffer. Six hundred microliters of the lysate was loaded per spin column and centrifuged for 2 minutes at 700 x g to bind the 6xHis-tagged proteins. The columns were then washed with 600 μl of wash buffer twice (50 mM NaH_2P0_4, 300 mM NaCl, 50 mM imidazole, pH 8.0) and finally the protein was eluted twice with 200 μl of elution buffer (50 mM NaH_2P0_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) (Qiagen). The eluates were dialyzed against 4 L of TKED buffer (150 mM KCl, 100 mM Tris-HCl pH 8, 1 mM EDTA, 0.1 mM DTT) using Slide-A-Lyzers (Pierce). To concentrate the samples, the dialyzed solutions were centrifuged for 2 hours at 7500 x g in Centricon YM-3 Amicron filter devices at 4˚C

(Millipore Billerica, MA). Glycerol was then added to 10% and the samples were stored at -20˚C.

Kinase Assays:

The procedure for these assays is essentially the same as those performed with similar proteins from *Streptococcus* and *Bacillus* species (12, 57). The reactions are set up to contain 1 μg of the purified HPr kinase/phosphatase, 5 μg HPr, fructose-1, 6-bisphosphate concentrations ranging from 0-7.5 mM, 5 mM $MgCl_2$, and 0.5 mM [γ ^{32}P]-ATP is added to a 1.5 ml Eppendorf tube to a final volume of 20 μl and incubated at 37°C for 5-15 minutes. The incubation is stopped using SDS quench buffer containing 10 mM Tris-HCl, 1 mM EDTA, 2.5% SDS, 5% β-mercaptoethanol, and 0.01% bromophenol blue and adjusted to pH 8. The 20 μl sample is then loaded to an SDS-polyacrylamide 15% gel and electrophoresed in a BioRad MiniProtean II under standard conditions. The electrophoresis chamber and buffers used during the protein separation and subsequent fixing/staining with Coomassie blue (0.1% in methanol-acetic acid-water, 5:2:5 by volume), were checked before disposal for radioactivity and disposed or cleaned properly based on their relative levels of radioactivity. After fixation/staining, the gels were wrapped in cellophane and placed on a blanked phosphorimager screen for 10-30 minutes. After exposure, the wrapped gels are removed and the screen was scanned on a STORM 860 phosphorimager scanner (Molecular Dynamics, Sunnyvale California) to quantify the fluorescence created by the radioactivity of the gel. Imagequant software was then used to quantify the density of the bands on the resulting gel pictures.

Results:

Protein Purification:

The protein samples were verified by SDS-PAGE separation on homogenous 20% acrylamide Phastgel minigels. Figure 4 illustrates a 20% polyacrylamide gel containing cell lysates of the three protein induction cultures before and after Ni^{2+}-NTA purification and concentration.

Kinase Assays: Optimal Fructose-1,6-bisphosphate Concentration:

To perform HPr K/P kinase assays, conditions were based on conditions set forth in previous assays of kinase activity of the HPr K/P of other species of bacteria (20 mM Tris-HCl, 5 mM ATP, and 5 mM $MgCl_2$) (13, 18). Reactions were then set up with varying levels of fructose-1, 6- bisphosphate to identify the intermediate's relationship to kinase activity. The results indicated that fructose-1, 6- bisphosphate does activate the kinase activity of HPr K/P to phosphorylate HPr. This enzyme, however, appears more sensitive to lower concentrations of fructose-1, 6- bisphosphate than the corresponding HPr K/P enzymes of other bacteria. In these assays, 8 mM was shown as the optimal concentration for *C. perfringens* HPr K/P. This contrasts the results seen in other *B. subtilis*, where 40 mM has been tested as the optimal concentration for the activation of the *B. subtilis* HPr K/P (54). Figure 5 illustrates the results of these assays.

Kinase Assays: HPr-Ser-46 vs. HPr-Ala-46

To verify the phosphorylation site of HPr, PCR mutagenesis was undertaken to mutate the codon encoding serine-46 of HPr to an alanine. The new overproduced HPr-46-Ala was then used in the same kinase assays utilizing γ-P^{32} ATP that were used to characterize the wild-type *C. perfringens* HPr protein. The results of these assays agree

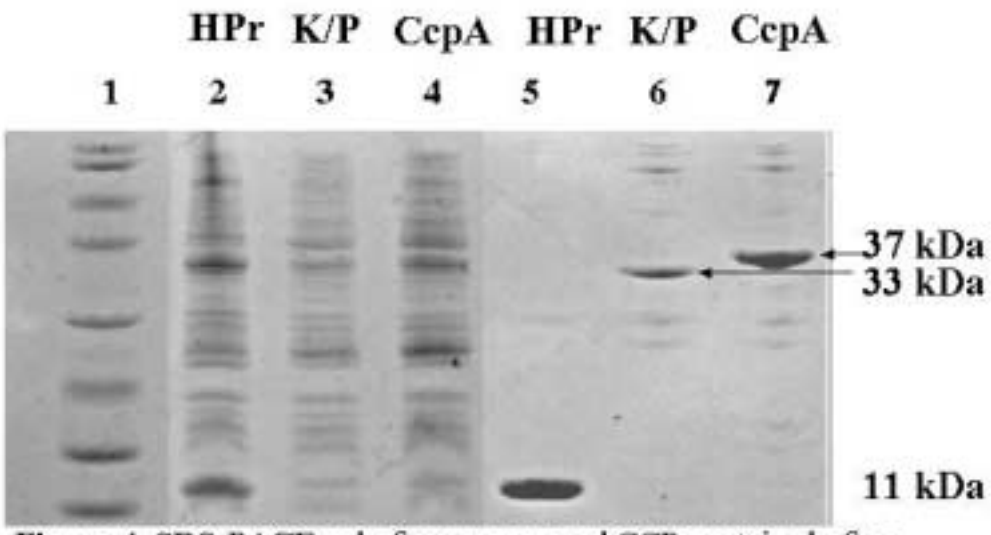

Figure 4. SDS-PAGE gel of overexpressed CCR proteins before and after Ni^{2+}-NTA purification. Lanes 2-4 contain whole cell extracts of *E. coli* strain BL21 Codonplus RIL transformed with pBAD30 vectors encoding HPr, K/P, and CcpA and lanes 5-7 contain samples taken after purification through Ni^{2+}-NTA chromatography. Lane 1: Molecular size markers: 6.5 kDa, 14.4 kDa, 21.5 kDa, 31 kDa, 45 kDa, 66.2 kDa, 97.4 kDa, 116.25 kDa

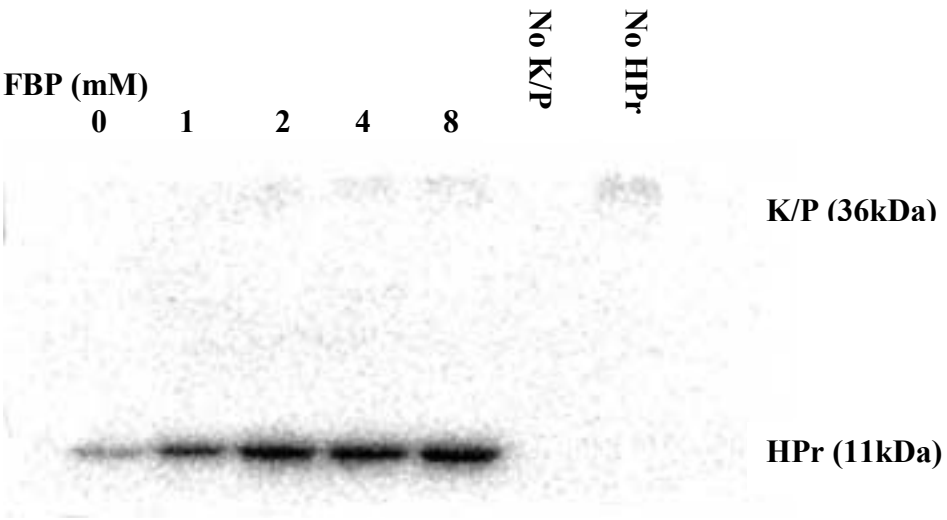

Figure 5: Phosphorylation Assay to identify the transfer of ^{32}P containing phosphate from ATP to HPr. Fructose-1, 6-bisphosphate levels were varied in the first five lanes, while both control lanes contained 8 mM FBP. SDS 15% acrylamide PAGE gels were electrophoresed under standard conditions and exposed on a phosphorimager screen for 30 minutes. The phosphorimager screen was then scanned for fluorescence on a STORM 860 phosphorimager scanner.

with the belief that the serine residue of position 46 in HPr is the phosphorylation site. Even in concentrations of fructose-1, 6- bisphosphate that provided for the optimal level of phosphorylation of HPr by HPr K/P, HPr-46-Ala remains unphosphorylated by HPr K/P (Figure 6).

Discussion:

C. perfringens strain SM101 is a highly saccharolytic bacterium capable of using a diverse number of sugars and is also equipped with genes allowing it to make use of multiple amino acids. Depending on the environment, the bacteria may or may not need any one of these genes actively transcribed and translated and would benefit greatly from a system of regulation that could coordinate these metabolic genes based on carbon source availability. Carbon catabolite repression, the system of gene regulation found in many other low G+C Gram-positive bacteria, is believed to perform this function. Genes encoding the three major components of CCR were found in *C. perfringens* strain SM101. I have PCR amplified the genes encoding HPr kinase/phosphatase and its substrate HPr with primers designed to incorporate a 6X-Histidine tag onto the C-terminal end of the protein and cloned them into an inducible expression vector. After purification of the proteins from induced cultures containing these plasmids, I have been able to conduct biochemical assays in order to determine some basic features of the proteins and compare them with results of similar assays conducted on HPr kinase/phosphatase and HPr expressed in other low G+C Gram-positive bacteria. The kinase assays were conducted to determine whether *C. perfringens* HPr K/P phosphorylated HPr and if so, is stimulated by increased levels of fructose-1, 6-bisphosphate. The exact residue of phosphorylation was also determined. The results

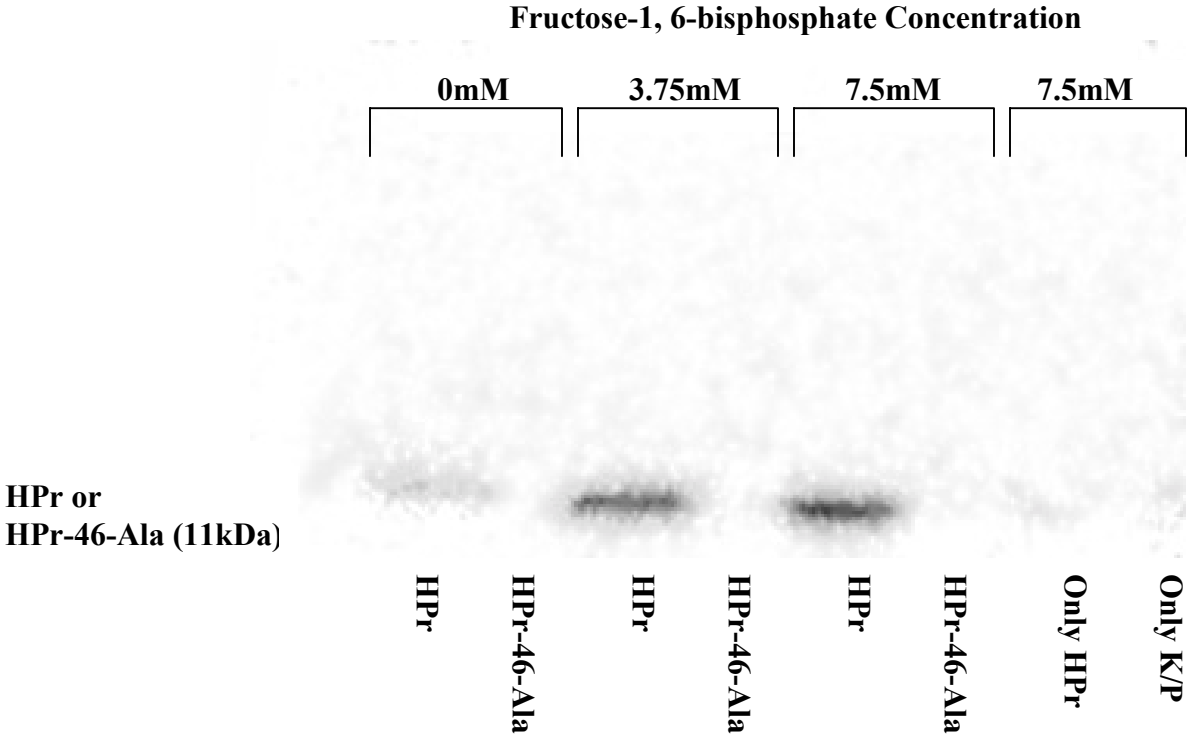

Figure 6: Kinase Assay to identify the transfer of ^{32}P containing phosphate from ATP to the putative phosphate-receiving residue in the proteins HPr and HPr-46-Ala. HPr and HPr-46-Ala both ran to a position corresponding to 11kDa when electrophoresed on SDS 15% acrylamide PAGE gels. After electrophoresis the gels were fixed and exposed on a phosphorimager screen for 30 minutes. The phosphorimager screen was then scanned for fluorescence on a STORM 860 phosphorimager scanner.

indicated that *C. perfringens* strain SM101 HPr K/P is able to phosphorylate HPr and is stimulated by fructose-1, 6- bisphosphate. However, 7.5 mM fructose-1, 6- bisphosphate in the reactions provided the optimal level of phosphorylation as opposed to the 40 mM fructose-1, 6- bisphosphate needed to achieve optimal phosphorylation as seen in similar assays with *B. subtilis* (54). This indicates the CCR sensing in *C. perfringens* may be more sensitive and more quickly activated to produce HPr-Ser-P. After PCR mutagenesis of the gene encoding HPr-Ser to encode HPr-46-Ala, kinase assays were performed by a similar method to those performed to identify the optimal concentration of fructose-1, 6-bisphosphate. Phosphorylation reactions were set up with HPr K/P with wild-type HPr or with HPr-Ala for concentrations of 0 mM, 3.75 mM, and 7.5 mM of fructose-1, 6-bisphosphate. The assays confirmed the idea that HPr is phosphorylated on the serine residue 46 of HPr, as the bands pertaining to HPr-Ala on the SDS-PAGE gels did not contain detectable levels of P^{32}-PO_4 after undergoing the same phosphorylation reaction conditions that will phosphorylate HPr-Ser.

CHAPTER THREE

Electrophoretic Mobility Shift Assays of ORF54 and *ccpA* CRE-sites and Loss of Glucose Repression in *ccpA*$^-$ *C. perfringens*

Abstract:

Clostridium perfringens is a Gram-positive anaerobic pathogen capable of causing, amongst other diseases, severe food poisoning and antibiotic associated diarrhea. The pathogenesis of *C. perfringens* food poisoning has been found to be intimately connected with sporulation; a pathway that is itself controlled through sensing of the nutritional state of the cell. Sensing and subsequent regulation of the carbon situation in many Gram-positive bacteria has been shown to be governed by a pathway known as carbon catabolite repression (CCR) (67). In this pathway, catabolite control protein A (CcpA) and seryl-phosphorylated HPr (histidine containing phosphocarrier protein) form a complex that is capable of binding to cis-elements near the promoters of regulated genes, regulating transcription. Two such sites that may function as CcpA binding sites were found: one preceding ORF54, a gene encoding a putative lactose permease and part of an operon encoding β-galactosidase in *C. perfringens* ATCC 13124 and another upstream of the gene encoding *ccpA* in *C. perfringens* strain SM101. After identifying the putative CRE-sites in the promoter region and performing electrophoretic mobility shift assays using purified CcpA and HPr on DNA fragments containing these putative CRE-sites, we were able to conclude that CcpA+HPr-Ser-P was able to bind to these DNA fragments. To determine the role of CcpA in the regulation of the β–galactosidase encoding operon, we compared the levels of β–galactosidase in wild-type versus *ccpA*$^-$ *C.*

perfringens strain SM101, providing correlating *in vivo* evidence to support the biochemical evidence of CCR in *C. perfringens*.

Introduction:

Carbon catabolite repression (CCR) is a regulatory pathway based upon the need to sequentially transport and break down the highest energy carbon substrate possible while repressing genes involved in the transport/breakdown of less energetically favorable carbon sources (67). In *C. perfringens*, a common soil and inhabitant of the human intestines that is capable of utilizing a diverse number of sugars, amino acids, and proteins, the ability to regulate the numerous genes responsible for the breakdown and metabolism of secondary carbon sources is a high priority (64). In the accepted model CCR is accomplished through the action of HPr kinase/phosphatase, which is stimulated through high levels of fructose-1, 6-bisphosphate, the result of high glycolytic activity, to phosphorylate HPr on the serine-46 residue. HPr-Ser-P then forms a tetramer complex in conjunction with a dimer of the protein CcpA, which is then able to bind DNA at sites known as CRE-sites near promoters of regulated genes or operons and regulate transcription (61).

Lactose utilization is a characteristic in *C. perfringens* strain SM101 that has been shown to be regulated through a CcpA-dependent mechanism (Melville, unpublished). Examination of the DNA sequence in and around the promoter of the operon ORF54 encoding β-galactosidase showed a sequence resembling a consensus CRE-site of *B. subtilis* (TGWNANCGNTNWCA) (31). A second CRE-site was identified based on information that in many species of low G+C Gram-positive bacteria, the gene encoding CcpA is autoregulated (9, 48). By locating and examining the *ccpA* gene of *C.*

perfringens strain SM101, a second candidate CRE-site was identified with sequence homology to the CRE-site of *B. subtilis* (3, 30). This second CRE-site was also of interest based on its high degree of dyad symmetry (AGAAAACGTTTTTT**),** a characteristic of CRE-sites in many other species of low G+C Gram-positives (50, 51). Figure 7 illustrates the CRE-site of *C. perfringens* strain SM101 *ccpA* and its relation to the putative promoter of the gene. By utilizing purified *C. perfringens* strain SM101 HPr, HPr K/P, and CcpA proteins produced through recombinant *E. coli* and two radiolabeled DNA fragments containing these two *C. perfringens* candidate CRE-sites, we were able to show the basic functions of CCR in *C. perfringens* strain SM101. This technique will distinguish if CcpA binds the two CRE-sites and if so, whether this interaction is enhanced by HPr-Ser-P. Proving that these candidate CRE-sites bind CcpA will further the search to create a consensus CRE-site used in finding genes regulated through CCR, and perhaps an insight into the regulation of pathogenesis and sporulation in the pathogenic *Clostridia*.

A *ccpA*$^{-}$ *C. perfringens* is unable to produce the CcpA protein to bind to CRE-elements and regulate the transcription of its regulon. If the CRE-site preceding the operon containing β–galactosidase in *C. perfringens* is used to negatively regulate *lacZ* transcription from its –1 position, a *ccpA*$^{-}$ strain should produce recognizably more LacZ, as glucose repression of the lactose degrading enzyme is abolished (see Figure 8 for a map of ORF54 CRE-site in relation to the promoter and start codon of the operon). β–galactosidase assays were used to gauge LacZ levels in a *ccpA*$^{-}$ and wild-type *C. perfringens* in glucose rich, lactose rich, or sugarless media to illustrate the loss of

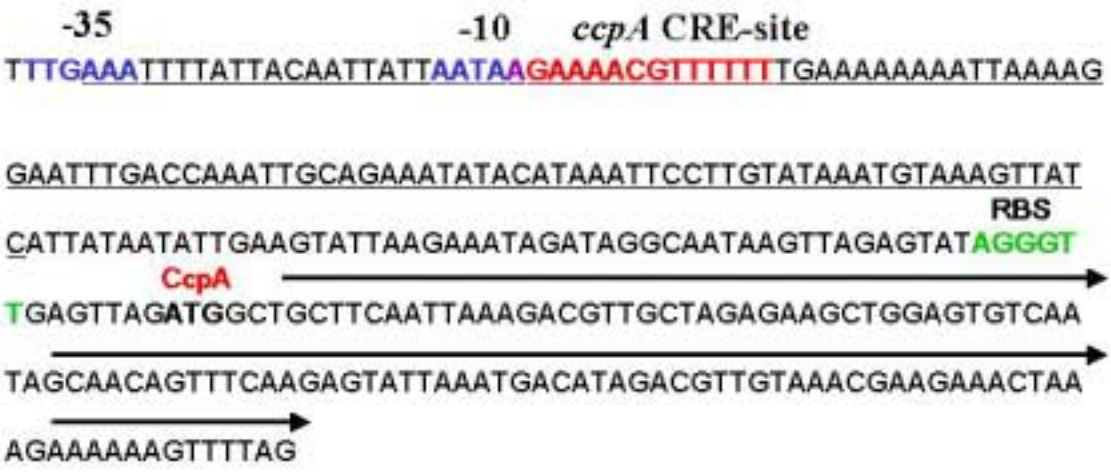

Figure 7. Diagram illustrating the CRE-site of the *ccpA* gene of *C. perfringens* strain SM101 in relation to the putative promoter and ribosomal binding site of the *ccpA* gene. The underlined region indicates the fragment amplified for gel retardation assays.

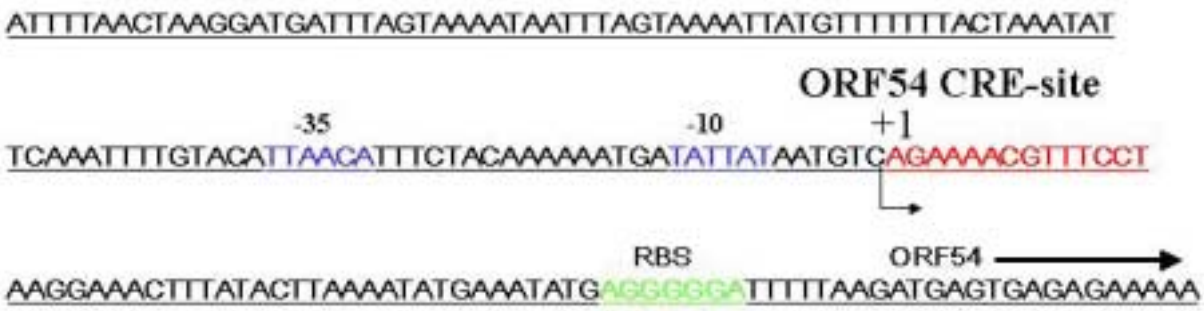

Figure 8. Diagram illustrating the CRE-site of the ORF54 operon of *C. perfringens* ATCC 13124 in relation to the promoter and ribosomal binding site of the *pbg* containing operon. The entire underlined region was amplified for gel retardation assays.

glucose repression over *lacZ* expected if the gene is regulated through CCR. A table of all plasmids and pertinent strains used in these experiments is included as Table 3.

Methods and Materials:

Electrophoretic Mobility Shift Assay:

A 261 bp DNA fragment containing the catabolite responsive element (CRE) of an operon encoding a lactose permease and β-galactosidase (ORF54) from *C. perfringens* type strain 13124 was PCR amplified and cloned into the pKS^- cloning vector. Similarly a 120 bp DNA fragment containing the CRE-site containing region of the promoter region of *ccpA* was amplified from *C. perfringens* strain SM101 and cloned into the pKS^- cloning vector. After restriction digestion to remove the DNA fragments from the vector backbone, gel electrophoresis, and purification through a Qiagen gel extraction kit, the DNA fragments were radioactively labeled with γ-P^{32} ATP via T4 polynucleotide kinase in a reaction adjusted to 5% PEG 8000 and incubated at 37˚C for 10 minutes. After heat inactivation at 90˚C for 2 minutes, the radiolabeled fragments were extracted with phenol: chloroform: isoamyl alcohol (25:24:1), ethanol precipitated, air dried, and resuspended in dH_2O.

Purified HPr is then phosphorylated by HPr kinase/ phosphatase under the conditions determined through kinase assays: 20 mM Tris-HCl (pH 7.5), 7.5 mM fructose -1, 6-bisphosphate, 5 mM $MgCl_2$, 1 mM DTT, 10 mM ATP, in a 20 µl reaction containing ~12 µg HPr, and ~2 µg K/P. The reaction was incubated at 37˚C for 15 minutes and stored on ice.

Table 3. Bacterial strains and plasmids used in the study of CRE-site binding of CcpA of *C. perfringens* CRE-sites and study of glucose repression in *ccpA*$^{-}$ *C. perfringens*

Strain/Plasmid	Relevant Characteristics	Reference/Source
E. coli		
DH10B	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) F80d *lacZ* ΔM15 *lacX74 deoR recA1 araD139 (ara, leu*)7697 *galU galK λ-rpsL endA1 nupG*	Gibco/BRL Corp.
C. perfringens		
SM101	*hpr, ptsK, ccpA*	Zhao and Melville, 1998
SM120	*ccpA*$^{-}$ SM101, *Cm*^{R}	Varga et al, 2004
Plasmids		
pKS^{-}	General cloning vector providing blue/white screen, Ampicillin resistance, high copy number pUC *E. coli* origin	Stratagene
pWH5	261bp DNA fragment containing the upstream region of the ORF54 operon of *C. perfringens* ATCC 13124 cloned into the 5' *BamHI* site and 3' *PstI* site of pKS-	This Study
pWH6	111bp DNA fragment containing the upstream region preceding the ccpA gene of *C. perfringens* strain SM101 cloned into the 5' *BamHI* site and 3' *PstI* site of pKS-	This Study

Tubes were then arranged containing binding buffer: 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% glycerol, 50 mM KCl, 0.05% Igepal 630, and ~2 μg of the now phosphorylated HPr-Ser-P. CcpA was added to the tubes to final concentrations of 0 μM, 1.95 μM, 4 μM, 10 μM, and 19.5 μM. A control was also performed containing no HPr-Ser-P. The radiolabeled CRE containing DNA fragment was then added to the reaction and incubated for 15 minutes at 37˚C. Cold competitor DNA (where needed) was added at a concentration of ~60X the amount of radiolabeled DNA (2.4 μg). Nondenaturing sample buffer (10% glycerol, 0.25% bromophenol blue) was then added and the samples were added directly to a nondenaturing Tris-Borate-EDTA (TBE) 6% polyacrylamide, 10% glycerol gel and electrophoresed for 1 hour at 150 V. The gel was dried onto Whatman paper on a BioRad 583 gel drier under vacuum for 30 minutes. The gel/paper was then exposed to a phosphorimager screen for ~10 minutes and visualized on a STORM 860 Phosphorimager (Molecular Dynamics, Sunnyvale California). The resulting images were then examined for the shift of the DNA + CcpA band characteristic of DNA bound by a DNA-binding protein as it electrophoresed through the gel.

β-galactosidase Assays:

Wild-type *C. perfringens* strain SM101 and *ccpA- C. perfringens* strain SM101 (71) were grown in PGY (30 g/L proteose peptone #3 (Difco), 111 mM D-glucose, 10 g/L yeast extract, 1 g/L sodium thioglycolate, and resazurin as redox indicator), PLY (same as PGY, except 111 mM D-lactose instead of D-glucose), and PY (PGY, but with no glucose) under anaerobic conditions at 37°C. These cultures were subcultured into their corresponding media and an $O.D._{600}$ measurement was immediately taken and 1 ml aliquots were pelleted and stored at -20°C. The subcultures were incubated under

anaerobic conditions at 37°C with samples being taken every hour for $O.D._{600}$ measurement and 1 ml aliquots removed for pelleting and storage at -20°C. These samples were taken for 8 hours for each strain and media type. The frozen pellets of these samples were resuspended in 800 µl of assay buffer (50 mM NaH_2PO_4, 1 mM EDTA, 5 mM DTT) and 8 µl toluene was added. Samples were vortexed for 1 minute and incubated on ice for 10 minutes and incubated in a 37°C waterbath for 30 minutes with caps open. In 15-second intervals, 160 µl O-nitrophenyl-β-D-glucuronide was added to the tubes, recording the start times. Tubes were incubated at 37°C until yellow color developed and at that point 400 µl 1 M Na_2CO_3 was added to stop the reaction (recording the stop time). Tubes were centrifuged for 10 minutes at 10,000 x g, and 900 µl of the supernatant is transferred to a disposable cuvette for a A_{405} reading in a spectrophotometer. Specific activity for each sample time point is determined by the equation $(A_{405} \times 1000)/(O.D._{600} \times \text{time} \times \text{culture volume})$.

Results:

Electrophoretic Mobility Shift Assays:

Electrophoretic Mobility Shift Assays (EMSAs) were employed for the purpose of showing that CcpA will bind regions of DNA considered to contain CRE-sites. By utilizing these assays we were first able to show that *C. perfringens* strain SM101 CcpA is capable of binding the two candidate CRE-sites, identified through sequence homology to accepted CRE-site sequences taken from *B. subtilis*. Both the CRE-site taken from the gene encoding CcpA in *C. perfringens* strain SM101 as well as the CRE-site amplified from the upstream region of an operon encoding a lactose permease from *C. perfringens* ATCC 13124 were retarded during electrophoresis through a 5% polyacrylamide gel by

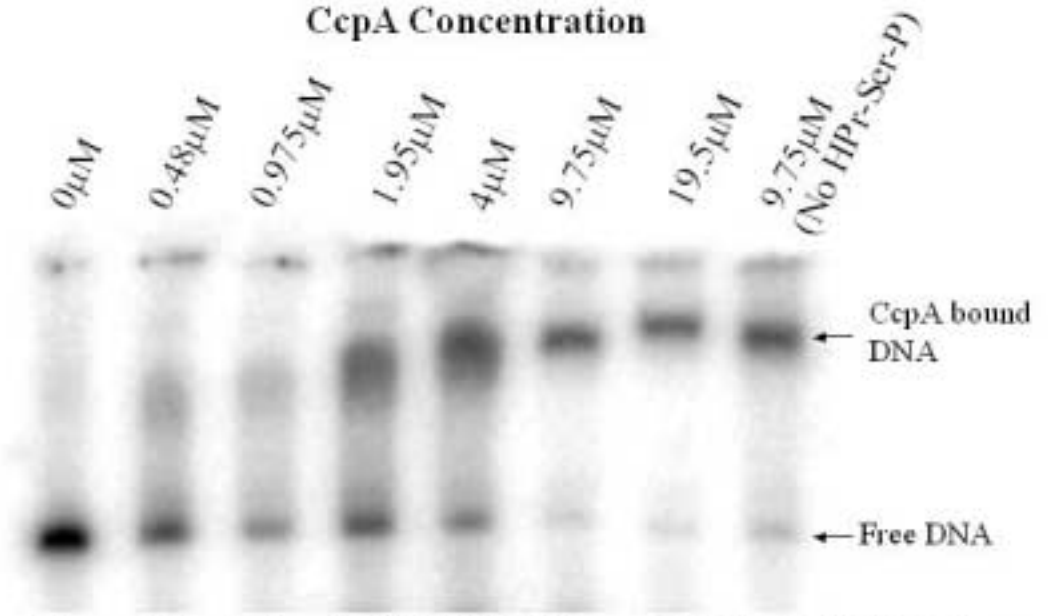

Figure 9. Electrophoretic Mobility Shift Assay of a P^{32} radiolabelled 261bp DNA fragment containing the CRE site preceding ORF54 of *C. perfringens* ATCC 13124. Reactions contained 100mM Tris-HCl (pH 7.5), 1mM EDTA, 10% glycerol, 50mM KCl, 0.05% Igepal 630, and ~2 μg of the HPr-Ser-P and CcpA as indicated.

CcpA binding. Figure 9 illustrates the binding of CcpA to the 261bp DNA fragment containing the ORF54 CRE-site tested with increasing concentrations of CcpA and as a control, one reaction where no HPr-Ser-46-P was included in the binding reaction. In this control, there is no distinctive evidence that HPr-Ser-46-P is necessary for binding of CcpA to this CRE-site, although more experimental evidence will be necessary to verify this conclusion. Another EMSA, utilizing a ~111bp DNA fragment bearing the CRE-site found near the *ccpA* gene of *C. perfringens* strain SM101 was performed in which concentrations of CcpA were included in reactions to retard the DNA as it is moves through a 5% polyacrylamide gel and also in corresponding reactions where cold unlabeled DNA containing the *ccpA* CRE-site was added to compete with CcpA binding. By competing for CcpA binding, the competitor DNA shifted the labeled band to the same position as reactions with no CcpA included (Figure 10). This competition assay was also performed for the DNA fragment containing the ORF54 CRE-site (results not shown).

β-galactosidase Assays

β-galactosidase assays used to gauge differences in the production of the lactose cleaving enzyme between wild-type and *ccpA*$^{-}$ *C. perfringens* strain SM101 distinctly illustrate the loss of glucose repression through CCR of β-galactosidase in the *ccpA*$^{-}$ *C. perfringens*. Even in glucose rich media, the *ccpA*$^{-}$ *C. perfringens* is unable to bind the CRE-site regulating the genes involved in lactose degradation under conditions where they are unnecessary to the metabolism of the bacteria. Figure 11 illustrates the β-galactosidase specific activities of *ccpA*$^{-}$ and wild-type *C. perfringens* in both glucose rich/lactose free and lactose rich/glucose free media.

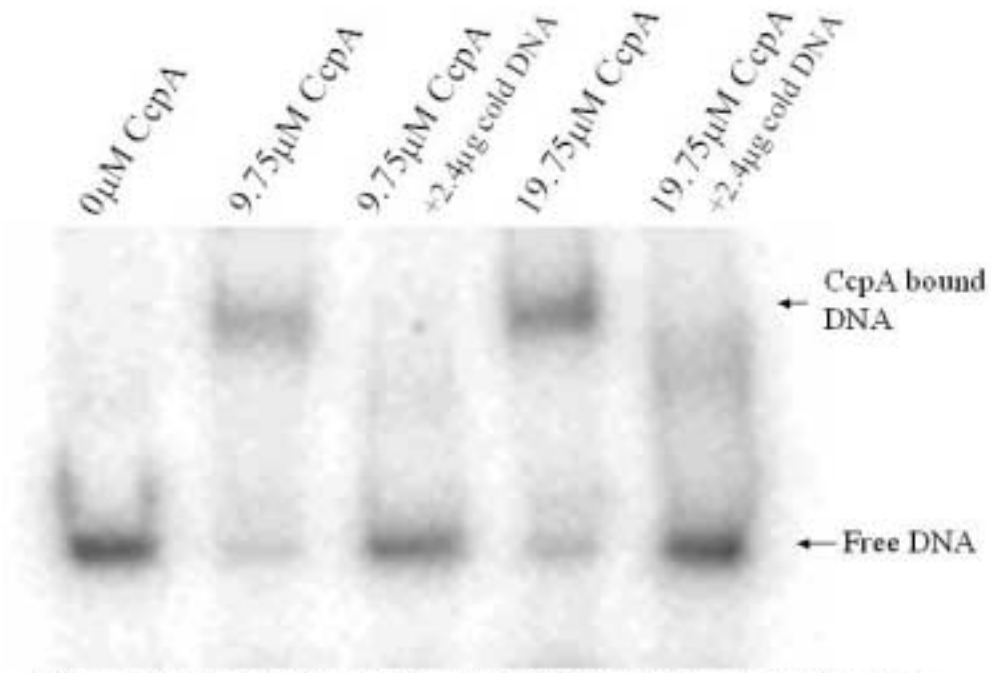

Figure 10. Phosphorimage of an EMSA showing the retardation of a 111bp DNA fragment containing the CRE-site preceding *ccpA* of *C. perfringens* strain SM101 and the corresponding reduction in retardation after the same unlabelled, fragment is added in excess.

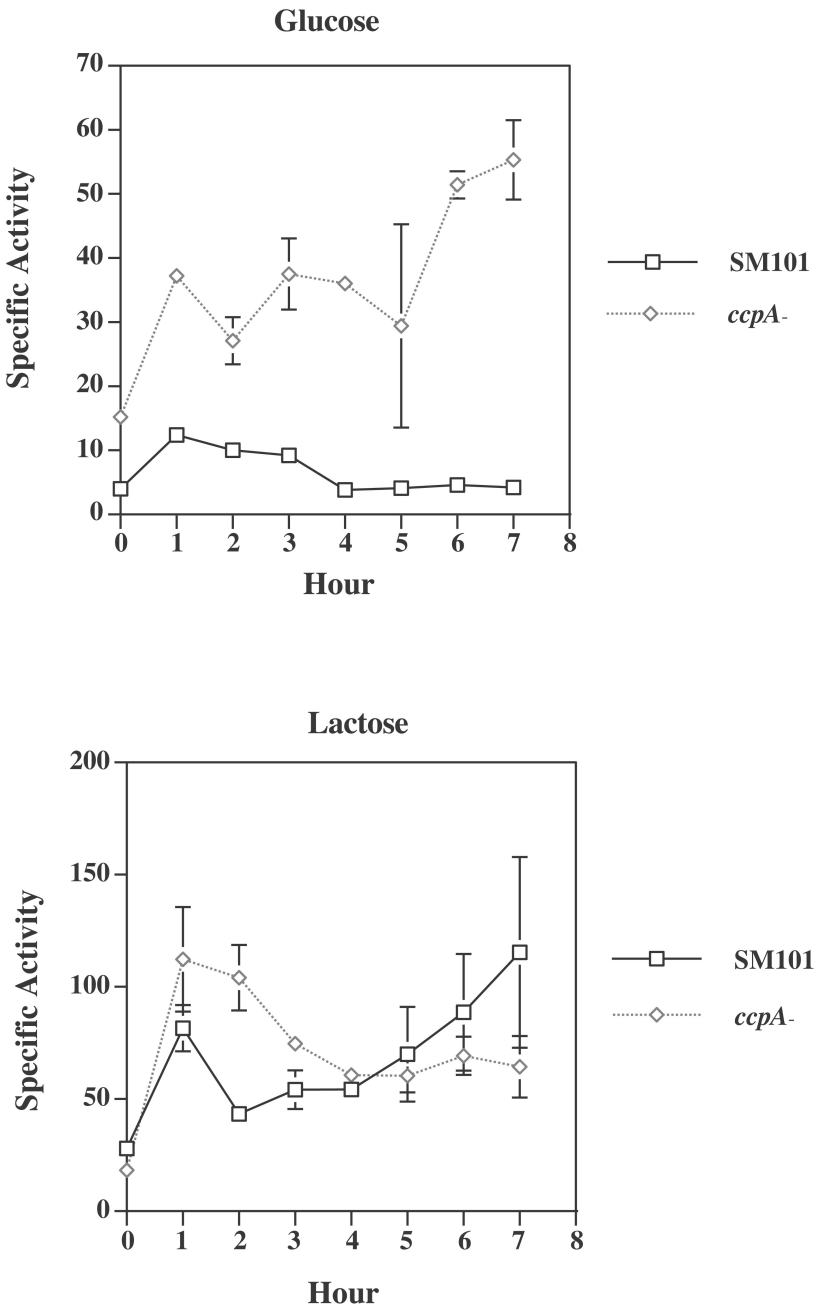

Figure 11. β-galactosidase assay results for wild-type and *ccpA*$^{-}$ *C. perfringens* when grown in PGY (top) or PLY (bottom). Cultures were grown simultaneously at 37 C anaerobically, with samples taken every hour for O.D. $_{600}$ measurement and measurement of β-galactosidase activity. The results illustrate a lack of glucose repression over β-galactosidase production in the *ccpA*$^{-}$ mutant, demonstrating the role of CcpA in carbon catabolite repression. Error bars are included, indicating standard deviation for the three replications of the experiment.

Discussion:

We were able to show experimentally the binding of purified recombinant CcpA to DNA fragments containing CRE-sites amplified from upstream of the *C. perfringens* strain ATCC 13124 operon encoding the ORF54 lactose permease and β-galactosidase, as well as a region of DNA found upstream of the *ccpA* gene of *C. perfringens* strain SM101. These findings indicate that these genes may be regulated during CCR in *C. perfringens*. The DNA fragment containing the CRE-site of ORF54 was bound by CcpA in the EMSA procedure, indicating that the operon encoding the proteins needed for the transport and breakdown of lactose may be regulated through CCR. To phenotypically test for evidence of this assumption, we made use of a *ccpA*- strain of *C. perfringens* to compare β-galactosidase levels of the mutant versus wild-type. The findings of these assays support the idea that the genes involved in lactose utilization in *C. perfringens* are repressed by glucose in a CcpA dependent manner as glucose repression of β-galactosidase is abolished in the *ccpA*- mutant.

An interesting result of the EMSA assays performed was the lack of effect the HPr-Ser-P concentration had on the binding of CcpA to CRE-sites. One possible explanation for this is that at the C-terminus of CcpA there is a binding site for HPr-Ser-P that could be affected by the addition of the 6X-His tag encoded for the purification of the proteins (36). This hypothesis could be disproven with the creation of an expression vector encoding CcpA with an added protease cleavage site preceding the 6X-His tag that would allow for its removal after purification. However, 6X-His tagged CcpA has been

used in similar experiments with no reported unresponsiveness to HPr-Ser-P, so the more likely explanation is that *C. perfringens* may control CCR in a yet undescribed fashion.

CHAPTER FOUR

Identifying CRE-sites through Computer Based Searches

Abstract:

Examination of the genomes of sequenced strains of *Clostridium perfringens* for genes involved in metabolism illustrates a species of bacteria capable of utilizing a variety of carbon sources. In order to coordinate this array of genes, CRE-sites, ~14bp cis-elements, are found near promoters of genes to be regulated through carbon catabolite repression. To these CRE-sites, the transcriptional regulator CcpA will bind and either repress or activate transcription based on the CRE-sites position relative to the promoter. This CcpA binding site has been elucidated in *Bacillus subtilis* by compiling the genes shown to be controlled through CCR, examining the promoter regions for CRE-sites and aligning these CRE-sites to arrive at a consensus sequence for a CRE-site (46). Similarly, in *C. perfringens* we can take CRE-sites shown to be bound by CcpA to make a rough consensus CRE-site for use in finding even more CCR regulated genes. As more CRE-sites are found, our predicted *C. perfringens* strain SM101 CRE-site will arrive closer to a true consensus.

Introduction:

The first reference to CcpA came through work dedicated to studying the α-amylase gene of *Bacillus subtilis*, a gene known to be transcriptionally regulated under glucose repression (23, 29). During this study, a transposon insertion mutation was found that abolished the glucose repression of the α-amylase gene. The sequence surrounding the transposon insertion was examined and was found to encode a transcriptional

regulator with similarity to the LacI/GalR family of repressors. It had been identified earlier as the transcriptional regulator *alsA*, a transcriptional regulator involved with acetoin biosynthesis of *B. subtilis* (23). Many studies have utilized this CRE-site, known as *amyO*, for studies investigating the binding properties of CcpA with CRE-sites (29, 30). It was also shown that by knocking out or changing the sequence of sites resembling the *amyO* found preceding the *xyl*, *hut* and *gnt* operons of *B. subtilis* also knocked out glucose repression of these metabolic operons (10, 25, 35). Later work, aimed at the newly broadening scope of genes regulated by CCR in low G+C Gram-positive bacteria, involved creating a *ccpA*- mutant and comparing phenotypic characteristics of this strain versus the wild-type of the same bacteria (5, 14, 16, 70).

The CRE-sites of the *B. subtilis* CCR mechanism discovered through research of the amylase, histidine utilization, and xylose utilization genes/operons are the best studied examples of these CcpA binding sequences. With the new tool of a putative CRE-site consensus, Moreno et al were able to identify many genes regulated by CcpA dependent (and independent) CCR by utilizing a whole-transcriptome approach through a cDNA microarray based experiment comparing the transcriptional pattern of wild type *B. subtilis* versus a *ccpA*- mutant of the same strain under various growth conditions. These results were then compared to the results of a computer based search designed to identify possible CRE sites in or around promoters of genes found in the *B. subtilis* chromosome (49). For this search, a weighted matrix consensus CRE site (TGNAANCGNWNNCW) (47) was used to search the *B. subtilis* chromosome using the GRASP-DNA program (60). It was determined that the genes of the computer based search correlated well with the results of the microarray-based experiment for genes

regulated by CCR (glucose repressed, CcpA repressed with or without glucose, and CcpA activated without glucose and repressed with glucose) (49). The results of the experiments showed evidence that this technique was efficient at distinguishing CRE-sites based on sequence alone and could be similarly performed in other bacteria.

In the case of *C. perfringens*, however, there is no easily accessible microarray available for use in determining the differences in transcriptional patterns between a *ccpA*$^-$ mutant and wild-type *C. perfringens* then comparing these results to those of the CRE-site searches. However, since Moreno et al gave proof of concept for this procedure we can use a modified version of this method to search for CRE-sites and then experimentally show CcpA binding on a gene-by-gene basis, rather than through a transcriptional profile (49).

Methods and Materials:

CRE Searching:

Searches were performed on the newly sequenced genome of *C. perfringens* strain SM101 for CRE-sites with similarity (two base degeneracy allowed) to the CRE sites found preceding the ORF54 operon and the *C. perfringens* strain SM101 *ccpA* gene. The findpattern program provided by SeqWeb (Accelrys) was used for searches of the genome of *C. perfringens* strain SM101 for possible CRE-sites based on these *ccpA* and ORF54 CRE-sites. 2000 bp of sequence taken upstream and downstream from the CRE-site was then searched through databases using the blastx program provided by NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). CRE-sites that were found to be in or within 400 bp (arbitrarily chosen distance) of the promoters of these nearby genes were considered potential candidate CRE-sites. Primers were then designed to amplify small DNA

fragments containing these candidate CRE-sites and these amplified fragments were then used in electrophoretic mobility shift assays to again determine the binding affinity of CcpA for these sites and determine if these genes may be regulated under the control of CCR in *C. perfringens* strain SM101.

DNase Protection Assay:

To determine the protein-DNA sequence binding specificity of CcpA to a CRE-site containing DNA fragment, DNase footprinting was performed as described by Galas and Schmitz, with modifications (11). To perform this assay, HPr is phosphorylated by HPr K/P in 20 mM Tris-HCl (pH 7.5), 7.5 mM fructose -1, 6-bisphosphate, 5 mM $MgCl_2$, 1 mM DTT, 10 mM ATP, in a 20 µl reaction containing ~12 µg HPr, and ~2 µg K/P for 15 minutes at 37˚C. Varying concentrations of CcpA varying from 0 mM to 12.5 mM and ~1.5 µg of the phosphorylated HPr were added to a reaction mixture containing 10 mM Tris-HCl pH 8, 1 mM EDTA, 50 µg BSA, 0.05% Igepal 630, 10% glycerol, 1 mM DTT, and 10 µg poly dAdT and P^{32} radiolabeled CRE containing DNA measuring ~50,000 cpm of activity (28). One unit of RQ1 DNase was then added to each reaction along with 2 µl 1X DNase buffer and incubated for 30 seconds at 37˚C. The reactions were stopped with 90 µl of DNase stop solution containing 3 M sodium acetate, 0.25 M EDTA, and 0.15 mg/ml herring sperm DNA. The samples were then phenol:chloroform:isoamyl alcohol (25:24:1) precipitated twice, ethanol precipitated, washed in 70% ethanol twice, air dried and resuspended in loading buffer (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol) (Promega). After heating to 85˚C for 2 minutes, the samples along with Sequenase sequencing reactions of the DNA fragment (USB) were loaded onto a 6% polyacrylamide 7 M urea sequencing gel on a Gibco model S2 sequencing gel

apparatus where it was electrophoresed in TBE buffer at 1500 V, 40 mA, and 45 W for 3 hours. The gel was then dried to Whatman filter paper on a BioRad 583 gel dryer under vacuum for 2 hours. The gel/paper was then visualized using a STORM 860 Phosphorimager (Molecular Dynamics).

Electrophoretic Mobility Shift Assay:

~100 bp DNA fragments containing possible catabolite responsive elements (CRE) found in the genome of *C. perfringens* type strain SM101 through searches for sites homologous to CRE-sites known to bind CcpA, were PCR amplified. After gel electrophoresis, and purification through a qiagen gel extraction kit, the DNA fragments were radioactively labeled with γ-P^{32} ATP via T4 polynucleotide kinase in a reaction adjusted to 5% PEG 8000 and incubated at 37˚C for 10 minutes. After heat inactivation at 90˚C for 2 minutes, the radiolabelled DNA fragments were extracted with phenol: chloroform: isoamyl alcohol (25:24:1), ethanol precipitated, air dried, and resuspended in dH_2O.

Purified HPr is then phosphorylated by HPr kinase/ phosphatase under the conditions determined through kinase assays: 20 mM Tris-HCl (pH 7.5), 7.5 mM fructose -1, 6-bisphosphate, 5 mM $MgCl_2$, 1 mM DTT, 10 mM ATP, in a 20 µl reaction containing ~12 µg HPr, and ~2 µg K/P. The reaction was incubated at 37˚C for 15 minutes and stored on ice.

Tubes were then arranged containing binding buffer: 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% glycerol, 50 mM KCl, 0.05% Igepal 630, and ~2 µg of the now phosphorylated HPr-Ser-P. A control was setup containing only radiolabeled DNA and CcpA was then added to the other tubes to final concentrations of 10 µM. Cold unlabeled

competitor DNA (where needed) was added at a concentration of ~60 X the amount of radiolabeled DNA (2.4 μg). The radiolabelled CRE containing DNA fragment was then added to the reaction and incubated for 15 minutes at 37˚C. Nondenaturing sample buffer (10% glycerol, 0.25% bromophenol blue) was then added and the samples were added directly to a nondenaturing Tris-Borate-EDTA (TBE) 6% polyacrylamide, 10% glycerol gel and electrophoresed for 1 hour at 150 V. The gel was exposed to a phosphorimager screen for ~12 hours and visualized on a STORM 860 Phosphorimager (Molecular Dynamics, Sunnyvale California).

Results:

CRE Search:

Two separate searches were performed on the genome of *C. perfringens* strain SM101: a search for sites homologous to the CRE-site found upstream of ORF54 of *C. perfringens* ATCC 13124, and a search for sites homologous to the CRE-site found near the promoter of the *ccpA* gene itself of *C. perfringens* strain SM101. Both of these searches allowed a two base pair mismatch to expand the search results.

The search of the *C. perfringens* strain SM101 genome for sites similar to the CRE-sites of ORF54 and *ccpA* provided several results immediately of interest based on their similarity to CRE-site searches done in *B. subtilis*, as well as experimental evidence of genes known to be controlled through CCR in *B. subtilis*, including genes involved in the acetate production pathways, several types of sugar and amino acid/peptide transporters, and various other metabolically involved genes (5, 16, 17).

We have taken the ~150 genes found through this method and identified the genes which possess a CRE-site in or within 400 bp from the promoter of the candidate gene.

45 CRE-sites were found through this manner that satisfied these criteria, and I have designed primers to PCR amplify ~100 bp DNA fragments containing these CRE-sites from the genome of *C. perfringens* strain SM101 for use in gel retardation assays screening for CcpA binding. These results (which are not finished as of the publication of this text) will allow us to narrow the suspected consensus CRE-site of *C. perfringens* to a truer to life model, which will then be used to identify other genes regulated through CCR. Table 4 contains the gene/ gene products (found through sequence homology searches through blastx (NCBI) putatively regulated by CcpA dependent CCR as well as the location of the CRE-site within the *C. perfringens* strain SM101 genome. Also included in Table 4 are The Institute for Genomic Research (TIGR) assigned Open Reading Frame designations for *C. perfringens* strain SM101 for genes that could be definitively identified (single copy gene, with correct flanking genes). Genes that could not be given ORF designations because of redundancy (transposons, hypothetical proteins) will be given ORF numbers after the *C. perfringens* strain SM101 genome becomes available through NCBI for blast searches to correctly identify the genes as identified by TIGR.

DNase Protection Assay:

DNase protection assays designed to show where CcpA binds to CRE-sites (shown earlier to bind CRE-sites) were attempted several times for both the ORF54 and the *ccpA* CRE-site containing fragments. Unfortunately, no valid results were returned from this procedure that helped elucidate the binding position of CcpA. Figure 12 illustrates the inconclusive results of the DNase protection assay for CcpA using a *ccpA* CRE-site containing fragment.

Table 4: Genes identified as candidates for regulation through CCR in *C. perfringens* strain SM101. ORF designations are given where applicable. The underlined gene product indicates the CCR regulated gene candidate and arrows indicate gene orientation in relation to the CRE-site.

CRE#	TIGR assigned ORF in *C. perfringens* strain SM101	Location in *C. perfringens* SM101 Genome	CRE-sites found through homology to *ccpA* or ORF54 CRE-sites Regulated Gene(s)
1	ORF00071	72,351	fatty acid synthesis← **CRE** ←acetate kinase
2	ORF00091	90,678	hypothetical protein ←**CRE** ←RNA binding Sun protein
3	ORF00149	150,226	**CRE** →nitrate reductase cat. Subunit
4	ORF01313	220,327	**CRE** →hypothetical protein (fucA)
5	ORF01314	221,642	glycolate oxidase← **CRE** ←stage V sporulation protein
6	ORF00214	224,140	**CRE** →sporulation protein V D
7	ORF02315	328,129	**CRE** →nucleoside diphosphate transporter
8		438,318	**CRE** →DNA topoisomerase IV subunit A
9	ORF02008	616,295	**CRE**→acyl-CoA thioesterase
10	ORF00710	781,079	**CRE** →50S ribosomal L13
11	ORF00778	836,013	**CRE** →NAD dependent glyceraldehyde 3-phosphate dehydyrogenase
12		871,522	**CRE** →transcriptional regulator
13	ORF00887	955,274	**CRE** →*ccpA*
14	ORF01098	1,208,767	**CRE**→phosphoglycerate mutase
15		1,370,312	hypothetical protein→ **CRE** →hypothetical protein
16	ORF01313	1,443,504	lactate permease →**CRE** →beta subunit electron transfer protein
17	ORF01365/ORF01366	1,506,188	*veeA* homolog →**CRE**←transposase
18	ORF01393	1,550,854	**CRE** →xanthine permease
19	ORF01466	1,622,380	**CRE**→ UDP N-acetyl-D-mannosaminuronic acid dehydrogenase
20		1,717,526	**CRE**→two component histidine kinase
21	ORF00815	1,753,816	cell division protein *(ftsH)* →**CRE** →hypothetical protein
22		1,739,281	**CRE**→glutaminase
23	ORF01244	1,777,572	**CRE**→capsular polysaccharide synthesis protein
24		1,823,545	**CRE**→*moxR* ATPase
25		1,955,383	transposase←**CRE** →sugar transporter
26		2,008,689	transposase →**CRE** →hydrolase
27	ORF01806	1,996,959	cyclomaltodextrinase→**CRE** →hypothetical protein
28	ORF01828/ORF0	2,009,329	transposase →**CRE** →hypothetical protein

	1830		
29	ORF01833	2,024,768	Beta-galactosidase →**CRE** →hypothetical protein
30	ORF01854	2,056,926	**CRE** →gluconate permease
31	ORF01955	2,148,981	**CRE** →PTS permease for NAG
32		2,267,728	hypothetical protein →**CRE** →tetracycline resistance protein
33	ORF02100	2,302,614	HPr K/P →**CRE** →cation transporter
34		2,218,047	**CRE** →hypothetical protein FOG PAS/PAC
35		2,235,042	permease →**CRE** →hypothetical protein
36		2,235,108	permease →**CRE** →hypothetical protein
37		2,313,138	hypothetical protein →**CRE** →transposase
38	ORF02194	2,392,109	citrate lyase→ **CRE** ←malate oxidoreductase
39	ORF02229	2,426,814	dihydroortase ←**CRE** ←aspartate carbamoyl transferase
40	ORF02439	2,658,829	FeS cofactor synthesis←**CRE** ←chorismate mutase
41	ORF02441	2,662,462	**CRE** →sugar hydrolase
42		2,756,101	hypothetical protein→ **CRE** →hypothetical protein
43	ORF02550	2,773,836	transcriptional regulator ←**CRE** ←sucrose PTS permease
44		2,826,211	ABC transporter ←**CRE** ←hypothetical protein
			CRE-sites similar to ORF54 CRE-site only
45		1,269,314	**CRE** →transporter protein
46		1,677,068	Two component response regulator→ **CRE** →hypothetical protein
47		2,577,308	**CRE** →amino acid transporter
48	ORF00315	2,615,593	**CRE** →acetyl transferase

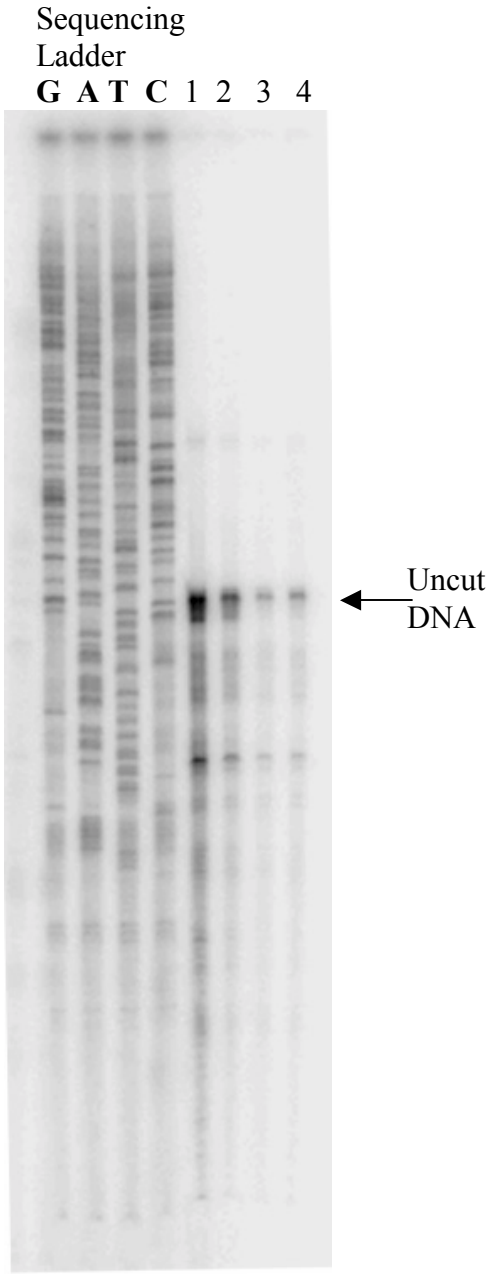

Figure 12. DNase protection assay in the presence of CcpA of a DNA fragment containing the CRE-site preceding *ccpA* from *C. perfringens* strain SM101. The result is similar to the DNase protection assays done with both the *ccpA* CRE-site and the ORF54 CRE-site, showing what appears to be insufficient digestion by the RQ1 DNase. A Sequenase sequencing reaction (USB) was run alongside the assay (GATC). Lane 1: no CcpA, Lane 2: 1.25 μM CcpA, Lane 3: 6.25 μM CcpA, Lane 4: 12.5 μM CcpA

Discussion:

The computer based searches of the genome of *C. perfringens* strain SM101 for a degenerate CRE-site sequence based on CRE-sites determined in this study to bind CcpA, provided a group of genes that intuitively could be regulated by CCR and many which are known to be regulated in *B. subtilis* through CCR. The screening of the ~100 bp DNA fragments containing these CRE-sites will prove to be a decisive tool for separating true CRE-sites from false positives identified through accidental homology. These resulting positively identified CRE-sites will be used to create a new consensus for a continued search of the genomes of *C. perfringens* as we continue the search for a model for the sequence identified and bound by *C. perfringens* CcpA.

The DNase protection assays provided no evidence of the binding site of CcpA on the CRE-site. This may be attributed to the AT-richness of the *C. perfringens* DNA, and the fact that DNase is sometimes unable to effectively degrade low G+C DNA. To avoid this problem DNase protection assays can be performed utilizing hydroxy radicals rather than DNase to degrade the DNA. To determine the CcpA binding site, we may also be able to turn to a new mutagenesis procedure that will produce CRE-sites containing 1 bp, 2 bp, 3 bp…etc. mutations in succession in the DNA fragment containing the CRE-site, creating an increasingly unidentifiable CRE-site. This series of mutated CRE-sites can then be used to identify at what point CcpA can no longer bind to the CRE-site, thereby determining the region necessary for CcpA to bind the DNA and effectively defining a footprint without the DNase footprinting procedure.

CHAPTER FIVE

Concluding Remarks

In *Bacillus subtilis*, the discovery of the carbon catabolite repression pathway and its massive scope of regulation (~30% of the total genes) has led to a better understanding of the metabolism, physiology, and genetic regulation of the bacteria. If similar developments in the study of CCR are made in the study of *C. perfringens*, a bacteria whose quick metabolism and ability to sporulate make it a successful pathogen, this information could be used to prevent the pathogenesis of many clostridial diseases.

We have been able to demonstrate many similarities between the accepted model of CCR in other low G+C Gram-positives and *C. perfringens*. The HPr kinase/phosphatase of *C. perfringens* was able to phosphorylate HPr and was stimulated by high levels of fructose-1, 6-bisphosphate. The serine-46 residue of HPr was also shown to be the residue responsible for accepting the phosphate in this phosphorylation reaction. The role of HPr-Ser-P as a cofactor for CcpA in *C. perfringens* CCR, however, has been more inconclusive. The results of EMSAs examining the role of HPr-Ser-P on the binding of CcpA to CRE-sites gave no direct evidence that HPr-Ser-P influenced CcpA's binding affinity for CRE-sites. Controls containing CcpA and no HPr-Ser-P were as effective for binding CRE-sites as those reactions containing up to 2 μg HPr-Ser-P.

To demonstrate that CcpA can bind to CRE-sites to regulate transcription and also that the predicted CRE-sites were effective at binding CcpA, electrophoretic mobility shift assays (EMSA) were performed. The candidate CRE-sites tested in the EMSAs were a predicted CRE-site taken from a region of DNA preceding an operon encoding a lactose permease and a CRE-site preceding the gene encoding CcpA itself. Both CRE-

sites bound CcpA effectively in the assays, supporting the idea that these were both genuine CRE-sites and also that CcpA binds DNA to regulate transcription in *C. perfringens*. DNase assays were performed to identify where CcpA bound to the CRE-site, but were inconclusive, as it appeared the labeled DNA remained uncut by the DNase. Other strategies could be used to get around this set back, including PCR mutagenesis of the CRE-site or nested deletions by exonuclease of the CRE-site containing DNA followed by EMSAs to identify the exact region of DNA needed to bind CcpA to the fragment.

Putative CRE-sites have also been identified through computer based searches of the genomes of *C. perfringens* strain SM101 for sites similar to the CRE-sites we have shown to bind CcpA. Primers have been designed to amplify small DNA fragments containing these CRE-sites for use in EMSAs to screen for CcpA binding. Sequences identified as CRE-sites can then be compiled and compared to determine a consensus CRE-site that can be used to search the genomes of *C. perfringens* species for CRE-sites more efficiently.

Through this project many characteristics of CCR in *C. perfringens* have been elucidated through the study of the proteins of the pathway. Taken together, the results of these experiments, as well as those yet to be performed will provide a better understanding of the metabolism, physiology, and potential virulence of the pathogen *C. perfringens*.

CHAPTER SIX

References:

1. **Abrahao, C., R. J. Carman, H. Hahn, and O. Liesenfeld.** 2001. Similar frequency of detection of *Clostridium perfringens* enterotoxin and *Clostridium* difficile toxins in patients with antibiotic-associated diarrhea. Eur J Clin Microbiol Infect Dis **20:**676-7.
2. **Asha, N. J., and M. H. Wilcox.** 2002. Laboratory diagnosis of *Clostridium perfringens* antibiotic-associated diarrhoea. J Med Microbiol **51:**891-4.
3. **Aung-Hilbrich, L. M., G. Seidel, A. Wagner, and W. Hillen.** 2002. Quantification of the influence of HPrSer46P on CcpA-cre interaction. J Mol Biol **319:**77-85.
4. **Behari, J., and P. Youngman.** 1998. A homolog of CcpA mediates catabolite control in *Listeria monocytogenes* but not carbon source regulation of virulence genes. J Bacteriol **180:**6316-24.
5. **Belitsky, B. R., H. J. Kim, and A. L. Sonenshein.** 2004. CcpA-dependent regulation of *Bacillus subtilis* glutamate dehydrogenase gene expression. J Bacteriol **186:**3392-8.
6. **Czeczulin, J. R., R. C. Collie, and McClane.** 1996. Regulated expression of *Clostridium perfringens* enterotoxin in naturally *cpe*-negative type A, B, and C isolates of *C. perfringens*. Infect. Immun. **64:**3301-3309.
7. **Davison, S. P., J. D. Santangelo, S. J. Reid, and D. R. Woods.** 1995. A *Clostridium acetobutylicum* regulator gene (*regA*) affecting amylase production in *Bacillus subtilis*. Microbiology **141:**989-96.
8. **Deutscher, J., and M. H. Saier, Jr.** 1983. ATP-dependent protein kinase-catalyzed phosphorylation of a seryl residue in HPr, a phosphate carrier protein of the phosphotransferase system in *Streptococcus pyogenes*. Proc Natl Acad Sci U S A **80:**6790-4.
9. **Egeter, O., and R. Bruckner.** 1996. Catabolite repression mediated by the catabolite control protein CcpA in *Staphylococcus xylosus*. Mol Microbiol **21:**739-49.
10. **Fujita, Y., and Y. Miwa.** 1994. Catabolite repression of the *Bacillus subtilis gnt* operon mediated by the CcpA protein. J Bacteriol **176:**511-3.
11. **Galas, D. J., and A. Schmitz.** 1978. DNAse footprinting: a simple method for the detection of protein-DNA binding specificity. Nucleic Acids Res **5:**3157-70.
12. **Galinier, A., J. Haiech, M. C. Kilhoffer, M. Jaquinod, J. Stulke, J. Deutscher, and I. Martin-Verstraete.** 1997. The *Bacillus subtilis crh* gene encodes a HPr-like protein involved in carbon catabolite repression. Proc Natl Acad Sci U S A **94:**8439-44.
13. **Galinier, A., M. Kravanja, R. Engelmann, W. Hengstenberg, M. C. Kilhoffer, J. Deutscher, and J. Haiech.** 1998. New protein kinase and protein phosphatase families mediate signal transduction in bacterial catabolite repression. Proc Natl Acad Sci U S A **95:**1823-8.

14. **Gaudu, P., G. Lamberet, S. Poncet, and A. Gruss.** 2003. CcpA regulation of aerobic and respiration growth in *Lactococcus lactis*. Mol Microbiol **50:**183-92.
15. **Groves, D. J., and A. F. Gronlund.** 1969. Carbohydrate transport in *Clostridium perfringens* type A. J Bacteriol **100:**1256-63.
16. **Grundy, F. J., A. J. Turinsky, and T. M. Henkin.** 1994. Catabolite regulation of *Bacillus subtilis* acetate and acetoin utilization genes by CcpA. J Bacteriol **176:**4527-33.
17. **Grundy, F. J., D. A. Waters, S. H. Allen, and T. M. Henkin.** 1993. Regulation of the *Bacillus subtilis* acetate kinase gene by CcpA. J Bacteriol **175:**7348-55.
18. **Gunnewijk, M. G., P. W. Postma, and B. Poolman.** 1999. Phosphorylation and functional properties of the IIA domain of the lactose transport protein of *Streptococcus thermophilus*. J Bacteriol **181:**632-41.
19. **Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol **177:**4121-30.
20. **Hanson, K. G., K. Steinhauer, J. Reizer, W. Hillen, and J. Stulke.** 2002. HPr kinase/phosphatase of *Bacillus subtilis*: expression of the gene and effects of mutations on enzyme activity, growth and carbon catabolite repression. Microbiology **148:**1805-11.
21. **Hatheway, C. L.** 1990. Toxigenic clostridia. Clin Microbiol Rev **3:**66-98.
22. **Henkin, T. M.** 1996. The role of CcpA transcriptional regulator in carbon metabolism in *Bacillus subtilis*. FEMS Microbiol Lett **135:**9-15.
23. **Henkin, T. M., F. J. Grundy, W. L. Nicholson, and G. H. Chambliss.** 1991. Catabolite repression of alpha-amylase gene expression in *Bacillus subtilis* involves a trans-acting gene product homologous to the Escherichia coli *lacl* and *galR* repressors. Mol Microbiol **5:**575-84.
24. **Hobbs, B. C.** 1974. *Clostridium welchii* and *Bacillus cereus* infection and intoxication. Postgrad Med J **50:**597-602.
25. **Jacob, S., R. Allmansberger, D. Gartner, and W. Hillen.** 1991. Catabolite repression of the operon for xylose utilization from *Bacillus subtilis* W23 is mediated at the level of transcription and depends on a cis site in the *xylA* reading frame. Mol Gen Genet **229:**189-96.
26. **Johnson, C. C.** 1989. *Clostridium perfringens* food poisoning, p. 629-638. *In* W. L. George (ed.), Anaerobic infections in humans. Academic Press, London.
27. **Johnson, S., and D. N. Gerding.** 1997. Enterotoxemic infections. *In* R. W. Titball (ed.), The Clostridia: Molecular biology and pathogenesis. Academic Press, Inc., San Diego, CA.
28. **Kim, H. J., C. Jourlin-Castelli, S. I. Kim, and A. L. Sonenshein.** 2002. Regulation of the *Bacillus subtilis ccpC* gene by *ccpA* and *ccpC*. Mol Microbiol **43:**399-410.
29. **Kim, J. H., and G. H. Chambliss.** 1997. Contacts between *Bacillus subtilis* catabolite regulatory protein CcpA and *amyO* target site. Nucleic Acids Res **25:**3490-6.
30. **Kim, J. H., Z. T. Guvener, J. Y. Cho, K. C. Chung, and G. H. Chambliss.** 1995. Specificity of DNA binding activity of the *Bacillus subtilis* catabolite control protein CcpA. J Bacteriol **177:**5129-34.

31. **Kobayashi, T., T. Shimizu, and H. Hayashi.** 1995. Transcriptional analysis of the beta-galactosidase gene (*pbg*) in *Clostridium perfringens*. FEMS Microbiol Lett **133:**65-9.
32. **Kokai-Kun, J. F., K. Benton, E. U. Wieckowski, and B. A. McClane.** 1999. Identification of a *Clostridium perfringens* enterotoxin region required for large complex formation and cytotoxicity by random mutagenesis. Infect Immun **67:**5634-41.
33. **Kokai-Kun, J. F., and B. A. McClane.** 1997. The *Clostridium perfringens* enterotoxin. *In* R. W. Titball (ed.), The Clostridia: Molecular biology and pathogenesis. Academic Press, Inc., San Diego, CA.
34. **Kokai-Kun, J. F., and B. A. McClane.** 1996. Evidence that a region(s) of the *Clostridium perfringens* enterotoxin molecule remains exposed on the external surface of the mammalian plasma membrane when the toxin is sequestered in small or large complexes. Infect Immun **64:**1020-5.
35. **Kraus, A., and W. Hillen.** 1997. Analysis of CcpA mutations defective in carbon catabolite repression in *Bacillus megaterium*. FEMS Microbiol Lett **153:**221-6.
36. **Kraus, A., E. Kuster, A. Wagner, K. Hoffmann, and W. Hillen.** 1998. Identification of a co-repressor binding site in catabolite control protein CcpA. Mol Microbiol **30:**955-63.
37. **Kuster, E., T. Hilbich, M. K. Dahl, and W. Hillen.** 1999. Mutations in catabolite control protein CcpA separating growth effects from catabolite repression. J Bacteriol **181:**4125-8.
38. **Kuster-Schock, E., A. Wagner, U. Volker, and W. Hillen.** 1999. Mutations in catabolite control protein CcpA showing glucose-independent regulation in *Bacillus megaterium*. J Bacteriol **181:**7634-8.
39. **Larson, H. E., and S. P. Borriello.** 1988. Infectious diarrhea due to *Clostridium perfringens* [letter]. J Infect Dis **157:**390-1.
40. **Luesink, E. J., C. M. Beumer, O. P. Kuipers, and W. M. De Vos.** 1999. Molecular characterization of the *Lactococcus lactis ptsHI* operon and analysis of the regulatory role of HPr. J Bacteriol **181:**764-71.
41. **Luesink, E. J., R. E. van Herpen, B. P. Grossiord, O. P. Kuipers, and W. M. de Vos.** 1998. Transcriptional activation of the glycolytic *las* operon and catabolite repression of the *gal* operon in *Lactococcus lactis* are mediated by the catabolite control protein CcpA. Mol Microbiol **30:**789-98.
42. **McClane, B. A.** 2001. The complex interactions between *Clostridium perfringens* enterotoxin and epithelial tight junctions. Toxicon **39:**1781-91.
43. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg Infect Dis **5:**607-25.
44. **Melville, S. B., R. E. Collie, and B. A. McClane.** 1997. Regulation of enterotoxin production in *Clostridium perfringens*, p. 471-490. *In* R. W. Titball (ed.), The Clostridia: Molecular biology and pathogenesis. Academic Press, Inc., San Diego, CA.
45. **Melville, S. B., R. Labbe, and A. L. Sonenshein.** 1994. Expression from the *Clostridium perfringens cpe* promoter in *C. perfringens* and *Bacillus subtilis*. Infect Immun **62:**5550-8.

46. **Miwa, Y., and Y. Fujita.** 2001. Involvement of two distinct catabolite-responsive elements in catabolite repression of the *Bacillus subtilis* myo-inositol (*iol*) operon. J Bacteriol **183:**5877-84.
47. **Miwa, Y., A. Nakata, A. Ogiwara, M. Yamamoto, and Y. Fujita.** 2000. Evaluation and characterization of catabolite-responsive elements (*cre*) of *Bacillus subtilis*. Nucleic Acids Res **28:**1206-10.
48. **Morel, F., J. Frot-Coutaz, D. Aubel, R. Portalier, and D. Atlan.** 1999. Characterization of a prolidase from *LactoBacillus delbrueckii* subsp. *bulgaricus* CNRZ 397 with an unusual regulation of biosynthesis. Microbiology **145:**437-46.
49. **Moreno, M. S., B. L. Schneider, R. R. Maile, W. Weyler, and M. H. Saier, Jr.** 2001. Catabolite repression mediated by the CcpA protein in *Bacillus subtilis*: novel modes of regulation revealed by whole-genome analyses. Mol Microbiol **39:**1366-81.
50. **Muscariello, L., R. Marasco, M. De Felice, and M. Sacco.** 2001. The functional *ccpA* gene is required for carbon catabolite repression in *LactoBacillus plantarum.* Appl Environ Microbiol **67:**2903-7.
51. **Nakano, M. M., P. Zuber, and A. L. Sonenshein.** 1998. Anaerobic regulation of *Bacillus subtilis* Krebs cycle genes. J Bacteriol **180:**3304-11.
52. **Postma, P. W., J. W. Lengeler, and G. R. Jacobson.** 1993. Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. Microbiol Rev **57:**543-94.
53. **Ramstrom, H., M. Bourotte, C. Philippe, M. Schmitt, J. Haiech, and J. J. Bourguignon.** 2004. Heterocyclic bis-cations as starting hits for design of inhibitors of the bifunctional enzyme histidine-containing protein kinase/phosphatase from *Bacillus subtilis*. J Med Chem **47:**2264-75.
54. **Ramstrom, H., S. Sanglier, E. Leize-Wagner, C. Philippe, A. Van Dorsselaer, and J. Haiech.** 2003. Properties and regulation of the bifunctional enzyme HPr kinase/phosphatase in *Bacillus subtilis*. J Biol Chem **278:**1174-85.
55. **Reizer, J., C. Hoischen, F. Titgemeyer, C. Rivolta, R. Rabus, J. Stulke, D. Karamata, M. H. Saier, Jr., and W. Hillen.** 1998. A novel protein kinase that controls carbon catabolite repression in bacteria. Mol Microbiol **27:**1157-69.
56. **Reizer, J., M. J. Novotny, W. Hengstenberg, and M. H. Saier, Jr.** 1984. Properties of ATP-dependent protein kinase from *Streptococcus pyogenes* that phosphorylates a seryl residue in HPr, a phosphocarrier protein of the phosphotransferase system. J Bacteriol **160:**333-40.
57. **Reizer, J., S. L. Sutrina, M. H. Saier, G. C. Stewart, A. Peterkofsky, and P. Reddy.** 1989. Mechanistic and physiological consequences of HPr (ser) phosphorylation on the activities of the phosphoenolpyruvate:sugar phosphotransferase system in gram-positive bacteria: studies with site-specific mutants of HPr. Embo J **8:**2111-20.
58. **Saier, M. H., Jr., and R. D. Simoni.** 1976. Regulation of carbohydrate uptake in gram-positive bacteria. J Biol Chem **251:**893-4.
59. **Sambrook, J., and D. Russel.** 2001. Molecular cloning: A laboratory manual, 3 ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

60. **Schilling, C. H., L. Held, M. Torre, and M. H. Saier, Jr.** 2000. GRASP-DNA: a web application to screen prokaryotic genomes for specific DNA-binding sites and repeat motifs. J Mol Microbiol Biotechnol **2:**495-500.
61. **Schumacher, M. A., G. S. Allen, M. Diel, G. Seidel, W. Hillen, and R. G. Brennan.** 2004. Structural basis for allosteric control of the transcription regulator CcpA by the phosphoprotein HPr-Ser46-P. Cell **118:**731-41.
62. **Shandera, W. X., C. O. Tacket, and P. A. Blake.** 1983. Food poisoning due to *Clostridium perfringens* in the United States. J Infect Dis **147:**167-70.
63. **Shih, N.-J., and R. G. Labbe.** 1994. Effect of glucose on sporulation and extracellular amylase production by *Clostridium perfringens* type A in a defined medium. Curr. Micobiol. **29:**163-169.
64. **Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi.** 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc Natl Acad Sci U S A **99:**996-1001.
65. **Sparks, S. G., R. J. Carman, M. R. Sarker, and B. A. McClane.** 2001. Genotyping of enterotoxigenic *Clostridium perfringens* fecal isolates associated with antibiotic-associated diarrhea and food poisoning in North America. J Clin Microbiol **39:**883-8.
66. **Stulke, J., and W. Hillen.** 1998. Coupling physiology and gene regulation in bacteria: the phosphotransferase sugar uptake system delivers the signals. Naturwissenschaften **85:**583-92.
67. **Titgemeyer, F., and W. Hillen.** 2002. Global control of sugar metabolism: a gram-positive solution. Antonie Van Leeuwenhoek **82:**59-71.
68. **Tobisch, S., D. Zuhlke, J. Bernhardt, J. Stulke, and M. Hecker.** 1999. Role of CcpA in regulation of the central pathways of carbon catabolism in *Bacillus subtilis*. J Bacteriol **181:**6996-7004.
69. **Todd, E. C.** 1989. Costs of acute bacterial foodborne disease in Canada and the United States. Int J Food Microbiol **9:**313-26.
70. **Turinsky, A. J., F. J. Grundy, J. H. Kim, G. H. Chambliss, and T. M. Henkin.** 1998. Transcriptional activation of the *Bacillus subtilis ackA* gene requires sequences upstream of the promoter. J Bacteriol **180:**5961-7.
71. **Varga, J., V. L. Stirewalt, and S. B. Melville.** 2004. The CcpA Protein Is Necessary for Efficient Sporulation and Enterotoxin Gene (*cpe*) Regulation in *Clostridium perfringens*. J Bacteriol **186:**5221-9.
72. **Weickert, M. J., and S. Adhya.** 1992. A family of bacterial regulators homologous to Gal and Lac repressors. J Biol Chem **267:**15869-74.
73. **Wieckowski, E. U., J. F. Kokai-Kun, and B. A. McClane.** 1998. Characterization of membrane-associated *Clostridium perfringens* enterotoxin following pronase treatment. Infect Immun **66:**5897-905.
74. **Wilcox, M. H., and E. T. Smyth.** 1998. Incidence and impact of *Clostridium difficile* infection in the UK, 1993-1996. J Hosp Infect **39:**181-7.
75. **Yoshida, K., K. Kobayashi, Y. Miwa, C. M. Kang, M. Matsunaga, H. Yamaguchi, S. Tojo, M. Yamamoto, R. Nishi, N. Ogasawara, T. Nakayama, and Y. Fujita.** 2001. Combined transcriptome and proteome analysis as a

powerful approach to study genes under glucose repression in *Bacillus subtilis*. Nucleic Acids Res **29:**683-92.

76. **Zhao, Y., and S. B. Melville.** 1998. Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (*cpe*) of *Clostridium perfringens*. J Bacteriol **180:**136-42.

William H.C. Horton
Department of Biology
Virginia Tech, Blacksburg
2119 Derring Hall
Blacksburg, Virginia 24061-0406
(540) 231-8937 (w) (919) 933-7729 (h)
whorton@vt.edu

Education:

Virginia Polytechnic Institute and State University-Blacksburg 2002-Present
Principle Investigator: Dr. Stephen Melville.

Area of research: Mechanism of Carbon Catabolite control in *Clostridium perfringens*

Emory & Henry College- Emory, Virginia 1998 to 2002
Bachelor of Science earned in Biology, with a minor earned in Chemistry.

Experience:

Graduate Teaching Assistant, Virginia Polytechnic Institute and State University 2002 to 2003

Professional Experience:

Invited speaker for the Microbiology Club of Virginia Tech Fall 2002
Invited tour guide for Microbiology Club of Virginia Tech Fall 2003
Invited tour guide for Microbiology Club of Virginia Tech Winter 2004

Seminars and Posters Presented:

1.Virulence Control Mechanisms of *Clostridium perfringens*.
Department seminar given to Department of Biology, Virginia Tech. Blacksburg VA. October 2003

2.Components of Catabolite Repression in *Clostridium perfringens*.
Poster presented at Mid-Atlantic Microbial Pathogenesis Conference. Wintergreen, VA. February 2004

Awards Received:

Received travel grant for Mid-Atlantic Microbial Pathogenesis Conference. February 2004
Applied for Graduate Research Development Project grant, Fall 2004