Effect of Autoregulated TxeR on the Expression of
*Clostridium difficile* Toxins

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ABSTRACT

Clostridium difficile is a major nosocomial pathogen responsible for causing pseudomembranous colitis. It is estimated that 25% of antibiotic-associated diarrhea is due to C. difficile. These diseases result from intestinal tissue damage caused by two of the largest known bacterial toxins, A and B. Molecular studies of the C. difficile toxins have identified a 19.6 kb toxigenic element that contains both toxin genes flanked by three small open reading frames (ORFs). The focus of this study is to elucidate the function of the ORF, designated txeR, which is located at the beginning of the toxigenic element. The deduced amino acid sequence of txeR predicts a 22-kDa protein that contains a helix-turn-helix motif characteristic of DNA binding regulatory proteins. To determine if the protein TxeR regulates expression from the toxA, toxB, and txeR promoters, gene fusions were constructed that contained the various promoter regions and a reporter gene. The immunodominant region of toxin A located at the carboxy-terminus, termed the repeating units (ARU), was selected as the reporter gene. Expression studies were performed in Escherichia coli host strains. Levels of ARU expression were measured by enzyme-linked immunosorbent assay using an ARU-specific monoclonal antibody.

Expression levels of ARU from the toxin B promoter region with TxeR supplied on the same plasmid (in cis) or on a different plasmid (in trans) were determined. In cis, ARU levels were 50-fold higher than strains without txeR. In trans, expression of ARU from the toxin B promoter region increased over 800-fold. When TxeR was supplied in trans to a toxin A promoter region-ARU fusion, expression levels of ARU increased over 500-fold. To test for autoregulation, TxeR was supplied in trans to the txeR promoter region fused to ARU. The effect was an increase of ARU expression up to 20-fold over background. These results suggest that TxeR is a trans-acting regulator that stimulates expression of the C. difficile toxins and is subjected to autoregulation.
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# Table of Contents

ABSTRACT ................................................................................................................................................... II

ACKNOWLEDGEMENTS ......................................................................................................................... III

TABLE OF CONTENTS ............................................................................................................................ V

LIST OF FIGURES ................................................................................................................................... VI

Chapter 1 ............................................................................................................................................... vi

Chapter 2 ............................................................................................................................................... vi

Chapter 3 .............................................................................................................................................. vii

LIST OF TABLES .................................................................................................................................... VIII

CHAPTER 1: AN OVERVIEW OF CLOSTRIDIUM DIFFICILE ........................................................... 1

INTRODUCTION ........................................................................................................................................ 2

ISOLATION AND IDENTIFICATION OF CLOSTRIDIUM DIFFICILE .................................................... 3

DISCOVERY OF TWO TOXINS FROM C. DIFFICILE.............................................................................. 4

BIOLOGICAL PROPERTIES OF TOXIN A AND B. ............................................................................ 5

MOLECULAR PROPERTIES OF TOXINS A AND B. .............................................................................. 6

MECHANISM OF ACTION. ....................................................................................................................... 9

COMPONENTS OF THE TOXIGENIC ELEMENT. .................................................................................. 10

C. DIFFICILE GENE EXPRESSION SYSTEM ....................................................................................... 12

SPECIFIC OBJECTIVES AND RATIONAL OF THE EXPERIMENTAL APPROACHES. ...................... 13

CHAPTER 2: REGULATION OF C. DIFFICILE TOXA AND TOXBGENE EXPRESSION BY TXER .... 14

INTRODUCTION ........................................................................................................................................ 15

MATERIALS AND METHODS ................................................................................................................ 17

RESULTS .................................................................................................................................................. 29

DISCUSSION .......................................................................................................................................... 44

CHAPTER 3: REGULATORY CONTROL OF TXER ........................................................................... 51

INTRODUCTION ........................................................................................................................................ 52

MATERIALS AND METHODS ................................................................................................................ 53

RESULTS .................................................................................................................................................. 58

DISCUSSION .......................................................................................................................................... 66

REFERENCES .......................................................................................................................................... 70

CURRICULUM VITAE ............................................................................................................................. 77
List of Figures

**Chapter 1**

**Figure 1.1** Toxigenic element and conserved regions between the toxin genes of Clostridium difficile VPI strain 10463.

**Chapter 2**

**Figure 2.1** A schematic representation of the cloning strategy for TxeR in *cis*-orientation to toxin B promoter.

**Figure 2.2** The cloning strategy for TxeR in *trans*-orientation to toxin B promoter.

**Figure 2.3** A schematic overview of the cloning of toxin A promoter region (AP) in *trans* with TxeR.

**Figure 2.4** Characteristics of TxeR common to response regulators.

**Figure 2.5** Alignment of TxeR with UviA and Orf-22.

**Figure 2.6** Immunoblot analysis of the activation of ARU expression by TxeR in *cis*-orientation with toxin B promoter.

**Figure 2.7** Effects of TxeR in *cis* orientation to the toxin B promoter and the expression of ARU.

**Figure 2.8** SDS-PAGE analysis of β-galactosidase expression under the control of toxin B promoter and TxeR.

**Figure 2.9** Immunoblot analysis of TxeR effects in *trans* to the toxin B promoter (BP).

**Figure 2.10** Effects of TxeR in *trans*-orientation to the toxin B promoter and the expression of ARU.
Figure 2.11 Immunoblot analysis of activation of ARU expression by TxeR in *trans* to the toxin A promoter (AP).

Figure 2.12 Effects of TxeR in *trans*-orientation to the toxin A promoter on the expression of ARU.

Figure 2.13 Proposed model of extracytoplasmic function (ECFs) sigma factors from Missiakas and Raina (1998).

Chapter 3

Figure 3.1 Cloning strategy of *txeR* promoter region in the *in-trans* expression system.

Figure 3.2 Evaluation of *txeR* for possible autoregulation.

Figure 3.3 Immunoblot analysis of ARU expressed when TxeR is *trans* to either *toxA*, *toxB* or *txeR* promoter regions fused to ARU.

Figure 3.4 Summary of the expression levels of ARU during stationary phase (20 h) from *toxA*, *toxB* or *txeR* promoters.
List of Tables

Chapter 2

Table 2.1 Positive effect of TxeR on the activation of β-gal expression controlled by the toxin B promoter.

Chapter 3

Table 3.1 List of combination strains and controls based on their plasmids.

Table 3.2 Sequence alignment of C. difficile promoter regions.

Table 3.3 Expression levels of ARU during stationary phase (20 h).
Chapter 1: An Overview of *Clostridium difficile*
Introduction

Antibiotic therapy revolutionized the medical treatment of bacterial infections and has become a useful tool in other medical practices, such as the prevention of bacterial infections during chemotherapy, following organ transplantation, and in immunosuppressed patients. Unfortunately, antibiotics can alter the natural gastrointestinal microflora. Without the natural microflora, patients undergoing antibiotic therapy are more susceptible to infections from enteric pathogens. Intestinal complications due to antibiotic treatment range from mild cases of antibiotic-associated diarrhea to the life-threatening condition of pseudomembranous colitis. Pseudomembranous colitis is characterized by excessive fluid secretion and formation of yellow plaques on the colonic mucosa that are filled with fibrin, dead epithelial cells, mucus and leukocytes. The disease was first associated with antibiotic therapy with the use of clindamycin in the 1970s and was referred to as clindamycin colitis. Subsequently, pseudomembranous colitis was found to be a consequence of treatment with many antibiotics.

The causative agent of an estimated 25% of antibiotic-associated diarrhea and nearly all cases of pseudomembranous colitis is *Clostridium difficile* (Bartlett 1994, Bartlett et al. 1978, Lyerly et al. 1988, Lyerly and Wilkins, 1995). It is a Gram-positive, spore-forming anaerobic bacterium and is considered a major nosocomial pathogen. *C. difficile* is a common problem in hospitals and nursing homes because of the high number of persons undergoing antibiotic therapy, the contamination of hospital environments...
with spores excreted by infected patients, and the lack of stringent sanitation practices (McFarland et al. 1989).

**Isolation and Identification of *Clostridium difficile*.**

In 1935, Hall and O’Tool described an organism they had isolated from the feces of healthy infants. The culture filtrates from the organism showed toxicity when injected subcutaneously into guinea pigs. However, since the infants showed no sign of disease, it was incorrectly assumed that this organism was not a problem in humans. Apparently, the isolation process of the organism was extremely difficult, hence the name, *Bacillus difficilis* (Hall and O’Tool 1935). Eventually, the family Bacillaceae was subdivided into the genera *Bacillus* and *Clostridium* and that led to *B. difficilis* being renamed *C. difficile*.

Reported cases of antibiotic-associated diarrhea and pseudomembranous colitis have increased since the use of antibiotics in the late 1940s and specifically the introduction of clindamycin in 1969 (Allen et al. 1977, Randolf and Morris 1977, Tedesco et al. 1974). Stool samples of patients suffering from pseudomembranous colitis were reported to contain a toxin that caused a rounding effect of tissue culture cells (Larson et al. 1977). It was soon discovered that the cytotoxic effect of these samples could be neutralized by gas gangrene antitoxin. In an attempt to further identify the neutralization agent, it was shown that *Clostridium sordellii* antitoxin, a component of the gas gangrene antitoxin, was responsible for inactivating the cytotoxin (Larson and Price 1977, Rifkin et al. 1977). Scientists were unable to culture *C. sordellii* from pseudomembranous colitis patients, but had routinely isolated what was thought to be
“nonpathogenic” *C. difficile*. Culture filtrates prepared from the *C. difficile* isolates from infected patients were shown to be cytotoxic to tissue culture cells and the cytotoxicity could be reduced by *C. sordellii* antitoxin (Bartlett and Gorbach 1977, Bartlett et al. 1978). Those findings led to *C. difficile* being identified as the causative agent of pseudomembranous colitis. The neutralization of *C. difficile* culture filtrates by *C. sordellii* antitoxin was due to similarities between toxins produced by each of the organisms.

**Discovery of two toxins from *C. difficile***.

Two research teams independently isolated two distinct toxins from pathogenic cultures of *C. difficile* by ion-exchange chromatography. The toxins were designated A and B by Taylor et al. (1980), or D-1 and D-2 by Banno et al. (1981), based on their relative position to each other during elution. Toxin A (308,000 Da) and toxin B (269,696 Da) are two of the largest known single polypeptide bacterial toxins. Pathogenic strains of *C. difficile* produced both toxins in approximately equal amounts, but the amount of toxins produced varied over 100,000-fold among strains (Lyerly et al. 1988). About 25% of *C. difficile* isolates do not produce toxins and do not cause pseudomembranous colitis or antibiotic-associated diarrhea. A few variant strains, like strain 8864, produce a modified toxin B and no toxin A (Borreilo et al. 1992, Lyerly et al. 1992, Torres 1991). The ability of these strains to cause disease has only been documented recently. An outbreak of antibiotic-associated diarrhea and pseudomembranous colitis in a Canadian hospital in 1999 was attributed to a toxin A-/toxin B+ strain of *C. difficile* (Alfa et al. 1999, Al-Barrak et al. 1999).
Biological Properties of Toxin A and B.

Toxin A is commonly referred to as the enterotoxin because purified toxin A is highly enterotoxic when tested in the ligated intestinal loop assay. Toxin A causes a hemorrhagic fluid secretion, extensive tissue damage, and mucosal inflammation of intestinal tissues (Lyerly et al. 1982, Sullivan et al. 1982). The destruction of epithelial cells is due to the ability of toxin A to bind to specific carbohydrate receptors on the cell surface that are required for entry into cells (Krivan et al. 1986, Tucker et al. 1991). Toxin A also has chemotactic properties that heighten the inflammatory response in the colon by stimulating the release of cytokines and neurokinins, inducing the loss of macrophages, and causing T-lymphocyte apoptosis (Castagliuolo et al. 1994, Castagliuolo et al. 1997, Castagliuolo et al. 1998, Mahida et al. 1998, Pothoulakis et al. 1994).

Both toxins A and B are cytotoxic to mammalian cells, but since toxin B is 1000-fold more active on most cell lines it is often referred to as the cytotoxin. Subpicogram amounts of toxin B have been shown to cause a rounding effect of tissue culture cells (Sullivan et al. 1982). In contrast to toxin A, toxin B does not bind to epithelial cells and has no enterotoxic activity. If toxin B was administered with a small amount of toxin A or if the intestine was mechanically injured, a fluid response was detected (Lyerly et al. 1985). Lyerly et al. (1985) proposed that toxin B may gain access to the underlying cells after toxin A has destroyed the epithelial cells. The minimum lethal dose of both toxin A and B was determined to be 50 ng when injected intraperitoneally in mice (Lyerly et al. 1982).
Molecular properties of toxins A and B.

Both toxins were sequenced from overlapping genomic DNA fragments and cloned as complete genes in *Escherichia coli* host strains (Barroso et al. 1990, Dove et al. 1990, Johnson et al. 1990, Phelps et al. 1991). The toxin A gene is an open reading frame of 8,130 nucleotides, encoding a protein of 2,710 amino acids (Dove et al. 1990). The toxin B gene is located upstream of the toxin A gene and contains 7,098 nucleotides, encoding a protein of 2,366 amino acids (Barroso et al. 1990). Comparison of the deduced amino acid sequences of toxin A with B revealed that there is an overall identity of 49% and a similarity of 63% if conserved substitutions are included in the analysis (Eichel-Streiber et al. 1992). The high degree of conservation suggests that the toxin A and B genes may be the result of gene duplication.

The proteins encoded by the toxin genes share several structural features (Fig. 1.1). Each toxin contains a putative nucleotide binding site at the N-terminus, a central hydrophobic region, four conserved cysteine residues located in almost identical sites between the two toxins, and a series of contiguous repeating units at the C-terminus (Barroso et al. 1994, von Eichel-Streiber 1992, von Eichel-Streiber et al. 1992). In toxin A, the repeating units comprise approximately one-third of the molecule and bind to I, X and Y carbohydrate antigens on the human intestinal epithelium (Tucker et al. 1991). The toxin A repeating units (ARU) are also the immunodominant portion of the molecule, containing at least two epitopes for the monoclonal antibody PCG-4 (Frey et al. 1992). A specific cell receptor for toxin B has not been determined.
Figure 1.1. Toxigenic element and conserved regions between the toxin genes of *Clostridium difficile* VPI strain 10463. The arrows above the open reading frames (ORFs) indicate the direction of transcription. In addition to the toxin A and B genes, pathogenic strains contain 3 ORFs for putative proteins TxeR, Txe2 and Txe3. The glucosyltransferase domain has been confirmed in toxin B and is assumed to be located in a similar region for toxin A. The repeating units in toxin A, termed ARU, function as the receptor binding domain. The receptor and function of the similar region in toxin B is unknown.
Barroso et al. (1994) evaluated the importance of the conserved regions between the toxins by the use of deletion analysis and site-directed mutagenesis with recombinant toxin B in *E. coli*. Complete elimination of the repeating units only reduced cytotoxic titers of cell lysates by 10-fold on tissue culture cells. A similar effect was observed when the repeating units of recombinant toxin A were eliminated (Barroso and Wilkins, unpublished data). These findings suggested that the toxins are capable of entering cells by less specific mechanisms than binding to a specific receptor. The loss of cytotoxicity due to elimination of the repeating units was not unexpected with toxin A. The CHO-K1 cell lines do not express the carbohydrate receptor to which the toxin A repetitive region binds.

The site-directed mutagenesis studies of toxin B revealed that replacing the second and fourth cysteine residues with serine residues resulted in a 90% decrease in cytotoxicity for Chinese Hamster Ovary K1 (CHO) cells. Since the toxins are not affected by reducing agents, the loss of activity could not caused by structural changes due to the loss of disulfide bridges. When the histidine residue within the putative nucleotide binding site was changed to glutamine, 99% of the cytotoxicity on CHO cells was eliminated. Elimination of the central hydrophobic region of the toxin B gene resulted in a mutant protein that still retained a small level of cytotoxic activity. These results supported the idea that the N-terminal region was necessary for cytotoxic activity. This assumption has recently been confirmed by Hofmann et al. (1997) and will be discussed in the next section.
Mechanism of Action.

Several bacterial toxins, like the C2 and C3 toxins of Clostridium botulinum and Pseudomonas aeruginosa exotoxin, disrupt eukaryotic cells by targeting guanine triphosphate (GTP) binding proteins (Aktories 1997, Eichel-Streiber et al. 1996). The GTP-binding proteins serve as molecular switches for various signaling pathways in the cell and as regulators of cytoskeleton proteins. The superfamily of Ras proteins contain the Rho subfamily proteins called GTPases. The Rho proteins (e.g., Rho, Rac, and Cdc42) are essential for the polymerization of filamentous actin (F-actin) from globular actin (G-actin) in the cytoskeleton. Actin is responsible for stress fiber formation, cell adhesion, cell morphology and cell motility. In the active state, Rho is bound to GTP and an effector molecule specific for a particular function. If the effector site is blocked or modified, the Rho proteins are inactivated. Bacterial toxins inactivate GTPases by ADP-ribosylation, or deamidation (Aktories 1997). A novel mechanism of inactivating GTPases was revealed by studies of C. difficile toxins A and B.

Toxins A and B from C. difficile have been shown to inactivate Rho proteins by transferring a glucose molecule from uridine diphosphate (UDP)-glucose to Rho (Just et al. 1995a, b). Both toxins add glucose to threonine-37 in the effector binding domain of Rho and threonine-35 of Rac and Cdc42. Glucosylation of the Rho GTP-binding proteins causes disaggregation of the actin filaments in the cytoskeleton which results in the characteristic cell rounding (Dillon et al. 1995, Just et al. 1995 a, b). The glucosyltransferase activity of toxin B has been localized to the N-terminal end between amino acids 1-546 (Hofmann et al. 1997). A deletion clone of toxin B (amino acids 1-516) showed a 1000-fold decrease in enzyme activity and no detectable cytotoxic
activity. The conclusion of Hofmann et al. (1997) from other deletion studies was that residues 516-546 are important either structurally or functionally for both enzymatic and cytotoxic activities.

The similarities between \textit{C. difficile} toxins and other large clostridial toxins includes both physical properties (e.g., size) as well as similar enzymatic activities. In a recent review by Aktories (1997), the enzymatic functions of several large clostridial toxins were compared. Like \textit{C. difficile}, \textit{C. sordellii} HT toxin also glucosylates Rho, Rac and Cdc42 proteins using UDP-glucose as a substrate. On the other hand, \textit{C. sordellii} LT toxin glucosylates different GTP-binding proteins: Rac, Cdc42, Ras, and Rap proteins. \textit{Clostridium novyi} alpha-toxin is also a glucosyltransferase, except UDP-N-acetyl-glucosamine is the co-substrate (Aktories 1997).

\textbf{Components of the toxigenic element.}

The toxin genes of \textit{C. difficile} are located in a 19.6 kb insert on the chromosome that was identified by Hammond and Johnson (1995) and termed the toxigenic element (Fig 1.1). This same region has also been referred to as the pathogenicity locus by vonEichel-Streiber et al. (1992). The corresponding region in nonpathogenic strains is represented by a 127 bp fragment that contains inverted repeats that may act as a target for insertion of the toxigenic element (Hammond and Johnson 1995).

The genes for toxins A and B are separated by a small open reading frame \textit{(txe2)}. There also is a small open reading frame (ORF) upstream of the toxin B gene, termed \textit{txeR} by Moncrief et al. (1997), and one downstream of the toxin A gene, termed \textit{txe3}.
Several research teams have performed transcriptional analysis of the toxigenic element (Dupuy and Sonenshein 1998, Hammond et al. 1997, Hundsberger et al. 1997). All studies detected major populations of messenger RNA that corresponded to transcription from the promoters of each toxin gene and the three ORFs. These studies also revealed a minor polycistronic message that began with the txeR promoter and included both the toxin genes. The large transcripts are likely the result of insufficient termination of transcription during high level expression since each gene has been shown to have a functional promoter. The ORF of txe3 is transcribed in the opposite direction of the other genes in the toxigenic element. Hundsberger et al. (1997) have shown that transcripts of txe3 (referred to as tcdC) appeared early in the exponential growth phase, when the transcripts of txeR, txe2, and the toxin genes were at a low level. The txe3 transcript then disappeared during stationary growth when the other genes were being transcribed at higher levels.

The function of the three proteins encoded by the ORFs flanking the toxin genes has not been determined. The txeR gene is 555 bp in length, predicting a protein of 184 amino acids with a molecular mass of 22 kDa. The possible function of TxeR will be discussed in later chapters. The txe2 gene is 426 bp encoding a protein of 142 amino acids, with an estimated molecular weight of 17 kDa. It is predicted to be a hydrophobic protein (Dove et al. 1990). The function of this putative protein is unknown. The txe3 gene is 696 bp, encoding a protein of 232 amino acids. Txe3 is an acidic protein that contains repetitive amino acids stretches of different lengths (Dove et al. 1990, Hundsberger et al. 1997). Based on the concentration of mRNA levels of Txe3 during early stages of growth, Hundsberger et al. (1997) suggested that the putative protein
might be acting as a negative regulator of transcription of the other genes in the toxigenic element.

**C. difficile Gene Expression System.**

Research on the regulation of gene expression in *C. difficile* has been hampered by the lack of a gene transmission system. The only reported success with gene transfer in *C. difficile* used conjugative transposons and a *Bacillus subtilis* intermediate host (Mullany et al. 1990, Mullany et al. 1991, Mullany et al. 1994). One of the limitations of the conjugative transponson system was the ability to transfer large DNA fragments (>1 kb). The transposon system was not considered adequate to study the effects of potential regulatory elements on toxin gene expression since the DNA fragment sizes were greater than 2 kb. Since the toxin genes had been cloned in *E. coli* and shown to be biologically active, it seemed reasonable to develop an *E. coli* expression system to study gene regulation. I was particularly interested in studying the importance of the first ORF, termed *txeR*, on expression of the toxin genes.
Specific Objectives and Rational of the Experimental Approaches.

My research objectives were to:

(1) develop an expression system in \textit{E. coli} to evaluate the function of the \textit{txeR} gene product, TxeR

(2) determine the effect of TxeR on the promoter regions of the toxin A and B genes using promoter-reporter gene fusions in the \textit{E. coli} expression system above, and

(3) determine whether or not TxeR is autoregulated by using a promoter-reporter gene fusion in the expression system designed above.
Chapter 2: Regulation of *C. difficile* toxA and toxB gene expression by TxeR


Note: All technical work reported in the publication and this thesis were performed by Lisa A. Barroso and the following text has been newly written for this thesis.
Introduction

Bacterial virulence genes are frequently organized in clusters. These clusters or cassettes, termed pathogenicity islands, are presumed to be acquired by gene transmission from bacteriophages or other bacteria (Groisman and Ochmann 1996, Hacker et al. 1997). The pathogenicity island of \textit{C. difficile} was initially described by Hammond and Johnson (1995) as a 19.6 kb toxigenic element containing the toxin A and B genes and three small open reading frames (ORFs). Most other pathogenicity islands contain a large number of genes, so the toxigenic element of \textit{C. difficile} may be more accurately referred to as a pathogenicity islet.

The presumptive product of the ORF located upstream of the toxin B gene, termed TxeR, was predicted to be a basic protein of 184 amino acids with a molecular weight of 22,158 Da (GenBank accession number U25131, Hammond and Johnson 1995). The positively charged carboxy-terminus contained an unusually high content of lysine residues. This type of carboxy-terminus was known to be a characteristic of DNA binding proteins involved in the regulation of gene expression. Therefore, I proposed that TxeR was a DNA binding protein that affected expression of the toxin A and B genes. To test this hypothesis, I created an expression system to evaluate the role of TxeR on the expression of the toxin genes.

The only \textit{C. difficile} gene transmission system currently available relies on conjugative transposons and requires a \textit{B. subtilis} intermediate (Mullany et al. 1990, 1991, 1994). I
did not consider this system adequate for this project because it has limitations on the fragment size that can be transferred. Therefore, I created an expression system in *E. coli*. My initial experiments were designed with the *txeR* promoter region and gene next to, or in *cis*-orientation to, the toxin B promoter region fused to a reporter gene. I chose the toxin A repeating units (ARU) as the reporter gene because it is from *C. difficile* and there are specific polyclonal and monoclonal antibodies available to this portion of toxin A. Also, I could measure levels of ARU expression easily with a commercially available ELISA kit.

To further explore the role of TxeR as a regulatory protein, I developed a *trans*-activating expression system. These experiments were designed to eliminate the possibility that increased expression from the toxin promoter regions were due to a *cis*-acting element in the DNA of the *txeR* region. To test this, I cloned the *txeR* gene on a separate plasmid, or in *trans* orientation to another plasmid that contained the promoter region of *toxB* or *toxA* fused to the ARU reporter gene. In the following experiments, I used these constructs to determine if TxeR is a response regulator of the toxigenic element of *C. difficile*. 
Materials and Methods

Bacterial strains, plasmids and reagents.  *E. coli* strain DH5α and cloning vector pUC19 were purchased from Life Technologies, Inc. (Rockville, MD).  *E. coli* strain BL21-DE3, plasmids pACYC184, pT7-7, and pLKC480 were kindly provided by Dr. D. Dean (Department of Biochemistry, Virginia Tech, Blacksburg, VA).  Previously described plasmids pCDtoxB and pCDtoxA.03 containing the toxin B gene and toxin A gene, respectively, were the source for *C. difficile* gene fragments (Johnson et al. 1990, Phelps et al. 1991).  Plasmids pCD21, used for subcloning the txeR gene, and pCD19 used for subcloning the toxin A promoter region were generous gifts from Dr. J. L. Johnson (Department of Biochemistry, Virginia Tech, Blacksburg, VA).  Bacterial strains were prepared with calcium chloride and used for transformations according to the method described by Cohen et al. (1972).  All restriction endonucleases and modifying enzymes were purchased from New England Biolabs (Beverly, MA), Promega (Madison, WI), or Life Technologies and used according to protocols recommended by the manufacturer.  All general chemicals were molecular biological grade (when available) and obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Protein database search.  PIR-protein data libraries were searched with Lasergene (DNASStar, Inc., Madison, WI).  Motif library searches were done using the software program MacPattern (Fuchs).  A BLAST search was also performed with the GenBank database (Altschul et al. 1997).
General cloning and Polymerase Chain Reaction (PCR). Methods for DNA manipulations have been previously described by Sambrook et al. (1989). PCR products were generated in a Hybaid Omn-E thermal cycler (Franklin, MA) using the high fidelity enzyme Ultma polymerase (Perkin-Elmer Cetus, Norwalk, CT) according to the protocol recommended by the manufacturer. All primers used in amplification reactions were generated by DNAgency (Malvern, PA) or Genosys (The Woodlands, TX). Prior to restriction endonuclease digestions, PCR products were purified with the PCR Clean Up kit from Boehringer Mannheim (Indianapolis, IN). All DNA fragments, including digested PCR products, were isolated from 0.7% agarose gels with the Elu-Quick DNA purification kit (Schleicher and Schuell, Keene, NH) following electrophoresis.

Cloning of txeR in cis to the toxin B promoter. Refer to Figure 2.1 for an overview of the cloning strategy. In Step 1, the 317 bp fragment containing the toxin B promoter region was amplified from pCDtoxB with a 5’ end primer (5’ GCGCGAATTC-TCTAGACAAGCTGTTAATAAGGC-3’) containing an EcoRI site and a 3’ end primer (5’-CGCGGATCCCTCATAAAATTTTCTCCTTTAC-3’) containing a BamHI site. The resulting PCR product, termed BP, was digested with EcoRI/BamHI and subcloned in pUC19 to create pBP. In Step 2, a 2.89 kb fragment, containing the repeating units of toxin A (ARU) and the transcriptional termination signal of the toxin A gene, was isolated from a Sau3A/HindIII digestion of pCDtoxA.03. The ARU fragment was ligated to a BamHI/HindIII digest of pBP to create pBP-ARU. In Step 3, a 3.6 kb SapI fragment
containing the txeR gene from pCD21 was ligated to a 3.1 kb Sapi fragment from pBP-ARU to generate ptxeR-BP-ARU. Both ptxeR-BP-ARU and the control strain pBP-ARU were separately transformed into *E. coli* strain DH5α. Clones containing the constructs were selected after growth on media containing ampicillin and were confirmed by plasmid isolation and restriction enzyme analysis (data not shown).

**Construction of a transcriptional fusion vector containing txeR, toxB promoter region and lacZ as the reporter gene.** The pLKC480 transcriptional fusion vector contains a multiple cloning region (MCR) and the *lacZ* gene without a promoter. The ptxeR-BP-ARU clone described above was the source of txeR and the toxin B promoter. To facilitate cloning of the txeR-BP region, the *Bam*HI site within the MCR of ptxeR-BP-ARU was eliminated by digesting the plasmid with restriction enzymes *Sma*I and *Hinc*II (present in the MCR). After ligation of the blunt ends, the new clone was digested with *Eco*RI/*Bam*HI to liberate a 1.8 kb fragment containing txeR-BP. The txeR-BP fragment was subcloned to corresponding sites in pLKC480 to generate pLKC-txeR-BP. For comparison, pLKC-BP was created by eliminating a 250 bp *Xba*I fragment of txeR from pLKC-txeR-BP which inactivated TxeR. Both plasmids were independently transformed into *E. coli* strain DH5α. Growth of recombinant strains in the presence of ampicillin and the restriction enzyme analysis of isolated plasmids were used to confirm the plasmid constructs (data not shown).
Figure 2.1. A schematic representation of the cloning strategy for \textit{txeR} in \textit{cis} orientation to toxin B promoter. Step 1: The toxin B promoter region (BP) was amplified by PCR and subcloned in pUC19. Step 2: The repeating units of toxin A (ARU) were digested from pCDtoxA0.3 and used as the reporter gene under the control of BP. Step 3: The promoter region (TP) and open reading frame of \textit{txeR} were digested from pCD21 and cloned with BP-ARU. The resulting clone, ptxeR-BP-ARU was used for expression studies. Restriction sites: E, \textit{EcoRI}; B, \textit{BamHI}; S, \textit{Sau3A}; H, \textit{HindIII}; Sp, \textit{SapI}; H*, \textit{HindIII} restriction site from pUC19 vector.
Expression studies using *txeR* and *txoB* promoter region in *trans*. Figure 2.2 depicts the cloning strategy used to construct the plasmids that supply *txeR* in *trans* to the toxin B promoter. The coding region of *txeR* was amplified from pCD21 using a primer at the 5’ end with an *Nde*I site (5’-AGGGTGATCATATGCAAAAGTCTTT-3’) that contained the ATG start codon of *txeR* to allow for in-frame expression from the T7 promoter. The primer complementary to the 3’ end of the *txeR* gene contained a *Pst*I site (5’-CTCTGCAGTTACAAGTTAAAATAAT-3’) for subcloning to pT7-7 to produce pT7-txeR. A 3.5 kb *XbaI*-HindIII fragment from pBP-ARU containing the toxin B promoter region fused to ARU was subcloned to pACYC184, resulting in pAC-BP-ARU. For expression studies, both plasmids were co-transformed into *E. coli* strain BL21-DE3 using the calcium chloride method (Cohen et al. 1972). Another strain, containing pT7-7 and pAC-BP-ARU was created to use as a control strain to correctly identify the effects of TxeR on the toxin B promoter region. Identification of clones that contained both plasmids were initially screened by growth in selective media containing ampicillin and chloramphenicol. Restriction enzyme analysis of isolated plasmid preparations before and after a growth study also confirmed the presence of both plasmids throughout the experiment.

Expression studies using *txeR* and toxin A promoter region in *trans*. Refer to Figure 2.3 for a depiction of the cloning strategy. The 570 bp intergenic region containing the toxin A promoter was amplified from pCD19 with a 5’ primer containing a *XbaI* site (5’-TGATCTAGATGCTAAGGATGAAAAG-3’) and a 3’ primer with a *BamHI* site (5’-GGGGATCCGACATAAAAAACCTCTTAGTAT-3’). The PCR product was subcloned to pACYC184 and termed pAC-AP. A 3.24 kb *BamHI* fragment, containing the ARU
gene fragment, was isolated from pAC-BP-ARU and subcloned to pAC-AP, resulting in pAC-AP-ARU. The plasmid containing txeR described above, pT7-txeR, was co-transformed with pAC-AP-ARU into *E. coli* strain BL21-DE3 for expression studies. A negative control strain containing pT7-7 and pAC-AP-ARU was also created in order to accurately compare the expression results with the strain containing *txeR*. The presence of both plasmids in the recombinant strains were confirmed by restriction enzyme analysis of isolated plasmids from the above strains after growth in media containing ampicillin and chloramphenicol. Plasmid preparations before and after each growth study confirmed the presence of both plasmids throughout the experiment.
Figure 2.2. The cloning strategy for *txeR* in *trans* orientation to toxin B promoter. The coding region of *txeR* was amplified by PCR and cloned in expression vector pT7-7. A second plasmid was created to contain the toxin B promoter region (BP) and ARU reporting gene in the low copy number vector pACYC184. Both plasmids were co-transformed into *E. coli* BL21-DE3 for expression studies. Restriction sites: H, *HindIII*; N, *NdeI*; P, *PstI*; X, *XbaI*. 
Figure 2.3. A schematic overview of the cloning of toxin A promoter region (AP) in trans with TxeR. The AP region was amplified by PCR and cloned in pACYC184 (pAC-AP). The reporter gene, ARU, was isolated from pAC-BP-ARU and cloned in pAC-AP to create pAC-AP-ARU. Both pT7-txeR and pAC-AP-ARU were transformed in *E. coli* BL21-DE3 in order to study the affect of TxeR on the toxin A promoter. Restriction sites: H, *Hind* III; E, *Eco*RI; X, *Xba*I; and B, *Bam*HI.
Growth of recombinant strains and generation of protein lysates. Recombinant bacterial strains described above were grown at 37°C in a shaking incubator in 400 ml of Terrific Broth (Tartof 1987) containing 100 µg ampicillin/ml and/or 100 µg chloramphenicol/ml as appropriate. All cultures were inoculated with cells from a starter culture equal to an OD<sub>600</sub> of 0.05. Samples were collected every 2 h and cells harvested by centrifugation (5 min at 15,800 x g). Cells were suspended in an equal volume of phosphate buffered saline, pH 7.4 (PBS), containing Complete™ protease inhibitor cocktail (Boehringer Mannheim). Cells were lysed by either (1) sonication (Virtis, Gardinier, NY) with a pulse cycle of 0.6 sec on and 1.0 sec off for 2 min, or (2) bead-beating with a Mini-BeadBeater from BioSpec Products (Bartlesville, OK) that vortexed the cells four times for 30 sec at high speed with an equal weight/volume of 0.12-0.18 mm glass beads. Cellular debris was removed by centrifugation (5 min at 15,800 x g) and the supernatants were collected and stored at -20°C. The total protein concentrations were determined with the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) using bovine serum albumin to generate the standard curve.

Enzyme-Linked Immunosorbent Assay (ELISA). The level of ARU in recombinant lysates was determined with the ToxA Test (TechLab, Inc., Blacksburg, VA) following the 2 h protocol. The test consists of affinity-purified polyclonal antibody specific for toxin A immobilized to microtiter wells and a horseradish peroxidase labeled monoclonal antibody (PCG-4) specific for the repeating units of toxin A (Lyerly et al. 1983) as the detecting antibody. Addition of the two component substrate solution from the kit, that contains a mixture of tetramethylbenzidine and hydrogen peroxide in a citric acid buffer, generated a color reaction in lysate samples containing ARU. The reaction was stopped with the addition of 1 M sulfuric acid. Units of ELISA reactivity were arbitrarily defined as the A<sub>450</sub> multiplied by the reciprocal of the highest dilution with an absorbance of 0.2-2.5 nm. The levels of ARU are expressed as units/mg of total protein in the recombinant lysate.

β-Galactosidase Assay for lacZ fusion proteins. The following protocol is an adaptation from Experiments in Molecular Genetics, page 352-359 (Miller 1977)
provided by Dr. T. Larson (Department of Biochemistry, Virginia Tech, Blacksburg, VA). Briefly, 2 ml samples were collected every 2 h over the course of 30 h and OD<sub>600</sub> recorded. The cells were centrifuged (5 min at 15,800 x g) and suspended in an equal volume of Z buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>, 50 mM 2-mercaptoethanol, pH 7.0). The cells were lysed by sonication (1.5 min for three cycles). Cellular debris was removed by centrifugation and the supernatants were collected and diluted in Z buffer for the assay. After a 5 min incubation at 28<sup>0</sup>-30<sup>0</sup> C, 0.16 ml of o-nitrophenyl-β-D-galactoside (ONPG) at 4 mg/ml was added. Initial reactions were allowed to continue until a yellow color developed (5-10 min). Once an appropriate reaction time had been determined, it was used as a standard for the rest of the assay. The total reaction time (10 min) was recorded and the reaction stopped with the addition of 0.4 ml of 1 M Na<sub>2</sub>CO<sub>3</sub>. Any precipitated material was removed by centrifugation for 2 min at room temperature. Absorbance values were recorded at 420 nm and 550 nm. Units of β-galactosidase activity were determined using the following equation:

\[
\text{Units} = 1000 \times \frac{[\text{OD}_{420} - 1.75(\text{OD}_{550})]}{\text{(time min) (volume of cells) (OD}_{600})}
\]

**SDS-PAGE and Western blot analysis.** Lysates from recombinant bacterial strains were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). For comparison, depending on the group of samples to be assayed, the same amount of total protein (20-40 µg) of a particular time point were denatured with 2.5% sodium dodecyl sulfate and reduced with 5% 2-mercaptoethanol. Samples were heated in a boiling water bath for 3 min and separated by electrophoresis in 7.5% separating-4% stacking SDS-PAGE gels. Proteins were transferred from the gel to nitrocellulose in 0.025 M Tris-0.192 M glycine buffer, 20% methanol, pH 8.3 (16 h, 13 V). The membranes were rinsed in PBS and blocked for 1 h in PBS containing 10% Carnation nonfat dry milk and 0.3% Tween-20. The primary antibody used to detect ARU was the monoclonal antibody PCG-4 at a concentration of 1:3000 in blocking buffer. After a 1 h incubation, the membranes were washed 3 times for 5 min each in PBS. A 1:2000 dilution of anti-goat IgG-peroxidase in blocking buffer
was used as the secondary antibody. The membranes were incubated for 1 h and washed as stated above. Detection was performed with TMB membrane peroxidase substrate from Kirkegaard & Perry Laboratories (Gaithersburg, MD). After sufficient color development, the membranes were washed with ddH₂O and allowed to air dry.
Results

Similarity of TxeR to transcriptional activators. A motif search of the deduced amino acid sequence of TxeR with the PIR protein data bases using DNAStar identified a helix-turn-helix motif sequence at the carboxy-terminus. This particular motif is characteristic of bacterial DNA binding proteins that serve as response regulators (Fig. 2.4). Additional database searching of the TxeR protein sequence revealed a 28% sequence identity with UviA, a putative positive response regulator of *Clostridium perfringens* that regulates bacteriocin production (Garnier and Cole, 1988). In addition, TxeR also has 25% sequence identity with Orf-22 from *Clostridium botulinum* C 468 that regulates the expression of botulinum neurotoxin (BoNT) C1 responsible for animal botulism (Hauser, 1994). Alignments of TxeR, UviA, and Orf-22 protein sequences are shown in Figure 2.5. A Blast search revealed another regulator of BoNT, termed P-21 that is 25% identical to TxeR and 51% similar with conserved substitutions. P-21 is analogous to Orf-22, but found in *C. botulinum* types A and B that are responsible for food-borne and infant botulism (East et al. 1996).

Positive regulation of the toxin B promoter by TxeR in *cis*. To determine if TxeR affected expression of toxin B, the repeating units of toxin A (ARU) were fused to the toxin B promoter. The promoter and coding regions of the *txeR* gene was then cloned upstream of the toxin B promoter region (Fig 2.1). The resulting plasmid, ptxeR-BP-ARU, reflects the genomic organization of the 5’ end of the *C. difficile* toxigenic element. In Figure 2.6, an immunoblot using PCG-4 monoclonal antibody detected the 94 kDa ARU protein as expressed from ptxeR-BP-ARU. There also was a truncated protein or breakdown product of approximately 89 kDa. Expression of *C. difficile* proteins in *E. coli* often results in unstable products (Barroso and Wilkins, unpublished data). Cole and Garnier (1993) speculated that the difficulty in expressing *C. perfringens* genes in *E. coli* was due in part to the difference of codon usage between the species. Clostridial species are rich in adenine and thymine nucleotides that can lead to the depletion of charged
tRNA molecules for the codons of arginine and leucine. Consequently, the limited availability of minor tRNAs can alter the expression of clostridial genes in *E. coli*. The result can be premature termination of proteins during translation.

The expression levels of ARU from the recombinant strains were monitored by ELISA over a 24 h period in order to determine at what point during the growth cycle TxeR had an effect on the toxin B promoter. In Fig. 2.7A, the ELISA results show that there was a striking affect of TxeR on expression of ARU from the toxin B promoter in *cis*. Significant expression of ARU did not occur until 8 h, when cultures approached stationary phase growth (Fig. 2.7B). Expression levels continued to rise during stationary phase and by 18 h, the level of ARU (2552 Units/mg) was over 50-fold higher than in cultures lacking TxeR (43 Units/mg).
Figure 2.4 Characteristics of TxeR common to response regulators. DNA binding motif contains a large number of positively charged amino acids indicated with a (+).
Figure 2.5. Alignment of TxeR with UviA and Orf-22. Identical amino acids are boxed. The helix-turn-helix DNA binding motifs correspond to TxeR amino acids 149 to 168, UviA amino acids 156 to 175, and Orf-22 amino acids 144 to 163.
Figure 2.6. Immunoblot analysis of the activation of ARU expression by \textit{txeR} in \textit{cis} orientation with the toxin B promoter. Samples were analyzed at 20 h.

Lane 1: Low molecular weight protein standards (kDa);
Lane 2: pUC vector;
Lane 3: BP-ARU only;
Lane 4: BP-ARU + \textit{txeR};
Lane 5: high molecular weight protein standards (kDa).
Figure 2.7. Effects of TxeR in cis-orientation to the toxin B promoter on the expression of ARU. **(A)** Comparison of the level of ARU expression with and without TxeR was determined throughout the time course study. **(B)** Growth of the cultures were also monitored.
Transcriptional activation of lacZ by TxeR and the toxin B promoter. To study the activation of the toxin B promoter (BP) by TxeR in a different reporter system, I cloned the txeR-BP fragment in the vector pLKC480. This expression vector, pLKC480, lacks a functional promoter for lacZ. The expression system is designed to measure levels of β-galactosidase (β-gal) from transcriptional fusions with the lacZ gene. I evaluated the pLKC480 fusion clones that contained BP with and without txeR. In multiple time course studies the expression levels of β-gal were highest after 24 h (data not shown). The results from one experiment of samples collected at 24 h, 26 h, 28 h, and 30 h are shown in Table 2.1. At 30 h, the activity of β-gal was 657-fold higher in the presence of TxeR. Only a small amount of the β-gal protein was expressed, but a definite increase was detected by SDS-PAGE (Fig. 2.8).

Table 2.1. Positive effects of TxeR on the activation of β-gal expression controlled by the toxin B promoter.

<table>
<thead>
<tr>
<th>Hour</th>
<th>Units of β-gal activity</th>
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<tbody>
<tr>
<td></td>
<td>BP - TxeR</td>
</tr>
<tr>
<td>24</td>
<td>2.3</td>
</tr>
<tr>
<td>26</td>
<td>2.4</td>
</tr>
<tr>
<td>28</td>
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<td>30</td>
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Figure 2.8. SDS-PAGE analysis of β-galactosidase expression under the control of the toxin B promoter and TxeR. The increasing amount of protein seen at 115 kDa in lanes 2-5 corresponds to increasing levels of β-gal expression over time.

Lane 1: High molecular weight protein standards (kDa);
Lanes 2-5: lacZ + TxeR at 24 h, 26 h, 28 h, and 30 h, respectfully;
Lane 6: lacZ - TxeR at 30 h.
Positive regulation of the toxin B promoter in *trans*. To further investigate the ability of TxeR to activate the toxin B promoter, regardless of its proximity to the promoter region, additional vectors were constructed to supply TxeR in *trans*. To accomplish this, a fragment containing the BP-ARU was subcloned to the vector pACYC184 and the coding region of *txeR* was subcloned to the vector pT7-7. For comparison, a control strain was created that contained the plasmid with BP-ARU, but lacked the *txeR* containing plasmid. Expression of ARU in the presence of TxeR was confirmed by immunoblot analysis of 22 h samples (Fig. 2.9).

When TxeR was present, the levels of ARU detected by ELISA increased over 800-fold by 18 h (Fig. 2.10A). Induction of expression did not occur until about 8 h, as the cultures approached stationary phase (Fig. 2.10B). The expression levels from the unactivated promoter were lower in the *trans* experiment, as compared to the *cis* experiments, because ARU was present on a low copy vector.

Since *txeR* was cloned in pT7-7, an inducible expression vector, the initial *trans* experiments were performed to evaluate the necessity to induce the T7 RNA polymerase gene to activate the T7 promoter to express high levels of TxeR. The use of isopropyl β-D- thiogalactoside (IPTG) to induce the T7 RNA polymerase did not significantly contribute to the ability of TxeR to activate expression of ARU (data not shown). Therefore, to minimize the number of parameters present during growth of the cultures, I decided to perform all *trans* experiments without induction of T7 RNA polymerase by IPTG. Uninduced levels of *txeR* expression from the T7 promoter were enough to allow TxeR to exert its positive effect on ARU expression.
Figure 2.9. Immunoblot analysis of TxeR effects in trans to the toxin B promoter (BP). Samples of the strains with and without TxeR were analyzed for time point 22 h.

Lane 1: Low molecular weight protein standards (kDa);
Lane 2: BP - TxeR
Lane 3: BP +TxeR
Lane 4: High molecular weight protein standards (kDa).
Figure 2.10. Effects of TxeR in trans-orientation to the toxin B promoter on the expression of ARU. (A) Levels of ARU expression as detected by ELISA and (B) growth of cultures with and without TxeR.
Positive regulation of the toxin A promoter in trans. Additional vectors were constructed to test the effect of TxeR on the expression of ARU from the toxin A promoter (AP). The trans experiment should accurately predict the effect of TxeR on the expression of the toxin A gene in C. difficile since the promoter for toxin A is located far downstream of txeR on the chromosome. Using the in-trans cloning strategy for the toxin B promoter outlined above, the toxin A promoter region was fused with ARU. Subsequently, the expression levels of ARU from the toxin A promoter, with or without TxeR supplied in trans, were compared.

Initially, the cultures were grown for 24 h and cell lysates tested by immunoblot analysis to confirm the expression of ARU from the toxin A promoter with TxeR present (Fig 2.11). Next, a growth study was designed to analyze samples every 2 h over the course of 18 h. The ELISA results showed a dramatic increase in the expression of ARU from the toxin A promoter (Fig. 2.12A). As with the toxin B promoter, significant activation of expression from the toxin A promoter did not occur until stationary phase (Fig. 2.12B). By 18 h, the level of ARU expression was over 500-fold higher in the presence of TxeR (3516 Units/mg versus 6.2 Units/mg).
Figure 2.11. Immunoblot analysis of activation of ARU expression by TxeR in trans to the toxin A promoter (AP) after 24 h.

Lane 1: Low molecular weight protein standards (kDa);
Lane 2: AP - TxeR;
Lane 3: AP + TxeR;
Lane 4: High molecular weight protein standards (kDa).
Figure 2.12  Effects of TxeR in *trans*-orientation to the toxin A promoter on the expression of ARU.  (A) Levels of ARU expression and (B) growth of cultures with and without TxeR are compared over time.
Discussion

The *E. coli* expression systems used for this research were designed to determine if TxeR affected transcription of the *toxA* and *toxB* genes in either a *cis* or *trans*-orientation to the toxin gene promoter regions. The assumption that regulation of the toxin genes seen in *E. coli* is reflective of what occurs in *C. difficile* has been confirmed by other researchers (Dupuy and Sonenshein 1998, Song and Faust 1998). Dupuy and Sonenshein (1998) demonstrated that the *toxA* and *toxB* promoter regions, as well as the TxeR promoter region, were able to express β-glucuronidase from an *E. coli* fusion vector transformed into another clostridial species, *Clostridium perfringens*. Their data suggests that the promoter regions would be susceptible to similar regulation in *C. difficile*. Song and Faust (1998) demonstrated that *C. difficile* promoters are functional in *E. coli* by detecting mRNA levels of the transcripts generated from the toxin gene promoters.

The *E. coli* expression systems developed for this research project confirmed that TxeR acts as a positive element of transcription affecting both *toxA* and *toxB* promoter regions. The *trans* data indicates that it is the protein product of *txeR* that acts as a positive response regulator of transcription (Moncrief et al. 1997). The data presented here has also been confirmed by Hundsberger et al. (1997). They detected transcripts of *txeR* late in the growth phase, along with the expression of the toxin genes, and postulated that TxeR activates transcription of both genes. Their findings support the hypothesis that TxeR functions as a positive regulator of toxin gene expression.

To further investigate the role of TxeR in the regulation of *toxA* and *toxB* gene expression, a collaborative study was established with Dr. A. L. Sonenshein at the School of Medicine, Boston, MA. His research team noted that during purification of RNA
polymerase from \textit{C. difficile}, TxeR co-purified with the enzyme through every step Department of Molecular Biology and Microbiology, Tufts University. A sequence comparison of TxeR with regulatory proteins from \textit{Bacillus subtilis} showed some degree of homology with a family of alternative sigma factors (Sonenshein, personal communication). A proposed model for regulation of expression of the toxin genes by TxeR can be postulated, based on the combined data presented here (Fig 2.13).

Bacteria have evolved several mechanisms to detect changes in and adapt to their environment. One system that has been recently characterized utilizes alternative sigma factors and anti-sigma inner membrane proteins to respond to various extracellular changes. The subfamily of alternative sigma factors named extracytoplasmic function (ECF) sigma factors are bound to inner membrane proteins which serve as the sensor and signaling molecules (Missiakas and Raina 1998). The anti-sigma inner membrane protein releases the sigma factor after undergoing a conformational change resulting in the release of a repressor protein in response to an environmental change (Fig. 2.13). The liberated sigma factor is then capable of combining with the RNA polymerase to stimulate transcription of responsive genes. The alternative sigma factors have been identified in several bacterial species, including \textit{E. coli}, various \textit{Mycobacterium} species and \textit{Pseudomonas aeruginosa} (Missiakas and Raina 1998).
Figure 2.13. Proposed model of extracytoplasmic function (ECF) sigma factors from Missiakas and Raina, 1998. The sigma factor is bound by an inner membrane protein, an
anti-sigma factor. The ECF is released due to a signal from the periplasm as a result of environmental stress. Once released, the ECF stimulates transcription of other genes.
Evidence to support the alternate sigma factor model for TxeR is based on additional work recently completed by Sonenshein and his research group. Initially, purified TxeR was shown to stimulate \textit{in vitro} transcription from the toxins A and B promoters in the presence of RNA polymerase holoenzymes isolated from \textit{C. difficile}, \textit{B. subtilis}, and \textit{E. coli}. In control experiments using only core enzymes (without a sigma factor) transcription from the toxin promoters was not observed. The stimulation of transcription from the toxin promoters was restored with the addition of purified TxeR (Sonenshein, personal communication). Scientists analyzing a sigma factor common to several species of \textit{Mycobacterium} also demonstrated the same phenomena when recombinant \textit{M. tuberculosis} sigma factor was combined with core RNA polymerase from \textit{M. smegmatis} (Wu et al. 1997). The ability of TxeR to function with different RNA polymerases, including \textit{C. difficile} and \textit{E. coli}, validates the results of the \textit{E. coli} expression system previously described in this report.

In \textit{B. subtilis} and Mycobacterial species, sigma factors participate in responses to survival following stress (Wu et al. 1997). The signal for production of the toxins in \textit{C. difficile} is unknown but stressful situations have been shown to result in increased expression. The dialysis sac method used for the growth of \textit{C. difficile} cultures restricts the transport of nutrients, which causes an increase in toxin production. The toxins are produced in higher quantities \textit{in vitro} under the following conditions: the presence of small amounts of antibiotics, increased oxygen tension, elevated temperature and limitation of nutrients such as biotin (Nakamura et al. 1982, Onderdonk et al. 1979, Yamakawa et al. 1996). Some of these factors are potential environmental stress signals.
encountered by *C. difficile* in the human colon during infection. Alternatively, growth of *C. difficile* under favorable conditions, such as in the presence of glucose or rapid growth without the dialysis sac in rich media, decreases or totally suppresses the amount of toxin produced (Dupuy and Sonenshein 1998, Haslam et al. 1986). The identification of an environmental signal that starts the cascade of events remains to be determined.

In some cases, bacterial toxin production is thought to be a result of an environmental signal altering a response regulator that affects transcription of toxin genes. Currently, putative response regulators of toxin production in clostridial species are being identified. Recent studies involving the regulation of toxin production in *Clostridium tetani* and *Clostridium botulinum* revealed that each organism contains a protein with features common to DNA binding response regulators. (Marvaud et al. 1998). The putative regulatory protein (BotR) from *C. botulinum C* and *C. botulinum D* showed homology to TxeR from *C. difficile* and UviA from *C. perfringens*. Based on similar structure and function between the botulinum neurotoxins and tetanus toxin, a gene homologous to BotR was identified in *C. tetani* and termed TetR. Both TetR and BotR were shown to activate transcription of tetanus toxin in *C. tetani*. Thus, the potential for a common mechanism of clostridial toxin gene regulation is emerging.

Whether or not the mechanism of regulation is that of a response regulator acting as an alternative sigma factor remains to be determined. One characteristic of alternative sigma factors that can be explored is their ability to autoregulate their expression in response to a variety of environmental stresses. The genes encoding the alternative sigma factors in *E. coli* and *P. aeruginosa* are the first genes of an operon and are autoregulated (Missiakas and Raina 1998). Likewise, TxeR is also the first gene in the toxigenic
element of *C. difficile*. To investigate whether or not TxeR has an effect on its own promoter will provide more evidence to confirm the function of TxeR as an alternative sigma factor.
Chapter 3: Regulatory control of *txeR*
Introduction

Bacteria have evolved a variety of methods to regulate expression of genes, many of which remain to be elucidated. Many genes are constantly transcribed and their corresponding proteins are constitutively expressed. Some genes are transcribed as a result of a complex two-component regulatory system that involves modifications of sensory molecules that signal DNA binding regulatory proteins. These DNA binding proteins can exert a positive or negative effect on transcription of genes. Other DNA binding proteins have been classified as alternative sigma factors that respond to environmental conditions, bind to RNA polymerase core enzymes, and affect transcription of a gene or genes.

TxeR displays structural and functional characteristics of DNA binding proteins (Results, Chapter 2). Research that included the isolation of RNA polymerase from *C. difficile* performed by Dr. A. L. Sonenshein revealed that TxeR was a contaminating protein throughout the process (personal communication). Additional experiments were performed by their research team that suggested TxeR might act as a specific type of DNA binding protein that is known as an alternative sigma factor. Certain sigma factors from *E. coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* have been shown to be autoregulated (Missiakas and Raina 1998). In order to characterize TxeR as a DNA binding protein and potential alternative sigma factor, the previously described in-trans expression system using an *E. coli* host was used to determine if TxeR is autoregulated.
Materials and Methods

**Bacterial strains, plasmids and reagents.** *E. coli* strain BL21-DE3, plasmids pACYC184 and pT7-7 were kindly provided by Dr. D. Dean (Department of Biochemistry, Virginia Tech, Blacksburg, VA). Transformations of calcium chloride treated bacterial strains were performed according to the method described Cohen et al (1972). The reporter gene for these experiments, ARU from *C. difficile* toxin A, was cloned from pCDtoxA.03 (Phelps et al. 1991). The plasmid pCD21 used for subcloning the region upstream of *txeR* was a generous gift from Dr. J. L. Johnson (Department of Biochemistry, Virginia Tech, Blacksburg, VA). All restriction endonucleases and modifying enzymes were purchased from Promega (Madison, WI) or Life Technologies and used according to protocols recommended by the manufacturer. All chemicals were molecular biological grade (when available) and obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

**General cloning and Polymerase Chain Reaction (PCR).** General molecular biology techniques, such as plasmid isolation, were performed according to methods described by Sambrook et al. (1989). The high fidelity DNA polymerase enzyme, Pwo (Boehringer Mannheim, Indianapolis, IN) was used to generate PCR products in a Hybaid Omn-E thermal cycler (Franklin, MA). All primers used in amplification reactions were generated by Genosys (The Woodlands, TX). Prior to restriction endonuclease digestions, PCR products were purified with the PCR Clean Up kit from Boehringer Mannheim. All DNA fragments, including digested PCR products, were isolated from 0.7% agarose gels with the Elu-Quick DNA purification kit (Schleicher and Schuell, Keene, NH) following electrophoresis.
**Autoregulation studies using TxeR and txeR promoter region in trans.** The promoter region of *txeR* was amplified by PCR from pCD21 (Fig. 3.1). The primer at the 5’ end contained a *Hind*III site (5’-GCGCAAGCTTTTAGATGGTTGCAGAGT-3’) and the 3’ primer contained a *Bam*HI site (5’-CGGGATCCTGCATAAAATCACCCCTCT-3’). The resulting 0.85 kb PCR product was subcloned in pACYC184 and termed pAC-TP. The clone, pAC-BP-ARU described in Chapter 1, was the source for the ARU gene fragment. The ARU fragment was isolated from as a 3.24 kb *Bam*HI fragment and subcloned to pAC-TP to create pAC-TP-ARU. For expression studies, pT7-txeR and pAC-TP-ARU were co-transformed using the calcium chloride method (Cohen et al., 1972) into *E. coli* strain BL21-DE3. As a negative control and to monitor the effects of TxeR, the pT7-7 vector and pAC-TP-ARU were co-transformed into *E. coli* strain BL21-DE3. Recombinant strains containing both plasmids were confirmed by growth on selective media containing ampicillin and chloramphenicol and restriction enzyme analysis of plasmid isolation preparations.
Figure 3.1. The cloning approach used to evaluate if txeR responds to autoregulation. **Step 1:** The txeR promoter region was amplified by PCR and cloned in pACYC184 (pAC-TP). **Step 2:** The reporter gene, ARU from toxin A, was digested from the toxin B promoter clone and subcloned to pAC-TP. The resulting clone, pAC-TP-ARU, was co-transformed with pT7-txeR in *E. coli* and used in expression studies. Restriction sites: H, *Hind*III; B, *Bam*HI.
Growth of recombinant strains and generation of protein lysates.

Recombinant bacterial strains described above for TxeR in trans to its own promoter were grown as outlined in Chapter 1. For comparison, all the recombinant strains from the in trans experiments and the appropriate control strains listed in Table 3.1 were grown simultaneously.

Table 3.1 List of recombinant strains and controls based on their plasmids.

<table>
<thead>
<tr>
<th>txeR supplied</th>
<th>Promoter</th>
<th>Plasmid (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>T7</td>
<td>pT7-7 alone</td>
</tr>
<tr>
<td>in trans</td>
<td>None</td>
<td>pT7-txeR and pACYC184</td>
</tr>
<tr>
<td>No</td>
<td>Toxin B</td>
<td>pT7 and pAC-BP-ARU</td>
</tr>
<tr>
<td>in trans</td>
<td>Toxin B</td>
<td>pT7-txeR and pAC-BP-ARU</td>
</tr>
<tr>
<td>No</td>
<td>Toxin A</td>
<td>pT7 and pAC-AP-ARU</td>
</tr>
<tr>
<td>in trans</td>
<td>Toxin A</td>
<td>pT7-txeR and pAC-AP-ARU</td>
</tr>
</tbody>
</table>
Samples were collected every 4 h and cells harvested by centrifugation (5 min. at 15,800 x g). Cells were suspended in an equal volume of phosphate buffered saline (PBS), pH 7.4, containing Complete™ protease inhibitor cocktail (Boehringer Mannheim). Cells were lysed by sonication (Virtis, Gardinier, NY) using a pulse cycle of 0.6 sec on/1.0 sec off for 2 min. Cellular debris was removed by centrifugation and the supernatants were collected and stored at 20°C. The total protein concentrations were determined with the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) using bovine serum albumin to generate the standard curve.

**Enzyme-Linked Immunosorbent Assay (ELISA).** The level of ARU in recombinant lysates was determined with the ToxA Test (TechLab, Inc., Blacksburg, VA) following the 2 hour protocol. Units of ELISA reactivity were arbitrarily defined as the A₄₅₀ multiplied by the reciprocal of the highest dilution with an absorbance of 0.2-2.5 nm. The level of ARU is expressed as units/mg of total protein in the recombinant lysate.

**SDS-PAGE and Western blot analysis.** Lysates prepared from recombinant strains were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Forty µg of total protein from each sample at the 20 h time point were denatured with 2.5% sodium dodecyl sulfate and
reduced with 5% 2-mercaptoethanol. After heating the samples in a boiling water bath for 3 min, the samples were electrophoresised in 7.5% separating-4% stacking SDS-PAGE gels. Proteins were transferred as outlined in Chapter 1. The membranes were rinsed in PBS, pH 7.4 and blocked for 1 h in PBS containing 10% carnation nonfat dry milk and 0.3% Tween-20 (blocking buffer). The primary antibody used to detect ARU was the monoclonal antibody PCG-4 at a concentration of 1:1000 in blocking buffer. After a 1 h incubation, the membranes were washed 3 times for 5 min each in PBS. A 1:2000 dilution of anti-goat IgG-peroxidase conjugate in blocking buffer was used as the secondary antibody. The membranes were incubated for 1 h and washed as stated above. Detection was performed with TMB membrane peroxidase substrate from Kirkegaard & Perry Laboratories (Gaithersburg, MD). After sufficient color development, the membranes were washed with ddH₂O and allowed to air dry.

**Results**

**Sequence similarities of promoter regions from TxeR, toxin A and toxin B of* C. difficile*.** The proposed promoter sites of the toxin A and B genes are located approximately 200 nucleotides before the start codons and show 76% sequence identity in the –10 and –35 regions (von Eichel-Streiber 1992). Transcriptional analysis of the toxigenic element confirms that these sites are functional promoters in* C. difficile* (Hammond et al. 1997, Dupuy and Sonenshein 1998). Sequence alignment of a similar region upstream of the* txeR* gene also shows homology to the promoter regions of the toxin genes (Table 3.2). The promoter region of* txeR* was compared to the functional promoters* toxA* and* toxB*. The degree of similarity shown in Figure 3.2 of the in the –35 and –10 regions between the promoters and the positive regulation of expression from the
toxin promoters shown in Chapter 1, suggest that expression of \textit{txeR} may be autoregulated.

Table 3.2. Sequence alignment of the –35 and –10 promoter regions of \textit{txeR}, \textit{toxA} and \textit{toxB} of \textit{C. difficile}.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Sequence Homology</th>
<th>Bp to Start</th>
</tr>
</thead>
<tbody>
<tr>
<td>txeR</td>
<td>TTTACAatactttattaataaaag--TTATTG</td>
<td>182</td>
</tr>
<tr>
<td>toxB</td>
<td>TTTACAatatttctagacaacgtatTTATTC</td>
<td>169</td>
</tr>
<tr>
<td>toxA</td>
<td>TTTACAaattactatcagacaatctccTTATCT</td>
<td>239</td>
</tr>
</tbody>
</table>

\textbf{Autoregulation of txeR promoter by TxeR.} Based on the success of previous in \textit{trans} expression system in \textit{E. coli}, the same system was used to evaluate the \textit{txeR} promoter region for autoregulation. To accomplish this, the \textit{txeR} promoter region was fused with the ARU region to create pAC-TP-ARU. Plasmids pT7-txeR and pAC-TP-ARU were co-transformed into \textit{E. coli}. In comparison with the strain lacking TxeR, levels of ARU expression increased substantially after 8 h (Fig 3.2A). At 14 h, the influence of TxeR on its own promoter generated a 20-fold increase of ARU expression when compared to the control strain. Differences were not due to alteration in growth
(Fig 3.2B). The level of ARU expression from the txeR promoter without the activation by TxeR was considerably higher than the observed levels of ARU expressed from the toxin A and B promoter regions. The decline in ARU levels after 14 h in this experiment was not consistently observed in all growth studies. The difference in detectable levels of ARU, in comparison to those the toxA and toxB constructs, could be a result of the txeR promoter region responding to another level of regulation by E. coli.

**Direct comparison of the effects of TxeR on the toxin A, toxin B and TxeR promoter constructs.** To control for any effects of experimental variation (e.g. ELISA lots used to measure ARU levels) resulting in ARU expression differences, all strains created for the evaluation of promoter regions (toxA, toxB, and txeR) in trans expression systems were grown simultaneously under the same conditions. Samples were collected at 4 h intervals to monitor levels of ARU expression. Immunoblot analysis of samples at 20 h demonstrated the activation of the different promoter regions in the presence or absence of txeR (Fig 3.3). As previously reported in Chapter 1, ARU expression remained highest during stationary phase for all strains (data not shown).
Figure 3.2. Evaluation of txeR for possible autoregulation. With TxeR in *trans* orientation to the *txeR* promoter, (A) levels of ARU expression were detected by ELISA and (B) growth of cultures were monitored over an 18 h period.
The ELISA results presented in Figure 3.4 confirmed the immunoblot analysis showing that expression of ARU from the \textit{toxA} and \textit{txeR} promoters were not tightly controlled by TxeR. There were detectable levels of ARU in the (-)TxeR plasmids even though the plasmids are in low copy (Fig 3.3 and 3.4). Regardless of the baseline expression levels of the different control strains, all the promoter regions responded to TxeR activation. The \textit{txeR} promoter region appeared to be more easily activated by other regulators or signal proteins present in \textit{E. coli}. Alternatively, TxeR may be constitutively expressed at low levels. Table 3.3 shows ELISA results of the combined growth experiment directly comparing all promoter constructs. Expression levels in the presence of TxeR ranged from 14 to 352-fold increase over control strains.
Figure 3.3. Immunoblot analysis of ARU expressed when TxeR is trans to either the toxA, toxB or txeR promoter regions fused to ARU. Samples at 20 h were analyzed.

Lane 1: Low molecular weight protein standards (kDa);
Lane 2: Control strain pT7-7 vector
Lane 3: AP promoter - TxeR
Lane 4: AP promoter + TxeR
Lane 5: BP promoter - TxeR
Lane 6: BP promoter + TxeR
Lane 7: TP promoter - TxeR
Lane 8: TP promoter + TxeR
Lane 9: Control strain pT7-txeR and pACYC184 vector
Figure 3.4. Summary of the ability of TxeR to activate transcription of ARU from various promoters regions during stationary phase.
Table 3.3 Expression levels of ARU during stationary phase (20 h).

<table>
<thead>
<tr>
<th>txe R supplied</th>
<th>Promoter</th>
<th>Plasmid(s)</th>
<th>Units/mg</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>No in trans</td>
<td>Toxin B</td>
<td>pT7 and pAC-BP-ARU</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Toxin B</td>
<td>pT7-txeR and pAC-BP-ARU</td>
<td>6302</td>
<td>352</td>
</tr>
<tr>
<td>No in trans</td>
<td>Toxin A</td>
<td>pT7 and pAC-AP-ARU</td>
<td>68</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Toxin A</td>
<td>pT7-txeR and pAC-AP-ARU</td>
<td>3516</td>
<td>52</td>
</tr>
<tr>
<td>No in trans</td>
<td>TxeR</td>
<td>pT7 and pAC-TP-ARU</td>
<td>177</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>TxeR</td>
<td>pT7-txeR and pAC-TP-ARU</td>
<td>2484</td>
<td>14</td>
</tr>
</tbody>
</table>
Discussion

In the case of *C. difficile*, the environmental signal that begins the cascade of events from transcription of toxin genes to toxin production is unknown. One mechanism used by bacteria to sense environmental changes are the use of response regulators. A class of response regulators is the alternative sigma factors that recognize specific promoter regions. They interact with RNA polymerase to increase transcription that elevates protein production. Several putative response regulators have been identified in clostridial species however, classification of these proteins has not been accomplished. My research presented in Chapter 2 identified the potential function of TxeR as a positive regulator of toxin gene expression from the pathogenicity islet of *C. difficile*.

Autoregulation of gene expression is a control mechanism that is characteristic of some regulatory proteins. The autoregulated expression of the *bv*gAS operon in *Bordetella pertussis* produces BvgA that positively regulates expression of the virulence genes (Roy and Falkow 1991). The ability of a protein to autoregulate expression enables a system to quickly respond to an environmental stimulus. In *Vibrio fischeri*, the positive autoregulation of LuxR is an essential part of the complex regulation of genes involved in bioluminescence (Shadel and Baldwin 1992). Results presented in this chapter show that TxeR activates transcription from its own promoter. Also, the promoter region of *txeR* exhibited low level activation in the absence of TxeR. These
results suggest that expression of \( tx{e}R \) in \( E. \ coli \) is enhanced by, but not completely dependent on, TxeR. Evaluation of the promoter regions of \( C. \ difficile \) by Hunsberger et al. (1997) identified two potential transcriptional start sites for \( tx{e}R \). One site may be for expression of \( tx{e}R \) by a less specific RNA polymerase holoenzyme while the other site is susceptible to activation by TxeR under certain environmental stimuli. By maintaining a low concentration of a regulatory protein, the bacteria are poised to initiate the cascade of events that leads to high level expression of target genes.

The promoter regions recognized by a class of regulatory proteins are usually conserved. Initial sequence alignments of the promoter regions from \( C. \ difficile \) toxin genes and \( tx{e}R \) showed homology in the –10 and –35 regions. Additional comparison of promoter regions in the \( C. \ difficile \) pathogenicity islet led to a proposal for a consensus sequence among these unique genes. The ultraviolet-light-inducible promoters of \( C. \ perfringens \) and the promoter of \( Corynebacterium \ diphtheriae \ ptox \) gene also contain the same consensus sequence (Hundsberger et al. 1997). The similarities of these promoter regions suggest the presence of corresponding homologous regulators.

To date, four clostridial regulators that positively activate gene expression have been identified. UviA, the regulator of ultraviolet-light-inducible promoters in \( C. \)
*perfringens* was the first to register amino acid similarities to TxeR. Recently, putative response regulators BotR from *C. botulinum* and TetR from *C. tetani* were shown to have similarity to UviA and TxeR. These transcriptional regulators represent a class of proteins that may be shared amongst all clostridial species. In the case of BotR, it was shown to stimulate expression of genes with tetanus toxin promoters. The BotR and TetR regulatory proteins were interchangeable in *C. tetani* (Marvaud et al. 1998). Based on similarities between regulatory proteins of these bacteria, it is possible that these proteins originated from a common ancestor.

In an attempt to classify these regulatory proteins, I have discovered that they do not easily fall into one category. TxeR shares some characteristics of alternative sigma factors. Mainly, it has the ability to bind to RNA polymerase and stimulate transcription from the toxin promoters. The differences between TxeR and classified sigma factors can be found when the protein sequences are compared. A database search of completed genome sequences revealed no known class of proteins that are similar to TxeR. For example, structural features such as four specific regions with highly conserved sequences amongst sigma factors are absent in TxeR. The only similarity is the helix-turn-helix motif that is conserved in all classes of DNA binding proteins. TxeR is considerably smaller in size than most sigma factors. It has been shown that sigma factors
truncated at the N-terminal region could bind, with reduced specificity, to promoter DNA regions. If TxeR is a sigma factor, it may represent a novel class of altered sigma factors that are specific for clostridial species. Continued research on any one of these regulatory proteins from Clostridia will provide more evidence as to the classification and mechanism of regulation used by these bacteria.
References


Curriculum Vitae

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Education

Master of Science, Department of Biology, Microbiology and Immunology Option, Virginia Polytechnic Institute and State University. 1996-1999.
Thesis Title: Effect of Autoregulated TxeR on the Expression of Clostridium difficile Toxins.

Bachelor of Science, Department of Biology, Virginia Polytechnic Institute and State University. 1979-1984.

Professional Positions

Laboratory Specialist Senior. Department of Biochemistry, VPI & SU. Nov. 1988-present.

Development of mucosal immunization strategies to enteric pathogens
  • Murine model to evaluate the efficacy of intranasal and/or oral immunizations
  • ELISA to detect the levels of antigen-specific antibodies
  • ELISPOT to determine antigen-specific secreting cells in spleen and lamina propria
• Cloning, expression and purification of recombinant proteins
• PAGE, Western analysis and Capillary electrophoresis

**Molecular characterization of human pathogenic anaerobic bacteria**
• Perform research in Biosafety Level 3 (BL3) containment facility
• Cloning, DNA sequencing and PCR amplification of toxin genes
• Site-directed mutagenesis
• Investigation of the regulation of toxin gene expression
• Use of Southern hybridizations to evaluate toxin gene heterogeneity
• Mammalian tissue culture for cytotoxicity studies

**Managerial/Supervisory Responsibilities**
• Technical training and supervision of lab personnel, graduate and undergraduate students
• Laboratory maintenance and inventory purchases
• Monitor budgets, record expenditures, and reconcile research grants
• Proficient in computer programs: Microsoft Office, Canvas, etc.

**Laboratory Specialist A.** Department of Pathobiology, Virginia-Maryland Regional College of Veterinary Medicine, VPI & SU. March 1986 - November 1988.

**Gene regulation and gene expression in Escherichia coli**
• General recombinant DNA techniques
• Enzymatic assays

**Supervisory Responsibilities**
• Technical training of graduate students and technicians
• Equipment maintenance and supply purchases
• Radioisotope disposal and inventory control

**Laboratory Specialist.** Department of Plant Pathology, Physiology and Weed Science, VPI & SU. November 1984 - March 1986.

**Purified and characterized plant viruses.**
• Monitored plant responses to viral infections
• Developed ELISA diagnostic tests for detection of plant viruses
• Electron microscopy
Supervisory Responsibilities

• Manage laboratory operations
• Trained lab personnel
• Prepared materials for courses in plant virology

Publications


**Meeting Abstracts**


Moncrief, J. S., **L. A. Barroso**, D. M. Lyerly, and T. D. Wilkins. 1993. Expression and characterization of the repeating units of *Clostridium difficile* toxin A fused to glutathione s-transferase. 93rd General Meeting of ASM.


Hahn, P., C. J. Phelps, and **L. A. Barroso**. 1992. Expression of a recombinant peptide containing the receptor binding portion of toxin A of *Clostridium difficile* using the Baculovirus system. 92nd General Meeting of ASM.

**University Awards and Appointments at VPI & SU**

1999  Staff Woman of the Year Award, presented by The Women’s Center at VPI & SU  
1995-96  President, Women’s Network at VPI & SU  
1994-95  Program chair for Women's Network at VPI & SU  
1993  College of Agriculture and Life Sciences (CALS) Staff Versatility Award  
1992-93  University Steering Committee for Leadership Excellence  
1992-93  CALS Staff Senate Representative  
1991-92  Vice chair of the University Classified Staff Affairs Committee (CSAC)  
1990-92  Classified Staff Representative to University Council.  
1990-92  CALS Representative to University CSAC  
1990-91  Secretary,  CSAC Subcommittee on University Council, Commissions and Committees  
1990-91  Chairperson of the Department of Anaerobic Microbiology Staff Association  
1989-90  Support Personnel Leadership Committee for CALS Steering Committee
References

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