

**THE ROLE OF CCPA IN REGULATING THE CARBON-STARVIATION
RESPONSE OF *CLOSTRIDIUM PERFRINGENS***

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(Abstract)

Clostridium perfringens is a significant human pathogen, causing 250,000 cases of food poisoning in addition to several thousand potentially lethal cases of gas gangrene each year in the United States. Historically, work in this field has centered around toxin production, as *C. perfringens* can produce over 13 toxins. This work expands the knowledge of the starvation-response of *C. perfringens*, which includes several potential virulence factors, sporulation, motility and biofilm formation. Sporulation protects cells from a variety of stresses, including starvation. Efficient sporulation requires the transcriptional regulator CcpA, mediator of catabolite repression. Sporulation is repressed by glucose, but, surprisingly, in a CcpA-independent fashion. *C. perfringens* cells in a biofilm are resistant to a number of environmental stresses, including oxygen and antibiotics. Biofilm formation is repressed by glucose, and other carbohydrates, independently of CcpA. Gliding motility, a type four pili (TFP)-dependent phenomenon, affords *C. perfringens* with a mechanism for moving across a solid surface in response to carbohydrate starvation, while carbohydrates supplementation at high levels delay the initiation of the motility response. CcpA is required for the proper initiation of motility, a *ccpA*⁻ *C. perfringens* strain showed a considerable increase in the time to initiation of motility on lactose and galactose, and was unable to move at all in the presence of

glucose. Gliding motility represents the most significant finding of this work. TFP were previously undescribed in any Gram-positive bacterial species, and this work produced genetic evidence suggesting their presence in all members of the clostridia, and physical evidence for TFP-dependent gliding motility in a second species, *C. beijerinckii*.

DEDICATION

I dedicate this dissertation to my parents, Charles and Mary Lou Varga, for providing me with an excellent set of genes, and to my entire family, for giving me an environment for their proper expression.

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TABLE OF CONTENTS

FIGURES	viii
TABLES	ix
Chapter 1 - General Overview	1
Introduction to the clostridia and <i>Clostridium perfringens</i>	2
Disease pathogenesis of <i>C. perfringens</i>	7
Relevant responses to carbohydrate conditions.....	17
Chapter 2 - The CcpA protein is necessary for efficient sporulation and enterotoxin gene (<i>cpe</i>) regulation in <i>Clostridium perfringens</i>	28
Abstract.....	29
Introduction.....	31
Results.....	34
Discussion.....	46
Materials and Methods.....	52
Acknowledgments.....	61
Chapter 3 - Type IV pili-dependent gliding motility in the Gram-positive pathogen <i>Clostridium perfringens</i> and other clostridia	62
Abstract.....	63
Introduction.....	64
Results.....	66
Discussion.....	86
Materials and Methods.....	91
Acknowledgments.....	99
Chapter 4 - The Role of CcpA in Regulating the Formation of <i>Clostridium perfringens</i> Biofilms	100
Abstract.....	101
Introduction.....	102
Results.....	105
Discussion.....	117
Acknowledgements.....	122
Materials and Methods.....	123
Chapter 5 - The role of CcpA in regulating <i>Clostridium perfringens</i> gliding motility	128
Introduction.....	129
Results.....	131
Discussion.....	135
Materials and Methods.....	137
Acknowledgements.....	140
Chapter 6 - Comparative genomics of <i>Clostridium perfringens</i> strains	141
Abstract.....	142
Introduction.....	143
Results	146
Discussion.....	158
Materials and Methods	161
Chapter 7 - Transposon mutagenesis of <i>Clostridium perfringens</i>	164

Introduction	165
Results and Discussion	169
Materials and Methods	172
Acknowledgements	177
Chapter 8 – Overall conclusions	178
Bibliography	182
<i>Curriculum Vitae</i>	206

FIGURES

Figure 1- 1. Model of <i>C. perfringens</i> AFP pathogenesis.....	10
Figure 1- 2. Model of CcpA regulation of carbohydrate utilization.....	18
Figure 1- 3. A simplified model of the phosphorelay initiating sporulation in <i>B. subtilis</i>	20
Figure 1- 4. Schematic representing an overview of the TFP complex.....	25
Figure 2- 1. Generation of a <i>ccpA</i> - strain of <i>C. perfringens</i>	36
Figure 2- 2. Raffinose utilization by <i>C. perfringens</i>	39
Figure 2- 3. Expression from the <i>cpe-gusA</i> fusion in wild-type and <i>ccpA</i> - strains of <i>C. perfringens</i>	43
Figure 2-4. Levels of synthesis of collagenase, polysaccharide capsule, and PLC.....	45
Figure 2-5. Model illustrating the involvement of CcpA in regulation of sporulation and <i>cpe</i> transcription.....	50
Figure 3- 1. <i>C. perfringens</i> genes involved in the synthesis or function of TFP.....	67
Figure 3- 2. Movement of <i>C. perfringens</i> on agar plates.....	70
Figure 3- 3. Visualization of TFP on the surface of <i>C. perfringens</i>	74
Figure 3- 4. Pili on the surface of <i>C. perfringens</i> strains.....	76
Figure 3- 5. Immunoblot of whole cells of <i>C. perfringens</i> strain 13 using anti-pilin sera	77
Figure 3- 6. <i>pilT</i> and <i>pilC</i> mutant strains are non-motile on plates	79
Figure 3- 7. . <i>pilT</i> and <i>pilC</i> mutant strains do not express pili on the cell surface	80
Figure 3- 8. TFP biosynthesis genes in other clostridial species	84
Figure 3- 9. Proposed evolution of type IV pili	87
Figure 4- 1. Biofilm formation by <i>C. perfringens</i> strains	106
Figure 4- 2. Oxygen protection in biofilms	107
Figure 4- 3. Survival of <i>C. perfringens</i> against antibiotic challenge	109
Figure 4- 4. Effect of glucose on biofilm formation.....	111
Figure 4- 5. Low lactose concentrations fail to repress biofilm formation.....	112
Figure 4- 6. Microscopy of <i>C. perfringens</i> biofilms.....	115
Figure 4- 7. Localization of PilA in <i>C. perfringens</i> biofilms.....	116
Figure 4- 8. Biofilm formation by multiple <i>C. perfringens</i> toxinotypes	120
Figure 5- 1. <i>C. perfringens</i> genes involved in the synthesis or function of TFP.....	130
Figure 5- 2. Measurement of the time to initiation of motility	133
Figure 5- 3. Primer extension for <i>pilB</i>	134
Figure 6- 1. Comparison of <i>spo0A</i> sequences from multiple <i>C. perfringens</i> strains	149
Figure 6- 2. Capsule production by <i>C. perfringens</i> strains	153

TABLES

Table 1- 1. Toxins produced by <i>C. perfringens</i>	4
Table 1- 2. <i>C. perfringens</i> toxinotypes	5
Table 1- 3. Animal diseases caused by <i>C. perfringens</i>	16
Table 1- 4. Genes encoding components of TFP	26
Table 2- 1. Spore production by a <i>ccpA</i> - strain of <i>C. perfringens</i>	38
Table 2- 2. Carbohydrate repression of <i>C. perfringens</i> sporulation	41
Table 2- 3. Bacterial strains and plasmids used in chapter 2	54
Table 3- 1. <i>C. perfringens</i> pilin homologs	71
Table 3- 2. Strains and plasmids used in chapter 3	92
Table 4- 1. Bacterial strains used in chapter 4.	124
Table 5- 1. Strains and plasmids used in chapter 5	138
Table 6- 1. Maximum sporulation efficiency of <i>C. perfringens</i> strains	147
Table 6- 2. Comparison of sporulation gene composition	152
Table 6- 3. Biolog assay of carbohydrate metabolism	157
Table 6- 4. Toxin differences in sequenced strains of <i>C. perfringens</i>	159
Table 6- 5. Strains used in chapter 6.	162
Table 7- 1. Summary of experimental results with transposon mutagenesis in <i>C.</i> <i>perfringens</i>	171
Table 7- 2. Table of strains used in chapter 7.	173

Chapter 1 - General Overview

Introduction to the clostridia and *Clostridium perfringens*

The *clostridia* are Gram-positive spore-forming, rod-shaped, anaerobic bacteria, initially discovered by Pasteur in 1861 ((260) referenced in (353)). Members of the *clostridia* belong to the phylum *Firmicutes*, which is thought to have diverged from the *Actinobacteria* approximately 3 billion years ago (29). The class *clostridia* is estimated to have separated from the other class of bacteria in the *Firmicutes*, the *Bacilli*, around 2.6 billion years ago (29), prior to the great oxidation event which occurred approximately 2.4 – 2.3 billion years ago (29, 34).

Historically, bacteria were placed in the genus *Clostridium* due to physiological traits (i.e. rod-shaped cells, anaerobic metabolism, Gram-positive cell walls and formation of endospores) (315). However, as molecular genotyping techniques have been developed, the genetic relationships between *clostridial* species have not always represented the phenotypic relationship (315). The genus *Clostridium* is broken down into 19 rRNA clusters (68), which contain more than 39 pathogens (315). Most of the major pathogens are located in cluster I (*C. botulinum*, *C. perfringens*, *C. tetani*, and *C. novyi*), and in cluster II (*C. histolyticum*, *C. chauvoei* and *C. septicum*), with *C. difficile* and *C. sordelli* located in cluster XI (68). While the members of cluster I are somewhat related, they are quite distantly related to bacteria making up the other 18 clusters (315).

General overview of *C. perfringens*

C. perfringens, a Gram-positive, endospore forming anaerobic bacterium, was first described in 1892 (354), after isolation from a tissue infection. *C. perfringens* is a prolific toxin producer, with identification of over 13 toxins produced by various strains

(282). Due in part to the toxins produced by the species, as well as a number of other potential virulence factors, *C. perfringens* is capable of causing a broad range of diseases in a vast host-range. The arsenal of toxins produced by this pathogen is summarized in Table 1-1. Subsets of these toxins are produced by individual strains of *C. perfringens*, and the variation in toxin makeup of each strain affects what diseases the strain can potentially cause. *C. perfringens* strains are placed into 5 toxinotypes (A-E) based on their ability to produce the 4 major toxins: α , β , ϵ , and ι , shown in Table 1-2 (208, 210). The *C. perfringens* enterotoxin (CPE) can be potentially found in any of the toxinotypes.

1. Major toxins produced by *C. perfringens*

The chromosomally-encoded *C. perfringens* α -toxin is a phospholipase C, and was the first bacterial toxin to be identified as having an enzymatic function (190). It breaks down sphingomyelin and phosphatidyl-choline (168) in addition to its phospholipase activity. The amino-terminus of the protein possesses the phospholipase activity (158), while the carboxy-terminus gives the hemolytic and lethal activity (235).

The β -toxin, encoded on a plasmid (54), was first identified in the 1930s (214). It is dermonecrotic (290) and causes the destruction of the intestinal vili (140).

C. perfringens ϵ -toxin is plasmid-borne, and associated with IS1151 insertion sequences (69); ϵ -intoxication leads to a fatal enterotoxaemia (54). The toxin causes edema (52), and in sheep brains has been observed to form lesions (52, 210).

ι -toxin is an ADP-ribosylating binary toxin (310) that is encoded by a plasmid (266). It disrupts the actin cytoskeleton (6, 199), resulting in edema and hemorrhaging.

Table 1-1. Toxins produced by *C. perfringens*.

Toxin	Function	Location	Reference
α	Phospholipase C	Chromosome	(190)
β	Destruction of intestinal vili	Plasmid	(54) (214)
β -2	Intestinal Necrosis	Plasmid	(364)
δ	Hemolysin	???	(251)
ϵ	Lesions in brain	Plasmid	(52, 210)
θ	Cholesterol dependant cytolysin	Chromosome	(345)
ι	ADP-ribosylating binary toxin	Plasmid	(266) (310)
κ	Collagenase	Chromosome	(123)
λ	Metallo-protease	Plasmid	(266)
μ	Hyaluronidase	Chromosome	(308)
ν	DNAse	Chromosome	(254)
Sial ¹	Sialidase (NanHIJ)	Chromosome	(234, 303)
CPE ²	Lyses intestinal epithelial cells	Chromosome or Plasmid	(212, 213, 305, 326)

Based on from Rood and Cole (282)

1. Sialidase
2. *C. perfringens* enterotoxin

Table 1-2. *C. perfringens* toxinotypes.

Type	α	β	ϵ	ι
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

Based on (311).

2. Accessory toxins produced by *C. perfringens*

C. perfringens can also produce a number of accessory toxins, in addition to the major toxins. The β -2 toxin is encoded on a plasmid and can cause intestinal necrosis (364), and is lethal when injected into mice (107). The δ -toxin is a hemolysin (14) that is active against the red blood cells of sheep, goats and pigs, but not horses, humans or rabbits (251). The *C. perfringens* chromosome encodes a cholesterol-dependant cytolysin called perfringolysin O (PFO), or θ -toxin. PFO is similar to other bacterial cholesterol-dependant cytolysins (345) as it binds cholesterol on the host-cell membrane (253) and forms a pore by oligamerizing after binding to cholesterol, and leading to cytolysis (121, 344). The κ -toxin, encoded on the chromosome of *C. perfringens* (24), is a collagenase that degrades the most abundant protein of the host's cellular matrix (123). λ -toxin is a plasmid-encoded metallo-protease (266) that breaks down fibrinogen and fibronectin (266). Some *C. perfringens* strains produce a hyaluronidase, known as μ -toxin and is encoded on the chromosome. μ -toxin breaks down haluronic acid, made up of repeated N-acetyl-D-glucosamine, and is thought to aid in the diffusion of α -toxin (308). The ν -toxin is a cell-wall anchored DNase (254), encoded by the *cadA* gene on the *C. perfringens* chromosome (303). *C. perfringens* strains also produce a number of sialidases, NanI, NanH and NanJ (234, 303). Sialidases break down N-acetylated neuraminic acids (295), and are thought to facilitate the spreading of *C. perfringens* infections (296) by breaking down connective tissue (210).

3. *Clostridium perfringens* enterotoxin

When sporulating, some strains of *C. perfringens* produce an enterotoxin, CPE, that has been studied extensively, and has been proven to be the causative agent of food

poisoning (212, 213, 305, 326). CPE is a 319 amino acid (72), 35-kDa monomeric protein (203), with no known homology to identified protein toxins (126), which causes the symptoms associated with food poisoning. A high number ($\sim 10^6$) of CPE molecules can bind to individual intestinal epithelial cells (165, 207, 209, 358), in a one to one ratio with a 50 kDa receptor protein on the cell surface (358). The binding leads to pore formation in the cell membrane by forming a complex with a 70 kDa host protein (164). This pore then allows for a loss of ions and solutes from the cell, an influx of water into the cell. The effects of the pore on the cell include inhibition of macromolecule synthesis, morphologic damage (202), and eventual cell lysis (206).

Early research, consisting of mostly biochemical assays, was directed towards verifying the role of sporulation and its linkage to CPE production. In 1968, Duncan and Strong developed a sporulation medium for *C. perfringens*, which allowed for the investigation of sporulating cells (87). Using this medium, Hauschild *et al.* (124) were able to demonstrate that while sporulating cells produce CPE, vegetative cells do not. Using kinetic analysis of sporulating cells, Duncan (85) then demonstrated that exponentially growing cells did not produce spores or CPE. Duncan's work also showed that sporulation is required for CPE production, and that as long as the initial stage of sporulation occurs, some CPE would be produced (85). However, many *C. perfringens* strains can sporulate while not producing CPE, which correlates with the low percentage of environmental isolates that actually carry the *cpe* gene (217).

Disease pathogenesis of *C. perfringens*

1. Human diseases caused by CPE producing *C. perfringens* Type A strains.

C. perfringens causes a number of diseases of the human GI tract involving CPE, including acute food poisoning (AFP), antibiotic-associated diarrhea (AAD), infectious diarrhea (ID) and sudden infant death syndrome (SIDS). *C. perfringens* was first identified as a cause of food poisoning in England in 1943 and in the United States 1945 (135, 149), since then it has become known as a major cause of food poisoning throughout the world.

The CDC estimates that in the United States there are approximately 14 million cases of food poisoning from known sources each year. *C. perfringens* is the 4th highest causative agent of these cases, with an estimate of approximately 250,000 cases of AFP each year (30, 31) resulting from more than 600 individual outbreaks, with a fatality rate less than one percent (216). In England, *C. perfringens* is the second leading cause of food poisoning, with over 170,000 cases each year (3). The annual economic costs of *C. perfringens* acute food poisoning have been estimated to be over ten billion dollars in the United States alone (53, 242, 338). *C. perfringens* food poisoning is relatively mild, resulting in symptoms that include diarrhea and abdominal pain, with nausea and fever being less common symptoms (299). Fortunately, the disease is self-limiting and usually lasts less than 24 hours (149).

C. perfringens is a common food contaminant, and can be found in a wide range of types of food (289). However, beef or other meat products are the main sources of food poisoning outbreaks (149). Food poisoning manifests after an individual consumes food contaminated with a high number of vegetative cells (at least 10^9 cells). While most bacteria die from the acidic conditions of the stomach (134), surviving bacteria are carried to the small intestine, and complete the sporulation process that was triggered by

the very low pH in the stomach (222). When *C. perfringens* sporulates, the previously described enterotoxin CPE is produced (209), and as a result of the autolysis at the end of the sporulation cycle, CPE is released into the surrounding environment (120). Other research has shown that non-sporulating cells do not produce CPE (85, 86, 125).

In addition to the cell death caused by CPE, *C. perfringens* infections also cause histopathological damage to the small intestines (211, 213). Damage can be detected rapidly, starting from within 15 to 30 minutes after exposure to CPE, in the vili tips of the small intestine (300), and with time the entire small intestinal villus shows signs of damage (211, 300). CPE has been shown to bind to the claudins of the tight-junctions in the intestine (99, 155, 156, 313), which become exposed as cell damage accumulates (203, 204). Sonoda (313) also observed that the binding of CPE to the claudins results in destruction of the tight-junctions. The destruction of the tight-junctions causes a loss of integrity of the intestinal villus, which along with the emptying of the intestinal cells' contents, and the accompanying cell death, causes the diarrhea associated with a *C. perfringens* infection (287). Figure 1-1 shows a model encompassing both the effects of CPE on individual cells as well as on the structure of the tight-junctions.

The *cpe* gene has many characteristics that may be important in the epidemiology of the disease. *C. perfringens* strains can and do exist without the *cpe* gene, and are not hindered in sporulation (217). It has been observed that the *cpe* gene is located within a naturally occurring transposon (49, 54). Furthermore, work by Cornillot *et al.* (69) has shown that the *cpe* gene can be located on either a large plasmid or the chromosome in some strains of *C. perfringens*. The location of the CPE gene has been shown to affect the disease caused by the pathogen. When AFP isolates are examined, CPE is almost

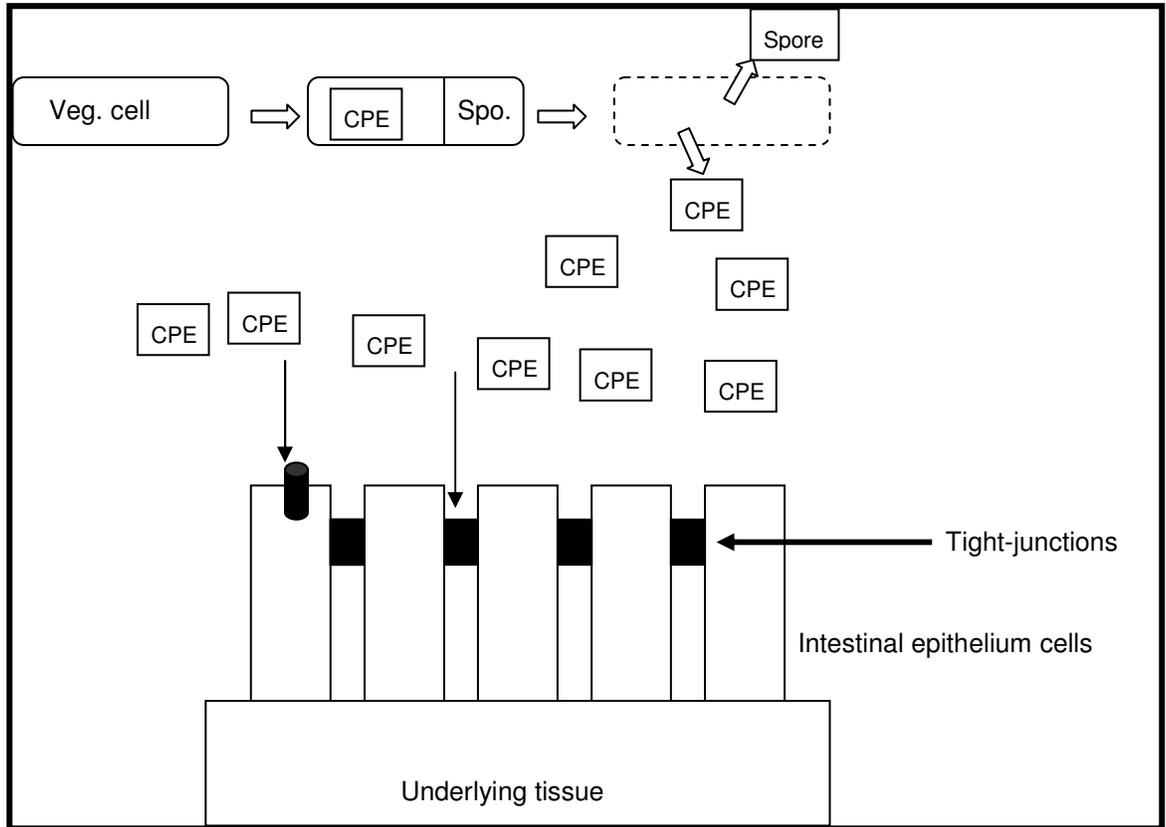


Figure 1-1. Model of *C. perfringens* AFP pathogenesis. *C. perfringens* cells enter into the small intestine and initiate sporulation. Upon completing sporulation, cell autolysis releases spores and CPE into the intestinal lumen. CPE then binds to, and forms pores in, intestinal epithelial cells. Cell lysis further exposes the tight-junctions, which CPE can then bind to, resulting in loss of integrity. Cell death, coupled with breakdown of the tight junctions results in the histopathological damage associated with AFP (205).

always located on the chromosome (69), when AAD or infectious diarrhea isolates are examined, the gene is always located on a plasmid (67). While a recent publication (331) describes the first known case of an AFP outbreak being caused by a *C. perfringens* isolate with an extra-chromosomally located *cpe* gene, it has been suggested that such an event may only occur when the food is contaminated after cooking (294). This differs from AFP caused by chromosomal *cpe* strains, in which the bacteria contaminate the food prior to cooking, which is then improperly cooked and stored.

The locus of the *cpe* gene has also been implicated as having a role in the properties of spores produced by those strains. Both vegetative cells and spores of chromosomal *cpe* strains have higher heat resistance (294) and cold resistance (180) than the plasmid *cpe* strains. The spores of chromosomal *cpe* strains were also observed to have thicker spore coats, and higher dipicolinic acid levels than the spores of plasmid *cpe* strains (242).

Clostridium difficile is most often associated with antibiotic associated diarrhea (AAD) observed in hospitals (359). Standard testing for AAD is an enzyme-linked immunoabsorbent assay (ELISA) assay for the presence of *C. difficile* Toxins A and B, with no further testing if a negative result occurs (224). However, when individuals are properly tested for *C. perfringens*, using special sensitive ELISA assays (224), there is a similar rate of *C. perfringens* detection to *C. difficile* detection in cases of AAD (1, 41). As a result of studies such as those by Modi *et al.* (224) and Sparks *et al.* (314), *C. perfringens* has recently been recognized as a major cause of non-food borne (i.e., antibiotic-associated) diarrhea.

People suffering from AAD tend to be elderly and hospitalized. They have often received treatment with cephalosporins, erythromycin or trimethoprim for a preexisting infection (224). The symptoms of *C. perfringens* AAD include abdominal pain and diarrhea, with blood and mucus in the stool, and tend to be prolonged, more severe and sporadic when compared to AFP (224).

A model for *C. perfringens* AAD has been proposed by Wrigley (361) based on the observation that the short-chain fatty acid (SCFA) levels in patients with AAD are lower than the levels in non-afflicted individuals (65). SCFAs are important to the health of the colonic environment by providing an energy source for epithelial cells (74).

During antibiotic treatment, there is a reduction in the normal flora, including members of the genus *Bacteroides*. The SCFAs acetate and isobutyrate, both by-products of *Bacteroides* fermentation were able to significantly inhibit sporulation in a *C. perfringens* isolate (361). Therefore, as the antibiotic therapy causes a reduction in SCFA production in the intestine, local populations of *C. perfringens* are able to sporulate efficiently, and if they are CPE+, potentially resulting in AAD.

The strains in the gut could either have always been CPE+, or could become CPE+ by several mechanisms including colonization by aerosolized spores in a clinical setting aided by the depleted normal flora (224). A second possibility is that the plasmid bearing *cpe* strains are already present in the environment, and when internalized by the patients they mobilize their plasmids to the *C. perfringens* already present in the gut. This could take place either before, or after, antibiotic therapy, as long as the end result was a high number of *C. perfringens* with the *cpe* plasmid present in the digestive tract (224).

C. perfringens is also known to cause a form of infectious diarrhea found primarily in elderly, institutionalized patients (173). *C. perfringens* infectious diarrhea mainly occurs in women (72%) and in people greater than 60 years of age (86%) (143).

The symptoms of *C. perfringens* infectious diarrhea are more severe than those associated with AFP. In addition to the profuse diarrhea associated with AFP, 50% of infectious diarrhea sufferers have abdominal pain and 35% having blood in their stool (149), and is protracted, with infectious diarrhea lasting an average of 7 days (149).

Sudden infant death syndrome (SIDS) is the cause given for asymptomatic, deaths of babies for which no obvious cause of death is discernable through an autopsy (230). Research has recently indicated a possible role of bacterial toxins, initially *C. botulinum* neurotoxin (18, 265, 312), with further recognition of a correlation of the presence of other bacterial toxins including *Staphylococcus aureus* toxic shock syndrome toxin (240), *Escherichia coli* heat-labile enterotoxin and Vero-toxin (37), and *C. perfringens* enterotoxin (151, 152, 183, 233)

2. Human tissue infections caused by *C. perfringens* Type A strains

Type A *C. perfringens* can cause a very serious tissue infection known as gas gangrene or clostridial myonecrosis. Historically, *C. perfringens* has been a significant cause of battlefield and battlefield-related deaths. In more recent times, proper medical treatments have reduced the military significance of gas gangrene. However, the disease persists as a health problem in the United States. Each year there are approximately 3,000 cases of gas gangrene each year, with a fatality rate of 25% (122, 274).

Gas gangrene typically has a short incubation time after wound contamination, from 6 h to 8 h (320), followed by a rapid progression to fulminant disease (191), with

fatality rates approaching 100% in untreated (19, 322). After wound contamination (309), surviving *C. perfringens* are thought to either persist and multiply in the wound or multiply after being phagocytosed by macrophages at the site of infection (245). As the infection progresses, α - and θ -toxins mediate the leukostasis and cytotoxicity (321, 335, 343) leading to swelling and anaerobiasis (250).

As the infection worsens, α - and θ -toxins enter the bloodstream, leading to organ failure, shock and ultimately death (48). Treatment options are limited in the case of gas gangrene. If the infection is caught early enough, massive doses of antibiotics, including penicillin G (320), can be administered with some success (320). If treatment is not received until the infection is well established, drastic treatment options including massive tissue debridement or limb amputation are required to save the life of the victim (320).

3. Human disease caused by *C. perfringens* Type C

C. perfringens type C causes a deadly enteric infection in humans known variously as enteritis necroticans (EN) (177), Darmbrand (369), or pigbel (232). EN is endemic in nature, Darmbrand broke out among very young and very old people in post WWII Germany, both groups suffering from malnutrition (177). Pigbel is known to occur amongst young children in the Highlands region of Papua New Guinea (176, 184). Pigbel is associated with diets that are low in protein, and occurs after traditional meals containing large amounts of undercooked pork and sweet potatoes (177). The large protein meal, and the synergistic effect of the protease inhibitors of the sweet potatoes coupled with the low protease levels of under-nourished children, provides an environment for contaminating *C. perfringens* to flourish (177). Symptoms of EN

include abdominal pain, vomiting and bloody diarrhea, frequently leading to intestinal blockage (283). While minor cases can resolve without significant treatment, severe cases can require surgical repair of the jejunum and ileum (177).

4. Animal diseases caused by *C. perfringens*

In addition to its significance as a human pathogen, *C. perfringens* can cause a very wide range of diseases in other animals. The toxinotypes cause distinct diseases in specific hosts, representative diseases and hosts are summarized in Table 1-3.

Type A strains of *C. perfringens* can cause necrotic enteritis (NE) in a fowl and other animals (8, 162). *C. perfringens* type A infections cause significant economic loss in poultry, estimated at \$2 billion world-wide (346). Recent changes in the legal environment prohibiting the inclusion of antibiotics in feed has resulted in a resurgence of NE (347). The α -toxin is thought to be the primary virulence factor (347), and the disease progresses from intestinal damage (236), to necrosis and rapid death (310).

C. perfringens type B strains can cause hemorrhagic enteritis in horses (97), cows (278), and lambs (327). The disease begins as ulceritis in the small intestine, leading to death of the animal (97).

C. perfringens type C can cause enterotoxaemia in a variety of animals, including calves (111) and lambs (112). Symptoms of type C enterotoxaemia include diarrhea, bleeding, necrosis and gas accumulation (17). The β -toxin is thought to be responsible for the severity of type C infections.

Table 1-3. Animal diseases caused by *C. perfringens*.

Toxinotype	Host ¹	Disease	Primary toxin
A	Fowl	Necrotic enteritis	A
B	Lambs	Hemorrhagic enteritis	α β ϵ ?
C	Pigs	Enterotoxaemia	B
D	Sheep	Enterotoxaemia	ϵ
E	Rabbits	Enterotoxaemia	ι

1. Representative host. Table based on (311)

Type D strains of *C. perfringens* cause enterotoxaemia, primarily in sheep (334), and also in calves (4) and goats. The ϵ -toxin produced by *C. perfringens* type D infections spreads from the gastrointestinal tract to the central nervous system, leading to sudden death (310).

C. perfringens type E strains are most known for their ability to cause enteritis in rabbits (28). While they can also be found as the causative agent of enterotoxaemia in cows and sheep (310), the extent of the host range is uncertain (311). It is thought that the ι -toxin (311), acting on actin microfilaments (7) is responsible for pathogenesis of the disease.

Relevant responses to carbohydrate conditions

1. Catabolite repression in the *Firmicutes*

The low G+C Gram-positive bacteria (*Firmicutes*) maintain a hierarchy of carbohydrate utilization through the action of the transcriptional-regulator carbohydrate control protein A (CcpA) (33, 336, 337). CcpA belongs to the LacI/GalR family of DNA binding proteins (100), it recognizes imperfect palindromic repeats termed catabolite response elements (*cre*) (341), where it can either activate or repress gene expression based on where the *cre* site is located with respect to the promoter region (81).

The mechanism of CcpA action is shown in Figure 1-2. Certain sugars are phosphorylated during translocation into cells (known as PTS sugars) with a phosphate from the EIIAB complex. This phosphate originates from phosphoenol-pyruvate which phosphorylates a histidine residue on a small effector molecule, Hpr (His-phos-Hpr), which in turn phosphorylates EIIAB (187). In the presence of PTS sugars and high energy conditions, His-phos-Hpr is constantly donating its phosphate group to EIIAB.

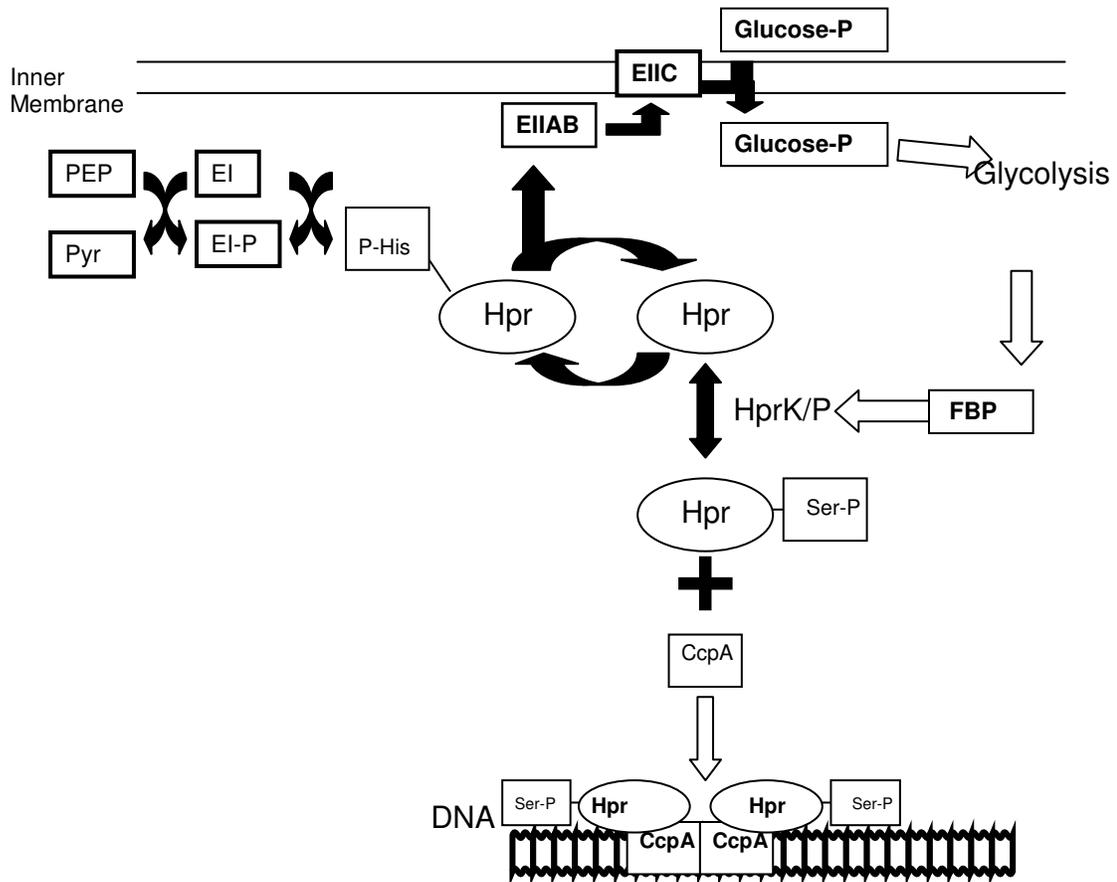


Figure 1-2. Model of CcpA regulation of carbohydrate utilization. Black arrows represent pertinent flow of phosphate in the mechanism, from phosphoenol-pyruvate (PEP) to EI, which in turn phosphorylates a histidine residue on Hpr (P-H-Hpr). The phosphate from P-H-Hpr is transferred to EIIAB of the phosphotransferase system (PTS), which replaces the phosphate EIIIC donates to incoming PTS sugars during translocation into the cell. In the presence of PTS sugars, non-phosphorylated Hpr accumulates, which can then be phosphorylated on a serine residue (P-Ser-Hpr) by the fructose-bisphosphate (FBP) induced HprK/P. P-Ser-Hpr binds to CcpA dimers, greatly increasing its affinity for DNA, resulting in activation or repression of target genes. In the absence of PTS sugars, P-His-Hpr accumulates, and CcpA fails to bind DNA, resulting in loss of activation or repression of target genes. Figure is based on (187).

The resulting high levels of fructose-bisphosphate activate a kinase, HprK/P, which leads to the phosphorylation of a serine residue of Hpr, resulting in a form of Hpr which can bind to CcpA and greatly increase its DNA binding capability (187). In the absence of PTS sugars, His-phos-Hpr accumulates, resulting in CcpA with a lowered affinity for DNA, relieving catabolite repression (187).

2. Sporulation by *B. subtilis* and *C. perfringens*

Bacillus subtilis sporulation is a well-studied phenomenon. Sporulation is induced by starvation, most efficiently when the cells are at high density (269), in a pathway known as the phosphorylation-cascade (Figure 1-3). The inducers of sporulation activate a number of kinases (319) that phosphorylate the protein Spo0F (136), which can be dephosphorylated by proteins in the Rap family (263). In the absence of Rap phosphatases, Spo0F~P donates its phosphate group to Spo0B which then transfers the phosphate to Spo0A, the master regulator of sporulation (269). Spo0A is subject to dephosphorylation by Spo0E (252), and the presence of phosphorylated Spo0A is what induces sporulation in a cell (62).

In contrast to the state of knowledge of *B. subtilis* sporulation, significantly less is known about *C. perfringens* sporulation, and much of what is known is inferred from *B. subtilis* data. Owing in part to their common genetic lineage, *C. perfringens* sporulation does share many similarities to *Bacillus subtilis* sporulation, (29, 172). However, despite the similarities, there are a number of differences in sporulation between the two genera. The most prominent difference between *C. perfringens* (and all *clostridial* species) sporulation and *B. subtilis* sporulation is in the mechanism of initiation. Whereas *B. subtilis* has a phospho-relay that activates Spo0A, *clostridial* species do not have a

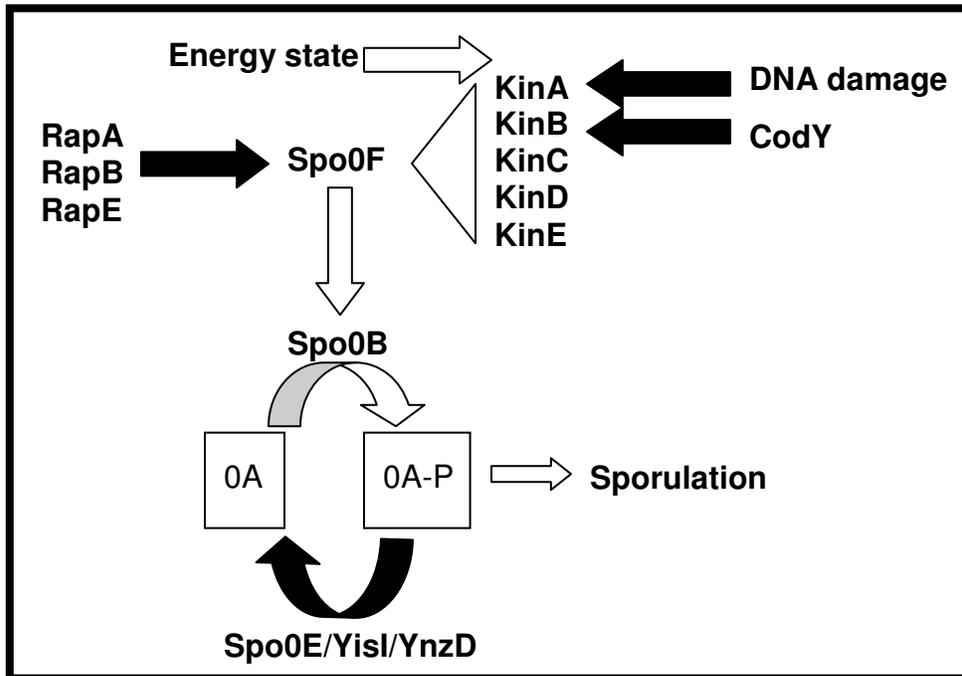


Figure 1-3. A simplified model of the phosphorelay initiating sporulation in *B. subtilis*. White arrows represent the activity that induces sporulation, black arrows represent activity that represses sporulation. Phosphates enter the system through the action of 5 kinases, KinA-E, which respond to environmental signals and are repressed by DNA damage. Phosphates from KinA-E accumulate on Spo0F, which can be dephosphorylated by RapABE. Phosphate passes from Spo0F to Spo0B to Spo0A (0A and 0A-P in the figure), which can be dephosphorylated by Spo0E, YisI and YnzD. Phosphorylated Spo0A activates sporulation genes, beginning the sporulation process. Figure based on (91, 270).

phospho-relay (89, 234, 303), and activate Spo0A through currently unknown mechanisms.

Early work attempting to optimize *C. perfringens* sporulation rates examined the role of carbohydrates in repressing sporulation. Starch or raffinose are primarily used as the main carbohydrate in sporulation media (169, 171). More recent work by Shih and Labbe (302) indicated that 15 mM of glucose, sucrose, mannose and maltose significantly inhibited sporulation, while 30 mM of ribose and fructose had no impact of sporulation of tested strains. Similar to *B. subtilis*, exogenous purines in sporulation medium lower the sporulation levels of *C. perfringens*, and purine analogs can increase sporulation efficiency (288).

In 2004, Wrigley (361) performed an analysis of the effect of *Bacteroides fragilis*, a very numerous member of the gut flora, on *C. perfringens* sporulation levels. *B. fragilis* co-cultures with *C. perfringens* resulted in repression of sporulation, analysis of metabolic end-products of *B. fragilis* showed that the succinate and acetate were the responsible factors for the repression (361). This provides a scenario for the repression of *C. perfringens* in the human gut during normal conditions.

Recent work by Philippe *et al.* (268) demonstrated the role of inorganic phosphate in *C. perfringens* sporulation. Cells of *C. perfringens* strain SM101 were shown to maximally sporulate at inorganic phosphate levels of 15-30 mM. While this also impacted the pH of the media, modifying the pH with either 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) or 3-(N-Morpholino)propanesulfonic acid (MOPS) in the absence of added phosphate resulted in very low levels of sporulation (268). Intriguingly, the levels of inorganic phosphate in the human gut of a healthy adult are approximately 30

mM (268), which may represent an evolutionary adaptation of mammalian gut isolates of *C. perfringens*, as the sporulation of the environmental isolate strain 13 increases 1000-fold in the absence of phosphate buffer (234).

Recent work by Durre *et al.* (88) with the solvent-producing *Clostridium acetobutylicum*, suggested that sporulation in the *clostridia* was a response to lethal environmental conditions, such a low pH. It was reasoned that a response of this nature would not require the integration of a number of environmental signals that *B. subtilis* utilizes, and explains the lack of a complex phosphorelay for initiation of sporulation (88).

3. Biofilm formation by *B. subtilis* and related bacteria

Biofilms are dense bacterial communities encased in a matrix consisting of polysaccharides and proteins (44), and are thought to be the predominant state of bacterial existence in nature (78). Biofilm formation is a commonly occurring trait in the *Firmicutes*, however virtually no research has been done on biofilm formation by the *clostridia*, almost all research has been directed towards the *Bacilliales*, primarily in the genera of *Bacillus*, *Staphylococcus* and *Streptococcus*.

B. subtilis is a soil bacterium, which has recently been shown to form complex biofilms (118). The decision to form a biofilm is controlled by the master regulator of sporulation *spo0A* (118) and the starvation response sigma factor, σ^H (316). Production of the matrix, consisting of poly-D-glutamic acid (317) and the protein TasA (43) results from genes that are indirectly regulated by Spo0A (119).

B. subtilis biofilm formation is responsive to carbohydrate levels in the environment as high levels of glucose repress biofilm formation in a *ccpA*-dependant

manner (316). However, the absence of glucose also results in minimal biofilm formation (316). *B. subtilis* can form biofilms in response to high salinity and heavy metal ions (225).

In *Staphylococcus aureus*, biofilms are implicated in a number of diseases, including eye infections (179) and nosocomial infections (256). Biofilm formation is dependant on special peptidoglycan synthesis and fibrinogen binding genes (277). *S. aureus* biofilm formation requires the availability of carbohydrates, it was observed that 25 mM glucose was necessary for formation *in vitro* (82).

Streptococci are frequently found in the oral environment causing dental carries (reviewed in (116) and (71), a process in which biofilm formation is thought to be of paramount importance. *S. gordonii* biofilm formation requires the *comEC/comD* environmental sensing system (108), and is induced by low carbohydrate levels (108). It was demonstrated that CcpA was required for biofilm formation (355), and that biofilms increased the acid resistance of *S. mutans* (141).

Members of the *Bacilliales* also utilize the LuxS/AI-2 quorum sensing system to regulate biofilm formation, however, the role of quorum sensing varies in each species. In *B. cereus*, quorum sensing represses biofilm formation (20), but in *B. subtilis* (186) and *S. gordonii* (215) LuxS/AI-2 is required for biofilm formation.

Surface translocation by means of type IV pili

Type IV pili (TFP) are filamentous proteins (360) that fulfill a number of cellular functions, including DNA transformation (166) and motility (350). TFP are frequently important virulence factors, examples include the pathogens *P. aeruginosa* (153), *E. coli* (38), and *Neisseria* species (219).

The filaments are typically 6 nm wide and up to 3 μ m long (298), and comprised of the pilin subunit, PilA (38). PilA is initially translated as a 145-160 residue protein, with a short conserved N-terminal signal sequence (363) that is cleaved by the signal peptidase PilD (197). The PilA fiber is extended by the polymerization of mature pilin by PilB (83), passing through an outer-membrane pore composed of multiple PilQ proteins (83). The pilus-complex is shown in Figure 1-4. Motility is achieved by retraction of the TFP, actuated by the retraction motor, PilT, depolymerizing the pilus (328), while the tip remains firmly adhered to the surface (50). The force of this retraction has been measured at 10 pN (304). TFP and the associated structural proteins are encoded by a large number of genes with a range of complex functions (summarized in Table 1-4), typically arranged in gene clusters (350).

TFP mediated motility is colonial in nature, individual cells are typically non-motile (197), and has been best studied in *Myxococcus xanthus* (gliding motility) and the twitching motility of *Neisseria* and *P. aeruginosa*. However, the difference between gliding motility and twitching motility is minimal at best (197, 298). Twitching motility was first described in 1961 (175) as colony expansion across a solid surface. The short intermittent jerks observed by Henrichsen (130) were attributed to the polar pili (129), as cells possessing and lacking flagella were capable of twitching motility (129). In *Neisseria gonorrhoea* PilA diversity (264) and phase variation (220) were observed as a by-product of the strong host response to TFP (264).

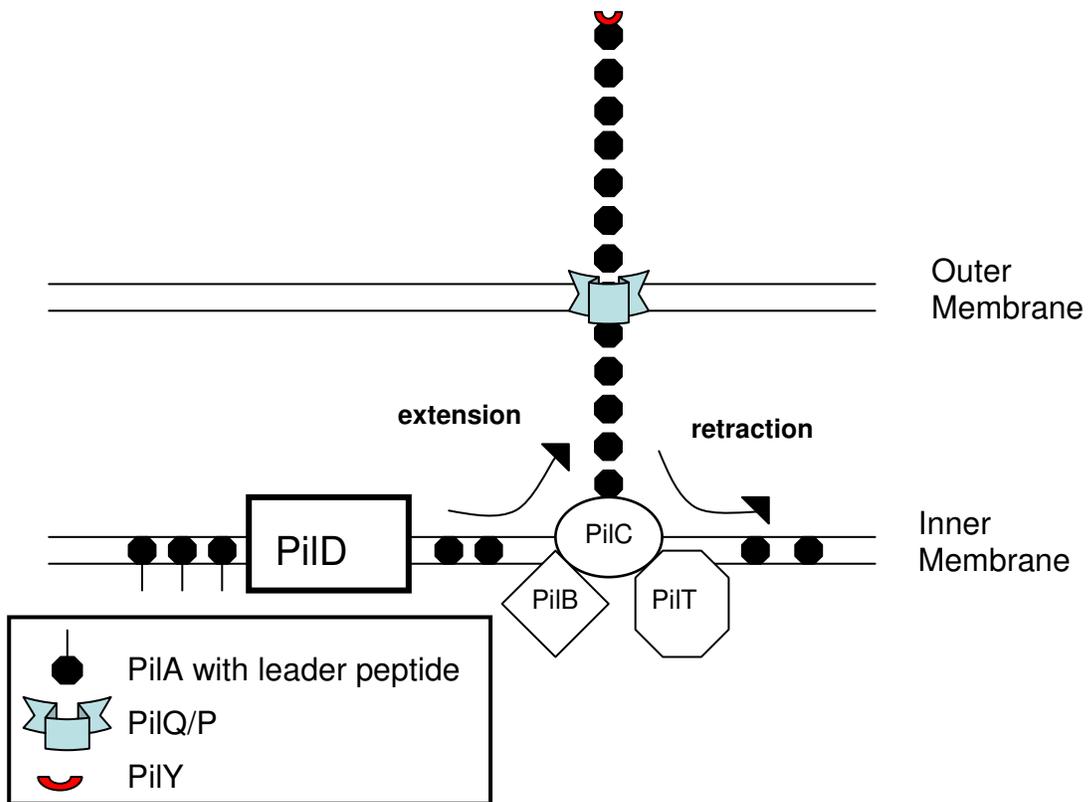


Figure 1-4. Schematic representing an overview of the TFP complex. Based on Mattick (197), this shows the organization of the TFP complex. PilD processes the PilA prepilin by cleaving the signal sequence. PilB then polymerizes PilA into a mature pilus, extending it through the outer membrane by means of a pore made up of PilQ stabilized by PilP. The tip, PilY, adheres to a surface and PilT depolymerizes the pilus, pulling the cell towards the point of adherence.

Table 1-4. Genes encoding components of TFP.

<i>pil</i> gene	(Proposed) function	Reference
A	Pilin subunit	(159)
B	Extension motor	(342)
C	Innermembrane protein, stabilizes PilD	(161)
D	Prepilin peptidase	(244)
E	Minor pilin	(12)
F	“bridgehead” protein	(160)
G	CheY homolog	(198, 357)
H	FrzZ homolog	(198, 357)
I	CheW homolog	(198, 357)
J	MCP homolog	(73, 76)
K	Transcriptional regulator	(73, 76)
L	CheA homolog	(197)
M	Multimeric NTP binding complex	(195)
N	Cytoplasmic membrane anchor	(84)
O	Cytoplasmic membrane anchor	(84)
P	Stablizes PilQ	(84)
Q	Oligomeric secretory pore, outer membrane	(13)
R	Transcriptional regulator	(13)
S	Transcriptional regulator	(13)
T	Retraction motor	(221)
U	Related to pilus retraction	(356)
V	Minor pilin	(197)
W	Minor pilin	(197)
X	Minor pilin	(12)
Y	Tip Adhesin	(12)
Z	Cell cycle based regulation	(11)

In his early review on gliding motility, Henrichsen (129) described 3 groups of bacteria that engaged in gliding motility: the filamentous *Cyanobacteria*, *Myxobacteria*, and *Cytophaga*. Gliding motility characteristically resulted in flat colony edges, with groups of cells forcing the expansion across the solid surface (129). Work with *M. xanthus* has shown that the polar TFP bind to fibril material surrounding neighboring cells (243) and that the process is regulated by nutrient availability (133).

**Chapter 2 - The CcpA protein is necessary for efficient
sporulation and enterotoxin gene (*cpe*) regulation in
*Clostridium perfringens***

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Abstract

Clostridium perfringens is the cause of several human diseases, including gas gangrene (clostridial myonecrosis), enteritis necroticans, antibiotic-associated diarrhea, and acute food poisoning. The symptoms of antibiotic-associated diarrhea and acute food poisoning are due to sporulation-dependent production of *C. perfringens* enterotoxin, CPE. Glucose is a catabolite repressor of sporulation by *C. perfringens*. In order to identify the mechanism of catabolite suppression by glucose, a mutation was introduced into the *ccpA* gene of *C. perfringens* by conjugational transfer of a non-replicating plasmid into *C. perfringens*. CcpA is a transcriptional regulator known to mediate catabolite repression in a number of low G-C Gram positive bacteria, of which *C. perfringens* is a member. The *ccpA*⁻ strain sporulated at a 60-fold lower efficiency than the wild-type strain in the absence of glucose. In the presence of 5 mM glucose, sporulation was repressed about 2,000-fold in the wild-type strain and 800-fold in the *ccpA*⁻ strain, in comparison to the same strains grown in the absence of glucose. Therefore, while CcpA is necessary for efficient sporulation in *C. perfringens*, glucose-mediated catabolite repression of sporulation is not due to the activity of CcpA. Transcription of the *cpe* gene was measured in the wild-type and *ccpA*⁻ strains grown in sporulation medium, using a *cpe*::*gusA* fusion (*gusA* is an *Escherichia coli* gene encoding the enzyme β -glucuronidase). In the exponential growth phase, *cpe* transcription was 2 times higher in the *ccpA*⁻ strain than in the wild-type strain. Transcription of *cpe* was highly induced during the entry into stationary phase in wild-type cells but was not induced in the *ccpA*⁻ strain. Glucose suppressed *cpe* transcription in both the wild-type and *ccpA*⁻ strain. Therefore, CcpA appears to act as a repressor of *cpe* transcription in exponential growth but is required for efficient sporulation and *cpe* transcription upon entry into stationary phase. CcpA was also required for maximum synthesis of collagenase (kappa toxin),

acted as a repressor of polysaccharide capsule synthesis in the presence of glucose, but did not regulate synthesis of the phospholipase, PLC (alpha toxin).

Introduction

Clostridium perfringens is a Gram positive anaerobic bacterium, readily found in soil, sediments and the intestinal contents of humans and animals (123). It is the cause of several human diseases, including gas gangrene (clostridial myonecrosis), and enteritis necroticans (282). *C. perfringens* is also the third most common source of bacterial food poisoning in the U. S. (216, 255). After ingestion of contaminated food containing vegetative cells, food poisoning symptoms are caused by production of a potent enterotoxin protein (CPE) made by sporulating cells in the gastro-intestinal tract. The enterotoxin interacts with epithelial cell tight junction proteins in a series of steps, leading to cell death and the symptoms of diarrhea and intestinal cramping characteristic of the disease (204). Enterotoxin positive strains of *C. perfringens* have increasingly been identified as a significant cause of non-food-born and antibiotic-associated diarrhea (1, 137, 149, 314). A strong correlation between the location of the *cpe* gene and disease has been observed: acute food poisoning isolates carry the *cpe* gene on the chromosome while isolates obtained from cases of non-food-born or antibiotic-associated diarrhea have a plasmid-born copy of the *cpe* gene (67, 69, 314). Whether located on the chromosome or on a plasmid, CPE production is always correlated with sporulation by *C. perfringens* (217, 293).

Since sporulation and enterotoxin production are inextricably linked, one approach to dealing with the disease is to identify agents that block sporulation, and therefore, CPE production and disease. Glucose has been shown to act as a catabolite repressor of sporulation in *C. perfringens* (172, 301). The mechanism of catabolite repression of sporulation by glucose in *C. perfringens* has not been determined, but in

Bacillus subtilis, another Gram positive spore-forming bacterium, many catabolite repressor (CR) effects from glucose, including sporulation have been shown to be mediated by the transcriptional regulator CcpA, a member of the LacI/GalR family of repressor proteins (57, 128). Homologues of CcpA have been found across a broad spectrum of low G-C Gram positive bacteria, including the clostridia (47, 80). CcpA binds to *cis* elements termed *cre* (catabolite responsive elements) and functions either as a transcriptional repressor or activator (337). However, CcpA often has weak, non-specific affinity for DNA when added alone in in vitro experiments (100). A co-repressor of CcpA is HPr-ser-phosphate (100). The HPr protein is phosphorylated at Ser 46 by HPr serine kinase/phosphatase, and the kinase function is activated by fructose 1,6-bis-phosphate (FBP). The HPr-ser-P-CcpA complex then binds to *cre* elements and regulates transcription of genes in the CcpA regulon. By regulating HPr ser kinase/phosphatase activity, the intracellular concentration of FBP provides a link between the metabolic state of the cell and CcpA transcriptional activity.

A *ccpA*- strain of *B. subtilis* exhibits partial relief of CR effects of glucose on sporulation, but mutants lacking active CcpA still show some glucose-mediated repression of sporulation (57, 223), suggesting other mechanisms of CR are involved. Using a whole-genome transcriptional analysis approach, Moreno *et al.* identified many genes that were subject to CR by glucose in a CcpA-independent manner and found that most of these were involved in sporulation (229). Since sporulation is necessary for enterotoxin synthesis (i.e., cause food poisoning) by *C. perfringens* (217), we investigated the role that CcpA plays in CR of sporulation and CPE synthesis. Unlike the situation in *B. subtilis*, where CcpA partially mediates CR of sporulation by glucose,

CcpA is needed for efficient sporulation and CPE synthesis in *C. perfringens* whether glucose is present or not, establishing a novel role for CcpA in sporulating bacteria.

Results

The *C. perfringens* *ccpA* gene.

The *C. perfringens* *ccpA* gene from strain SM101 was predicted to encode a protein of 332 amino acids with a molecular weight of 37, 200 Daltons. The CcpA protein of *C. perfringens* exhibited the highest level of identity (~70%) to CcpA orthologs from *C. acetobutylicum* (termed RegA) (80) and *C. tetani* (47) but exhibited significantly less identity ($\leq 44\%$) to other CcpA orthologs from Gram positive bacteria in the sequence databases. Kraus *et al.* (167) noted the significant sequence difference between the RegA protein of *C. acetobutylicum* and other members of the CcpA subfamily of the LacI/GalR family of transcriptional regulators.

The coding region for the *ccpA* gene in strain SM101 lies 717 bp downstream from a divergently transcribed conserved hypothetical protein and 250 bp upstream of a hypothetical gene transcribed in the same direction as *ccpA* (<http://www.tigr.org/tdb/mdb/mdbinprogress.html>). Thirty bp downstream of the coding region for *ccpA* there is a potential 28 bp stem-loop structure followed by a string of 7 thymidines, possibly indicating the presence of a rho-independent terminator that can function in *C. perfringens* (370).

Allele replacement of the wild-type *ccpA* gene with an inactivated copy of *ccpA*.

Strain SM101 was used to study the effects of a *ccpA* mutation because it sporulates well, is CPE+, and is relatively easily transformed by electroporation. A conjugational system developed in our laboratory was used to transfer the suicide plasmid, pSM225, from *E. coli* into *C. perfringens*. The allele replacement strategy, following integration of pSM225 into the chromosome, is shown in Figure. 2-1B. To confirm the recombination

events had taken place as shown in Figure. 2-1B, chromosomal DNA was isolated from the *ccpA*- strain SM120, digested with restriction enzymes and subjected to a Southern blot analysis, which confirmed that the recombination events shown in Figure. 2-1 B had occurred (data not shown). To our knowledge, this is the first report of a mutation made in a gene in *C. perfringens* by using conjugational transfer of non-replicating plasmids from *E. coli* to *C. perfringens*. This technique may be useful for constructing mutations in *C. perfringens* strains that are not efficiently transformed by electroporation.

The CcpA protein is needed for efficient sporulation in *C. perfringens* but does not mediate glucose CR of sporulation.

To determine the role CcpA plays in regulating glucose-mediated CR of sporulation in *C. perfringens*, the wild-type (SM101) and *ccpA*- (SM120) strains were grown in DSSM-raffinose sporulation medium with or without added glucose. In the absence of glucose, the wild-type strain sporulated at 68.2%, while the *ccpA*- strain produced spores at 1.13%, a 60-fold decrease compared to wild-type (Table 2-1). The presence of a plasmid (pSM257) carrying a wild-type copy of the *ccpA* gene in the *ccpA*- strain restored sporulation only to 11.5%, still 6-fold less than the wild-type level (Table 2-1). However, the presence of plasmid pSM257 in the wild-type strain lowered sporulation to a level (6.52%) similar to that seen with the complemented *ccpA*- strain, suggesting multiple copies of the *ccpA* gene are inhibitory to normal sporulation functions (Table 2-1). The addition of 5 mM glucose to the DSSM-raffinose medium lowered the sporulation of the wild-type strain to 0.0332%, a 2,054-fold reduction, while the *ccpA*- strain sporulated at 0.00139%, an 812-fold reduction compared to the *ccpA*-

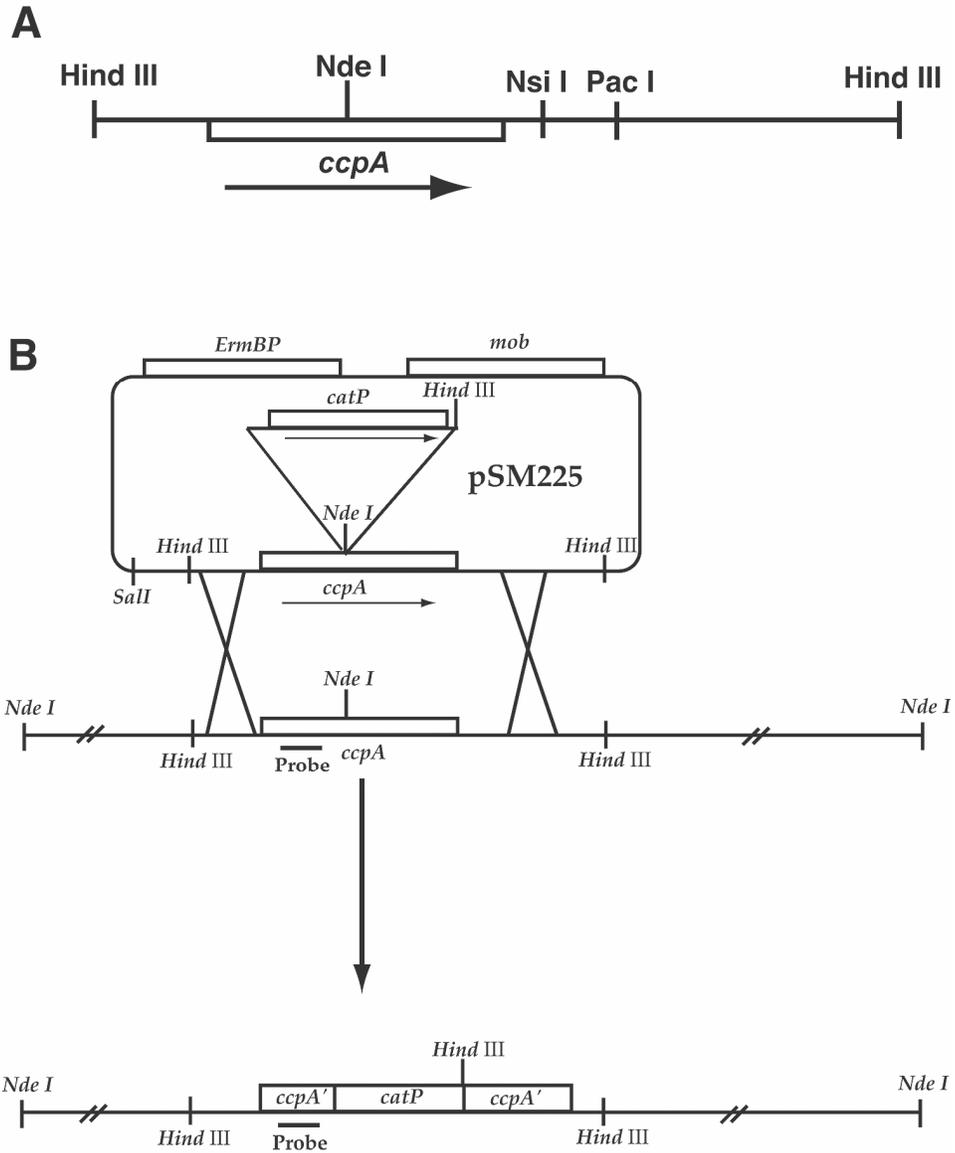


Figure 2-1. (A) Schematic diagram showing restriction sites and *ccpA* gene orientation in the 3.8 kb HindIII insert in pSM310. (B) Diagram showing the strategy used for allele replacement of the wild-type copy of the *ccpA* gene of *C. perfringens* with an insertionaly inactivated copy. The location of the *ccpA*-specific probe is shown beneath the N-terminal coding region of the *ccpA* gene.

strain in the absence of glucose (Table 2-1). Since the *ccpA*- strain shows a similar level of CR due to glucose as did the wild-type strain, it appears that CcpA does not mediate CR effects of glucose on sporulation. The presence of pSM257 in the wild-type strain caused a 26-fold reduction in sporulation when glucose was present, which is in the same range as the 10-fold decrease seen in the absence of glucose (Table 2-1). When compared to the *ccpA*- strain, however, the *ccpA*- strain with pSM257 exhibited a 10-fold increase in sporulation in the absence of glucose, but a 12-fold decrease seen in the absence of glucose (Table 2-1). We do not have an explanation for these contrasting results, but it is probably due to aberrant CcpA regulation due to multi-copy effects, since it was seen only at very low levels of sporulation with strains containing plasmid pSM257.

A *ccpA*- strain is able to utilize raffinose during growth in DSSM-raffinose sporulation medium.

The medium used to induce sporulation of strain SM101 contains 7.9 mM raffinose, which we have shown previously is necessary for efficient sporulation and CPE production by the parent strain of SM101, NCTC 8798 (218). One possibility as to why the *ccpA*- strain failed to sporulate efficiently in DSSM-raffinose medium was that it could not utilize the raffinose as a carbohydrate source to sporulate. This hypothesis was tested by measuring raffinose consumption by SM101 and the *ccpA*- strain during growth in DSSM-raffinose. These strains used raffinose at the same rate and to the same extent, beginning in late-exponential phase of growth (Figure. 2-2). Therefore, an inability to metabolize raffinose was not the reason the *ccpA*- strain failed to sporulate efficiently in DSSM-raffinose medium.

Table 2-1. Spore production by *C. perfringens* wild-type and *ccpA*- strains in sporulation medium in the presence or absence of 5 mM glucose.

Strain	- Glucose			+ Glucose		
	Total no. cfu	Spores	Sporulation	Total no. cfu	Spores	Sporulation
SM101	3.18x10 ⁷ +/- 2.06x10 ⁷	2.17x10 ⁷ +/- 2.17x10 ⁷	68.2%	2.86x10 ⁷ +/- 1.85x10 ⁷	9,520 +/- 4,450	0.0332%
SM101(pSM257)	1.11x10 ⁷ +/- 3.52x10 ⁶	7.24x10 ⁵ +/- 6.84x10 ⁵	6.52%	1.78x10 ⁷ +/- 1.19x10 ⁷	227 +/- 178	0.00127%
SM120	5.25x10 ⁶ +/- 4.50x10 ⁶	5.94x10 ⁴ +/- 1.00x10 ⁵	1.13%	5.56x10 ⁶ +/- 3.49x10 ⁶	78.3 +/- 56.4	0.00139%
SM120(pSM257)	9.14x10 ⁶ +/- 8.70x10 ⁶	1.05x10 ⁶ +/- 2.97x10 ⁶	11.5%	2.68x10 ⁷ +/- 3.11x10 ⁷	28.0 +/- 17.9	0.000105%

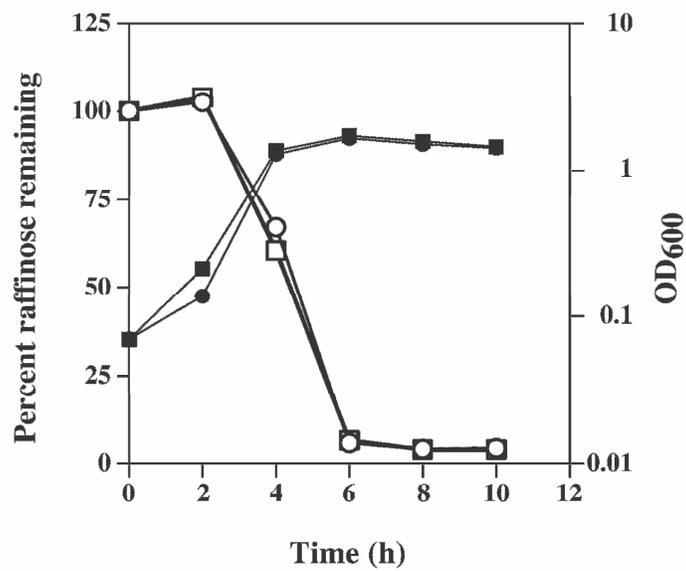


Figure 2-2. Growth (closed symbols) and utilization of raffinose (open symbols) by strains SM101 (wild-type) (squares) and SM120 (*ccpA*⁻) (circles). Representative data shown from one of two identical experiments.

Other sugars show different effects on sporulation by wild-type and *ccpA*- strains of *C. perfringens*.

Shih and Labbe (301) demonstrated that other sugars besides glucose could have a CR on sporulation in *C. perfringens*. Therefore, we examined the effects of the addition of 5 mM each of mannose, lactose and galactose had on sporulation by the wild-type and *ccpA*- strains of *C. perfringens* (Table 2-2). For the wild-type strain, mannose and lactose repressed sporulation 2,850-fold and 29-fold, respectively, in comparison to sporulation seen in the absence of sugars, while galactose stimulated sporulation 2.4-fold (compare top row in Table 2-2 to top row in Table 2-1). The extremely high sporulation efficiency seen with galactose, 167%, probably represents incomplete germination of spores in the experiment that measured the total cfu, in which the cells were not subjected to germination-inducing heat treatment (see Materials and Methods). Using the *ccpA*- strain, we saw that mannose repressed sporulation 5,650-fold in comparison to the same strain in the absence of sugars, while ≤ 10 spores/ml could be detected in the medium with added lactose and galactose (compare the bottom row in Table 2-2 to the third row in Table 2-1). The results seen with galactose were surprising, since galactose stimulated sporulation in the wild-type strain, as described above.

Transcription of the *cpe* gene is repressed in exponential phase but induced in stationary phase by CcpA in *C. perfringens* grown in sporulation medium.

We used a *cpe-gusA* protein fusion on a plasmid (pSM237) to measure *cpe* expression in wild-type and *ccpA*- strains of *C. perfringens*. Transcription and translation

Table 2-2. Sporulation by *C. perfringens* wild-type and *ccpA*- strains in the presence of sugars other than glucose.

Strain	Mannose			Lactose			Galactose		
	Total no. cfu	Spores	Sporulation	Total no. cfu	Spores	Sporulation	Total no. cfu	Spores	Sporulation
SM101	7.47×10^7 +/- 1.02×10^8	1.78×10^5 +/- 1.43×10^5	0.0239%	2.00×10^7 +/- 2.56×10^6	4.77×10^5 +/- 8.08×10^5	2.38%	2.23×10^7 +/- 2.00×10^7	3.02×10^7 +/- 1.02×10^7	166.7%
SM120	4.7×10^7 +/- 2.61×10^7	7.17×10^1 +/- 5.39×10^1	0.0002%	9.67×10^6 +/- 5.77×10^6	≤10	~0%	5.67×10^6 +/- 4.06×10^6	≤10	~0%

from the *cpe* promoter was measured as units of β -glucuronidase activity, which is the gene product of the *E. coli gusA* gene (see Materials and Methods). In early and mid-exponential phase the wild-type and *ccpA*- strains showed relatively low levels of β -glucuronidase activity (but not the same, see below) (Figure. 2-3A and 2-3B). Beginning in late log phase and extending throughout stationary phase, there was a dramatic induction in β -glucuronidase activity in the wild-type strain, but not in the *ccpA*- strain; the activity in the wild-type strain was as much as 230 times as high as that seen in the *ccpA*- strain. In contrast, in early and mid-exponential phase, the levels of β -glucuronidase activity in the *ccpA*- strain were actually about twice that seen in the wild-type strain (Figure 2-3C and 2-3D), suggesting that CcpA may act as a repressor of *cpe* transcription in exponential phase. The addition of 5 mM glucose to the sporulation medium prevented the strong induction seen in stationary phase in the wild-type strain and abolished the 2-fold difference seen in exponential phase between the *ccpA*- and wild-type strains (Figure 2-3C and 2-3D). The presence of glucose resulted in a slower growth rate in the *ccpA*- strain in comparison to the wild-type strain, but similar levels of growth were achieved by the time the cells reached stationary phase (Figure 2-3D). Since we observed that the *ccpA*- strain had higher levels of expression of the *cpe-gusA* fusion in exponential phase than did the wild-type strain in sporulation medium, we measured the levels of expression in PGY medium, which does not induce sporulation in *C. perfringens* (Figure 2-3E and 2-3F). Throughout the exponential phase, the *ccpA*- strain exhibited a 2-fold higher level of expression than did the wild-type strain, suggesting that CcpA can act as a repressor of *cpe* expression when the cells are growing in the vegetative state.

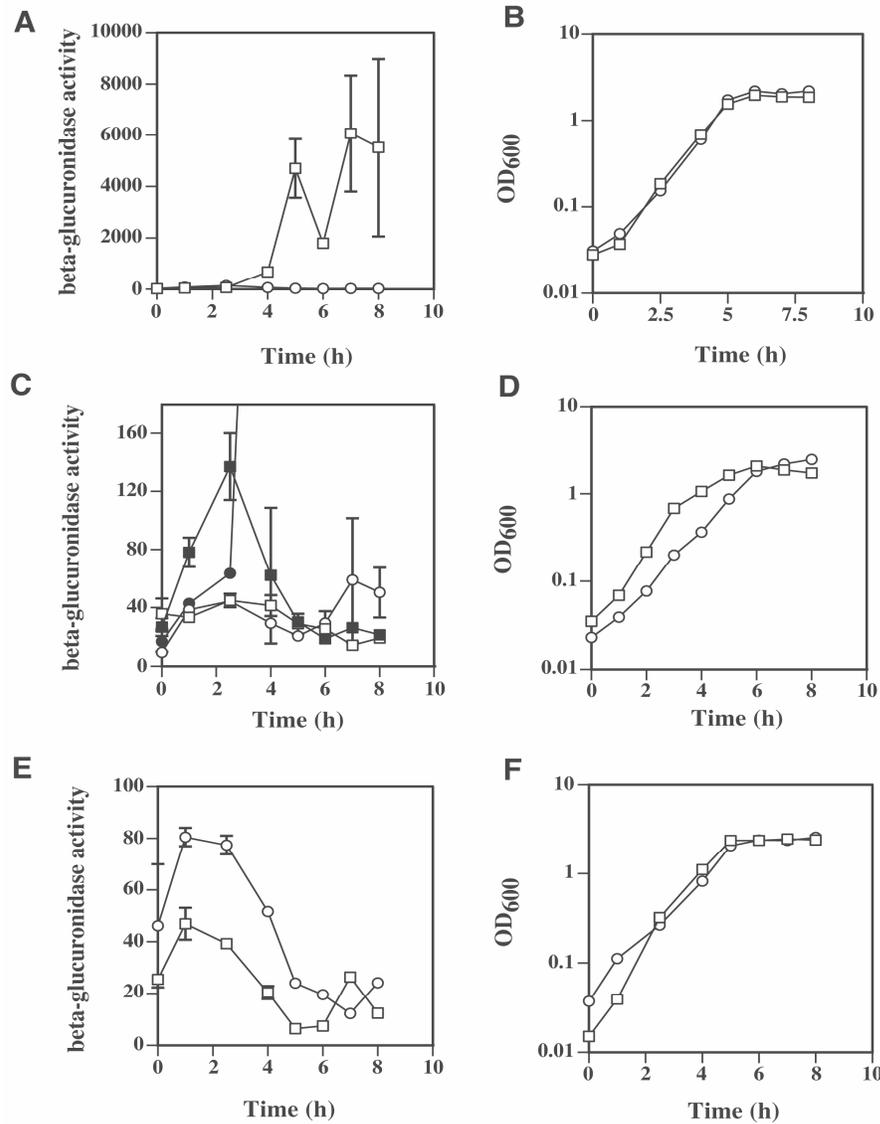


Figure 2-3. Expression from the *cpe-gusA* fusion in wild-type and *ccpA*- strains of *C. perfringens*. Expression (panel A) and growth (panel B) of the wild-type (squares) and *ccpA*- strain (circles) in DSSM sporulation medium. Expression (panel C) and growth (panel D) of the wild-type (open squares) and *ccpA*- strain (open circles) in DSSM sporulation medium with 5 mM glucose added to the medium. Superimposed in panel C are the values shown in panel A (closed symbols, with wild-type (squares) and *ccpA*- (circles)) so that direct comparisons can be made at these low levels of expression. Expression (panel E) and growth (panel F) of the wild-type (squares) and *ccpA*- strain (circles) in PGY medium. Note the difference in scale between panel A and panels C and E. The mean and SD of triplicate samples are shown in panels A, C, and E. Representative growth curves are shown in panels B, D, and F.

Regulation of synthesis of collagenase (kappa toxin) and polysaccharide capsule are dependent on CcpA, but PLC (alpha toxin) is not.

We examined whether other virulence factors besides CPE are subjected to CR by glucose and whether CcpA mediates these effects. In the absence of glucose the wild-type strain produced, on average, 12 times as much collagenase as the *ccpA*- strain (Figure 2-4A). Production of collagenase by *C. perfringens* was repressed by the addition of glucose to the medium, but this effect was not relieved in the *ccpA*- strain (Figure 2-4A).

Strain SM101 produces a polysaccharide capsule (data not shown), as does its parent strain, NCTC 8798 (Hobbs serotype 9) (60). The polysaccharide capsule of strain NCTC 8798 has been determined to be composed of glucose, galactose, and galactosamine in a molar ratio of 1:1.6:1.1 (60). Without glucose, the *ccpA*- strain produced about 1.2-fold more capsule than the wild-type strain (Figure 2-4B). With the addition of glucose, the *ccpA*- strain produced 2.8 times as much capsule material as the wild-type, suggesting CcpA exhibits glucose-mediated CR on capsule synthesis. In the wild-type strain, PLC synthesis increased about 3-fold with the addition of glucose but the *ccpA*- strain exhibited the same pattern and levels of activity, suggesting CcpA was not the mediator of the activation effect shown by glucose (Figure 2-4C).

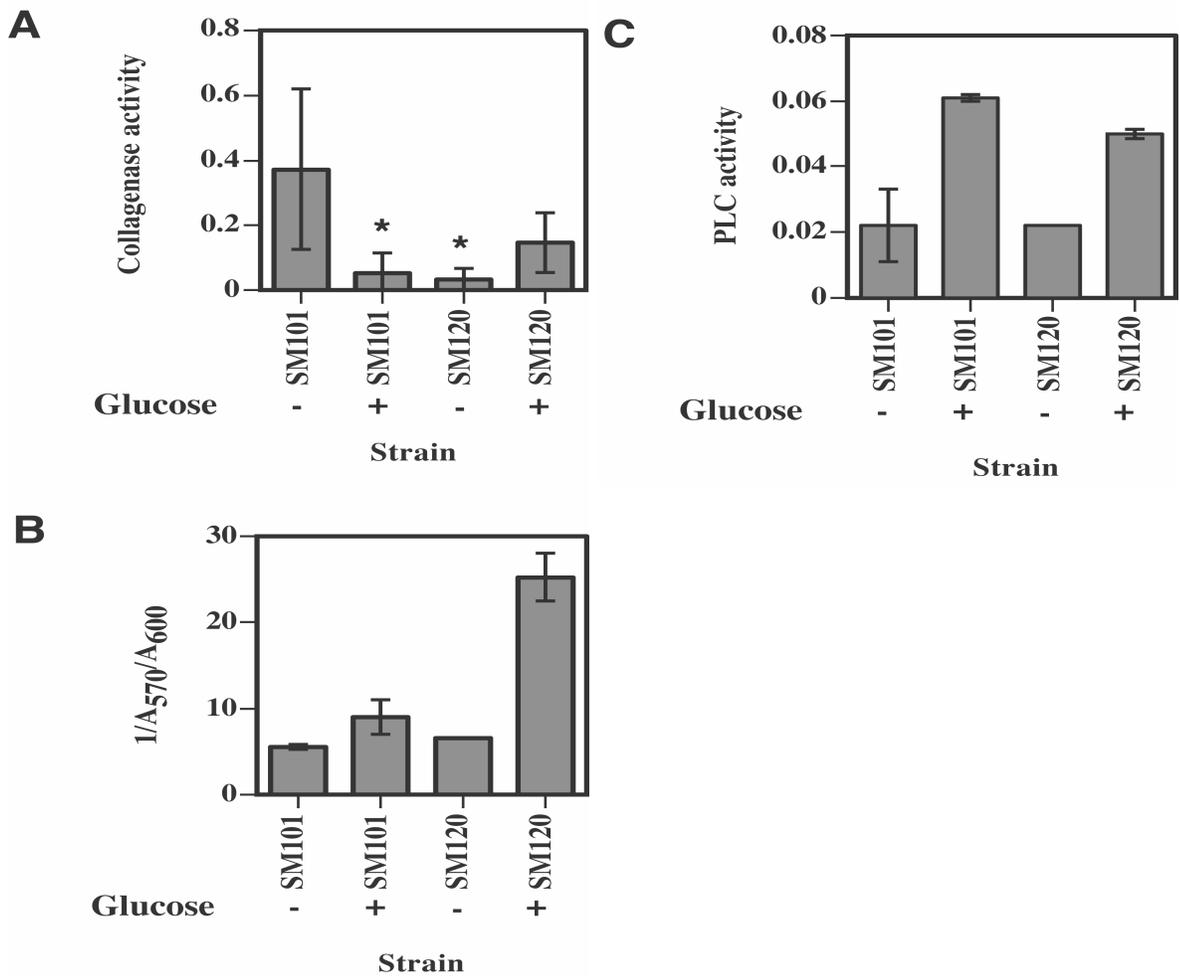


Figure 2-4. Levels of synthesis of collagenase (A), polysaccharide capsule (B), and PLC (C) in the wild-type (SM101) and *ccpA*- (SM120) strains under the conditions shown in the figure. For panels A and C, the medium was PGY or PY (PGY without glucose) while in panel B the medium was T-soy broth with 14 mM glucose added as indicated. Shown are the mean and SD of triplicate (panel A) and duplicate (panels B and C) sample values. The asterisk denotes a statistically significant difference ($P < 0.05$) between strain SM101 without glucose and the indicated values, using the *t*-test. There was no statistically significant difference in the values compared with any of the other possible combinations shown in panel A.

Discussion

The goal of this work was to investigate the role of the transcriptional regulator CcpA in CR of sporulation and enterotoxin synthesis in *C. perfringens*. The *ccpA* gene of *C. perfringens* was cloned and sequenced. It exhibited a high level of sequence homology (~70%) to other Clostridium CcpA orthologs, but considerably less homology (~40%) to CcpA orthologs from other low G-C Gram positive bacteria, including *B. subtilis*. An allele replacement strategy was used to introduce a mutation into the *ccpA* gene of *C. perfringens* and our analysis of this mutant suggests that CcpA regulates sporulation in a different manner than in *B. subtilis*: CcpA was necessary for efficient sporulation in *C. perfringens* (Table 2-1) whereas in *B. subtilis* it only mediates CR by glucose and is not directly involved in sporulation (57, 223).

The *ccpA*- strain sporulated at a frequency 60-fold less than the wild-type strain in the absence of glucose (Table 2-1). The sporulation medium we used in these experiments, DSSM, contains ~8 mM raffinose. This sugar has been shown to be necessary for efficient sporulation and CPE production by the parent strain (NCTC 8798) of the strain used here, SM101 (218). Typically, *C. perfringens* sporulation media, including DSSM, contain moderate amounts of nutrient-rich ingredients (e.g., proteose peptone and yeast extract) in combination with a slowly utilizable carbohydrate source (e.g., starch or raffinose) (169-172, 301). Therefore, carbohydrate metabolism appears to be an important part of the initiation and/or completion of sporulation by *C. perfringens*. However, the utilization rate of raffinose did not differ between the wild-type and *ccpA*-strains (Fig. 2-2), indicating there must be another CcpA-mediated effect that regulates sporulation. The question of why CcpA is necessary for sporulation remains to be

answered but one approach to solving the problem will be to look for second site mutations that restore sporulation to wild-type levels in the *ccpA*⁻ strain.

The addition of 5 mM glucose to the sporulation medium resulted in a ~2,000-fold reduction and ~800-fold reduction in sporulation by the wild-type and *ccpA*⁻ strains, respectively (Table 2-1). Since the amount of repression was similar in the two strains, we interpret these results to mean that CcpA did not mediate the glucose-mediated CR effect seen with sporulation. This parallels the CcpA-mediated CR effect by glucose seen in *B. subtilis*, where a *ccpA*⁻ strain was only partially derepressed for sporulation in the presence of glucose (57, 223). As mentioned in the Introduction, Moreno *et al.*, identified many genes in *B. subtilis* that were subject to CR by glucose in a CcpA-independent manner and found that most of these were involved in sporulation (229). A similar situation seems to exist in *C. perfringens*.

We attempted to complement the *ccpA*⁻ strain with a plasmid (pSM257) carrying a wild-type copy of the *ccpA* gene, but found that it only restored sporulation to 11.5%, in comparison to the 68.2% seen with the wild-type strain (Table 2-1). We believe the partial complementation was due to a multicopy effect, where abnormally high levels of CcpA in the cell activated or repressed genes involved in sporulation in an inappropriate manner, leading to lower levels of sporulation. This hypothesis was supported by the results seen when pSM257 was transformed into the wild-type strain and found to repress sporulation to 6.52%, similar to that seen with the complemented *ccpA*⁻ strain (Table 2-1). While failing to adequately complement the *ccpA*⁻ mutation, the results with pSM257 do provide support for our hypothesis that CcpA is directly involved in sporulation in the absence of glucose.

As seen with glucose, the addition of mannose, lactose and galactose showed decreasing levels of CR on sporulation in the *C. perfringens* wild-type strain, in the order: mannose or glucose > lactose > galactose (Table 2-1 and Table 2-2). In fact, galactose provided a 2.4-fold increase in sporulation efficiency in comparison to the wild-type strain. In the *ccpA*⁻ strain, the results were very different, where sporulation was much lower under all conditions but the level of CR was, in order : lactose or galactose > mannose > glucose (Table 2-1 and Table 2-2). One model to partially explain these results is shown in Fig. 2-5A. In this model, the presence of sugars signals to an unknown regulator (X in the figure) to suppress sporulation. CcpA has dual roles in this model, where it can negatively affect the activity of X and it can act directly on the sporulation cycle as an activator. Therefore, in a *ccpA*⁻ strain, X would be derepressed and CcpA would not activate sporulation genes, leading to a powerful repressive effect on sporulation.

During sporulation, the level of transcription of the *cpe* gene generally was in agreement with the amount of sporulation that occurred under each condition (Table 2-1 and Fig. 2-2). By far the highest amount of *cpe* transcription occurred during sporulation by the wild-type cells in the absence of glucose. This is consistent with our previous results where the highest level of *cpe* induction always occurred in concert with sporulation (217, 218, 370). Transcription of the *cpe* gene was greatly reduced in the *ccpA*⁻ strain in the absence of glucose and in both strains in the presence of glucose (Fig. 2-3A, 2-3C, 2-3E). During the vegetative stage of growth in sporulation medium or in growth in PGY, although low, the *cpe* promoter was twice as active in the *ccpA*⁻ strain than in the wild-type strain (Fig. 2-3C, 2-3E). This is the first time, to our knowledge,

that a regulator of *cpe* transcription during vegetative growth has been identified. A model summarizing the effects of CcpA on *cpe* transcription is shown in Fig. 2-5B. During vegetative growth CcpA acts directly or indirectly to repress *cpe* transcription. However, under sporulating conditions, CcpA is an activator of sporulation functions which leads to synthesis of active forms of the mother cell-specific sigma factors, SigE and SigK. We have identified 3 promoters upstream of *cpe* that are responsible for the majority of sporulation-dependent transcription and the -10 and -35 promoter recognition sequences of these promoters have a high level of homology to consensus -10 and -35 recognition sequences from σ^E - and σ^K -dependent promoters identified in *B. subtilis* (115, 370). Therefore, our hypothesis is that CcpA activates *cpe* transcription during sporulation indirectly by activating or de-repressing genes that lead to the synthesis of SigE and SigK (Fig. 2-5B).

We also compared the *ccpA*- and wild-type strains for differences in expression of the toxin collagenase. Collagenase activity was repressed by glucose in the wild-type strain but not in the *ccpA*- strain, but CcpA was needed for expression even in the absence of glucose (Fig. 2-4A). This suggests that other factors are involved in regulating collagenase activity. Transcription of the gene encoding the collagenase of *C. perfringens*, *colA*, has been shown to be regulated by the global two-component regulatory factors VirR/VirS as well as a regulatory RNA, VR-RNA (25, 27, 303). It may prove valuable to determine if these alternative regulators are subjected to CR effects mediated by CcpA.

The role of the capsule as a virulence factor in *C. perfringens* pathogenicity has been controversial (309). Polysaccharide capsule synthesis was induced only 1.2-fold by

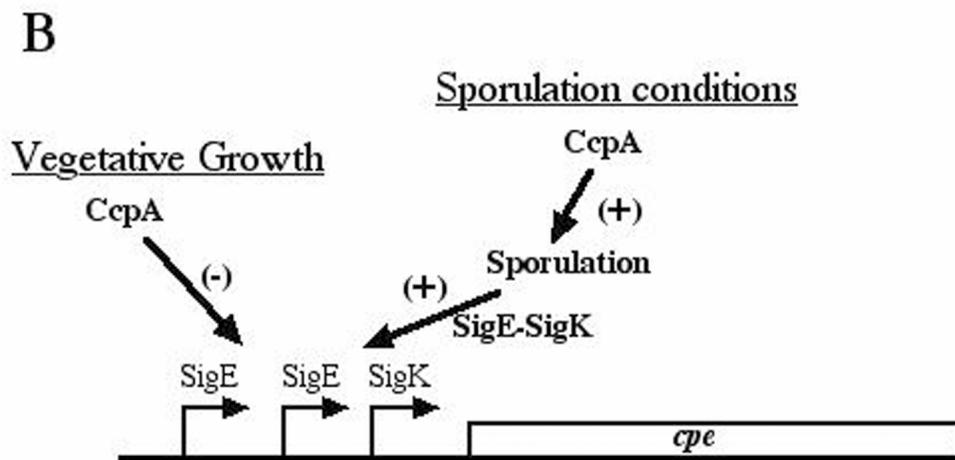
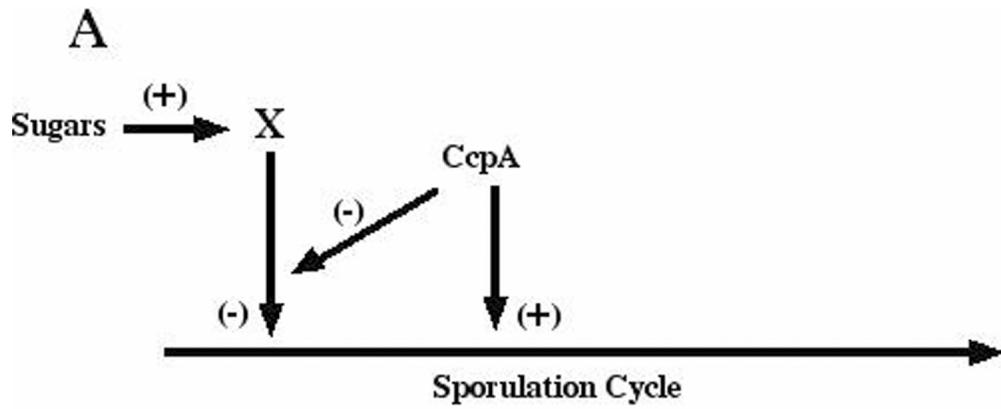


Figure 2-5. Model illustrating the involvement of CcpA in regulation of sporulation (A) and *cpe* transcription (B). See text for detailed descriptions of the models.

the addition of glucose to the medium in which the wild-type strain was grown but was induced 3.8-fold in the *ccpA*- strain when glucose was added (Fig. 2-4B). This suggests that capsule synthesis is more strongly induced by glucose in the absence of CcpA. Since glucose is one of the components of the capsule of strain SM101 (60), it is not surprising that it induced the synthesis of more capsular material but the regulatory mechanism appears to involve more functions than CcpA.

Synthesis of PLC, the phospholipase/sphingomyelinase of *C. perfringens* was induced about 3-fold by the addition of glucose to the medium, but this effect was independent of CcpA. As with *colA*, transcription of the *plc* gene has been shown to be regulated by VirR/VirS and VR-RNA (25, 27, 189, 303). Since PLC has been shown to be the most important virulence factor in gas gangrene infections caused by *C. perfringens* (22, 23, 323), it would be of interest to determine the mechanism of glucose-mediated induction of this virulence factor.

In summary, we have identified CcpA as an important regulator that is necessary for sporulation and CPE production in *C. perfringens*. This could provide a valuable insight into potential therapeutic strategies to block sporulation and CPE production and relieve the symptoms of patients infected with non-food-borne enteritis caused by *C. perfringens*.

Materials and Methods

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table 2-3. *C. perfringens* was grown in a Coy anaerobic chamber (Coy Laboratory Products) in PGY medium (30 g of proteose peptone, 20 g of glucose, 10 g of yeast extract and 1 g of sodium thioglycolate per liter) as described (218). *E. coli* was grown in Luria-Bertani broth (LB) (10 g of tryptone, 5 g of NaCl and 5 g of yeast extract per liter) on plates or in liquid.

Sporulation assays were done based on previously described methods (16, 171). Briefly, overnight cultures grown at 37 °C in fluid thioglycollate medium (FTG) were added to prewarmed serum bottles containing 50 ml of Duncan-Strong sporulation medium with raffinose (DSSM), which contained, per liter: 4 g yeast extract, 15 g proteose peptone, 5.4 g Na₂HPO₄, 1g sodium thioglycollate, and 4 g raffinose (171). After 24 hours, the cultures grown in DSSM were serially diluted, and plated on PGY medium to determine the total number of colony forming units (cfu). To determine the number of spores in the culture, samples were also heated at 75° C for 15 minutes prior to plating on PGY medium. In order to ascertain the effect of sugars on sporulation, stock solutions of each sugar were added to give a final concentration of 5 mM.

Cloning the *ccpA* gene of *C. perfringens*.

The published sequences of *ccpA* genes from several *Bacillus* species and the sequence encoding the CcpA homolog from *Clostridium acetobutylicum* (named RegA) by Davison *et al.* (80) were used to design two degenerate primers to amplify, by PCR, a ~250 base pair region near the N-terminus of the *ccpA* gene of *C. perfringens* strain

Table 2-3. Strains and plasmids used in this study.

Strain/Plasmid	Relevant characteristics	Source or reference
<u><i>E. coli</i></u>		
DH10B	F- <i>mcrA</i> D(<i>mrr-hsdRMS-mcrBC</i>) F80 <i>dlacZ</i> ΔM15 <i>ΔlacX74 deoR recA1</i> <i>araD139 Δ(ara, leu)7697</i> <i>galU galK λ- rpsL endA1</i> <i>nupG</i>	Gibco/BRL Corp
HB101	<i>supE44 ara14 galK2 lacY1</i> <i>Δ(gpt-proA)62 rpsL20</i> (Str ^r) <i>xyl-5 mtl-1 recA13</i> <i>Δ(mcrC-mrr) HsdS-(r-m-)</i>	D. Ohman
<u><i>C. perfringens</i></u>		
NCTC 8798	Cpe+	C. Duncan
SM101	High frequency electroporation derivative of NCTC 8798	(370)
SM120	<i>ccpA</i> - derivative of SM101	This study
<u>Plasmids</u>		
pBluescript SK-	<u>Parent/relevant genotype</u>	Stratagene Corp.
pBluescript SK+		Stratagene Corp.

pSF3	Source of <i>mob</i> region	D. Ohman
pJIR750	<i>C. perfringens</i> - <i>E. coli</i> shuttle vector	(26)
pJIR751	<i>C. perfringens</i> - <i>E. coli</i> shuttle vector	(26)
pYZ67	pBluescriptSK- <i>catP</i>	This study
pSM300	pBluescript SK- <i>ermBP</i>	This study
pSM300M	pSM300/mobilization (<i>mob</i>) region from pSF3	This study
pSM310	pBluescript SK- <i>ccpA</i>	This study
pSM310Cm	pSM310/ <i>catP</i>	This study
pSM225	pSM300M/ <i>ccpA-catP</i>	This study
pSM237	pJIR751/ <i>cpe-gusA</i> fusion	This study
pSM257	pJIR751/ <i>ccpA</i>	This study
pDOB13	pJIR751/ <i>ccpA</i>	This study

NCTC 8798 (S. B. Melville, unpublished results). After DNA sequencing confirmed the fragment was part of the *ccpA* gene, the fragment was used as a probe for Southern blot analyses of chromosomal DNA isolated from strain NCTC 8798. For Southern blot analyses, 10 µg of chromosomal DNA was digested to completion, and agarose gel electrophoresis and transfer to a nylon membrane were done as previously described (291). To detect hybridization to the probes, the Phototope Star detection system was used according to the manufacturers' instructions (New England Biolabs). A single 3.8 kb HindIII fragment was found to hybridize to the probe. A plasmid library was constructed in *E. coli*, containing *C. perfringens* Hind III-digested chromosomal DNA fragments 3-5 kb in size, cloned into the vector pBluescript SK+. *E. coli* clones containing the library were then screened by colony hybridization using the ³²P-labelled *ccpA* gene fragment as probe. One clone was identified that contained a 3.8 kb HindIII insert, and named pSM310. Sequencing of the insert showed it contained the entire *ccpA* gene and flanking sequences (Figure 2-1A).

Plasmid constructs.

The 3.8 kb HindIII fragment from pSM310 was subcloned into the *E. coli*-*C. perfringens* shuttle vector, pJIR751 (26) to make plasmid pDOB13. Plasmid pDOB13 was digested with PacI and KpnI, the overhanging ends were removed by T4 DNA polymerase, and the plasmid self-ligated. This gave plasmid pSM257, which contains only the *ccpA* gene and its promoter region (Figure 1A). Plasmid pYZ67 was made by PCR amplification of the *catP* gene from plasmid pJIR750 (26), using primers with an EcoRI site at the upstream end and HindIII at the downstream end, digesting the PCR product with EcoRI and HindIII and ligating the *catP* gene to pSK- at the EcoRI-HindIII

sites of the polylinker. Plasmid pSM310Cm was made by cloning the EcoRI-Hind III *catP* gene fragment from pYZ67, blunt ended using the Klenow fragment of DNA polymerase, and ligating it to pSM310 digested with NdeI that also had the ends filled in using the Klenow fragment. The unique NdeI site in pSM310 lies near the center of the *ccpA* gene (Fig. 1A). The entire 5.1 kb *ccpA-catP* construct was cloned into the mobilizable suicide vector pSM300M by digesting pSM310Cm with BamHI and Sall, isolating the *ccpA-catP* fragment and ligating it to BamHI-Sall digested pSM300M, to make pSM225. pSM300M was made in two steps. First, the *bla* gene from pBluescript SK- was replaced with the *ermBP* gene from pJIR751 by ligating the blunt ended *ermBP* gene fragment into DraI (at position 1912) and SspI (at position 442) digested pBluescript SK-, to create pSM300. Next, the *mob* locus from plasmid pSF3 (297) cut with EcoRI and BglII, was blunt ended and ligated to pSM300 (which had been partially digested with PvuII) at the PvuII site at position 997, to make pSM300M.

The *cpe-gusA* fusion vector, pSM237, was made by digesting pSM104 (218), which encodes chloramphenicol resistance, with PstI and EcoRI and isolating the fragment containing the *cpe-gusA* gene fusion. This fragment was then ligated to the *E. coli-C. perfringens* shuttle vector, pJIR751 (26), which encodes erythromycin resistance, that had been digested with PstI and EcoRI. Plasmid pSM237 was used to quantify *cpe* promoter activity by measuring β -glucuronidase enzyme activity, the product of the *gusA* gene (146).

Construction of a *ccpA* mutation in *C. perfringens*.

A *ccpA*- mutant strain was constructed using allele replacement techniques by conjugational transfer of plasmid pSM225 from *E. coli* into *C. perfringens* strain SM101

(370). pSM225 lacks an origin of replication that functions in *C. perfringens*. The recombination strategy used is shown in Fig. 1B. First, plasmid pSM225 was transformed into *E. coli* strain HB101 carrying plasmid pRK2013 (93). pRK2013 provided all of the *tra* gene functions in trans to mobilize pSM225 into *C. perfringens*. Plasmid pSM225 was conjugationally transferred by biparental mating of *E. coli* strain HB101 carrying pRK2103 and pSM225 with *C. perfringens* strain SM101. Conjugation was carried out on filters at a ratio of donor to recipient of 100:1. Filters were then placed on PGY plates and incubated in an anaerobic chamber overnight. The following day, the filters were washed with PGY liquid media to detach the bacteria, the cells pelleted by centrifugation and plated on the *C. perfringens* selective media, tryptose sulfite cycloserine (TSC) agar, containing erythromycin (30 mg/l) and chloramphenicol (20 mg/l). Several transformants were isolated from the mating. Two of these transformants were then grown in PGY liquid cultures with chloramphenicol only, and spread on PGY chloramphenicol plates. Replica plating techniques were used to screen >3,000 colonies for the loss of erythromycin resistance and maintenance of chloramphenicol resistance, the pattern expected if the cell had undergone the double-recombination event shown in Fig. 1B. Two erythromycin-sensitive chloramphenicol-resistant isolates were obtained, and results are described for one of them, SM120.

Enzyme and polysaccharide capsule assays.

Cell cultures grown for extracellular toxin assays were incubated in either PGY or PY (PGY without added glucose) media. All cultures were inoculated with a 1% aliquot of a culture grown overnight in PGY and grown to late log phase before samples were removed for toxin assays. Alpha toxin activity was measured through a slightly modified

version of a previously published method (322). Ten microliter samples of the appropriate filtered cell culture supernatants were added to 100 μ l of a reaction mixture containing 1.0 mM CaCl₂, 0.1 mM ZnCl₂ and 10 mM *p*-nitrophenylphosphorylcholine (NPPC) in phosphate buffered saline (pH 7.2). Reaction mixtures were incubated at 37 °C for one hour, then quenched with 1 ml cold 0.02 N NaOH, and the A₄₁₀ determined.

Kappa toxin activity assays were based on previously described methods (24, 196). Overnight cultures of *C. perfringens* were transferred into 50 ml of either PGY or PY at a 1% inoculum, and incubated anaerobically for 4 hours at 37 °C. The cultures were centrifuged and the supernatants collected into 250 ml centrifuge bottles.

Ammonium sulfate (Fisher Scientific) was added to the solution to saturation to precipitate proteins. After 1 hour incubation at room temperature, the samples were centrifuged at 10,000 x *g*, and the supernatants poured off. The pellets were resuspended in 25 ml of sodium borate buffer (0.2 M boric acid, 0.15 M NaCl), and transferred to 50 ml centrifuge bottles. Ammonium sulfate was added again until saturation was achieved, at which time samples were incubated for 1 hour at room temperature. Upon centrifugation, the supernatant was removed, and the pellet resuspended in 500 μ l of sodium borate buffer supplemented with 30 μ M ZnCl₂. These concentrated protein solutions were dialyzed four times against 1 l volumes of zinc-supplemented sodium borate buffer. After dialysis, collagenase activity was determined by measuring the release of azo-dye from Azocoll (Sigma). For each sample, 6 mg of Azocoll was placed in a microcentrifuge tube, and washed with sodium-borate buffer. To each tube, 400 μ l of concentrated culture supernatant was added, along with 800 μ l of sodium-borate buffer. The reactions were incubated for 3 hours at 37° C with horizontal shaking. After

incubation, samples were centrifuged at 13,000 x g for 15 minutes, and the supernatants transferred to new tubes. The A₅₉₅ for each sample was determined in a Thermospectronics Genesys spectrophotometer. Specific activity for collagenase is shown as A₅₉₅ units per mg of protein. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, California)

β-glucuronidase enzyme assays to detect *cpe-gusA* transcriptional activity in *C. perfringens* were done as previously described (218). Glucose was added to the growth medium at a final concentration of 5 mM as described for some experiments.

The levels of polysaccharide capsule produced by SM101 and the *ccpA* mutant were measured by the ability of cellular polysaccharides to bind trypan blue by the method previously published by Black and Yang (39) with slight modifications. Cells were grown overnight in T-Soy broth with and without 14 mM glucose (Difco, Detroit, Michigan), and transferred into fresh media at a 5% inoculum size. After 4-6 hours of growth, cultures were centrifuged, the pellets washed once with modified MOPS (morpholinepropanesulfonic acid) buffer (10 mM MOPS (pH 7.6), 2 mM MgSO₄, 1 mM CaCl₂), and resuspended in 3.5 ml of modified MOPS buffer, and the OD₆₀₀ was obtained for each sample. Duplicate assays were performed by adding 1.35 ml of cell suspension to a 2 ml microcentrifuge tube with 150 μl of a 50 μg/ml trypan blue solution (Biowhittaker, Walkersville, MD). The tubes were incubated with horizontal shaking for 30 minutes at room temperature, and centrifuged at 13,000 x g. For each sample, 200 μl of the supernatant was loaded in triplicate in a 96-well plate, and the A₅₇₀ was obtained using a SPECTRAfluor plus plate reader (Tecan, Salzburg, Austria). The amount of dye bound (the more dye that bound to the capsule, the lower the A₅₇₀ observed) was divided

by the OD₆₀₀ values for each sample to give dye binding units as A₅₇₀/OD₆₀₀. In order to provide a positive correlation to the amount of capsule material, the results were presented as the ratio 1/A₅₇₀/OD₆₀₀ in Fig. 2-4

Measurement of raffinose utilization.

Raffinose concentrations were determined as described in protocols published by Boehringer Mannheim for the UV spectroscopic determination of raffinose in foodstuffs (catalog number 428167). Samples were taken from the cell cultures at the designated times. Supernatants containing soluble carbohydrates were separated from cells by centrifugation at 10,000 X g for five minutes, followed by filtration through a 0.2 µm syringe filter. Filtered supernatants were stored at -20 °C until analyzed.

Nucleotide sequence accession number.

The *C. perfringens ccpA* gene sequence from strain SM101 has been deposited in Genbank, with the accession number AF309566. Since the time of this submission, the entire sequence of the genome of strain SM101 has been determined by The Institute of Genomic Research (<http://www.tigr.org/tdb/mdb/mdbinprogress.html>).

Acknowledgments

We thank Wesley Black for assistance with the polysaccharide capsule assays. This work was supported by grants #98-02844 and #2000-02621 from NRICGP/USDA awarded to S.B.M

**Chapter 3 - Type IV pili-dependent gliding motility in the
Gram-positive pathogen *Clostridium perfringens* and other
*clostridia***

This work has been submitted for publication with *Molecular Microbiology*. Movies referenced in this work are available at <http://www.blackwell-synergy.com/loi/MMI>

Abstract

Bacteria can swim in liquid media by flagella rotation and can move on surfaces via gliding or twitching motility. One type of gliding motility involves the extension and retraction of type IV pili (TFP), which pull the bacterium in the direction of attachment. TFP-dependent gliding motility has been seen in many Gram-negative bacteria but not in Gram-positive bacteria. Recently, the genome sequences of three strains of *C. perfringens* have been completed and we identified gene products involved in producing TFP in each strain. Here we show that *C. perfringens* produces TFP and moves with an unusual form of gliding motility involving groups of densely packed cells moving away from the edge of a colony in curvilinear flares. Mutations introduced into either the *pilT* or *pilC* genes of *C. perfringens* abolished motility and surface localization of TFP. Genes encoding TFP are also found in the genomes of all nine clostridial species sequenced thus far and we demonstrated that *Clostridium beijerinckii* can move via gliding motility. It has recently been proposed that the clostridia are the oldest Eubacterial class and the ubiquity of TFP in this class suggests that a clostridia-like ancestor possessed TFP, which evolved into the forms seen in many Gram-negative species.

Introduction

Bacterial swimming motility in liquid is mediated by flagella rotation, but bacteria have evolved other mechanisms for moving across surfaces. These include gliding and twitching motility, terms more relevant to the type of motion seen under a microscope than the underlying mechanism of movement (197, 200). Twitching motility is widespread in Gram-negative bacteria, including pathogens such as *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae* and the anaerobic bacterium *Dichelobacter nodosus* (197). Gliding motility in Gram negative bacteria is also seen in the social (S) motility of *Myxococcus xanthus* and some cyanobacteria (197, 200). Each of these motility systems has been shown to depend on the production of type IV pili (TFP). TFP are thin filaments that are extended out from the cell, attach to a surface and then are retracted back into the cell, thereby pulling the bacterium in the direction of the point of attachment (50, 243, 360). While flagella-mediated swimming is wide-spread among Gram-positive bacteria, gliding motility via TFP has not been directly observed in these bacteria. Pili have recently been described on the surface of the Gram-positive pathogens *Corynebacterium diphtheriae*, *Streptococcus pyogenes* and *Streptococcus pneumoniae*, but these pili appear to be anchored to the peptidoglycan cell wall in a sortase-dependent fashion and are not retractable (174, 228, 339). There are older reports that *Streptococcus sanguis/parasanguis* possess pili, and colony migration was seen on agar plates (94, 106, 131). However, whether individual bacteria exhibited twitching motility was not reported and later references to pili in *S. parasanguis* do not discuss the twitching motility phenotype (362).

The Gram-positive anaerobic pathogen *Clostridium perfringens*, which causes gas gangrene and food poisoning (281) has previously been described as a nonmotile bacterium (55). Recently, the complete genome sequences of three different strains of *C. perfringens* have been determined: strains 13, ATCC 13124, and SM101 (234, 303). Annotated lists of the potential proteins encoded on the genomes indicated a complete lack of flagella proteins and chemotaxis-associated gene products (234, 303). However, genes that might encode the constituents of TFP (such as *pilA-D*, *pilT*, etc.) were identified in each of the three sequenced strains. Therefore, we examined these strains for a motility phenotype and found that each one is capable of gliding motility in a TFP-dependent manner. These results represent the first definitive example of a Gram-positive bacterium utilizing TFP for gliding motility. We also present evidence suggesting that all nine clostridial species in which a genome sequence has been determined are capable of TFP-dependent motility, including such pathogens as *C. botulinum*, *C. difficile*, and *C. tetani*. These finding will necessitate a new approach to understanding the host colonization and invasive capacity of pathogenic clostridia.

Results

Type IV pili-encoding genes in *C. perfringens*

We compared the putative TFP biosynthesis genes in the genome sequences of the three sequenced strains (strain 13, SM101 and ATCC 13124) and found a high degree of synteny and sequence similarity in these gene regions (Fig. 3-1). Three separate loci were identified in which TFP-associated genes were present. These include: (1) a putative monocistronic *pilT* gene (Fig. 1A), (2) a putative four-gene operon containing *pilB* and *pilC* genes followed by two coding regions encoding proteins of unknown function (Fig. 3-1B), (3) a set of 12-14 genes comprising two potential transcription units, a monocistronic *pilA1* gene and a large operon beginning with a *pilD* gene (Fig. 3-1C), which encodes a putative pre-pilin peptidase. Downstream of the *pilD* gene are second copies of *pilB* and *pilC* genes, followed by a second *pilA* gene (*pilA2*). The *pilM*, *pilN* and *pilO* genes encode proteins whose precise function are unknown but are associated with production of a functional TFP (195), pilin glycosylation (307) and secretin assembly (243). ORFs 2280-2277 of strain 13 encode proteins of unknown function, but the N-terminal region of the first three proteins contain conserved motifs associated with pili assembly or function: CPE 2280 has a PilV conserved motif, CPE 2279 has a PulG conserved motif, and CPE2278 has a PilW motif. The presence of these motifs indicates the gene products could play a role in pilin biosynthesis or protein secretion.

Genes encoding the major structural component of TFP filaments, PilA, were identified by examining the sequence in the ~60 amino acid N-terminal region, which includes the site where pre-pilin peptidase (PilD) cleaves the PilA proteins during the secretion process (197).

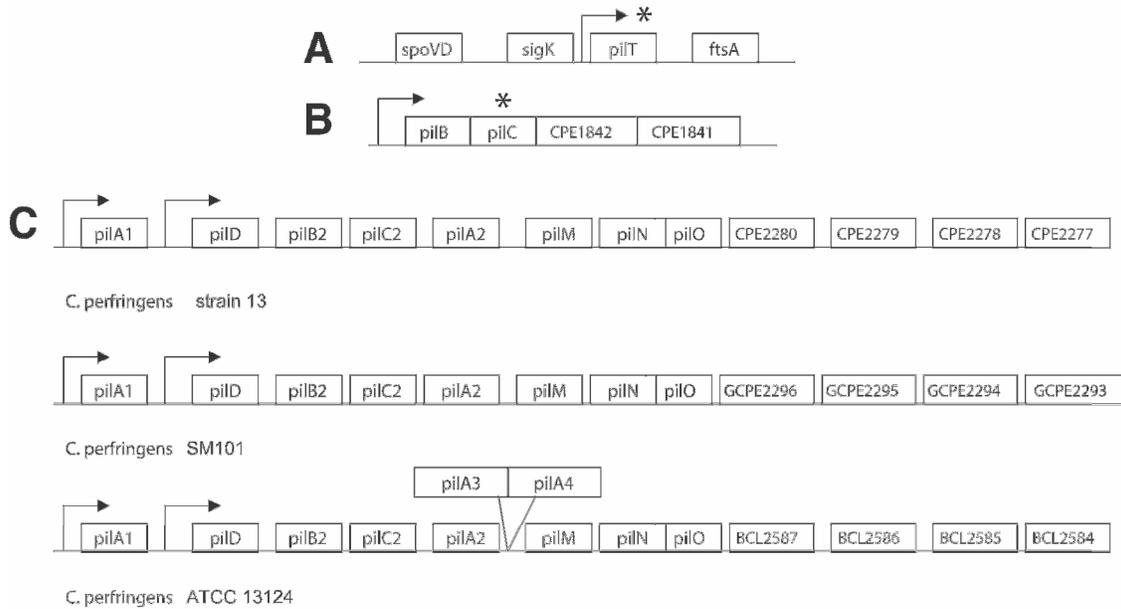


Figure 3-1. *C. perfringens* genes involved in the synthesis or function of TFP. A. *pilT* gene; B. *pilB* operon; C. large pilin locus. The gene order shown in A and B was identical in the three strains. For the *pilB* operon (B), the numbering system for the ORFs shown refers only to strain 13, the other strains have different numerical designations for the orthologs shown in the figure. Arrows indicate putative transcriptional start sites for the operons shown in the figure. Asterisks denote genes disrupted by insertion mutagenesis procedures.

While two *pilA* genes were identified in strains 13 and SM101, four were recognized in strain ATCC 13124 (Figure 3-1C).

The genome sequences also indicate that PilQ orthologs are absent in these strains. PilQ is a secretin family protein found in the outer membrane of Gram-negative bacteria where it forms a multimeric pore (243). The ring-like PilQ oligomer functions to both regulate the extension of the pilus through the outer membrane by opening and closing and also to act as a stabilizer for the pilus-outer membrane point of contact (243). The lack of this protein in the Gram-positive bacterium *C. perfringens* is logical, since it lacks an outer membrane, as do all Gram-positive bacteria.

Characteristics of *C. perfringens* motility

Since each of the three sequenced strains of *C. perfringens* contained sufficient genes to encode a complete TFP and the appropriate accessory proteins (Figure 3-1), we screened for motility by placing a suspension of each bacterial strain on brain heart infusion (BHI) agar medium. We then observed that the edge of each colony moved away from the source of the initial inoculum, with the spreading bacteria apparent as thick mucoid extensions (Figure 3-2).

The characteristics of individual cell movement by *C. perfringens* strains were determined by recording video microscopic images of the bacteria on BHI agar surfaces under anaerobic conditions and with a heated stage (set at 40°C). The bacteria were not motile under aerobic conditions and the rate of motility was very slow at room temperature (22°C). Single bacteria were never seen to move with any strain examined. When the edge of a colony from strain 13 (as well as strains SM101 and ATCC 13124) was observed, the bacteria could be seen moving away as multicellular flares that are

attached at the base to the colony and nearly always develop a curvilinear shape (inset Fig. 3-2A and Movie S1). Measurements of the rate of movement of the leading edge of the flares in strain 13 (see Experimental procedures) showed an average rate of 85 $\mu\text{m}/\text{h}$. The continued migration of the curved flares usually resulted in the flare making contact with the leading edge of the colony or another flare, thus giving the impression of engulfment of the intervening space by the bacterial movement (Movie S1). Subsequently, the engulfed region would be filled by movement and growth by the surrounding bacteria (Movie S1). Although the two bacteria are phylogenetically very distantly related, *C. perfringens* motility has some similarities in appearance and mobility rates to that seen with TFP-dependent S-motility in *M. xanthus* (333).

***C. perfringens* PilA1 and PilA2 are predicted to have protein folds highly similar to pilin proteins found in Gram-negative bacteria**

The pilin proteins comprising the major subunits of TFP are unusual in that they exhibit a very large degree of sequence divergence after the first 60 amino acids but maintain a similar 3 dimensional fold (21). In fact, this appears to be the case with the PilA1 and PilA2 proteins from strain 13, which have only 27% identity in their amino acid sequences (data not shown). In order to determine if the *C. perfringens* PilA proteins from the sequenced strains might have folds similar to those seen in pilins from Gram-negative bacteria, we used the FUGUE protein prediction proGram (368) to compare the *C. perfringens* PilA proteins to those in the database (available on the FUGUE server at <http://www-cryst.bioc.cam.ac.uk/~fugue/>). All of the PilA proteins from the three *C. perfringens* strains were predicted to have matches to structures in the database with a confidence level >99% (Table 3-1). The PilA1 predicted fold had only a single match, to

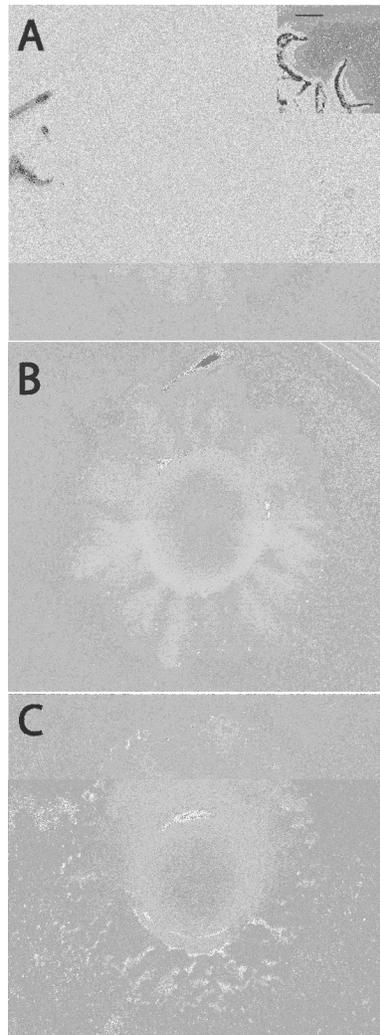


Figure. 3-2. Movement of *C. perfringens* on agar plates.. A. Strain 13; B. strain ATCC 13124; C, strain SM101. Central rings of cells in each panel is ~ 0.8 cm in diameter, which shows the location of the initial boundary of the inoculum. Inset in panel A: Edge of a colony of strain 13 on BHI plates, showing the curvilinear flares at the leading edge of the colony (image obtained from Movie S1). Bar = 12 μ m.

Table 3-1. Predicted structural homologs to *C. perfringens* PilA proteins identified using the FUGUE structural prediction proGram (368).

Pilin protein	Structural homologs with >99% confidence		
	1T92 ^a	1HPW, 1DZO ^b	2PIL ^c
strain13-PilA1	X		
strain 13-PilA2	X	X	X
SM101-PilA1	X		
SM101-PilA2	X	X	X
ATCC 13124-PilA1	X		
ATCC 13124-PilA2	X	X	X
ATCC 13124-PilA2A		X	X
ATCC 13124-PilA2B	X	X	X

a. PulG (pseudopilin) from *Klebsiella pneumoniae* (163)

b. Pilins from *P. aeruginosa* (127, 272)

c. *N. gonorrhoeae* phosphorylated-pilin (258)

the pseudopilin PulG from *Klebsiella pneumoniae*, while the PilA2 proteins had matches to PulG and pilins from *P. aeruginosa* and *N. gonorrhoeae* (Table 3-1). The significance of the match to the PulG pseudopilin is unknown, since pseudopilins, to our knowledge, have not been previously described in Gram-positive bacteria. It may be that the pseudopilin fold is similar enough to the pilin folds for *C. perfringens* pilins to have a similar (predicted) structure.

Visualization of type IV pili

The presence of pili on the surface of the *C. perfringens* strains was seen when the cells were observed in a field emission-scanning electron microscope (FE-SEM). The pili produced by strain 13 (Fig. 3-3A) are similar in appearance to those produced by strains ATCC 13124 and SM101 (Fig. 3-4). They are thin (5-7 nm in width), relatively short in length (200-300 nm), appear to be stiff (i.e., they do not show much curvature over their length) and cover the entire surface of the cell (Fig. 3-3A). We identified two genes in strain 13 that encode the major subunits of the type IV pili, PilA (Fig. 3-1C), called *pilA1* and *pilA2*. In order to determine if these pilin proteins are incorporated into the pili seen on the surface of strain 13 in FE-SEM images, we had peptides specific for each pilin protein synthesized and used as an antigen for production of antibodies in rabbits (see Experimental procedures). The rabbit antisera was tested for its specificity to the respective pilin proteins using immunoblots. Although the predicted mol wt of the PilA1 protein is 14.9 kD, the anti-sera detected a high mol wt species of ~125 kD (Fig. 3-5). This was seen despite boiling the proteins in sample buffer for 15 min, indicating PilA1 was either not dissociated into monomers by this treatment or is covalently attached to a larger moiety. The rabbit antisera was found to bind specifically to a protein of

somewhat less (14 kD) than the predicted molecular mass for PilA2, 19.5 kD (Fig. 3-5), suggesting the protein may be processed by the pre-pilin peptidase (PilD) in *C.*

perfringens.

The pilin antisera was used in immunogold labeling experiments to localize the PilA1 and PilA2 proteins (Fig. 3-3 B-E), using 10 nm gold beads covalently linked to Staphylococcal Protein A, which binds to mammalian IgG molecules. The gold beads bound in a random pattern over the entire surface of the cells, and overlap the location of the pili seen in FE-SEM images (e.g., Fig. 3-3A). The random pattern of bead distribution can be expected if there are hundreds of short pili on the surface, since the chances are remote of multiple beads binding to a single pilus. With pre-immune serum, very few beads were associated with the bacterial cells (Fig 3-3C and 3-3E).

The rabbit antisera was also used in indirect immunofluorescence experiments to localize the PilA1 and PilA2 proteins in intact bacteria (Fig. 3-3F). The fluorescent signal overlapped the pattern seen in both the FE-SEM and immunogold labeling (i.e., over the entire surface of the cell), providing further evidence that the pili observed in the FE-SEM have the PilA1 and/or PilA2 proteins associated with them.

***C. perfringens pilT* and *pilC* mutants are non-motile**

In order to determine if the putative TFP-encoding genes were involved in motility, we introduced mutations into the *pilT* and *pilC* genes of strain 13. The *pilT* gene product belongs to the AAA family of ATPases, which are usually oligomeric and hydrolyze ATP to drive the assembly or disassembly of macromolecular complexes (50). The *pilC* gene product appears to play a role in synthesis of TFP, since PilC mutants do

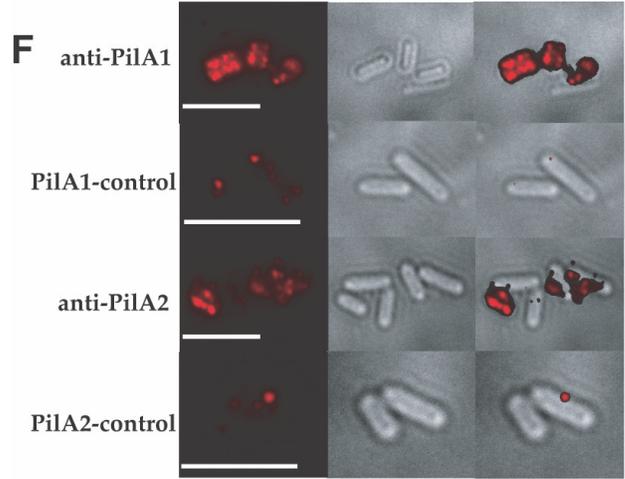
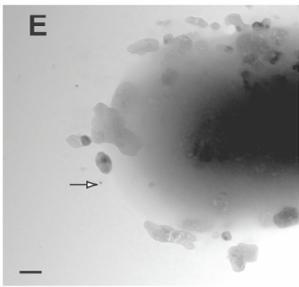
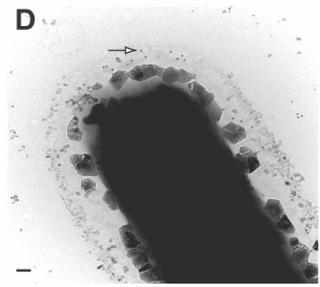
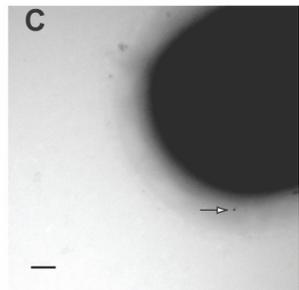
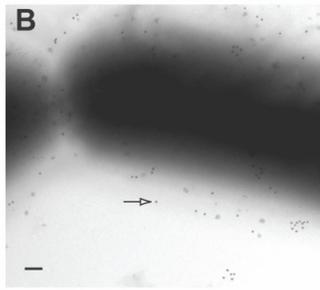
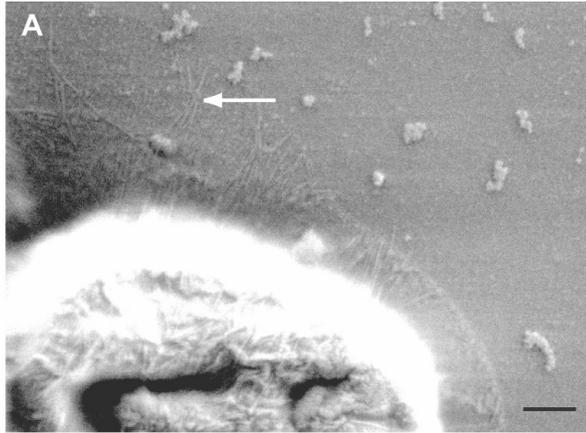


Figure 3-3. Visualization of TFP on the surface of *C. perfringens*. A. FE-SEM image of pili on strain 13 (arrow). B-E. Immunogold labeling of TFP on strain 13 using 10 nm gold beads (arrows) coupled to Protein A. Sera used were anti-PilA1 (B), pre-immune sera for anti-PilA1 (C), anti-PilA2 (D), pre-immune sera for anti-PilA2 (E). Note the significantly higher level of beads visible in panels B and D in comparison to panels C and E. Bar = 250 nm. F. Indirect immunofluorescence labeling of strain 13 cells. In each row, the first image shows the fluorescent signal, the middle figure the differential interference contrast (DIC) image, and the right figure the combined image. Control sera are the pre-immune sera from each rabbit. The fluorescent signal for the combined images were all set to the same intensity so that they can be directly compared. White bars = 10 μ m.

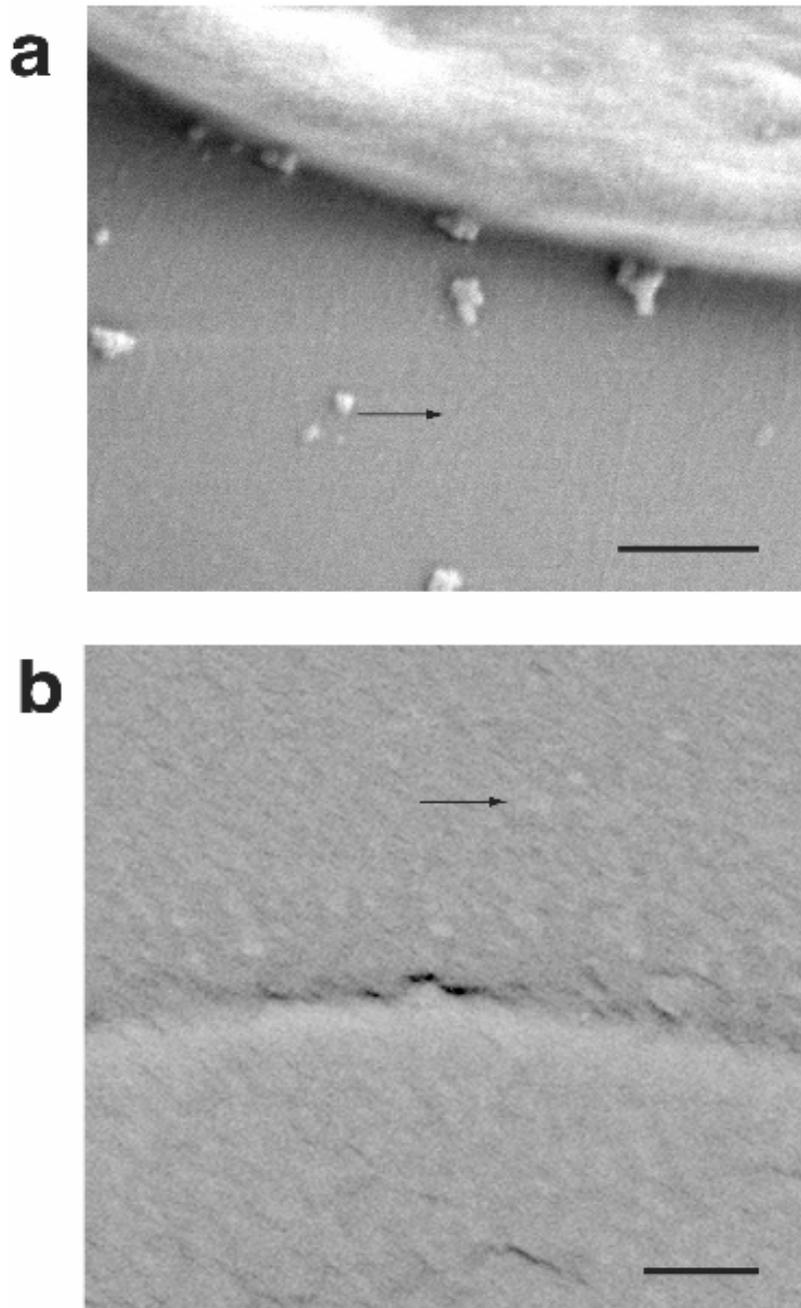


Figure 3-4. Pili on the surface of *C. perfringens* strains. A. strain ATCC13124; B. strain SM101. Arrows point to pili extended out onto the electron microscopy grid. Note that strain SM101 produces a significant quantity of capsular material, which give the edge of the cell an indistinct appearance. Bars = 200 nm.

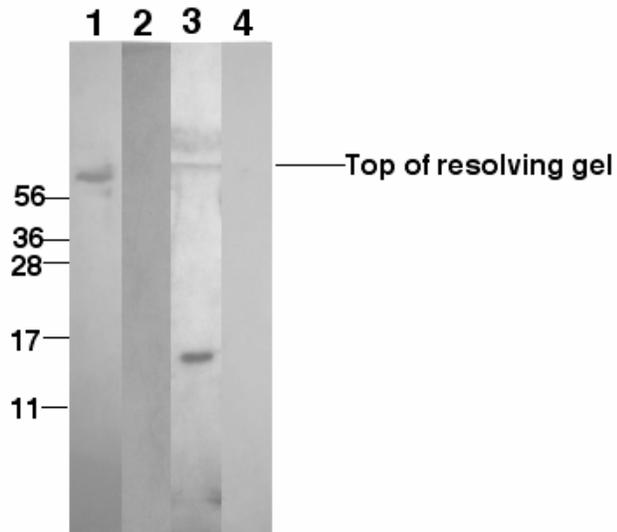


Figure 3-5. Immunoblot of whole cells of *C. perfringens* strain 13 using anti-pilin sera. Lane 1, anti-PilA1; lane 2, pre-immune rabbit sera before PilA1 peptide inoculation; lane 3, anti-PilA2; lane 4, pre-immune rabbit sera before PilA2 peptide inoculation.

not produce pili on the surface of the bacteria (244). The motility phenotype of the *C. perfringens pilT* and *pilC* mutant strains were nearly identical (Figure 3-6). The *pilT* and *pilC* mutants failed to migrate away from the source of inoculum on BHI plates (Figure 3-6 B-C). The colony edges of the *pilT* mutant strain showed none of the flares characteristic of the parent, strain 13 (data not shown), but motility on plates was restored when the intact *pilT* gene was introduced into the *pilT* mutant strain (Figure 3-6D). Due to the inability to clone an intact *pilC* gene in *E. coli*, we were unable to complement the *pilC* mutant strain (see Experimental procedures). Pili were not observed on the surface of the *pilT* or *pilC* mutants using the FE-SEM (Figure 3-7A-B). Immunofluorescent staining using PilA1 and PilA2 antisera showed that intact cells do not have these proteins on their surface, but lysed cells and cell debris gave a strong fluorescent signal (Figure 3-7C). Indirect immunofluorescent staining directed against both PilA1 and PilA2 was restored to intact cells when the wild-type *pilT* gene was used to complement the *pilT* mutant strain (Figure 3-7C). These results indicate the *pilT* mutant (and probably the *pilC* mutant) can still produce the PilA proteins but cannot assemble pili on the surface of the cell.

Differentiating cell growth from motility

C. perfringens is one of the fastest dividing bacteria on record, with doubling times as short as 10 minutes (148). Since bacterial growth is undoubtedly a component of the rate of extension of the flares away from a colony, having the non-motile *pilT* mutant allowed us to determine what role growth played in the motility process. Two

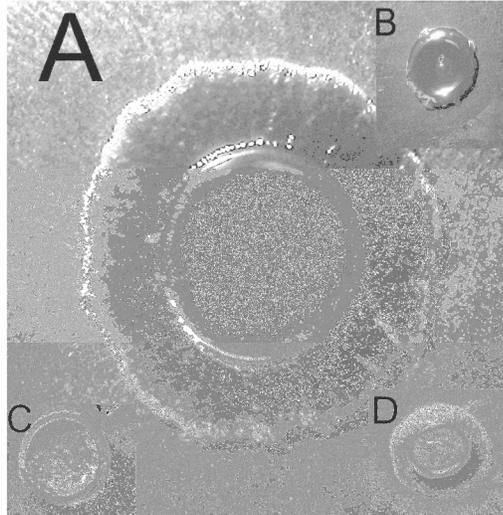


Figure 3-6. *pilT* and *pilC* mutant strains are non-motile on plates. A. Strain 13. B. Strain SM126 (*pilC* mutant). C. Strain SM125 (*pilT* mutant). D. Strain SM126 transformed with plasmid pSM271 (*pilT*⁺). In panels B and C, note the absence of migration of cells away from the original source of inoculation. Central ring of cells in each panel, where the initial inoculum was placed, is ~ 0.8 cm in diameter

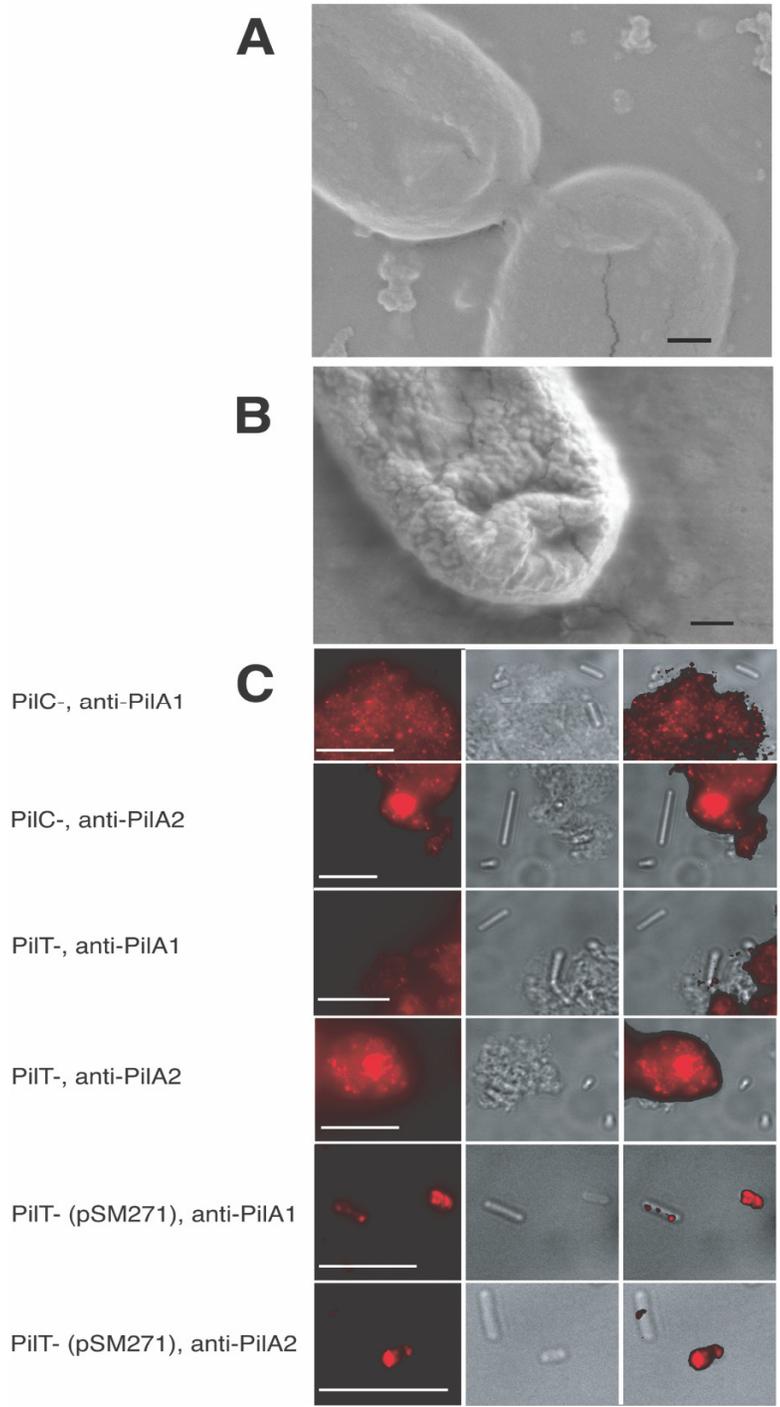


Figure 3-7. *pilT* and *pilC* mutant strains do not express pili on the cell surface. A. Representative FE-SEM image of *pilT* mutant cells. Note the absence of visible pili. B. Representative FE-SEM image of a *pilC* mutant cell, showing the absence of visible pili. C. Immunofluorescent labeling of *pilT* and *pilC* mutant cells using the antisera listed for

each panel. Left to right in each row, are fluorescent, DIC and combined images. The fluorescent signal for the combined images were all set to the same intensity so that they can be directly compared. Note that in the top 4 rows, the fluorescence is associated with lysed cells and cell debris and does not co-localize with intact cells. Pre-immune sera was used as a control for each mutant and the cells exhibited minimal fluorescence under these conditions. White bars = 10 μ m.

hour-old microcolonies from strain 13 and the *pilT* mutant strain were filmed using video microscopy, microcolonies were filmed since it is easier to observe the motion of individual cells in this state than the leading edge of a mature colony. The wild-type strain 13 cells were observed coiling and moving away from their original location (Movie S2), while the *pilT* mutant bacteria remained in place but continued to divide and formed round colonies (Movie S3). We interpret these differences in behavior as indicating that strain 13 moves by a combination of motility and cell division while the *pilT* mutant strain “moves” by cell division only.

TFP-encoding genes in other *clostridial* species

Since TFP-mediated gliding motility has not been reported in Gram-positive bacteria, we analyzed the TFP-encoding genes of *C. perfringens* to determine if they had unusual characteristics. The guanosine and cytosine (G+C) content of the pili-encoding genes shown in Fig. 3-8 is 26-27%, compared to an overall G + C content of 28.2-28.6% for the three complete genomes (234). The lack of any significant difference in base composition suggests the pili-encoding genes are either of ancient origin or have been recently acquired from another low G + C bacterium, e.g., another *Clostridium* species. To determine if other clostridia and closely related genera possessed TFP-encoding genes, the BLAST proGram (15) was used to identify homologs to the *C. perfringens* TFP-related proteins in other clostridial species for which genome sequence data was available. We discovered that all of the clostridial species examined possess orthologs of the core TFP proteins PilA-D and PilT, and that a significant level of synteny was preserved between the TFP-encoding genes of each species, including two neurotoxin producing pathogens, *C. botulinum* and *C. tetani* (Figure 3-8). The presence of these

conserved genes leads us to hypothesize that all of the clostridial species shown in Figure 3-8 are capable of TFP-mediated motility. With the exception of *C. perfringens*, all of the clostridial species shown in Figure 3-8 also possess flagella-encoding genes (data contained in websites listed in Acknowledgments), indicating that these species should be motile in liquid via flagella rotation. As a partial test of our hypothesis that all of the clostridia species sequenced thus far are capable of TFP-mediated gliding motility, we obtained *C. beijerinckii* strain NRRL B592 and examined its motility phenotypes (the strain in which the genome sequence has been determined, shown in Figure 3-8, is NCIMB 8052). *C. beijerinckii* are non-pathogenic bacteria that have been used for the production of industrial solvents such as acetone and butanol but, to our knowledge, have never been reported to move via gliding motility. *C. beijerinckii* strain NRRL B592 was highly motile via flagella-mediated swimming when placed in an environment with high liquid content (i.e., BHI medium with 0.5% agar (Movie S4)). However, when it was spread out on a semi-solid surface with low moisture content (i.e., on BHI medium with 4% agar), the *C. beijerinckii* cells exhibited gliding motility strongly resembling that seen with *C. perfringens*, with multiple curvilinear flares extending from the colony and eventual engulfment of the enclosed space (Movie S5).

Since TFP-encoding genes are present in *C. perfringens* and the other clostridia, we used the *C. perfringens* TFP-encoding genes for a BLASTP search of the entire NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST/>) to determine if homologs to these proteins are present in other bacteria. For the BLASTP proGram analyses, we used the protein sequences of the core proteins needed to produce TFP: PilB, PilC, PilD, and PilT (PilA orthologs can be difficult to identify due to sequence divergence (21, 197)). The

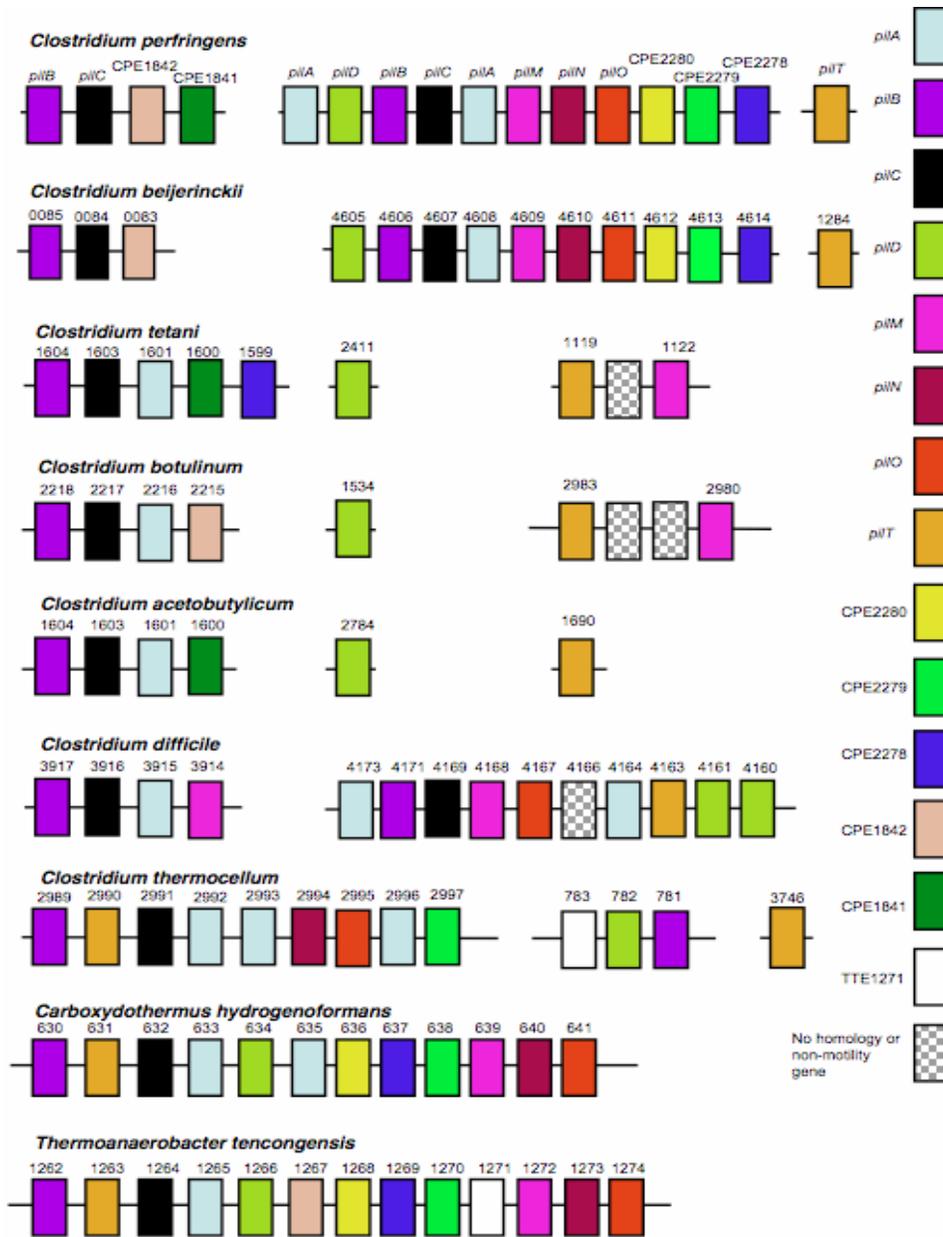


Figure 3-8. TFP biosynthesis genes in other clostridial species. BLASTP comparisons were made using *C. perfringens* TFP-encoding genes (shown in Fig. 3-1) to other *Clostridium* species and species in closely related genera. The putative TFP-encoding genes for the other species are aligned to show the maximum level of synteny to *C. perfringens* and each other. On the right side of the figure, the *C. perfringens* ORF designations are shown in color code to illustrate the synteny in the genes. Above each ORF, the currently assigned ORF number for each species is shown.

bacterial species possessing the 30 orthologs with the highest level of homology to each *C. perfringens* ORF were then placed into a phylogenetic tree using the AroPath proGram (<http://aropath.lanl.gov/index.html>), which uses 16S rRNA sequences to construct phylogenetic relationships. We found orthologs to the four *C. perfringens* TFP-related proteins across the entire spectrum of Gram-negative Eubacterial genomes, including the deeply rooted Aquificiae and Thermus/Deinococci families (Data not shown). The amino acid sequence similarity between the orthologs is highly conserved, as shown by the fact that the E values for the least similar of the 30 orthologs examined for PilB, PilC, PilD, and PilT were e^{-110} , e^{-84} , e^{-21} , e^{-84} , respectively. However, of all the major groups of Firmicutes (i.e., low G-C Gram-positive bacteria) for which sequence data are available, only the *clostridia* class possesses significant numbers of orthologs similar to those found in *C. perfringens* TFP (Data not shown).

Role of *C. perfringens* TFP in virulence

We examined the *pilT* and *pilC* mutant strains for altered virulence phenotypes. In a mouse gangrene infection model (246), the *pilT* and *pilC* mutant strains exhibited a unique phenotype: the mice succumbed to the infection at doses similar to the wild-type strain, but exhibited few outward signs of a gangrene infection, i.e., blackness and swelling of the infected limb (B. Therit and S. B. Melville., unpublished data). The underlying mechanisms for this pathogenic phenotype are currently under investigation.

Discussion

The evolution of TFP in Eubacteria

We have demonstrated in this report that the Gram-positive pathogen *C. perfringens* produces TFP and utilizes these for gliding motility. The ability to produce TFP appears to be ubiquitous in the clostridia, as shown by gene profiles and the ability of *C. beijerinckii* to exhibit gliding motility. The results from a recently published phylogenetic tree of life, which used genes involved in protein synthesis to construct the tree, indicated that the clostridia were the most ancient identifiable Eubacterial class (63). Although Archaeal flagella function mechanistically in a manner similar to Eubacterial flagella (i.e., they are rotational motors), the component proteins of Archaeal flagella have sequence similarities to the TFP found in Eubacteria (e.g., (66, 262), leading many authors to suggest that Archaeal flagella and TFP evolved from structures that predated the evolutionary split between these organisms. Since all nine of the clostridia species for which a complete chromosomal DNA sequence is available appear to have the capacity to move via TFP-mediated gliding motility, we propose that a clostridia-like progenitor of the Eubacteria possessed TFP and that this evolved into the TFP that are now found widely dispersed in Gram-negative bacteria (Figure 3-9). Supporting evidence for this hypothesis comes from the high level of sequence homology seen between the *C. perfringens* TFP-associated proteins PilB-D and PilT and their homologs in phylogenetically diverse Gram-negative bacteria (Data not shown). Additional evidence comes from the percent G + C of the TFP-encoding genes, which is identical to

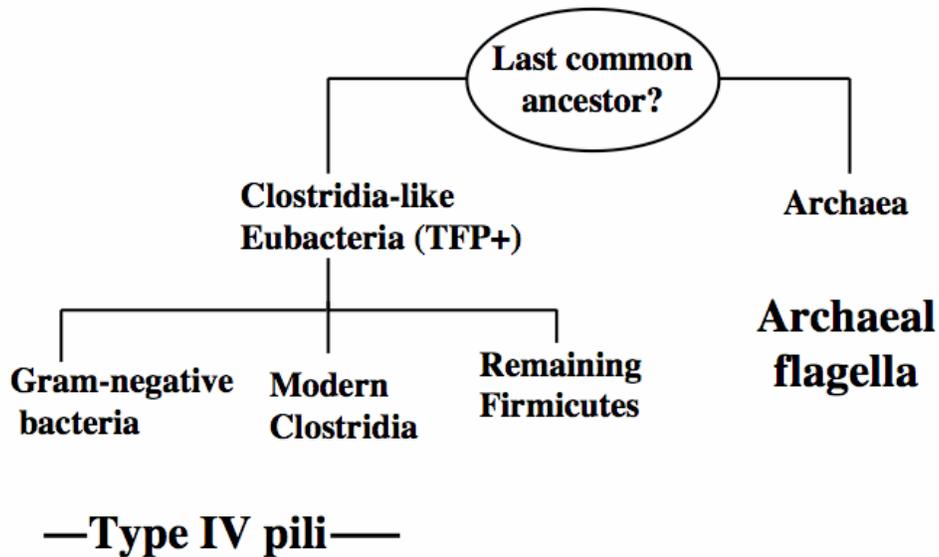


Figure 3-9. Proposed evolution of type IV pili. A putative last common ancestor of Eubacteria and Archaea likely possessed surface components that evolved into TFP in Eubacteria and flagella in Archaea. An early Eubacterial species, similar to modern clostridia, possessed TFP and the TFP were maintained in modern clostridia and Gram-negative bacteria but lost in the remaining genera in the *Firmicutes* (i.e., low G+C Gram-positive bacteria).

the rest of the extremely A-T rich genome of *C. perfringens*, suggesting the genes either came from another clostridial species or were obtained at an early stage of *C. perfringens* evolution. The phylogenetic data used by Ciccarelli *et al.* to assemble their tree of life also led the authors to conclude that the clostridia are one of the slowest evolving groups of Eubacteria (63), which may partly account for the ubiquity and sequence similarity of the TFP-associated genes in the clostridia family.

Interestingly, the TFP-mediated gliding motility we have seen with the clostridia appears to be absent for the most part in the rest of the Firmicutes (i.e., low G + C Gram-positive bacteria). This observation may not hold true when additional Firmicutes species are sequenced, but it does suggest that at the point in which the clostridia branched from the other Firmicutes, TFP gliding motility was maintained in the clostridia but soon lost in the other members of the Firmicutes. This suggests that the clostridia were under evolutionary pressure to maintain gliding motility functions, but the other Firmicutes were not. This may provide some clues as to what role the TFP-dependent motility plays in clostridial ecology and pathogenic lifestyle.

The presence of flagella in all the sequenced strains of the clostridia except *C. perfringens* does not seem to have precluded the bacteria from maintaining the capacity for TFP-mediated gliding motility, as we demonstrated with *C. beijerinckii* (Movies S4-S5). We were only able to demonstrate gliding motility in *C. beijerinckii* when the water content of the medium they were growing on was low. If there was sufficient moisture to form a water film of any thickness, the bacteria moved via flagella. Switching between flagella-mediated swimming and TFP-mediated gliding has been seen in many Gram-

negative bacteria, including *P. aeruginosa*. Other Gram-negative bacteria, such as *N. gonorrhoeae*, like *C. perfringens*, have TFP-mediated motility only and lack both flagella and chemotaxis systems.

Behavioral characteristics of gliding motility in *C. perfringens*

C. perfringens moves away from the colony in an unusual manner: the bacteria are contained in a tightly packed flare which, because of its curvilinear shape, always makes contact with either a colony edge or another flare. The movement most closely resembles social (S) motility in *M. xanthus*, but there are significant differences as well. With *M. xanthus*, while the leading edge of A-S+ cells (i.e., cells lacking adventurous motility but possessing S motility) move away from the colony in flares, individual cells can be seen reversing motion within the flare itself (333). This is not the case with *C. perfringens*. If the base of a flare becomes dislodged from the edge of the colony, as occurs on occasion, the cells at the point of dislodgement can be seen reversing direction as a group, but never as individual cells. An example of this behavior can be seen in the lower right center of Movie S1. These motility characteristics may have evolved to allow the bacterium to move away from the vicinity of an established colony, where low nutrient levels and toxic metabolic byproducts are expected to be present.

Components of TFP biosynthesis and function in *C. perfringens*

The array of TFP-encoding genes seen in *C. perfringens* (Fig. 3-1) indicates they have several features in common with Gram-negative bacteria and some important differences. The core proteins involved in pilin synthesis and function, PilA-D, and PilT are highly conserved, suggesting the assembly and disassembly of PilA polymers might work in a similar fashion as that proposed in Gram-negative bacteria (360). The apparent similarity

in the 3-dimensional fold of the *C. perfringens* PilA proteins to pilins from Gram-negative bacteria suggests a common origin and mechanism of pilus assembly.

As mentioned in the Results section, the absence of a PilQ homolog is expected for a Gram-positive bacterium lacking an outer membrane. However, *C. perfringens* does have a thick peptidoglycan layer in the cell wall that must be traversed by the pili filament. This opening in the peptidoglycan layer must also be maintained when the basal apparatus for pili are synthesized but the pili are not extended, since extension of the pili themselves are unlikely to have sufficient force to penetrate the peptidoglycan layer. Some of the predicted peptides seen associated with the TFP genes (e.g., in strain 13, CPE1842-CPE1841 or CPE 2280-CPE2277) may play a role in this function.

The PilT protein has been shown to be involved in retraction of the extended pilus (221), and *pilT* mutant strains of Gram-negative bacteria usually exhibit a lack of motility but an excess of extended pili emanating from the bacterial surface, since they cannot be retracted (50). The phenotype of the *C. perfringens pilT* mutant matches one expected for a *pilB* mutant (PilB is involved in extension of the pilus (198)) and PilB proteins and PilT proteins share a high degree of homology in other bacterial species. However, the *C. perfringens pilT* gene product contains motifs that are highly conserved in other PilT proteins but absent in PilB proteins (21, 259), giving us confidence in our assignment of the protein as a PilT ortholog. The lack of pili on the surface of our *pilT* mutant strain suggests that either PilT plays a different role in *C. perfringens* than retracting pili or that pili assembly has a feedback sensing mechanism that prevents the bacteria from assembling non-functional pili. Complementation of the *C. perfringens pilT* mutant strain with *pilT* genes from Gram-negative bacteria may help resolve this issue

Materials and Methods

Bacterial strains, plasmids and growth conditions

Table 3-2 lists the bacterial strains, plasmids and oligonucleotides used in this study. *E. coli* strain DH10B was used for routine cloning steps. *E. coli* was grown on Luria-Bertani broth supplemented with 15 g/l of agar (Difco) for solid media. As appropriate, antibiotics were added at concentrations of 300 µg/ml of erythromycin and 20 µg/ml of chloramphenicol.

C. perfringens strains were grown in an anaerobic environment in a Coy anaerobic chamber (Coy Labs). Routine growth was performed on PGY medium (218). As necessary, 30 µg/ml of erythromycin and 20 µg/ml of chloramphenicol were added to the PGY medium. Motility assays were performed on brain heart infusion (BHI) medium (Difco) supplemented with 1 g/l sodium thioglycollate and 10 g/l agar. *C. beijerinckii* strain NRRL B592 was grown on BHI medium under anaerobic conditions.

For observing motility on agar plates, *C. perfringens* strains were grown overnight in PGY and then concentrated to an equivalent of 10 OD₆₀₀ units. Fifteen µl of the cell suspension were spotted on the appropriate media and incubated at 37°C under anaerobic conditions. Macroscopic colony images were taken with a Nikon Coolpix 4500 camera.

Generation of *pilT* and *pilC* mutant strains of *C. perfringens*

To construct strains lacking functional pili, mutations were introduced into the *pilT* and *pilC* genes of strain 13 by insertion of a non-replicating suicide vector via homologous recombination. Oligonucleotide primers OSM174 and OSM175 were used to amplify a

Table 3-2. Strains and plasmids used in the study.

Strain/Plasmid/Oligo nucleotide	Relevant characteristics/Sequence	Source or reference
<u>Strain</u>		
<i>E. coli</i>		
DH10B	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) F80d <i>lacZ</i> Δ M15 <i>lacX74 deoR recA1 araD139 (ara, leu)7697 galU galK λ-rpsL endA1 nupG</i>	Gibco/BRL Corp.
JM107	F' <i>traD36 lacI^q Δ(lacZ)M15 proA+/B+/e14-</i> (McrA) Δ (<i>lac-proAB</i>) <i>thi gyrA96(Nal^r) endA1 hsdR17 (r_K-m_K+)</i> <i>relA1 glnV44</i>	(365)
<i>C. beijerinckii</i>	NRRL B592	J. S. Chen
<i>C. perfringens</i>		
Strain 13	Gangrene-associated strain	C. Duncan
SM101	Acute food poisoning-associated strain	(370)
ATCC 13124	Gangrene-associated strain	ATCC
SM125	<i>pilT</i> - mutant of strain 13	This study
SM126	<i>pilC</i> - mutant of strain 13	This study
<u>Plasmids</u>		
pSM300	<i>E. coli</i> origin, erythromycin resistance	(348)
pSM305	pBluescriptSK- with a 3 kb HindIII insert containing complete <i>sigK</i> and <i>pilT</i> genes and gene fragments of putative <i>spoVD</i> (5') and <i>ftsA</i> (3') homologs from <i>C. perfringens</i> strain NCTC 8798	(insert sequence accession number: AF218835)
pDOB20	pSM300 with <i>pilT</i> gene internal fragment	This study
pDOB21	pSM300 with <i>pilC</i> gene internal fragment	This study
pJV5	<i>E. coli-C. perfringens</i> shuttle vector, chloramphenicol and kanamycin resistance	This study
pSM271	pJV5 with complete <i>pilT</i> gene from plasmid pSM305	This study
pJIR750	<i>E. coli-C. perfringens</i> shuttle vector, chloramphenicol resistance	(26)
<u>Oligonucleotides</u>		
OSM174	5'-CGATACGAGCTCTTCAATACATGGTAAAGG-3' (SacI)	
OSM175	5'-GTGATAGGTACCTTTGTAAAACAGAAGCTAAC-3' (KpnI)	
OSM176	5'-TCCAGAGAGCTCGAAGGATATGATGAGGATTG-3' (SacI)	
OSM177	5'-CATTTATGGTACCTATACTACTGCTTAAAGTAG-3' (KpnI)	

589 bp *pilT* DNA fragment by PCR methods using chromosomal DNA as the template. The PCR product contained DNA sequences internal to the *pilT* gene of strain 13 and had SacI and KpnI restriction recognition sites designed into the primer sequences. The PCR product was digested with restriction enzymes SacI and KpnI and ligated to plasmid pSM300 (348) digested with SacI and KpnI. The resulting plasmid, pDOB20, was transformed into *E. coli* strain JM107 to produce multimer forms of the plasmid (strain JM107 is *recA+*). We have previously observed that multimer forms of recombinant suicide plasmids preferentially recombine into the chromosome of *C. perfringens* (246). Plasmid pDOB20 was purified from strain JM107 and used to transform *C. perfringens* strain 13 by electroporation followed by selection of erythromycin resistant transformants, as previously described (246). An erythromycin resistant isolate was identified and screened by Southern blot analysis to confirm that the plasmid had inserted at the *pilT* locus in the chromosome. The Southern blot experiments indicated that a multimer form (6-7 copies) of the plasmid had inserted into the chromosome and disrupted the *pilT* coding sequence. An identical strategy was used to construct a *pilC* mutant of strain 13, except primers OSM176 and OSM177 were used to amplify a 587 bp fragment internal to *pilC* to make pDOB21. Southern blot experiments also showed a multimer form (6-7 copies) of pDOB21 had inserted into the chromosome at the *pilC* locus of strain 13. Based on our prediction that *pilC* is the second gene in a 4 gene operon (Figure 3-1), it is likely this insertion event resulted in a polarity-induced reduction in expression of the two downstream genes in the operon, which have unknown functions.

Complementation of the *pilT* mutant strain was achieved by isolating a fragment containing the entire *pilT* gene and ~240 bp of upstream DNA, with flanking XbaI restriction sites, from plasmid pSM305. This fragment was ligated to the *E. coli*-*C. perfringens* shuttle vector pJV5 to produce plasmid pSM271. The *pilT* gene located on plasmid pSM305 was isolated from the chromosome of *C. perfringens* strain NCTC 8798, the parent of strain SM101 (370). Plasmid pSM271 was transformed into strain SM125 and this resulted in restoration of motility on BHI plates (data not shown). Multiple attempts to complement the *pilC* mutation were made using PCR products which contained the entire *pilB* operon (Figure 3-1B) or the *pilC/CPE1842/CPE1841* genes in the *E. coli*-*C. perfringens* shuttle vectors pJV5 and/or pJIR750 (26) but no clones could be recovered from transformed *E. coli* strains. Because the *pilC/CPE1842/CPE1841* gene products are all predicted to be membrane bound proteins (data not shown), *E. coli* may not be able to tolerate their presence in the cell.

Video microscopy

Two ml of BHI with various concentrations of agar were poured into 50 ml tissue culture flasks to provide a thin layer of medium for bacterial motility. *C. perfringens* strains were streaked onto the agar surface and incubated at 37°C under anaerobic conditions. The cap on the flask was then tightened to maintain anaerobic conditions and the flask removed from the anaerobic chamber. Bacterial movement was observed by phase contrast microscopy, using a Nikon TE200 inverted microscope equipped with 10X, 20X LW, and 40X LW phase objectives and an ELWD phase condenser. A Tokai Hit ThermoPlate was used to maintain stage temperature at 40°C. Images were acquired with a Dage-MTI CCD100 camera and recorded onto S-VHS videotape through a Panasonic

AG-1970 videocassette recorder. A Silicon Graphics O2 computer running ISEE software was used to capture frames (for figures) and image sequences (for movie files) from videotape. For movie files, frames were captured at 30 sec or 1 minute intervals and the resulting image sequences were converted to QuickTime movie format. Measurements of the rate of movement of the leading edge of the flares in strain13 was done using video microscopy and the manual tracking feature of Image-Pro Plus 5.1 (Media Cybernetics, Inc.). For Movie S5 only, time lapse images were captured using an Olympus IX81 upright microscope linked to a Hamamatsu Model C4742 CCD camera. Digital image collection and conversion to QuickTime video format was done using SlideBook 4.1 imaging software (Intelligent Imaging Innovations, Inc.)

Field emission-scanning electron microscopy (FE-SEM)

C. perfringens strains were grown for 2-4 h in BHI broth, and a 100 µl droplet of the cell culture was added to a Formvar and carbon-coated copper grid and allowed to incubate anaerobically at 37°C for 12-24 h, depending on the experiment. Evaporation was prevented by sealing the copper grids in a plastic container with water reservoirs. The copper grids were then immersed in 2.5% glutaraldehyde to fix the cells, rinsed two times in distilled water and allowed to dry under anaerobic conditions. Samples were sputter coated under vacuum with a 5 nm thick layer of gold using a Cressington 208 Hr rotary sputter coater. The grids were then observed on a Leo 1550 field emission scanning electron microscope (FE-SEM) with a beam acceleration of 1 kV.

PilA specific antibodies

Putative surface-exposed peptide sequences were derived by comparing the PilA1 and PilA2 predicted protein sequences to the published crystal structures of other PilA protein

sequences (reviewed in (50, 197, 243)). For PilA1, predicted to have 140 amino acids, the peptide sequence is DKPQSGDSYNVDIE (amino acids 117-130) and for PilA2, predicted to have 182 amino acid residues, the sequence is DLNGDGTGTPKEE (amino acids 146-158). BLASTP comparisons of the peptide sequences to predicted protein sequences in strain 13 indicated that no significant matches to the peptides were present in other proteins. The peptides and antisera were produced by Affinity Bioreagents Co. The peptides were conjugated to keyhole limpet hemocyanin (KLH) and the conjugates were injected into rabbits to produce antisera. Before immunization, serum was drawn from the rabbits and was used as the source for the pre-immune serum described in this report.

Immunoblotting, immunofluorescence, and immunogold labeling studies

For immunoblots, *C. perfringens* cells were grown on BHI 1% agar plates and the cells were scraped off and placed in sample buffer before boiling for 15 minutes. SDS-PAGE gels and immunoblotting were done as described previously (291). Primary anti-sera was diluted 1:2,000 and secondary sera (goat anti-rabbit conjugated to horse radish peroxidase) was diluted 1:5,000. PilA1 and PilA2 proteins in *C. perfringens* were localized using indirect immunofluorescence. *C. perfringens* strains were grown on BHI 1% agar plates and the cells at the leading edge of a motile colony (if present) were scraped off the plate and fixed in 2.5% paraformaldehyde in Dulbecco's phosphate buffered saline without calcium and magnesium (DPBS). After 10 min in the fixative, the cells were washed 2 times in 2% bovine serum albumin (BSA) in DPBS. The bacteria were then suspended in a 1:100 dilution of rabbit anti-sera in 2.5% BSA/DPBS and incubated for 30 min at 37 °C. The cells were then washed 2 times in 2.5%

BSA/DPBS and suspended in a 1:100 dilution of goat anti-rabbit sera conjugated to Alexafluor 594 (Molecular Probes, Inc.) in 2.5% BSA/DPBS and incubated for 30 min at 37 °C. The cells were then washed 2 times in 2.5% BSA/DPBS and placed on a glass slide with a coverslip and images were captured using an Olympus IX81 upright microscope linked to a Hamamatsu Model C4742 CCD camera. For immunogold, studies, the cells were grown and fixed as described for immunofluorescence and then washed in blocking solution (1% BSA, 5% goat serum). A drop of bacterial suspension in blocking solution was placed on a Formvar-carbon coated copper electron microscope grid and the bacteria allowed to settle at room temperature for 30 min. The grids were then incubated in a 1:100 dilution of anti-pilin sera in blocking solution for 30 min at room temperature and then washed 2 times in blocking solution. The grids were then floated on a drop containing a 1:20 dilution of 10 nm gold beads linked to Staphylococcal Protein A (Jackson Immunoresearch) for 30 min at room temperature. After washing the grids once in DPBS and once in distilled water, they were air dried and then examined in a JEOL 200EX electron microscope at 60 kV.

Legends to Movies

Movie location. Movies are available at <http://www.blackwell-synergy.com/loi/MMI>.

Movie S1. Strain 13 making flares that converge. Filmed under anaerobic conditions at 40°C on BHI medium with 0.5% agar. The field of view seen in the video is 48 µm wide by 36 µm. Total elapsed time depicted in the video is 121.5 min.

Movie S2. Movement of two hour-old microcolonies of strain 13. Notice that the cells appear to migrate and move in loops while also continuing to increase in number. Filmed under anaerobic conditions at 40°C on BHI medium with 1% agar. The field of view seen in the video is 48 µm wide by 36 µm. Total elapsed time depicted in the video is 63 min.

Movie S3. Movement of two hour-old microcolonies of strain SM125, a *pilT* mutant of strain 13. Note that the cells appear to increase in number but do not show significant amounts of movement, in comparison to the cell movement shown in Movie S2, which illustrates the movement of the wild-type strain, 13. Filmed under anaerobic conditions at 40°C on BHI medium with 1% agar. The field of view seen in the video is 48 µm wide by 36 µm. Total elapsed time depicted in the video is 61.5 min.

Movie S4. Flagella mediated motility in *C. beijerinckii* strain NRRL B592. Filmed under anaerobic conditions at 37°C on BHI medium with 0.5% agar. The field of view seen in the video is 48 µm wide by 36 µm. Total elapsed time depicted in the video is 4.2 sec.

Movie S5. Gliding motility in *C. beijerinckii* strain NRRL B592. Filmed under anaerobic conditions at 35°C on BHI medium with 4% agar. A 10 µm scale bar and time stamp (h:min:sec) are included in the video images.

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**Chapter 4 - The Role of CcpA in Regulating the Formation of
C. perfringens Biofilms**

Abstract

Biofilm formation represents a significant bacterial survival mechanism as they have been shown to increase bacterial resistance to a variety of stresses. In this work, *C. perfringens* was shown to form biofilms as a response to low (>1 mM) carbohydrate conditions. Higher levels of carbohydrates (25-100 mM) repress biofilm formation in a CcpA independent process. As is the case in many bacteria, type four pili (TFP) were necessary for *C. perfringens* biofilm formation, as demonstrated by confocal and electron microscopy. Despite their necessity in biofilm formation, TFP were not associated with the bacteria in the biofilm, but with the extra-cellular matrix. Biofilms afforded *C. perfringens* protection from environmental stress, increasing surviving a 24 hour exposure to atmospheric oxygen and exposure to H₂O₂. Biofilms also increased survival against a 16 x MIC of penicillin G ~10-fold over planktonic cells. However, when treated with metronidazole, *C. perfringens* in biofilms were completely killed by 24 hours, while planktonic cells showed a 1000-fold increase in CFU from the starting time and a 10⁷-fold increase in CFU from the 6 h time point.

Introduction

Clostridium perfringens is a Gram-positive, anaerobic pathogen which causes a number of important human diseases including gas gangrene and acute food poisoning, and a variety of animal infections. Due to its spectrum of diseases caused, research interest in this organism has been focused on the ability of the species to produce more than 13 toxins (282) and their role in pathogenesis. In addition to its toxigenic potential, *C. perfringens* is known to form resilient endospores in response to adverse environmental conditions including carbohydrate starvation (348).

One physiological aspect of *C. perfringens* that contributes to its widespread pathogenic capability is its environmental ubiquity. *C. perfringens* can be found anywhere mammals are present, including virtually all soils world-wide (185, 238, 310). In order to persist in the environment, *C. perfringens*, an obligate anaerobe, must have a means to deal with oxidative stress. *C. perfringens* can tolerate short exposures to oxygen, and recent research has helped to determine the mechanism for this oxygen survival. Oxygen exposure induces expression of several proteins that protect *C. perfringens* strain 13 from oxidative stress (145), including rubredoxin and rubrerythrin (102, 178). Failure to generate mutants of a third gene, encoding a superoxide dismutase (SOD), indicates that *sod* is an essential gene in *C. perfringens* (145). Several species of *Clostridium*, including *C. acetobutylicum*, are capable of micro-aerophilic growth. Growth in the presence of minute oxygen concentrations is dependant on a NAD(P)H oxidase, and a NADH/NAD(P)H-dependant H₂O₂ reductase (157).

Long term resistance to oxygen, and thus environmental persistence, can be achieved by the formation of endospores, the resilient and inert products of

differentiation. However, endospores represent a drastic response, whereas the ability to form biofilms is a survival mechanism that can contribute to environmental persistence in a short-term, flexible fashion (51, 78).

Biofilms are adherent bacterial populations that are encapsulated in a matrix composed of polysaccharides, nucleic acids, and proteins (44), and are the predominant state of bacteria in nature (78). In addition to environmental considerations, biofilms are also a factor in pathogenesis. The ability to form biofilms is a well-known trait of a number of pathogens such as *Vibrio cholerae* (367), *Escherichia coli* (273), and *Legionella pneumophila* (280), and a major factor in the pathogenesis of *Pseudomonas aeruginosa* lung infections (70) and *Staphylococcus epidermidis* infections from medical implants (114). *C. perfringens* has never been reported to form biofilms; however, there is one report in the literature of the identification of *C. perfringens* spores in biofilm material isolated from PVC water storage containers in South Africa (144).

Recently, we have described the type-four pilus (TFP) mediated gliding motility of *C. perfringens* (Varga, manuscript submitted, see Chapter 3) which is inducible by carbohydrate starvation (Varga, manuscript in progress, see Chapter 3). TFP are filamentous proteins, consisting of polymers of a single peptide subunit, PilA, and a complex of proteins including PilD (signal peptidase that recognizes PilA), PilB (extension motor), PilC (membrane protein), and PilT (retraction motor) (95, 197). TFP are involved in a number of cellular activities besides motility, including DNA transfer (101) and biofilm formation (154, 257, 333).

Since cells can exist as either planktonic (free floating) cells, or sessile biomass (biofilm), we measured biofilm formation as the ratio of sessile biomass to planktonic

cells. As TFP are frequently involved in biofilm formation, we investigated the ability of *C. perfringens* to form biofilms, the necessity of TFP for the biofilm formation process, the contribution of biofilms to stress resistance, and the role of carbohydrates and catabolite repression in the regulation of biofilm formation.

Results

All sequenced strains of *C. perfringens* can form biofilms

C. perfringens strains ATCC 13124, 13 and SM101 were assayed for the ability to form biofilms in several concentrations of glucose. Biofilm cells (sessile biomass) were measured by staining the biofilm with crystal violet, extracting the stain with methanol and measuring the OD₅₇₀. The formation of biofilms was measured as the ratio of sessile biomass (OD₅₇₀) to the OD₆₀₀ of free floating, planktonic, cells. All 3 strains demonstrated the ability to form biofilms (Figure 4-1). Additionally, glucose functioned as a repressor of biofilm formation in all 3 strains (Figure 4-1). While all strains were capable of forming biofilms, and showed the same response to glucose, the value of the OD₅₇₀/OD₆₀₀ varied for each strain. In 0 mM glucose, ATCC 13124 had a ratio ~4-fold higher than strain 13 and ~1.3-fold higher than SM101, while in 100 mM glucose ATCC 13124 had a value ~10-fold and ~4-fold higher than strain 13 and SM101, respectively.

C. perfringens biofilms protect cells from environmental stresses

The ability of *C. perfringens* biofilms and planktonic cells to survive stresses in the form of atmospheric oxygen, hydrogen peroxide, penicillin and metronidazole, was assayed. *C. perfringens* is known to be aerotolerant (45), and our results indicated that while planktonic cells could survive in atmospheric oxygen at 30.6% and 9.2% for 6 h and 24 h, respectively, biofilm cells actually increased in number by 6 hours, and by 24 h biofilm survival was 76.6% (Figure 4-2). Exposure to H₂O₂ showed that sessile cells had a 1-log higher rate of survival than planktonic cells, 10.5% to 1.5% (Figure 4-2).

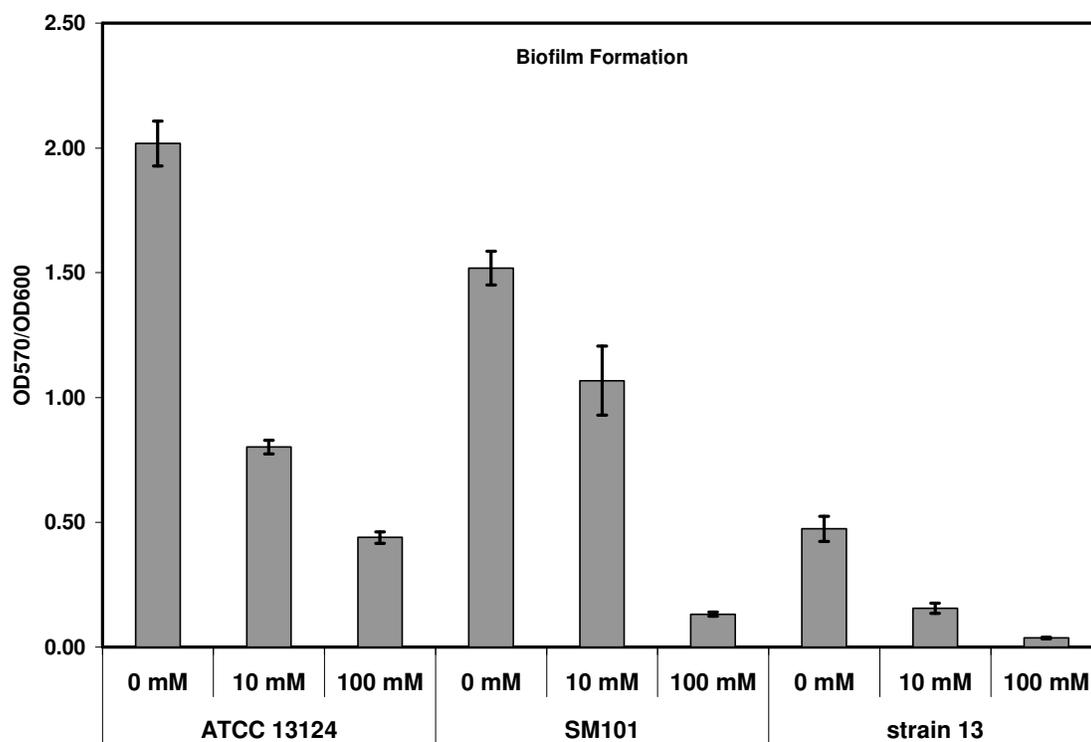


Figure 4-1. Biofilm formation by *C. perfringens* strains. *C. perfringens* strains ATCC 13124, 13, and SM101 were assayed for biomass distribution between sessile and planktonic cells (OD₅₇₀/OD₆₀₀) in 24-well plates. Quadruplicate samples were measured, and the error bars represent +/- 1 standard deviation.

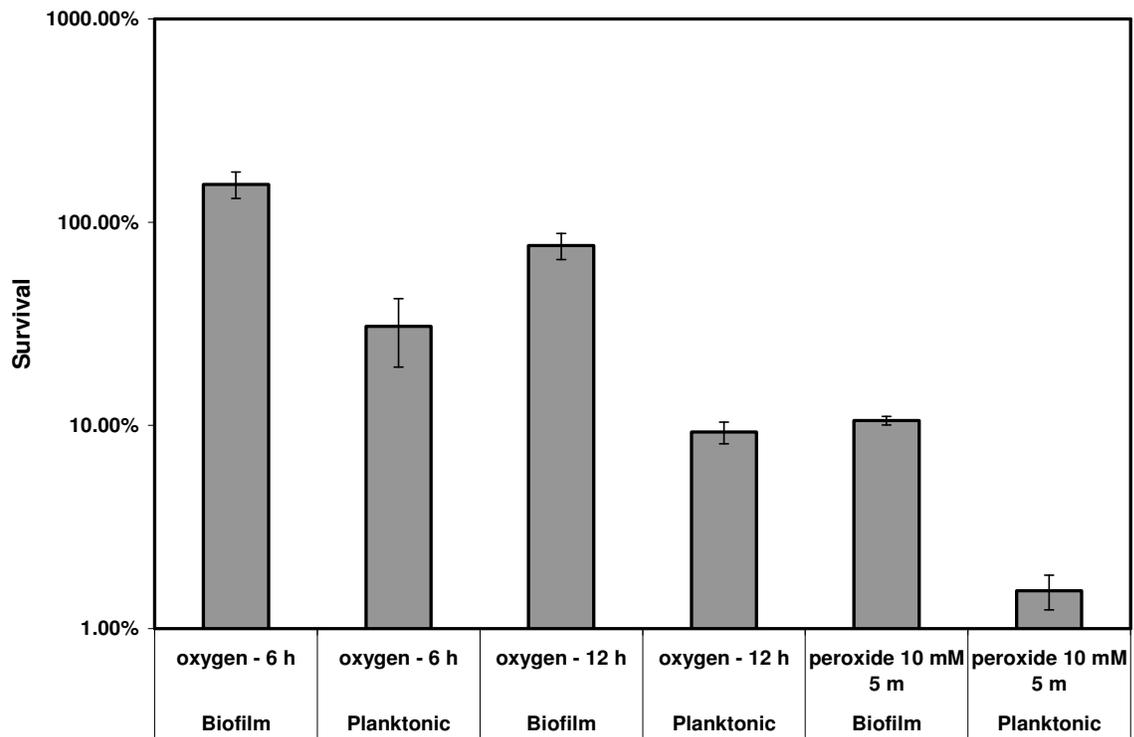


Figure 4-2. Oxygen protection in biofilms. *C. perfringens* 3 day old biofilms were exposed to various oxidative stresses. Samples were exposed to atmospheric oxygen for 6 h and 24 h, and to 10 mM hydrogen peroxide for 5 minutes. In all tested conditions, biofilms provided an approximately 10-fold increase in survival.

C. perfringens is extremely sensitive to penicillin, a bactericidal antibiotic that inhibits cell wall synthesis. The MIC of penicillin for greater than 95% of strains has been reported as low as 0.125 – 0.25 µg/ml (194, 279). Figure 4-3 details the survival of *C. perfringens* to commonly used antibiotics, penicillin and metronidazole. Exposure to high levels of penicillin (20 µg/ml) for 6 h failed to kill *C. perfringens* cells in a biofilm, while identical exposure with planktonic cells resulted in a survival rate of 15%. However, by 24 h survival of *C. perfringens* in a biofilm dropped to 4%, while planktonic survival decreased to 0.8%.

Metronidazole is a bactericidal antibiotic that functions exclusively against anaerobes (330) by damaging DNA after reduction by pyruvate-ferridoxin oxidoreductase (104). *C. perfringens* is highly susceptible to metronidazole, with an MIC of 4 µg/ml (64, 194). The results of metronidazole treatment did not follow the same trend as penicillin treatment did. Interestingly, at 6 hours, a similar reduction of viable cells was observed in biofilms and planktonic cells, however, at 24 h it became impossible to recover viable cells from biofilms, while the planktonic cells had shown a 10-fold increase in CFU over the starting time point (Figure 4-3). This phenomenon has not, to our knowledge, been observed before, and no satisfying explanation has presented itself to explain these aberrant results.

Role of glucose and *ccpA* in limiting biofilm formation

In *Bacillus subtilis*, a *ccpA* mutation was shown to alleviate glucose-repression of biofilm formation (316). As the initial assays suggested that glucose has the ability to limit biofilm formation, a more detailed investigation was performed utilizing a previously described *ccpA*⁻ derivative of *C. perfringens* SM101, SM120 (348).

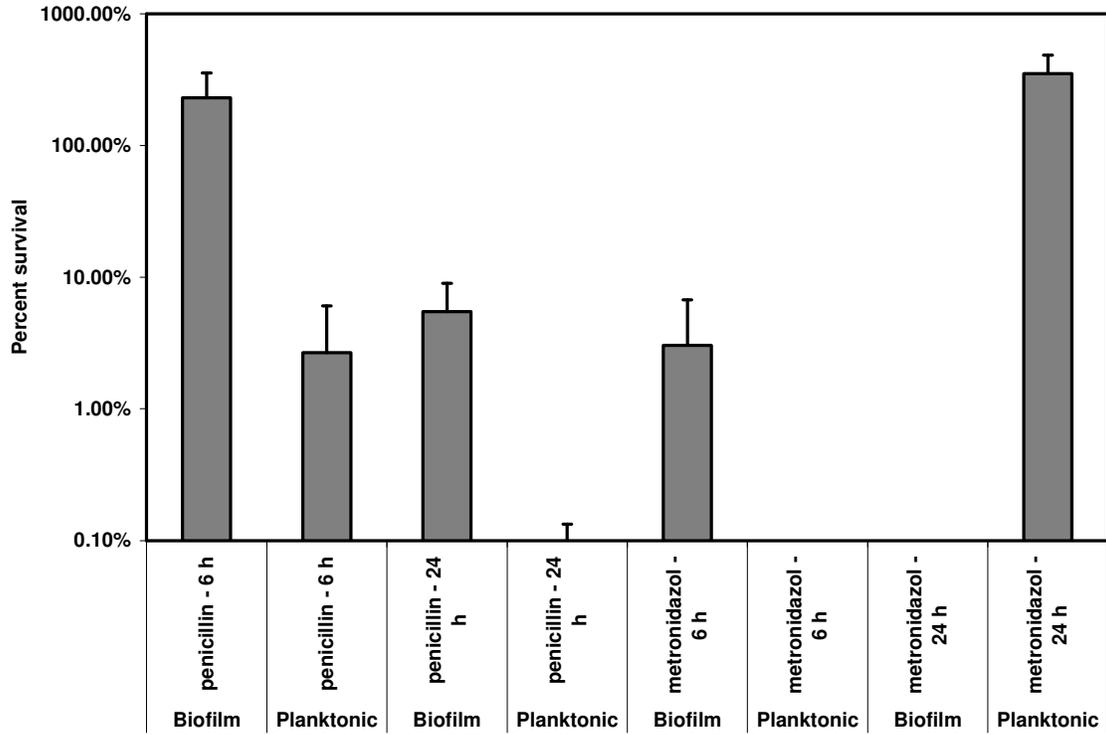


Figure 4-3. Survival of *C. perfringens* against antibiotic challenge. *C. perfringens* biofilms were exposed to penicillin or metronidazole for 6 h and 24 h. After exposure to penicillin, biofilm cells survived at levels at least 10 fold higher than planktonic cells. However, metronidazole eradicated biofilms by 24 hours, while planktonic populations increased in number in the same time period.

C. perfringens SM120 has already been shown to be deficient in sporulation, and to have aberrant glucose-regulation of several virulence factors including capsule synthesis and enterotoxin production (348), in addition to having a defect in the initiation of gliding motility (Varga, manuscript in progress).

Figure 4-4 shows the results of a 5 day time course study of biofilm formation in the presence of varying amounts of glucose. For both strains, the low glucose conditions showed a consistently higher ratio of sessile biomass to planktonic cells when compared with the samples with high glucose levels. The apparent increase in the OD₅₇₀/OD₆₀₀ on day 5 for SM101 is a result of a large decrease in the OD₆₀₀ coupled with an increase in the OD₅₇₀ (data not shown).

Further attempts to determine a role for *ccpA* in *C. perfringens* biofilm formation were made by testing the effect of adding lactose (Figure 4-5), maltose, or fructose (data not shown) to biofilm experiments in concentrations of 10 mM and 100 mM. With the exception of 10 mM lactose, all three carbohydrates showed the same overall trend as glucose, increased carbohydrate levels decreased the ratio of sessile biomass to planktonic cells. There was a marked increase in the biofilm formation values for 10 mM lactose compared with 10 mM glucose for *C. perfringens* SM101 and SM120.

Interestingly, the *ccpA*⁻ mutant did not show any altered biofilm phenotypes relative to the parent strain under any of the tested glucose concentrations (Figure 4-4). This is in contrast to observations in *B. subtilis*, where *ccpA* mediates glucose-repression of biofilms (316) and in *Streptococcus mutans*, where *ccpA* acts as an activator of biofilm formation (355).

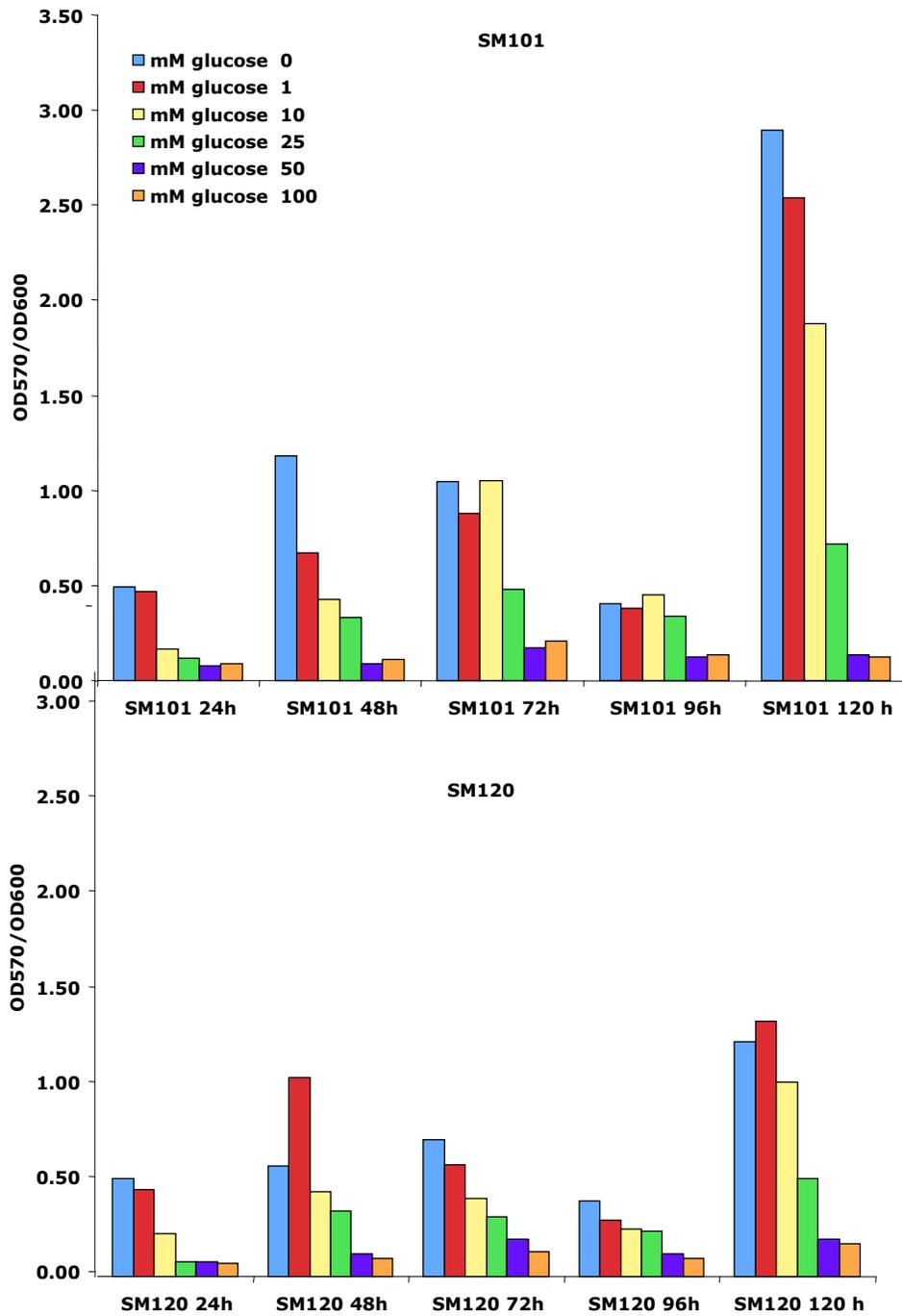


Figure 4-4. Effect of glucose on biofilm formation. *C. perfringens* SM101 and SM120 were assayed for biofilm formation in the presence of various glucose concentrations for a period of 120 hours. Both strains were subject to glucose repression of biofilm formation.

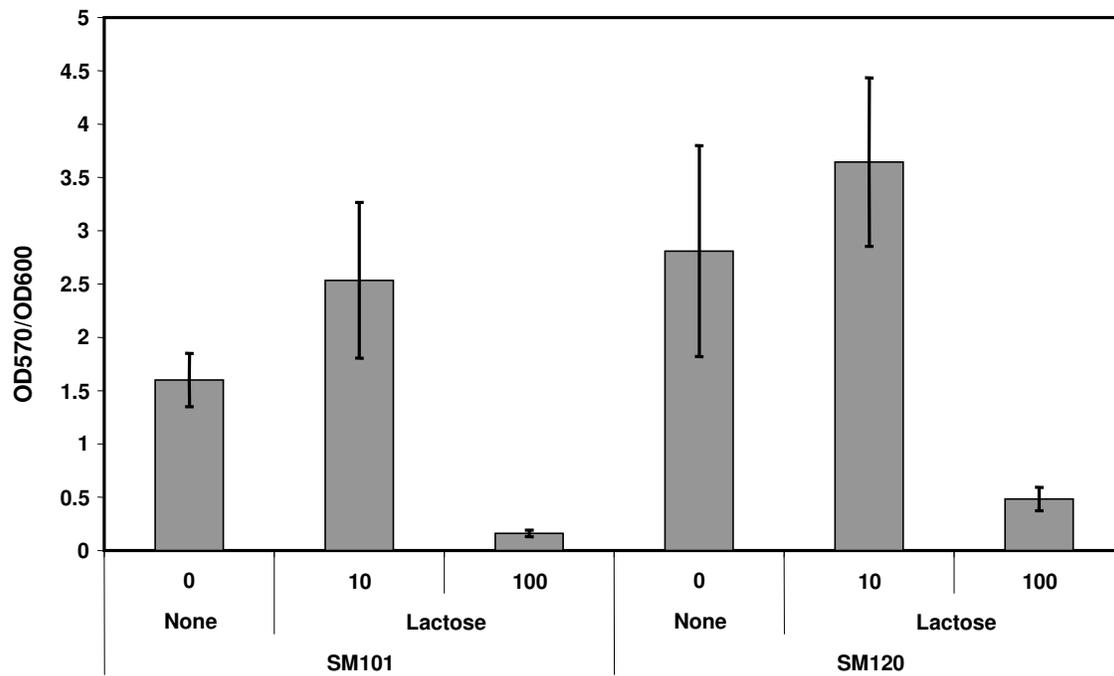


Figure 4-5. Low lactose concentrations fail to repress biofilm formation. When compared with the effect of 10 mM glucose, 10 mM lactose showed no repression of biofilm formation, while 100 mM lactose did repress biofilm formation.

Biofilm visualization and the requirement of TFP for biofilm formation

In other micro-organisms, such as *P. aeruginosa* (249), *Aeromonas caviae* (32) and *Vibrio cholerae* (227), TFP are required for biofilm formation, leading us to test the possibility that TFP are required for *C. perfringens* biofilm formation as well. *C. perfringens* strain 13, a *pilC* mutant (SM125), and a *pilT* mutant (SM126) were incubated in BHI broth on glass coverslips and examined for evidence of biofilm formation using FE-SEM, and incubated in 8-chamber microscope slides for laser confocal microscopy (Figure 4-6). In the FE-SEM images, strain 13 appeared to form a layer of bacteria several cells thick encased in a dense matrix material (Figure 4-6A), while SM126 (Figure 4-6C) and SM125 (data not shown) appeared in the FE-SEM to have much fewer cells present but to still produce a significant amount of matrix material. Three dimensional laser confocal microscopy images of fluorescently labeled bacteria revealed that the overall thickness (30-40 μm) of the biofilms was similar between the wild-type and *pilT* mutant strain. However, strain 13 had, on average, 5 times the number of bacteria present in the biofilm (Figure 4-6B) than did SM125 (data not shown) or SM126 (Figure 4-6D).

Pilin subunits do not co-localize with *C. perfringens* in biofilms

Since TFP were shown to be required for efficient biofilm formation, biofilms were stained with previously described polyclonal antibodies to *C. perfringens* strain 13 PilA1 and PilA2 pilin subunits (Varga, manuscript submitted, see Chapter 3). Figure 4-7 shows representative images displaying the result of the staining, panels A and C show PilA1 and PilA1 pre-bleed staining, respectively. Panels B and D show the PilA2 and PilA2 pre-bleed staining, respectively. For both samples, the antibodies show intriguing

binding patterns, the areas of heavy binding are spatially distinct from bacteria in the sample, while the pre-bleed shows little to no binding for both samples.

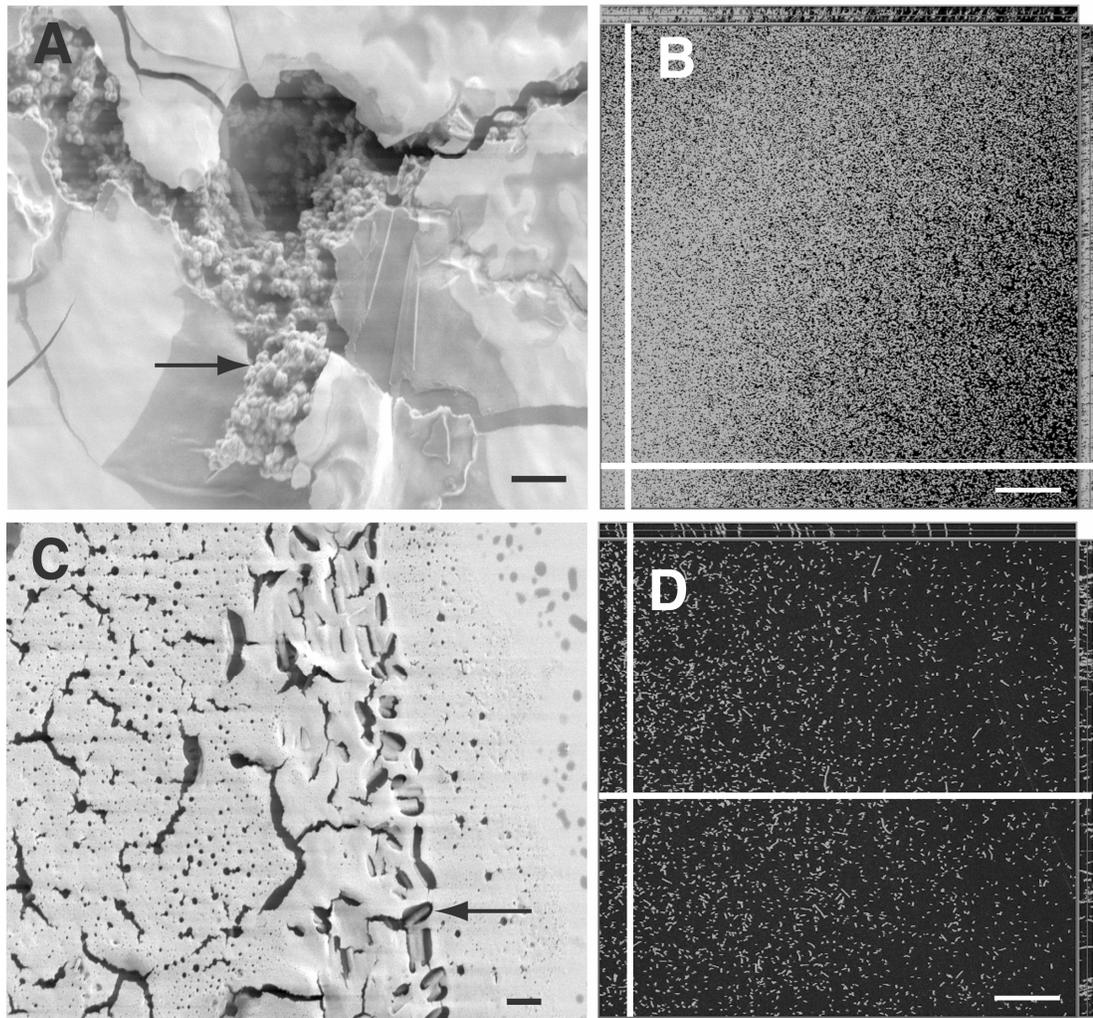


Figure 4-6. Microscopy of *C. perfringens* biofilms. FE-SEM images of strain 13 (panel A) and SM126 (panel C) show that both strains are encased in a matrix, however the strain 13 biofilm contains more cells (arrows). Confocal microscopy images of strain 13 (panel B) and SM126 (panel D) show fluorescently stained bacteria in the biofilms.

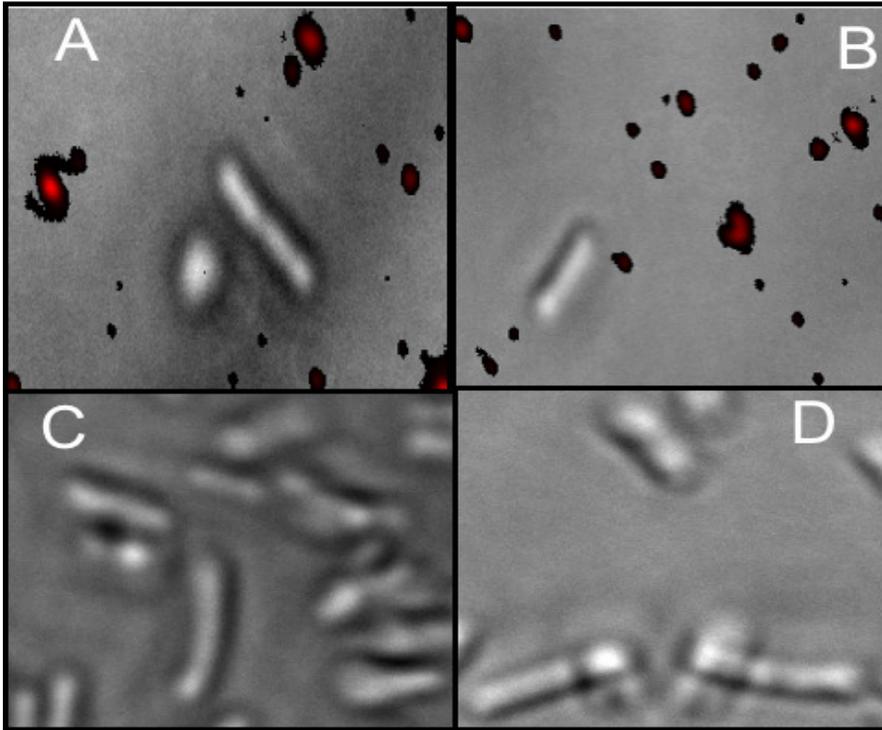


Figure 4-7. Localization of PilA in *C. perfringens* biofilms. *C. perfringens* strain 13 3 day old biofilms were fixed in paraformaldehyde and stained with antibodies to either the *C. perfringens* PilA1 or PilA2 protein, and then stained with Alexafluor 594 conjugated goat-anti rabbit antibodies. Fluorescent microscopy revealed that the PilA1 (panel A) and PilA2 (panel B) antibodies bound to the biofilm matrix, independent of the bacterial cells. Panels C and D are the pre-bleed serum for PilA1 and PilB2, respectively.

Discussion

Biofilm formation by *C. perfringens* represents a previously undescribed survival strategy by this lethal pathogen. Similar to biofilm formation in other pathogens (70, 114) the necessity of TFP in biofilm formation was established in *C. perfringens* strain 13 through confocal microscopy of wild-type and TFP- strains, which showed wild-type strains deposited significantly more bacteria on the surface than the TFP deficient strains (Figure 6). Fluorescent antibody staining demonstrated an interesting pattern of independent localization of pilin subunits and the bacteria. This pattern was not verifiable in the other two strains examined in this work, ATCC 13124 and SM101, due to antibody constraints. The peptide sequences that the antibodies recognize are not conserved in the ATCC 13124 PilA1-4 peptides, and while they are conserved in the SM101 peptides (Varga manuscript submitted) (234). The pre-bleed serum shows extensive recognition of the bacteria, thus limiting the ability to interpret the results of antibody staining.

Published work on the role of catabolite repression in biofilm formation shows that there is no unifying theme for the carbohydrate-based regulation of biofilm formation. In *Streptococcus gordonii*, carbohydrates induce biofilm formation (108), in *S. mutans* the phosphotransferase system component EIIAB^{man} activates biofilm formation in the presence of glucose (2), and in *B. subtilis ccpA* represses biofilm formation under high glucose levels, but some glucose is required for biofilm formation (316). In the Gram-negative bacterium *P. aeruginosa*, catabolite repression controlling protein (Crc) is required for both biofilm formation and TFP-dependent twitching motility (248), and in *E. coli*, glucose repression of biofilm formation is mediated by

cyclic-AMP-receptor protein (142). With this in mind, our results with *C. perfringens* and *ccpA* represent another permutation to the possibilities of the impact of catabolite repression on biofilm formation (Figures 4-1 and 4-4).

C. perfringens has been previously reported to be extremely sensitive to H₂O₂ (45), which was corroborated by the observed low survival rate of *C. perfringens* SM101 in H₂O₂. The genomes of *C. perfringens* strains ATCC 13124 and 13 both encode an alkyl-hydroperoxidase which is absent in the genome of SM101 (234). The lack of a hydroperoxidase may be a factor responsible for the low survival of SM101 observed in our experiments. However, there was a marked increase in survival against the 5 minute 10 mM H₂O₂ exposure, 10.5% in biofilms compared with 1.5% in planktonic cultures, which demonstrates the protective effect imbued on cells in the biofilm.

The role of biofilm formation in pathogenesis of *Clostridium perfringens* remains unknown at this time, but the experiments that have been described give clues as to the environmental conditions and cues for biofilm formation. Simple mono- and di-saccharides can repress biofilm formation (Figures 4-1, 4-4, and 4-5). The tested carbohydrates, glucose (241), fructose (132, 275), lactose (340), and maltose (241) are hydrolyzed and absorbed in the small intestine, and are typically absent in the large intestine. Due to the lack of repressors of biofilm formation, the potential exists for *C. perfringens* to form biofilms in the large intestine.

C. perfringens is known to cause a form of non-food borne enteritis associated with antibiotic use (AAD) (41, 42, 359). Biofilms are well known for their ability to confer antibiotic resistance to the constituent bacteria through a variety of mechanisms (79, 261), and in our work biofilms appear to contribute to antibiotic resistance of *C.*

perfringens against some antibiotics (Figure 4-3). This evidence leads to a hypothesis that biofilm formation can potentially contribute to *C. perfringens* AAD by aiding in bacterial persistence through antibiotic treatment. However, as the *C. perfringens* enterotoxin (CPE) is the causative effect of the symptoms of AAD (224), and CPE is only expressed by sporulating cells (124), for biofilms to play a role in AAD, cells must either be sporulating in the biofilm, as has been observed in *B. subtilis* (118, 182) and *B. cereus*(349) or escaping the biofilm and sporulating after antibiotic treatment.

C. perfringens type C, D and E strains are known to colonize the gut of mammals (310). In addition *C. perfringens* causes enteric infections in many animals, including economically important animals such as cattle, pigs and sheep (reviewed in (311)). In addition to type A strains tested (Figure 4-1), representative type C, D and E strains were also found to be capable of biofilm formation (Figure 4-8). Biofilm formation may represent a mechanism for colonization and persistence in the gut of these animals until a change occurs that allows for pathogenesis to take place.

Environmentally, biofilm formation may provide for a less drastic stress response than differentiation into an inert endospore. Both responses require carbohydrate limiting environments (Figures 4-1, 4-4, 4-5 and (348)). However, there have been additional factors reported for sporulation in *C. perfringens*, including phosphate concentration (268). The temporary nature of a biofilm may be a response to a different set of stimuli than those that trigger sporulation, including oxygen stress, which was tested as survival in atmospheric oxygen and H₂O₂ (Figure 4-2). *C. perfringens* in biofilms exhibited significantly better survival in atmospheric O₂ than planktonic cultures (Figure 4-2). This environmental persistence may contribute to *C. perfringens* pathogenesis by

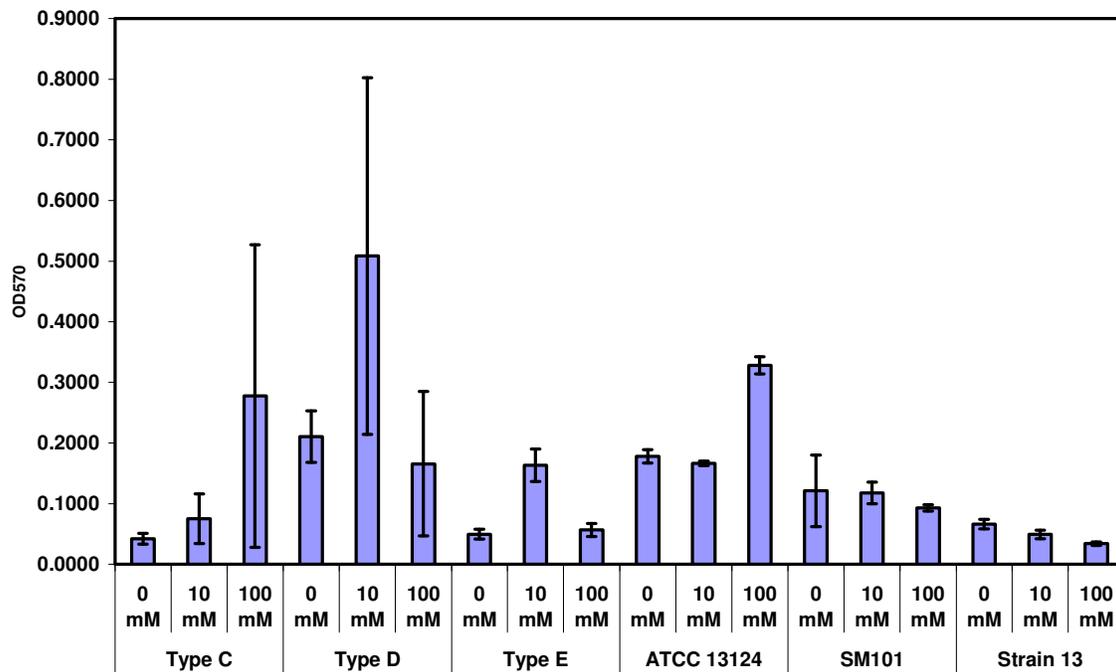


Figure 4-8. Biofilm formation by multiple *C. perfringens* toxinotypes. *C. perfringens* types C, D and E were assayed for biofilm formation. For comparative purposes, the values from three type A strains (13, ATCC 13124, SM101) are also included.

acting as a reservoir of vegetative cells, potentially able to colonize and infect wounds.

The ability of *C. perfringens* to form biofilms results in a multi-faceted response by the bacterium to environmental stresses. Three aspects of this response are that sporulation can occur in any environment, but motility is constrained to solid surfaces (Varga, manuscript submitted), and biofilm formation takes place at liquid/solid interfaces. All 3 responses include a different carbohydrate-repressive aspect, sporulation has both *ccpA*-independent glucose-mediated repression, and *ccpA*-dependant activation, motility has *ccpA*-dependant activation and carbohydrate-mediated repression, and biofilm formation utilizes *ccpA*-independent, carbohydrate-mediated repression. Spores and biofilms both protect against hazardous environmental conditions while motility provides no physical protection, but sporulation is a long term response, and biofilms and motility can be temporary.

These factors hint at a complex regulatory network that is required in *C. perfringens* to manage three distinct stress responses and to utilize the most advantageous response for the particular environment.

Acknowledgements

We thank Blair Therit and Kristi DeCourcy for their assistance with confocal microscopy, and Steve McCartney for help with the FE-SEM. We also thank Dr. Wesley Black for discussions concerning TFP.

Materials and Methods

Stand growth of bacterial strains

All bacterial strains used in this study are listed in Table 4-1. *C. perfringens* strains were routinely grown in PGY (per liter: 30 g proteose peptone #3, 20 g glucose, 10 g yeast extract, 1 g sodium thioglycollate) (218), with 15 g / l of agar for solid medium. When appropriate, media was supplemented with 20 µg / ml of chloramphenicol or 30 µg / ml of erythromycin. All cultures were incubated in an 80% nitrogen / 10% hydrogen / 10% CO₂ in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, Michigan), at 37° C unless otherwise noted.

Biofilm growth

For generation of biofilms, overnight cultures of *C. perfringens* were washed in PBS and resuspended to an OD₆₀₀ of 0.1 in a Genesys spectrophotometer, using different types of media and vessels depending on the experiment. For microscopy, 200 µl cultures were grown in Lab-tek II Cell Culture treated 8-chamber microscope slides (Nalgene Nunc International, Rochester, NY) using Brain Heart Infusion broth (BHI) (Difco, Livonia, MI), or 400 µl cultures were grown in 24-well polystyrene tissue culture plates using T-Soy Broth (Difco) supplemented with filter sterilized carbohydrates for determination of biomass distribution. Cultures were incubated at 30° C for up to 5 days anaerobically.

Quantitation of biofilm biomass

One to five day old biofilms were analyzed in the following manner, developed from published methods (43, 103, 316):

Table 4-1. Bacterial strains used in this study.

<i>C. perfringens</i> strain	Relevant characteristics	Source
ATCC 13124	Type A strain, gangrene	ATCC
JGS 1495	Type C strain	G. Songer
JGS 1721	Type D strain	G. Songer
JGS 1987	Type E strain	G. Songer
Strain 13	Gangrene, highly transformable	(192)
SM101	Food poisoning, transformable	(370)
SM120	<i>ccpA</i> - derivative SM101	(348)
SM125	<i>pilC</i> - derivative of strain 13, TFP-, non-motile	(348)
SM126	<i>pilT</i> - derivative of strain 13, TFP-, non-motile	(348)

The supernatant from each well was transferred to 96-well plates. The wells of the 24-well plate were then gently washed twice with PBS. The plates were then incubated for 30 minutes at room temperature with 400 μ l of filter sterilized 1% crystal violet. Excess crystal violet was removed from the wells, followed by two additional PBS washes. The dye was then extracted during a 30 minute room temperature incubation with 100% methanol, which was then transferred to a second 96-well plate. The OD₆₀₀ of the culture supernatants and the OD₅₇₀ of the methanol-extract dye were measured in a SPECTRAfluor plus plate reader (Tecan, Salzburg, Austria). The value of the OD₅₇₀ divided by the OD₆₀₀ was used to represent the ratio of sessile biomass (10³) to planktonic cells.

Assessment of biofilm resistance characteristics

Biofilms of *C. perfringens* SM101 were prepared as described above. T-soy broth was supplemented with 10 mM lactose to optimize biofilm formation. After 3 days, the sessile and planktonic populations were quantitated from one set of tissue culture plates. Initial planktonic cell counts were determined by washing the pooled supernatants of 3 wells in PBS, diluting the samples, and plating for CFU. Initial biofilm cell counts were determined by adding PBS to the wells of the plate, detaching the cells by scraping with a sterile pipette tip, pooling the contents of 3 wells, washing the cells in PBS, diluting the samples, and plating for CFU.

To determine the resistance capabilities of sessile and planktonic cells, the supernatants from biofilm cultures were washed and resuspended in PBS (for H₂O₂ and oxygen resistance) or T-Soy with lactose (for antibiotic resistance) and transferred to fresh tissue culture plates. The supernatants were replaced with PBS (for H₂O₂ and

oxygen resistance) or T-Soy with lactose (for antibiotic resistance) in the original biofilm culture. Samples were subjected to several stresses similar to what *C. perfringens* might encounter, including exposure to air for 6 h and 24 h, 10 mM H₂O₂ for 5 m (90), 20 µg/ml of penicillin G for 6 h and 24 h (306), and 4 µg/ml of metronidazole for 6 h and 24 h.

After the stress treatment, the samples were processed as described for the initial cell counts. Percent survival was calculated by dividing the post-treatment CFU by the initial CFU. All samples were pools of 3 wells, data are presented as the average of at least two separate pools, with error bars representing the standard deviation of the samples.

Biofilm visualization

Confocal microscopy was performed on biofilms formed by *C. perfringens* strain 13, and its TFP- derivatives SM125 (*pilC*) and SM126 (*pilT*). After 3 days of growth, biofilms contained in 8-chamber microscope slides were gently washed twice with PBS prior to staining with BacLIGHT Live/Dead stain (Molecular Probes), and visualized with a Zeiss LSM 510 Laser Scanning Microscope on an inverted Axiovert 100 M.

Field-emission scanning electron microscopy (FE-SEM) was performed to visualize the surface of *C. perfringens* biofilms. *C. perfringens* strains were grown for 2-4 h in BHI broth, and a 100 µl droplet of the cell culture was added to either a glass coverslip or a Formvar-coated copper grid and allowed to incubate anaerobically at 37°C for 12 h to 24 h, depending on the experiment. Evaporation was prevented by sealing the coverslips and copper grids in a plastic container with water reservoirs. The glass coverslips or copper grids were then immersed in 2.5% gluteraldehyde to fix the cells,

rinsed two times in distilled water and allowed to dry under anaerobic conditions.

Samples were sputter coated under vacuum with a 5 nm thick layer of gold using a Cressington 208 Hr rotary sputter coater. The coverslips or grids were then observed on a Leo 1550 field emission scanning electron microscope with a beam acceleration of 1 kV.

Fluorescent microscopy to visualize antibody staining also utilized 3-day-old biofilms formed in 8-chamber slides. Samples were fixed anaerobically for 15 minutes in 2.5% paraformaldehyde dissolved in 2% bovine serum albumin/PBS (BSAPBS). The fixative was removed, and the samples were washed twice with BSAPBS. The samples were then stained with 1:100-diluted rabbit antibodies against *C. perfringens* strain 13 pilin subunits PilA1 and PilA2 (Varga, manuscript in submission) for 1 hour at 37° C. Unbound antibodies were removed with 2 additional BSAPBS washes, prior to staining with the secondary antibody, goat anti-rabbit conjugated Alexafluor 588 (Molecular Probes). The fluorophore was diluted 1:50 in BSAPBS, and incubated with the sample for 1 hour at 37° C and washed twice with BSAPBS to remove unbound antibodies. 200 µl of BSAPBS was added to samples after the final wash. Samples were visualized on a Olympus IX81 upright fluorescent microscope at 400x magnification, controlled by SlideBook software (Intelligent Imaging Innovations, Inc) operating a Hamamatsu OHC1 ccd camera. To standardize images, exposure times were uniformly 700 ms, and the intensity cutoff value was 400.

**Chapter 5 - The role of CcpA in regulating *C. perfringens*
gliding motility**

Introduction

C. perfringens is a Gram-positive, anaerobic pathogen that causes a number of diseases in humans, including gas-gangrene and acute food poisoning (AFP) (135). In order for *C. perfringens* to cause food poisoning, strains must carry the gene encoding an enterotoxin, *cpe*, and sporulate. It is during sporulation that these strains express the enterotoxin (212, 213, 305, 326) which causes the symptoms of AFP (202). Previously, we have determined that the transcriptional regulator CcpA, a member of the LacI/GalR family of DNA binding proteins, is required for *C. perfringens* to efficiently sporulate and produce CPE (348).

Recently, it was determined that *C. perfringens* possessed the ability to utilize type four pili (TFP) to produce surface translocation (Varga, manuscript submitted, see Chapter 3). During that study, it was observed that glucose could repress motility, leading us to consider the role of catabolite repression in *C. perfringens* gliding motility.

TFP are long, thin filaments consisting of polymerized pilin subunits (38, 360). In *C. perfringens*, the TFP encoding genes are located in 3 locations on the chromosome (Figure 5-1 is reproduced from chapter 3, Figure 2), but nothing is known of the regulation of any genes in the pathway.

Utilizing our previously described *ccpA* mutant of *C. perfringens* SM101, experiments were performed to analyze the nature of the carbohydrate repression of motility, and attempts were made to locate promoters for TFP encoding genes in SM101 and strain 13 by reporter gene fusion and primer extension.

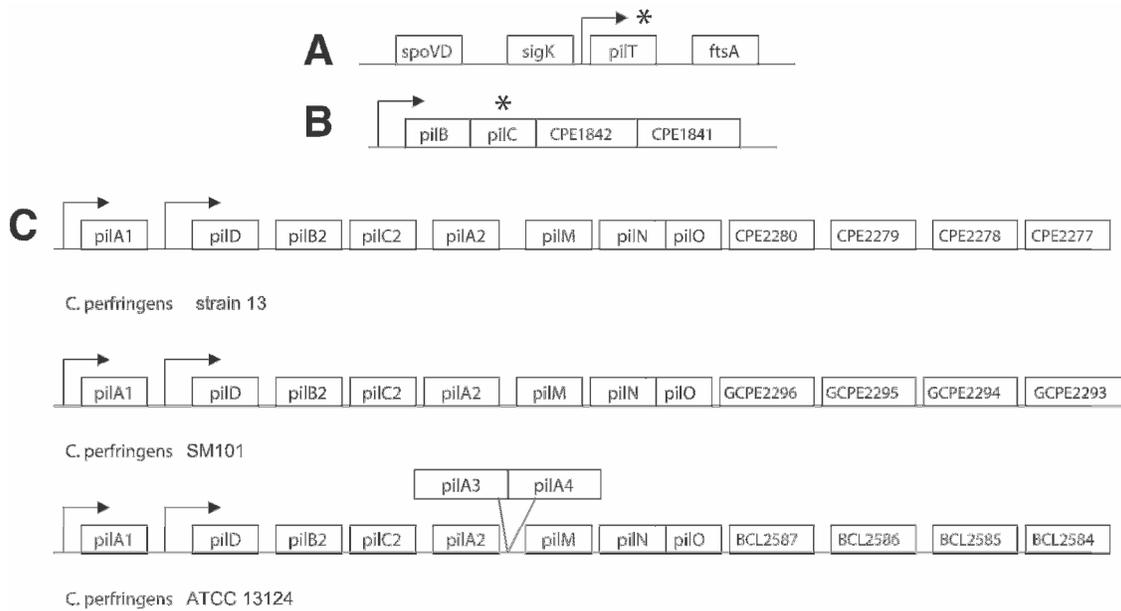


Figure 5-1. *C. perfringens* genes involved in the synthesis or function of TFP. A. *pilT* gene; B. *pilB* operon; C. large pilin locus. The gene order shown in A and B was identical in the three strains. For the *pilB* operon (B), the numbering system for the ORFs shown refers only to strain 13, the other strains have different numerical designations for the orthologs shown in the figure. Arrows indicate putative transcriptional start sites for the operons shown in the figure.

Results

CcpA is required for proper initiation of motility

The time of motility initiation in *C. perfringens* SM101 was measured on PY medium supplemented with glucose, lactose or galactose, in concentrations of 1 mM, 5 mM, 10 mM, 50 mM and 100 mM. SM101 initiated motility in low glucose conditions (1-10 mM) by 8 hours, but higher glucose repressed the initiation until 22 hours after inoculation. Lactose and galactose caused the same trends, with low carbohydrate conditions allowing for earlier initiation of motility than the high carbohydrate conditions permitted.

The *ccpA*⁻ strain showed significant defects in motility initiation. In the presence of glucose, it was unable to move during the 72-hour time span of observation. At lactose and galactose concentrations over 1 mM it took 46 hours for motility to begin. At carbohydrate concentrations of 1 mM, motility took 72 hours to initiate. The motility data for SM101 and SM120 is summarized in Figure 5-2.

Identification of promoters and transcription start sites

The upstream regions of *pilA1*, *pilA2*, *pilB1* and *pilD* from SM101 were successfully cloned in pSM240, and verified by sequencing. However, multiple attempts to quantify activity from the *gusA* reporter gene fusion failed (data not shown).

RNA was harvested from motile SM101 and incubated with the *pilA1*, *pilB1* and *pilD* primers. RNA from strain 13 was also used, however, the *pilA1* primer target was not present in strain 13, therefore only *pilB1* and *pilD* primers were utilized. In SM101, +1 site for *pilA1* and for both strains +1 sites for *pilD* were not identified. However,

for both strains the *pilB1* primer gave a product of **73** bases (Figure 5-3), indicating the location of the transcription start site.

These results are paradoxical, as the motile cells should have been activating some promoters in the assayed region. This is particularly true for *pilA1* as the PilA1 protein (the structural component of the TFP) is present in many copies. It is possible that a very low percentage of cells were actually motile at the time of RNA purification, leading to a proportionally small amount of target RNA for the primers to bind to, resulting in too low of a signal to be detected. In the future, the primer extension will be repeated with more RNA in an attempt to generate a signal.

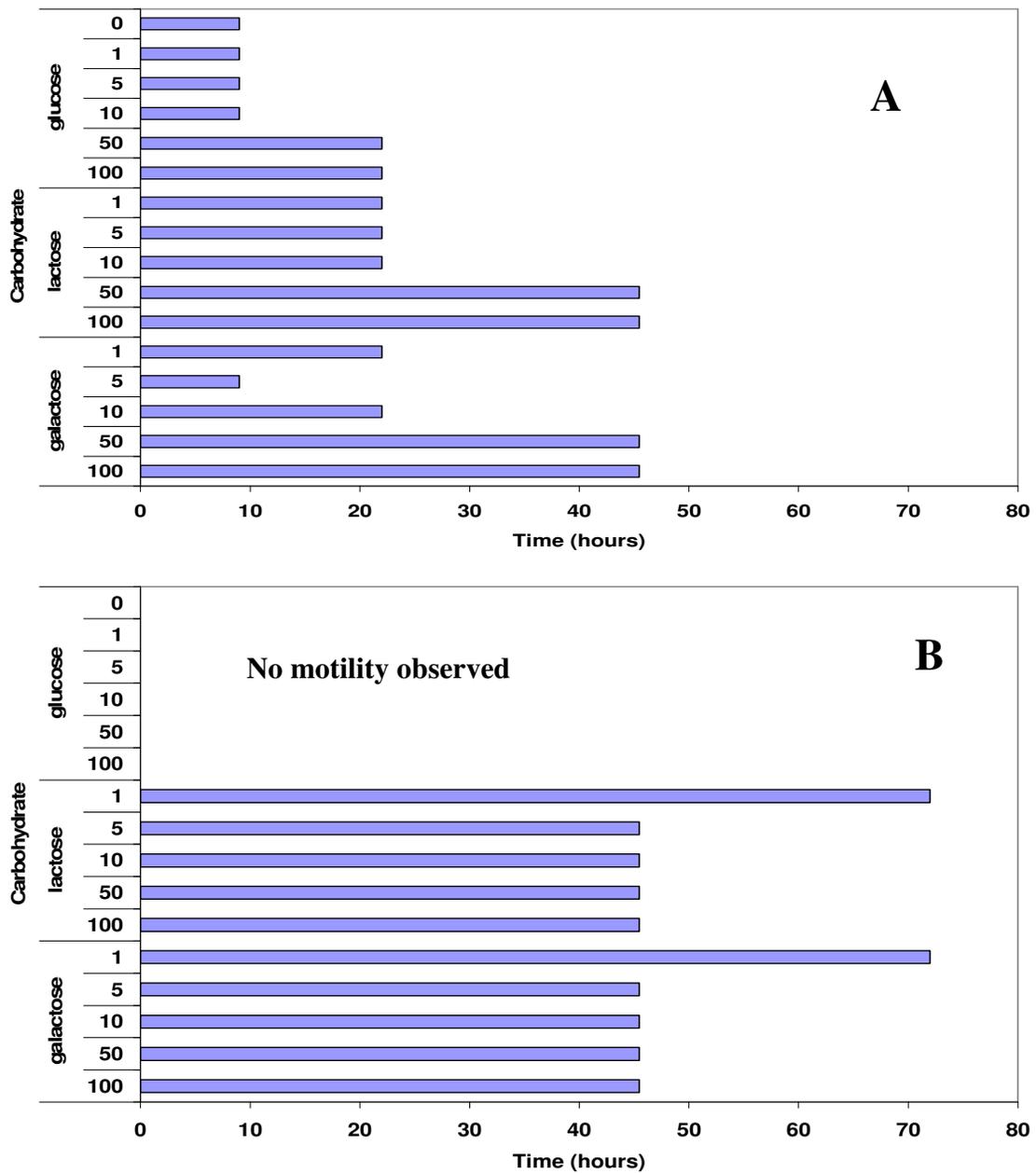


Figure 5-2. Measurement of the time to initiation of motility. *C. perfringens* strains SM101 (A) and SM120 (*ccpA*-) (B) were grown overnight in PGY medium, washed in PBS and concentrated ~15-fold. India ink was added to the cultures which were then plated on PY medium supplemented with various carbohydrates. The samples were observed for an initial expansion from the stained point of inoculum.

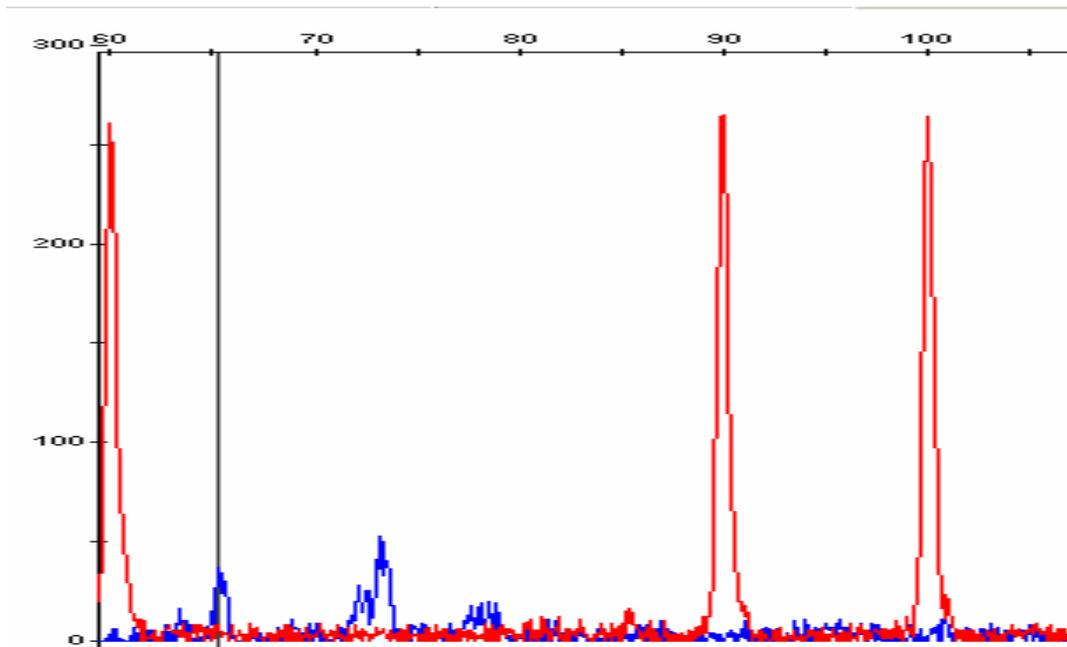


Figure 5-3. Primer extension for *pilB*. Primer extension was performed utilizing a fluorescently labeled primer, fluorescently labeled size standards and capillary analysis of fragment size in order to locate the transcriptional start site for several *pil* genes. *pilB*, the only one to give a product, is shown above. The red peaks are the ROX-400HD size standard, the blue peak at 73 bases represents the +1 site. Data was analyzed using GENEMAPPER software.

Discussion

CcpA is a master regulator in low-G + C Gram-positive bacteria that responds to glucose in the environment. Previous work in *C. perfringens* established the necessity for CcpA in order for cells to efficiently sporulate (348), a starvation response. TFP-mediated motility appears to be another response to starvation. The motility rate of the bacteria remains very similar regardless of the carbohydrate concentration in the media (data not shown), however the bacteria in the assays with lower carbohydrate concentrations initiated motility earlier than the 50 mM and 100 mM experiments (Figure 5-2A). This suggests that the trigger for motility is nutrient limitation, which was supported by experiments with a *ccpA*- strain of *C. perfringens*, SM120 (Figure 5-2B). SM120 was unable to initiate motility on glucose, and suffered from a delay in initiation in the presence of other carbohydrates, when compared with SM101. The simplest explanation is that CcpA is an activator of motility, but that given enough time, the motility response can begin in its absence, and that glucose possesses a CcpA-independent ability to repress motility. The loss of the activator, coupled with the glucose-repression results in a total inability to initiate motility. This is not an unfounded hypothesis, as sporulation is also repressed by glucose independently of CcpA (348).

The failure of both primer extension and *gusA* fusion to identify promoters is troubling, as the genetic organization of the TFP genes is complex (Figure 5-1). Knowing the status of genes as being mono- or poly-cistronic is important for mutational analysis, as demonstrated by the difficulties with analyzing a *pilC* mutant in strain 13 (Varga, manuscript submitted). CcpA is known to bind to imperfect palindromic repeats

(341). Analysis of the sequences upstream of the TFP genes did not reveal any close matches to known *C. perfringens* CcpA binding sites.

In order to better understand the mechanism of gliding motility in *C. perfringens*, it is imperative that the genetic organization be better understood. Promoters need to be identified so that quantitative assays can be performed to truly understand the role of carbohydrate repression of motility.

Materials and Methods

Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 5-1. *Escherichia coli* strains were grown in LB broth, supplemented with 20 µg/ml of chloramphenicol as necessary. *C. perfringens* strains were routinely grown in PGY (218), supplemented with 20 µg/ml of chloramphenicol as necessary. For motility assays, strains were grown on either PY, brain heart infusion (BHI) or T-Soy (TSA) agar. PY agar was supplemented with filter sterilized sugars after autoclaving.

Motility assays

In order to assay motility, *C. perfringens* strains were grown overnight in PGY, washed and concentrated 15 fold in PBS, and spotted on PY plates supplemented with appropriate carbohydrates. Samples were incubated at room temperature for 30 minutes to allow the culture time to dry out prior to inversion and incubation at 37° C anaerobically. For measuring the time to initiation of motility, 0.1% India Ink was added to the bacterial resuspension to mark the initial inoculum. Samples were observed, and the time of motility initiation, indicated by a 0.5 mm expansion of the colony, was noted.

Promoter identification

Promoter fusions of ~500 bp fragments of DNA upstream of the start codon of *pilA1*, *pilA2*, *pilB1*, and *pilD* from SM101 were amplified by PCR and cloned into the reporter plasmid, pSM240, upstream of a promoter-less β-glucuronidase gene. *C. perfringens* SM101 was electroporated with the resulting plasmids, and motile cells were harvested and assayed for β-glucuronidase activity (218).

Table 5-1. Strains and plasmids used in this study

<i>E. coli</i>	Relevant characteristics	Source
DH10B	Cloning strain	
<i>C. perfringens</i>		
13		C. Duncan
SM101		(370)
Plasmids (parent)		
pSM240	GusA reporter	
pSM240A1 (pSM240)	<i>pilA1</i> promoter region	This study
pSM240A2 (pSM240)	<i>pilA2</i> promoter region	This study
pSM240B1 (pSM240)	<i>pilB1</i> promoter region	This study
pSM240D (pSM240)	<i>pilD</i> promoter region	This study

Primer extension was performed with 5' fluorescently labeled (6-FAM) oligonucleotides, as communicated by Dr. Wesley Black (40). Downstream primers were designed to bind at least 50 bases downstream of the start codon. RNA was harvested from motile cells with the triazole reagent (Invitrogen) and used in a reverse transcriptase (Access RT-PCR kit, Promega) reaction set up as per the manufacturer's instructions, except that it contained 1 µg of RNA and 50 pg primer. After the incubation period, the samples were co-loaded with the ROX-400HD fluorescent size standard (ABI), run through a capillary column. The resulting data was analyzed with GENEMAPPER software at the Virginia Bioinformatics Institute Core Laboratory Facility. The size of the fluorescently labeled product indicates the location of the +1 site of transcription.

Acknowledgements

We thank Dr. Wesley Black for assisting with the primer extension protocol, and members of the VBI core lab for assisting with the use of GENEMAPPER.

Chapter 6 - Comparative genomics of *Clostridium perfringens* strains

Abstract

With the completion of the determination of the genome sequence of three strains of *C. perfringens*, studies were undertaken to analyze potential virulence factors. Biology substrate utilization assays were performed to characterize carbohydrate use by *C. perfringens*. Of the 95 substrates tested, 17 substrates were utilized by all three strains, 65 of the substrates were not utilized by any of the strains, and 13 substrates were utilized by 1 or 2 of the *C. perfringens* strains, which may be influenced by the different environments the three tested strains were isolated from.

The ability to form endospores is a defining characteristic of the genus *Clostridium*. The spore formation of all three strains was examined under a variety of conditions. None of the tested conditions supported observable spore counts for ATCC 13124, while SM101 sporulated in all conditions, with the highest efficiency in sporulation media with raffinose and phosphate buffer. Strain 13 sporulated extremely poorly in identical conditions; however, when the phosphate buffer was removed there was a 1000-fold increase in spore production. The sporulation gene content of ATCC 13124 and SM101 was identical, and the only differences in the sporulation gene complement was that strain 13 lacked two spore coat protein-encoding genes, *cotJB* and *cotJC*.

All three strains tested positive for capsule production. Investigation of the genome sequence revealed evidence of putative capsule-encoding genes in all three strains. However, the exact gene content, and arrangement in the chromosome, differed in each strain.

Introduction

C. perfringens is a widely distributed pathogen, and can be found anywhere that mammals inhabit (185, 238, 310). *C. perfringens* can cause gas gangrene and lethal enteric infections in humans (177), as well as acute food poisoning (135) and non-food borne enteritis (41, 173). The species can produce at least 17 toxins (311), of which any strain can produce a subset. *C. perfringens* strains are grouped into 5 biotypes (A-E) based on their ability to produce the major toxins α , β , ϵ , and ι . In addition to toxins, *C. perfringens* produces a number of other potential virulence factors, including a polysaccharide capsule (59-61, 139), motility (Varga, manuscript submitted, see Chapter 3) and biofilm formation (Varga, manuscript in progress, see Chapter 4).

The genome sequence of three *C. perfringens* type A strains has been determined. The first sequenced strain, 13, is a canine gangrene isolate (193), which was sequenced in 2002 (303). More recently (234), the genome sequences of the *C. perfringens* type strain (ATCC 13124), a human gangrene isolate (226), and SM101, a derivative of the well studied acute food poisoning isolate NCTC 8798 (370), have been determined.

As part of the genome sequence determination project several characteristics of the *C. perfringens* strains were compared, including sporulation and substrate utilization (234), in order to demonstrate the validity of the genome analysis. In addition to those characteristics, the capsule production of the three sequenced strains is examined in this work.

As a bacterium that can be found in environments ranging from the gut of mammals to soil (282), *C. perfringens* isolates will be in contact with a large number of potential growth substrates. While the Shimizu group described the presence of multiple

phosphotransferase-system carbohydrate utilization operons (303), minimal research has been directed toward *C. perfringens* metabolism, outside of work related to sporulation and inhibition of sporulation (170, 288, 348).

Bacterial capsules typically consist of polysaccharides and in pathogens can frequently serve as an anti-phagocytic defense (77, 324). For a number of pathogens, including *Streptococcus pneumoniae* (117), the polysaccharide capsule is an important virulence factor, and its loss can lead to a decrease in lethality to the host (231). *C. perfringens* strains typically make capsules, which can be distinct enough for use in serotyping isolates (325) and the published genome sequences (234, 303) demonstrate a genetic basis for the presence and uniqueness of the capsule.

Sporulation plays a key role in the physiology and disease pathogenesis of *C. perfringens*. The ability to form inert, resilient endospores allows *C. perfringens* to persist in otherwise lethal environments, and contributes to its wide spread nature. In addition to the contribution of spores to environmental persistence, sporulation of enterotoxin producing strains is linked to acute food poisoning (85, 87, 124), the 4th highest cause of bacterial food poisoning in the United States (30, 31).

Type-IV pili (TFP) are filamentous protein fibers composed of repeating monomers of the Pila subunit, which typically provide a motive force for bacteria by extending, attaching to a substrate and retracting, pulling the cell forward (For review see (197)). TFP are required for virulence in *Francisella tularensis* (96) and *Neisseria meningitidis* (366) by mediating host-pathogen contact. As previously described, *C. perfringens* strains ATCC 13124, 13 and SM101 all possess a complement of genes

encoding TFP (234) and initial analysis of the TFP loci showed identical composition of putative TFP-encoding ORFs (Varga, manuscript submitted).

Results

Variance in sporulation ability of sequenced strains

Initial heat resistance assays on the *C. perfringens* strains indicated that while SM101 sporulated efficiently, there was little to no sporulation in ATCC 13124 and strain 13 (Table 6-1). Due to this, a number of other methods were attempted to determine if sporulation was occurring and producing spores with less heat resistance than SM101. Tests using chloroform, desiccation or UV radiation all gave similar results: in standard DSSM, strain 13 sporulates extremely poorly, and ATCC 13124 sporulates at undetectable levels (Table 6-1).

While attempting to increase sporulation of ATCC 13124, the phosphate buffer for the sporulation media was removed. This did not result in an increase in ATCC 13124 sporulation, but did result in a 1000-fold increase in spore formation by strain 13 (Table 6-1). Strain 13 was previously reported to have a nonsense mutation in the master regulator of sporulation, *spo0A* (303), which was thought to be the reason for its historically observed poor sporulation. As a *spo0A* mutation in *C. perfringens* has been shown to completely abolish sporulation (138), we sequenced the *spo0A* gene on our lab stock of strain 13 in order to determine that status of the mutated codon in our isolate. The sequences of *spo0A* from published *C. perfringens* genomes (234, 303) and from other *C. perfringens* strains (46) were aligned with CLUSTALW (Figure 6-1). The “T” highlighted in red is the mutated base in the published strain 13 sequence, in our

Table 6-1. Maximum sporulation efficiency of *C. perfringens* strains.

Strain	Spores produced	Sporulation efficiency
SM101	10^7	70%
strain 13	10^5	0.1%
ATCC 13124	<10	0%

8239_spo0A_revcomp	TAAATGTGGCATAATTATATCTAATACAACATAAATCTGGTTGTTTTCTTCTATAAGTTTTAAAGCTTCCA
strain_13_spo0A (S)	TAAATGTGGCATAATTATATCTAATACAACATAAATCTGGTTGTTTTCTTCTATAAGTTTTAAAGCTTCCA
F4969_spo0A_revcomp	TAAATGTGGCATAATTATATCTAATACAACATAAATCTGGTTGTTTTCTTCTATAAGTTTTAAAGCTTCCA
B11_spo0A_revcomp	TAAATGTGGCATAATTATATCTAATACAACATAAATCTGGTTGTTTTCTTCTATAAGTTTTAAAGCTTCCA
SM101_spo0A_revcomp	TAAATGTGGCATAATTATATCTAATACAACATAAATCTGGTTGTTTTCTTCTATAAGTTTTAAAGCTTCCA
13124_spo0a_revComp	TAAATGTGGCATAATTATATCTAATACAACATAAATCTGGTTGTTTTCTTCTATAAGTTTTAAAGCTTCCA
8239_spo0A_revcomp	ATCCATCCTTTGCAATTCCTGTAACAACAATATCCCTTTGACTTAATAAATAGTCATTTAAAACCTTTGT
strain_13_spo0A (S)	ATCCATCCTTTGCAATTCCTGTAACAACAATATCCCTTTGACTTAATAAATAGTCATTTAAAACCTTTGT
F4969_spo0A_revcomp	ATCCATCCTTTGCAATTCCTGTAACAACAATATCCCTTTGACTTAATAAATAGTCATTTAAAACCTTTGG
B11_spo0A_revcomp	ATCCATCCTTTGCAATTCCTGTAACAACAATATCCCTTTGACTTAATAAATAGTCATTTAAAACCTTTGT
SM101_spo0A_revcomp	ATCCATCCTTTGCAATTCCTGTAACAACAATATCCCTTTGACTTAATAAATAGTCATTTAAAACCTTTGT
13124_spo0a_revComp	ATCCATCCTTTGCAATTCCTGTAACAACAATATCCCTTTGACTTAATAAATAGTCATTTAAAACCTTTGT
8239_spo0A_revcomp	TATCATCAGCAATTAATACAGATATTTTTGATTCCTTCAT
strain_13_spo0A (S)	TATCATCAGCAATTAATACAGATATTTTTGATTCCTTCAT
F4969_spo0A_revcomp	TATCATCAGCAATTAATACAGATATTTTTGATTCCTTCAT
B11_spo0A_revcomp	TATCATCAGCAATTAATACAGATATTTTTGATTCCTTCAT
SM101_spo0A_revcomp	TATCATCAGCAATTAATACAGATATTTTTGATTCCTTCAT
13124_spo0a_revComp	TATCATCAGCAATTAATACAGATATTTTTGATTCCTTCAT

Figure 6-1. Comparison of *spo0A* sequences from multiple *C. perfringens* strains. The *spo0A* sequences from strains 8239, 13, F4969 and B11 were retrieved from NCBI, ATCC 13124 and SM101 from www.tigr.org. Sequences were aligned with CLUSTALW on Biology Workbench (Workbench.sdsc.edu). The base highlight in red is the nonsense mutation present in the published strain 13 sequence, but in our stock the mutation is not present. Bases in blue are conserved, the stop codon is highlighted in yellow.

lab stock of strain 13, sequencing revealed that base as a “G”, the same as in the other *C. perfringens* strains.

A detailed analysis of the sporulation gene composition of the three genomes was performed in order to detect any differences that might account for the vastly different sporulation capabilities of the highly sporulating SM101 and the two poor sporulating samples. However, of the 63 sporulation associated genes found in SM101, all 63 were found in ATCC 13124 and strain 13 was only missing two spore coat proteins (Table 6-2) (234, 303).

Capsule

C. perfringens strains were assayed for capsule production by measuring the trypan blue-binding capability of cells (Figure 6-2). Trypan blue binds to the bonds in polysaccharides, therefore the amount of dye removed from solution could be used to represent the amount of polysaccharide capsule present. All three strains produced similar amounts of capsule. Cherniak performed experiments to determine the capsule composition of *C. perfringens* Hobbs serotype 9 (NCTC 8798, the parent strain of SM101) (60). The Hobbs 9 capsule consisted primarily of glucose, galactose and glucosamine. The strain 13 capsule was analyzed, and determined to mainly consist of glucose, galactose, mannose, glucuronic acid, and *N*-acetylgalactosamine (247). As the capsule composition of two of the sequenced strains proved to be quite different, an analysis of the genetic basis for capsule production was undertaken (234). The genome sequences of all three strains revealed an interesting capsule trait. While all three strains possessed a set of putative capsular genes, each strain had a unique set of genes, and

Table 6-2. Comparison of sporulation gene composition.

SM101 annotation name	SM101 orf	strain 13 orf	13124 orf
FtsW-RodA-SpoVE family	353	350	3008
Penicillin binding protein transpeptidase	354	351	3007
stage V sporulation protein D	552	564	2779
probable spore germination protein	641	647	2688
spore germination protein, GerABKA	642	648	2687
spore germination protein, GerABKC	643	649	2686
relA-spoT family protein	648	654	2681
spore coat assembly protein CotJB	960	ABSENT	2216
CotJC	961	ABSENT	2215
GerA spore germination protein	1079	984	2029
stage V sporulation protein B (spoVB)	1249	1203	1845
stage V sporulation protein R	1357	1302	1707
small, acid-soluble spore protein	1436	1423	1564
spoVK domain protein	1542	1431	1547
stage V sporulation protein S (spoVS)	1669	1271	1288
stage IV sporulation protein A (spoIVA)	1750	1253	1201
Sporulation factor SpoIIGA (spoIIGA)	1760	1763	1189
Penicillin binding protein transpeptidase	1765	1769	1183
sporulation protein, putative	1804	1809	1139
Spo0A	1807	1813- 1812	1136
stage IV sporulation protein B (spoIVB)	1808	1814	1135
stage III sporulation protein AH	1820	1826	1132
stage III sporulation protein AG	1821	1827	1121
stage III sporulation protein AF	1822	1828	1120
stage III sporulation protein AE	1823	1829	1119
stage III sporulation protein AD	1824	1830	1118
stage III sporulation protein AC	1825	1831	1116
stage III sporulation protein AB	1826	1832	1115
stage III sporulation protein AA	1827	1833	1113
stage V sporulation protein E (spoVE)	1853	1859	1085
stage V sporulation protein D, spoVD	1857	1863	1081
stage V sporulation protein D, spoVD	1872	1880	1065
small, acid-soluble spore protein, alpha-beta family	1897	1903	1042
related to <i>B. subtilis</i> spore coat protein COTS	2006	2010	928
stage IV sporulation protein	2020	2020	917

stage II sporulation protein P	2040	2040	896
spore protease (gpr)	2041	2041	895
SpoVA family protein	2045	2045	890
stage V sporulation protein AD family protein	2046	2046	889
SpoVA family protein	2047	2047	888
RNA polymerase sigma-F factor (sigF)	2048	2048	887
anti-sigma F factor (spoIIAB)	2049	2049	886
anti-sigma f factor antagonist	2050	2050	885
small, acid-soluble spore protein (sspC1)	2063	2064	871
Spo0B associated GTP-binding protein (obg)	2122	2127	805
cell cycle protein, FtsW-RodA-SpoVE family	2131	2136	796
small, acid-soluble spore protein 1	2156	2161	769
stage III sporulation protein D (spoIIID)	2184	2181	746
stage II sporulation protein D (spoIID)	2186	2183	744
stage II sporulation protein R	2213	2211	716
spore coat protein CotS (cots)	2220	2218	709
spore coat protein, putative	2222	2220	707
spore coat protein, putative	2224	2222	705
spore-cortex-lytic enzyme, putative	2378	2353	553
stage V sporulation protein B (spoVIB)	2523	2481	414
stage V sprulation protein T (spoVT)	2524	2482	412
stage V sporulation protein G	2533	2491	399
spore maturation protein B	2574	2532	355
spore maturation protein	2575	2533	354
spore cortex-lytic enzyme SleC (sleC)	2599	2560	324
stage 0 sporulation protein J	2699	2651	226
sporulation initiation inhibitor protein soj (soj)	2700	2652	225
SpoIIJ-associated protein (jag)	2704	2657	220

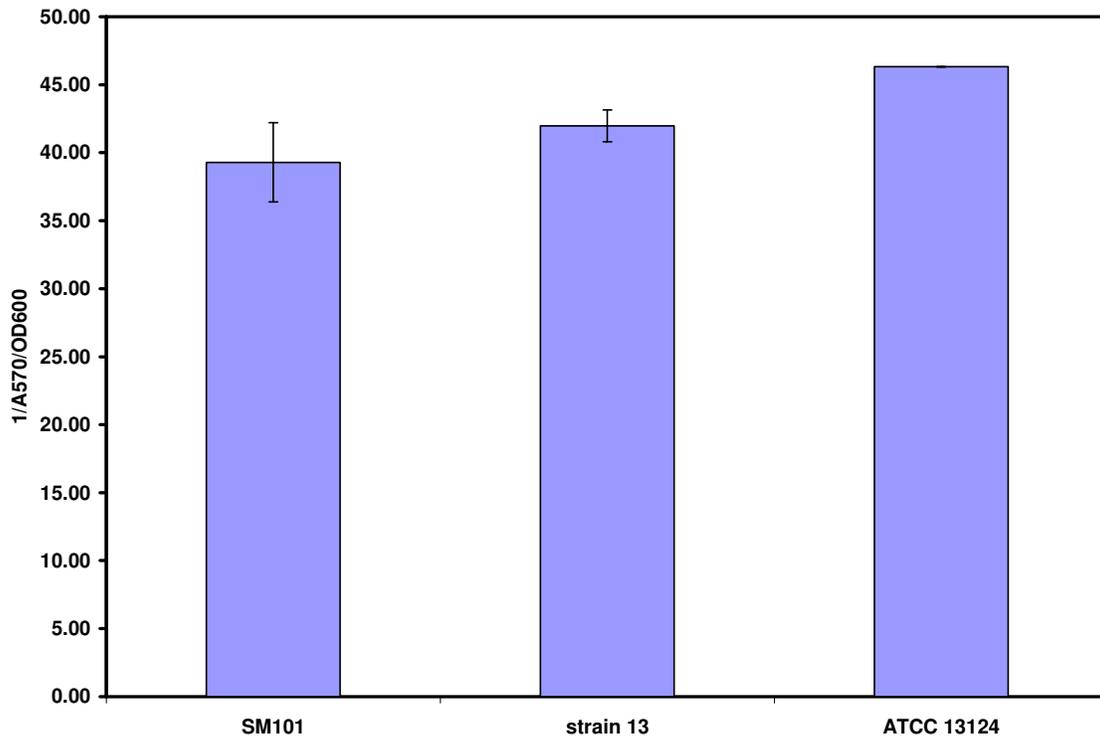


Figure 6-2. Capsule production by *C. perfringens* strains. The ratio of trypan blue-binding to OD₆₀₀ shows that all 3 strains tested produce similar amounts of exopolysaccharide capsule. Error bars represent standard deviations of triplicate assays.

while ATCC 13124 and strain 13 both have capsule genes located on the same region of the chromosome, the capsule genes of SM101 are distributed throughout the chromosome (234). The gene content of the strain 13 capsule-encoding regions included genes that potentially encode for the utilization of mannose, glucuronic acid, and *N*-acetylgalactosamine, while the genes that appear to encode the SM101 capsule include putative glucose, galactose and glucosamine utilization functions (234).

Biolog analysis reveals minimal differences in carbon-substrate utilization.

Biolog analysis results are summarized in Table 6-3. All 3 strains were very similar in their substrate utilization. ATCC 13124 was unique in its inability to utilize lactulose, α -methyl-D-glucoside, and raffinose. Strain 13 was the only strain unable to utilize *N*-acetyl-D-mannosamine. SM101 proved to be the strain with the most differences, being able to utilize amygdalin, D-cellobiose, gentibiose, mannitol, D-melibiose, β -methyl-D-glucoside and stachyose, and being the only one of the three strains unable to utilize thymidine-5-monophosphate and uridine-5-monophosphate.

Table 6-3. Biolog assay of carbohydrate metabolism.

substrate	ATCC13124	strain 13	SM101
Water	-	-	-
N-acetyl-D-galactosamine	-	-	-
N-acetyl-d-glucosamine	+	+	+
N-acetyl-D-mannosamine	+	-	+
adonitol	-	-	-
amygdalin	-	-	+
D-arabitol	-	-	-
arbutin	-	-	-
d-cellobiose	-	-	+
A-cyclodextrin	-	-	-
B-cyclodextrin	-	-	-
dextrin	+	+	+
dulcitol	-	-	-
i-erythritol	-	-	-
d-fructose	+	+	+
l-fucose	-	-	-
d-galactose	+	+	+
d-galacturonic acid	-	-	-
gentibiose	-	-	+
d-gluconic acid	-	-	-
d-glucosaminic acid	-	-	-
a-d-glucose	+	+	+
glucose-1-phosphate	-	-	-
glucose-6-phosphate	-	-	-
glycerol	-	-	-
d,l-a-glycerol phosphate	-	-	-
m-inositol	-	-	-
a-d-lactose	-	+	+
lactulose	-	+	+
maltose	+	+	+
maltotriose	+	+	+
d-mannitol	-	-	+
d-mannose	+	+	+
d-melezitose	-	-	-
d-melibiose	-	-	+
3-methyl-d-glucose	+	+	+
a-methyl-d-galactoside	-	-	-
b-methyl-d-galactoside	-	-	-
a-methyl-d-glucoside	-	+	+

b-methyl-d-glucoside	-	-	+
palatinose	+	+	+
d-raffinose	-	+	+
l-rhamnose	-	-	-
salicin	-	-	-
d-sorbitol	-	-	-
stachyose	-	-	+
sucrose	-	+	+
d-trehalose	+	+	+
turanose	+	+	+
acetic acid	-	-	-
formic acid	-	-	-
fumaric acid	-	-	-
glyoxylic acid	-	-	-
a-hydroxybutyric acid	-	-	-
b-hydroxybutyric acid	-	-	-
itaconic acid	-	-	-
a-ketobutyric acid	-	-	-
a-ketovaleric acid	-	-	-
d,l-lactic acid	-	-	-
l-lactic acid	-	-	-
d-lactic acid methyl ester	-	-	-
d-malic acid	-	-	-
l-malic acid	-	-	-
propionic acid	-	-	-
pyruvic acid	+	+	+
pyruvic acid methyl ester	-	-	-
d-scccharic acid	-	-	-
succinamic acid	-	-	-
succinic acid	-	-	-
succinic methyl ester	-	-	-
m-tartaric acid	-	-	-
urocanic acid	-	-	-
l-alaniamide	-	-	-
l-alanine	-	-	-
l-alanyl-l-glutamine	-	-	-
l-alanyl-l-histidine	-	-	-
l-alanyl-l-threonine	-	-	-
l-asparagine	-	-	-
l-glutamic acid	-	-	-
l-glutamine	-	-	-
glycyl-l-aspartic acid	-	-	-
glycyl-l-glutamine	-	-	-

glycyl-l-methionine	-	-	-
glycyl-l-proline	-	-	-
l-methionine	-	-	-
l-phenylalanine	-	-	-
l-serine	-	-	-
l-threonine	-	-	-
l-valine	-	-	-
l-valine plus l-aspartic acid	-	-	-
2;-deoxy adenosine	+	+	-
inosine	+	+	+
thymidine	+	+	+
uridine	+	+	+
thymidine-5'-phosphate	+	+	-
uridine-5'-phosphate	+	+	+

Discussion

The genomes of *C. perfringens* strains 13, ATCC 13124 and SM101 showed a number of differences (234). Toxin differences between the strains were already known, and are summarized in Table 6-4. The completion of two additional *C. perfringens* genome sequences has allowed for an investigation of known and potential virulence factors of the species.

Sporulation is a defining characteristic of the clostridia, and in *C. perfringens* plays a role in disease pathogenesis and environmental persistence. Genetically, the strains were nearly indistinguishable from each other in terms of sporulation gene composition (Table 6-2), however, as shown in Table 6-1, there was a very large difference in sporulation ability of the strains tested.

It has been known that some *C. perfringens* strains are better able to sporulate with raffinose as the carbohydrate instead of starch. The fact that, at least in this sampling, raffinose-utilization is not a trait possessed by all *C. perfringens* strains might explain why raffinose does not increase sporulation for all strains (Table 6-3). The lack of raffinose metabolism by ATCC 13124 validates observations from the genome sequences of all three strains. Strains 13 and SM101 have a copy of the multiple sugar metabolism (MSM) operon. The MSM operon, first described in 1992 (285) is found in a number of streptococci including some strains *S. mutans* and *S. gordonii* (332). The operon encodes a number of genes with carbohydrate transport and catabolism functions, conferring a broad utilization specificity, including the ability to metabolize raffinose (332). The MSM operon has not been described outside of the streptococci previously.

Table 6-4. Toxin differences in sequenced strains of *C. perfringens*

Strain	Toxin			
	Alpha	Theta	Enterotoxin	Sialidase
13	Yes	Yes	No	NanI , NanJ (303)
ATCC 13124	Yes ¹	Yes	No	NanH, NanI, NanJ (234)
SM101	Yes ²	No	Yes	NanH (234)

1 ATCC 13124 produces very high levels of the alpha toxin (234)

2 SM101 produces low levels of the alpha toxin (unpublished results)on the cell

The capsule composition for strains 13 and SM101 are known to be different, and genetically the differences in all three strains are evident in both gene make-up and organization. ATCC 13124 contains a large locus encoding putative capsule genes, in the same location on the chromosome strain 13 contains a completely different set of genes, and SM101 has capsule genes widely distributed across the chromosome. However, despite the genetic differences, all three strains behaved similarly in the trypan blue-dye binding assay, measuring exopolysaccharide capsule levels in the cells.

Recent studies have demonstrated the vast genetic diversity of *C. perfringens* strains (105, 147, 150, 181, 239). The genome sequences of 3 type A strains corroborates this diversity, with strains showing differences in genome size, make-up and extra-chromosomal elements (data not shown). While toxin differences between strains are known, the differences in accessory virulence factors (motility, capsule, sporulation, biofilm formation (Varga, manuscript in preparation)) are not well studied. The wide array of diseases caused by *C. perfringens*, with a large host-range, merits further study of virulence factors beyond analysis of the well-known toxins.

Materials and Methods

Bacterial strains and routine culture conditions

All bacterial strains used, and their relevant characteristics are listed in Table 6-5. Strains were routinely grown in PGY liquid medium (218) or with 15 g/l of agar for solid medium, in a Coy anaerobic chamber maintaining an environment of 80% N₂ 10% CO₂ and 10% H₂.

Sporulation assays

Sporulation assays were performed as previously described (348). Cells were grown overnight in fluid thioglycollate (FTG) (Difco), and subcultured at 5% into pre-warmed Duncan-Strong Sporulation Medium (171) with either raffinose or starch, and incubated for 24 h and 37°C.

Spores were enumerated by several methods. Heat resistance was measured by heating dilutions at 75°C for 15 minutes, and plated for CFU/ml. Resistance to solvents was measured by adding chloroform to samples, mixing thoroughly, incubating at room temperature for 10 min, followed by dilution and plating. Desiccation resistance was measured by spotting 100 µl of culture on a sterile Petri dish, and allowing the samples to dry and incubate at room temperature for 7 days. In order to ascertain ultraviolet radiation resistance, cells were diluted in PBS in 24 well plates, and exposed to short wave UV light with gentle horizontal shaking, prior to plating.

spo0A from our lab stock of strain 13 was sequenced using BigDye reaction mix according to the manufacturer's instructions (ABI, Foster City, CA). *spo0A* sequences from *C. perfringens* SM101 and ATCC 13124 were taken from draft genome sequences (234), sequences for B11, F4969 and 8239 were acquired from a BLAST

Table 6-5. Strains used in this study.

Strain	Phenotype	Source
ATCC 13124	<i>plc+</i> , <i>pfo+</i>	ATCC
13	<i>plc+</i> , <i>pfo+</i> , highly transformable	C. Duncan
SM101	<i>plc+</i> , <i>cpe+</i> , transformable	(370)

analysis (www.ncbi.nlm.nih.gov/BLAST). ClustalW sequence alignments were performed at the Biology Workbench (workbench.sdsc.edu).

Sporulation gene composition comparison was performed by visually searching the SM101 genome annotation (234) for sporulation genes. Peptide sequences were used in a BLAST analysis performed on *ClostriDB* (clostri.bham.ac.uk) against the strain 13 and ATCC 13124 genomes. An expect value of e^{-20} was used as the cutoff for considering a gene to be the same as in SM101.

Capsule production

Levels of exopolysaccharide capsule were assayed as previously described (348) and (39). Briefly, cells were grown overnight in PGY and subcultured in T-Soy with and without glucose (Difco). The ability of the polysaccharide-specific dye trypan blue (TB) to bind subcultured cells was assayed by measuring the decrease in OD₅₄₅ of a TB solution. In order to account for differing levels of cells in each assay, the OD₅₄₅ of each sample was normalized by dividing by the OD₆₀₀ of each sample. The data is presented as the inverse of the OD₅₄₅/OD₆₀₀ in order to show a positive correlation.

Substrate utilization

Biolog substrate utilization assays were performed as per the manufacturer's instructions (Biolog, Hayward, CA). Assays were performed between 3 and 5 times per strain, and the presence of a single positive result is construed as that strain being positive for metabolization of that substrate.

Chapter 7 - Transposon mutagenesis of *Clostridium perfringens*

Introduction

In the United States, *C. perfringens* causes approximately 250,000 cases of acute food poisoning (AFP) each year (30, 31). The symptoms of AFP are caused by an enterotoxin (CPE) which is produced only when *C. perfringens* sporulates (85, 124). Since *C. perfringens* must sporulate in order to produce CPE, and strains lacking the *cpe* gene are not hindered in sporulation (217), identification of mutations that either allow CPE production to occur while at the same time preventing sporulation, or allow sporulation to occur while preventing CPE production, may give insight into regulatory genes in the toxin production pathway. The fact that sporulation and enterotoxin production are the basis for two diseases caused by *C. perfringens*, acute food poisoning (209) and antibiotic associated diarrhea (41), merit research into the linkage of sporulation with enterotoxin production. Insights into the genetic linkage of the two traits could result in the development of treatments for the diseases.

In addition to its significance in causing disease, *C. perfringens* has potential to counteract disease as well. The ability of *C. perfringens* to express vast quantities of a single protein while sporulating has resulted in its recent investigation as a possible vector for a human immunodeficiency virus (HIV) vaccine (58). The vaccine strategy is to encode viral coat proteins on a plasmid, under control of the *cpe* promoter, sporulating *C. perfringens* in the GI tract then deliver large quantities of the protein to the gastric-associated lymphatic tissue (58).

While the *cpe* gene has been cloned (72), there may be other genes that have not yet been identified or characterized, which may play a role in *cpe* regulation or expression. Limited other work has been published that has helped identify aspects of the regulation of enterotoxin synthesis. Zhao and Melville (370) identified SigE-like and SigK-like dependent promoter sequences upstream of the *cpe* gene. Varga and Melville (348) demonstrated that the transcriptional regulator CcpA is required for the repression of *cpe* synthesis during vegetative growth, and for activation of expression during sporulation. Huang *et al.* showed that the *spo0A*, the master regulator of sporulation in *Bacillus subtilis*, is required for sporulation and enterotoxin synthesis in *C. perfringens* (138).

However, one reason for the lack of research into the co-regulation of sporulation and enterotoxin synthesis has been a lack of molecular genetic tools in *C. perfringens*. One notable absence is that of a transposon mutagenesis system which could reliably create a mutation in a single, randomly selected gene. In order to be of value in identifying the genes that are involved in a given pathway, a transposon mutagenesis system must have several traits. To ensure that any genes involved in the process or pathway have been identified, a transposon must insert with very little, or no, bias into the host chromosome, and preferably have the potential to insert into a large number of sites. It is also necessary for a single insertion event to occur in order to properly identify the gene that was disrupted; this is necessary so that any phenotypic changes observed can be attributed to a single gene. Previously, Awad and colleagues (24) have used Tn916 to mutagenize *C. perfringens*. However, Tn916 is strikingly unsuited for efficient use in *C. perfringens*. Transformant strains frequently had multiple transposon

insertions, the transposon was prone to transposition after isolation of the transformants, and transformants were frequently found to have suffered large deletions of the chromosome (24).

The *mariner* transposons, common among eukaryotic organisms, fulfill many of the desired traits; these transposons have an insertion sequence of “TA” and insert with no reported site specificity or bias (5). The *mariner* transposons are also useful because they have the ability to transpose, using a single transposase gene, without the addition of any extra compounds to the cell (284). Mutants obtained using *mariner* transposons have been generated in many diverse organisms such as insects (188), protozoa (113), and vertebrate organisms (92). The *mariner* transposon system has also been successfully used in a variety of pathogenic bacteria including *Haemophilus influenzae* (5), *Streptococcus pneumoniae* (5), *E. coli*, and *Mycobacterium smegmatis* (284). The *mariner* transposon has been used by Rubin *et al.* (284) and Golden *et al.* (109, 110), to identify and characterize genes in *Campylobacter jejuni*, the most common cause of gastrointestinal disease in the United States (286), causing over 2 million cases per year (98).

A second transposon being investigated for use in *C. perfringens* is Tn4351. Originally found in *Bacteroides fragilis*, Tn4351 has been used in a variety of bacterial genera including *Cytophaga*, *Flavobacterium*, and *Porphyromonas* (201, 329, 351).

Tn10, originally isolated from *Shigella flexneri* (56, 237, 318) and *Salmonella typhimurium*, as a multi-drug resistance factor (352), and has been used for over 30 years in *E. coli* mutagenesis. In its natural form, Tn10 has a strong bias in insertions (36),

mutations were made in the transposase gene by Bender and Kleckner (35) that significantly reduced the site specificity for insertion.

Tn10 was further modified to encode for spectinomycin resistance and move the *E. coli* origin of replication inside the transposon inverted repeats. A temperature sensitive origin of replication recognizable by *B. subtilis*, and a general Gram-positive erythromycin resistance gene (75, 318), were added to a plasmid containing the modified Tn10 by Petit and colleagues (267) creating the mutagenesis vector pIC333.

We attempted to mutagenize *C. perfringens* with *mariner*, Tn4351 and Tn10 transposons in an attempt to determine the nature of the genetic linkage between enterotoxin synthesis and sporulation. This work produced a useful tool for genetic analysis in *C. perfringens*, a functional temperature sensitive plasmid origin of replication.

Results and Discussion

Transposon mutagenesis with *mariner*.

Over 50 electroporations were performed with pSM262, pSM264 and pJV16, all electroporations failed to yield erythromycin resistant colonies that contained the transposon. It is unknown why these *mariner*-containing plasmids failed to mutagenize *C. perfringens*, as both transposons were functional in *E. coli* (data not shown). This result was not unique to SM101, strain 13 also failed to undergo transposon mutagenesis with any of the plasmids.

Tn4351 transposon mutagenesis.

Multiple electroporations with Tn4351 failed to produce transformant colonies. This was not entirely unexpected, as no cloning was done to optimize promoters or antibiotic resistance markers in Tn4351 for use in *C. perfringens*.

Tn10 mutagenesis.

Initial electroporations with Tn10 produced spectinomycin and chloramphenicol resistant colonies that grew in 3-4 days at room temperature. These colonies were grown overnight at 30° C in PGY and remained resistant to both antibiotics. After the cultures were subjected to growth at 43° C, hundreds of spectinomycin-resistant colonies arose after plating and subsequent incubation at 37° C. In order to verify loss of the plasmid, 100 colonies were patched onto chloramphenicol plates, only 6 colonies grew, indicating that loss of the plasmid was prevalent in the population.

Attempts to recover the transposon by re-ligating digested chromosomal DNA failed, transformant *E. coli* were not produced through multiple attempts. Southern blotting performed on chromosomal DNA from spectinomycin resistant, chloramphenicol

sensitive *C. perfringens* strains failed to show hybridization to the spectinomycin resistance gene.

It is possible that the original *C. perfringens* transformants possibly contained a spontaneous spectinomycin resistance trait, but they were also carrying the Tn10 derivative plasmid. This is due to the observations that the strains could initially grow on chloramphenicol, but after the high temperature growth period, most strains lost the ability to grow on chloramphenicol.

Further attempts to use Tn10 to mutagenize *C. perfringens* have failed, for reasons that are unclear to us. However, this work did produce a plasmid containing a temperature sensitive origin of replication, which is a new tool for *C. perfringens* genetic analysis. Table 7-1 summarizes the results of this, and previously published, transposon mutagenesis in *C. perfringens*

Table 7-1: Summary of experimental results with transposon mutagenesis in *C. perfringens*

Transposon	Original Host	Functional in <i>C. perfringens</i>	Efficient	Reference
Tn916	<i>Enterococcus faecalis</i>	Yes	No	(24)
<i>mariner</i>	<i>Drosophila</i>	No	NA	This study
Tn4351	<i>Bacteroides fragilis</i>	No	NA	This study
Tn10	<i>Shigella flexneri</i>	No	NA	This study

NA: Not applicable

Materials and Methods

Bacterial strains and standard growth conditions.

All bacterial strains and plasmids used are listed in Table 7-2. *E. coli* strains were grown in Luria-Bertani Broth (LB) at 37° C with shaking (10 g of tryptone, 10 g of NaCl, and 5 g of yeast extract per liter). For general use, *C. perfringens* strains were grown anaerobically in PGY medium at 37° C [in an anaerobic chamber (Coy Laboratory Products, Inc.)] (30 g proteose peptone (Difco), 20 g glucose, 10 g yeast extract (Difco) and 1g of sodium thioglycollate per liter) (218). When necessary, LB was supplemented with 300 µg/ml erythromycin, 20 µg/ml chloramphenicol, 100 µg/ml spectinomycin or 40 µg/ml x-gal. *C. perfringens* media was supplemented with 30 µg/ml erythromycin, 20 µg/ml chloramphenicol or 150 µg/ml spectinomycin.

Modification of *mariner* transposon for *C. perfringens*

The original *mariner* vector, pFlyingDutchman (284) was modified by replacing the kanamycin resistance gene with the *C. perfringens* erythromycin resistance gene *ermBP*, from pJIR750 (26), resulting in the vector pSM262. In pSM262, the transposase promoter was P_{tac}, it was replaced with the chloramphenicol resistance gene *cat(P)* promoter from pJIR751 (26), creating the plasmid pJV16.

Modification of Tn10 transposon for *C. perfringens*

The Tn10 vector pIC333 was digested with SacI and HindIII to remove the non-functional erythromycin resistance gene. The chloramphenicol resistance gene from pJIR750 was PCR amplified with primers engineered to contain SacI and HindIII restriction sites. The *cat(P)* gene was ligated to pIC333 and electroporated into XL1-blue

Table 7-2. Table of strains used in this study.

<i>E. coli</i> strains	Notes	Source
DH10B	General cloning strain	Invitrogen
XL1-Blue	LacI ^q cloning strain	Stratagene
S17	λ <i>piR</i> strain	
<i>C. perfringens</i> strains		
13	Gangrene, transformable	C. Duncan
SM101	Food poisoning, transformable	(370)
Plasmid (parent)		
pFlyingDutchman	Contains <i>mariner</i>	(284)
pJIR750	<i>catP</i> gene	(26)
pJIR751	<i>ermB</i> (P) gene	(26)
pSM262 (pFD)	<i>mariner</i> with erythromycin resistance	This study
pSM264 (pSM262)	Ampicillin from backbone replaced with kanamycin	This study
pJV16(pSM262)	Transposase under control of <i>catP</i> promoter	This study
pIC333	Mini-Tn10 transposon	(267)
pIC333S/C (pIC333)	Chloramphenicol resistance encoded on plasmid backbone	This study
pIP4351	TN4351	Dr. Wesley Black

E. coli. XL1-blue is a strain of *E. coli* which does not express the *lac* promoter unless it is induced with IPTG (Stratagene).

Transposon mutagenesis protocol

The transposons were introduced into *C. perfringens* through the procedure modified from Allen and Blaschek (9, 10):

C. perfringens strains were grown anaerobically in 5 ml of liquid PGY overnight. Cells were then centrifuged and washed twice in 5 ml of electroporation buffer (0.31M sucrose, 6mM sodium-phosphate, 60 μ M MgCl₂) and resuspended in 3 ml of electroporation buffer. 400 μ l of washed cells were added to a 0.4 cm electrode gap cuvette (BTX) and transformed with 1-20 μ g of plasmid DNA using a BTX ECM 630 electroporator (Genetronics, Inc.) set at 2500 V with a resistance of 125 ohms and a capacitance of 50 μ F. After the electroporation, the cuvette was placed on ice for a 15-minute recovery period, then transferred to 1.5 ml of brain heart infusion broth (BHI) and grown anaerobically at 37°C (for *mariner* and Tn4351) or room temperature or 30° C (for Tn10) for a 3-hour phenotypic expression stage. Following the 3-hour phenotypic expression stage, the cells were concentrated in a volume of 200 μ l and spread on two BHI plates with spectinomycin and chloramphenicol and incubated at 37°C (for *mariner* and Tn4351) or room temperature or 30° C (for Tn10) for 2-5 days, until colonies developed.

Tn10 colonies were restreaked on fresh chloramphenicol/spectinomycin PGY plates to verify antibiotic resistance and to isolate individual colonies. Candidate colonies were transferred to PGY broth and incubated at room temperature for 2 days, at which time the cultures were diluted one hundred-fold in fresh PGY broth and incubated

in gas pack jars (Oxoid) at 43° C for 3 hours to prevent plasmid replication. After the 3 hour incubation, cultures were diluted and plated on PGY with either spectinomycin or spectinomycin and chloramphenicol and incubated overnight at 37° C. Colonies from the spectinomycin plates were streaked onto fresh PGY spectinomycin plates and PGY plates with chloramphenicol and spectinomycin to determine if the plasmid persisted in the cells through the heat treatment.

Evaluation of transposon mutagenesis.

Erythromycin resistant colonies that were generated from electroporations using the mariner transposon and colonies that arose from electroporations using Tn10 derivatives were selected for further study by Southern blotting (292). Chromosomal DNA was prepared by the method of Pospiech and Neumann (271). Cells were grown overnight in 30 ml of PGY, concentrated and washed in STE (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.4). The washed pellet was resuspended in 5 ml of STE, supplemented with 2 mg/ml lysozyme and incubated at 37° C for 2 hours. After incubation, 500 µl of 10% SDS was added to the cells, and 2 µl of 100 mg/ml proteinase K was added prior to an additional 2 h incubation at 55° C. To this solution, 1.83 ml of 5 M NaCl and 5.5 ml of chloroform were added. The mixture was incubated at room temperature for 30 minutes, with frequent inversions, and then centrifuged at 4500 g for 15 minutes in order to separate the phases. The aqueous phase was transferred to a new tube, and the DNA was precipitated by the addition of 7.5 ml of isopropanol. The DNA was spooled, transferred to a new tube, and washed with ethanol prior to resuspension in TE. Southern blotting was performed using either biotinylated erythromycin resistance gene as a probe for mariner transformants, or biotinylated spectinomycin resistance gene

as a probe for Tn10 transformants, with the NEB phototope and star detection kits (New England Biolabs).

Recovery of Tn10 from mutagenized strains.

2 μ l of chromosomal DNA was used in a 50 μ l overnight HindIII digest, after which the enzyme was heat inactivated as per the manufacturer's instructions (New England Biolabs). The digest was added to a 50 μ l ligation mixture and incubated overnight at room temperature. 15 μ l of the ligation was dialyzed on a 0.022 μ m filter, and used to electroporate *E. coli*. If plasmid DNA was produced by the self-ligation of chromosomal DNA, it would be used as template for DNA sequencing, in order to identify the interrupted gene.

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Chapter 8 – Overall conclusions

The experiments described in this dissertation begin to illuminate the complex mechanisms of the starvation response by *C. perfringens*. Prior to the initiation of these experiments, little was known about this response. Previous work was limited to demonstrating that *C. perfringens* sporulation was responsive to carbohydrate availability (170, 288).

Our initial experiments demonstrated the nature of glucose repression of sporulation (348). While it had previously been determined that glucose represses sporulation, it was shown that this is independent of the transcriptional regulator CcpA, which is known to mediate catabolite repression (and activation) in *B. subtilis* (187). CcpA, however, was utilized by *C. perfringens* to repress enterotoxin (CPE) synthesis during vegetative growth, and to activate CPE synthesis during sporulation. The exact mechanism by which CcpA regulates CPE production is unknown, as there are no identifiable CcpA binding sites in the vicinity of *cpe*.

As *C. perfringens* has historically been considered non-motile (354), the discovery of motility in *C. perfringens* was a significant finding in its own right. However, the fact that *C. perfringens* motility is mediated by type four pili (TFP) is even more intriguing, as TFP have never been reported in Gram-positive bacteria. Carbon-starvation also plays a CcpA-mediated role in regulating motility in *C. perfringens*. However, inspection of the promoter regions of the TFP genes does not reveal any putative CcpA binding sites, indicating that motility is also indirectly activated by CcpA.

Biofilm formation, like motility, is another trait that had not previously been identified in *C. perfringens*. However, after identifying TFP-mediated motility, since TFP are well known for their contribution to biofilm formation, we investigated the

possibility that *C. perfringens* could form biofilms. *C. perfringens* was observed to be able to form biofilms, and the process was repressed by carbohydrates. However, like sporulation, carbohydrate repression of biofilm formation was also CcpA independent, and the mechanism for repression of biofilm formation is unknown.

This work has expanded the view of the *C. perfringens* starvation response from simply consisting of endospore formation, to also include the formation of biofilms and the development of gliding motility. All three responses are mediated by the availability of carbohydrates, primarily glucose for sporulation but non-specifically for motility and biofilm formation.

The three responses are significantly different from each other. Sporulation can occur in any environment and protects the cells from a number of lethal conditions, but it is very drastic, since at least temporarily, it removes the bacterium from the replicating population. Gliding motility requires a solid surface and is energy intensive, but it does not protect the cells from stresses other than starvation. Biofilm formation requires a solid/liquid interface, allows for protection from stresses, including oxygen and antibiotics, and is much less drastic a response than sporulation.

Work remains to identify regulatory aspects of these divergent starvation responses. The first response made by the cell must be the nature of the stresses it encounters. If there are potentially lethal components in the environment, gliding motility will do nothing to protect the bacteria, unless the bacteria are able to move faster than the substance can diffuse.

If the only stress present is starvation, the bacteria must have some mechanism for determining the fluidity of their environment, as the three responses explored in this work

take place in environments of differing fluidity (*ie* liquid culture, liquid/solid interface, and solid surface). One possible mechanism for modulating the response based on the fluid nature of the environment is to measure the density of an exported molecule. In a fluid environment, the molecule would diffuse away from the cells, but on a solid surface it would accumulate to high density surrounding the cells. This mechanism is similar to one that proposed the use of acyl-homoserine lactone rings as diffusion sensing by Redfield (276). Another possible explanation is that a given environment simply cannot physically support the behavior. For example, gliding motility requires tightly packed, organized cells, and a fluid environment might introduce too much disorder to allow for motility along the interface.

This research has provided considerable insights into the physiology of *C. perfringens*. However, all of the information learned from this work has left us with many significant questions that remain to be answered. These include elucidating the role of CcpA in the regulation of *cpe* expression, during both vegetative growth and sporulation. It is also important to identify the signals used by the bacteria to initiate the three distinct responses, this will aid in the understanding of why certain responses are favored over others. Answering those questions will require the further refinement of genetic tools in *C. perfringens*, for example, a viable transposon mutagenesis system.

The discovery of motility in *C. perfringens* is the most intriguing finding of this research, not only for its implications on the physiology of a lethal pathogen, but because of the identification of homologous TFP-encoding gene clusters in all other members of the clostridia, and the potential impact on the understanding of bacterial evolution.

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Curriculum Vitae

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Area of research: The role of catabolite repression in pathogenesis and physiology of *Clostridium perfringens*.
Date of Ph.D. Defense: September 1st, 2006
- 1997 to 2001:** Niagara University-Niagara University, NY, 14109
Bachelor of Science earned in Biology, with a minor earned in Chemistry.

Experience:

- 2000 to 2001:** Undergraduate Research, Niagara University
- 2001 to 2002:** Graduate Teaching Assistant, General Microbiology Lab, Virginia Polytechnic Institute and State University
- 2004:** Graduate Teaching Assistant, Pathogenic Bacteriology Lab, Virginia Polytechnic Institute and State University
- 2002 to 2005:** Mentored multiple undergraduate research students.
- 2006:** Post-doctoral fellow, The Institute for Genomic Research, Rockville MD

Professional Experience:

- Fall 2002:** Invited speaker for the Microbiology Club of Virginia Tech
- Spring 2006:** Invited speaker for the Virginia Tech Department of Biological Sciences Research Day
- May 2006:** Invited speaker, The Institute for Genomic Research, Rockville MD

Seminars and Posters Presented:

1. CcpA Mediates Multiple Forms of the *Clostridium perfringens* Carbon Starvation Stress Response.

PowerPoint presentation given as an invited speaker at The Institute for Genomic Research, Rockville, MD, May 2006.

2. Proper Developmental Stress-Response of *Clostridium perfringens* Depends on the Metabolic Regulator CcpA.

Poster presented and the Virginia Tech College of Science Research Roundtable, Blacksburg, VA, April 2006.

3. The Role of *ccpA* in *Clostridium perfringens* stress response.

PowerPoint presentation given as an invited speaker for Virginia Tech Department of Biological Sciences Research Day, Blacksburg, VA, February 2006.

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5. The Role of Carbohydrates in Regulating Type IV pili-Mediated Motility in *Clostridium perfringens*.

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6. Sporulation Ability of Three Strains of *Clostridium perfringens*.

Poster presented at the Virginia Tech Biology Department Alumni research Presentation, Blacksburg, VA February 2004

7. Sporulation Ability of Three Strains of *Clostridium perfringens*.

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8. The Role of the Sigma-factor *sigK* in *Clostridium perfringens* Sporulation Poster presented at the ASM General Meeting, Washington, DC, May 2003

9. Molecular Genetics tools in *Clostridium perfringens*

Poster presented at the *Clostridium* Pathogenesis Conference, Wood's Hole, MA, April 2003

10. Molecular Genetics tools in *Clostridium perfringens*

Poster presented at the Mid-Atlantic Microbial Pathogenesis Meeting, Wintergreen, VA, February 2003

11. Molecular pathogenesis of food poisoning

Slide presentation given at the Department of Biology, Virginia Tech, Blacksburg, October 2002.

Seminars and Posters as Non-presenting Author:

1. Characterization of Type 4 Pili-Dependent Social Motility by *Clostridium perfringens*. Non-presenting author for a poster presentation at Microbial Pathogenesis and Host Response, Cold Spring Harbor, NY, September 2005.
2. Motility in a “non-motile” bacterium: Type 4 pili dependent movement of *Clostridium perfringens* resembles social motility in *Myxococcus xanthus*. Non-presenting author for a PowerPoint presentation at the Mid-Atlantic Microbial Pathogenesis Meeting, Wintergreen, Virginia, February 2005.
3. Characterization of the Hpr Kinase/Phosphatase system regulating the activity of the carbon catabolite repressor in CcpA in *Clostridium perfringens*. Non-presenting author on poster presented at the Mid-Atlantic Microbial Pathogenesis Meeting, Wintergreen, VA, February 2004

Publications:

Varga J, Stirewalt VL, Melville SB. The CcpA Protein Is Necessary for Efficient Sporulation and Enterotoxin Gene (*cpe*) Regulation in *Clostridium perfringens*. J Bacteriol. 2004 Aug;186(16):5221-9.

Varga J, O'Brien DK, Ngyuen V, Walker RA, Melville SB. Evidence for an Ancient and Ubiquitous Mechanism of Bacterial Movement: Type IV Pili-dependent Gliding Motility in the clostridia. Manuscript submitted.

Myers GS, Varga J, Melville SB, Paulsen IT and 29 others. Skewed genomic variability in strains of the toxigenic bacterial pathogen *Clostridium perfringens*. Genome Research. 2006 August.

Varga J, Melville SB. The Transcriptional Regulator CcpA is Required for Proper Activation of Gliding Motility in *Clostridium perfringens*. Manuscript in progress.

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Awards and Honors:

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