AN ABSTRACT OF THE THESIS OF

Colton B. Bond for the degree of Master of Science in Microbiology presented on June 3, 2011.

Title: Role of Plc in the Germination of *Clostridium perfringens* Food Poisoning Isolate SM101

Abstract approved:

Mahfuzur R. Sarker

Clostridium perfringens is the causative agent of gas gangrene and the 3rd most common cause of type A food borne disease in the United States. Critical to the pathogenicity of *C. perfringens* is the ability of this bacterium to produce highly resistant, metabolically dormant spores that can resume metabolic function through the process of germination. Once germinated, vegetative cells can release toxins, such as the alpha toxin, the causative agent of gas gangrene. Alternatively, in type A food poisoning strains, vegetative cells can sporulate in the human gastrointestinal tract

releasing *C. perfringens* enterotoxin (CPE) the causative agent of type A food poisoning.

Recent work in *Bacillus subtilis* has identified YcsK, a phospholipase B, as having a role in the germination of *B. subtilis* spores possibly by modifying and disrupting one or more spore membranes. Although there are no YcsK homologues in *C. perfringens*, the *C. perfringens* alpha toxin has phospholipase C activity that also may modify and disrupt spore membranes. Therefore, our goal was to characterize the germination phenotype of *C. perfringens* spores lacking Plc. The novel findings of this study are five-fold: [1] Plc is expressed under sporulation conditions but is not under regulation by Spo0A, [2] Plc is necessary for normal rates of spore germination [3] Plc mutant spores incubated with dodecylamine and 100mM AK are able to release DPA, during the initial stages of germination, in a congruent fashion to wild-type spores, [4] Plc is required for normal levels of spore viability and outgrowth for a given population of phase bright spores, [5] Finally, pro-SleC/SleC Western blot analysis in this study showed that Plc mutant spores have less active SleC during germination than wild-type spores. Collectively, these results suggest that Plc is an important element of *C. perfringens* spore germination and that it acts by aiding in cortex hydrolysis by either directly or indirectly regulating the processing of SleC through an unknown mechanism.

June 3, 2011

Role of Plc in the Germination of *Clostridium perfringens* Food Poisoning Isolate SM101

by

Colton B. Bond

A THESIS

Submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Master of Science

Presented June 3, 2011

Commencement June 2011.

Master of Science thesis of Colton B. Bond presented on June 3, 2011.

APPROVED:

Major Professor, representing Microbiology

Chair of the Department of Microbiology

Dean of the Graduate School

I understand that my thesis will become a part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Colton B. Bond, Author

ACKNOWLEDGEMENTS

I would like to thank Dr. Mahfuz Sarker for his guidance, patience, and for providing me the opportunity to pursue my degree.

I would also like to thank Dr. Daniel Paredes-Sabja for his guidance and mentoring over the course of my time working in Dr. Sarker's lab.

Additionally, I'd like to thank my fellow lab members Pathima Udompijitkul, Maryam Alnoman, Saeed Banawas, and Shaina Hewitt for their camaraderie and support during the planning and execution of experiments.

A special thanks to my Mom and Dad, my sister Mckenzie, Anthony Pettigrew, and Kelly Hanslovan, who provided the emotional support without which I would not have finished my degree.

Finally, I'd like to thank the love of my life, Kise Rosen, for her unwavering positive attitude and support. She has given my life perspective and has helped me through this trying time.

CONTRIBUTION OF AUTHOR

Dr. Mahfuzur R. Sarker as a major professor provided the laboratory facilities needed for the research done on this study.

Dr. Mahfuzur R. Sarker and Dr. Daniel Paredes-Sabja contributed to the experimental design, data analysis and preparation of the manuscript in chapter 2.

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF TABLES

Role of Plc in the Germination of *Clostridium perfringens* Food Poisoning Isolate SM101

Chapter 1

General Introduction and Literature Review

Clostridium perfringens is a spore forming, anaerobic, rod shaped bacterium that is ubiquitous in nature and is part of the normal microflora in the human intestinal tract (4, 33). Previously known as *Clostridium welchii* , *C. perfringens* is thought to be the most widely occurring pathogenic bacterium of the genus *Clostridium*, of which, *Clostridium difficile*, *Clostrdium botulinum*, *Clostridium tetani*, and *Clostridium acetobutylicum* are members (15). It is the causative agent of *C. perfringens* type A food poisoning and is ranked as the 3rd most common cause of food borne illness in the United States with ~250,000 cases annually (36). Additionally, *C. perfringens* is the causative agent of non-food-borne gastrointestinal (GI) disease, gas gangrene, necrotic enteritis, septicemia, and cellulitis (18). *C. perfringens* has a documented repertoire of ~17 toxins and isolates have been categorized (A-E) by their ability to produce the toxins alpha, beta, iota, and epsilon, respectively (15, 35).

1.1 Major Toxins of *C. perfringens*

Alpha toxin

Alpha toxin is the toxin implicated in *C. perfringens* gas gangrene (12). Gas gangrene is caused when vegetative cells or spores from the environment come in contact with an open wound or are ingested. Upon entry into the affected area, the vegetative cells grow or the spores germinate and begin vegetative growth and release toxin (65). Though the exact mechanism is not known, it is believed that the alpha toxin exerts its deleterious effect by hydrolysis of host membranes and by perturbing the metabolism of host cells to the advantage of the pathogen (6, 19). Alpha toxin is a zinc metalloprotease that has both phospholipase C and sphingomeilinase activity which catalyze the cleavage of the polar head group resulting in a charged head group and a diacylglycerol group or ceramide, respectively (21, 61, 67).

All strains of *C. perfringens* have the alpha toxin structural gene (35), *plc*, which is controlled by a single promoter which has the classic -35 and -10 sequences upstream of the shine dalgarno sequence (5). Expression of *plc* is known to be indirectly regulated by the VirRS two component regulatory system which includes for VirS, a sensor histidine kinase, that in response to unknown molecule(s) phosphorylates VirR a response regulator. However, the putative gene(s) that VirRS directly regulates which, in turn, directly regulate the expression of *plc* are not known (1, 28). A purine rich region of ~77bp just upstream of the -35 box contains several $d(A)_{5\text{-}6}$ sequence tracts and progressive deletion of these tracts results in lower expression of *plc* (32). Sequencing and bioinformatic analysis of *plc* from isolates spanning type A-E have shown that the *plc* gene is highly conserved (96-98% sequence identity) among the toxinotypes (11).

Bioinformatic analysis of the 398 amino acid primary protein sequence shows that the alpha toxin is composed of three domains (62, 63). The first domain is consists of the first 28 amino acids comprising a classic signal peptide sequence, indicative of a secreted protein (26, 62). The second domain consists of amino acid residues 1-248 which has ~29% sequence identity to the nontoxic *Bacillus cereus* phospholipase C (26). This second domain has been individually identified to possess full PLC activity and reduced sphingomeylinase activity (63). Finally, the third domain consists of amino acid residues 249 – 370 and has ~29% sequence identity to human arachadonate-5'-lipoxygenase. It has been shown that without the third domain the lethality of the toxin is severely attenuated and indeed, it is this domain that non-lethal homologues, such as *B. cereus* phospholipase C, are missing. The third domain is thought to be responsible for binding of the toxin to phospholipids in a Ca^{2+} dependent manner (39, 67). Studies have shown that the alpha toxin can degrade phospholipids in liposome arrangements and that this degradation can leads to the leakage of molecules not normally able to pass the lipid bilayer, such as fluorescent dye (40, 41).

Beta toxin

The *C. perfringens* beta toxin is the major pathogenic determinant of enteritis necroticans and Pig Bel (25). The beta toxin is produced by *C. perfringens* type B and type C strains and the toxin gene, *cpb*, is carried on a plasmid (53, 59). The toxin

causes hemorrhagic necrosis and significant destruction of the intestinal villi and can be lethal (12). However, the beta toxin is rapidly inactivated by enzymes such as trypsin which is normally found in the human gastrointestinal tract. Enteritis necroticans and Pig Bel are generally associated with the ingestion of high protein meals by undernourished individuals because of the lower levels of pancreatic enzymes such as trypsin (24).

Epsilon toxin

The *C. perfringens* epsilon toxin is the major virulence factor of *C. perfringens* type B and type D strains and it is the third most potent toxin in clostridia after the tetanus and botulinum neurotoxins (51). The epsilon toxin gene, *etx*, is located on a plasmid (20). The toxin is secreted in its inactive 32.7 kDa prototoxic form and upon proteolytic cleavage by enzymes in the gastrointestinal tract or by the *C. perfringens* lambda toxin the toxin is activated (18, 37). Though the mechanism of action is not known, the toxin is lethal and it appears to increase vascular permeability in the brain, intestine, and kidneys (51).

Iota toxin

Iota toxin is produced by the *C. perfringens* type E strains. The toxin genes, *iap* and *ibp*, are found on a plasmid and code for two separate polypeptides that each have specific functions. The 47.5 kDA polypeptide, Ia, has actin specific ADP-

ribosyltransferase activity and the 71.5kDa polypeptide, Ib, comprises the binding unit (58). The iota toxin is known to cause sporatic diarrheic outbreaks among domesticated cattle (3).

Clostridium perfringens Enterotoxin (CPE)

C. perfringens enterotoxin is a heat and pH labile protein that is the causative agent in *C. perfringens* type A food poisoning and non-foodborne (NFB) gastrointestinal diseases (54). Less than 5% of these type A isolates produce CPE (33). The *cpe* gene has been shown to be located in the chromosome of food poisoning strains and on a plasmid in NFB strains (8). The toxin's expression is regulated by three promoters with the P1 promoter under the control of RNA polymerase sigma factors, sigma K and the latter two under the control of sigma E (68). CPE is expressed in the mother cell compartment and can be found in the supernatant of sporulating culture ~9-10 h after inoculation in a sporulating media (9). The pathogenicity cycle begins with the ingestion of spore infected food or non-food substrate. The spores germinate in the food, especially after cooking, or upon entry into the gastroinstestinal tract where they later spontaneously sporulate. Upon lysis of the mother cell, during the final stage of sporulation, CPE is released into the environment (9). In the human GI tract the CPE bind to the tight junction between epithelial cells and modulates membrane permeability resulting in fluid and electrolyte loss (34).

1.2 Sporulation

Bacillus and *Clostridium* can initiate sporulation by sensing nutrient deficient conditions (52). In *Bacillus*, sensor histidine kinases sense these conditions and the signal is transduced to the response regulator, Spo0A, which becomes phosphorylated. Once Spo0A is phosphorylated it triggers the start of asymmetric division of vegetative cells forming two compartments the prespore and the mother cell. Sporulation genes are regulated by a series of sporulation specific RNA polymerase sigma factors, sigma F, sigma E, sigma G, and sigma K which are activated in an ordered fashion (27). Sigma F and G regulate expression in the forespore and Sigma E and K regulate expression in the mother cell. First sigma F becomes active in the prespore and then, upon septum formation, pro-sigma E is proteolytically cleaved and becomes active in the mother cell (13, 66). Sigma F then promotes the transcription of Sigma G which also regulates its own transcription (60). Finally, sigma K is transcriptionally activated by sigma E and co-regulated by itself (23). The major steps are conserved in *C. perfringens* (16, 57). However, CPE is uniquely expressed during sporulation by some *C. perfringens* type A isolates and CPE is released into the environment upon lysis of the mother cell (14).

1.3 Bacterial Spore Germination

The germination of endospores has been well studied in *Bacillus subtilis* and to a lesser extent in other *Bacillus* and *Clostridium* species, including *C. perfringens* SM101 (38, 42, 48, 49). Germination can be initiated by a variety of germinants which are compounds such as nutrients, a 1:1 chelate of C^{2+} and DPA, and the cationic surfactant dodecylamine. Nutrient germinants for *C. perfringens* include amino acids such as L-Asn, KCl, and inorganic phosphate (Pi) with sodium as cogerminants (49, 50). Upon binding of nutrient germinants by the germinant receptor located in the spores inner membrane, an unknown signal is transduced to downstream effectors signaling the release of monovalent cations, such as H^+, K^+, and Na^+. This release causes an increase in pH in the spore core from 6.5 to 7.7, which is essential for enzymatic activity later on (55). Next, the spore releases its large depot (~10% of the spores dry weight) of pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)) in the form of a 1:1 chelate with primarily Ca^{2+} (but also with magnesium and manganese) which simultaneously results in an influx of water resulting in partial core hydration (55). At this point the increase in core hydration has reduced the spore resistance to moist heat. Following the release of Ca-DPA, the spore cortex is hydrolyzed by cortex lytic enzymes (SCLE), allowing for further influx of water, resulting in the resumption of metabolic activity. In *C. perfringens*, the SCLE SleC is produced as an inactive zymogen (56). Upon cleavage the N terminal proregion of pro-SleC by CspB, a serine protease, SleC becomes active and begins hydrolyzing the spore cortex peptidoglycan (29, 46).

Objective of this study

C. perfringens is the causative agent of gas gangrene and the 3rd most common cause of food borne disease in the United States. Critical to the pathogenicity of *C. perfringens* is the ability of this bacterium to produce highly resistant, metabolically dormant spores that can resume metabolic function through the process of germination. Recent work with *Bacillus subtilis* has shown that YcsK, a phospholipase B, has a role in *B. subtilis* spore germination. In this study it is hypothesized that phospholipases may also play a role in the germination response of *C. perfringens* spores. Knowledge pertaining to the mechanism of *C. perfringens* spore germination will be instrumental to the development of strategies to prevent *C. perfringens* diseases.

Specific Aims

1. **Construction of an alpha toxin (*plc*) knock-out mutant in *C. perfringens* type A food poisoning strain SM101.**

2. **Characterization of *plc* mutant spores with nutrient and non-nutrient germinants.**

Chapter 2

Role of Plc in the Germination of *Clostridium perfringens* Food Poisoning Isolate SM101

Colton Bond, Daniel Paredes-Sabja, Mahfuzur R. Sarker

To be submitted to the Journal of Bacteriology

2.1 Abstract

Clostridium perfringens is the causative agent of gas gangrene and several gastrointestinal (GI) diseases in humans and animals. Critical to the pathogenicity of *C. perfringens* is the ability of this bacterium to produce highly resistant, metabolically dormant spores that can resume metabolic function through the process of germination (44). Once spores germinate, vegetative cells can proliferate and release toxins, such as the alpha toxin, the causative agent of gas gangrene. Alternatively, vegetative cells can sporulate in the human gastrointestinal tract releasing *C. perfringens* enterotoxin (CPE) the causative agent of type A food poisoning (33, 35). The novel findings of this study are five-fold: [1] Plc is expressed under sporulation conditions but is not under regulation by *spo0A*, [2] Plc is necessary for normal rates of spore germination [3] Plc mutant spores incubated with dodecylamine and 100mM AK are able to release DPA, during the initial stages of germination in a congruent fashion to wild-type spores, [4] Plc is required for normal levels of spore viability and outgrowth for a given population of phase bright spores, [5] Finally, pro-SleC/SleC Western blot analysis in this study shows Plc mutant spores have less active SleC during germination than wild-type spores. Collectively, these results suggest that Plc is an important element of *C. perfringens* spore germination and that it acts by aiding in cortex hydrolysis by either directly or indirectly regulating the processing of SleC through an unknown mechanism.

2.2 Introduction

Clostridium perfringens is a gram positive, anaerobic, spore former that is ubiquitous in nature (33). *C. perfringens* isolates are categorized into five types (A-E) based on their ability to produce alpha, beta, epsilon, and iota toxins (15). A small percentage of type A isolates produce the *C. perfringens* enterotoxin (CPE) and cause type A food poisoning which is the 3rd most common cause of food borne disease in the United States (36). *C. perfringens* type A is also the most important causative agent of gas gangrene which is mediated by the enzymatic activity of alpha toxin, a phospholipase C, secreted during vegetative growth (35). However, in order for *C. perfringens* to release toxins and cause disease, its highly resistant dormant spores must resume metabolic function and 'return to life' through a process termed, germination, outgrow, and return to vegetative growth (55). Thus, the ability of spores to germinate is an essential factor in *C. perfringens* pathogenesis.

In *Bacillus* and *Clostridium* species, germination can be initiated by a variety of germinants which are compounds such as nutrients, a 1:1 chelate of Ca^{2+} and DPA, and the cationic surfactant dodecylamine (38, 42, 48, 49). Upon binding of nutrient germinants by the germinant receptor located in the spores inner membrane, an unknown signal is transduced to downstream effectors signaling the release of monovalent cations, such as H^+, K^+, and Na^+ (55). Next, the spore releases its large depot (~10% of the spores dry weight) of pyridine-2,6-dicarboxylic acid (dipicolinic

acid (DPA)) in the form of a 1:1 chelate with primarily Ca^{2+} which simultaneously results in an influx of water resulting in partial core hydration (43, 44). Following the release of Ca-DPA, the spore cortex is hydrolyzed by cortex lytic enzymes (SCLE), allowing for further influx of water, resulting in the resumption of metabolic activity (38).

Recent work in *Bacillus subtilis* has identified the protein YcsK as a critical component of spore germination. YcsK mutant spores were shown to be deficient in their ability to germinate with a 77% reduction spore germination and a 60 to 70% decrease in Ca-DPA release compared to wild-type (30, 31). Furthermore, *ycs*K-GFP fusion assays have shown that YcsK is present in the mother cell and associates with the spore coat during the late stages of sporulation. Finally, YcsK has been shown to have phospholipase B activity (cleavage of ester linkages of the sn-1 & sn-2 positions of phospholipids) and the supernatant of YcsK mutant spores has significantly less free fatty acids than wild-type suggesting that YcsK modifies the spores outer membrane (30, 31).

Bioinformatic analysis of the *C. perfringens* SM101 genome shows no homologues of YcsK. However, alpha-toxin, a phospholipase C (Plc) is present in all strains (including food poisoning isolates). Although, Plc has a different enzymatic activity than phospholipase B (Plc is a phosphodiesterase that cleaves phosphomonoesters off the glycerol backbone of lipids) (30, 40), we hypothesize that the alpha-toxin might also modify one or more spore membranes and possibly play an

important role in the ability of *C. perfringens* spores to germinate. Consequently, we have constructed *plc-gusA* fusions and *plc* mutants and we have characterized the expression and germination phenotype, respectively. Our results indicate that *plc* is expressed under sporulation and vegetative growth conditions but is not regulated by the sporulation master regulator *spo0A*. *plc* mutant spores germinate less rapidly than wild-type spores in response to nutrient germinants. However, DPA release is not inhibited or slowed during dodecylamine or AK induced germination. Interestingly, *plc* mutant spores did have a slower outgrowth and did have a reduced colony forming ability. Finally, *plc* mutant spores were observed to have lower levels of active SleC during AK triggered germination.

2.3 Methods and Materials

Bacterial strains, plasmids, and primers. The *C. perfringens* and *E. coli* strains, in addition to, the plasmids and primers used in the study are listed in Table 1.

DNA Isolation. Single colonies or 0.5ml of stock culture were inoculated into 10ml TGY (3% trypticase broth, 2% glucose, 1% yeast extract, and 0.1% cysteine) media and incubated overnight at 37°C. 1.5ml aliquots of overnight culture were resuspended in 1ml of lysis solution (50ul 1M Tris pH8.0, 25ul 0.5M EDTA pH8.0, 500ul 20% glucose, 0.02g lysozyme, 5ul 20mg/ml proteinase K, and 420ul of distilled H2O) for 2 h at 37°C. Next, the samples were resuspeded in 500ul of 100mM Tris + 10mM EDTA pH 8.0, 125ul of 10% sarkosyl was added and samples were incubated at 60°C for 10 min. After that, a phenol:chloroform (1:1) extraction was performed and the top layer was relocated to a new tube and incubated with 5ul of 10ml/ml RNase A at room temperature for 30 min. Another phenol:chloroform (1:1) extraction was performed and the top layer was relocated to a new tube. DNA was then precipitated by adding 40ul 5M NaCl and 1ml 200proof EtOH. Samples were centrifuged at 13,200 rpm for 10 min at 4°C, the supernatant was removed, and the DNA pellet was allowed to dry thoroughly. Finally, the DNA was dissolved in 75ul of TE (10mM Tris, 1mM EDTA pH8.0) and was stored at -20°C.

Construction of an expression vector and expression of plc. *plc-gusA* fusion constructs were made by PCR amplifying the region immediately 521 bp upstream of

plc using primers CPP881/882 (Table 2) and cloning it into TOPO-XL resulting in pCB41. pCB41 was then digested with SalI-PstI and the 521bp promoter region was cloned into pMRS127 upstream of promoter less *gus*A resulting in pCB42. pCB42 was electroporated into SM101 and IH101 (*spo0A*::*catP*) and two separate clones were assayed for beta-glucoronidase activity at various time points under vegetative growth conditions (TGY media) and Sporulation conditions (Duncan-Strong (DS) media) (10).

Construction of *plc*::intron mutant. The *plc* mutator plasmid (pDP239) was constructed by inserting an erythromycin resistance gene into the EcoRV restriction site within the *catP* cassette of pJIR750ai (sigma-alderich). pDP239 was then electroporated into *C. perfringens* SM101 and cells were plated on BHI agar with erythromycin (50ug/ml). Each transformant clone was then passaged 8 times in TGY media with erythromycin (50ug/ml) by growing cultures for 12h and then inoculating an additional tube of TGY media. Cultures that had been passaged 8 times were then streaked for colony isolation on 5% egg yolk agar plates. Colonies exhibiting no darkened halo (no beta hemolysis activity indicative of Plc activity (62)) around the colony were then confirmed to have the mutant genotype by PCR. *plc* mutants were then passaged an additional 10-14 times in TGY media (without selection) to cure the plasmid. Plasmid curing was confirmed by streaking passaged cultures for colony isolation and patching single colonies on BHI agar plates with 5% egg yolk or BHI agar plates with erythromycin (50ug/ml). The mutant genotypes of colonies that

exhibit erythromycin sensitivity (Er^S) and also exhibited no beta hemolysis activity on the egg yolk plates were further confirmed by southern blot.

Construction of *plc*::*catP* mutant. The *plc* mutator plasmid was constructed by utilizing a suicidal vector already used for *in-cis* complementation of genes in the *plc* locus that contains the *erm*B antibiotic resistance marker (pDP129). pDP129 contains a ~1.7kb fragment upstream of *plc* and an ~1.3kb downstream fragment. pDP129 was modified by cloning an ~1.3kb catP cassette into the KpnI-SalI multiple cloning site located between the upstream and downstream *plc* fragments on the plasmid resulting in pDP275, the mutator plasmid. pDP275 was electroporated into SM101 and transformants were confirmed for single integration of the plasmid via PCR. Single integrants (Er^R, Cm^R) were passaged up to 10 times in TGY without selection. Single colonies were isolated for each passage and Er^S, Cm^R colonies were screened for by patching onto BHIA with Cm or Er. Mutant genotypes were confirmed by PCR.

Construction of a *plc* mutant strain complemented with wild-type *plc*. A 1,704bp PCR amplicon of the entire *plc* ORF and the promoter region was amplified using primers CPP847/848 (Table 2), containing a KpnI and SalI restriction site at the 5' ends, respectively. The KpnI-SalI amplicon was cloned into a multicopy vector pJIR751 making pCB37. pCB37 was electroporated into CB102 cells, plated on BHI agar plates supplemented with 5% egg yolk and erythromycin (50ug/ml) and incubated anaerobically at 37C for 24h. Colonies that overexpress *plc* have a very

pronounced halo around them. These colonies were isolated and the genotype was confirmed by PCR using primers CPP 847/848 (Table 2).

Southern Blot Analysis. DNA from SM101 (parential wild-type strain), CB102 (*plc*::intron), CB102 (pCB37), and pDP239 (mutator plasmid) were digested with BamHI at 37°C for 24 h. Digested DNA was run on a 1% agarose gel and the gel was treated with 0.2M HCl 20 min, Denaturing solution (5M NaOH, 1.5M NaCl) 20 min, and Neutralization buffer (5M THAM, 1.5M NaCl, pH7.5) 20 min before being transferred overnight onto an Amersham Hybond^{TM}-N+ nylon membrane (GE Healthcare). The DNA was crosslinked to the membrane by treating with UV (150 KJ) and was hybridized with the probe for 36 h at 55°C. Southern blots were washed with primary wash buffer (2M Urea, 0.1% (w/v) SDS, 50mM Monobasic Sodium Dihydrogen Phosphate pH 7.0, 1mM MgCl2, 0.2% (w/v) Blocking reagent) at 55°C for 10 min twice and then washed with secondary wash working solution (0.05M Tris base, 0.1M NaCl, 2mM MgCl2) at room temperature once for 5 min. Alkaline phosphatase activity was assayed for using the chemiluminescent detection system (Molecular Imager ChemiDoc XRS+ System, BioRad) and by using CDP-Star chemiluminescent detection system (GE Healthcare).

The targetron probe was made by PCR amplifying a 343bp fragment of the intron from the targetron mutator plasmid using primers CPP811/812 (Table 2). The purified probe was labeled using the AlkPhos labeling kit (GE Healthcare) and stored at -20°C.

Spore preparation. Starter culture were obtained by inoculating 0.1ml of a cooked meat stock into 10ml fluid thioglycolate (FTG) media (Difco) and incubating overnight at 37°C. 0.5ml of overnight culture was inoculated into 10ml of FTG media and was incubated at 37°C for ~8-9 hours. To obtain larger quantities of spores, 10-15, 10ml FTG tubes were inoculated at the same time. To obtain sporulating culture 50ml of 8-9 h FTG culture was inoculated into 1L of pre-warmed Duncan-Strong (DS) media and was incubated at 37°C for 24 h. Sporulating cultures were then concentrated by centrifugation (8,000 rpm, 4°C) and were purified by either washing several times with sterile distilled H_2O or using the density gradient Histodenz (Nycodenz, Sigma). Purified spore suspensions were confirmed by phase contrast microscopy to be >97% free of vegetative cells, germinated spores and debris. Purified spores were suspended in sterile distilled H_2O to an OD_{600nm} of ~6 and were stored at -80°C.

Germination assay. Purified spore suspensions of OD_{600nm} of ~1 were heat shocked at 80°C for 10 min, cooled to RT for 5 min, and incubated at 40°C for 10 min before being added to germinants. At various time points, the OD_{600nm} reading was recorded as previously described (49). For maximum rate assays, an OD_{600nm} reading was taken every 2.5 min for the first 15 min and the maximum rate is expressed as the maximum decrease in OD_{600nm}/min.

DPA release. Spore suspensions of a OD_{600nm} ~1.5 were heat activated at 80°C for 10min, cooled down to room temperature 5 min, and preincubated at 40°C for 10 min

before the addition of the spores to preheated (40°C) 100mM Asparagine +100mM KCl pH 7.5 or 25mM tris-HCl buffer pH 7.5. At various time points, the samples were vortexed, 1 ml aliquots were taken, and the spore suspensions were washed 5 times with sterile distilled H_2O. Samples were then boiled for 60 min, centrifuged at 14,000 rpm for 5 min, and 400ul aliquots of the supernatant were then measured at OD_{270nm} as previously described (49). The spores initial DPA content was measured by boiling 1ml aliquots of spore suspension in tris-HCl buffer pH 7.5 at an OD_{600nm} ~1.5 for 60 min, centrifugation of the samples at 14,000 rpm for 5 min, and measuring the supernatant at OD_{270nm}.

In some experiments, *C. perfringens* spores were incubated with 1mM dodecylamine + 25mM tris-HCl buffer pH 7.4. In this case, spores that were not heat activated were preincubated at 60°C for 10 min before the addition of the spores to preheated (60°C) dodecylamine solution. At various time points, the samples were vortexed, 1 ml aliquots were taken, and they were centrifuged at 14,000 rpm for 5 min. 400ul aliquots of the supernatant were then measured at OD_{270nm} as previously described (49). The spores initial DPA content was measured by boiling 1ml aliquots of spore suspension in tris-HCl buffer pH 7.5 at an OD600nm ~1.5 for 60 min, centrifugation of the samples at 14,000 rpm for 5 min, and measuring the supernatant at OD_{270nm} (49).

Colony forming efficiency and population density. Purified spore suspensions of an OD_{600nm} of 0.950 to 1.050 were heat activated at 80°C for 10 min, cooled to room

temperature 5 min, and warmed to 40°C for 10 min. The heat activated spores were then serially diluted in 25mM tris-HCl buffer pH 7.5 and plated onto BHI agar plates. In some experiments, heat activated spores suspensions were incubated at 40°C with 100mM KCl + 25mM tris-HCl buffer pH 7.5 for 20 min, serially diluted, and plated on BHI agar plates with an additional 100mM KCl added. In other experiments, spores were decoated with (50mM Tris-HCl pH 8.0, 8M Urea, 1% (w/v) SDS, and 50mM DTT), serially diluted and plated on BHI agar plates with 1ug/ml lysozyme. All plates were incubated at 37°C for 24 hours and colonies were counted using a plate counter.

For each experiment above, 10ul samples of purified spore suspensions were loaded into a Z30000 Helber Counting Chamber and at least 400 phase bright spores were counted. Spore population densities were obtained by multiplying the volume of the large squares on the slide by the average number of spores counted in each large square and values are expressed as phase bright spores/ml/OD_{600nm}.

Outgrowth and growth curve. For outgrowth experiments, heat activated purified spore suspensions were inoculated into 10ml of pre-warmed (37°C) TGY media resulting in an OD600nm of ~0.150. At various time points, the OD_{600nm} was recorded. For growth curve experiments, 0.5ml of overnight culture was inoculated into 10ml TGY media and incubated at 37°C for 3 h. After incubation the OD_{600nm} of the cultures was recorded and ~0.5ml of culture was inoculated into a pre-warmed 10ml TGY and incubated at 37°C. At various time points the OD_{600nm} was recorded as previously described (47).

Western blot analysis. Purified spores of an OD_{600nm} of ~18 were heat activated at 80°C 10 min, cooled to room temperature 5 min, pre-warmed at 40°C for 10 min and added to 100mM AK (100mM Asparagine, 100mM KCl, 25mM Tris-HCl pH 7.5). Samples incubated with AK were incubated at 40°C and at various time points 20ul samples were taken, mixed with 20ul of lamelli loading buffer and boiled for 60 min. After boiling the samples were centrifuged at 13,200 rpm for 5min and the supernatant was removed to another tube and stored on ice. As a control spores that were not heat activated were decoated (50mM Tris-HCl pH 8.0, 8M Urea, 1% (w/v) SDS, and 50mM DTT). Decoated spores were incubated at 40°C for 90 min, and boiled for 60 min. After boiling the samples were centrifuged at 13,200 rpm for 5 min and the supernatant was removed to another tube and stored on ice. Once all samples had been obtained they were removed from ice, boiled for 3 min to dissolve any precipitating protein, run on a SDS-PAGE gel (12% acrylamide) and proteins were transferred to a PVDF membrane (Millipore). Western blots were probed with 1:20,000 dilution of anti-SleC polyclonal antibodies in PBS (25mM Sodium phosphate pH 7.4, 150mM NaCl) with 1% BSA and 1% tween, at room temperature for 24 h as previously described (46). Next, the membrane was probed with 1:10,000 dilution of goat anti-mouse IgG-Horseradish peroxidase conjugate (Promega) for 1h at room temperature. Horseradish peroxidase activity was assayed for with a chemiluminescence detection system (Molecular Imager ChemiDoc XRS+ System, BioRad) and by using the horseradish peroxidase sensitive substrate PicoMax (Rockland Immunochemicals). Each western blot also included a prestained ladder (Page Ruler Plus, Fermentas).

Statistical analysis. Student's *t*-test was used for specific comparisons between wild-type and mutant spores.

2.4 Results

***C. perfringens plc* is expressed during sporulation but is not regulated by *spo0A*.** To assess whether the *C. perfringnes plc* gene, encoding a phospholipaseC, is expressed during sporulation, the promoter region immediately upstream of the *plc* ATG start codon (which included the three poly(A) tracts, the -35, and -10 promoter sequences) was fused to *E. coli gusA* (Fig. 2.1A) and was introduced into *C. perfringnes* SM101. Upon introduction, GUS activity was measured. High GUS activity was seen under vegetative conditions as has been previously described (7). However, GUS activity of cells containing the *plc-gusA* fusions under sporulating conditions showed an approximately two-fold increase in activity as compared to vegetative conditions ($P < 0.01$) and both conditions showed peak expression at 3h (Fig 2.1B).

Since previous studies have shown *spo0A* to be the master regulator of sporulation specific genes (17) the *plc-gusA* fusions were also introduced into IH101 (*spo0A*::*catP*) and GUS activity was measured. Unexpectedly, similar expression levels were observed in IH101 as in SM101 with peak expression at 3 h and *plc* expression under sporulation conditions being at least two-fold higher ($P < 0.005$) than under vegetative growth conditions (Fig 2.1C). Collectively, these results suggest that *plc* is expressed under sporulation conditions but is not under regulation by *spo0A*.

Confirmation of *plc* mutant genotype and phenotype. Recent studies with *Bacillus subtilis* have shown that a phospholipase B plays a major role in the ability of spores to germinate (30, 31) since *C. perfringens plc* is expressed during sporulation, it was of interest to determine whether *C. perfringens* Plc also plays a role in spore germination. To this end, two *plc* mutant strains were constructed; a *plc* deletion mutant (CB101) (Fig 2.2A) and a *plc* mutant made by the Targetron gene knock out system (CB102) (Fig 2.3A) as described in the materials and methods (7). To confirm the *plc* deletion mutant had the mutated genotype we conducted PCR on the *plc* ORF. As expected, CB101 had a ~900bp DNA fragment inserted into the *plc* ORF but the wild-type strain did not (Fig 2.2B). It has been documented that Plc activity will create a zone of turbidity on egg yolk agar (61). Therefore we also grew our mutants on this media and observed that CB101 but not the parental wild-type strain had a halo of turbidity surrounding bacterial growth (Fig 2.2C). As expected, similar results on egg yolk agar were observed for the targetron mutant as well (Fig 2.3B). In addition, a *plc*::targetron mutant (CB102) containing a multicopy plasmid with the wild-type *plc* allele was also plated on egg yolk agar and increased halo of turbidity was observed consistent with their being multiple wild-type alleles expressed (Fig 2.3B). Finally, the mutant genotype of CB102 was analyzed by southern blot using BamHI digested DNA and hybridization with a 343bp intron fragment (see Methods and Materials). As expected, the probe only hybridized to CB102 mutant chromosomal DNA and the mutator plasmid control but not SM101 wild-type chromosomal DNA. Additionally, there was only one site of probe hybridization (~3258bp chromosomal, ~3083bp

targetron plasmid) for those samples to which it bound which is consistent with a single insertion of the *plc* targetron into the *plc* ORF (Fig 2.3C).

***C. perfringens plc* mutant spores germinate less rapidly than SM101 wild-type spores.** Preliminary analysis of CB101 showed that spores incubated with the nutrient media BHI (Fig 2.4A) germinated to a significantly reduced extent ($p< 0.005$) over 90 min as compared to the parental wild-type strain, with CB101 spores exhibiting an ~9% smaller decrease in their OD_{600nm} after 90 min. Additionally, incubation of CB101 with the nutrient germinants, AK, KCl, and Pi also resulted in significantly reduced germination responses ($P < 0.01$, $P < 0.005$, $P < 0.05$, respectively) as compared to wild-type after 90min (Fig 2.4B-D).

For a more thorough analysis, CB102 spores were also incubated with the nutrient germinants BHI, AK, KCl, and Pi and their decrease in OD_{600nm} was measured. As expected, CB102 spores exhibited an ~14%, 18% and 15% smaller decrease in their OD_{600nm} after 90 min as compared to the parental wild-type spores in response to BHI, AK and Pi, respectively (Fig 2.5A-C). Indeed these germination deficits with BHI, AK, and PI were significant ($P < 0.005$, $P < 0.00005$, $P < 0.000005$, respectively). Unexpectedly, the drop in OD_{600nm}, in response to KCl, of CB102 spores was not significantly different from that of wild-type spores after 90 min. However, there was an ~5% reduction ($P < 0.005$) in the ability of CB102 spores to decrease in their OD_{600nm} after 20 min of incubation with KCl as compared to wild-type spores. However, efforts to complement the germination phenotype by expressing the wild-

type allele *in-trans* in CB102 resulted in an even smaller decrease in the OD_{600nm} over 90 min than with CB102 spores (Fig 2.5A-D).

***C. perfringens* spores lacking Plc and wild-type SM101 spores release their DPA in a congruent manner.** Previous studies in *B. subtilis* and *C. perfringens* have shown that DPA release can be stimulated through activation of the nutrient germinant receptors and also by the cationic surfactant dodecylamine, which directly stimulates the opening of DPA channels (64). Since *plc* mutant spores germinated less rapidly then next question was whether Plc plays a role in DPA release during the early phase of germination. Unexpectedly, *C. perfringens* spores lacking Plc released DPA at similar rates and levels as wild-type spores in response to incubation with dodecylamine (Fig 2.6A) and 100mM AK (Fig 2.6B).

Effect of *plc* mutation on spore population density and colony forming efficiency. After releasing DPA during the initial stages of germination, spores of *B. subtilis* and *C. perfringens* begin cortex hydrolysis which is done by SCLEs (22). Since *plc* mutant spores germinated less rapidly than wild-type spores but released their DPA like wild-type spores, it was of interest to see whether *plc* mutant spores had a deficiency in their ability to form colonies. Interestingly, *plc* mutant spores plated on BHI agar yielded similar titers (Fig 2.7A). However, upon counting the number of phase bright spores in a suspension at an OD_{600nm} of 1, the *plc* mutant spores preparations contained ~two-fold more spores ($P < 0.005$) (Fig 2.7B). To see if the ~50% lower percentage of titers (in relation to the number of phase bright spores

available to form a colony) of *plc* mutant spores was a result of them germinating poorly with BHI, the spores were incubated with 100mM KCl and then plated on BHI agar supplemented with 100mM KCl (Fig 2.7A). However, similar titers were observed suggesting that lower titer counts were not the result of poor germinant stimulation. To see if *plc* spores were deficient in their ability to commence cortex hydrolysis, *plc* mutant spores and wild-type SM101 spores were decoated, heat activated, and plated on BHI supplemented with 1ug/ml lysozyme as described in the materials and methods. Intriguingly, wild-type spore titers remained the same but *plc* mutant spores had titer levels ~85.5% greater than wild-type.

***C. perfringens plc* mutant spore outgrowth is slower than wild-type.** Since *plc* mutants had a two-fold lower colony forming efficiency (relative to the density of phase bright spores in mutant versus wild-type suspensions) it was also of interest to see if Plc might have a role in outgrowth. After inoculating TGY media with similar levels of phase bright spores the OD_{600nm} was monitored for three hours. Interestingly, *plc* mutant spores went through outgrowth more slowly than wild-type spores ($P < 0.01$) (Fig 2.8A). To see if this was due to different rates of growth between wild-type and *plc* mutant strains a growth curve was conducted as described in the materials and methods. However, no significant difference in vegetative cell growth was observed between the strains (Fig 2.8B).

Plc spores have less active SleC during germination. In *C. perfringens* the SCLE SleC is in an inactive form (pro-SleC) with a short amino acid pro-region. Once this

pro-region is cleaved off by CspB, SleC begins to break down the cortex peptidoglycan which ultimately results in complete germination (22, 46, 48). Since *plc* mutant spores had lower colony forming ability and outgrowth capability and that incubation with lysozyme caused greatly increased the colony forming ability of *plc* mutant spores, it was of interest to see whether there were any detectable differences in the SCLEs. Spores were germinated with 100mM AK and samples were taken at 5, 10, and 30 min. Additionally, the protein extracts of decoated spores (DS), not incubated with AK, were also collected and subjected to western blot analysis with anti-SleC polyclonal antibodies. Similar levels of the inactive zymogen pro-SleC (34kDa) were present in all the samples and, as expected, no SleC (31kDa) was observed with decoated spores. However, small bands of SleC were observed after 10 and 30 min of AK germination of wild-type SM101 spores but no SleC bands were observed at 5, 10, or 30 min with *plc* mutant spores incubated with AK.

2.5 Discussion

Clostridium perfringens is the causative agent of gas gangrene and several gastrointestinal diseases in humans and animals. The ability of this bacterium to produce highly resistant, metabolically dormant spores that can resume metabolic function through the process of germination is a critical to its ability to cause disease (44). Once dormant spores germinate, outgrow, and vegetatively proliferate they can release toxins and cause disease. Alternatively, vegetative cells can sporulate in the human gastrointestinal tract releasing *C. perfringens* enterotoxin (CPE), causing type A food poisoning (33, 35).

A major conclusion of this study is that *plc* is expressed under sporulation conditions in a Spo0A independent fashion and that this expression in ~two-fold higher than under vegetative growth conditions. Therefore, it is then reasonable that Plc could have an active role in germination. Although, further studies into whether the alpha toxin is expressed in the mother cell or in the developing forespore are warranted. The two-fold higher expression suggests that the *plc* promoter is being recognized by RNA polymerase sigma factors associated with sporulation and vegetative growth. However, this plc regulator is unlikely to be any anything under the sole control of Spo0A since expression conditions in a Spo0A mutant were identical to those under wild-type conditions. It might be that expression is suppressed by an unknown regulator under vegetative conditions and this suppressor is suppressed under sporulation conditions allowing for greater *plc* expression.

A second major conclusion of this work is that *plc* mutant spores germinated less rapidly than wild-type spores and that DPA release was identical with wild-type and *plc* mutant spores. This indicates that germinant access to germinant receptors through the outer membrane was not a limiting factor since the germinant receptors transduced signals that resulted wild-type levels of DPA being released. This is in contrast to the recent findings that a phospholipase B has a role in *B. subtilis* germination and decreases the DPA release by ~70% (31).

The third conclusion is that *plc* mutant spores had slower outgrowth and a lower colony forming efficiency (when comparing the percentage of phase bright spores in a suspension at an OD_{600nm} 1). Since *plc* mutant spores were smaller than wild-type spores, it made sense that more phase bright spores would be necessary in a suspension to obtain an OD_{600nm} of 1. However, the colony forming efficiency had to be adjusted to account for the two fold increase in phase bright spores in an OD_{600nm} 1 suspension. It was also interesting to see that incubation and plating with supplemental KCl did not increase the titers for mutant or wild-type. This suggests that even though spores did not germinate as well with BHI as they did with KCl, after 24h incubation on BHI agar most spores in a population did germinate. Decoated *plc* spores incubated with lysozyme gave rise to significantly more colonies than decoated wild-type spores, suggesting that *plc* spores have a deficiency in their ability to go through cortex hydrolysis. However, the increase in colony forming efficiency to nearly 100% suggests that cortex hydrolysis deficiencies alone cannot account for this increase in viability. Since *plc* mutant spores were observed to be smaller in size, it may well be

that Plc plays structural roles in the spore and might contribute to the decoated wild-type spore cortex peptidoglycan being less accessible to the lysozyme than Plc mutant spores

The last conclusion is that small bands of SleC were observed after 10 and 30 min of AK germination of wild-type SM101 spores but no SleC bands were observed at 5, 10, or 30 min with *plc* mutant spores incubated with AK. This evidence further suggests that *plc* mutant spores are deficient in their ability to go through cortex hydrolysis. It is possible that Plc indirectly activates SleC processing by activation of another enzyme but this seems unlikely since Plc activity is has been pretty well confined to phospholipids. Another possibility is that Plc attaches to the spore outer membrane and provides a small steric environment where regulatory enzymes, like CspB, are more likely to encounter and process SleC. Further investigation into the role of Plc in *C. perfingens* spore germination is certainly warranted as the mechanism may shed light on gaps in our knowledge of the germination process and lead to new prophylactic treatments.

Figures

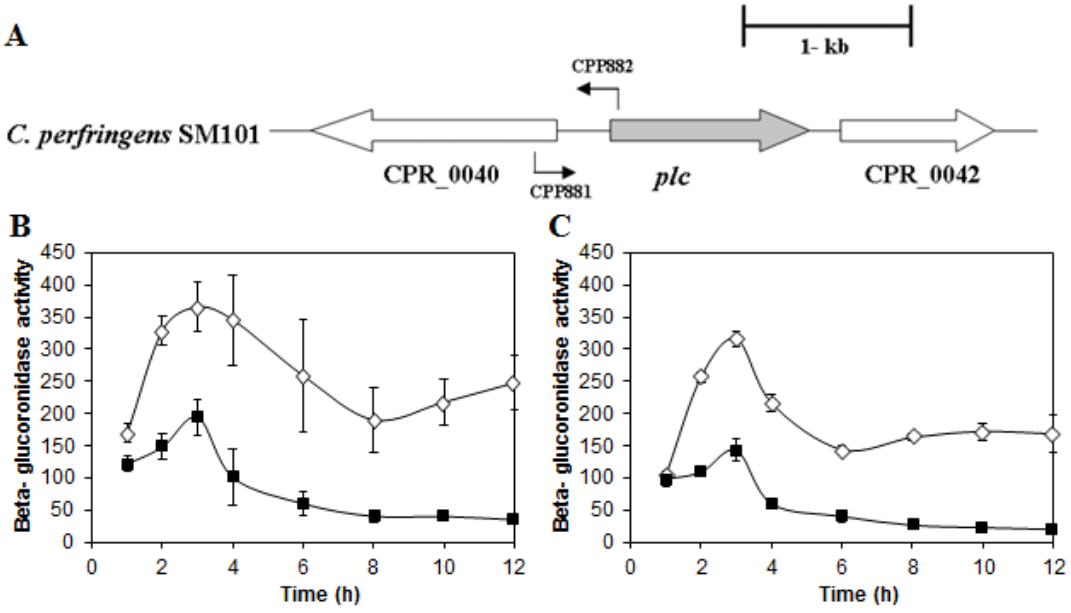

Fig 2.1. Arrangement and expression of *plc*. (A) *plc* orientation in the *C. perfringnes* SM101 genome. Arrows signify the primers used to amplify the promoter region which was cloned upstream of a promoterless *gus*A as described in the materials and methods. *plc-gusA* fusion construct expression in SM101 (wild-type) (B) or IH101 (*spo0A*::*catP*) (C) grown in TGY vegetative media (■) and DS sporulation media (◊). These results represent the average of two independent experiments. Standard error values were used to compute deviations from the mean.

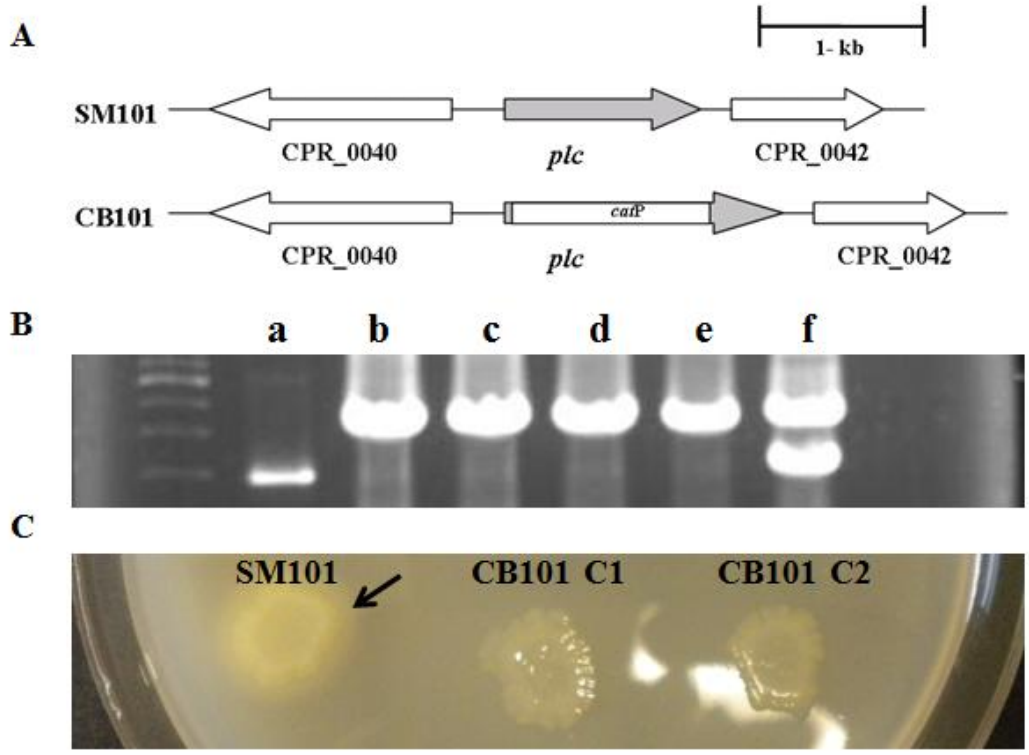

Fig 2.2. Construction of *plc* mutant using homologous recombination. (A) Diagram of wild-type (SM101) and the mutated allele (*plc*::*catP*, CB101). An ~764 bp fragment of the *plc* ORF was deleted and replaced with a 1.3 kb *catP* cassette. (B) Genotyping of *plc* mutant by PCR analysis. Lane a, SM101 control; lane b-e, clones with mutated allele; lane f, single integrant control (wild-type and mutated allele). (C) SM101 and CB101 grown on 4% egg yolk agar plates for 24 h at 37°C. Phospholipase C activity can be clearly seen by the darkened halo around bacterial growth (black arrow).

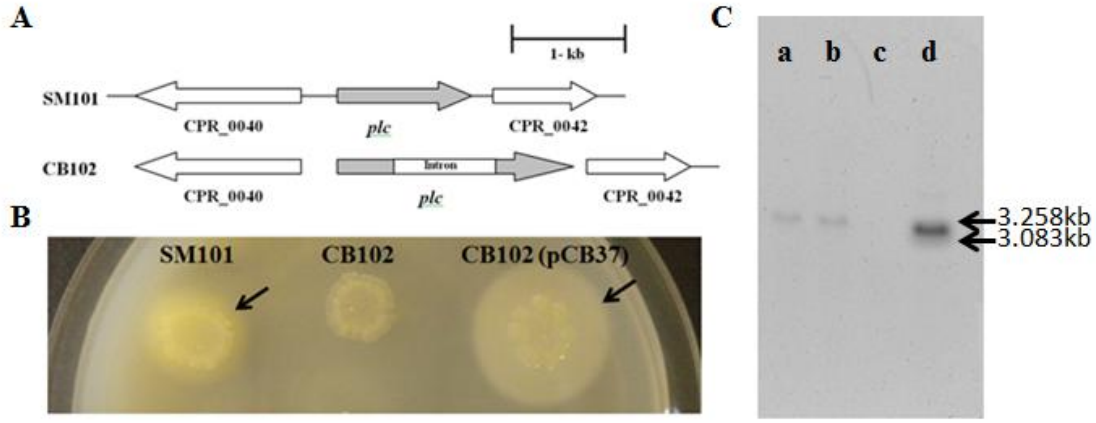

Fig 2.3. Construction of *plc* mutant using targetron method. (A) Diagram of wild-type (SM101) and the mutated allele (Δ*plc*::intron, CB102). An ~900 bp intron fragment was inserted into the antisense strand of the *plc* ORF between the 50 and 51bp from the start codon. (B) SM101, CB102 (Δ*plc*::intron), and CB102 (pCB37) (*plc* mutant complemented with wild-type *plc*) were grown on 4% egg yolk agar plates for 24h at 37 C. Phospholipase C activity can be clearly seen by the darkened halo around bacterial growth (black arrow). (C) Southern blot analysis of *plc* mutant. Lane a, CB102 (Δ*plc*::intron); lane b, CB102 (pCB37) (*plc* mutant complemented with wild-type *plc*); lane c, SM101 (wild type) negative control, pDP239 (mutator plasmid) positive control.

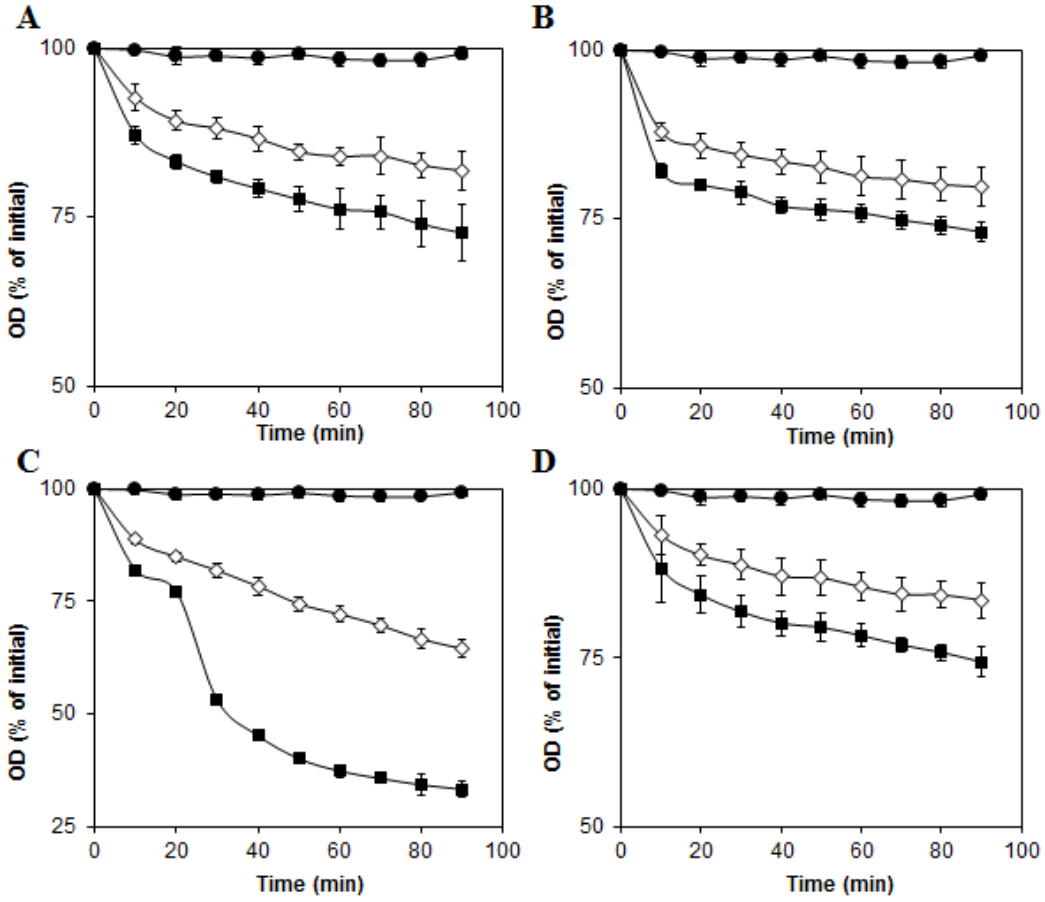

Fig 2.4. Germination of *C. perfringens* spores with nutrient germinants. Heat activated spores of strains SM101 (wild-type) (■) and CB101 (Δ*plc*:: *catP*) (◊) were germinated at 40°C with (A) BHI broth pH 7.5, (B) 100mM Asparagine + 100mM KCl pH 7.5, (C) 100mM KCl pH 7.5, and (D) 250mM inorganic phosphate (Pi) pH 6.0. Spores were also incubated with 25mM tris-HCl buffer pH 7.5 (●). After the addition of germinant, OD_{600nm} readings were recorded at various times. Data points represent the average of at least 3 independent experiments and error bars represent the standard deviation.

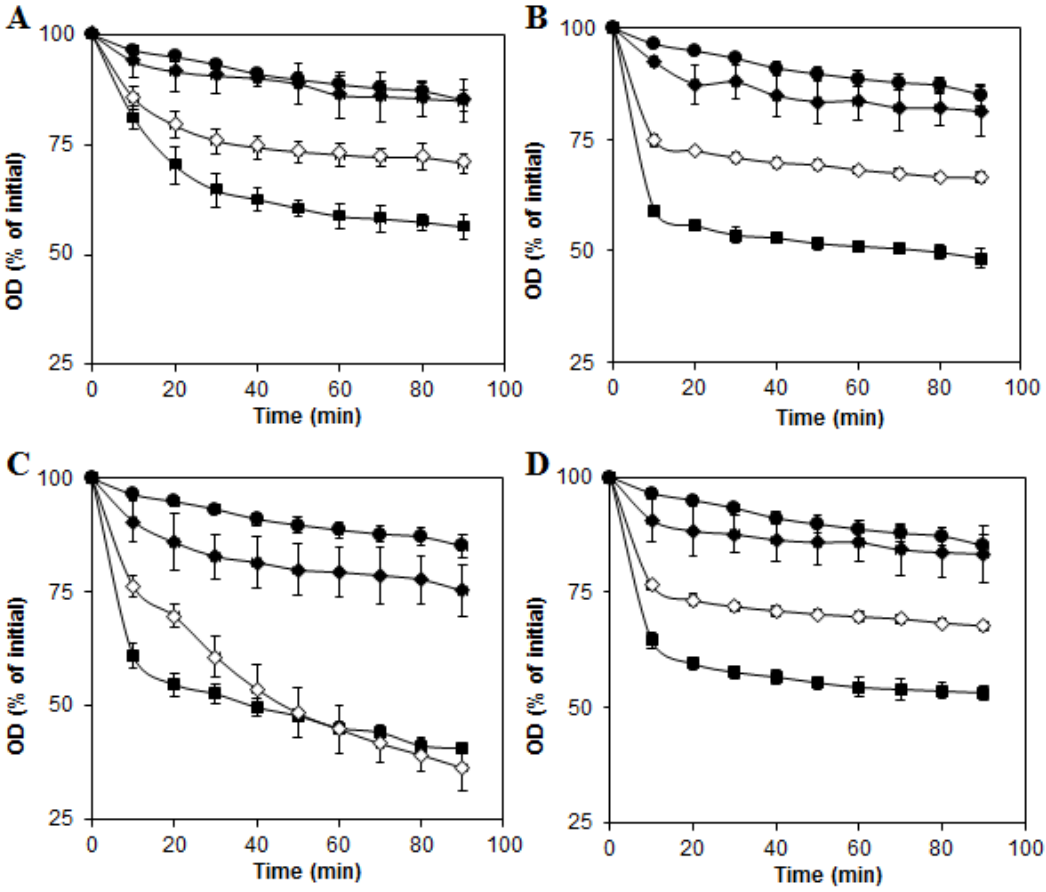

Fig 2.5. Germination of *C. perfringens* spores with nutrient germinants. Heat activated spores of strains SM101 (wild-type) (■), CB102 (Δ*plc*:: intron) (◊) and CB102 (pCB37) (*plc* mutant complemented with wild-type *plc*) (◆) were germinated at 40°C with (A) BHI broth pH 7.5, (B) 100mM Asparagine + 100mM KCl pH 7.5, (C) 100mM KCl pH 7.5, and (D) 250mM inorganic phosphate (Pi) pH 6.0. Spores were also incubated with 25mM tris-HCl buffer pH 7.5 (●). After the addition of germinant, OD_{600nm} readings were recorded at various times. Data points represent the average of at least 3 independent experiments and error bars represent the standard deviation.

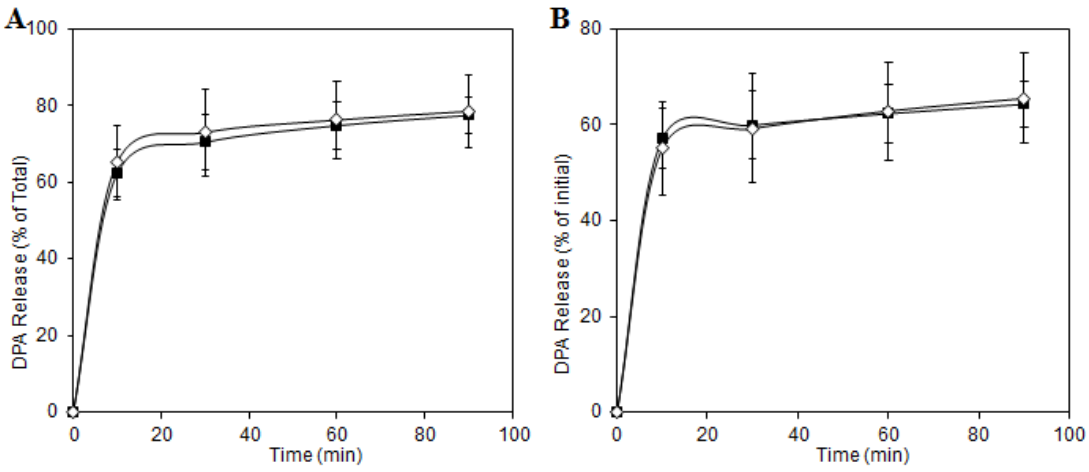

Fig 2.6. DPA release of *C. perfringens* spores incubated with non nutrient and nutrient germinants. (A) non heat-activated spores of strains SM101 (wild-type) (■) and CB102 (Δ*plc*:: intron) (◊) were germinated at 60°C with 1mM dodecylamine or (B) heat activated spores were germinated at 40°C with 100mM Asparagine + 100mM KCl pH 7.5, After the addition of germinant, OD_{405nm} readings were recorded at various times as described in the materials and methods. Data points represent the average of at least 3 independent experiments and error bars represent the standard deviation.

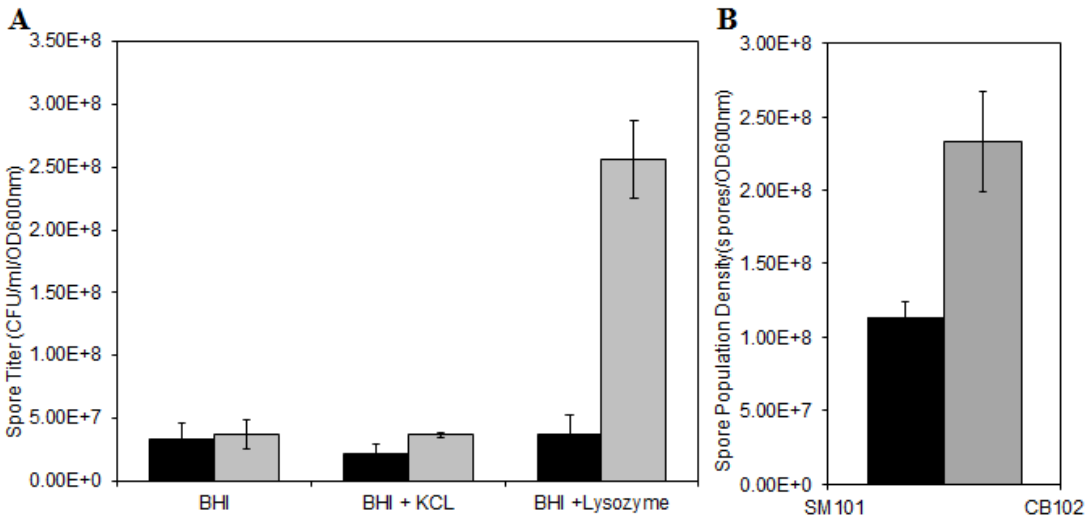

Fig 2.7. Colony forming efficiency and population density of spores of *C. perfringens* strains. (A) Heat-activated spores of strains SM101 (wild-type) (■) and CB102 (*plc*::intron) (◊) of an OD_{600nm} ~1 were serially diluted and plated on BHIA, BHIA supplemented with 100mM KCL or decoated spores were serially diluted and plated on BHIA supplemented with 1ug/ml lysozyme. (B) Phase bright spores were counted via phase contrast microscopy and the spore population density was calculated as described in the materials and methods. Data points represent the average of at least 3 independent experiments and error bars represent the standard deviation.

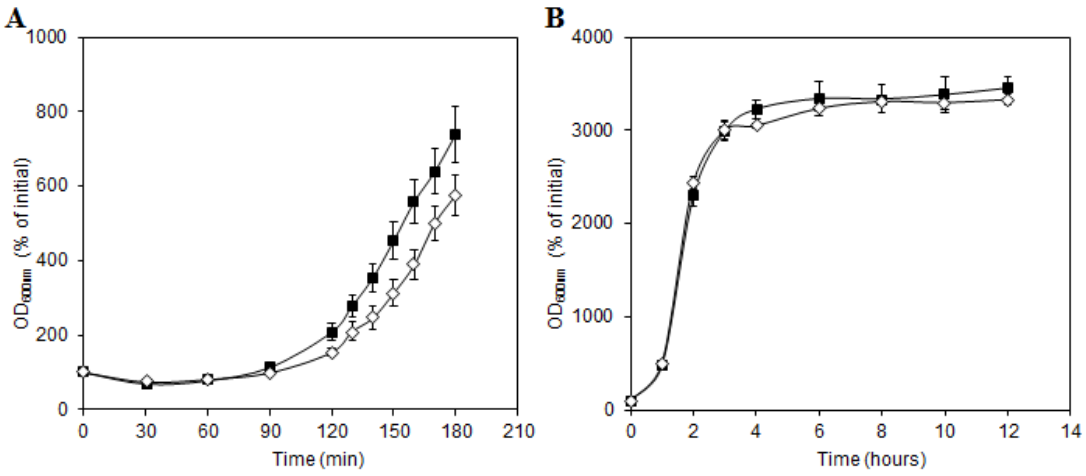

Fig 2.8. Outgrowth of spores and vegetative growth of *C. perfringens* strains. (A) Heat-activated spores of strains SM101 (wild-type) (■) and CB102 (Δ*plc*:: intron) (◊) were inoculated into TGY media resulting in an initial OD_{600nm} ~1 the optical density was measured at various time points. (B) three hour TGY growth of both strains were inoculated into fresh TGY media and the OD_{600nm} was measured at various time points as described in the materials and methods. Data points represent the average of at least 3 independent experiments and error bars represent the standard deviation.

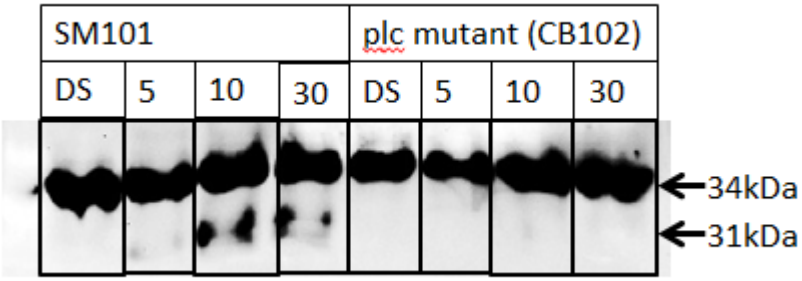

Fig 2.9. SleC detection by western blot in *C. perfringens* strains. Heat-activated spores of strains SM101 (wild-type) (■) and CB102 (Δ*plc*:: intron) (◊) were incubated with 100mM Asparagine + 100mM KCl for 5, 10, and 30 min at 40°C and the inactive pro-SleC (34kDa) and the active SleC (31kDa) was detected by western blot as described in the materials and methods. Additionally, as a control, spores that were not heat activated were decoated (DS) and the spore coat protein extracts were probed for SleC by western blot.

Tables

Table 2.1. *C. perfringens* isolates and plasmids used in this study

Strain	Relevant characteristics	Source/Reference
SM101	Electroporatable derivative of human food poisoning type A isolate, NCTC8798, chromosomal *cpe*$^{+}$	(45, 69)
CB101	A delta *plc*::*catP* mutant derived from parental wild-type strain SM101	This Study
CB102	A delta *plc*::intron mutant (targetron method) derived from parental wild-type strain SM101	This Study
Plasmid		
pDP239	pJIR750ai (plc targeted targetron mutator plasmid) with *ermBP* cloned into the EcoRV site within the *catP* cassette	This Study
pDP275	A KpnI-SalI ~1.3kb *catP* cloned into pDP129 between the up and downstream *plc* regions.	This Study
pCB37	KpnI-SalI 1.905kb *plc* ORF and promoter region into pJIR751	This Study
pCB41	SalI-PstI 521bp *plc* promoter region cloned into pMRS127	This Study
pDP129	A suicidal vector targeting the *plc* locus.	(45)
pJIR750ai	*C. perfringens*/*E. coli* shuttle vector containing a *Ll.LtrB* retargeted to the *plc* gene.	(49)
pJIR751	*C. perfringens*/*E. coli* shuttle vector, Er^{R}	(2)

Table 2.2. Primers used in this study

Primer name	Primer sequence	Region	Position[a]	Use
CPP811	ATAATTATCCTTACCAGCCCATAGGG TGCGCCCAGATAGGGTG	Intron	+444 to +493	SP[b]
CPP812	ACAAAGAAAGGTAAGTTAAGCCTATGGA TCACCACATTTGTACA	Intron	+733 to +793	SP[b]
CPP847	GGTACCGCCCAAGTTCTTTAGCAAGAGAAG	plc	-482 to -458	CP[c*]
CPP848	GTCGACGCCAGCTCCTAGGAATCCTG	plc	+1403 to +1453	CP[c*]
CPP881	GTCGACGCCCAAGTTCTTTAGCAAGAGAAG	plc	-482 to -458	GUS
CPP882	CTGCAGCGTGGCACAAACAAGCGC	plc	+20 to +39	GUS

[a] Relative to the *plc* ATG start codon or origin of replication on pDP239 (southern blot probe).

[b] Construction of southern blot targetron probe.

[c] Construction of complementation plasmid.

[*]Used as *plc*::*catP* detection primers.

Chapter 3

Conclusion

Clostridium perfringens is the causative agent of gas gangrene and several gastrointestinal (GI) diseases in humans and animals. Critical to the pathogenicity of *C. perfringens* is the ability of this bacterium to produce highly resistant, metabolically dormant spores that can resume metabolic function through the process of germination (44). Once spores germinate, vegetative cells can proliferate and release toxins, such as the alpha toxin, the causative agent of gas gangrene. Alternatively, vegetative cells can sporulate in the human gastrointestinal tract releasing *C. perfringens* enterotoxin (CPE) the causative agent of type A food poisoning (33, 35).

In this present work we assayed for the expression of *plc* and compared the germination phenotypes of *plc* mutants to their parental wild-type strains. We found that Plc is expressed under sporulation conditions independent of Spo0A. Additionally, we found that Plc plays a role in the germination of spores with BHI, AK, inorgainic phosphate (Pi), and to a lesser extent KCl. Plc also plays a role in outgrowth and colony forming efficiency. Finally, SleC western blot analysis suggests that Plc plays its role in germination by indirectly regulating the processing of inactive pro-SleC into active SleC. Further investigation into the role of Plc in *C. perfingens* spore germination is certainly warranted as the mechanism may shed light on gaps in our knowledge of the germination process and lead to new prophylactic treatments.

Bibliography

1. **Ba-Thein, W., M. Lyristis, K. Ohtani, I. T. Nisbet, H. Hayashi, J. I. Rood, and T. Shimizu.** 1996. The virR/virS locus regulates the transcription of genes encoding extracellular toxin production in Clostridium perfringens. J Bacteriol **178:**2514-20.
2. **Bannam, T. L., and J. I. Rood.** 1993. Clostridium perfringens-Escherichia coli shuttle vectors that carry single antibiotic resistance determinants. Plasmid **29:**233-5.
3. **Barth, H., K. Aktories, M. R. Popoff, and B. G. Stiles.** 2004. Binary bacterial toxins: biochemistry, biology, and applications of common Clostridium and Bacillus proteins. Microbiol Mol Biol Rev **68:**373-402, table of contents.
4. **Brynestad, S., M. R. Sarker, B. A. McClane, P. E. Granum, and J. I. Rood.** 2001. Enterotoxin plasmid from Clostridium perfringens is conjugative. Infect Immun **69:**3483-7.
5. **Bullifent H. L., M. A., Awad M.M., Scott P.T., Rood J.I, Titball R.W.** 1996. The level of expression of alpha-toxin by different strains of *Clostridium perfringens* is dependent on differences in promoter structure and genetic background. Anaerobe **2:**365-371.
6. **Bunting, M., D. E. Lorant, A. E. Bryant, G. A. Zimmerman, T. M. McIntyre, D. L. Stevens, and S. M. Prescott.** 1997. Alpha toxin from Clostridium perfringens induces proinflammatory changes in endothelial cells. J Clin Invest **100:**565-74.
7. **Chen, Y., B. A. McClane, D. J. Fisher, J. I. Rood, and P. Gupta.** 2005. Construction of an alpha toxin gene knockout mutant of Clostridium perfringens type A by use of a mobile group II intron. Appl Environ Microbiol **71:**7542-7.
8. **Collie, R. E., and B. A. McClane.** 1998. Evidence that the enterotoxin gene can be episomal in Clostridium perfringens isolates associated with non-food-borne human gastrointestinal diseases. J Clin Microbiol **36:**30-6.
9. **Duncan, C. L.** 1973. Time of enterotoxin formation and release during sporulation of Clostridium perfringens type A. J Bacteriol **113:**932-6.
10. **Duncan, C. L., and D. H. Strong.** 1968. Improved medium for sporulation of Clostridium perfringens. Appl Microbiol **16:**82-9.
11. **Effat, M. M.** 2008. Restriction enzyme analysis and DNA sequencing comparison for alpha-toxin gene among different types of Clostridium perfringens. Pak J Biol Sci **11:**380-5.
12. **Flores-Diaz, M., and A. Alape-Giron.** 2003. Role of Clostridium perfringens phospholipase C in the pathogenesis of gas gangrene. Toxicon **42:**979-86.
13. **Gholamhoseinian, A., and P. J. Piggot.** 1989. Timing of spoII gene expression relative to septum formation during sporulation of Bacillus subtilis. J Bacteriol **171:**5747-9.

14. **Harry, K. H., R. Zhou, L. Kroos, and S. B. Melville.** 2009. Sporulation and enterotoxin (CPE) synthesis are controlled by the sporulation-specific sigma factors SigE and SigK in Clostridium perfringens. J Bacteriol **191:**2728-42.
15. **Hatheway, C. L.** 1990. Toxigenic clostridia. Clin Microbiol Rev **3:**66-98.
16. **Hilbert, D. W., and P. J. Piggot.** 2004. Compartmentalization of gene expression during Bacillus subtilis spore formation. Microbiol Mol Biol Rev **68:**234-62.
17. **Huang, I. H., M. Waters, R. R. Grau, and M. R. Sarker.** 2004. Disruption of the gene (spo0A) encoding sporulation transcription factor blocks endospore formation and enterotoxin production in enterotoxigenic Clostridium perfringens type A. FEMS Microbiol Lett **233:**233-40.
18. **J I Rood, B. A. M., J G Songer, R W Titball.** 1997. The Clostridia:Molecular Biology
and Pathogenesis. Academic Press Inc, San diego,California.
19. **Kald, B., R. M. Boll, C. Gustafson-Svard, R. Sjodahl, and C. Tagesson.** 1994. Phospholipase C from Clostridium perfringens stimulates acetyltransferase-dependent formation of platelet-activating factor in cultured intestinal epithelial cells (INT 407). Scand J Gastroenterol **29:**243-7.
20. **Katayama, S., B. Dupuy, G. Daube, B. China, and S. T. Cole.** 1996. Genome mapping of Clostridium perfringens strains with I-CeuI shows many virulence genes to be plasmid-borne. Mol Gen Genet **251:**720-6.
21. **Krug, E. L., and C. Kent.** 1984. Phospholipase C from Clostridium perfringens: preparation and characterization of homogeneous enzyme. Arch Biochem Biophys **231:**400-10.
22. **Kumazawa, T., A. Masayama, S. Fukuoka, S. Makino, T. Yoshimura, and R. Moriyama.** 2007. Mode of action of a germination-specific cortex-lytic enzyme, SleC, of Clostridium perfringens S40. Biosci Biotechnol Biochem **71:**884-92.
23. **Kunkel, B., K. Sandman, S. Panzer, P. Youngman, and R. Losick.** 1988. The promoter for a sporulation gene in the spoIVC locus of Bacillus subtilis and its use in studies of temporal and spatial control of gene expression. J Bacteriol **170:**3513-22.
24. **Lawrence, G.** 1979. The pathogenesis of pig-bel in Papua New Guinea. P N G Med J **22:**39-49.
25. **Lawrence, G. W.** 1997. The pathogenesis of enteritis necroticans, p. 197-207. *In* M. B. A. Rood J. I., Songer J. G, Titball R. W. (ed.), *The Clostridia: Molecular Biology and Pathogenesis*. London: Academic.
26. **Leslie, D., N. Fairweather, D. Pickard, G. Dougan, and M. Kehoe.** 1989. Phospholipase C and haemolytic activities of Clostridium perfringens alpha-toxin cloned in Escherichia coli: sequence and homology with a Bacillus cereus phospholipase C. Mol Microbiol **3:**383-92.
27. **Losick, R., and P. Stragier.** 1992. Crisscross regulation of cell-type-specific gene expression during development in B. subtilis. Nature **355:**601-4.

28. **Lyristis, M., A. E. Bryant, J. Sloan, M. M. Awad, I. T. Nisbet, D. L. Stevens, and J. I. Rood.** 1994. Identification and molecular analysis of a locus that regulates extracellular toxin production in Clostridium perfringens. Mol Microbiol **12:**761-77.
29. **Masayama, A., K. Hamasaki, K. Urakami, S. Shimamoto, S. Kato, S. Makino, T. Yoshimura, M. Moriyama, and R. Moriyama.** 2006. Expression of germination-related enzymes, CspA, CspB, CspC, SleC, and SleM, of Clostridium perfringens S40 in the mother cell compartment of sporulating cells. Genes Genet Syst **81:**227-34.
30. **Masayama, A., S. Kato, T. Terashima, A. Molgaard, H. Hemmi, T. Yoshimura, and R. Moriyama.** 2010. Bacillus subtilis spore coat protein LipC is a phospholipase B. Biosci Biotechnol Biochem **74:**24-30.
31. **Masayama, A., R. Kuwana, H. Takamatsu, H. Hemmi, T. Yoshimura, K. Watabe, and R. Moriyama.** 2007. A novel lipolytic enzyme, YcsK (LipC), located in the spore coat of Bacillus subtilis, is involved in spore germination. J Bacteriol **189:**2369-75.
32. **Matsushita, C., O. Matsushita, S. Katayama, J. Minami, K. Takai, and A. Okabe.** 1996. An upstream activating sequence containing curved DNA involved in activation of the Clostridium perfringens plc promoter. Microbiology **142 (Pt 9):**2561-6.
33. **McClane, B. A.** 2007. Clostridium perfringens, p. 351-372. *In* L. R. B. M. P. Doyle, and T. J. Montville (ed.), Food Microbiology: fundamentals and frontiers, 2 ed. ASM Press, Washington, D.C.
34. **McClane, B. A., and G. Chakrabarti.** 2004. New insights into the cytotoxic mechanisms of Clostridium perfringens enterotoxin. Anaerobe **10:**107-14.
35. **McDonnell, J. L.** 1986. Toxins of Clostridium perfringens type A, B, C, D, and E., p. 477-517. *In* F. D. J. Drews. (ed.), In Pharmacology of Bacterial Toxins. Oxford: Pergamon Press.
36. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg Infect Dis **5:**607-25.
37. **Minami, J., S. Katayama, O. Matsushita, C. Matsushita, and A. Okabe.** 1997. Lambda-toxin of Clostridium perfringens activates the precursor of epsilon-toxin by releasing its N- and C-terminal peptides. Microbiol Immunol **41:**527-35.
38. **Moir, A., B. M. Corfe, and J. Behravan.** 2002. Spore germination. Cell Mol Life Sci **59:**403-9.
39. **Moreau, H., G. Pieroni, C. Jolivet-Reynaud, J. E. Alouf, and R. Verger.** 1988. A new kinetic approach for studying phospholipase C (Clostridium perfringens alpha toxin) activity on phospholipid monolayers. Biochemistry **27:**2319-23.

40. **Nagahama, M., K. Michiue, and J. Sakurai.** 1996. Membrane-damaging action of Clostridium perfringens alpha-toxin on phospholipid liposomes. Biochim Biophys Acta **1280:**120-6.
41. **Nagahama, M., A. Otsuka, M. Oda, R. K. Singh, Z. M. Ziora, H. Imagawa, M. Nishizawa, and J. Sakurai.** 2007. Effect of unsaturated bonds in the sn-2 acyl chain of phosphatidylcholine on the membrane-damaging action of Clostridium perfringens alpha-toxin toward liposomes. Biochim Biophys Acta **1768:**2940-5.
42. **Paidhungat, M., and P. Setlow.** 2000. Role of ger proteins in nutrient and nonnutrient triggering of spore germination in Bacillus subtilis. J Bacteriol **182:**2513-9.
43. **Paredes-Sabja, D., B. Setlow, P. Setlow, and M. R. Sarker.** 2008. Characterization of Clostridium perfringens spores that lack SpoVA proteins and dipicolinic acid. J Bacteriol **190:**4648-59.
44. **Paredes-Sabja, D., P. Setlow, and M. R. Sarker.** 2011. Germination of spores of Bacillales and Clostridiales species: mechanisms and proteins involved. Trends Microbiol **19:**85-94.
45. **Paredes-Sabja, D., P. Setlow, and M. R. Sarker.** 2009. GerO, a putative Na+/H+-K+ antiporter, is essential for normal germination of spores of the pathogenic bacterium Clostridium perfringens. J Bacteriol **191:**3822-31.
46. **Paredes-Sabja, D., P. Setlow, and M. R. Sarker.** 2009. The protease CspB is essential for initiation of cortex hydrolysis and dipicolinic acid (DPA) release during germination of spores of Clostridium perfringens type A food poisoning isolates. Microbiology **155:**3464-72.
47. **Paredes-Sabja, D., P. Setlow, and M. R. Sarker.** 2009. Role of GerKB in germination and outgrowth of Clostridium perfringens spores. Appl Environ Microbiol **75:**3813-7.
48. **Paredes-Sabja, D., P. Setlow, and M. R. Sarker.** 2009. SleC is essential for cortex peptidoglycan hydrolysis during germination of spores of the pathogenic bacterium Clostridium perfringens. J Bacteriol **191:**2711-20.
49. **Paredes-Sabja, D., J. A. Torres, P. Setlow, and M. R. Sarker.** 2008. Clostridium perfringens spore germination: characterization of germinants and their receptors. J Bacteriol **190:**1190-201.
50. **Paredes-Sabja, D., P. Udompijitkul, and M. R. Sarker.** 2009. Inorganic phosphate and sodium ions are cogerminants for spores of Clostridium perfringens type A food poisoning-related isolates. Appl Environ Microbiol **75:**6299-305.
51. **Payne D., O. P.** 1997. The Clostridium perfringens epsilon-toxin, p. 439-447. *In* M. B. A. Rood J. I., Songer J. G, Titball R. W. (ed.), The Clostridia: Molecular Biology and Pathogenesis. London: Academic.
52. **Piggot, P. J., and J. G. Coote.** 1976. Genetic aspects of bacterial endospore formation. Bacteriol Rev **40:**908-62.

53. **Rood, J. I., and S. T. Cole.** 1991. Molecular genetics and pathogenesis of Clostridium perfringens. Microbiol Rev **55:**621-48.
54. **Sarker, M. R., R. J. Carman, and B. A. McClane.** 1999. Inactivation of the gene (cpe) encoding Clostridium perfringens enterotoxin eliminates the ability of two cpe-positive C. perfringens type A human gastrointestinal disease isolates to affect rabbit ileal loops. Mol Microbiol **33:**946-58.
55. **Setlow, P.** 2003. Spore germination. Curr Opin Microbiol **6:**550-6.
56. **Shimamoto, S., R. Moriyama, K. Sugimoto, S. Miyata, and S. Makino.** 2001. Partial characterization of an enzyme fraction with protease activity which converts the spore peptidoglycan hydrolase (SleC) precursor to an active enzyme during germination of Clostridium perfringens S40 spores and analysis of a gene cluster involved in the activity. J Bacteriol **183:**3742-51.
57. **Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi.** 2002. Complete genome sequence of Clostridium perfringens, an anaerobic flesh-eater. Proc Natl Acad Sci U S A **99:**996-1001.
58. **Simpson, L. L., B. G. Stiles, H. H. Zepeda, and T. D. Wilkins.** 1987. Molecular basis for the pathological actions of Clostridium perfringens iota toxin. Infect Immun **55:**118-22.
59. **Steinthorsdottir, V., V. Fridriksdottir, E. Gunnarsson, and O. S. Andresson.** 1995. Expression and purification of Clostridium perfringens beta-toxin glutathione S-transferase fusion protein. FEMS Microbiol Lett **130:**273-8.
60. **Sun, D. X., R. M. Cabrera-Martinez, and P. Setlow.** 1991. Control of transcription of the Bacillus subtilis spoIIIG gene, which codes for the forespore-specific transcription factor sigma G. J Bacteriol **173:**2977-84.
61. **Titball, R. W.** 1993. Bacterial phospholipases C. Microbiol Rev **57:**347-66.
62. **Titball, R. W., S. E. Hunter, K. L. Martin, B. C. Morris, A. D. Shuttleworth, T. Rubidge, D. W. Anderson, and D. C. Kelly.** 1989. Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase C) of Clostridium perfringens. Infect Immun **57:**367-76.
63. **Titball, R. W., D. L. Leslie, S. Harvey, and D. Kelly.** 1991. Hemolytic and sphingomyelinase activities of Clostridium perfringens alpha-toxin are dependent on a domain homologous to that of an enzyme from the human arachidonic acid pathway. Infect Immun **59:**1872-4.
64. **Vepachedu, V. R., and P. Setlow.** 2007. Role of SpoVA proteins in release of dipicolinic acid during germination of Bacillus subtilis spores triggered by dodecylamine or lysozyme. J Bacteriol **189:**1565-72.
65. **Willis, T. A.** 1969. *Clostridia of wound infection*. Butterworth and Co., London.
66. **Wu, J. J., M. G. Howard, and P. J. Piggot.** 1989. Regulation of transcription of the Bacillus subtilis spoIIA locus. J Bacteriol **171:**692-8.

67. **Young, P. R., W. R. Snyder, and R. F. McMahon.** 1991. Kinetic mechanism of Clostridium perfringens phospholipase C. Hydrolysis of a thiophosphate analogue of lysophosphatidylcholine. Biochem J **280 (Pt 2):**407-10.
68. **Zhao, Y., and S. B. Melville.** 1998. Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (cpe) of Clostridium perfringens. J Bacteriol **180:**136-42.
69. **Zhao, Y., and S. B. Melville.** 1998. Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (*cpe*) of *Clostridium perfringens*. J. Bacteriol. **180:**136-42.