AN ABSTRACT OF THE DISSERTATION OF

Daniel Paredes-Sabja for the degree of Doctor of Philosophy in Food Science and Technology presented on June 4 , 2009.

Title: Molecular Mechanism of Germination of *Clostridium perfringens* Spores.

Abstract approved:

_____

Mahfuzur R. Sarker J. Antonio Torres

Clostridium perfringens is the causative agent of a wide variety of diseases in animals and humans. *C. perfringens* can produce more than 15 toxins. However, individual strains produce a subset of these toxins. Although a small percentage of *C. perfringens* isolates (mostly belonging to type A) produce *C. perfringens* enterotoxin (CPE), these are very important human gastrointestinal (GI) pathogens, causing *C. perfringens* type A food poisoning (FP) and nonfood-borne GI diseases (NFBGID). Due to its anaerobic nature and the ability to form extremely resistant spores found ubiquitously in the environment, to cause the wide array of C. perfringens-associated diseases (CPAD), these *C. perfringens* spores must germinate, release the nascent cell, grow and produce their toxins. Therefore, germination of *C. perfringens* spores is the initial and perhaps most important step for the progression of diseases in animals and humans. Although extensive research has been conducted on the mechanism of spore germination of *Bacillus* species, very few studies of spore germination have been conducted in *Clostridium* species mainly due to the lack of molecular genetic tools. Genomic comparisons reveal significant differences in the backbone of the

germination apparatus between *Bacillus* and *Clostridium* species. Consequently, a detail understanding of the molecular mechanism of germination of *C. perfringens* spores is essential for the development of novel preventive strategies for CPAD as well as diseases caused by other pathogenic *Clostridium* species.

The first focus of this work was to identify and characterize the germinants and the receptors involved in *C. perfringens* spore germination. Result from these studies found differential germination requirements between spores of FP and NFBGID isolates in that: (i) while a mixture of L-asparagine and KCl was a good germinant for spores of FP and NFBGID isolates, KCl and, to a lesser extent, L-asparagine triggered spore germination in FP isolates only; and ii) L-alanine and L-valine induced significant germination of spores of NFBGID but not FP isolates. In contrast to *B. subtilis*, *C. perfrinegns* genomes sequenced to date possess no tricistronic *gerA*-like operon, but has a monocistronic *gerAA* that is far from a *gerK* locus. The *gerK* locus contains a bicistronic *gerKA-gerKC* operon and a monocistronic *gerKB* upstream and in the opposite orientation to *gerKA-gerKC*. Consequently, through the construction of mutations into strain SM101, a *C. perfringens* FP isolate, the role of *gerAA*, *gerKA-gerKC* and *gerKB* genes in *C. perfringens* spore germination were investigated. Results indicated that KCl, L-asparagine and Ca-DPA required GerKA and/or GerKC receptors, while GerAA and GerKB played an auxiliary role in germination. Lack of GerKA and/or GerKC, and GerKB significantly reduced spores colony forming efficiency, indicating a role in spore viability.

The fact that *C. perfringens* spores lacking the main germinant receptor(s) proteins, GerKA and/or GerKC, are still able to germinate albeit poorly compared to

wild-type, and that *C. perfringens* spores germinate with K^+ ions alone, raises the hypothesis that GrmA-like antiporters might also play some role in *C. perfringens* spore germination. Two putative GrmA-like antiporters (i.e., GerO and GerQ) are encoded in the genome of all *C. perfringens* sequenced to date. This study shows that *gerO* and *gerQ* genes are expressed uniquely during sporulation and the mother cell compartment of the sporulating cell. Complementation studies of K^+ uptake and Na^+ sensitive *E. coli* mutants indicate that while GerO is capable of translocating K^+ and Na^+, GerQ is only capable of translocating, to a small extent, Na^+ ions. Spores lacking GerO had defective germination in rich medium, KCl, L-asparagine, and Ca-DPA, but not with dodecylamine, defect that might be prior to DPA release during germination. In contrast, loss of GerQ had a much smaller effect on spore germination. Two adjacent Asp residues, important in ion transcloation of the *E. coli* Na^+/H^+ antiporter NhaA were also present in GerO, but not GerQ, and replacement of these residues for Asn reduced the protein's ability to complement *gerO* spores. Although results from this study indicate that putative antiporters have some role on *C. perfringens* spore germination, it is unclear whether their role is direct or during spore formation.

C. perfringens type A FP spores are capable of germinating with K^+ ions, an intrinsic mineral of meats commonly associated with FP. Inorganic phosphate (Pi) is also intrinsically found in meat products. Consequently, we hypothesized that FP spores are capable of germinating in presence of Pi. Results from this study show that spores of the majority of FP, but not NFBGID isolates, are able to germinate in presence of Pi. Pi-induced germination of FP spores is primarily through the GerKA and/or GerKC protein, while GerAA and to a much lesser extent, GerKB, play

auxiliary roles. The putative Na^+/K^+-H^+ antiporter, GerO, is also required for normal Pi-induced germination. These results suggest that the differential germination phenotypes between spores of FP and NFGID isolates is tightly regulated by their adaptation to different environmental niches.

A second focus of this work was to investigate the mechanism of signal transduction between the germinant receptors and the downstream effectors. In *B. subtilis*, the SpoVA proteins have been associated with Ca-DPA uptake and subsequent release during sporulation and germination, respectively. In addition, Ca-DPA acts as a signal molecule for cortex hydrolysis during *B. subtilis* spore germination, activating the cortex lytic enzyme (CLE) CwlJ. Results from this study show that in contrast to *B. subtilis spoVA* mutants, where spores lyse quickly during purification, *C. perfringens spoVA* spores were stable and germinated similarly as wild-type spores. These results suggest major differences in the regulation of the germination pathway between *C. perfringens* and *B. subtilis*, and suggest that activation of CLEs in *C. perfringens* might be through a different pathway than the Ca-DPA pathway of *B. subtilis*.

A third focus of this work was to investigate the *in vivo* role of the CLE involved in peptidoglycan (PG) spore cortex hydrolysis during *C. perfringens* spore germination. Two *C. perfringens* CLEs (i.e., SleC and SleM) degrade PG spore cortex hydrolysis *in vitro*, however, due to lack of genetic tools, their *in vivo* role in spore germination remains unclear. Results from this study show that *C. perfringens sleC* spores released their DPA slower than wild-type and were not able to germinate with nutrients and non-nutrient germinants. In contrast, *sleM* spores germinated similar as

wild type in presence of nutrient and non-nutrient germinants, indicating that while SleC is essential for cortex hydrolysis and viability of *C. perfringens* spores, SleM although can degrade cortex PG in vitro, is not essential.

A fourth focus of this work was to investigate the *in vivo* role of the Csp proteases in the initiation of cortex hydrolysis. *In vitro* work has shown that Csp proteins process the inactive proSleC into the mature enzyme, SleC. However, the *in vivo* role of the Csp proteins has not been established. In this study, spores a *C. perfringens cspB* mutant exhibited significantly less viability than wild-type spores, and were unable to germinate with either rich medium or Ca-DPA. Germination of *cspB* spores was blocked prior to DPA release and cortex hydrolysis. Results from this study indicate that CspB is essential to generate active SleC and allow cortex hydrolysis early in *C. perfringens* spore germination. In contrast to *B. subtilis*, Ca-DPA did not activate the CLEs during spore germination present in *cspB* spores supporting previous results that Ca-DPA acts trough the GerKA and/or GerKC receptor.

A final focus of this work was to develop a strategy to inactivate *C. perfringens* spores in meat products. *C. perfringens* spores posses high heat and pressure resistance, however, they loss their resistance properties during early stages of germination. In contrast to *B. subtilis* spores, germination of *C. perfringens* spores could not be triggered with low pressures. However, they germinated efficiently when heat activated in presence of L-asparagine and KCl at temperatures lethal for vegetative cells, and these germinated spores were efficiently inactivated by subsequent treatment with pressure assisted thermal processing (586 MPa at 73ºC for

10 min). This study shows the feasibility of a novel strategy to inactivate *C. perfringens* spores in meat products formulated with germinants.

Collectively, the present study contributes to the understanding of the mechanism of spore germination in the pathogenic bacterium *C. perfringens*, and developed an alternative and novel strategy to inactivate *C. perfringens* spores in meat products.

Molecular Mechanism of Germination of *Clostridium perfringens* Spores

by
Daniel Paredes-Sabja

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented June 4, 2009
Commencement June 2010.

Doctor of Philosophy dissertation of Daniel Paredes-Sabja presented on June 4, 2009

APPROVED:

Co-Major Professor, representing Food Science & Technology

Co-Major Professor, representing Food Science & Technology

Head of the Department of Food Science & Technology

Dean of the Graduate School

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

Daniel Paredes-Sabja, Author

ACKNOWLEDGEMENTS

I would like to thank Dr. Mahfuzur Sarker and Dr. J. Antonio Torres for being such great advisors, mentors, and for their support and friendship during all this period.

I would like to thank Dr. Mahfuz Sarker for giving me the opportunity to work in his lab, for being patient in guiding me throughout the period of this degree.

I would like to thank Dr. J. Antonio Torres for inviting me to join the graduate program of Food Science and Technology.

I would like to thank Dr. Peter Setlow for being an excellent collaborator and for the excellent discussions we have had throughout this project.

Special thanks to former students of Dr. Sarker's lab: Deepa Raju and I-Hsiu Huang; and to current members: Nahid Sarker, Pathima Udompijitkul, Maryam Alnoman, Colton Bond and Saeed Banawas for their friendship and the great collaborations we have made together.

Special thanks to Dr. Tom Shellhammer, who was very supportive during early stages of my Ph.D. degree.

I want to thank my parents Gonzalo Paredes and Ana María Sabja, and my brother Tomás Paredes for all their support during my graduate studies.

And I wish to specially thank my wonderful wife Mónica González for her unwavering support during these years, otherwise, none of these accomplishments would have been possible, and to my 1 year old son, Vicente Paredes-González, for making the last period of my graduate studies the best of all.

CONTRIBUTION OF AUTHORS

Dr. Mahfuzur R. Sarker as a co-major professor provided the laboratory facilities needed for the research done on Chapters 2 to 9.

Dr. J. Antonio Torres as a co-major professor provided the facility needed for the research done in Chapter 9.

Chapter 2: Dr. Mahfuzur R. Sarker and Mr. Daniel Paredes-Sabja contributed to the experimental design, data analysis and preparation of the manuscript. Dr. J. Antonio Torres and Dr. Peter Setlow contributed to data analysis and preparation of the manuscript.

Chapters 3 to 4: Dr. Mahfuzur R. Sarker, Dr. Peter Setlow and Mr. Daniel Paredes-Sabja contributed to the experimental design, data analysis and preparation of the manuscripts.

Chapter 5: Dr. Mahfuzur Sarker and Mr. Daniel Paredes-Sabja contributed to the experimental design, data analysis and preparation of the manuscript. Ms. Pathima Udompijitkul and Mr. Daniel Paredes-Sabja contributed equally to the experiments.

Chapter 6 to 8: Dr. Mahfuzur R. Sarker, Dr. Peter Setlow and Mr. Daniel Paredes-Sabja contributed to the experimental design, data analysis and preparation of the manuscripts.

Dr. Barbara Setlow contributed to some experiments and data analysis of Chapter 6

Chapter 9: Dr. Mahfuzur Sarker, Dr. J. Antonio Torres and Mr. Daniel Paredes-Sabja contributed to the experimental design, data analysis and preparation of the

manuscript. Dr. Saeed Akhtar and Mr. Daniel Paredes-Sabja contributed equally to the experiments of Chapter 9.

TABLE OF CONTENTS

Page

Chapter 1 ……..…..1

General Introduction and Literature review

1.1. Toxins of *C. perfrinegns* ……...…….…….1

1.2. *C. perfringens* food poisoning …… ……..……8

1.3. Resistance of *C. perfringens* spores ……..…….……9

1.4. Bacterial spore germination …….……11

1.4.1. Germinant receptors …….…….…….....13

1.4.2. Ion channels …….…….....14

1.4.3. DPA channels …….……15

1.4.4. Cortex lytic enzymes …….……16

1.5. Applications of bacterial spore germination …….……..……..17

Objective of this study …….……20

Chapter 2 ……..…..……...…21

Clostridium perfringens spore germination: characterization of germinants and their receptors.

2.1. Abstract ……..….22

2.2. Introduction ……....23

2.3. Materials and Methods ……...25

2.4. Results ……....31

2.5. Discussions ……..…40

TABLE OF CONTENTS (Continued)

Page

Chapter 3 ……..……...62

Role of GerKB in germination and outgrowth of *Clostridium perfringens* spores.

3.1. Abstract ……63

3.2. Short Note……63

3.3. Supplementary Information ……..77

Chapter 4 ……..……..…79

GerO, a putative Na^+/H^+-K^+ antiporter, is essential for normal germination of spores of the pathogenic bacterium *Clostridium perfringens* .

4.1. Abstract ……80

4.2. Introduction ……...81

4.3. Materials and Methods ……..83

4.4. Results ……...94

4.5. Discussions ……...……103

4.6. Supplementary Information ……118

Chapter 5 ……..…….…...128

Inorganic phosphate induces germination of spores of *Clostridium perfringens* type A food poisoning isolates.

5.1. Abstract ……....129

5.2. Introduction ……..129

5.3. Materials and Methods ……131

TABLE OF CONTENTS (Continued)

Page

5.4. Results ……..134

5.5. Discussions ……..…139

Chapter 6 ……..……..…….…..148

Characterization of *Clostridium perfringens* spores that lack SpoVA proteins and dipicolinic acid.

6.1. Abstract …….. ..149

6.2. Introduction ……..…150

6.3. Materials and Methods ……....152

6.4. Results ……..…160

6.5. Discussions ……..….169

Chapter 7 ……..……..…..186

SleC is essential for cortex peptidoglycan hydrolysis during germination of spores of the pathogenic bacterium *Clostridium perfringens.*

7.1. Abstract ……....187

7.2. Introduction ……..187

7.3. Materials and Methods ……....191

7.4. Results ……..198

7.5. Discussions ……...……207

7.6. Supplementary Information ……...…..219

TABLE OF CONTENTS (Continued)

Page

Chapter 8 ……..……222

The protease CspB is essential for initiation of cortex hydrolysis and DPA release during germination of spores of *Clostridium perfringens* type A food poisoning isolates.

8.1. Abstract ……....223

8.2. Introduction ……..223

8.3. Materials and Methods ……226

8.4. Results ……..…230

8.5. Discussions ……..235

8.6. Supplementary Information ……242

Chapter 9 ……..……246

Strategy to inactivate *Clostridium perfringens* spores in meat products.

9.1. Abstract ……247

9.2. Introduction ……..248

9.3. Materials and Methods ……..…249

9.4. Results and discussions ……....255

9.5. Conclusion ……...259

Chapter 10 ……...266

Conclusions

Bibliography ……....273

TABLE OF CONTENTS (Continued)

Page

Appendices ……...310

Appendix A: Scientific work published or in preparation during Mr. Daniel Paredes-Sabja's Ph.D. degree ……..…….310

LIST OF FIGURES

Figure Page

2.1 Germination of *C. perfringens* spores ……..47

2.2 KCl concentration dependence of *C. perfringens* spore germination ……. 48

2.3 Effect of temperature and pH on *C. perfringens* spore germination ……49

2.4 Analysis of nutrient receptors in *C. perfringens* ……..50

2.5 Germination of *C. perfringens* wild-type and *gerK* spores ……..51

2.6 Germination of *C. perfringens* wild-type and *gerAA* spores ……52

2.7 DPA-release during germination of *C. perfringens* spores ……...53

2.8 Germination of *C. perfringens* spores in BHI broth ……54

2.9 Ca-DPA germination of *C. perfringens* spores ……55

2.10 Dodecylamine germination of *C. perfringens* spores ……56

2.11 Putative model for *C. perfringens* spore germination ……57

3.1 Arrangement and expression of *gerKB* in *C. perfringens* SM101 ……72

3.2 Germination of spores of *C. perfringens* strains with various germinants …73

3.3 Germination of spores of *C. perfringens* strains with dodecylamine ……74

3.4 Outgrowth of spores of *C. perfringens* strains ……..75

4.1 Arrangement and expression of *gerO* and *gerQ* genes in *C. pergrinens* SM101 ……108

4.2 Effects of KCl and NaCl concentrations on growth of *E. coli* strains TK2420 and KNacb carrying *C. perfringens gerO* and *gerQ* ……110

LIST OF FIGURES (Continued)

Figure Page

4.3 Germination of mutant spores in BHI broth ……..…111

4.4 Germination of *C. perfringens* spores with various germinants ……...112

4.5 DPA release from *C. perfringens* spores incubated with various germinants ……..……114

4.6 Outgrowth of spores of *C. perfringens* strains ……..115

4.7 Effects of D161N and D162N subtitutions on GerO function during *C. perfringens* spore germination ……..…116

S4.1 Amino acid sequence alignment of Na^+/H^+ antiporter associated domains …..126

S4.2 Template-based homology models of *C. perfringens* GerO and GerQ with *E. coli* NhaA …….……....127

5.1 Effect of pH and Pi concentration in germination of *C. perfringens* spores ……..…….142

5.2 Germination of spores of *C. perfringens* in different buffers …….…...144

5.3 Germination of spores from various *C. perfringens* isolates with Pi …….145

5.4 Germination of spores of *C. perfringens ger* receptor mutant strains with Pi ……..146

5.5 Germination of spores of *C. perfringens* antiporter mutant strains with Pi …….……147

6.1 Arrangement and expression of the *spoVA* operon in *C. perfringens* ……175

6.2 Germination of intact and decoated *C. perfringens* wild-type and *spoVA* spores with various germinants ……...…176

6.3 DPA release from decoated *C. perfringens* spores incubated with various germinants ….……..……...177

LIST OF FIGURES (Continued)

Figure Page

6.4 Levels of α/β-type SASP in *C. perfringens* wild-type and *spoVA* spores ……..178

6.5 Resistance of *C. perfringens* wild-type and *spoVA* spores to heat and UV Radiation …..…….……..179

6.6 Resistance of *C. perfringens* wild-type and *spoVA* spores to various chemicals 180

7.1 Arrangement and expression of *sle* genes in *C. perfringens* SM101 …….211

7.2 *C. perfringens sle* deletion mutants and germination of their spores in BHI broth ……..…….212

7.3 Germination of *C. perfringens* spores over long periods on BHI agar plates …213

7.4 DPA release during germination of *C. perfringens* spores with BHI broth ….214

7.5 Germination of spores of *C. perfringens* strains with KCl ……215

7.6 Germination of *C. perfringens* spores with Ca-DPA …….216

7.7 Dodecylamine germination of *C. perfringens* spores ……217

8.1 Genomic arrangement and expression of *cspB* in *C. perfringens* strain SM101 ……239

8.2 Schematic representation of *cspB* deletion mutant in *C. perfringens* SM101 and germination of mutant spores in BHI broth …….……240

8.3 Germination of spores of *C. perfringens* strains with KCl …….…….241

S8.1 Sequence alignment of Csp proteins of *C. perfringens* strains ….……..…245

9.1 HPP-induced germination of *C. perfringens* spores …….……...260

9.2 Effect of incubation temperature on *C.perfringens* spore germination …….261

9.3 Heat inactivation of *C. perfringens* germinated spores in BHI broth ……...262

9.4 Heat-inactivation of *C. perfringens* spores in poultry meat ……..…263

LIST OF FIGURES (Continued)

Figure Page

9.5 HPP-inactivation of *C. perfringens* spores in poultry meat ……...…264

LIST OF TABLES

Table Page

2.1 Bacterial strains and plasmids used ……..……...58

2.2 Primers used in this study …….…...59

2.3 Germination of *C. perfringens* spores by various compounds ……..……...60

2.4 Germination of spores of *C. perfringens* isolates carrying *cpe* on the chromosome (C-*cpe*) or a plasmid (P-*cpe*) …….……..…..…....61

3.1 Colony formation by spores of *C. perfringens* strains …….…….....……76

S3.1 Bacterial strains and plasmids used ……..……....77

S3.2 Primers used in this study ……..……...78

4.1 Maximum germination rates of spores of various *C. perfringens* strains ……117

S4.1 Bacterial strains and plasmids used ……..……..…….....120

S4.2 Primers used in this study ……..…….…….....124

6.1 Bacterial strains and plasmids used ……..…….181

6.2 Primers used in this study …….…….183

6.3 Effects of *spoVA* mutation on *C. perfringens* spore properties ……...…..184

6.4 Colony formation by spores of *C. perfringens* strains ..……185

7.1 Colony formation by spores of *C. perfringens* strains …….……..… 218

S7.1 Bacterial strains and plasmids used ……..…....……..219

S7.2 Primers used in this study …….…….……..221

8.1 Colony formation by spores of *C. perfringens* strains ……242

S8.1 Bacterial strains and plasmids used …….....……..243

LIST OF TABLES (Continued)

Table Page

S8.2 Primers used in this study ……244

9.1 Germination of *C. perfringens* spores from food poisoning isolates at various temperatures ……...…….265

Molecular Mechanism of Germination of *Clostridium perfringens* spores.

Chapter 1

General Introduction and Literature review

Clostridium perfringens is a Gram-positive, anaerobic, rod-shaped and spore-forming bacteria that produces a wide array of toxins (117). *C. perfringens* is considered the most widely occurring pathogenic bacteria of the genus *Clostridium*, which includes approximately 120 species such as the industrial relevant *C. acetobutylicum*, and the pathogenic *C. botulinum*, *C. tetani* and *C. difficile* (60). *C. perfringens* is found ubiquitously in natural environments including soil, water sources and the intestinal tract of humans and animals (117).

C. perfringens has been associated with a variety of myonecrotic and gastrointestinal diseases in humans, domesticated livestock and birds (117, 222). The variety of diseases caused by *C. perfringens* is in part due to the following. i) Unique ability to produce an array of more than 15 toxins, though individual strains produce only a subset of these toxins (119). ii) A rapid doubling time (as fast as 8 min) (253). iii) High stress-resistant properties of their spores (95, 117, 193).

1.1 Toxins of *C. perfringens*

Based on the ability to produce the four major toxins, *C. perfringens* can be classified into 5 toxinotypes (i.e., A-E), and below is a summary of the characteristics of the major toxins.

Alpha toxin:

The alpha toxin is produced by all types of *C. perfringens* isolates, and in higher amounts by type A strains. Alpha toxin is chromosomally encoded by the *plc* gene and regulated at the transcriptional level by the VirR/VirS two component system and a small regulatory RNA molecule encoded by *virX* (136, 214). The toxin is a 43-kDa metalloenzyme dependent on Zinc and has two domains, the N-terminal domain exhibits phospholipase activity while the C-terminal domain is involved in membrane binding (188). Upon binding into the membrane of host cells, the toxin induces massive degradation of phosphatidylcholine and sphingomyelin followed by membrane disruption, causing tissue damage and lysis of blood cells and endothelial cells (188). This toxin has been implicated in many diseases, and is a main virulence factor of gas gangrene causing extensive tissue damage, hepatic toxicity and myocardial disfunction (141). The ability of alpha toxin to lyse blood cells is used in the reverse cAMP test for the identification of *C. perfringens* in diagnostic tools (141)

Beta toxin:

Beta toxin is mainly found in *C. perfringens* type B and C isolates in a large plasmid (60, 182), and is the major virulence factor of necrotic enteritis in many domesticated livestock including sheeps, lambs, and especially pigs with a high mortality rate (223). This toxin is a oligomerizing, pore forming toxin that forms cation dependent channels in susceptible membranes (131). Beta toxin alone is sufficient to cause necrosis of erytrocytes at the apices of jujenal villi which progress

to mucosal necrosis (245), and also is sufficient to induce typical necrotizing intestinal lesions in the rabbit ileal loop model (194). Human necrotic enteritis or pig-bel is caused by the consumption of under-cooked pork meat contaminated with *C. perfringens* type C mainly in immunocompromised individuals (99). Treatment consists of administration of β-toxoid (222). However, the mechanism of regulation remains unclear.

Epsilon toxin:

The epsilon is a pore forming toxin and the third most potent clostridial toxin after botulinum and tetanus neurotoxins and thus is considered by the CDC as a Category B Bioterrorist agent (79). The epsilon toxin (*etx*) is encoded by a large plasmid present in non-human pathogenic type B and D strains only (20), and has a limited host range including sheep, lambs, goats and cattle causing enterotoxemia and is of significant economic importance (78). Substantial evidence indicates epsilon is a pore forming toxin that increases cell permeability to small molecules and ions (163, 165, 166). Rapid progression of the disease generally leads to mortality, therefore vaccination is the preventive method of choice (185).

Iota toxin:

Iota-toxin, a binary toxin consist of two noncovalenttly lined components, where: Ia, with a molecular weight of ~ 47.5 kDa is the ADP-ribosyltransferase, and Ib with a molecular weight of ~ 71.5 kDa is involved in the binding and internalization of the Ia subunit into the cell (160, 217). Once inside the cell, Ia catalyzes ADP-

ribosylation of host cell actin at Arg-177, which induces cytoskeletal disarray and cell death (13). Both subunits (*iap* and *ibp*) are encoded in a large plasmid uniquely present in type E isolates (88). The diseases is manifested as diarrhea in animals, particularly domesticated livestock (79).

Other toxins:

Although the above are the main toxins used for toxinotyping *C. perfringens* isolates, there are other toxins that are not used for classification purposes, but have major roles in pathogenesis of certain diseases and are listed below.

NetB. The Necrotic Enteritis Toxin B-like (NetB) has bee recently identified as the major virulence factor in necrotic enteritis of poultry (89), a diseases that costs the international poultry industry more than US$ 2 billion per year (240). NetB is chromosomally encoded and present in ~ 15 % of *C. perfringens* type A isolates recovered from chickens (111). It's a 33 kDa protein with limited amino acid sequence similarity to other pore forming toxins including *C. perfringens* beta-toxin (38% identity) and displays cytotoxic activity against chicken leghorn male hepatoma cell line by forming hydrophilic pores in the cell membranes (89). The prominent feature of NE is acute death, with mortality rates that can reach 50% (178, 252), and the incidence of *C. perfrnigens* in the intestinal tract and in processed poultry meat being high (241). Clinical signs include depression, dehydration, somnolence, ruffled feathers, diarrhea and decreased feed consumption (2, 3, 48, 61).

CPE. The *Clostridium perfringens* enterotoxin (CPE) is the most important virulence factor for food poisoning (FP) and non-food-borne gastrointestinal (NFBGID) diseases in humans. However, a small percentage (< 5%) of the *C. perfringens* population, mostly type A isolates are able to produce CPE (117). The CPE is encoded by a *cpe* gene that can be located in the chromosome as well as in a large plasmid (32), with the majority of *C. perfringens* type A FP isolates carrying a chromosomal copy of the *cpe* gene, while all NFBGID isolates carry a plasmid copy of the *cpe* (32, 33, 97, 225). Interestingly, CPE is the only virulence factor associated uniquely with sporulation in the genus *Clostridium* (155). Its synthesis is under the control of the mother cells' sporulation-specific sigma factors SigK and SigE (59), and is synthesized as a 35 kDa protein in the mother cell compartment of the sporulating cell and released into the intestinal lumen when these sporulating cells lyse to release their spores (117).

Upon release of the CPE toxin, CPE binds via its C-terminal domain to surface receptor proteins of the host epithelial cells involved in tight junctions and belonging to the claudin family of proteins (46, 87). Next, CPE binding to the claudin and other co-receptors lead to formation of a 90 kDa small complex, which is sufficient for cytotoxicity (251), but is a precursor for formation of an SDS-resistant large-complex estimated to be ~ 155 kDa (218). This large complex corresponds to a pore, causing membrane permeability alterations in sensitive mammalians cells (115). These CPE-induced pores produce a extracellular Ca^{2+} influx, which at low dose of CPE results in activation of calmodulin and calpain leading to apoptosis of the host cell, while at high CPE-dose results in rapid increase of cellular Ca^{2+} levels resulting in oncosis (21). These CPE-induced membrane alterations causes direct histopathological damage to

the small intestine, especially the tips of the villi (118). The symptoms include acute abdominal pain and diarrhea; nausea, fever and vomiting (117).

Beta-2 Toxin. A beta-2 toxin has been discovered and found mainly in *C. perfringens* type C isolate (52), and also in type A isolates (62). Further studies have associated beta-2 toxin with necrotizing enterocolitis in a number of domestic animals and livestock (62, 90, 249). More recently, a study showed that beta-2 toxin along with the CPE is implicated in 75% of *C. perfringens* type A isolates causing antibiotic-associated diarrhea (AAD) and sporadic-diarrhea (SD) (42). The beta-2 toxin is a 28 kDa toxin encoded by the *cpb2* gene which is located in the same plasmid as the *cpe* gene in the majority of the isolates causing AAD and SD (42). This toxin has no homology to beta toxin of type B isolates (52), however little is known on its mechanism of action. The fact that *cpe* and cpb2 are in the same plasmid might explain in part why non-food-borne illnesses in humans caused by *C. perfringens* type A isolates carrying plasmid *cpe* lasts 1-2 weeks (22), while food-borne illness caused by *C. perfringens* type A isolates carrying chromosomal *cpe* lasts 24-48 h (22, 116), suggesting that beta-2 toxin has an important role in GI disease.

Other toxins that play a secondary role in pathogenesis of *C. perfringens* include: i) Kappa toxin, encoded by the chromosomally located *colA* gene, is a collagenase and gelatinase enzyme that facilitates tissue necrosis (114). However, studies indicate that *colA* mutants were still able to produce gas gangrene and clostridial myonecrosis in mouse (9); ii) Theta toxin, also known as perfringolysin, is

chromosomally encoded by the *pfoA* gene (238). This toxin binds to cholesterol rich-domains (termed rafts) in the membrane of host cells triggering the conformational changes that effect oligomerization and initiate pore formation (43, 63). Consistent with a secondary role in pathogenesis, a recent study indicates that it potentiates the lethal effects of epsilon toxin in a mouse model (41); iii) Delta toxin **i**s a 42 kDa hemolysin showing cytotoxic to various cell types such as erythocytes from even-toed ungulates (sheep, goats and pigs) (5), rabbit macrophages, human monocytes, and blood platelets from goat, rabbit, human and guinea pig (23, 83). This selective cytotoxicity is due to binding specificity of delta toxin with the ganglioside G_{M2} (5, 23, 83).

The ability of *C. perfringens* to produce a wide spectrum of diseases in humans and animals is due in part to its ability to produce at least 15 toxins (164, 183). However, it is not clear why *C. perfringens* produces illnesses that vary so greatly their severity and pathology. It is also not clear why certain isolates commonly associated with a certain diseases (i.e., gas gangrene) have never been found to cause another disease (food poisoning) and vice versa (184). One explanation is the presence of certain toxins, although it falls short in many instances. Strikingly, a recent study analyzed core housekeeping genes (i.e., the gyrase subunit A, *gyrA* gene; the 50S ribosomal protein, *rplL* gene; and the regulatory protein, *pfoS* gene) and virulence genes (i.e., alpha-toxin, *plc* gene; and the kappa-toxin, *colA* gene) of 247 *C. perfringens* isolates from various sources, revealing cryptic lineages of *C. perfringens* associated with different diseases (184). Pathogenic strains grouped into five distinct

evolutionary lineages, showing host adaptation beyond their toxinotype, and are the following. Lineage I include strains isolated from gas-gangrene, bovine hemorrhagic enteritis (β2+), antibiotic associated diarrhea, and retail meats (non-pathogenic); Lineage II includes strains isolated from human peritonitis and septicemia; Lineage III includes strains isolated from bovine and equine hemorrhagic enteritis (β2+); Lineage IV includes strains isolated from ovine enterotoxemia; Lineage V includes strains isolated from human food poisoning. Interestingly, these clusters based on genetic background are in well agreement with their source of disease. These results indicate that *C. perfringens* isolates of different lineages have adapted to best fit their host. One clear example, is the enterotoxin gene associated with food poisoning (FP), non-food-borne antibiotic associated diarrhea and sporadic diarrhea (AAD-SD) (42, 192). Although the genetic background of the *cpe* gene from the various sources of disease has no significant variation, the genetic background of the isolates from the various sources seems to go beyond the fact that FP isolates carry a chromosomal copy of the *cpe* gene while AAD-SD isolates carry a plasmid borne *cpe* gene (32, 192, 193), and involve traits that make them best fit to their specific niches. In this particular case, FP isolates form spores with 60-fold higher heat resistance than spores of AAD-SD isolates (193), making FP spores more suitable to FP environments. These examples clearly suggest that many of these lineages have recently evolved to best fit their host (184).

1.2 *C. perfringens* food poisoning

Clostridium perfringens type A food poisoning (FP) ranks as the third most commonly reported food-borne illness affecting more than 250,000 individuals annually and causing economical losses of over $120 million (138) in the USA. However, mainly *C. perfringens* type A carrying a chromosomal copy of the *cpe* gene are capable of causing the gastrointestinal symptoms of this food-borne illness (117). *C. perfringens* spores of FP isolates are the infectious morphotype that are metabolically dormant and resistant to various environmental stress factors. Various studies have given evidence that spores and vegetative cells of FP isolates are better adapted to FP environments than NFBGID isolates because the formers have higher resistance than those of NFBGID isolates to: i) moist heat; ii) osmotic, nitrite, and pH induced stress; iii) prolonged frozen storage; and iv) high pressure processing (HPP) (102, 103, 117, 150, 151, 193). These resistance properties of spores of FP isolates facilitate their survival in processed and poultry meats, products that are most commonly implicated in *C. perfringens* type A food poisoning outbreaks (117). Once conditions are favorable, these heat-resistant spores can germinate and outgrow into vegetative cells reaching high viable cell numbers (~10^6-10^7 cfu/g). After food is consumed, those viable vegetative cells that survived the stomach's acidity will initiate sporulation in the intestinal tract, release CPE upon cell lysis causing GI illness (117).

1.3 Resistance of *C. perfringens* spores.

C. perfringens FP pathogenesis involves survival of spores to food processing regimes, inadequate cooking, cooling or incomplete warming of the food (117).

Spores of FP isolates posses unique resistance properties that enable them to survive FP environments and cause disease. A genetic factor contributing to spore heat resistance is evident from findings that spores of different *C. perfringens* strains exhibit considerable variation in their heat resistance (193) and spores of FP isolates possess significantly greater heat resistance than spores of *C. perfringens* from other sources (174, 193). The high heat resistance of spores of FP isolates is not due to the presence of *cpe* because no difference in spore heat resistance was observed between wild-type and *cpe*-knock out mutant strains of FP isolate (173), but instead due to other factors with some still being unknown.

The genomes of *Clostridium* species contain multiple genes (termed *ssp*) encoding potentially DNA-protective α/β-type SASP, with 3 (*ssp1*, *2* and *3*) in *C. perfringens* (130, 213). Recent studies (151, 174-176), suggests that α/β-type SASP plays a role in the resistance of *C. perfringens* spores to moist heat and various chemicals. Recently, a novel α/β-type SASP has been identified, now named Ssp4, present in FP and NFBGID isolates (104). However, sporulating cultures of FP strains producing resistant spores consistently express a variant Ssp4 with an Asp substitution at residue 36. In contrast, Gly was detected at Ssp4 residue 36 in NFBGID strains producing relatively sensitive spores. The Asp 36 Ssp4 variant was demonstrated to be important for the exceptionally heat and nitrite resistant of most FP spores (104). The fact that the SM101 (a FP isolate) *ssp4* null mutant still remains substantially more heat resistant than wild-type F4969 (a NFBGID isolate) (104), together with previous studies (174-176) showing similar expression levels of Ssp1, 2 and 3 by SM101 and

F4969, may suggest that additional factors beyond the SASPs also contribute to the resistant phenotype of spores produced by many FP isolates.

Indeed, spore core water content is another major factor in *C. perfringens* spore resistance to moist heat (140, 152), as is also the case with *B. subtilis* spores (49, 208)**.** The core water content is directly affected by sporulation temperature; a higher sporulating temperature produces *C. perfringens* spores with higher heat resistance and vise versa (152). As in *B. subtilis* (169)**,** mutation in the operon encoding *spmA* and *spmB* leads to *C. perfringens* spores with a slight but significant increase in core water content (140, 152). This small increase in core water content leads to ~ 50% reduction in moist heat resistance; however, it does not affect spore resistance to UV and chemical treatments, presumably because the spores' DNA is saturated with α/β-type SASPs (152).

1.4 Bacterial spore germination

Spores of *Bacillus* and *Clostridium* species are formed in sporulation, a process triggered by integrating a wide range of environmental and physiological signals that arise form nutrient depletion, cell density, Krebs cycle (227). These spores are metabolically dormant and resistant to various environmental stress (150, 151, 174, 208). However, dormant spores monitor the environment, and under favorable conditions, spores germinate, outgrow, resume to vegetative growth and then release toxins and cause disease (117). Thus, spore germination has attracted research interest as it is through germination that spores ultimately cause a variety of life-threatening diseases (207). Over the past two decades, a great amount of research has been carried

out with *B. subtilis*, a model organism for spore-forming bacterium, and our current understanding of the model of spore germination are based in this organism.

Dormant spores initiate germination when they sense the presence of nutrients, termed germinants, in the environment. Germinants known to date include amino acids, sugars or purine nucleosides, yet combination of specific nutrients also trigger germination, for example a mixture for asparagine, glucose, fructose and K^+ (AGFK) triggers *B. subtilis* spore germination (207). Within seconds of sensing presence of germinants, the spore becomes committed to germinate, and even after removal of germinants, germinaton continues. However, the precise mechanism that triggers germination is unclear. Once spores initiate germination a series of biophysical and biochemical events take place:

- First, monovalent ions (H^+, K^+ and Na^+) are released from the spore core through a energy independent mechanism (229). The release of H^+ elevates the core pH from ~ 6.5 to 7.7, change that is crucial for spore metabolism once the hydration levels of the spore core are high enough for enzymatic activity (207).
- Second, the release of the spore core's large depot (~ 10% of spore dry wt) of pyridine-2, 6-dicarboxylic acid (dipicolonic acid [DPA]) as a 1:1 chelate with divalent cations, predominantly Ca^{2+} (Ca-DPA). This event follows ion release (229).
- Third, the released Ca-DPA is replaced by water resulting in increase core hydration, although not sufficient for protein mobility causes some decrease in wet-heat resistance (34, 200).

- Fourth, hydrolysis of the spore's peptidoglycan (PG) cortex is a later event in germination, and in *B. subtilis* is triggered at least in part by Ca-DPA release (146, 207).
- Fifth, degradation of the PG cortex eliminates the physical constraint allowing the core to expand and take up water to levels found in vegetative cells and resumption of metabolism (171).

These events comprise the germination process and take place in the absence of detectable energy metabolism (207).

Spores are also capable of germinating with "non-nutrient" agents including lysozyme, salts, high pressure, Ca^{2+}-DPA and cationic surfactants such as dodecylamine (54, 145, 201). These non-nutrient agents might well act through several of the components of the nutrient germination pathway (15, 146, 201). It is also likely that Ca-DPA released from one spores might stimulate germination of neighboring spores (207).

1.4.1 Germinant receptors

Spores sense the presence of germinants through germinants receptors. Genetic evidence strongly suggest that orthologuos proteins belonging to the GerA family form the nutrient germinant receptors (125, 127, 145). The genes of the GerA family are expressed uniquely during sporulation in the developing forespore (40). In *B. subtilis*, there are three germinant receptors encoded by three tricitronic operons,

termed, *gerA*, *gerB* and *gerK* (125, 257), with each operon encoding a single nutrient germinant receptor, which is a complex of the three proteins encoded by each cistron of their respective *gerA* family operon. Null mutations in any cistron within the operon results in inactivation of the respective receptor (127, 145). Genetic evidence also supports the model of the three proteins encoded in each operon physically interacting to form a nutrient receptor (69, 143). Hydropathy profiles show that proteins A and B (encoded by each operon) are integral membrane proteins, which is consistent with them being located in the spore's inner membrane (69, 147). In contrast, the C component is relatively hydrophilic and anchored to the membrane via a covalently attached diacylglyceryl moiety (71, 72). Despite the great body of research, it is still unclear which are the ligand binding sites and the mechanism of transduction of the germination signal to downstream effectors.

1.4.2 Ion channels

After binding of the germinants to their cognate receptors follows an efflux of ~ 80% of the spore's depot of Na^+, K^+ and H^+ through an energy independent process, causing a rise in the spore core's pH (229). The exact mechanism of release of monovalent ions during spore germination is not known, however, it is likely that cation:proton antiporters could be involved in this event. Indeed, a Na^+/H^+-K^+ antiporter termed GerN, identified in *B. cereus* as a possible participant in cation movement during spore germination (224, 233). The *gerN* shares high similarity to a *B. megaterium grmA* gene, and mutation blocks spore germination in nutrients (231). However, *B. cereus gerN* spores are still able to germinate with L-alanine and Ca-

DPA but not with inosine. Similar *gerN* mutation in *B. subtilis* had no role in spore germination (233). Therefore, it is not clear if GerN works in spore germination, and instead is directly involved in spore formation.

Water is another small molecule that exhibits significant movement during spore germination. The volume of *B. subtilis* spore core increases 2- to 2.5-fold through germination by water uptake (207). The mechanism of water uptake is not clear, and no homolog to aquaporins, except for a facilitator of glycerol uptake, GlpF is present in *B. subtilis*. However, the role of this protein in spore germination is unclear.

1.4.3 DPA channels

Release of Ca-DPA during *B. subtilis* spore germination and DPS uptake into the developing spore during sporulation from the mother cell, the site of DPA synthesis requires the SpoVA proteins, which might form some sort of gated channel in the spore's inner membrane (242, 244). *B. subtilis*, SpoVA proteins are encoded by the hexacistronic *spoVA* operon (93). It is likely that most of these proteins are located in the spore's inner membrane as shown with SpoVAD proteins (243). These proteins are synthesized uniquely during sporulation in the developing forespore just prior to DPA uptake (236, 242). Deletion of the *spoVA* operon in *B. subtilis* produces strains that are able to initiate sporulation, but their spores are extremely unstable and quickly lyse during purification (236). It is unclear why DPA-less spores of *B. subtilis* are so unstable and lyse during sporulation, but presumably because they lack DPA and/or

because the CLE, SleB, is spontaneously activated in spores lacking DPA due to little constrain in the PG cortex (108, 146).

1.4.4 Cortex lytic enzymes

In *Bacillus* species, there are two redundant cortex-lytic enzymes (CLEs) that degrade the cortex PG during germination, SleB and CwlJ, and a third enzyme SleL (YaaH) that is unable to degrade the cortex by itself, though exhibits some cortex lytic activity (34, 98, 110, 207). Germination of *B. subtilis cwlJ* and *sleB* spores is relatively normal, however, spores of the double mutant are not able to degrade their cortex (34, 98, 110, 207). Both enzymes require muramic-δ-lactam for PG cleavage, which ensures that the germ cell wall of the outgrowing spore, which lacks this modification, is not degraded during germination and becomes the cell wall of the outgrowing spore (127, 172). Both enzymes are synthesized during sporulation. CwlJ in the mother cell and SleB in the forespore, and both are synthesized as a mature enzyme. CwlJ is localized in the spore outer cortex fraction, while SleB is present in the cortex as well as in the spore inner membrane/cortex boundary (207). The fact that both enzymes are synthesized in a mature form, suggest that there must be a mechanism to maintain these enzymes in an inactive state during spore dormancy, however to date this remains unclear. For CwlJ, the activation signal is Ca-DPA either released from the spore core as well as exogenous Ca-DPA (146). The signaling pathway for SleB is more elusive, with evidence suggesting that it might be activated by a change in the cortex PG stress (146, 203, 207).

The situation in *C. perfringens* is somehow different. Two CLE have been identified in *C. perfringens* S40, SleC and SleM, which are likely to be involved in cortex hydrolysis during spore germination. These enzymes have also been found in genomes of sequenced *C. perfringens* strains (130, 213). Both are synthesized in the mother cell (112), and localized in the spores' cortex PG (123). However, SleC is activated by proteolysis in the first minutes of spore germination (121, 137, 212). However, their *in vivo* role during *C. perfringens* spore germination remains unclear.

Analysis of all sequenced *Bacillus* identified orthologues of the GerA-family, GerN, SpoVA and CLE proteins with significant amino acid similarity to each other. In the *Clostridium* genomes sequenced so far, the majority of the species have all the components of the germination apparatus. The exceptions include *C. perfringens* and *Clostridium difficile*, with the former having no intact *gerA*-type operon and the latter species lacking orthologues of the GerA-family of receptors. This suggests that the backbone of the mechanism of spore germination has been conserved through the evolution of the different species, though with significant differences. Unfortunately, due to lack of easily implemented genetic tools for introducing mutation in most Clostridia species, there is limited knowledge on the mechanism of spore germination of Clostridia. Thus, studies in *Clostridium* are needed to address the difference in the spore germination mechanism between the aerobic Bacillus and the anaerobic *Clostridium* species.

1.5 Applications of bacterial spore germination

Understanding the molecular mechanism of spore germination of pathogenic bacteria holds profound implications in the identification of new drug targets, therapeutic development, decontamination and preventive measurements. For example, identification of compounds that trigger spore germination would allow the now germinated spore to loss their resistance to mild treatment and could be easily killed. Conversely, identifying key components of the germination apparatus might allow the development of a compound that would block spore germination, thus preventing progression of the disease.

Another attractive field for the control of spore germination is in the Food Industry. In response to the growing consumer demand for higher-quality, minimally processed, and additive free foods, the food industry needs to develop alternatives to conventional processing technologies. High hydrostatic pressure processing has evolved as a safe and effective technology meeting this demand with a great variety of products in the market (162). Although the application of 400 to 800 MPa has been reported to inactivate pathogenic and spoilage bacteria (80), the inactivation of bacterial spores has been a major challenge to HPP process developers (124, 161, 220).

Indeed, a recent report (150) shows that while *C. perfringens* vegetative cells were inactivated, spores were able to survive treatments of 650 MPa at 75ºC, posing a barrier for the development of high-pressure treated foods (i.e., meats) ubiquitously contaminated with *C. perfringens*. Several studies have successfully developed strategies to inactivate *Bacillus cereus* spores food industry settings. For example, high hydrostatic pressure and L-alanine can efficiently trigger germination of *B.*

cereus spore, which can be subsequently inactivated by a second high pressure treatment (177). More recently, a *in situ* strategy to inactivate adhered *B. cereus* spores to stainless steel of dairy milk equipment was developed by efficiently inducing germination with a mixture of L-alanine and inosine of adhered spores and subsequent inactivation with mild heat or alkali treatments typically used in decontamination of dairy pipelines (66). Unfortunately, to date there is little information of the molecular mechanism of *C. perfrinegns* spore germination to allow the development of high-hydrostatice pressure strategies to inactivate *C. perfringens* spores in meat products.

Objective of this study

In *C. perfringens*, spore germination is a crucial and early event in the development of any of the *C. perfringens*-associated disease. Due to its anaerobic nature and the fact that *C. perfringens* spores are ubiquitous in the environment, knowledge of *C. perfringens* spore germination is required for the development of new strategies to prevent *C. perfringens* diseases. To our knowledge the components and their role in *C. perfringens* spore germination is completely unknown.

The central goal of this research is to dissect the molecular mechanism of spore germination in *C. perfringens*.

The specific objectives of this research are:

1. To identify and characterize germinants and their receptors in *C. perfringens*.
2. To evaluate whether antiporters are involved in *C. perfringens* spore germination.
3. To investigate the mechanism of signal transduction in *C. perfringens* spore germination between the germinant receptors and the downstream effectors.
4. To investigate the *in vivo* role of CLEs involved in PG spore cortex hydrolysis during *C. perfringens* spore germination.
5. To investigate the *in vivo* role of the *cspB* protease in the initiation of PG spore cortex hydrolysis during *C. perfringens* spore germination.
6. To develop a strategy to inactivate *C. perfringens* spores in meat products.

Chapter 2

Clostridium perfringens spore germination: characterization of germinants and their receptors

Daniel Paredes-Sabja, J. Antonio Torres, Peter Setlow and Mahfuzur R. Sarker

Published in:

Journal of Bacteriology 2008 **190** (4): 1190-1201

2.1 Abstract

Clostridium perfringens food poisoning is caused by type A isolates carrying a chromosomal enterotoxin (*cpe*) gene (C-*cpe*), while *C. perfringens*-associated non-food-borne gastrointestinal (GI) diseases are caused by isolates carrying a plasmid-borne *cpe* (P-*cpe*). *C. perfringens* spores are thought to be the important infectious cell morphotype, and after inoculation into a suitable host, these spores must germinate and return to active growth to cause GI disease. We have found differences in the germination of spores of C-*cpe* and P-*cpe* isolates in that: (i) while a mixture of L-asparagine and KCl was a good germinant for spores of C-*cpe* and P-*cpe* isolates, KCl and, to a lesser extent, L-asparagine triggered spore germination only in C-*cpe* isolates; and (ii) L-alanine or L-valine induced significant germination of spores of P-*cpe* but not of C-*cpe* isolates. Spores of a *gerK* mutant of a C-*cpe* isolate in which two of the proteins of a spore nutrient germinant receptor are absent germinated slower than wild-type spores with KCl, did not germinate with L-asparagine, and germinated poorly with the non-nutrient germinants dodecylamine and a 1:1 chelate of Ca^{2+} and dipicolinic acid compared to wild-type spores. In contrast, spores of a *gerAA* mutant of a C-*cpe* isolate that lacks another component of a nutrient germinant receptor germinated at the same rate as wild-type spores with high concentrations of KCl, although germinated slightly slower with a lower KCl concentration, suggesting an auxiliary role for GerAA in *C. perfringens* spore germination. In sum, this study identified nutrient germinants for spores of both C-*cpe* and P-*cpe* isolates of *C. perfringens* and provided evidence that proteins encoded by the *gerK* operon are required for both nutrient- and non-nutrient-induced spore germination.

2.2 Introduction

Bacillus and *Clostridium* species have the ability to form metabolically dormant spores that are extremely resistant to environmental stresses such as heat, radiation and toxic chemicals (176, 208). As a consequence of this resistance, spores of a number of these species are significant agents of food spoilage and food-borne gastrointestinal (GI) diseases (210). However, to cause deleterious effects, dormant spores must first go through germination then outgrowth to be converted to vegetative cells. Spore germination has been most extensively studied in *B. subtilis* (127, 145, 207), and can be initiated by a variety of chemicals including nutrients, cationic surfactants and enzymes, as well as by hydrostatic pressure (148). Nutrient germinants for spores of *Bacillus* species include L-alanine, a mixture of L-asparagine, D-glucose, D-fructose and potassium ions (AGFK), and inosine (29, 126, 207). These nutrient germinants interact with cognate receptors located in the inner spore membrane (69, 146), stimulating the release of monovalent cations (H^+, Na^+, and K^+), divalent cations (Ca^{2+}, Mg^{2+}, and Mn^{2+}), and the spore core's large depot (~ 20% of core dry wt) of pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)) (207), accompanied by an increase in the water content of the spore core. DPA is released as a 1:1 chelate with divalent cations, predominantly Ca^{2+} (Ca-DPA) and Ca-DPA-release triggers further events in spore germination. Most important among the latter is the hydrolysis of the spore's peptidoglycan cortex by one or more spore cortex-lytic enzymes (SCLEs) that allows the core to expand and take up even more water to the level found in growing cells. This event in turn restores protein movement and enzyme action in the spore

core and leads to resumption of energy metabolism and macromolecular synthesis (34, 208).

Genetic evidence strongly suggests that orthologous proteins belonging to the GerA family form the nutrient germinant receptors through which the spore senses the presence of nutrients in the environment (125, 126, 145). In *B. subtilis* the genes of the GerA family are expressed only during sporulation in the developing forespore (40), and are in three tricistronic operons, termed *gerA, gerB* and *gerK* (125, 127). Each of these operons appears to encode a single nutrient germinant receptor which is a complex of the three proteins encoded by each operon, and null mutation in any cistron within the operon results in inactivation of the respective receptor (127, 145). There is also genetic evidence suggesting that the three proteins encoded by each operon physically interact to form a receptor (69, 143), and that these receptors interact with each other to some degree (6, 21, 143). Hydropathy profiling indicates that two proteins (A and B) encoded by each operon are integral membrane proteins, which is consistent with their being receptors for environmental stimuli (127, 145). However, the C component encodes a relatively hydrophilic product that is likely to be anchored to the membrane via a covalently attached diacylglyceryl moiety (71, 72, 127).

Spore germination in *Clostridium* species is less well studied than in *Bacillus*. Limited studies have shown that spores of proteolytic *C. botulinum* and *C. sporogenes* germinate in response to L-alanine, but not AGFK or inosine (17), but no such information is available for spores of *C. perfringens*, an important human GI pathogen. *C. perfringens* food poisoning is caused by type A isolates carrying a

chromosomal enterotoxin (*cpe*) gene (C-*cpe*), while *C. perfringens*-associated non-food-borne GI diseases are caused by isolates carrying a plasmid-borne *cpe* (P-*cpe*) (117, 192). However, exceptions have been reported in a recent study (96), which showed that P-*cpe* isolates can also be a common cause of food poisoning. *C. perfringens* spores are thought to be the important infectious cell morphotype, and after inoculation into a suitable host, these spores must germinate and return to active growth to cause GI disease (117). In this study, we have investigated the germination of spores of pathogenic *C. perfringens* C-*cpe* and P-*cpe* isolates. We have identified nutrient germinants for *C. perfringens* spores and identified a differential germination response in spores of C-*cpe* and P-*cpe* isolates. In addition, through construction of mutations in genes encoding nutrient germinant receptors, we have investigated the role of different receptors in spore germination in response to a number of nutrient and non-nutrient germinants.

2.3 Materials and Methods

Bacterial strains and plasmids. The *C. perfringens* strains and plasmids used in this study are described in Table 2.1.

Spore preparation. Starter cultures (10 ml) of *C. perfringens* isolates were prepared by overnight growth at 37°C in fluid thioglycollate broth (FTG) (Difco) as described (91). Sporulating cultures of *C. perfringens* were prepared by inoculating 0.2 ml of an FTG starter culture into 10 ml of Duncan-Strong (DS) sporulation medium (36), which was incubated for 24 h at 37°C to form spores as was confirmed by phase-contrast

microscopy. Spore preparations were prepared by scaling-up the latter procedure. Spores were purified by repeated washing with sterile distilled water until they were > 99% free of sporulating cells, cell debris and germinated spores, and were suspended in distilled water at an optical density at 600 nm (OD_{600}) of ~ 6 and stored at -20°C. Spores of *B. subtilis* strain JH642 were prepared by growth for ~72 h at 37°C on agar plates (132), and the spores were purified as described (180, 195).

Spore germination. After heat activation (70°C, 30 min for *B. subtilis*; 75°C, 10 min for P-*cpe*; 80ºC, 10 min for C-*cpe*), spores were cooled to room temperature and incubated at 30°C for 10 min (unless noted otherwise) before addition of germinants. Spores of C-*cpe* and P-*cpe* were heat activated at different temperatures because our preliminary germination assay demonstrated that C-*cpe* isolates germinated better when heat activated at 80ºC for 10 min, whereas P-*cpe* isolates at 75ºC for 10 min. Spore germination was routinely measured by monitoring the OD_{600} of spore cultures (Smartspec^{TM} 3000 Spectrophotometer, Bio-Rad Laboratories, Hercules, CA, USA) which falls ~ 60% upon complete spore germination, and levels of spore germination were confirmed by phase contrast microscopy. Germination was routinely carried out aerobically, since no differences in germination kinetics were detected under anaerobic conditions (data not shown). The extent of spore germination was calculated by measuring the decrease in OD_{600} and expressed as percentage of initial OD_{600}. The rate of germination was expressed as the maximum rate of loss of OD_{600} of the spore suspension, relative to the initial value. To evaluate the effects of pH on the rate of germination, germination was carried out in 25 mM sodium phosphate buffer (pH 5.7-

7.5) or 10 mM Tris-HCl buffer (pH 8.0 and 8.5) at 30°C. All values reported are averages of two experiments performed with two independent spore preparations, and individual values varied by less than 15% from the average.

Construction of *gerK* mutant. To isolate a derivative of *C. perfringens* strain SM101 with insertion of *catP* giving chloramphenicol resistance (Cm^r; 20 μg/ml) in the *gerK* operon, a *gerK* mutator plasmid was constructed as follows: A 3163-bp fragment carrying the *gerK* operon and 619-bp upstream of *gerKA* was PCR amplified with primers CPP213 and CPP214 that had KpnI and SalI cleavage sites, respectively (Table 2.2). The ~ 3.2-kb PCR fragment was cloned into plasmid pCR®-XL-TOPO® (Invitrogen, Carlsbad, CA) in *Escherichia coli* giving plasmid pDP9, excised from this plasmid by digestion with KpnI and SalI and the 3.2 kb fragment was ligated between the KpnI and SalI sites of plasmid pMRS104 giving plasmid pDP10. The latter plasmid was digested with SpeI that cuts only once within the *gerKA* ORF, the ends were filled, and a ~ 1.3-kb SmaI-NaeI fragment containing the *catP* gene from plasmid pJIR418 (11) was inserted giving plasmid pDP11. The latter plasmid contains an inactived *gerK* operon and since it contains no *C. perfringens* origin of replication, cannot replicate in this host. Plasmid pDP11 was introduced into *C. perfringens* strain SM101 by electroporation (35) and a *gerK* mutant, strain DPS101, was selected by allelic exchange as described (192). The replacement of the wild-type *gerKA* gene with the mutant allele in strain DPS101 and the loss of the plasmid from this strain were confirmed by PCR and Southern blot analyses (data not shown).

Construction of *gerAA* mutants. A derivative of strain SM101 with an intron inserted in the *gerAA* gene was constructed as follows: To target the *L1.LtrB* intron (25) to *gerAA*, the intron sequence in plasmid pJIR750ai was modified based upon the sequences of predicted insertion sites in the *gerAA* gene, using the InGex Intron Prediction Program (www.Sigma-Aldrich.com/Targetronaccess). For optimal gene interruption and stable insertion, the insertion site in the antisense strand, between positions 123/124 (Score: 9.4; E-value: 0.038) from the start codon, was chosen. Three short sequence elements from the intron RNA involved in base pairing with the DNA target site (25) were modified by PCR using *gerAA*-specific primers CPP235, CPP236, CPP237 (Table 2.2) and the LtrBAsEBS2 Universal primer (CGAAATTAGAAACTTGCGTTCAGTAAAC) provided with the Targetron™ Gene Knockout System (Sigma-Aldrich Corporation, St. Louis, MO). The 353-bp *gerAA* Targetron was then cloned into plasmid pCR®-XL-TOPO® generating plasmid pDP12, and a 353-bp HindIII-BsrGI fragment from pDP12 was cloned between the HindIII and BsrGI sites of the pJIR750ai vector giving plasmid pDP13. Plasmid pDP13 was electroporated into *C. perfringens* strain SM101 (35) and Cm^r colonies were screened for the insertion of the Targetron by PCR using *gerAA* specific primers CPP211 and CPP212 (Table 2.2). To cure the Cm^r-coding vector, one Cm^r-Targetron-inserted clone was subcultured daily for two days in FTG medium without Cm, and single colonies were patched onto Brain Heart Infusion (BHI) agar with or without Cm, giving strain DPS102.

To isolate a derivative of SM101 with a deletion of the entire *gerAA* gene, a Δ*gerAA* suicide vector was constructed as follows: A 1856-bp DNA fragment carrying

186-bp from the N-terminal coding region and 1670-bp upstream of *gerAA* was PCR amplified using primers CPP257 and CPP258 (Table 2.2), which had KpnI and SpeI cleavage sites at the 5' ends of the forward and reverse primers, respectively (Table 2.2). A 1994-bp fragment carrying 225-bp from the C-terminal coding region and 1769-bp downstream of *gerAA* was PCR amplified using primers CPP259 and CPP260 (Table 2.2), which had PstI and XhoI cleavage sites, respectively. These PCR fragments were cloned into plasmid pCR®-XL-TOPO® giving plasmids pDP18 and pDP19, respectively. A 1856-bp KpnI-SpeI fragment from pDP18 was cloned upstream of *catP* in pMRS99 (Sarker, unpublished data) giving plasmid pDP20, and an ~2.0-kb PstI-XhoI fragment from pDP19 was cloned downstream of *catP* in pDP20 giving pDP21. Finally, an ~4.5-kb fragment carrying Δ*gerAA*::*catP* was cloned into plasmid pMRS104 that cannot replicate in *C. perfringens* (67), giving plasmid pDP22. Plasmid pDP22 was introduced into *C. perfringens* strain SM101 by electroporation (35), and the *gerAA* deletion strain DPS103 was isolated by allelic exchange (192). The presence of the *gerAA* deletion in strain DPS103 was confirmed by PCR and Southern blot analyses (data not shown).

RT-PCR analyses. *C. perfringens* strains were grown in either DS sporulation medium (36) or TGY (3% trypticase, 2% glucose, 1% yeast extract, 0.1% cysteine) vegetative medium (91) at 37°C for 4 h, and total RNA was isolated as described (35, 68). The primer pairs CPP205 and CPP206, CPP207 and CPP208, and CPP283 and CPP284 (Table 2.2), which amplified 822-, 873- and 839-bp internal fragments from *gerAA*, *gerKA* and *gerKC*, respectively, were used to detect *gerAA*-, *gerKA*-, and

gerKC-specific mRNAs in RNA preparations by RT-PCR analysis as described (67, 68).

DPA release. DPA-release during nutrient-triggered spore germination was measured by heat activating a spore suspension (OD_{600} of 1.5) and incubating at 40°C with 5 mM KCl to allow adequate measurement of DPA release. For DPA-release during dodecylamine germination, spores were incubated at 60ºC with 1 mM dodecylamine, 25 mM Tris-HCl (pH 7.4). Aliquots (1 ml) of germinating cultures were centrifuged for 2 min in a microcentrifuge, and DPA in the supernatant fluid was measured by monitoring the OD_{270} as described (21, 201).

Measurement of spore core DPA content. Spores were germinated with and without heat activation, cooled to room temperature, diluted to an OD_{600} of 1.5 and incubated at 40°C with Ca^{2+}-DPA (50 mM $CaCl_2$, 50 mM DPA adjusted to pH 8.0 with Tris-base). At various times, aliquots (1 ml) were centrifuged for 2 min in a microcentrifuge, the spore pellet washed 4 times with 1 ml distilled water, and suspended in 1 ml of distilled sterile water. The residual spore core DPA content was determined by boiling samples for 60 min, centrifuging at 8,000 rpm in a microcentrifuge for 15 min, and measuring the OD_{270} of the supernatant fluid as described (21, 201). In *B. subtilis,* DPA compromises ~ 85% of the material absorbing at 270 nm released from spores by boiling (6, 21). The change in OD_{600} during spore germination by Ca-DPA was also measured as described above. However, since Ca-

DPA promotes spore clumping, spores were sonicated briefly to disrupt clumps before measuring the OD_{600}.

Colony formation assay. To assess the colony-forming ability of spores of strains SM101, DPS101 and DPS103, spores at an OD_{600} of 1 (~ 10^8 spores/ml) were heat activated at 80°C for 10 min, and aliquots of various dilutions were plated on BHI agar, incubated at 37°C anaerobically for 24 h, and colonies were counted.

Statistical analyses. Student's *t* test was used for specific comparisons.

2.4 Results

Ability of various compounds to trigger *C. perfringens* spore germination. Spores of *B. subtilis* 168 derivatives germinated well with either L-alanine, L-valine or AGFK, although not with the individual components of AGFK (Table 2.3), as expected (143). In contrast, *C. perfringens* SM101 spores germinated only slightly with two single amino acids, L-alanine and L-asparagine, although germinated well with AGFK (Table 2.3). However, much of the effect of AGFK appeared due to the K^+ ions, as KCl alone gave significant extent of germination of *C. perfringens* SM101 spores, glucose plus fructose were ineffective, and asparagine plus KCl was effective, albeit to a lesser extent than AGFK (Table 2.3; Fig 2.1). The rate of KCl-induced spore germination was dependent on the KCl concentration, with a maximum germination response at 100-200 mM (Fig. 2.2). In contrast to the stimulation of *C. perfringens* spore germination by KCl, NaCl was ineffective, while KI, KBr and

KH_2PO_4 were all effective (Table 2.3), as observed with spores of *B. megaterium* QM B1551 (28, 181).

To examine whether KCl with or without AGFK components is a universal germinant for *C. perfringens* spores, germination experiments were extended to spores of 6 additional isolates of *cpe*$^+$ *C. perfringens* type A, three C-*cpe* isolates (E13, NCTC8239, FD1041) and three P-*cpe* isolates (NB16, B40, F5603) (31, 32). As observed with spores of C-*cpe* isolate SM101 (Table 2.3), spores of the C-*cpe* isolates exhibited only minimal germination with L-alanine or L-valine, but some germination with L-asparagine (Table 2.4). However, spores of these isolates germinated well with KCl (Table 2.4), suggesting that KCl is a universal germinant for spores of C-*cpe* isolates. Interestingly, the germination of spores of the P-*cpe* isolates differed from that of spores of the C-*cpe* isolates, in that KCl alone did not induce significant germination of the P-*cpe* spores. The P-*cpe* spores also germinated fairly well with L-alanine and L-valine, as well as with AK, but not with L-asparagine alone (Table 2.4).

Effect of pH and temperature on *C. perfringens* spore germination. To define optimal conditions for *C. perfringens* spore germination, the temperature and pH of germination were varied, using spores of SM101 (a C-*cpe* isolate) and NB16 (a P-*cpe* isolate) and L-alanine, KCl and AK as germinants (Fig. 2.3A-D). While the optimum temperature for germination of SM101 and NB16 spores with all germinants tested was ~ 40°C, there were differences in the responses of spore germination with different germinants to temperature. In particular, germination of SM101 spores with KCl was much more sensitive to higher temperatures than was germination with AK.

The pH-dependence of germination of SM101 and NB16 spores with AK were also similar, with a pH optimum of 7.0-7.5. The responses of KCl and L-alanine germination to pH were also similar, but were not optimal at pH 7.0-7.5, rather exhibiting a gradual increase in germination rate as the pH was lowered to 5.7.

Identification of putative germination receptor homologues in *C. perfringens*. Studies with *B. cereus*, *B. anthracis* and *B. subtilis* have shown that the response of spores of these species to nutrient germinants is mediated through nutrient germinant receptor proteins encoded by the *gerA* operon family (29, 76, 143) . When the *C. perfringens* SM101 genome sequence was subjected to BLASTP analyses to identify genes encoding GerA-family nutrient germinant receptor protein homologues, four ORFs (CPR0614, CPR0615, CPR0616 and CPR1053) encoding proteins with high similarity (50-55%) to GerA-family proteins from *B. subtilis* were identified (Fig. 2.4A, B). CPR1053 is predicted to encode a 473 residue protein with a central region containing five transmembrane segments (TMS). Due to its high similarity with the 'A' proteins of all three *B. subtilis* GerA-type receptors, we termed CPR1053 *gerAA*. The *gerK* locus in *C. perfringens* (130) compromises three ORFs: CPR0614, CPR0615 and CPR0616. Based on the amino acid sequence similarity (39-56%) to the orthologues in *B. subtilis*, ORFs CPR0614, CPR0615 and CPE0616 were designated as *gerKB*, *gerKA* and *gerKC*, respectively (Fig. 2.4A, B). As in *B. subtilis*, *gerKA* and *gerKC* are adjacent, with *gerKA* the first gene in a putative bicistronic operon, but unlike the situation in *B. subtilis gerKB* is transcribed in the opposite direction from *gerKAC*, and is 96-bp upstream of *gerK*A. GerKA is predicted to be a 473 residue

protein with 5 TMS, and GerKC is predicted to be a 374-residue protein containing an N-terminal signal protein followed by a consensus sequence for diacylglycerol addition to a cysteine residue. GerKB is predicted to be a 362-residue protein with 10 TMS.

To assess the expression of *gerAA, gerKA* and *gerKC* homologues in *C. perfringens*, we performed RT-PCR analysis. As expected, the 822- 873- and 839-bp RT-PCR products specific for *gerAA*, *gerKA* and *gerKC*, respectively, were detected in RNA extracted from *C. perfringens* SM101 grown under sporulation conditions (Fig. 2.4C). The sizes of the RT-PCR amplified products matched the sizes of the products obtained in control PCR reactions (Fig. 2.4C). However, no *gerAA*-, *gerKA*- or *gerK*C-specific RT-PCR products were detected in RNA from SM101 vegetative cells (data not shown), indicating that *C. perfringens gerAA, gerKA* and *gerKC* are expressed only during sporulation.

Effect of a *gerK* mutation on nutrient germination of *C. perfringens* spores. As noted above, there are many studies with *Bacillus* species indicating that it is through nutrient germinant receptors of the GerA family that nutrients trigger spore germination. To assess whether the *gerKA* and the *gerKC* gene products have any role in *C. perfringens* spore germination, we constructed an insertion mutation in *gerKA*, giving strain DPS101. No *gerKA*- or *gerKC*-specific transcripts were detected in RNA isolated from sporulating DPS101 cells (Fig. 2.4C), indicating that the disruption of *gerKA* had a polar effect on the downstream *gerKC*. Strikingly, the germination of DPS101 spores with KCl, AK or L-asparagine was well below that of the parental

wild-type SM101 spores, in particular with L-asparagine, when spore germination was assessed by the OD_{600} of spore cultures (Fig. 2.5A-C). These differences were confirmed by examining spore cultures by phase contrast microscopy (data not shown), which showed in particular that after incubation for 60 min with L-asparagine, ≥ 95% of SM101 spores had germinated, while at most 5% of DPS101 spores had germinated.

Effect of a *gerAA* mutation on nutrient germination of *C. perfringens* spores. The only partial decrease in germination of spores lacking GerKA and GerKC with KCl and AK suggested that GerAA might also contribute to the *C. perfringens* spore germination with these germinants. Initial analysis of spores of a *gerAA* strain (DPS102) constructed using the Targetron-gene knockout system (25) found no difference in the kinetics of KCl, AK or L-asparagine germination of SM101 and DPS102 spores (data not shown). These results suggested that either GerAA has no role in spore germination or intron insertion leads to a C-terminal fragment of GerAA that retains activity in spore germination.

To more rigorously test the role of GerAA in spore germination, we constructed a derivative of strain SM101 (strain DPS103) in which the entire *gerAA* gene is deleted. Germination of DPS103 and SM101 spores in 100 mM KCl was similar, although slightly greater for the SM101 spores (Fig. 2.6A). However, the defect in the DPS103 spores was more evident at a suboptimal KCl concentration (10 mM), in which the extent of DPS103 spore germination was ≤ 50% of that of SM101 spores after 60 min of incubation (Fig. 2.6D). Although the *gerAA* spores again

showed no significant germination defect with 100 mM AK (Fig. 2.6B), at a lower AK concentration (10 mM) the extent of germination of DPS103 spores was significantly lower ($p < 0.01$) than for SM101 spores (Fig. 2.6E). However, there were no significant differences in the germination of SM101 and DPS103 spores with either high (100 mM) or low (10 mM) concentrations of L-asparagine (Fig. 2.6C, F).

Effect of *gerK* and *gerAA* mutations on DPA-release during *C. perfringens* spore germination. With *B. subtilis* spores, binding of nutrient germinants to specific receptors located in the spore's inner membrane triggers the release of a variety of compounds from the spore core, most notably DPA which comprises ~ 20% of the spore core's dry weight (144). Most of this DPA is released as Ca-DPA, and Ca-DPA release activates downstream germination events (207). Consequently, to gain more insight into the roles of GerAA, GerKA and GerKC in *C. perfringens* spore germination, we measured DPA release during KCl- and L-asparagine-triggered germination (Fig. 2.7A, B). During germination with 5 mM KCl, SM101 spores released nearly 67% of their DPA during the first 10 min, and 93% after 60 min of incubation, the latter as expected from fully germinated spores. DPS103 (*gerAA*) spores released slightly less DPA ($p < 0.01$) than SM101 spores after 60 min of incubation with 5 mM KCl, although SM101 and DPS103 spores exhibited similar DPA-release with L-asparagine (Fig. 2.7A, B). In contrast, DPS101 (*gerK*) spores released significantly less DPA during germination with either KCl or L-asparagine (Fig 2.7A, B). These results further support the hypothesis that GerAA plays an auxiliary role in KCl but not L-asparagine germination of *C. perfringens* spores, while

the products of the *gerK* operon are involved in both KCl and L-asparagine germination.

Effect of *gerK* and *gerAA* mutations on colony formation by *C. perfringens* spores. The germination defects observed in DPS101 and DPS103 spores suggested that these spores might have a lower colony-forming efficiency than SM101 spores, as spores need to sense the availability of nutrients to initiate germination and outgrowth. This hypothesis was tested by plating SM101, DPS101 and DPS103 spores on BHI agar and incubating for 24 h at 37°C under anaerobic conditions. No significant differences in colony formation efficiency were observed between SM101 (8.4×10^7 CFU/ml/OD_{600} unit [average of three experiments]) and DPS103 (8.3×10^7 CFU/ml/OD_{600} unit) spores, although DPS101 spores exhibited significantly lower colony-forming efficiency (1.6×10^6 CFU/ml/OD_{600} unit) than that of SM101 spores. No additional colonies appeared from DPS101 spores when plates were incubated up to 3 days. To evaluate whether the lower colony formation efficiency of DPS101 spores was due to their poorer germination, we compared the germination of DPS101, DPS103 and SM101 spores in BHI broth. As expected, DPS101 spores exhibited a significantly lower ($p < 0.01$) germination than wild-type (SM101) spores while there was only a minimal difference in germination between DPS103 and SM101 spores (Fig. 2.8). However, the germination difference in BHI broth between SM101 and DPS101 spores was nowhere near the 50-fold difference in colony formation. Therefore, spores of all three strains were germinated in BHI broth and examined by phase-contrast microscopy after 1 and 18 h of incubation. As expected ~ 65% of

SM101 and DPS103 spores, and ~ 30% of DPS101 spores were phase dark after 1 h of incubation, in agreement with the results from measurements of OD_{600} (Fig. 2.8). However, when spore suspensions were incubated for 18 h at 40ºC in BHI broth under aerobic conditions to prevent *C. perfringens* growth, ~ 99% of SM101 and DPS103 spores were phase dark, from which ~ 90% of these phase dark spores had released the nascent vegetative cell (data not shown). Strikingly, while ~ 70% of DPS101 spores were phase dark, ≤ 5% of these phase dark spores seemed to release the nascent vegetative cell (data not shown), which is in clear agreement with the lower colony formation observed from these spores (Table 2.5). These results suggest that the products of the *gerK* operon, but not *gerAA*, are not only essential for spore germination, but also in completing germination and outgrowth, and thus eventual colony formation in BHI medium.

Effect of *gerK* and *gerAA* mutations on Ca-DPA germination of *C. perfringens* spores. Previous work (145) has shown that *B. subtilis* spores lacking all nutrient germinant receptors are still able to germinate in the presence of exogenous Ca-DPA, which acts to promote cortex hydrolysis by activation of a spore cortex-lytic enzyme (SCLE) (146). Similar, albeit not identical spore cortex-lytic enzymes have also been found in other endospore-forming species, including *C. perfringens* (45, 77, 109, 122, 123, 212). When spores of strains SM101, DPS101 and DPS103 without prior heat activation were incubated with Ca-DPA and germination was measured, there were no significant changes in OD_{600} or spore refractility, and no release of DPA (data not shown). However, heat activated SM101 and DPS103 spores germinated significantly

as measured by both OD_{600} decrease and DPA-release (Fig. 2.9A, B). These results were confirmed by phase contrast microscopy, as ~ 80% of SM101 and DPS103 spores became phase dark after 60 min of incubation with Ca-DPA (data not shown). In contrast, no significant OD_{600} decrease and DPA-release was observed with heat-activated spores of DPS101 (*gerK*) spores incubated with Ca-DPA (Fig. 2.9A, B), and phase-contrast microscopy confirmed that after 60 min of incubation with 50 mM Ca-DPA, ~ 95% of the spores remained phase bright (data not shown). These results suggest that the putative *gerK* germinant receptor (but not the GerAA protein) plays a causal role in *C. perfringens* spore germination with Ca-DPA.

Effect of *gerK* and *gerAA* mutations on dodecylamine germination of *C. perfringens* spores. Dodecylamine, a cationic surfactant (201), can also germinate spores of many *Bacillus* and *Clostridium* species, and in *B. subtilis* spores dodecylamine may act by triggering spore core DPA release perhaps by opening a channel in the spore's inner membrane (201, 243, 244). Indeed, *B. subtilis* spores lacking all three nutrient germinant receptors release DPA in response to dodecylamine at a rate similar to that of wild-type spores (201). With spores of *C. perfringens*, wild-type (SM101) and *gerAA* (DPS103) spores exhibited similar rates of DPA release in response to dodecylamine (Fig. 2.10), indicating that GerAA is not required for dodecylamine germination. However, DPS101 (*gerK*) spores incubated with dodecylamine released DPA at a significantly slower rate than did wild-type (SM101) spores (Fig. 2.210), suggesting that *gerK*-encoded proteins are also involved in dodecylamine germination. Phase contrast microscopy of spores of all three strains

after 60 min of incubation with dodecylamine revealed that germinated spores were not as bright as dormant spores but not as dark as nutrient-germinated spores. This is in agreement with results with *B. subtilis* spores germinated with dodecylamine, where some but not all of the refractility of dormant spores in the phase contrast microscope is lost (201).

2.5 Discussion

While bacterial spores can remain in dormant for many years, they can return to life in as little as 20 min via spore germination and outgrowth if nutrients are added (for reviews see 29 and 46). There is much interest in these latter processes because: a) spores cause disease through germination and outgrowth in foodstuffs or in the body; and b) when spores germinate, they lose their resistance and are easy to kill. Thus, a detailed understanding of the mechanism(s) of spore germination may lead to the design of either inhibitors of germination or artificial germinants that could allow spore killing under mild conditions.

In this respect, our current study offers several significant contributions towards the understanding of the mechanism of germination of spores of *C. perfringens*, an anaerobic, toxigenic pathogen causing diseases in humans and animals (31, 32, 192, 249). Our studies suggests that *C. perfringens* C-*cpe* and P-*cpe* spores respond differently to germinants in that: (i) while AK is a universal germinant for all surveyed C-*cpe* and P-*cpe* spores, KCl and to a lesser extent L-asparagine can initiate germination of C-*cpe* but not P-*cpe* spores; and (ii) although L-alanine or L-valine are germinants for P-*cpe* spores, these amino acids gave no significant germination of C-

cpe spores. These different responses suggest that P-*cpe*, but not C-*cpe* spores, carry a functional L-alanine- receptor. The observation that L-alanine, a good germinant for spores of *B. subtilis*, *B. cereus*, and *C. botulinum* (4, 65, 145), was unable to trigger germination of spores of C-*cpe* isolates, further suggests that the germination response of C-*cpe* spores is different from that of *B. subtilis*, *B. cereus*, and *C. botulinum* spores, presumably due to differences in the complement of nutrient germinant receptors in these various species (130). Despite different germination responses, the optimum germination temperature for both C-*cpe* and P-*cpe* spores was ~ 40°C, slightly higher than the optimum growth temperature (37°C). The high optimum germination temperature for *C. perfringens* spores was not unexpected because the germination temperature optima for spores of *C. bifermentans* (246) and *C. botulinum* group IV type G (230) are 37-53°C and 37-45°C, respectively, significantly higher than the temperture optima for growth of these strains.

The germination of spores of C-*cpe* isolates by salt alone was a bit unexpected, but is by no means unique, since spores of at least some *B. megaterium* strains germinate well with salts alone, with KI better than KBr which is better than KCl (28, 181). In addition, K^+ ions are essential for the germination of *B. subtilis* spores with AGFK (181). Unfortunately, the precise mechanism of spore germination by salts alone is not known, nor what the advantage of this behavior might be.

Bacterial spores detect nutrient germinants through specific receptors (125, 126), and three tricistronic operons, *gerA*, *gerB*, and *gerK*, have been identified in *B. subtilis* as encoding the three functional receptors in this species (125, 126, 145). In contrast, the *C. perfringens* SM101 genome carries only monocistronic *gerA* and

gerKB and a bicistronic *gerKA-gerKC* operon (130). The products of the *gerK* operon are required for L-asparagine germination, presumably by acting as a receptor for L-asparagine. Since disruption of the *gerK* operon led to poorer spore germination and DPA release with KCl, this suggests that GerKA and/or GerKC play a significant role in *C. perfringens* spore germination by KCl. There also appears to be some interaction between the L-asparagine and KCl germination pathways, since *gerK* spores germinated more poorly with AK than with KCl. The responses of AK and KCl germination to pH and temperatures also suggest that L-asparagine interacts with a different receptor or different active site on the same receptor than does KCl, especially since AK allows significant germination at rather extreme temperatures (60°C). That individual nutrient germinant receptors and perhaps even individual germinant receptor proteins have multiple binding sites that recognize different germinants has been previously suggested from work on *B. subtilis* spore germination and more recently with *B. megaterium* spores (28).

Interestingly, the absence of GerAA slightly affected KCl germination and KCl-induced DPA release. An essential function of GerAA in recognition of germinants, in particular KCl, seems unlikely, since the maximum germination rate of *gerAA* spores with an optimal concentration of KCl was similar to that of wild-type spores. However, the slower germination of including DPA-release from *gerAA* spores using a sub-optimal KCl concentration suggest that GerAA may be involved in a peripheral or auxiliary fashion in KCl germination. However, this appears not to be the case when DPA-release is induced by exogenous Ca-DPA or dodecylamine, where the *gerAA* mutation had no effect.

The lower colony forming ability of *gerK* spores in the rich BHI medium compared to that of SM101 spores suggests that GerKA and/or GerKC are responsible for spore germination in this medium, and this was consistent with the slower germination of *gerK* spores in BHI medium. Interestingly, GerKA and/or GerKC also appear to be involved in the release of the nascent vegetative cell in germinated spores; perhaps they are responsible for the activation of either a cortex lytic enzyme to allow completion of germination, or some other enzyme that allows the nascent vegetative cell to be released from the coat/exosporium. The relatively high colony forming ability of the *gerK* spores is not due to *gerK* reversion because PCR did not detect the wild-type *gerKA-gerKC* operon in colonies obtained from *gerK* spores. While *gerK* spores had an ~ 50-fold lower colony forming ability than wild-type spores on BHI medium, this is much less of a decrease than observed with *B. subtilis* spores lacking all functional germinant receptors, in which colony forming ability was reduced to less than 0.1% of wild-type spores (145). However, the colony forming ability of *C. perfringens gerK* spores was significantly lower than that obtained with single *B. subtilis gerA*, *gerB* or *gerK* spores (145). The relatively high level of germination of *gerK C. perfringens* spores may be due to: i) contributions of remaining germinant receptor proteins such as GerAA and GerKB, even though no obvious "C" protein homologue remains; ii) the presence of germinant receptor proteins with significantly different sequences than those of the GerA family; and iii) stochastic activation of germination components downstream of the nutrient germinant receptors, such as SpoVA proteins that may comprise a channel involved in the DPA release (244) or an SCLE (207). Analysis of a strain with mutations not only in

gerKA-gerKC, but also in *gerAA* and *gerKB* may help in deciding between these alternatives.

In addition to nutrients, many non-nutrients also trigger spore germination (179, 201). We obtained several results with non-nutrient germination of *gerK C. perfringens* spores that were in contrast to results with *B. subtilis* spores that lack all nutrient germinant receptors (145, 146). First, *C. perfringens gerK* spores germinated extremely poorly with exogenous Ca-DPA, which in *B. subtilis* spores acts to promote cortex hydrolysis by activation of SCLEs (34), suggesting that products of the *gerK* operon are involved in Ca-DPA germination of *C. perfringens* spores, However, since the predicted amino acid sequences of GerKA and GerKC suggest that they are inner membrane proteins (in agreement with other GerA-family proteins), it is unlikely, although not impossible that they physically interact with the *C. perfringens* SCLEs, SleC and SleM, that are located within and at the outer boundary of the cortex (123, 212). Two possibilities can be envisioned: i) whether or not the cortex is degraded by SCLEs that are activated by exogenous Ca-DPA, the GerKA and GerKC proteins are essential for the opening of an inner membrane Ca-DPA channel perhaps composed of SpoVA proteins as is thought to be the case in *B. subtilis* spores (244); or (ii) there is indeed some physical interaction, either direct or indirect, between *gerK*-encoded proteins and SCLEs, and this is required for efficient SCLE activation. Genes encoding SCLEs as well as SpoVA proteins are indeed present in the *C. perfringens* genome (130), and studies examining the roles of these proteins in *C. perfringens* spore germination seem likely to be rewarding. Second, *C. perfringens gerK* spores released DPA at a significantly slower rate than wild-type spores with dodecylamine,

again in contrast to results with *B. subtilis* spores lacking all nutrient germinant receptors (201). These findings indicate that the *gerK*-encoded proteins are also involved in Ca-DPA-release triggered by dodecylamine: i) perhaps by directly interacting with and opening some Ca-DPA channel composed of SpoVA proteins; or ii) indirectly by interacting with GerKA and/or GerKC and activating these proteins (perhaps together with GerKB) which in turn results in Ca-DPA release which then activates downstream germination events. Again, analysis of *C. perfringens* spores with mutations in genes encoding all germinant receptor proteins, as well as SpoVA proteins and SCLEs should allow decisions between these alternative explanations.

In summary, the work reported in this communication allows us to propose a tentative working model explaining the effects of nutrient and non-nutrient germinants on *C. perfringens* spore germination (Fig. 2.11): i) some germinants (i.e. L-asparagine and KCl) bind to germinant receptors promoting the release of Ca-DPA, possibly through a channel composed at least in part of SpoVA proteins; ii) exogenous Ca-DPA requires the presence of GerKA and GerKC proteins for activation of SCLEs, which in turn degrade the spore cortex allowing completion of spore germination; and (iii) dodecylamine germination also requires the presence of the GerKA and GerKC proteins for proper Ca-DPA release through an inner membrane channel, and the released Ca-DPA would activate SCLEs, allowing cortex hydrolysis and again the completion of germination. Ongoing work is oriented towards understanding the important interactions between *gerK*-encoded proteins, SpoVA proteins and SCLEs and the role(s) these various components play in the germination of spores of

pathogenic *C. perfringens*. This understanding may well have applied implications in the areas of food safety and food preservation.

Fig 2.1: Germination of *C. perfringens* spores

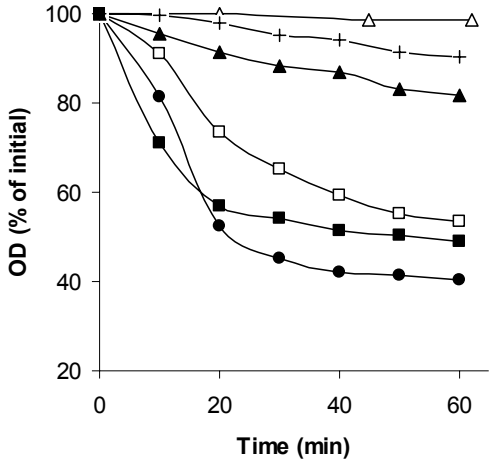


Fig 2.1 Germination of *C. perfringens* spores with various germinants. Spore of strain SM101 (wild-type) were heat activated and germinated at 30°C in 25 mM sodium phosphate buffer (pH 7.0) with no germinant (△), or with 100 mM: L-alanine (+); L-asparagine (▲);KCl (□); AK (■); AGFK (●), and the OD_{600} was measured as described in Materials and Methods.

Fig 2.2: KCl concentration dependence of *C. perfringens* spore germination

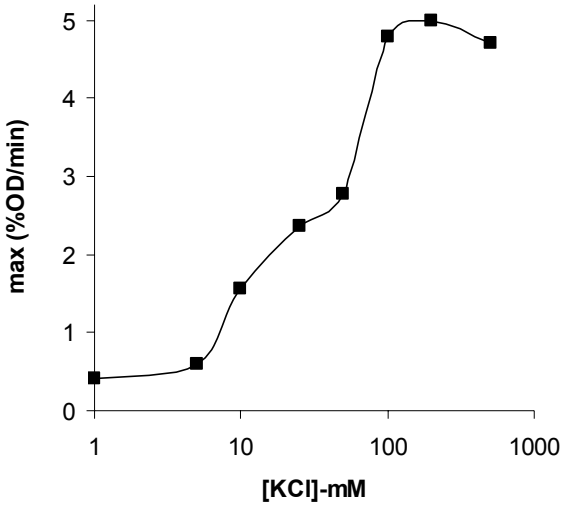


Fig. 2.2. KCl concentration dependence of *C. perfringens* spore germination. Heat activated SM101 spores (wild-type) were germinated with various KCl concentrations. The maximum rate of germination was calculated as described in Materials and Methods.

Fig 2.3: Effect of temperature and pH on *C. perfringens* spore germination

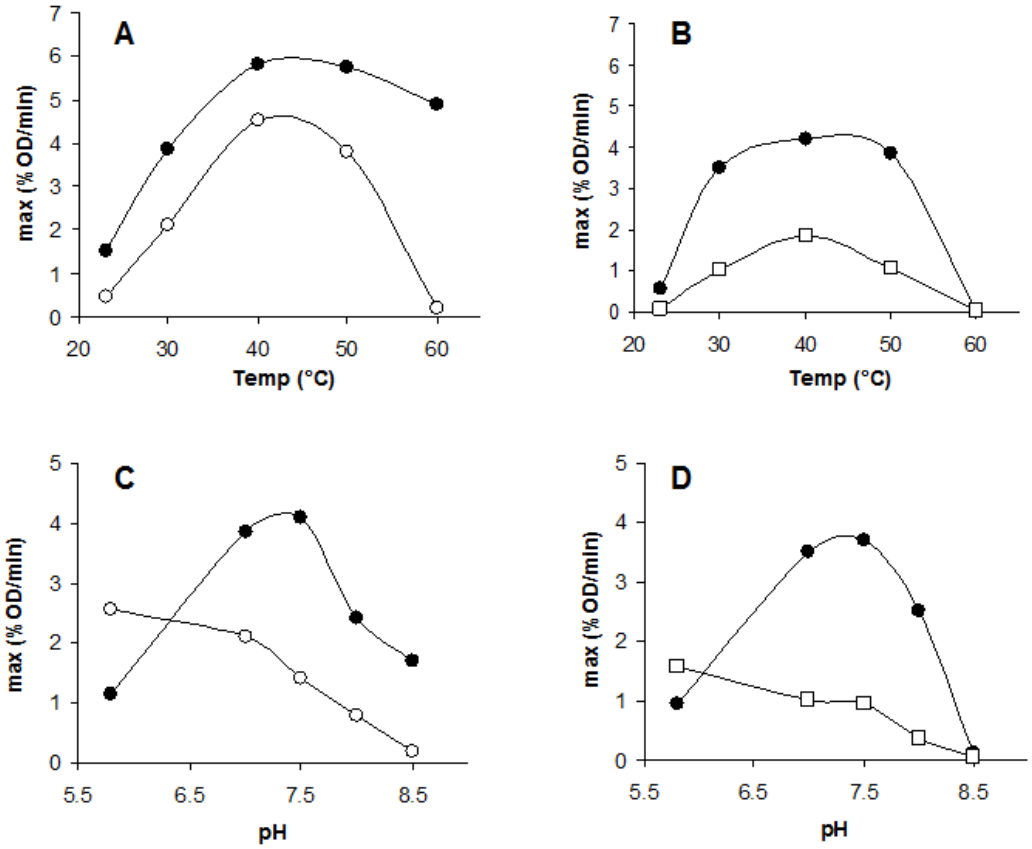
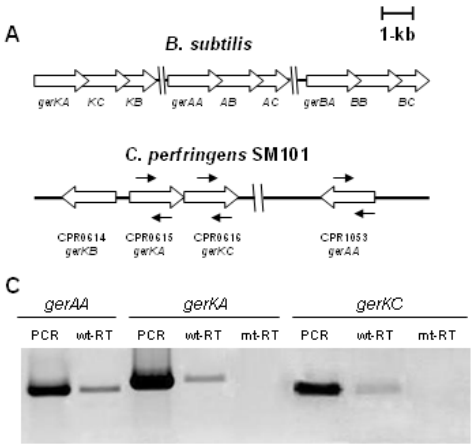


Fig. 2.3A-D. Effect of temperature (A and B) and pH (C and D) on germination of *C. perfringens* spores. Heat activated spores of strains SM101 (A, C) and NB16 (B, D) were germinated with: 100 mM AK (●), 100 mM KCl (○), or 100 mM L-alanine (□). The maximum rate of germination was calculated as described in Materials and Methods.

Fig 2.4: Analysis of nutrient receptors in *C. perfringens*



B

	Amino acid sequence similarity (%) to			
	CPR0614	CPR0615	CPR0616	CPR1053
B. subtilis				
gerAA		54		53
gerAB	56			
gerAC			ND	
gerBA		55		51
gerBB	ND[a]			
gerBC			39	
gerKA		56		51
gerKB	55			
gerKC			41	

[a]ND indicates no homology detected.

Fig. 2.4A-C. Analysis of genes encoding nutrient germinant receptors in *C. perfringens*. (A) Comparison of genes encoding nutrient germinant receptor proteins in *B. subtilis* and *C. perfringens*. Data were obtained from the Entrez Genome website (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi?view=1) (B) Percent amino acid sequence similarity between nutrient germinant receptor protein homologues from *B. subtilis* and *C. perfringens*. (C) RT-PCR analysis of *C. perfringens* genes encoding germinant receptor homologues. RNA from sporulating cells of strains SM101 (wild-type) and DPS101 (*gerK*) were subjected to RT-PCR analysis using *gerKA*-, *gerKC*- and *gerAA*-specific internal primers. Lanes labeled wt-RT and mt-RT contain RT-PCR products obtained from RNA from SM101 and DPS101 strains, respectively. Lanes labeled PCR contain PCR products obtained from SM101 DNA using *gerAA*-, *gerKA*- and *gerKC*-specific internal primers. The PCR and RT-PCR amplified products were analyzed by agarose (1%) gel electrophoresis and photographed under UV light. The presence of RT-PCR product can not be explained by the amplification from contaminated DNA because no PCR product was obtained from RNA in the absence of RT (data not shown).

Fig 2.5: Germination of *C. perfringens* wild-type and *gerK* spores

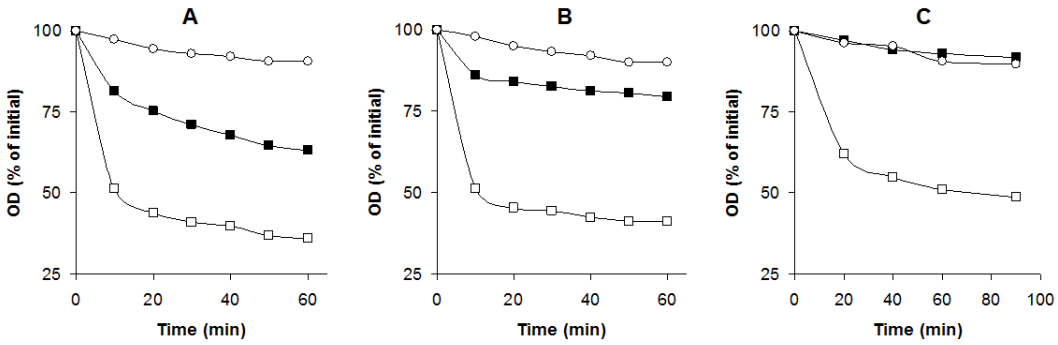


Fig. 2.5A-C. Germination of *C. perfringens* wild-type and *gerK* spores with various germinants. Heat activated spores of strains SM101 (wild-type) (□) and DPS101 (*gerK*) (■) were germinated with: A) 100 mM KCl; B) 100 mM L-asparagine plus 100 mM KCl; and C) 100 mM L-asparagine as described in Materials and Methods. The control germination (○) corresponds to heat activated spores incubated in 25 mM sodium phosphate buffer (pH 7.0); no difference between SM101 and DPS101 spores was seen.

Fig 2.6: Germination of *C. perfringens* wild-type and *gerAA* spores

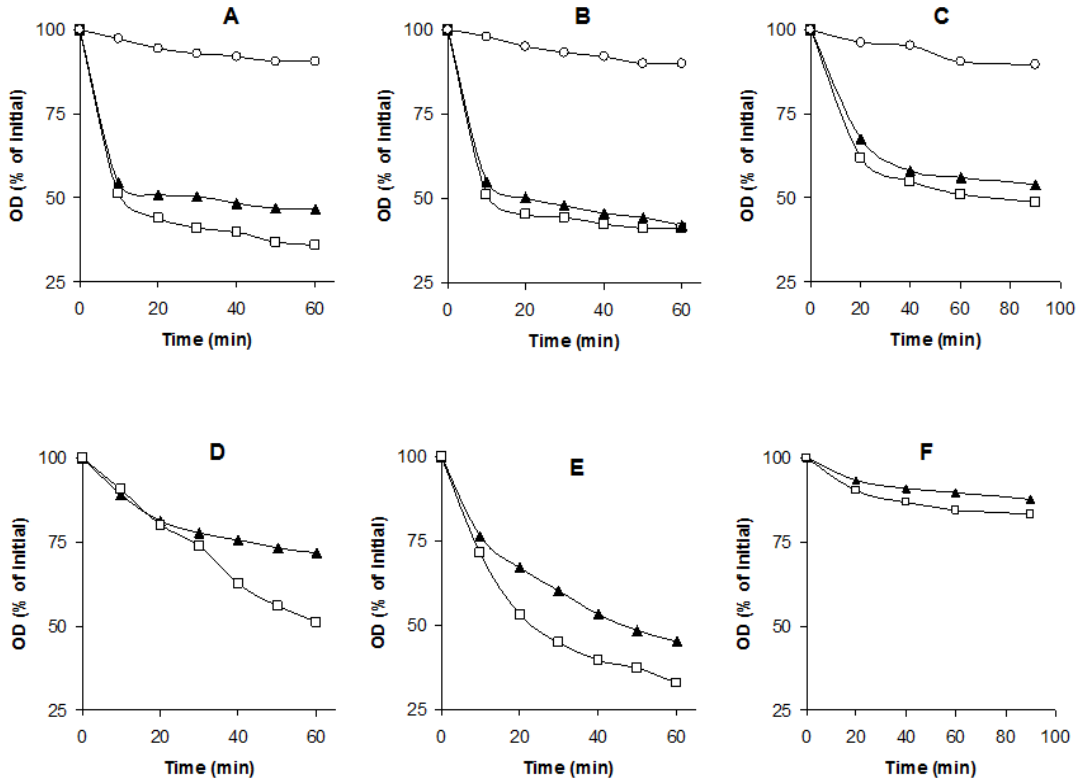


Fig. 2.6A-F. Germination of *C. perfringens* wild-type and *gerAA* spores with various germinants. Heat activated spores of strains SM101 (wild-type) (□) and DPS103 (*gerAA*) (▲) were germinated with: A) 100 mM KCl ; B) 100 mM L-asparagine and 100 mM KCl ; C) 100 mM L-asparagine; D) 10 mM KCl; E) 10 mM L-asparagine and 10 mM KCl; and F) 10 mM L-asparagine as described in Materials and Methods. The control germination (○) is heat activated spores incubated in 25 mM sodium phosphate buffer (pH 7.0), and no difference between spores of SM101 and DPS103 was observed.

Fig 2.7: DPA-release during germination of *C. perfringens* spores

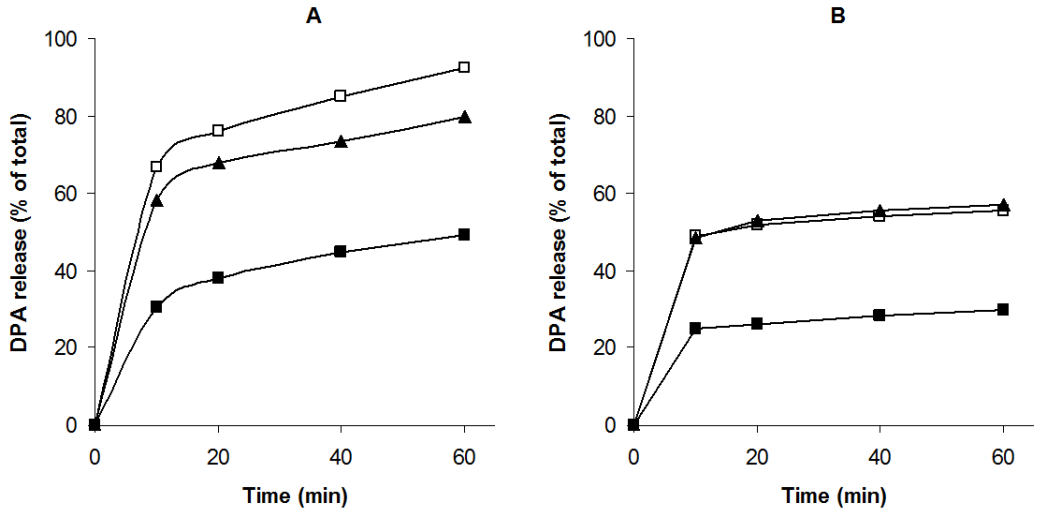


Fig. 2.7A,B. DPA-release during germination of *C. perfringens* spores. Heat activated spores of strains SM101 (wild-type) (□), DPS101 (*gerK*) (■) and DPS103 (*gerAA*) (▲) were germinated in 25 mM sodium phosphate buffer (pH 7.0) with: A) 5 mM KCl; or B) 100 mM L-asparagine. At various times, DPA release was measured as described in Materials and Methods.

Fig 2.8: Germination of *C. perfringens* spores in BHI broth

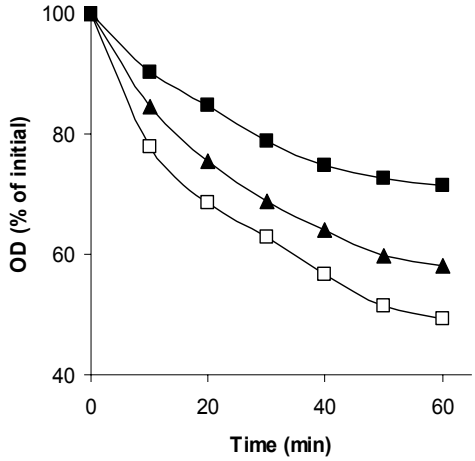


Fig. 2.8. Germination of spores of *C. perfringens* strains in BHI broth. Heat activated spores of strains SM101 (wild-type) (□), DPS101 (*gerK*) (■), and DPS103 (*gerAA*) (▲) were incubated at 40ºC with BHI broth and the OD_{600} was measured as described in Materials and Methods.

Fig 2.9: Ca-DPA germination of *C. perfringens* spores

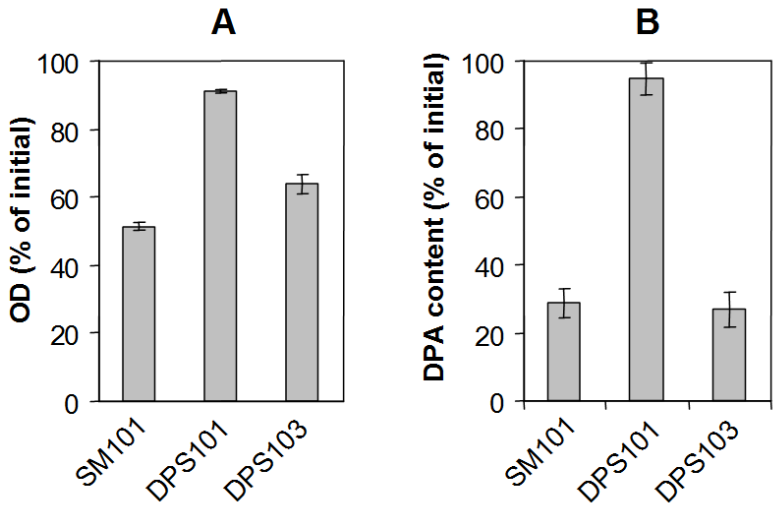


Fig. 2.9. Ca-DPA germination of spores of *C. perfringens* strains. Heat activated spores of strains SM101 (wild-type), DPS101 (*gerK*) and DPS103 (*gerAA*) were germinated with 50 mM Ca-DPA (pH 8.0) at 40°C for 60 min, and (A) changes in the OD_{600} of the culture and (B) DPA remaining in the spores were measured as described in Materials and Methods. The values shown are averages of two experiments with two independent spore preparations. Error bars show one standard deviation from the mean.

Fig 2.10: Dodecylamine germination of *C. perfringens* spores

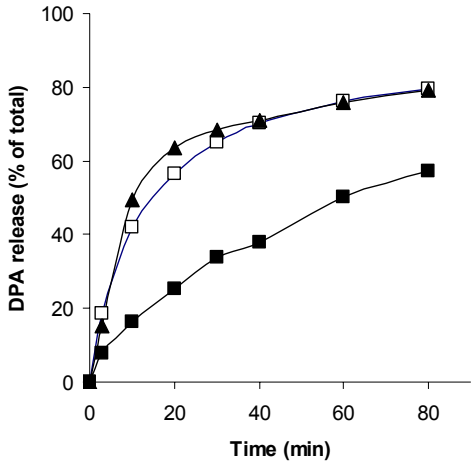


Fig. 2.10. Dodecylamine germination of spores of *C. perfringens* strains. Spores of strains SM101 (wild-type) (□), DPS101 (*gerK*) (■), and DPS103 (*gerAA*) (▲) were incubated at 60ºC with 1 mM dodecylamine (pH 7.4), and DPA release was measured as described in Materials and Methods.

Fig 2.11: Putative model for *C. perfringens* spore germination

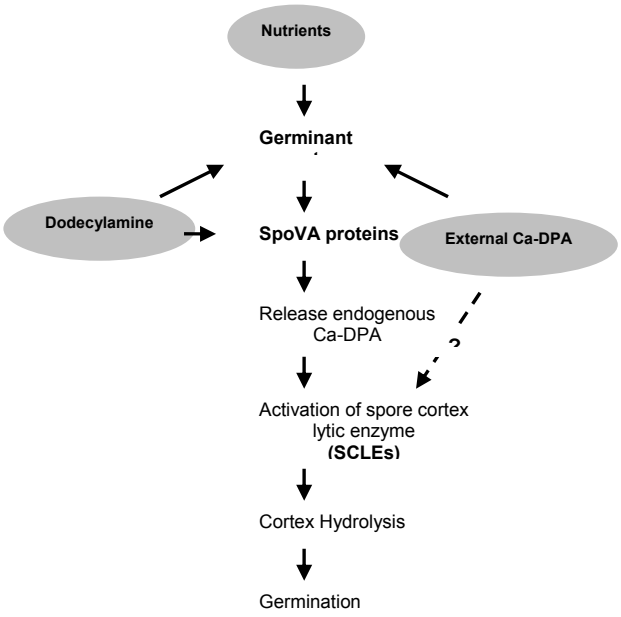


Fig. 2.11. Putative model for nutrient and non-nutrient germination of *C. perfringens* spores. Nutrients activate germinant receptors resulting in Ca-DPA release from the core, which triggers activation of SCLEs. External Ca-DPA induces germination through a mechanism that requires the GerK receptor to fully activate downstream germination events. Dodecylamine triggers DPA release by ultimately opening a DPA channel (composed of SpoVA proteins, by analogy with *B. subtilis* spores) in the spore's inner membrane. Since dodecylamine germination is unaffected by a *gerAA* mutation but is reduced by loss of GerKA and GerKC, dodecylamine presumably acts on both the GerK receptor to indirectly open a DPA channel, and directly on the DPA channel itself. SCLEs are then activated by the Ca-DPA release triggered by dodecylamine and SCLEs then promote cortex hydrolysis and completion of spore germination.

Table 2.1. Bacterial strains and plasmids used

Strain or Plasmid	Relevant characteristic(s)	Reference or source
B. subtilis		
JH642	*trpC2 pheAI*	(64)
C. perfringens		
SM101	Electroporatable derivative of a food poisoning type A isolate NCTC8798; carries a chromosomal *cpe* gene	(256)
DPS101	*gerKA::catP*	This study
DPS102	*gerAA::Intron*	This study
DPS103	Δ*gerAA::catP*	This study
NCTC8239	Food poisoning type A isolate; carries chromosomal *cpe* gene	(193)
E13	Food poisoning type A isolate; carries chromosomal *cpe* gene	(193)
FD1041	Food poisoning type A isolate; carries chromosomal *cpe* gene	(193)
NB16	Non-food-borne GI disease isolate; carries *cpe* gene on plasmid	(193)
B40	Non-food-borne GI disease isolate; carries *cpe* gene on plasmid	(193)
F5603	Non-food-borne GI disease isolate; carries *cpe* gene on plasmid	(193)
Plasmids		
pJIR418	*C. perfringens/E. coli* shuttle vector; carrying chloramphenicol (Cm^r) and erythromycin (Em^r) resistance	(11)
pMRS99	650-bp PCR fragment containing *catP* in pCR®-XL-TOPO®	M.R. Sarker
pMRS104	No origin of replication for *C. perfringens*; Em^r.	(67)
pJIR750ai	*C. perfringens/E.coli* shuttle vector containing an *L1.LtrB* intron re-targeted to *plc* gene	(25)
pDP9	~ 3.1-kb KpnI-SalI PCR fragment carrying *gerK* operon in pCR®-XL-TOPO®	This study
pDP10	~ 3.1-kb KpnI-XhoI fragment from pDP9 in pMRS104	This study
pDP11	~ 1.3-kb NaeI-SmaI *catP* fragment from pJIR418, in the SpeI site in the *gerK*A ORF in pDP10	This study
pDP12	~ 350-bp PCR fragment from pJIR750ai, containing target sites for intron to disrupt *gerAA* in pCR®-XL-TOPO®	This study
pDP13	pJIR750ai with IBS, EBS1d & EBS2 retargeted to insert in *gerAA*	This study
pDP18	~ 1.8-kb PCR fragment containing 1670-bp upstream of and 186-bp of the N-terminal coding region of *gerAA* in pCR®-XL- TOPO®	This study
pDP19	~ 2.0-kb PCR fragment containing 225-bp of the C-terminal coding region and 1769-bp downstream of *gerAA* in pCR®-XL-TOPO®	This study
pDP20	1827-bp KpnI-SpeI fragment from pDP18 in pMRS99	This study
pDP21	~ 2.0-kb PstI-XhoI fragment from pDP19 in pDP20	This study
pDP22	~ 4.5-kb KpnI-XhoI fragment from pDP21 in pMRS104	This study

Table 2.2. Primers used in this study

Primer name	Primer sequence[a]	Gene	Position[b]	Use[c]
CPP 205	5' GACAGACAGCATTAATTTTAGAAG 3'	*gerAA*	+304 to +328	PCR,
CPP 206	5' CAAGTATTAATCCTCCAATAACAG 3'	*gerAA*	+1102 to +1126	PCR
CPP 207	5' AGTGAGTACATAGTAAAACCATTGA 3'	*gerKA*	+133 to +157	PCR, RT
CPP 208	5' ATCATTATTATCACCTCTGCTACTAT 3'	*gerKA*	+980 to +1006	PCR, RT
CPP 211	5' CTTTAATGGGAATTATAGCA 3'	*gerAA*	-264 to -244	PCR
CPP 212	5' CAACAAATTTTGATTATTCTTC 3'	*gerAA*	+1430 to +1452	PCR
CPP213	5' GGGTACCCTTAAATATAGGAAGAAGAAGTGT 3'	*gerKA*	-619 to -595	MP
CPP214	5' GCGTCGACAACTTATTTTAAAGTGTATTTCCT 3'	*gerKA*	+2528 to +2544	MP
CPP235	5'AAAAAAGCTTATAATTATCCTTAGCCACCATGTATGTGCG CCCAGATAGGGTG 3'	*gerAA*	IBS 123/124	MP
CPP236	5'CAGATTGTACAAATGTGGTGATAACAGATAAGTCATGTA TTATAACTTACCTTTCTTTGT 3'	*gerAA*	EBS1d 123/124	MP
CPP237	TGAACGCAAGTTTCTAATTTCGATTGTGGCTCGATA GAGGAAAGTGTCT	*gerAA*	EBS2 123/124	MP
CPP257	GGGTACCCAACTTATGTTATTCCAGCAG	*gerAA*	-1650 to -1670	MP
CPP258	GACTAGTCTAAGGAAAAGAAGTCACTCA	*gerAA*	+166 to +186	MP
CPP259	GCTGCAGCGAACTTAGCTATGCCTTAAA	*gerAA*	+1195 to +1226	MP
CPP260	CCTCGAGGTGAATCAATGCTTTTAGAAT	*gerAA*	+3188 to +3208	MP
CPP283	GTTCTAAGTATTGTTTTATTACTGCC	gerKC	+927 to +953	RT
CPP284	GAAAATGAAGTGGGAAATATAGAC	*gerKC*	+114 to +138	RT

[a] Restriction sites are underlined.
[b] The nucleotide numbering begins at the first base of the translation codon of the relevant gene.
[c] PCR, polymerase chain reaction; MP, construction of mutator plasmid; RT, RT-PCR.

Table 2.3. Germination of *C. perfringens* spores by various compounds

Germinant (s)[a]	Mean % decrease in OD_{600} ± SD[b] in 60 min at 30°C with:	
	JH642	SM101
Control	0 ± 0.1	1 ± 0.2
L-Ala	41 ± 0.5	11 ± 1.6
L-Val	39 ± 0.1	6 ± 1.1
L-Asn	2 ± 0.5	18 ± 0.5
L-His	0 ± 0.5	4 ± 0.7
L-Lactate (50 mM)	1 ± 0.2	5 ± 2.2
Inosine (5 mM)	0 ± 0.3	6 ± 0.3
AGFK[c]	38 ± 0.9	60 ± 0.8
GFK	2 ± 0.5	55 ± 3.9
AGF	13 ± 1.8	16 ± 5.5
AK	1 ± 0.1	51 ± 0.9
GF	20 ± 0.2	6 ± 1.3
FK	0 ± 0.5	50 ± 1.8
GK	1 ± 0.7	50 ± 2.4
KCl	1 ± 0.4	47 ± 1.8
NaCl	1 ± 0.5	7 ± 1.9
KH_2PO_4 (pH 7.0)	1 ± 0.2	39 ± 1.3
KI	ND[d]	41 ± 1.4
KBr	ND	57 ± 1.4

[a]All compounds, except L-lactate and inosine, were used at 100 mM in 25 mM sodium phosphate (pH 7.0).
[b]Values are average of duplicate experiments with two different spore preparations; SD, standard deviation.
[c]AGFK is a mixture of L-asparagine, D-glucose, D-fructose and potassium ions.
[d]Not determined.

Table 2.4. Germination of spores of *C. perfringens* isolates carrying *cpe* on the chromosome (C-*cpe*) or a plasmid (P-*cpe*)

Germinants[a]	Mean % decrease in OD_{600} ± SD[b] in 60 min at 30°C					
	C-*cpe*			P-*cpe*		
	E13	8239	FD1041	NB16	B40	F5603
None	9 ± 0.1	5 ± 0.5	5 ± 4.4	4 ± 3.2	5 ± 0.5	2 ± 0.3
L-Ala	13 ± 0.6	8 ± 2.0	8 ± 3.1	42 ± 0.1	29 ± 2.1	49 ± 1.6
L-Val	7 ± 3.2	16 ± 6.6	7 ± 2.6	46 ± 3.2	49 ± 3.4	58 ± 3.6
L-Asn	17 ± 0.1	19 ± 0.4	20 ± 0.2	7 ± 1.1	3 ± 0.6	8 ± 2.5
AK	58 ± 0.1	52 ± 0.1	50 ± 0.4	50 ± 0.8	54 ± 0.5	65 ± 0.8
KCl	53 ± 0.4	56 ± 3.8	49 ± 1.1	2 ± 1.3	4 ± 0.5	8 ± 2.1

[a]All compounds were used at 100 mM in 25 mM sodium phosphate (pH 7.0).

[b]Values are average of duplicate experiments from two different spore preparations.; SD, standard deviation.

Chapter 3

Role of GerKB in germination and outgrowth of *Clostridium perfringens* spores.

Daniel Paredes-Sabja, Peter Setlow and Mahfuzur R. Sarker

Published as Short Note in:

Applied and Environmental Microbiology 2009 **75** (11): 3813-3817

3.1 Abstract

Previous work indicated that *C. perfringens gerKA gerKC* spores germinate significantly suggesting that *gerKB* also has a role in *C. perfringens* spore germination. We now find that i) *gerKB* was expressed only during sporulation, likely in the forespore; ii) *gerKB* spores germinated like wild-type spores with nonutrient germinants and high concentrations of nutrients, but more slowly with low nutrient concentrations; and iii) *gerKB* spores had lower colony forming efficiency and slower outgrowth than wild-type spores. These results suggest that GerKB plays an auxiliary role in spore germination under some conditions, and is required for normal spore viability and outgrowth.

3.2 Short Note

Spores of *Bacillus* and *Clostridium* species can break dormancy upon sensing a variety of compounds (termed germinants), including amino acids, nutrient mixtures, a 1:1 chelate of Ca^{2+} and pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) and cationic surfactants such as dodecylamine (207). Nutrient germinants are sensed by their cognate receptors, located in the spore's inner membrane (69), which are composed of proteins belonging to the GerA family (125, 126). In *Bacillus subtilis*, three tricistronic operons (*gerA*, *gerB*, and *gerK*) expressed uniquely during sporulation in the developing forespore each encode the three major germinant receptors, with different receptors responding to a different spectrum of nutrient germinants (40, 127, 207). Null mutations in any cistron in a *gerA*-family operon inactivate the function of the respective receptor (125, 127). In contrast, *Clostridium*

perfringens, a gram-positive, spore-forming, anaerobic pathogenic bacterium, has no tricistronic *gerA*-like operon but only a monocistronic *gerAA* that is far from a *gerK* locus. This locus contains a bicistronic *gerKA-KC* operon and a monocistronic *gerKB* upstream of and in the opposite orientation to *gerKA-KC* (Fig. 3.1A) (154). GerAA has an auxiliary role in the germination of *C. perfringens* spores at low germinant concentration, while GerKA and/or GerKC are required for L-asparagine-germination and have partial roles in germination with KCl and a mixture of KCl and L-asparagine (AK) (154). In contrast to the situation in *B. subtilis* where germinant receptors play no role in Ca-DPA germination (145, 146), GerKA and/or GerKC is required for Ca-DPA germination (154). The partial requirement for GerKA and/or GerKC in *C. perfringens* spore germination by KCl and AK suggests that the upstream gene product, GerKB, might also have some role in KCl and AK germination of *C. perfringens* spores. Therefore, in this study we have investigated the role of GerKB in the germination and outgrowth of *C. perfringens* spores.

To determine if *gerKB* is expressed during sporulation, 485 bp upstream of the *gerKB* coding sequence, including DNA between *gerKB* and *gerKA*, was PCR-amplified with primer pair CPP389/CPP391 that had SalI and PstI cleavage sites, respectively (see Table S3.2 in the supplementalmaterial). The PCR fragment was cloned between SalI and PstI cleavage sites in plasmid pMRS127 (174) to create a *gerKB-gusA* fusion in plasmid pDP84 (see Table S3.1 in supplemental material). This plasmid was introduced into *C. perfringens* SM101 by electroporation (35) and Em^r transformants were selected. The SM101 transformant carrying plasmid pDP84 was grown in TGY vegetative growth medium (3% trypticase soy, 2% glucose, 1% yeast

extract, 0.1% L-cysteine) (91) and in Duncan-Strong (DS) (36) sporulation medium, and assayed for β-glucuronidase (GUS) activity as described previously (256). Vegetative cultures of strain SM101 carrying plasmid pMRS127 (empty vector) or pDP84 (*gerKB-gusA*) exhibited no significant GUS activity, and strain SM101 grown in DS medium also exhibited no significant GUS activity (Fig. 3.1B and data not shown). However, GUS activity was observed in sporulating cultures of SM101(pDP84) (Fig. 3.1B), indicating that a sporulation-specific promoter is located upstream of *gerKB*. The expression of the *gerKB-gusA* fusion began ~ 3 h after induction of sporulation and reached a maximum after ~ 6 h of sporulation (Fig. 3.1B). The decrease in GUS activity observed after ~ 6 h is consistent with the GerKB-GusA protein being packaged into the dormant spore where it cannot be easily assayed, and thus that *gerKB* is expressed in the forespore compartment of the sporulating cell (113). These results confirm that, as with the *gerKA-KC* operon (154), *gerKB* is also expressed only during sporulation.

To investigate the role of GerKB in *C. perfringens* spore germination, we constructed a *gerKB* mutant strain (DPS108) as described previously (153, 154, 156). A 2,203-bp DNA fragment carrying 2,080-bp upstream and 123-bp from the N-terminal coding region of *gerKB* was PCR amplified using primers CPP369 and CPP367 (see Table S3.2 in the supplementary material), which had XhoI and BamHI cleavage sites at the 5' ends of the forward and reverse primers, respectively (see Table S3.2 in the supplementary material). A 1,329-bp fragment carrying 134-bp from the C-terminal and 1,195-bp downstream of coding region of *gerKB* was PCR amplified using primers CPP371 and CPP370 (see Table S3.2 in the supplementary

material), which had BamHI and KpnI cleavage site at the 5' ends of the forward and reverse primers, respectively (Table S3.2). These PCR fragments were cloned into plasmid pCR-XL-TOPO giving plasmids pDP67 and pDP69, respectively (Table S3.1). An ~ 2.2-kb BamHI-XhoI fragment from pDP67 was cloned into pDP1 (pCR-XL-TOPO carrying internal fragment of *gerAA*) giving plasmid pDP68, and a ~ 1.4-kb KpnI-BamHI fragment from pDP69 was cloned in pDP68 giving pDP73 (see Table S3.1 in the supplementary material). The latter plasmid was digested with BamHI, the ends filled, and an ~ 1.3-kb NaeI-SmaI fragment carrying *catP* from pJIR418 (11) was inserted, giving plasmid pDP74. Finally, a ~ 4.8-kb KpnI-XhoI fragment from pDP74 (Table S3.1) was cloned between the KpnI and SalI sites of pMRS104, giving pDP75, which cannot replicate in *C. perfringens*. Plasmid pDP75 was introduced into *C. perfringens* SM101 by electroporation (35), and the *gerKB* deletion strain DPS108 was isolated as described previously (192). The presence of the *gerKB* deletion in strain DPS108 was confirmed by PCR and Southern blot analyses (data not shown). Strain DPS108 gave ~ 70% sporulating cells in DS sporulation medium, similar to results with the wild-type strain, SM101 (data not shown).

Having obtained evidence for successful construction of the *gerKB* mutant, we compared the germination of heat activated (80°C; 10 min) *gerKB* and wild-type spores as previously described (154). Both the *gerKB* and wild-type spores germinated identically and nearly completely in 60 min at 40°C in Brain Heart Infusion (BHI) broth as determined by the fall in optical density at 600 nm (OD_{600}) of germinating cultures and phase contrast microscopy (data not shown). This result suggests that GerKB plays no essential role in spore germination in rich medium. The role of

GerKB in *C. perfringens* spore germination was also assessed with individual germinants identified previously (154). Heat activated wild-type and *gerKB* spores germinated similarly with high (100 mM) concentrations of KCl, L-asparagine, and AK, all in 25 mM sodium phosphate (pH 7.0), and in 50 mM Ca-DPA adjusted to pH 8.0 with Tris base (Fig. 3.2A-D). These results were also confirmed by phase-contrast microscopy (data not shown). However, with lower (10-20 mM) concentrations of KCl, L-asparagine and AK, *gerKB* spore germination was very slightly (Fig. 3.2A) to significantly (Fig. 3.2B,C) slower than that of wild-type spores. These results suggest that while GerKB is not essential for germination with high concentrations of KCl, L-asparagine or AK, it plays a significant role in germination with low L-asparagine and AK concentrations, and further that GerKB is not required for Ca-DPA germination. This latter finding is similar to the situation in *B subtilis* spores where germinant receptors play no role in Ca-DPA germination (201, 207). However, in *C. perfringens* spores GerKA and/or GerKC do play a significant role in Ca-DPA germination (154).

Bacterial spores can also germinate with dodecylamine, a cationic surfactant (201). In *B. subtilis* spores, dodecylamine induces germination most likely by opening channels composed, at least in part, of SpoVA proteins (244), allowing release of the spore's Ca-DPA (201). Spores of *B. subtilis* lacking all three functional germinant receptors release DPA as do wild-type spores upon incubation with dodecylamine (201), while *C. perfringens* spores lacking GerKA-KC incubated with dodecylamine release DPA slower than wild-type spores (154). However, when *C. perfringens gerKB* spores at an OD_{600} of 1.5 were incubated with 1 mM dodecylamine in Tris-HCl (pH 7.4) at 60ºC (21, 154), *gerKB* spores released their DPA slightly faster than wild-

type spores (Fig. 3.3), when DPA release was measured as described (154). These results suggest that GerKB has no role on dodecylamine germination.

Previous work (154) found that *C. perfringens* spores lacking GerKA-KC had lower viability than wild-type spores on rich medium plates, and it was thus of interest to determine *gerKB* spore viability, which was measured as previously described (153, 154). Strikingly, the colony forming ability of *gerKB* spores was ~ 7-fold lower ($P < 0.01$) than that of wild-type spores after 24 h on BHI plates (Table 3.1), and no additional colonies appeared when plates were incubated for up to 3 days (data not shown). The colony-forming ability of spores lacking GerKA and GerKC determined in parallel was ~ 12-fold lower than that of wild-type spores (Table 3.1). Phase-contrast microscopy of *C. perfringens* spores incubated in BHI broth for 24 h under aerobic conditions to prevent vegetative cell growth indicated that > 90% of wild-type spores had not only germinated but had also released the nascent vegetative cell, while > 85% of *gerKA gerKC* and *gerKB* spores remained as only phase dark germinated spores with no evidence of nascent cell release (data not shown), as found previously with *gerKA gerKC* spores (154). The fact that >85% of *gerKB* spores germinated in BHI medium in 24 h, but most of these germinated spores did not progress further in development strongly suggests that GerKB is needed for normal spore outgrowth (and see below) as well as for normal spore germination.

To evaluate whether preincubation with Ca-DPA could rescue apparently inviable *gerKB* spores via activation of GerKA and/or GerKC (154), *C. perfringens* spores of the wild-type and various *gerK* strains were heat activated, cooled and incubated in 50 mM Ca-DPA (made to pH 8.0 with Tris base) for 20 min at 40ºC,

plated on BHI medium agar plates with or without lysozyme, and counted after anaerobic incubation at 37ºC for 24 h. The preincubation of mutant spores with Ca-DPA gave no significant increase in colony forming efficiency (Table 3.1). To test whether spores with a lesion in either *gerKB* or the *gerKA-KC* operon could be recovered by digestion of the spore's peptidoglycan cortex, spores of various strains were decoated in 1 ml of 0.1 M sodium borate (pH 10)-2% 2-mercaptoethanol for 60 min at 37ºC, washed at least nine times with sterile distilled water (153), and plated on BHI plates containing lysozyme (1 μg/ml). While the viability of *gerKA gerKC* spores remained ~ 12-fold lower than that of wild-type spores, *gerKB* spores' viability increased slightly, but was still ~ 5-fold lower than that of wild-type spores (Table 3.1).

The results given above suggest that GerKB is essential not only for normal spore germination, but also for normal spore viability and outgrowth. To further examine if GerKB is involved in spore outgrowth, heat activated spores of DPS108 (*gerKB*) and SM101 (wild-type) strains were inoculated into 10 ml TGY broth to a final OD_{600} of 0.110 and 0.015 (one-seventh that of the *gerKB* spores), respectively, incubated anaerobically at 37ºC, and at various times the OD_{600} was measured. Although the initial wild-type spores were diluted to one-seventh the OD_{600} of *gerKB* spores to correct for the *gerKB* spores' lower viability, the wild-type spores' outgrowth was much faster than *gerKB* spores (Fig. 3.4), suggesting that GerKB is not only essential for normal spore germination and viability, but also for normal spore outgrowth, since the growth rates of wild-type and *gerKB* cells are identical (data not

shown). The difference in rates of outgrowth of wild-type and *gerKB* spores was even greater when the initial spores were at the same starting OD_{600} (data not shown).

The lack of effect of lysozyme on the viability of decoated *gerKB* spores (or *gerKA gerKC*) spores indicates that the defect in these spores is not the inability to degrade cortex peptidoglycan, since exogenous lysozyme restores viability to decoated *C. perfringens* spores that lack the essential cortex-lytic enzyme SleC (156). Indeed, *gerKB* spores degraded cortex peptidoglycan normally during spore germination with KCl (data not shown). The loss of GerKB (and perhaps GerKA and/or GerKC (154)) also slowed spore outgrowth noticeably. Some of this effect may be due to the low viability of the mutant spores, as the viability defect in these spores could manifest itself in spore outgrowth (and see below). However, when differences in spore viability were corrected for, *gerKB* spores still went through spore outgrowth more slowly than wild-type spores. The latter two findings are again different than the situation with *B. subtilis* spores, as while *B. subtilis* spores lacking known germinant receptors give low apparent viability on nutrient plates, the viability of these spores can be restored to almost wild-type spore levels by pre-incubation with Ca-DPA (145, 146).

The more novel conclusions from this work concern the role of GerKB in spore germination. GerKB is the only evident *C. perfringens* homolog of B proteins encoded by *gerA* operon homologs, and in *B. subtilis*, loss of the B protein from a GerA-type receptor eliminates the function of that receptor (207). One would therefore predict, based on the *B. subtilis* model, that loss of GerKB would largely eliminate *C. perfringens* spore germination. However, this was certainly not the case. There appear

to be a number of possible explanations for the marked difference in the germination behavior of spores of these two genera. First, the various GerA family proteins in *C. perfringens* spores may be able to function independently of each other, as opposed to the situation in *B. subtilis* spores. Second, perhaps there are additional *gerA*-family genes in the *C. perfringens* genome that encode proteins sufficiently different in sequence such that they are not detected by sequence alignment programs, and this is certainly possible. However, use of the *C. perfringens gerA*-family genes as query sequences also does not detect additional *gerA* family members (data not shown). Third, perhaps there is a radically different mechanism than activation of germinant receptors for triggering germination of *C. perfringens* spores. There is of course no evidence for this. However, recent work has identified a novel mechanism for triggering germination of spores of *Bacillus* species that does not involve the germinant receptors (211), and perhaps *C. perfringens* has some novel germination mechanism as well. At present we cannot decide definitively between these possible explanations. However, deletion of all known *gerA*-family genes from *C. perfringens* and examination of the germination of these multiply deficient spores would certainly help in deciding between these possibilities.

Fig 3.1: Arrangement and expression of *gerKB* in *C. perfringens* SM101.

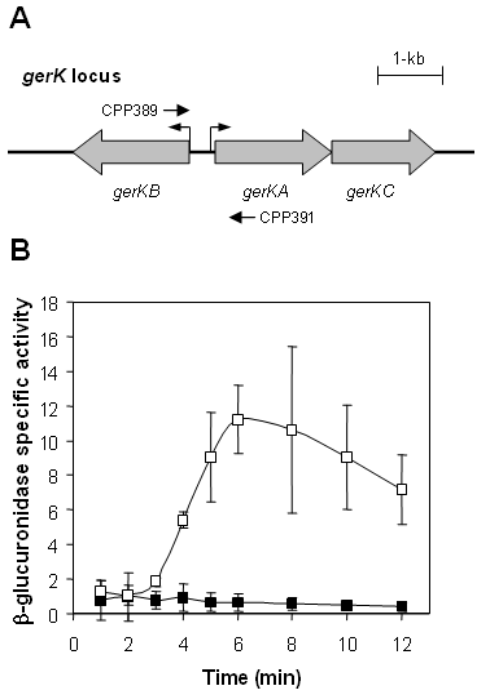


Fig. 3.1A-B. Arrangement and expression of *gerKB* in *C. perfringens* SM101. A) The arrangement of *gerK* locus in *C. perfringens* SM101 is shown and the locations of the primers used to amplify the upstream regions of the *gerKB* gene, and the putative promoters of *gerKB* and *gerKA* are indicated. The *gerKB* promoter was predicted to be within the intergenic regions between *gerKB* and the *gerK* operon. B) GUS specific activities from the *gerKB-gusA* fusion in strain SM101(pDP84) grown in TGY vegetative (filled squares) and DS sporulation (open squares) media were determined as described in the text. Data represent averages of three independent experiments with the error bars denoting standard deviations, and time zero denotes the time of inoculation of cells into either TGY or DS medium.

Fig 3.2: Germination of spores of *C. perfringens* strains with various germinants

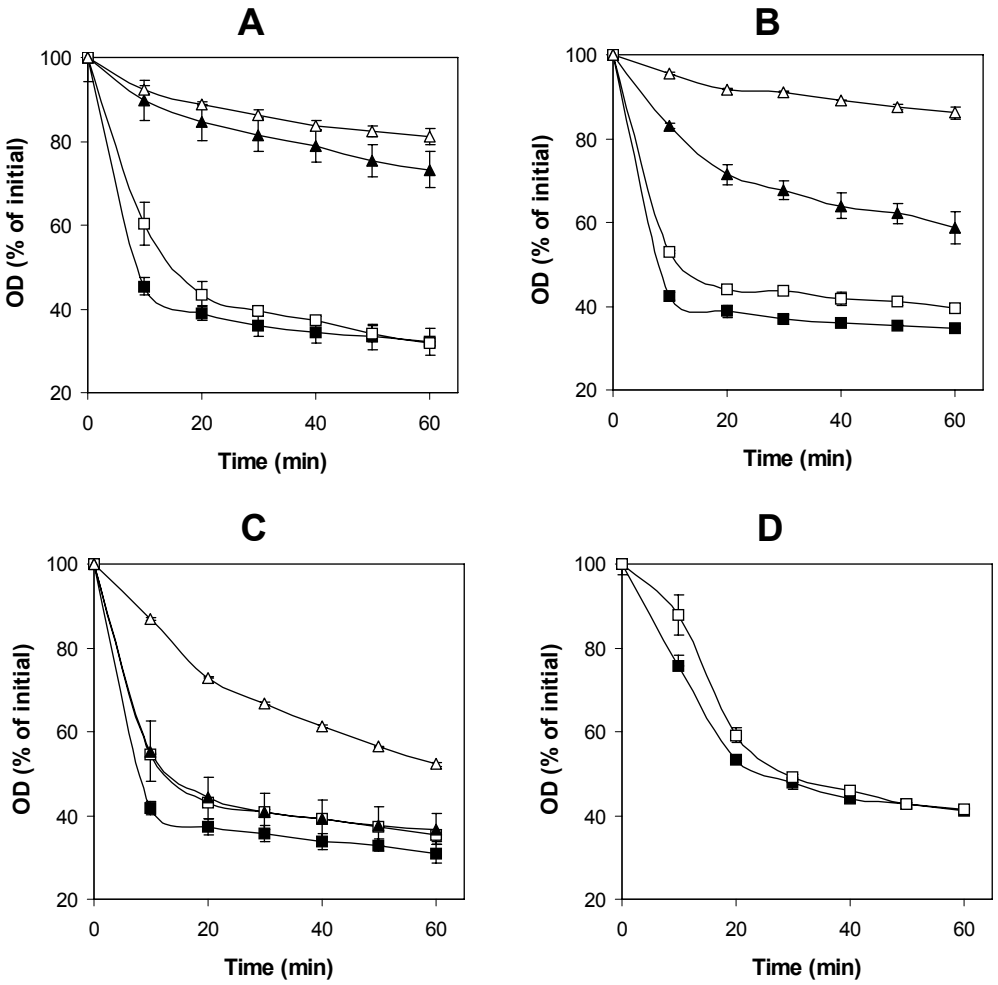


Fig. 3.2A-D. Germination of spores of *C. perfringens* strains with various germinants. Heat activated spores of strains SM101 (wild-type) (filled symbols) and DPS108 (*gerKB*) (open symbols) were incubated at an OD_{600} of 1 at 40°C with high (squares) and low (triangles) germinant concentrations of: A) 100 and 10 mM KCl; B) 100 and 20 mM L-asparagine; C) 100 and 10 mM AK; and D) 50 mM Ca-DPA as described in the text, and at various times the OD_{600} was measured. No significant germination was observed when heat activated spores of SM101 and DPS108 were incubated for 60 min at 40°C in 25 mM sodium phosphate buffer (pH 7.0) (data not shown). The data shown are averages of duplicate determinations with two different spore preparations, and error bars denote standard deviations.

Fig 3.3: Germination of spores of *C. perfringens* strains with dodecylamine.

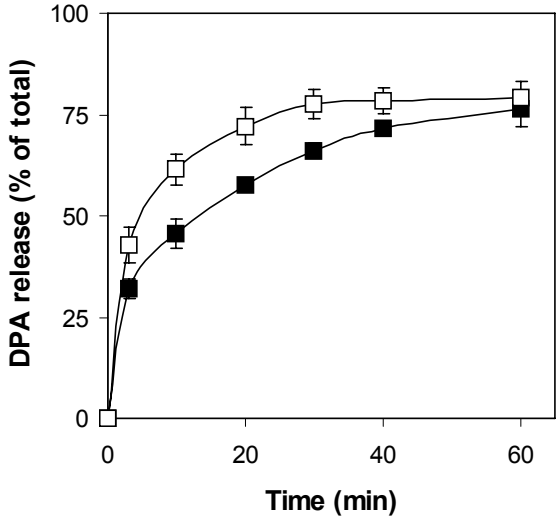


Fig. 3.3. Germination of spores of *C. perfringens* strains with dodecylamine. Spores of strains SM101 (wild-type) (filled squares) and DPS108 (*gerKB*) (open squares) were germinated with dodecylamine, and germination was monitored by measuring DPA release as described in the text. There was no significant DPA release in 60 min by spores incubated similarly but without dodecylamine (data not shown). Error bars denote standard deviations.

Fig 3.4: Outgrowth of spores of *C. perfringens* strains

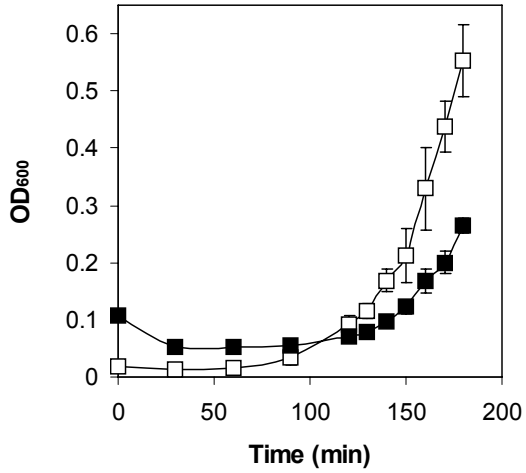


Fig. 3.4. Outgrowth of spores of *C. perfringens* strains. Heat activated spores of strains DPS108 (*gerKB*))Filled squares) and SM101 (wild-type) (open squares) were incubated anaerobically in TGY broth at an initial OD_{600} of 0.110 and 0.015, respectively, and the OD_{600} of the cultures was measured. Error bars denote standard deviations.

Table 3.1. Colony formation by spores of *C. perfringens* strains[a]

Strain/genotype	Spore titer ($CFU/ml/OD_{600}$)[b]		
	BHI	BHI + Ca-DPA[c]	BHI + Lyz[d]
SM101/wild-type	3.1×10^7	3.3×10^7	3.9×10^7
DPS101/*gerKA gerKC*	2.6×10^6	3.5×10^6	2.0×10^6
DPS108/*gerKB*	4.4×10^6	4.2×10^6	8.6×10^6

[a]Heat activated spores of various strains were plated on BHI agar, and colonies were counted after anaerobic incubation at 37ºC for 24 h.

[b]Titers are the average of colony forming units (CFU)/ ml/OD_{600} determined in three experiments and the variance was less than 15%.

[c]Heat activated spores were pre-incubated with Ca-DPA as described in the text and plated on BHI plates.

[d]Spores were decoated, heat activated, plated on BHI agar containing lysozyme (Lyz), and colonies were counted after anaerobic incubation at 37°C for 24 h.

3.3 Supplementary Information

Supplementary Tables

Table S3.1. Bacterial strains and plasmids used

Strain or Plasmid	Relevant characteristic(s)	Reference or source
C. perfringens		
SM101	Electroporatable derivative of food poisoning type A isolate, NCTC8798; carries a chromosomal *cpe*	(256)
SM101(pDP84)	Wild-type strain carrying *gerKB-gusA* fusion	This study
DPS108	*gerKB:catP*	This study
DPS101	*gerK::catP*	(154)
Plasmids		
pCR-XL-TOPO	*E. coli* vector; encodes resistance to kanamycin (Km^r; 50 μg/ml)	Invitrogen
pJIR418	*C. perfringens/E. coli* shuttle vector; encodes resistance to chloramphenicol (Cm^r; 20μg/ml) and erythromycin (Em^r; 50 μg/ml)..	(219)
pMRS127	*C. perfringens/E. coli* shuttle vector; Em^r, and has a promoterless *gusA*.	(174)
pMRS104	Em^r; has no *C. perfringens* origin of replication.	(67)
pDP1	*gerAA* internal fragment (~ 800-bp) cloned in EcoRI site of pCR-XL-TOPO	(153)
pDP67	~ 2.2-kb PCR fragment containing 123-bp of the *gerKB* N-terminal-coding region and 2080 upstream bp in pCR-XL-TOPO	This study
pDP68	~ 2.2-kb BamHI-XhoI fragment from pDP67 cloned between the BamHI and XhoI sites of pDP1	This study
pDP69	~1.3-kb PCR fragment containing 134-bp of the *gerKB* C-terminal-coding region and 1195 downstream bp in pCR-XL-TOPO	This study
pDP73	~ 1.3-kb KpnI-BamHI fragment from pDP69 cloned between the KpnI and BamHI sites of pDP68	This study
pDP74	An ~ 1.3-kb NaeI-SmaI *catP* fragment from pJIR418 in the BamHI site of pDP73	This study
pDP75	~ 4.8-kb KpnI-XhoI fragment from pDP74 cloned between the KpnI and SalI sites of pMRS104	This study
pDP84	485-bp PCR amplified region upstream of *gerKB* cloned in pMRS127	This study

Table S3.2. Primers used in this study

Primer name	Primer sequence[a]	Position[b]	Gene	Use[c]
CPP369	GCACCTCGAGGTACTTAATGCTCCTAAAACT AATAGATTTCC	-2081 to -2049	*gerKB*	MP
CPP367	CGCGGATCCGGAAAATCCAAGTATCTCTTCC GCC	+99 to +123	*gerKB*	MP
CPP371	CGCGGATCCGCTAAGAATGCCTATACCCTTTT TATAC	+955 to +982	*gerKB*	MP
CPP370	GCGGGTACCCGTCATACATAGGGTAGATACA CC	+2261 to +2284	*gerKB*	MP
CPP389	GGCAGCGTCGACGTTATTGGAATCCCAACAC CTC	-457 to -435	*gerKB*	GUS
CPP391	GCACGCTGCAGGTCTTGTATTTAGCTTTCCCA AAG	+4 to +28	*gerKB*	GUS

[a] Restriction sites are underlined

[b] The nucleotide position numbering begins from the first codon and refers to the relevant position within the respective gene sequence.

[c] MP, construction of mutator plasmid; GUS, construction of *gusA* fusion plasmid.

Chapter 4

GerO, a putative Na^+/H^+-K^+ antiporter, is essential for normal germination of spores of the pathogenic bacterium *Clostridium perfringens*.

Daniel Paredes-Sabja, Peter Setlow and Mahfuzur R. Sarker

Published in:

Journal of Bacteriology 2009 **191** (12): 3822-3831

4.1 Abstract

The pathogen *Clostridium perfringens*' genome encodes two proteins, GerO and GerQ, homologous to monovalent cation transporters suggested to have roles in germination of spores of some *Bacillus* species. GerO and GerQ were able to transport monovalent cations (K^+ and/or Na^+) in *Escherichia coli*, and *gerO* and *gerQ* were expressed only in the mother cell compartment during *C. perfringens* sporulation. *C. perfringens* spores lacking GerO were defective in germination with a rich medium, KCl, L-asparagine, and a 1:1 chelate of Ca^{2+} and dipicolinic acid (DPA), but not with dodecylamine, and the defect was prior to DPA release in germination. All defects in *gerO* spores were complemented by ectopic expression of wild-type *gerO*. Loss of GerQ had much smaller effects on spore germination, and these effects were most evident in spores also lacking GerO. A modeled structure of GerO was similar to that of the *E. coli* Na^+/H^+ antiporter NhaA, and GerO, but not GerQ contained two adjacent Asp residues thought to be important in the function of this group of cation transporters. Replacement of these adjacent Asp residues in GerO with Asn reduced the protein's ability to complement the germination defect in *gerO* spores, but not the ability to restore cation transport to *E. coli* cells defective in K^+ uptake. Together, these data suggest that monovalent cation transporters play some role in *C. perfringens* spore germination. However, it is not clear if this role is directly in germination, or perhaps in spore formation.

4.2 Introduction

Clostridium perfringens is a gram positive, spore-forming anaerobic pathogen that causes diseases in animals and humans (117). *C. perfringens* spores are metabolically dormant, are resistant to many environmental insults, and can survive for long periods. Once conditions are favorable, these spores can germinate, outgrow, return to vegetative growth and then release toxins and cause disease (119).

Bacterial spores initiate germination when they sense a variety of compounds termed germinants, which include nutrients, a 1:1 chelate of Ca^{2+} and pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)) (Ca-DPA) and cationic surfactants (154, 207). In spores of *Bacillus* species, nutrient germinants are sensed by specific germinant receptors located in the spore's inner membrane, each generally encoded by tricistronic operons of the *gerA* family. In *Bacillus megaterium* spores, the interaction of nutrient germinants with their cognate receptors leads to an energy independent efflux of ~ 80% of the spore's depot of Na^+ and K^+, as well as much H^+ efflux causing a rise of the spore core's pH, all within the first 5 min of germination; this efflux is followed by reuptake of K^+ by an energy-dependent system (229). The spores' large depot of Ca-DPA is also released shortly after monovalent cation release. The mechanism of release of monovalent cations during spore germination is not known, but monovalent cation antiporters could be involved somehow in this event. Indeed, a member of the CPA-2 monovalent cation:proton antiporter family of membrane transport proteins (187), GrmA, is essential for germination of *B. megaterium* ATCC 12872 spores (231), as *grmA* inactivation makes spores unable to release their DPA and complete germination with a variety of germinants. Similarly, in *Bacillus cereus*

ATCC 10876, a GrmA-type homologue, GerN, is essential for spore germination with inosine but not L-alanine (233), and studies with everted vesicles have shown that GerN possesses electrogenic Na^+/H^+-K^+ antiporter activity (224). The GerN homolog, GerT, also plays a minor role in *B. cereus* spore germination with inosine, as well as a major role in spore outgrowth under some conditions (198). However, in contrast to these latter results, GrmA-like antiporters appear to have no role in germination of spores of *B. megaterium* QM B1551 and *Bacillus subtilis* (28).

In *C. perfringens*, there is no intact tricistronic *gerA*-like operon, and the only locus that encodes the three proteins (A, B, and C) of a likely germinant receptor is the *gerK* locus, comprising a bicistronic *gerKA-gerKC* operon, and a *gerKB* gene located just upstream of *gerKA-gerKC* but in the opposite orientation (130). However, GerKA and GerKC appear able to function in spore germination in the absence of GerKB (157). The lack of a classical GerA-type germinant receptor and the fact that *C. perfringens* spores germinate with K^+ ions alone (154), raises the possibility that GrmA-like antiporters might also play some role in *C. perfringens* spore germination. The genome of *C. perfringens* strain SM101 has two genes encoding putative GrmA-like antiporters (Fig. S4.1) that we have termed *gerO* (CPR0227) and *gerQ* (CPR1038). Orthologs of the *gerO* and *gerQ* genes are also present in the genomes of nine additional *C. perfringens* strains (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). In current work we have constructed *gerO*, *gerQ* and *gerO gerQ* strains of *C. perfringens* and have examined the roles of GerO and GerQ in spore germination. The results of this work show that

GerO is essential for normal germination of *C. perfringens* spores, while GerQ plays at most only a minor role.

4.3 Materials and Methods

Bacterial strains and plasmids. *C. perfringens* and *Escherichia coli* strains, and plasmids used in this study are described in Table S4.1.

Construction of *gusA*-fusion plasmids and β-glucuronidase assay. DNA fragments (300-400 bp) upstream of *gerO* and *gerQ* from *C. perfringens* SM101, which include the 290 and 29-bp intergenic regions between *gerO* and CPR0226, and *gerQ* and CPR1039, respectively, which most likely contain these *ger* genes' promoters, were PCR-amplified using primers CPR383/CPR386 and CPR380/CPR385. The forward and reverse primers (the sequences of all primers used in this work are given in Table S4.2) had SalI and PstI cleavage sites at the 5'-ends, respectively. These PCR-fragments were digested with SalI and PstI and cloned between SalI and PstI sites in plasmid pMRS127 in *E. coli* DH5α, the host for all plasmid construction, as described (153, 174) to create *gerO*- and *gerQ-gusA* fusions, giving plasmids pDP81 and pDP82 (Table S4.1). These plasmids were introduced by electroporation (35) into *C. perfringens* SM101 and erithomycin-resistant (Em^r) transformants were selected. Transformants carrying plasmids with the *gerO*-and *gerQ-gusA* fusions were grown in TGY vegetative medium (3% trypticase soy, 2% glucose, 1% yeast extract, 0.1% L-cysteine) (91) and in Duncan-Strong (DS) (36) sporulation medium, and cells were extracted and assayed for β-glucuronidase (GUS) activity as described (256). GUS

specific activity was expressed in Miller Units that were calculated as described (174). Note that lysozyme was present in cell extraction prior to GUS assays, and while lysozyme treatment will likely not extract enzymes from intact dormant spores, it does allow extraction of enzymes from chemically decoated spores.

Decoating treatment of sporulating cultures. Cell pellets from 1 ml of DS sporulating cultures were treated to chemically decoat any spores present in 1 ml of 50 mM Tris-HCl (pH 8.0)-8 M urea-1% (wt/vol) SDS-50 mM dithiothreitol for 90 min at 37ºC and remaining spores were washed three times with 150 mM NaCl and twice with water (169). The decoated samples were then extracted and assayed for GUS activity as described above. Note that the decoating treatment will inactivate and/or remove any GUS not in dormant spores.

Construction of *gerO*- and *gerQ-gfp*-fusion plasmids and GFP visualization. Plasmids carrying the *gerO* or *gerQ* promoters fused to *gfp* were constructed as follows. A ~ 715-bp fragment was PCR-amplified from plasmid pEGFP (ClonTech, Mountain View, CA, USA) with Phusion™ High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA) using primers CPP602/CPP603 (forward primer had XbaI, PstI and SpeI sites, and the reverse primer had two extra T's and a BamHI site at the 5'-end). The PCR fragment was cloned into plasmid Zero-Blunt®-TOPO® (Invitrogen, Carsbad, CA, USA) giving plasmid pDP149. A 720-bp XbaI-BamHI fragment from plasmid pDP149 was cloned between the XbaI and BamHI sites of plasmid pET16b (Novagen, Gibbstown, NJ, USA), upstream of a strong

transcription terminator, giving plasmid pDP151. A 1023-bp PstI-HindIII fragment from plasmid pDP151, that likely contains the *gerO* promoter (Fig. 4.1B), was cloned between the PstI and HindIII sites in plasmid pDP81 giving plasmid pDP152. A 433-bp PCR fragment amplified from *C. perfringens* SM101 DNA using Phusion™ High-Fidelity DNA Polymerase and primers CPP380/CPP676, that carries 427-bp upstream and 6-bp from the N-terminal coding region of *gerQ* was digested with SalI and PstI and cloned between the SalI and PstI sites in plasmid pDP152, replacing the *gerO* promoter with the *gerQ* promoter and giving plasmid pDP182. As shown by assays of GUS activity (Fig. 4.1C), the 434-bp region upstream of *gerQ* contains a sporulation-specific promoter.

Plasmids pDP152 and pDP182 were introduced by electroporation (35) into *C. perfringens* strain SM101; Em^r transformants were selected, and the presence of plasmids pDP152 and pDP182 in SM101(pDP152) and SM101(pDP182) was confirmed by PCR (data not shown). 6-h TGY-vegetative and 6-h DS-sporulating cultures, and untreated and decoated purified spores of strains SM101(pDP152) and SM101(pDP182) prepared at 37ºC were examined on a DM4008B fluorescence microscope (Leica, Wetzier, Germany). For visualization of GFP, 5 μl of culture was applied to a poly-L-lysine coated glass microscope slide, and a dichroic mirror cube unit with a narrow-band-pass (450- to 490-nm) excitation filter, and a narrow-band-pass (500- to 550-nm) barrier emission filter were used. Photomicrographs were prepared with Adobe Photoshop and Microsoft Picture Manager Software.

Construction of *E. coli* strains carrying *gerO* and *gerQ*. *E. coli* strains TK2420 and KNabc carrying plasmids with *C. perfringens gerO* and *gerQ* were constructed as follows. An ~ 1.4-kb promoter-less fragment carrying 28 upstream bp, the coding region and 182-bp downstream of *gerO* was PCR amplified from *C. perfringens* SM101 DNA with Phusion™ High-Fidelity DNA Polymerase using primers CPP523/CPP524 (forward and reverse primers had KpnI and PstI sites at the 5'-ends, respectively). The ~ 1.4-kb PCR fragment was cloned into plasmid Zero-Blunt-TOPO giving plasmid pDP141. An ~ 1.4-kb KpnI-PstI fragment from plasmid pDP141 was then cloned between the KpnI and PstI sites of plasmid pGEM3zf(+) (Promega, Madison, WI, USA) behind the T7 promoter, giving plasmid pDP144. Expression of genes inserted in plasmid pGEM3zf(+) constructs in *E. coli*, even without concomitant expression of T7 RNA polymerase, results in levels of gene products sufficient for phenotypic effects of membrane transport proteins without the toxicity that often results from overexpression of such proteins (26, 57, 224). An ~ 1.6-kb promoter-less fragment carrying the coding region and 111-bp downstream of *gerQ* was PCR amplified from *C. perfringens* SM101 DNA with Phusion™ High-Fidelity DNA Polymerase using primers CPP533/CPP526 (forward primer had a KpnI site at the 5'-end and bases TTTATT at positions -7 to -2 relative to the start codon of *gerQ* were substituted for GGAGGGGA, to provide a better ribosome binding site (RBS) for *gerQ* mRNA as reported (224), and the reverse primer had a PstI site at the 5'-end) or with primers CPP525/CPP526 (forward and reverse primers had KpnI and PstI sites at their 5'-ends, respectively). Both ~ 1.6-kb PCR fragments were cloned into plasmid pGEM3zf(+) giving plasmids pDP147 and pDP148, respectively. Plasmids pDP144,

pDP147 and pDP148 were transformed into *E. coli* strains TK2420 and KNabc and Amp^r transformants were selected.

The K^+ uptake-deficient *E. coli* strain TK2420 has a normal complement of $Na^+(K^+)/H^+$ antiporters, and effects of increasing KCl concentration on growth of this strain and its derivatives were determined as described (12) in a defined medium containing Na^+. The Na^+/H^+ antiporter deficient KNabc strain has a normal complement of K^+ uptake proteins but reduced levels of K^+/H^+ antiporters mediating K^+ efflux; this strain is unable to grow at concentrations of $Na^+ > 75$ mM, but will grow in low-sodium medium, LBK (57). The effects of increasing Na^+ concentration on the growth of *E. coli* KNabc and its derivatives were measured using LBK with various concentrations of NaCl (57).

Construction of a *C. perfringens gerQ* deletion mutant. To isolate a derivative of *C. perfringens* SM101 with a deletion of *gerQ*, a Δ*gerQ* suicide vector was constructed as follows. An 832-bp DNA fragment carrying 583-bp upstream and 249-bp of the N-terminal coding region of *gerQ* was PCR amplified with primers CPP415/CPP417 (forward and reverse primers had KpnI and SpeI sites at the 5'-ends, respectively). A 1312-bp DNA fragment containing 80-bp of the C-terminal coding region and 1232-bp downstream of *gerQ* was PCR amplified with primers CPP411/CPP410 (forward and reverse primers contained PstI and XhoI sites at their 5'-ends, respectively). These fragments were cloned into plasmid pCR-XL-TOPO (Invitrogen, Carlsbad, CA, USA) giving plasmids pDP101 and pDP102. An ~ 0.8-kb KpnI-SpeI fragment from pDP101 was then cloned between the KpnI and SpeI sites just upstream of *catP* in plasmid

pDP25 (156), giving plasmid pDP103. An ~ 1.3-kb PstI-XhoI fragment from pDP102 was cloned between the PstI and XhoI sites downstream of *catP* in plasmid pDP103 giving plasmid pDP104. A 3.5-kb KpnI-XhoI fragment from plasmid pDP104, carrying the Δ*gerQ::catP* construct, was then cloned between the KpnI and SalI sites of plasmid pMRS104 (67), giving plasmid pDP105 which cannot replicate in *C. perfringens*. Plasmid pDP105 was introduced into *C. perfringens* SM101 by electroporation (35), and a chloramphenicol-resistant *gerQ* mutant was isolated as described (192). The identity of the *gerQ* strain DPS113 was confirmed by PCR and Southern blot analyses (data not shown).

Construction of a *C. perfringens gerO* deletion mutant. To isolate a derivative of *C. perfringens* SM101 with a deletion of *gerO*, a Δ*gerO* suicide vector was constructed as follows. A 1070-bp DNA fragment carrying 865-bp upstream and 205-bp of the N-terminal coding region of *gerO* was PCR amplified with primers CPP406/CPP408 (forward and reverse primers had KpnI and SpeI sites at their 5'-ends, respectively). A 1227-bp DNA fragment carrying 246-bp of the C-terminal coding region and 981-bp downstream of *gerO* was PCR amplified with primers CPP424/CPP430 (forward and reverse primers had PstI and XhoI sites at the 5'-ends, respectively). These PCR fragments were cloned into plasmid pCR-XL-TOPO giving plasmids pDP96 and pDP97, respectively. A 1.1-kb KpnI-SpeI fragment from plasmid pDP96 was then cloned between the KpnI and SpeI sites upstream of *catP* in plasmid pDP25, giving plasmid pDP98, and an ~ 1.2-kb PstI-XhoI fragment from plasmid pDP97 was cloned between the PstI and XhoI sites downstream of *catP* in plasmid pDP98, giving

plasmid pDP99. An ~ 3.7-kb KpnI-XhoI fragment from plasmid pDP99, carrying the Δ*gerO::catP* construct, was then cloned between the KpnI and SalI sites in plasmid pMRS104, giving plasmid pDP100 which cannot replicate in *C. perfringens*. Finally, a ~ 3.2-kb SpeI-PstI fragment from plasmid pDP35, carrying *tetM*, was cloned between the PstI and SpeI sites in plasmid pDP100, replacing the *catP* gene with *tetM* and giving plasmid pDP112. Plasmid pDP112 (carrying Δ*gerO*::*tetM*) was introduced into *C. perfringens* SM101 by electroporation, and a Tet^r *gerO* mutant was isolated as described (192). The identity of the *gerO* strain DPS116 was confirmed by PCR and Southern blot analyses (data not shown).

To isolate a derivative of *C. perfringens* SM101 with deletions of both *gerO* and *gerQ*, plasmid pDP112 was introduced into *C. perfringens* DPS113 (*gerQ*) by electroporation, and a Cm^r Tet^r *gerO gerQ* mutant was isolated as described (192). The identity of the *gerO gerQ* strain DPS115 was confirmed by PCR and Southern blot analyses (data not shown).

Construction of a Δ*gerO* strain complemented with *gerO*. To construct a *gerO* strain complemented with wild-type *gerO*, a suicide-complementing plasmid targeted to the *plc* locus was constructed as follows. A 1.8-kb DNA fragment carrying 396-bp upstream and the coding region of *gerO* was PCR amplified with Phusion™ High-Fidelity DNA Polymerase using primers CPP599/CPP600 (forward and reverse primers had KpnI and SalI sites at their 5'-ends, respectively). As shown by assays of GUS activity (Fig. 4.1B), the 396-bp region upstream of *gerO* contains a sporulation-specific promoter. This PCR fragment was digested with KpnI and SalI and cloned

between the KpnI and SalI sites of plasmid pDP129 (a suicide plasmid containing ~ 1.7- and 1.3-kb upstream and downstream of the *plc* locus) (156), giving plasmid pDP150 which cannot replicate in *C. perfringens*. Plasmid pDP150 was introduced into the *C. perfringens gerO* strain DPS116 by electroporation (35) and Em^r Tet^r transformants of strain DPS116(pDP150) were selected. The presence of both plasmid pDP150 and the original *gerO* deletion in the latter strain were confirmed by PCR and Southern blot analyses (data not shown).

Construction of a *gerO* strain containing *gerO*$^{D161N,\ D162N}$. The *gerO* strain containing *gerO*$^{D161N,\ D162N}$, with Asp161 and Asp162 replaced by two Asn residues, was constructed as follows. A 905-bp DNA fragment carrying 396-bp upstream and 509-bp of the N-terminal coding region of *gerO* was PCR amplified from *C. perfringens* SM101 DNA with Phusion™ High-Fidelity DNA Polymerase using primers CPP599/CPP662 (forward and reverse primers contained KpnI and HpaI sites at their 5'-ends, respectively). To produce the desired mutations, bases at positions 481 and 484 of *gerO* were altered to A residues in primer CPP662 (Table S4.2). An 879-bp DNA fragment carrying 697-bp of the C-terminal coding region and 182-bp downstream of *gerO* was PCR amplified from *C. perfringens* SM101 DNA with Phusion™ High-Fidelity DNA Polymerase using primers CPP663/CPP524 (forward and reverse primers had HpaI and PstI sites at their 5'-ends, respectively). These PCR fragments were cloned into plasmid Zero-Blunt-TOPO giving plasmids pDP169 and pDP167, respectively, and these plasmids were sequenced to confirm the presence of the mutations. A 0.9-kb KpnI-HpaI fragment from plasmid pDP169 was cloned

between the KpnI and HpaI sites in plasmid pDP25 giving plasmid pDP170, a 0.8-kb HpaI-PstI fragment from plasmid pDP167 was cloned between the HpaI and PstI sites in plasmid pDP170, giving plasmid pDP171, and a 1.6-kb KpnI-XhoI fragment from plasmid pDP171 was cloned between the KpnI and SalI sites in plasmid pDP129, giving plasmid pDP172. Plasmid pDP172 was sequenced to confirm that the construct was in-frame and contained the desired mutations (data not shown), and was introduced into *C. perfringens* strain DPS116 (*gerO*) by electroporation (35), and an Em^r Tet^r transformant (strain DPS116(pDP172)) was selected. The presence of plasmid pDP172 in strain DPS116(pDP172) was confirmed by PCR and Southern blot analyses (data not shown).

To attempt complementation of *E. coli* mutants with $gerO^{D161N, D162N}$, a ~1.2-kb DNA fragment was PCR amplified from plasmid pDP172 with primers CPP523/CPP524 (Table S4.2) and cloned between the KpnI and PstI sites in plasmid pGEM3zf(+), giving plasmid pDP173. Plasmid pDP173 was sequenced to confirm that the construct was in-frame and contained the desired mutations (data not shown), and was introduced into *E. coli* strains.

Spore preparation and purification. Spores of *C. perfringens* isolates were prepared and purified as described (153, 154). Briefly, *C. perfringens* sporulating cultures were prepared by inoculating 0.2 ml of an overnight culture grown at 37ºC in fluid thioglycollate broth (FTG) (Difco) into 10 ml of DS sporulation medium (36), incubated for 24 h at 37ºC and the presence of spores was confirmed by phase-contrast microscopy. Large amounts of spores were prepared by scaling-up the latter procedure

as described (154). Clean spore preparations were obtained by repeated centrifugation, washing with sterile distilled water until spore suspensions were > 99% free of sporulating cells, cell debris and germinated spores, suspended in distilled water at a final optical density at 600 nm (OD_{600}) of ~ 6 and stored at -20ºC. All mutant strains used in this work sporulated like the parental wild-type strain as observed by phase contrast microscopy (data not shown).

Spore germination. With the exception of dodecylamine germination, spore suspensions in water were heat activated (80°C, 10 min) prior to germination, cooled in water at ambient temperature for 5 min, and incubated at 40ºC for 10 min prior to addition of buffer and germinants as described (153, 154). Except for germination with dodecylamine (see below), spore germination was routinely measured by monitoring the OD_{600} of spore cultures (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA, USA), which falls ~ 60% upon complete spore germination, and levels of germination were confirmed by phase-contrast microscopy. The extent of spore germination was calculated from the percentage decrease in OD_{600} after 1 h, with 60% decrease set at 100% germination. All values reported are averages of two experiments performed on at least two independent spore preparations, and individual values varied by less than 10% from average values shown. In some experiments maximum rates of spore germination were determined by measuring the OD_{600} of germinating cultures every 2.5 min, the maximum slopes calculated and maximum rates of germination were expressed as the maximum rate of loss in the OD_{600} of the spore suspension relative to the initial OD_{600} of the culture.

Assessment of spores colony forming efficiency. The colony-forming efficiency of spores of various strains was assessed by plating aliquots of dilutions of heat activated spores on Brain Heart Infusion (BHI) agar, incubating plates anaerobically at 37ºC for 24 h, and colonies were counted. Outgrowth experiments were in TGY vegetative medium since it allows faster growth and maintains better anaerobiosis. Briefly, 300 μl of spore suspensions at an OD_{600} of 1.0, and in one experiment less were heat activated, cooled in water at room temperature, inoculated into 10 ml TGY medium at 37ºC; the cultures incubated at 37°C, and the OD_{600} was measured.

DPA release. DPA release during spore germination was measured as described (153, 154). Briefly, heat activated spore suspensions (OD_{600} of 1.5) were cooled and incubated at 40°C in BHI broth or in 25 mM sodium phosphate buffer with or without various germinants. After 60 min or 24 h, aliquots (1 ml) were centrifuged in a microcentrifuge (13,200 rpm, 3 min), the spore pellet was washed four times with 1 ml distilled water and suspended in 1 ml distilled water. The spore DPA remaining was determined by boiling samples for 60 min, cooling on ice for 5 min, centrifuging in a microcentrifuge for 5 min, and measuring the OD_{270} of the supernatant fluid as described (21, 201). The DPA content of the initial dormant spores was measured by boiling 1 ml aliquots for 60 min, centrifuging in a microcentrifuge for 5 min, and measuring the OD_{270} of the supernatant fluid as described (21, 154). Control experiments were done for each experiment to account for losses due to the multiple centrifugations, and corrections for such losses were made accordingly. In *C.*

perfringens spores ~ 90% of the material absorbing at 270 nm released from spores by boiling is DPA (156). In a few experiments the total DPA content of spores of various strains was measured by a colorimetric assay as described (153, 186).

Dodecylamine germination was assessed by measuring only DPA release by incubating spores (OD_{600} of 1.5) that had not been heat activated with 1 mM dodecylamine in 25 mM Tris-HCl (pH 7.4) at 60°C; aliquots (1 ml) of germinating cultures were centrifuged for 3 min in a microcentrifuge and DPA in the supernatant fluid was measured from the OD_{270} as described (21, 154). Initial DPA levels in dormant spores were measured as described above. No significant DPA release was observed when spores were incubated in 25 mM Tris-HCl (pH 7.4) at 60ºC for 1 h (data not shown).

4.4 Results

***C. perfringens gerO* and *gerQ* are expressed in the mother cell compartment during sporulation.** To evaluate whether the *C. perfringens gerO* and *gerQ* genes encoding putative antiporters are expressed during sporulation, upstream DNA from each gene, including the intergenic regions between these genes and the ones preceding them (Fig. 4.1A) that most likely contain these genes' promoters, was fused to *E. coli gusA*, and GUS activity was measured after introducing these fusions into *C. perfringens* SM101. No significant GUS activity was observed in vegetative cultures of SM101 carrying *gerO*- and *gerQ-gusA*, but significant GUS activity was detected in sporulating cultures carrying these *gusA*-fusions (Fig. 4.1B, C), indicating that sporulation-specific promoters are located upstream of *gerO* and *gerQ*. Note, however,

that we cannot rule out the possibility that *gerQ* is also transcribed with the immediately upstream ORF CPR1039 (Fig. 4.1A), perhaps even from a vegetative promoter (Fig. 4.1A). GUS expression from the *gerO*- and *gerQ-gusA* fusions began ~ 2 and 3 h after the start of sporulation, respectively, and reached maxima ~ 4 and 8 h (Fig. 4.1B,C). When sporulating cultures were treated with a decoating regimen (169) that inactivates mother cell enzyme activity but not enzymes within spores, no GUS activity was detected throughout sporulation of strains carrying *gerO*- and *gerQ-gusA* fusions or from purified dormant spores (data not shown), suggesting that *gerO* and *gerQ* are expressed only in the mother cell compartment of the sporulating cell.

To further confirm the site of expression of *gerO* and *gerQ*, transcriptional *gerO*- and *gerQ-gfp* fusions were constructed and introduced into *C. perfringens* SM101. As with the two *gusA* fusions, no significant GFP fluorescence was detected in vegetative cultures of strain SM101 carrying *gerO*- and *gerQ-gfp* fusions (data not shown). However, GFP fluorescence was detected after 4 h of sporulation in DS medium of strains carrying *gerO*- and *gerQ-gfp* fusions (data not shown), and this fluorescence was readily seen after 6 h of sporulation (Fig. 4.1D, E, F, G). No fluorescence was observed in a 6-h sporulating culture of strain SM101 (data not shown). Strikingly, GFP fluorescence from *gerO*- and *gerQ-gfp* was exclusively in the mother cell compartment of the sporulating cell, with no fluorescence observed in forespore compartments that were not fully refractile, developing refractile spores, or in purified dormant spores (Fig. 4.1D-G; and data not shown). An analysis of the intergenic region between CPR1039 and *gerQ* revealed a sequence very similar to those of sporulation-specific promoters dependent on SigK at -146 to -179 relative to

the *gerQ* translation start site, although no similarity to sporulation-specific SigK- and SigE-dependent promoters was found in the intergenic region between CPR0226 and *gerO* (data not shown). Collectively, these results suggest that GerO and GerQ are synthesized exclusively during sporulation and only in the mother cell compartment of the sporulating cell, and that GerQ might be under direct control of SigK.

Complementation of K^+ uptake and Na^+ sensitivity phenotypes in *E. coli* by *C. perfringens* GerO and GerQ. The sporulation-specific synthesis of GerO and GerQ was consistent with these proteins playing some role in spore germination, and the homology between these proteins and known antiporters (Fig. S4.1) suggested that GerO and GerQ might also be antiporters, To test this latter possibility directly, we examined whether plasmids carrying *C. perfringens gerO* or *gerQ* could complement the phenotype of *E. coli* strain TK2420 that is defective in K^+ uptake due to the absence of Kdp, TrkD1 and TrkA; TK2420 cells cannot grow in a defined medium (57) containing a low K^+ concentration. As expected, *E. coli* TK2420 cells carrying the control plasmid pGEM3zf(+) could not grow in defined medium containing less than 40 mM KCl (Fig. 4.2A). However, TK2420 strains carrying GerO grew in defined medium containing 15 and 20 mM KCl (Fig. 4.2A), suggesting that GerO is capable of promoting an inward flux of K^+ ions. In contrast, *E. coli* strain TK2420 cells expressing GerQ via either the native *gerQ* mRNA's RBS or a stronger RBS could only grow with 40 mM KCl (Fig. 4.2A; and data not shown).

We also examined the ability of GerO and GerQ to complement the Na^+-sensitive phenotype of *E. coli* strain KNabc that cannot grow in medium containing ≥

200 mM NaCl due to absence of Cha, NhaA and NhaB (75). As expected, growth of KNabc cells carrying the control plasmid decreased as the NaCl concentration of the LBK medium was increased from 0.06 to 0.15 M, and no growth was observed with 0.2 M NaCl (Fig. 4.2B). However, KNabc cells expressing GerO exhibited significantly ($p < 0.0001$) higher growth than KNabc cells in LBK medium with 0.1 and 0.15 M NaCl, although no growth was observed with 0.2 M NaCl (Fig. 4.2B). In addition, KNabc cells expressing GerQ via the native or an even stronger RBS grew significantly ($p < 0.0001$) more than KNabc(pGEM3zf+) cells in LBK medium with 0.08 and 0.1 M NaCl, although little and no growth were observed with 0.15 and 0.2 M NaCl, respectively (Fig. 4.2B; and data not shown). These results suggest that although both putative antiporters catalyze Na^+ efflux, GerO can translocate Na^+ against a greater Na^+ gradient. Collectively, these findings suggest that while GerO is capable of translocating K^+ and Na^+, GerQ is only capable of translocating Na^+, and perhaps only to a small extent. Note that while the actual levels of GerO and GerQ in *E. coli* are unknown, these complementation experiments were conducted as done successfully with the *B. cereus gerN* gene (224).

GerO, but not GerQ, is essential for normal germination of *C. perfringens* spores. Given that GerO can transport K^+ and Na^+, with GerQ perhaps having weak activity with Na^+, and the role of antiporters in at least some spore germination, it was of obvious interest to examine the function of GerO and GerQ in *C. perfringens* spore germination. Consequently, we constructed strains with deletions of *gerO* (strain DPS116), *gerQ* (DPS113), and both *gerO* and *gerQ* (strain DPS115). Wild-type and

gerQ spores germinated with similar kinetics and to similar extents in BHI broth as measured by changes in OD_{600} (Fig. 4.3). Phase contrast microscopy also showed that > 99% of wild-type and *gerQ* spores had become phase dark after 60 min of incubation in BHI broth (data not shown). However, *gerO* spores exhibited much poorer germination in BHI broth than wild-type and *gerQ* spores, with *gerO gerQ* spores germinating even more poorly ($P \leq 0.01$) (Fig. 4.3). Phase contrast microscopy further indicated that after 60 min of incubation in BHI broth, only ~ 30 and 20% of *gerO* and *gerO gerQ* spores, respectively, had become phase dark (data not shown). These results suggest that GerO plays a major role in spore germination in BHI broth, while significant effects of GerQ are seen only in the absence of GerO.

In *B. cereus* spores, GerN is involved in inosine-mediated germination but not in L-alanine-mediated germination (233). Therefore, to gain further understanding of the roles of GerO and GerQ in *C. perfringens* spore germination, assays were conducted with specific nutrient and non-nutrient germinants (154). The *gerQ* spores germinated slightly poorer than wild-type spores with KCl, while *gerO* spores germinated even more poorly ($P < 0.0001$), and *gerO gerQ* spores germinated poorest of all (Fig. 4.4A). These results were consistent with those from phase-contrast microscopy, where > 99, 90, 50 and 30% of wild-type, *gerQ*, *gerO* and *gerO gerQ* spores had become phase dark after 60 min of incubation with KCl (data not shown). In contrast to results with KCl, *gerQ* spores germinated like wild-type spores with L-asparagine, although *gerO* spores germinated more poorly than wild-type spores, while *gerO gerQ* spores germinated the poorest (Fig. 4.4B). Phase contrast microscopy showed that while ~ 70% of wild-type and *gerQ* spores had become phase

dark after 60 min of incubation with L-asparagine, only ~ 20 and 10% of *gerO* and *gerO gerQ* spores, respectively, had become phase dark. When a mixture of KCl and L-asparagine (AK) were used (Fig. 4.4C), the germination phenotypes observed were similar to those with KCl (Fig. 4.4A). Finally, the germination defects observed with *gerO* spores were eliminated when the *gerO* mutant spores also carried an ectopic wild-type *gerO*, indicating that the germination defects in strain DPS116 were due to specific inactivation of *gerO* (Fig 4.4A-C). Indeed, the *gerO* spores carrying an ectopic wild-type *gerO* actually germinated better than wild-type spores with L-asparagine (Fig. 4.4B). All these results suggest that GerO has a significant role in germination of *C. perfringens* spores, while the role of GerQ is secondary and notable primarily in the absence of GerO.

In contrast to the situation in *B. subtilis* spores where Ca-DPA triggers spore germination likely by activation of the cortex-lytic enzyme, CwlJ, and bypasses the germinant receptors (146), in *C. perfringens* spores Ca-DPA likely acts through the germinant receptors as do KCl and L-asparagine (154, 156). As expected, wild-type and *gerQ* spores germinated similarly with Ca-DPA (Fig. 4.4D), as was confirmed by phase contrast microscopy (data not shown). However, *gerO* spores germinated more poorly than wild-type and *gerQ* spores with Ca-DPA, and *gerO gerQ* spores germinated even more poorly (Fig. 4.4D). Phase contrast microscopy found that ~ 30 and 20% of *gerO* and *gerO gerQ* spores, respectively, had become phase dark after 60 min of incubation with Ca-DPA, while ~90% of wild-type and *gerQ* spores had become phase dark (data not shown). Again, the Ca-DPA germination defect of *gerO* spores was more than complemented by wild-type *gerO* (Fig. 4.4D). These results are

consistent with Ca-DPA acting on a germinant receptor and indicate that GerO, but GerQ only minimally in the absence of GerO, is required for normal Ca-DPA germination.

To more rigorously compare the effects of *gerO* and *gerQ* deletions on spore germination with various germinants, we measured the maximum rates of spore germination by monitoring the fall in OD_{600} every 2.5 min. As expected, *gerQ* spores exhibited a maximum germination rate similar to that of wild-type spores with L-asparagine and Ca-DPA and a slightly lower maximum rate than wild-type spores with KCl and AK (Table 4.1). However, *gerO* spores had a significantly lower maximum germination rate than wild-type spores with all germinants, and *gerO gerQ* spores had the lowest maximum germination rates (Table 4.1).

DPA release during germination of spores of various *C. perfringens* strains. The results noted above suggest that GerO and to a significantly lesser extent GerQ are essential for normal spore germination, and further that GerO and GerQ are likely involved in transport of Na^+ and/or K^+. With *B. megaterium* spores, release of H^+, Na^+, and K^+ from the spore core is a very early event in spore germination, preceding release of Ca-DPA (229). However, it is unclear if monovalent cation release is necessary for Ca-DPA efflux in germination. Since we found that dormant *gerO*, *gerQ* and *gerO gerQ* spores have similar levels of DPA (data not shown), and to measure how far the germination of these spores can progress, we assayed DPA release during germination with various spores. As expected, spores of all strains released only a small amount of DPA when incubated for 60 min in sodium phosphate buffer (Fig.

4.5). However, wild-type and *gerQ* spores released the majority of their DPA when germinated for 60 min with BHI broth, KCl or L-asparagine (Fig. 4.5). In contrast, while *gerO* spores released the majority of their DPA when germinated with KCl, only ~ half of their DPA was released upon germination with BHI broth or L-asparagine, and *gerO gerQ* spores generally released even less DPA than *gerO* spores upon incubation for 60 min with BHI broth, KCl or L-asparagine (Fig. 4.5). No significant further release of DPA was observed when spores of these strains were incubated for 24 h with BHI broth, KCl or L-asparagine (data not shown). However, spores of all four strains released 75-80% of their DPA when germinated with the cationic surfactant, dodecylamine (Fig. 4.5). The amount of DPA released is consistent with the extent of germination observed above, and suggest that a significant fraction of *gerO* and *gerO gerQ* spores, but not *gerQ* spores, cannot progress through Stage I of germination with BHI broth or L-asparagine.

Effects of *gerO* and *gerQ* mutations on *C. perfringens* spore outgrowth and colony forming efficiency. The severe germination defects of *gerO* and *gerO gerQ* spores suggested that outgrowth and colony forming efficiency of these spores might be lower than that of wild-type spores, since only a minority of the mutant spores became phase dark upon germination with BHI broth. As expected, wild-type (3.8×10^7 cfu/ml/OD_{600}) and *gerQ* (3.3×10^7 cfu/ml/OD_{600}) spores exhibited similar colony forming efficiency. However, the colony forming efficiency of *gerO* (2.0×10^7 cfu/ml/OD_{600}) and *gerO gerQ* (2.1×10^7 cfu/ml/OD_{600}) spores was significantly ($P < 0.001$) lower than that of wild-type spores, in agreement with results on spore

germination in BHI broth by phase contrast microscopy. The rate of increase in OD_{600} of wild-type spores incubated in TGY medium was also significantly faster than from all the mutant spores (Fig. 4.6), and this was also the case when less wild-type spores were used to compensate for the decreased viability of the *gerO* and *gerO gerQ* spores (data not shown). However, the *gerO*, *gerQ* and *gerO gerQ* strains grew vegetatively like the wild-type strain in TGY medium (data not shown). These results suggest that GerO and GerQ may also play roles in spore outgrowth and perhaps early growth of nascent vegetative cells released from germinated spores

Effect of D161N and D162N mutations on GerO function. The results noted above indicated that GerO: i) has cation transport activity; and ii) plays a significant role in *C. perfringens* spore germination. In order to potentially provide a direct connection between these two observations, we sought to generate a site directed *gerO* mutant that might be defective in cation transport. A cluster of amino acids (Asp164, Asp163, Asp133, and Thr132) that forms the putative Na^+-binding site in NhaA (70) is highly conserved among members of the NhaA protein family (142). Asp163 and Asp164 are essential for cation-translocation by *E. coli* and *Helicobacter pylori* NhaA and replacement of these Asp residues with Asn residues in *E. coli* NhaA leads to cells unable to grow in LBK broth with 0.6 M NaCl (75, 94). The alignment of amino acid sequences of GerO and GerQ with various other bacterial cation antiporters as well as a homology structural model (Fig. S4.1, S4.2) of GerO suggested that Asp161 and Asp162 in GerO might be involved in cation-translocation and thus important in germination of *C. perfringens* spores. To test this hypothesis, we changed these Asp

residues to Asn residues, and *gerO*$^{D161N,\ D162N}$ was expressed in *E. coli* strains defective in monovalent cation transport. Surprisingly, GerO$^{D161N,\ D162N}$ complemented the K^+ uptake defect of *E. coli* strain TK2420 as well as wild-type GerO (Fig. 4.2A). In addition, Na^+-sensitive *E. coli* KNabc cells expressing *gerO*$^{D161N,\ D162N}$ exhibited similar growth to that of this *E. coli* strain expressing wild-type *gerO* in LBK medium containing 0.1 M NaCl or less (Fig. 4.2B), but were unable to grow in LBK medium with 0.15 M NaCl (Fig. 4.2B). These results indicate that Asp161 and Asp162 are not essential for K^+ uptake by *C. perfringens* GerO, but are required at least in part for Na^+-translocation.

The *gerO*$^{D161N,\ D162N}$ gene was also expressed in *C. perfringens* DPS116 (*gerO*) to evaluate the importance of Asp161 and Asp162 in *C. perfringens* spore germination. As noted above, *gerO* spores germinated slower and to a lesser extent than wild-type spores with BHI broth, L-asparagine, KCl, or Ca-DPA (Fig. 4.7A-D). However, spores of strain DPS116(*gerO*$^{D161N,\ D162N}$) germinated faster and to a higher extent than *gerO* spores, but significantly slower and to a lesser extent than wild-type spores (Fig. 4.7A-D). Determination of maximum rates of spore germination (Table 4.1) revealed that while spores of strain DPS116 (Δ*gerO gerO*$^{D161N,\ D162N}$) exhibited higher maximum germination rates than *gerO* and *gerO gerQ* spores, these rates were significantly lower than those of wild-type and *gerQ* spores (Table 4.1).

4.5 Discussion

The work in this communication leads to a number of new conclusions, a few less notable or minor conclusions, and several more notable ones. The more minor

conclusions include the following. 1) While both *gerO* and *gerQ* were expressed only in *C. perfringens* sporulation, *gerQ* expression began ~1 h later than that of *gerO*. This suggests that the regulation of the expression of these two genes during sporulation is not identical, and that while transcription of *gerQ* might be under the control of the RNA polymerase sigma factor, SigK, transcription of *gerO* might be under the control of a different RNA polymerase sigma factor, and/or DNA binding proteins are involved in *gerO* and *gerQ* transcription. 2) GerO and GerQ appear to play no role in *C. perfringens* spore germination by dodecylamine, although are involved to at least some degree in spore germination by BHI broth, KCl, L-asparagine and Ca-DPA. This is similar to the effects of the only enzyme, SleC, essential for cortex hydrolysis during *C. perfringens* spore germination (156). Unfortunately the mechanism of spore germination with dodecylamine is not known, although it is possible that this cationic surfactant somehow opens a new or pre-existing DPA channel in the spore's inner membrane (153, 154, 156, 157, 201). In contrast, the other germinants noted above trigger *C. perfringens* spore germination by activation of at least the spore's GerK receptor (154, 157), and perhaps such different germination mechanisms have different requirements for cation transport proteins. Indeed, with *B. cereus* spores, inosine germination requires the antiporter GerN, while L-alanine germination does not (233). 3) GerO has two conserved adjacent Asp residues thought to be important at least in Na^+ transport by this class of proteins (75, 94). Conversion of these Asp residues in GerO to Asn residues greatly reduced the ability of ectopic expression of the *gerO* gene variant's ability to complement the germination defects of spores lacking a normal *gerO* gene. This result suggests that GerO exerts its effects in spore

germination through its ability to transport cations. However, the change of the Asp residues to Asn residues had little to no effect on cation transport by GerO in *E. coli*. The reason for the apparent contradiction between the results in *E. coli* and *C. perfringens* is not clear, but there are many unknowns in these experiments, in particular the affects of the double mutation on protein stability, and indeed, whether these Asp residues are even involved directly in cation transport by these proteins. 4) The final minor conclusion is that GerQ and GerO play roles not only in *C. perfringens* spore germination, but also in spore outgrowth. It is not clear if the role in spore outgrowth is a direct or indirect one, as it is not clear that either of these proteins are actually present in dormant spores (see below). However, a requirement for the likely cation transporter, GerT, in spore outgrowth has been observed with *B. cereus* spores (198). It is also known that germinating and outgrowing spores of at least *Bacillus* species are resistant to very high salt concentrations, much more resistant than are vegetative cells (237). Perhaps GerO and/or GerQ play some role in this resistance by transporting appropriate cations in spore outgrowth. It is also possible that GerO and GerQ are actually synthesized during spore outgrowth, perhaps in response to salt stress, although this has not been studied.

In addition to the minor conclusions noted above, there are three more notable conclusions: 1) the *C. perfringens* GerO and GerQ proteins exhibit sequence and predicted structural homology to monovalent cation antiporters, including such proteins that appear to be involved in germination of spores of at least some *Bacillus* species, and with at least some germinants; 2) GerO clearly can function in transport of K^+ and to a lesser extent Na^+ in *E. coli*, and GerQ appears to have at least weak Na^+

transport activity in *E. coli*; and 3) loss of GerO and to a lesser extent GerQ results in defects in *C. perfringens* spore germination. These results suggesting roles for one or more cation transport proteins in *C. perfringens* spore germination are similar to those suggested for spores of some *Bacillus* species, although not with all strains/species and not with all germinants (28, 198, 231, 233). The major questions provoked by these observations are how GerO and likely to a lesser degree GerQ function in spore germination and/or outgrowth, and whether the effects of these proteins are direct or indirect.

Loss of GerO and/or GerQ has no obvious effects on *C. perfringens* growth or sporulation. Thus the sporulation-specific expression of *gerO* and *gerQ* suggests that GerO and GerQ might be spore-specific proteins. There is also evidence suggesting that the spore germination-associated likely monovalent cation antiporters GerN and GerT of *B. cereus* are encoded by sporulation-specific genes (106, 198). While the latter results suggest that all these cation transport proteins are present in spores, *gerO* and *gerQ* appear to be expressed only in the mother cell compartment of sporulating *C. perfringens* cells, although this analysis has not been carried out with *gerN* and *gerT*. Synthesis of GerO and GerQ only in the mother cell compartment suggests that these proteins are most likely not present in the spore's inner membrane, as at least in spores of *Bacillus* species and probably *C. perfringens* spores as well, spore-specific inner membrane proteins such as germinant receptors and SpoVA proteins are synthesized in the developing forespore (147, 153, 154, 157, 207, 243). The likely absence of GerO and GerQ from the *C. perfringens* spore's inner membrane leaves the spore's outer membrane as the only likely spore-specific location for these proteins,

(although it is also possible that these proteins are only in the mother cell's plasma membrane). An outer membrane location for GerO and GerQ further means that these proteins could not be involved directly in cation transport across the spore's inner membrane in spore germination, and also makes it unlikely that these proteins could interact with the spore's germinant receptors. In addition, a decoating treatment applied to *C. perfringens* spores that likely removes much outer membrane protein (although this has not been shown directly) does not abolish spores' ability to release DPA in response to activation of germinant receptors by germinants, although cortex hydrolysis by the decoated spores is abolished due to extraction of SleC (123, 153). These findings suggest, although by no means prove, that the effects of GerO and GerQ on *C. perfringens* spore germination and outgrowth are not exerted directly during spore germination or outgrowth, but rather during spore formation in some way.

Fig 4.1: Arrangement and expression of *gerO* and *gerQ* genes in *C. pergrinens* SM101.

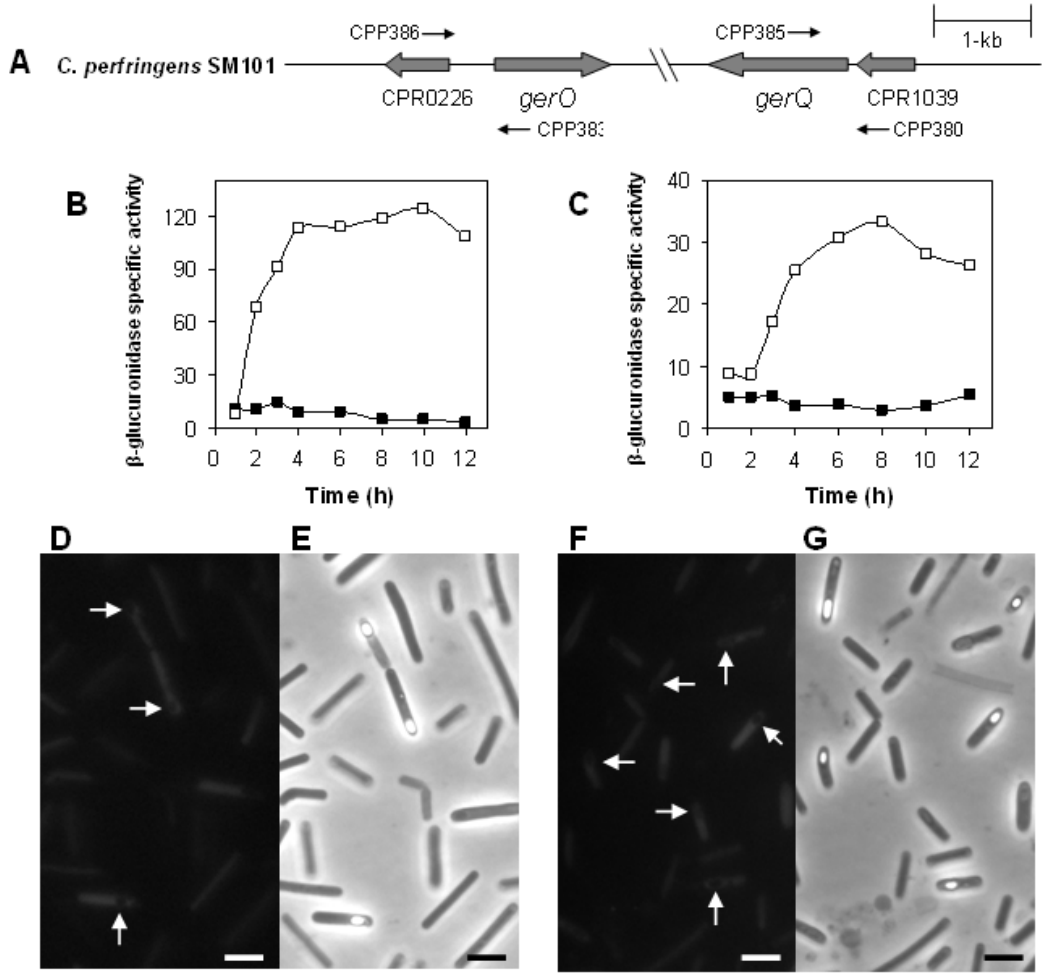


Fig. 4.1.A-G. Arrangement and expression of *gerO* and *gerQ* genes in *C. pergrinens* SM101. A) The arrangement of *gerO* and *gerQ* in *C. perfringens* SM101 and the location of the primers used to amplify the upstream regions of each gene are indicated including the intergenic regions between *gerO* and CPR0226, and *gerQ* and CPR1039. B, C) GUS specific activity from B) *gerO-gusA* and C) *gerQ-gusA* fusions in *C. perfringens* SM101 grown in TGY vegetative (filled squares) and DS sporulation (open squares) media were determined as described in Methods. Data are an average of three independent experiments, and time zero denotes the time of inoculation of cells into either TGY or DS media. D, F) Fluorescence microscopy and E, G) phase-contrast microscopy of *gerO-gfp* (D, E) and *gerQ-gfp* (F, G) fusions in *C. perfringens* SM101 grown for 6 h in DS sporulation medium as described in Methods.

Arrows indicate that there is no green fluorescence in the forespore compartment of the sporulating cell. Bar, 5 μm.

Fig 4.2: Effects of KCl and NaCl concentrations on growth of *E. coli* strains TK2420 and KNacb carrying *C. perfringens gerO* and *gerQ*.

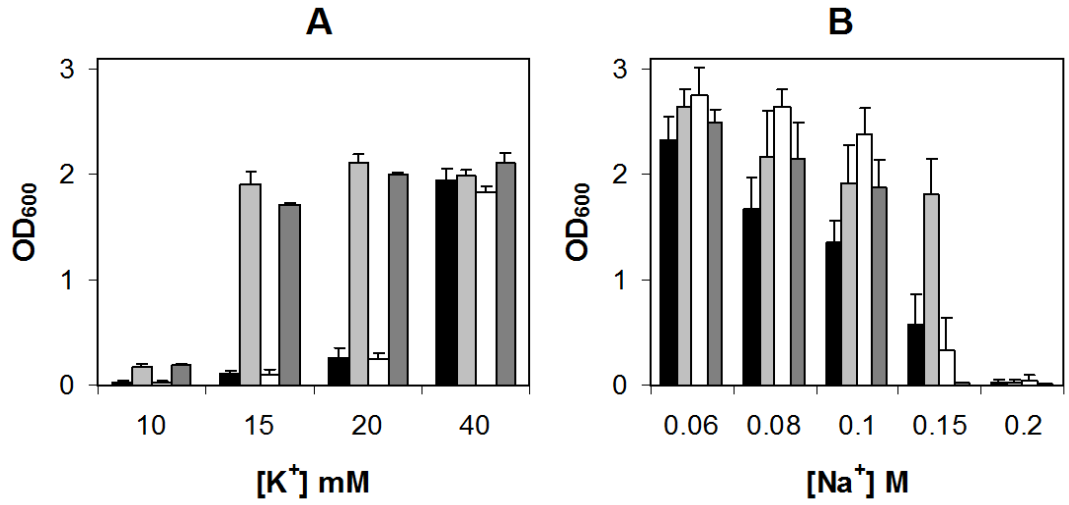


Fig. 4.2.A,B. Effects of KCl and NaCl concentrations on growth of *E. coli* strains TK2420 and KNabc carrying *C. perfringens gerO* and *gerQ*. A, B) Growth of *E. coli* strains A) TK2420 and B) KNabc transformed with the control plasmid (black bars), plasmid pDP144 (*gerO*) (light-grey bars), plasmid pDP147 (*gerQ*) (white bars) or plasmid pDP173 (*gerO* ^{D161N,D162N}) (dark-grey bars) was measured after incubation for 15 h at 37ºC in A) defined medium with various KCl concentrations, and B) LBK medium with increasing NaCl concentrations as described in Methods.

Fig 4.3: Germination of mutant spores in BHI broth.

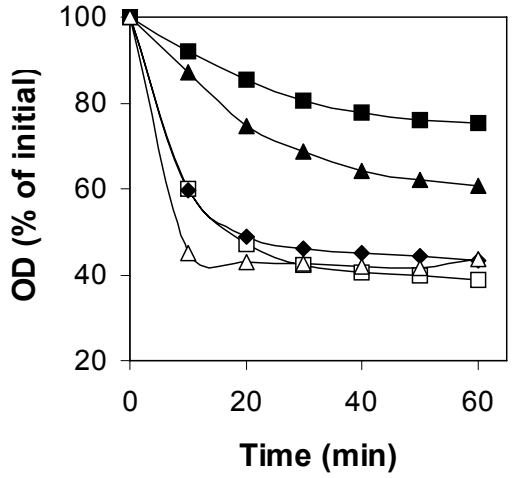


Fig. 4.3. Germination of mutant spores in BHI broth. Heat activated spores of *C. perfringens* strains SM101 (wild-type) (open squares), DPS113 (*gerQ*) (filled diamonds), DPS116 (*gerO*) (filled triangles), DPS115 (*gerO gerQ*) (filled squares) and DPS116(pDP150) (*gerO* mutant complemented with wild-type *gerO*) (open triangles) were germinated at 40ºC in BHI broth and the OD_{600} was measured as described in Methods.

Fig 4.4: Germination of *C. perfringens* spores with various germinants.

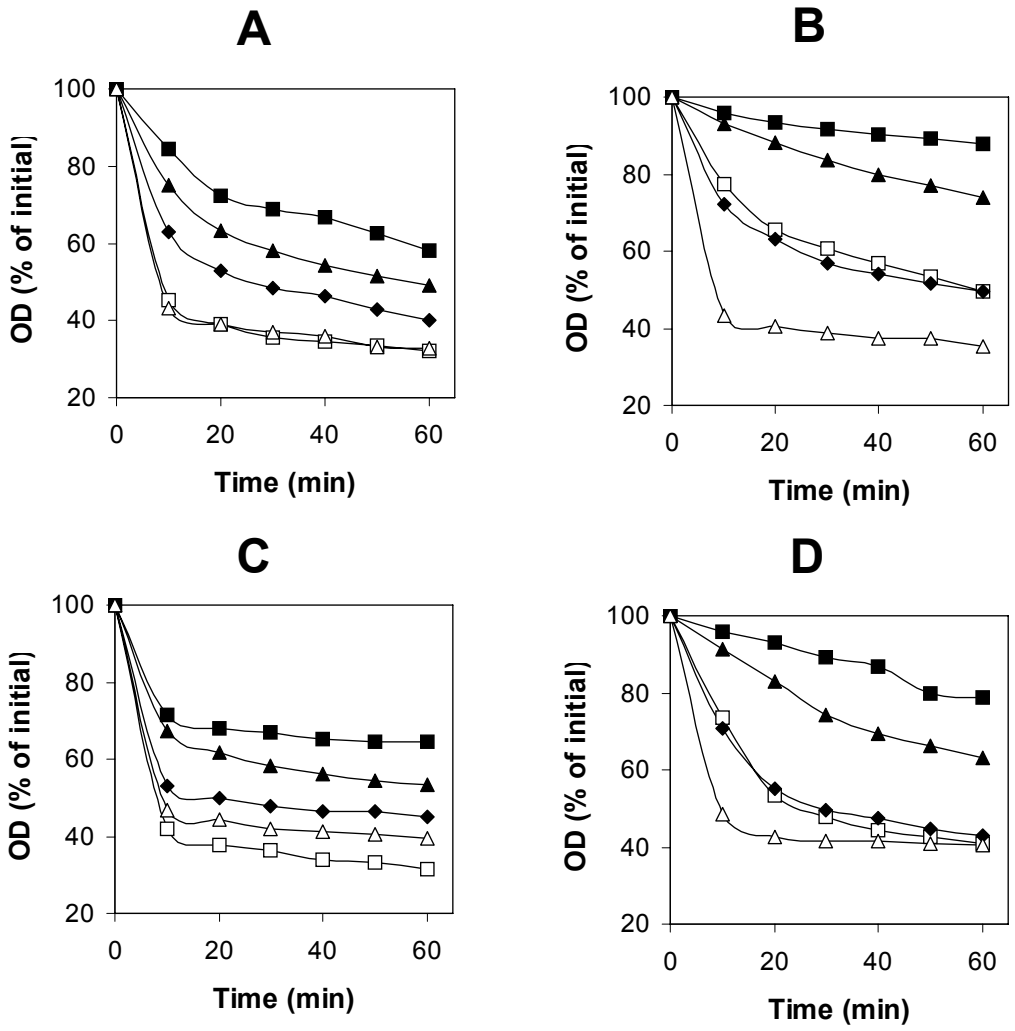


Fig. 4.4.A-D. Germination of *C. perfringens* spores with various germinants. Heat-activated *C. perfringens* spores of strains SM101 (wild-type) (open squares), DPS113 (*gerQ*) (filled diamonds), DPS116 (*gerO*) (filled triangles), DPS115 (*gerO gerQ*) (filled squares) and DPS116(pDP150) (*gerO* mutant complemented with wild-type *gerO*) (open triangles) were germinated at 40°C in 25 mM sodium phosphate buffer (pH 7.4) with: A) 100 mM KCl, B) 100 mM L-asparagine, or C) 100 mM L-asparagine and 100 mM KCl (AK), or at 40°C without sodium phosphate buffer in D) 50 mM Ca-DPA made to pH 8.0 with Tris base. At various times, the OD_{600} was measured as described in Methods.

Spores of various *C. perfringens* strains incubated in 25 mM sodium phosphate buffer (pH 7.0) at 40ºC exhibited less than 10% decrease in OD_{600} in 60 min (data not shown).

Fig 4.5: DPA release from *C. perfringens* spores incubated with various germinants.

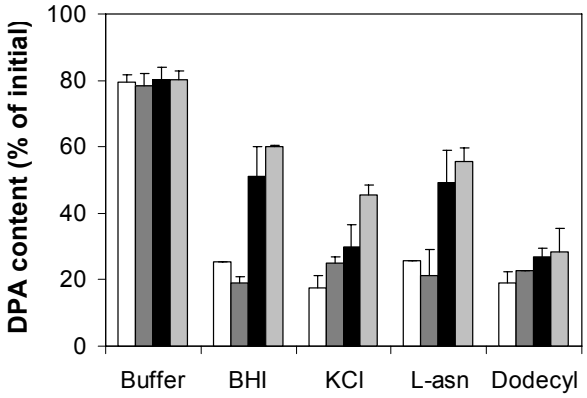


Fig. 4.5. DPA release from *C. perfringens* spores incubated with various germinants. Spores of *C. perfringens* strain SM101 (wild-type) (white bars), DPS113 (*gerQ*) (dark-grey bars), DPS116 (*gerO*) (black bars) and DPS115 (*gerO gerQ*) (light-grey bars) were germinated at 40ºC for 60 min with: Buffer, 25 mM sodium phosphate buffer (pH 7.0); BHI, BHI broth; KCl, 100 mM KCl - 25 mM sodium phosphate (pH 7.4); L-asn, 100 mM L-asparagine - 25 mM sodium phosphate (pH 7.4); or at 60ºC for 60 min with: Dodecyl, 1 mM dodecylamine - 25 mM Tris-HCl (pH 7.4), and DPA release was measured as described in Methods.

Fig 4.6: Outgrowth of spores of *C. perfringens* strains.

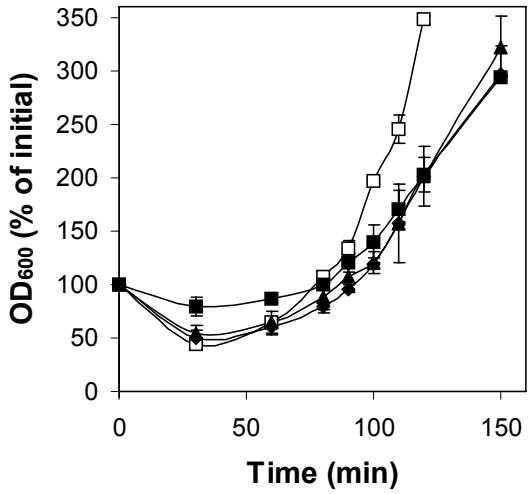


Fig. 4.6. Outgrowth of spores of *C. perfringens* strains. Heat activated spores of strains SM101 (wild-type) (open squares), DPS113 (*gerQ*) (filled diamonds), DPS116 (*gerO*) (filled triangles) and DPS115 (*gerO gerQ*) (filled squares) were incubated anaerobically in TGY vegetative medium at 37ºC and the OD_{600} was monitored as described in Methods.

Fig 4.7: Effects of D161N and D162N subtitutions on GerO function during *C. perfringens* spore germination.

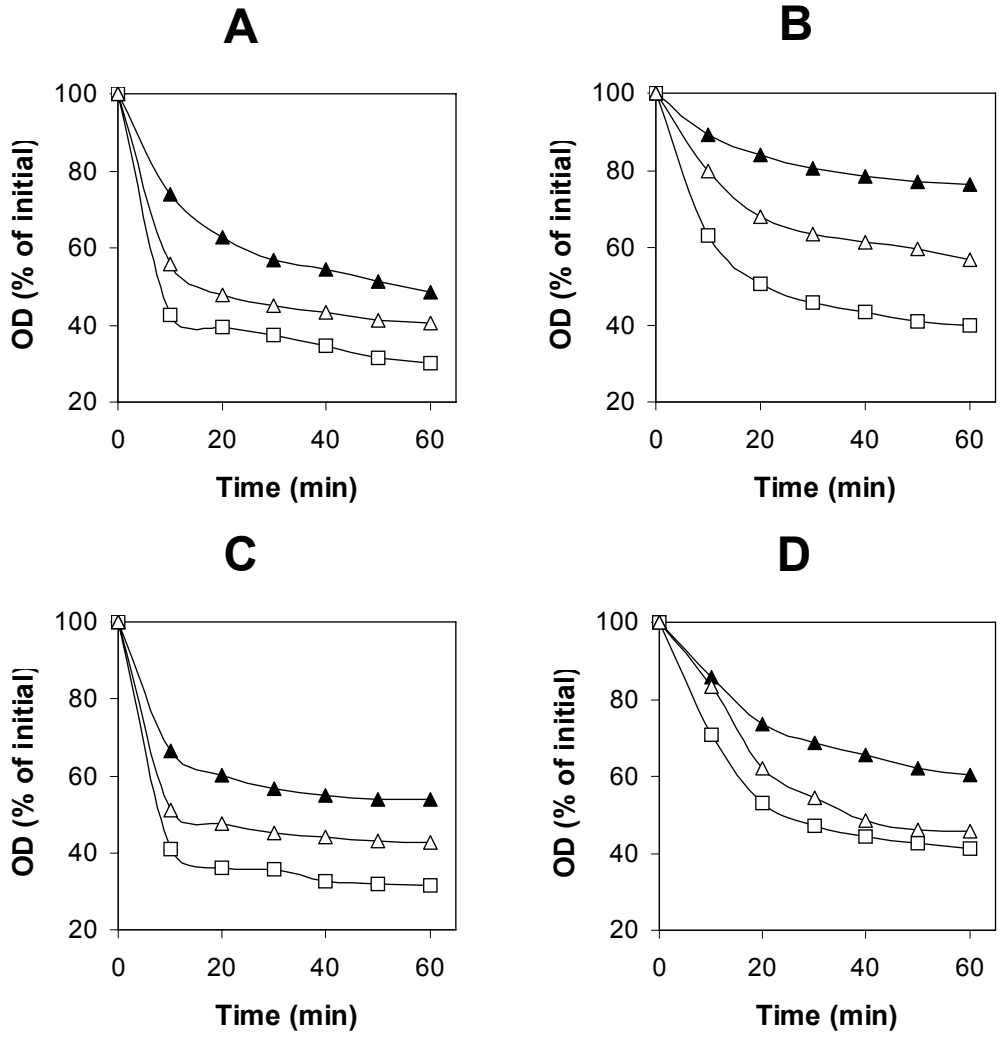


Fig. 4.7. A-D. Effects of D161N and D162N subtitutions on GerO function during *C. perfringens* spore germination. Heat activated spores of strains SM101 (wild-type) (open squares), DPS116 (*gerO*) (filled triangles), and DPS116(pDP172) (*gerO* mutant complemented with $gerO^{D161N,D162N}$) (open triangles) were germinated at 40ºC with: A) 100 mM KCl - 25 mM sodium phosphate (pH 7.4); B) 100 mM L-asparagine - 25 mM sodium phosphate (pH 7.4); C) AK – 25 mM sodium phosphate (pH 7.4); and D) 50 mM Ca-DPA made to pH 8.0 with Tris base; and the OD_{600} was measured as described in Methods.

.

Table 4.1. Maximum germination rates of spores of various *C. perfringens* strains

Strain (genotype)	Maximum rate of spore germination with the indicated germinant[a]			
	KCl	L-asparagine	AK	Ca-DPA
SM101 (wild-type)	100	100	100	100
DPS113 (*gerQ*)	85	97	71	101
DPS116 (*gerO*)	46	25	40	27
DPS115 (*gerO gerQ*)	37	25	40	21
DPS116 [Δ*gerO gerO* (D161N, D162N)]	67	70	82	52

[a] Spores were germinated at 40°C with 100 mM KCl, 100 mM L-asparagine, or 100 mM KCl and 100 mM L-asparagine (AK) each in 25 mM sodium phosphate (pH 7.0), or in 50 mM Ca-DPA made to pH 8.0 with Tris base, and maximum rates of spore germination were determined as percentage change in OD_{600}/min. All values are given relative to the value for SM101 spores with the respective germinant, and this latter value was set at 100.

4.6 Supplementary Information

Homology Modeling

Homology modeling of *C. perfringens* GerO and GerQ based on X-ray structure of the *E. coli* NhaA Na^+-H^+ antiporter. Amino acid sequence alignment between *C. perfringens* GerO and GerQ with *B. megaterium* ATCC 12872 GrmA and *B. cereus* ATCC10876 GerT and GerN reveals sequence identities of 20 to 33%, and sequence homologies of 43% to 73% (data not shown). Although BLASTP analysis shows no significant amino-acid sequence identity between either *C. perfringens* GerO or GerQ with NhaA from *E. coli* K12, *Helicobacter pylori* 26695 and *Vibrio parahaemolyticus* RIMD 2210633, a highly conserved cluster of residues involved in the cation-translocation machinery of *E. coli* NhaA is conserved in GerO, GerN, GerT and GrmA, but not in GerQ (Fig. S1). In *E. coli* NhaA Thr132, Asp133, Asp163 and Asp164 form the putative Na^+-binding site (70). These residues are also conserved in *H. pylori* and *V. parahaemolyticus* NhaA, and Asp163 and Asp164 are both essential for the function of *E. coli* and *H. pylori* NhaA (75, 94). While Asp133 is a Ser in *C. perfringens* GerO, *B. cereus* GerN and GerT and *B. megaterium* GrmA (Fig. S1), collectively, these findings suggest that likely germination-related antiporters of spore-forming bacteria possess a similar cation-translocation mechanism.

The *gerO* ORF is predicted to encode a 399 aa protein with 11 predicted transmembrane segments (TMS), while *gerQ* is predicted to encode a 484 aa protein with 13 predicted TMS. To gain more insight into the role of highly conserved residues of GerO (Fig. S1), homology template-based structures were constructed.

First, possible templates for Template-based modeling were searched at the META server (20), a portal to the several fold recognition method (20) and to the 3D Jury consensus method, where J-scores over 50 are highly significant (53). Both putative antiporters from *C. perfringens* SM101 were predicted to have a single match scoring above the 3D-Jury confidence threshold of 50 (53), and this match was to the crystal structure of the *E. coli* Na^+/H^+ antiporter, NhaA. Template-based modeling of the putative *C. perfringens* antiporters was carried out with Modeller (189). Significant predicted protein structures were obtained for GerO and GerQ with template modeling scores (TM-scores) (255) of 0.44 and 0.42, respectively. The predicted TMS were consistent with secondary structure predictions by PsiPred (84) at the Psipred server (http://bioinfo.cs.ucl.ac.uk/) (19) and HHpred (221) (server at http://toolkit.tuebingen.mpg.de/hhpred). Possible angular outliers of the predictive models of GerO and GerQ were calculated with RAMPAGE (107) (available on the RAMPAGE server at http://mordred.bioc.cam.ac.uk/~rapper/rampage.php). Ramachandran plots for the predicted models of GerO and GerQ suggest that 85-89% of the residues are in the favored region, 8.6-9.8% in the allowed region and 2.8-5.3% in the outlier region, results that were similar to Ramachandran plots of the NhaA antiporter crystal structure (pdb code, 1cdz) (data not shown). Although the RMSD and the TM-scores suggest that the predicted models are not reliable for drug-design experiments, they are sufficient to provide significant functional information (see below).

The predicted homology structures of GerO and GerQ show two discontinuous membrane helices (Fig. S2; TMS IV and X), similar to those observed in NhaA and in

the solved structures of other transporters (142). These discontinuous TMS are unusual α-helical structural elements conserved in various ion translocating transport proteins (196), and are defined as a "short helix-extended polypeptide chain-short helix" motif, crossing each other at the level of the extended peptides, where the partially charged short helices of both α-helices combine in an intertwined manner (196). In NhaA, these extended peptides with their backbone atoms, the helix termini and the polar/charged amino acid residues in close vicinity provide the basis for Na^+-H^+ recognition, binding and translocation (70). In *C. perfringens* GerO, the extended polypeptide chain of TMS X has positively charged Arg340 and negatively charged Glu342 (Fig. S2), suggesting that these residues might have a role in ion transport. Another striking finding is that residues Asp163 and Asp164 (Asp161 and Asp162 in GerO) that are involved in ion binding and translocation in NhaA (70), are predicted to be located in TMS V of both NhaA and GerO (Fig. S2). However, GerQ, as noted above, lacks these functionally important residues. Together, these results are consistent with *C. perfringens* GerO functioning as an ion transporter, while GerQ might have less activity in moving cations.

Supplementary Tables

Table S4.1. Bacterial strains and plasmids used

Strain or Plasmid	Relevant characteristic(s)[a]	Reference or Source
C. perfringens		
SM101	Electroporatable derivative of food poisoning type A isolate, NCTC8798; carries a chromosomal *cpe*	(256)
DPS113	*gerQ* mutant (*gerQ*::*catP*) derivative of SM101	This study
DPS115	*gerO gerQ* mutant (*gerO*::*tetM*, *gerQ*::*catP*) derivative of SM101	This study

DPS116	*gerO* mutant (*gerO*::*tetM*) derivative of SM101	This study
SM101(pDP81)	Wild-type strain carrying *gerO-gusA* fusion	This study
SM101(pDP82)	Wild-type strain carrying *gerQ-gusA* fusion	This study
DPS116(pDP150)	*gerO* mutant (*gerO*::*tetM*) complemented with wild-type *gerO*	This study
SM101(pDP152)	Wild-type strain carrying *gerQ-gfp* fusion	This study
DPS116(pDP172)	*gerO* mutant (*gerO::tetM*) carrying $gerO^{D161N, D162N}$	This study
SM101(pDP182)	Wild-type strain carrying *gerO-gfp* fusion	This study
E. coli		
TK2420	Δ*kdpABC* Δ*trkD1* Δ*trkA*	(57)
KNabc	Δ*cha* Δ*nhaA* Δ*nhaB*; Em^r, Cm^r, Kan^r.	(135)
TK2420(pGEM3zf(+))	TK2420 with plasmid pGEM3zf(+)	This study
TK2420(pDP144)	TK2420 carrying *gerO*	This study
TK2420(pDP147)	TK2420 carrying *gerQ* with modified RBS	This study
TK2420(pDP148)	TK2420 carrying *gerQ* with native RBS	This study
TK2420(pDP173)	TK2420 carrying $gerO^{D161N, D162N}$	This study
KNabc(pGEM3zf(+))	KNabc with plasmid pGEM3zf(+)	This study
KNabc(pDP144)	KNabc carrying *gerO*	This study
KNabc(pDP147)	KNabc carrying *gerQ* with modified RBS	This study
KNabc(pDP148)	KNabc carrying *gerQ* with native RBS	This study
KNabc(pDP173)	KNabc carrying $gerO^{D161N, D162N}$	This study
Plasmids		
pMRS104	No origin of replication for *C. perfringens*; Em^r	(67)
pMRS127	*C. perfringens/E. coli* shuttle vector carrying a promoterless *gusA*; Em^r	(174)

pEGFP	pEGFP carrying EGFP gene, Amp^r	ClonTech™
pET16b	*E. coli* overexpressing vector, Amp^r	Novagen™
pGEM3zf(+)	*E. coli* vevtor, phage f1 ori, Amp^r	Promega™
pDP25	pCR-XL-TOPO carrying chloramphenicol resistance gene (*catP*); Cm^r	(156)
pDP35	pCR-XL-TOPO carrying tetracycline resistance gene (*tetM*); Tet^r	(153)
pDP81	402-bp PCR fragment upstream of *gerO* in pMRS127	This study
pDP82	434-bp PCR fragment upstream of *gerQ* in pMRS127	This study
pDP96	1070-bp PCR fragment containing 865-bp upstream and 205-bp of the N-terminal-coding region of *gerO* in pCR-XL-TOPO	This study
pDP97	1227-bp PCR fragment containing 246-bp of the C-terminal-coding region and 981-bp downstream of *gerO* in pCR-XL-TOPO	This study
pDP98	1.1-kb KpnI-SpeI fragment from pDP96 cloned between KpnI/SpeI sites of pDP25	This study
pDP99	1.2-kb PstI-XhoI fragment from pDP97 cloned between PstI/XhoI sites of pDP98	This study
pDP100	3.7-kb KpnI-XhoI fragment from pDP99 cloned between KpnI/SalI sites of pMRS104	This study
pDP101	832-bp PCR fragment containing 583-bp upstream and 249-bp of the N-terminal-coding region of *gerQ* in pCR-XL-TOPO	This study
pDP102	1312-bp PCR fragment containing 80-bp of the C-terminal-coding region and 1232-bp downstream of *gerQ* in pCR-XL-TOPO	This study
pDP103	0.8-kb KpnI-SpeI fragment from pDP101 cloned between KpnI/SpeI sites of pDP25	This study
pDP104	1.3-kb PstI-XhoI fragment from pDP102 cloned between PstI/XhoI sites of pDP103	This study
pDP105	3.5-kb KpnI-XhoI fragment from pDP104 cloned between KpnI/SalI sites of pMRS104	This study
pDP112	3.2-kb SpeI-PstI fragment carrying *tetM* from pDP35 cloned between SpeI-PstI sites of pDP98	This study
pDP129	Δ*plc* locus; no origin of replication for *C. perfringens*; Em^r.	(156)
pDP141	1.4-kb PCR fragment containing *gerO* and its native RBS in Zero-Blunt TOPO	This study
pDP144	1.4-kb KpnI-PstI fragment from pDP141 cloned between KpnI/PstI sites of pGEM3f(+)	This study
pDP147	1.6-kb PCR fragment containing *gerQ* with modified RBS cloned between KpnI/PstI sites of pGEM3f(+)	This study

pDP148	1.6-kb PCR fragment containing *gerQ* with native RBS cloned between KpnI/PstI sites of pGEM3f(+)	This study
pDP149	715-bp PCR fragment carrying *gfp* in Zero-Blunt TOPO	This study
pDP150	1.8-kb PCR fragment containing 396-bp upstream and coding region of *gerO* cloned between KpnI/SalI sites of pDP129.	This study
pDP151	720-bp XbaI-BamHI fragment containing *gfp* from pDP149 cloned between XbaI/BamHI sites of pET16b	This study
pDP152	1023-bp PstI-HindIII fragment from pDP151 cloned between PstI/HindIII sites of pDP81	This study
pDP167	897-bp PCR fragment carrying 697–bp of C-terminal-coding region and 182-bp downstream of *gerO* cloned in Zero-Blunt TOPO	This study
pDP169	902-bp PCR fragment carrying 396-bp upstream and 509-bp of the N-terminal-coding region of *gerO* cloned into Zero-Blunt TOPO	This study
pDP170	0.9-kb KpnI-HpaI fragment from pDP169 cloned between KpnI/HpaI sites of pDP25	This study
pDP171	0.8-kb HpaI-PstI fragment from pDP167 cloned between HpaI/PstI sites of pDP170	This study
pDP172	1.6-kb KpnI-XhoI fragment from pDP171 cloned between KpnI/SalI sites of pDP129	This study
pDP173	1.2-kb PCR product carrying mutated *gerO* cloned between KpnI/PstI sites of pGEM3f(+)	This study
pDP182	433-bp PCR fragment carrying 427-bp upstream and 6-bp of the N-terminal-coding region of *gerQ* cloned between SalI/PstI sites of pDP152	This study

[a]Abbreviations used are: RBS - ribosome binding site; Amp^r – resistance to ampicillin (100 μg/ml); Cm^r – resistance to chloramphenicol (20 μg/ml); Em^r – resistance to erythromycin (50 μg/ ml); and Tet^r – resistance to tetracycline (2 μg/ml).

Table S4.2. Primers used in this study

Primer name	Primer sequence[a]	Position[b]	Gene	Use[c]
CPP380	GCAGCGTCGACGTTACAGGAATAGAGATGAATAACTT	-427 to -401	*gerQ*	GUS
CPP383	GCAGCGTCGACGCATCTAAAGCATTCTCAGCTGT	-396 to -373	*gerO*	GUS
CPP385	GACGCTGCAGCATTCATAAATAAATCATCTCTTTTCTTTAG	-24 to +7	*gerQ*	GUS
CPP386	GACGCTGCAGTATCATTACAAATCCTCCCTCATATAAT	-28 to +6	*gerO*	GUS
CPP406	GCGGGTACCCTACAGCCCTAGTTGTTCCTTCAAAG	-865 to -840	*gerO*	MP
CPP408	CGGACTAGTCACATAAGTACTATAACACCTAATTCTGCAAG	+174 to +205	*gerO*	MP
CPP410	GCACCTCGAGGGTTGCTGCTTTAGGAATAAAAAGTTTAGATG	+2656 to +2687	*gerQ*	MP
CPP411	GACGCTGCAGCTCCTTGCCGGAGTTAAGCATGATG	+1375 to +1399	*gerQ*	MP
CPP415	GCGGGTACCCGAATCTCTACATTACAATAGGTATGAACCTAC	-583 to -551	*gerQ*	MP
CPP417	CGGACTAGTCAAGCTCATCAAAGTCTATAGCAAGACC	+222 to +249	*gerQ*	MP
CPP424	GACGCTGCAGCGGAGCTAAGATATGTAGGTATAGTAATAT	+954 to +983	*gerO*	MP
CPP430	GCACCTCGAGGGTCTGCTGCTTTGTATCATAGG	+2159 to +2181	*gerO*	MP
CPP523	GATGGTACCATGTATATTATATGAGGGAGGATTTGTA**ATG**ATA	-28 to +6	*gerO*	CPcat
CPP524	GATCTGCAGTGCTCCCGCAACCACAAAAGG	+1361 to +1382	*gerO*	CPcat, SDM
CPP525	GATGGTACCCTAAAGAAAAGAGATGATTTATTT**ATG**AATG	-24 to +7	*gerQ*	CPcat
CPP526	GATCTGCAGCTCAGCTTTTTAACGTGATATTTTCTACG	+1537 to +1566	*gerQ*	CPcat
CPP533	GATGGTACCATGA*GGAGGGGA*T**ATG**AATGAAACTATAAATTATGATTCATTGTTAATC	-11 to +36	*gerQ*	CPcat
CPP599	GCGGTACCGCATCTAAAGCATTCTCAGCTGTTATAG	-396 to -373	*gerO*	CP, SDM
CPP600	GCGTCGACTGCTCCCGCAACCACAAAAGG	+1361 to +1382	*gerO*	CP
CPP602	TCTAGACTGCAGACTAGTGTGAGCAAGGGCGAGGAGCTGTT	+3 to +26	*gfp*	GFP
CPP603	GGATCCTTACTTGTACAGCTCGTCCATGCCGAG	+693 to +718	*gfp*	GFP
CPP662	GACGTTAACGCTATTATTCCTAAAATATTATTAATAATCGCAG	+469 to +509	*gerO*	SDM
CPP663	GACGTTAACCATAACAACTAGCTTAGCAGATC	+503 to +432	*gerO*	SDM
CPP676	GACCTGCAGATTCATAAATAAATCATCTCTTTTCTTTAG	-24 to +6	*gerQ*	GFP

[a] Restriction sites are marked by underlining, and translation start codons are emboldened. Note that there are three consecutive restriction sites in CPP602.

[b] The nucleotide numbering begins from the translation start codon and refers to the relevant position within the respective coding sequence.

[c] MP, construction of mutator plasmid; GUS, construction of *gusA* fusion plasmid; CP, construction of complementing plasmid; GFP, construction of *gfp* fusion plasmid; SDM, site directed mutagenesis of *gerO*; CPcat, construction of complementing plasmid for *E. coli* mutants; italicized bases in CPP533 correspond to substituted bases at positions -7 to -2 to provide a better translation start region for *gerQ*

Supplemental Figures

Fig. S4.1: Amino acid sequence alignment of Na^+/H^+ antiporter associated domains.

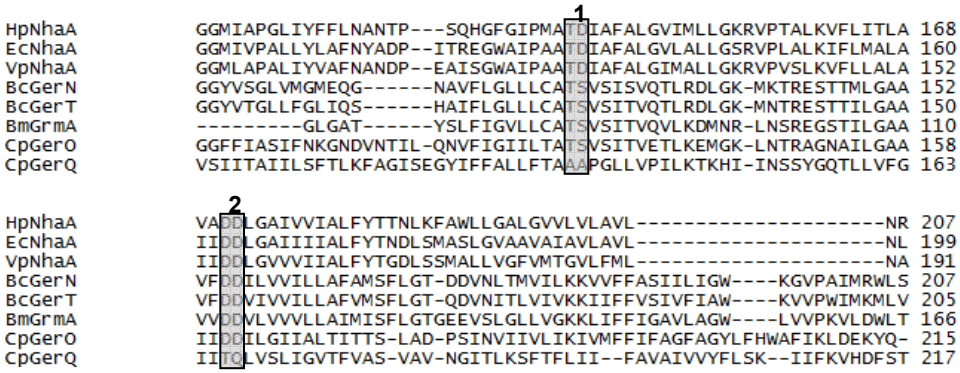


Fig. S4.1. Amino acid sequence alignment of Na^+/H^+ antiporter associated domains. The alignment was generated by use of T-Coffee (134). The grey boxes show the highly conserved amino acid residues Thr132, Asp133 (grey box 1) and Asp163, Asp164 (grey box 2), the latter being adjacent Asp residues involved in the Na^+/H^+-translocation machinery of *E. coli* NhaA (75). Bacterial species, proteins and accession numbers shown are: HpNhaA, *Helicobacter pylori* 26695 NhaA, NP_208343; EcNhaA, *E. coli* K12 NhaA, YP_001729002; VpNhaA, *Vibrio parahaemolyticus* NhaA RIMD 2210633; BcGerN, *B. cereus* ATCC10876 GerN, AF_246294; BcGerT, *Bacillus cereus* ATCC10876 GerT, EU_789572; BmGrmA, *B. megaterium* ATCC 12872 GrmA, AAB40041; CpGerO, *C. perfringens* GerO, YP_697562; CpGerQ, *C. perfringens* GerQ, YP_698361.

Fig. S4.2: Template-based homology models of *C. perfringens* GerO and GerQ with *E. coli* NhaA.

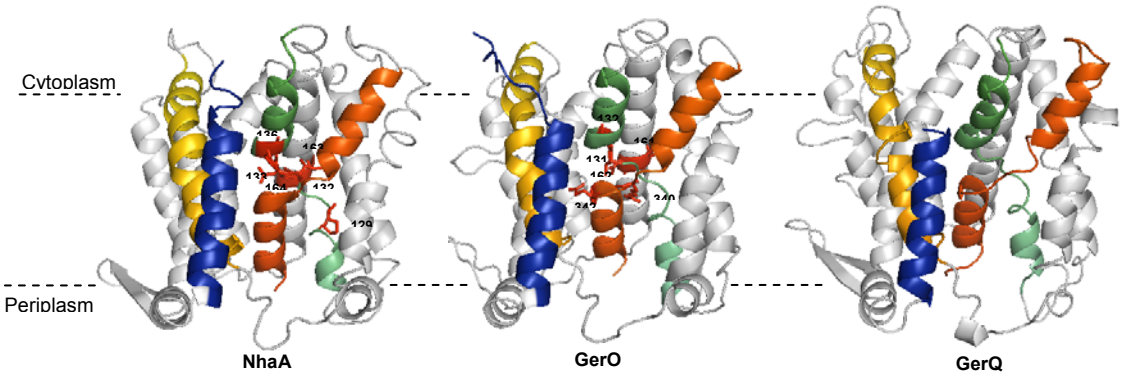


Fig. S4.2. Template-based homology models of *C. perfringens* GerO and GerQ with *E. coli* NhaA. The homology models were prepared using Pymol (http://pymol.sourceforge.net/). Transmembrane segment (TMS) XII is removed to show the functional organization of the interrupted helices TMS IV-XI of *E. coli* NhaA and *C. perfringens* GerO and GerQ. The overall root mean square deviation (RMSD) (86) between NhaA and GerO and GerQ is 11.2 Å and 14.0 Å, respectively. A stick-and-ball representation of functionally important residues of NhaA in the putative active site (black) is shown. Highly conserved residues that likely have functional roles in GerO and GerQ are shown.

Chapter 5

Inorganic phosphate induces germination of spores of *Clostridium perfringens* type A food poisoning isolates.

Daniel Paredes-Sabja*, Pathima Udompijitkul* and Mahfuzur R. Sarker

*These authors contributed equally to this manuscript

Published in:

Applied and Environmental Microbiology: **Submitted for Publication**

5.1 Abstract

Clostridium perfringens type A isolates carrying a chromosomal copy of the enterotoxin (*cpe*) gene are involved in the majority of food poisoning (FP) outbreaks, while type A isolates carrying plasmid-borne *cpe* gene are involved in *C. perfringens*-associated non-food-borne (NFB) gastrointestinal (GI) diseases. To cause diseases, *C. perfringens* spores must germinate and return to active growth. Previously, we showed that only spores of FP isolates were able to germinate with K^+ ions. We now found that the spores of the majority of FP isolates, but none of the NFB isolates, germinated with inorganic phosphate (Pi) at pH 6.0. Spores of *gerKA-KC* and *gerAA* mutant germinated slower and released less DPA than wild-type spores with Pi. Although *gerKB* spores germinated to a similar extent as wild-type spores with Pi, their rate of germination was slower. Similarly, *gerO* and *gerO gerQ* mutant spores germinated slower and released less DPA than wild-type spores with Pi. In contrast, *gerQ* spores germinated slightly slower than wild-type spores, but released all of their DPA during Pi germination. In sum, this study identified Pi as a novel nutrient germinant for spores of most FP isolates and provided evidence that proteins encoded by the *gerKA-KC* operon, *gerAA*, and *gerO* are required for Pi-induced spore germination.

5.2 Introduction

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming and pathogenic bacterium that causes a wide array of gastrointestinal (GI) diseases in both animals and humans (117, 119). However, *Clostridium perfringens* type A food poisoning (FP) is the most common *C. perfringens* associated illnesses among

humans, and is currently ranked as the 3rd most commonly reported food borne-disease (117). Only type A isolates that produce the *C. perfringens* enterotoxin (CPE) have been associated with *C. perfringens* GI-illnesses (117). *C. perfringens cpe*-positive isolates can carry the *cpe* gene on either the chromosome or a plasmid (32, 33). Interestingly, the majority of *C. perfringens* type A FP isolates carry a chromosomal copy of the *cpe* gene, while all non-food-borne (NFB) GI disease isolates carry a plasmid copy of the *cpe* (32, 33, 97, 225). The genetic differences involved in the pathogenesis differences between *C. perfringens* FP versus NFB isolates seems to involve more factors than the simple location of the *cpe* gene. For example, spores of FP isolates are strikingly more resistant than spores of NFB isolates to heat (193), cold (4ºC) and freezing (-20ºC) temperatures (103), and chemicals used in food industry settings (102); making FP spores more suited for FP environments than NFB spores. However, to cause diseases, these dormant spores, under favorable environmental conditions, germinate to return back to life, proliferate to high numbers and then produce toxins to cause disease (117).

Bacterial spores germinate when they sense the presence of nutrients (termed germinants) in the environment through their cognate receptors located in the spore's inner membrane (147). In *C. perfringens*, some nutrients that initiate germination include L-asparagine, KCl, a mixture of L-asparagine and KCl (AK), a 1:1 chelate of Ca^{2+} and dipicolinic acid (DPA) (Ca-DPA) (154). The main receptor involved in sensing these compounds is the GerKA and/or GerKC receptor(s) which is required for L-asparagine and Ca-DPA, and only partially required for KCl and AK (154, 157). Upon binding of the germinant to their cognate receptor, a variety of biophysical

events take place, including the release of monovalent ions (i.e., Na^+, K^+ and Li^+) followed by the release of the spore's large depot of Ca-DPA (207). In *Bacillus subtilis*, release of Ca-DPA acts as signal for activation of the cortex-lytic enzyme (CLE) CwlJ (146). In contrast, Ca-DPA release from the spore core has no role in triggering cortex hydrolysis during *C. perfringens* spore germination (153, 156, 159); instead, Ca-DPA induces germination via GerKA and/or GerKC receptor(s) (154, 157). Degradation of the cortex in both species leads to hydration of the spore core up to levels found in growing bacteria, allowing resumption of enzymatic activity and metabolism, and consequently outgrowth (156, 207).

The ability of bacterial spores to sense different nutrients appears to be tightly regulated by their adaptation to different environmental niches. For example, spores of FP, but not NFB isolates, are capable of germinating with KCl (154), an intrinsic mineral of meats that are most commonly associated with FP, suggesting an adaptation of FP isolates to FP environments. In addition, the level of inorganic phosphate (Pi) is also significant in meat products (42-60 mM) (USDA; http://www.nal.usda.gov/fnic/food-comp/search/). Consequently, in this study we found that Pi at pH 6.0 is a unique germinant of spores of *C. perfringens* type A FP isolates and acts through the GerKA and/or GerKC and GerAA receptors, and also requires the presence of the putative Na^+/K^+-H^+ antiporter, GerO, for normal germination.

5.3 Materials and methods

Bacterial strains and plasmids. *C. perfringens* isolates used in this study included the following. Six FP isolates carrying a chromosomal *cpe* gene: SM101 (electroporatable derivative of FP type A, NCTC8798) (256), NCTC8798 (37), NCTC10239, E13, FD1041 (32) and 6263 (58). Three NFB isolates carrying a plasmid copy of the *cpe* gene: F4969, NB16 and B40 (32). One type C *cpe*-negative isolate: JGS1495 (from diarrheic pig) (47). *C. perfringens* SM101 germinant mutants: DPS101 (*gerKA-KC*) and DPS103 (*gerAA*) (154), DPS108 (*gerKB*) (157). *C. perfringens* SM101 antiporter mutants: DPS113 (*gerQ*), DPS116 (*gerO*) and DPS115 (*gerO gerQ*) (158).

Spore preparation and purification. Starter cultures (10 ml) of *C. perfringens* isolates were prepared by overnight growth at 37°C in fluid thioglycollate broth (FTG) (Difco) as described (91). Sporulating cultures of *C. perfringens* were prepared by inoculating 0.2 ml of an FTG starter culture into 10 ml of Duncan-Strong (DS) sporulating medium (36); this culture was incubated for 24 h at 37ºC to form spores as confirmed by phase-contrast microscopy. Spore preparations were prepared by scaling-up the latter procedure. Spore preparations were cleaned by repeated centrifugation and washing with sterile distilled water until spores were > 99% free of sporulating cells, cell debris and germinated spores, and suspended in distilled water at a final OD_{600} of ~ 6 and stored at -20ºC (154).

Germination assays. Spores at an OD_{600} of ~ 1.0 were routinely heat shocked (80ºC; 10 min) and cooled in water at ambient temperature and germinated with 100 mM

sodium phosphate (pH 6.0), 100 mM citric acid (pH 6.0), 100 mM 3-morpholinopropanesulfonic acid (pH 6.0), or 100 mM maleic acid (pH 6.0) as previously described (154). Spore germination was routinely measured by monitoring the OD_{600} of spore cultures (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA), which falls ~ 60% upon complete spore germination and levels of germination were confirmed by phase-contrast microscopy. All values reported are averages of two experiments performed on at least two independent spore preparations, and individual values varied by less than 10% from average values shown. The maximum rates of spore germination were determined by measuring the OD_{600} of germinating cultures every 2.5 min, the maximum slopes calculated and maximum rates were expressed as the maximum rate of loss in OD_{600} of the spore suspension relative to the initial OD_{600} of the culture.

DPA release. DPA release during Pi-germination was measured by incubating a heat activated spore suspension of an OD_{600} of 1.0 at 40ºC with 250 mM sodium phosphate (pH 6.0). Aliquots (1 ml) of germinating cultures were centrifuged for 3 min in a microcentrifuge and DPA in the supernatant fluid was measured by monitoring the OD_{270} as described previously (21, 154). To measure the total spore DPA content, a 1 ml aliquot of the germination incubations was boiled for 60 min, centrifuged for 5 min in a microcentrifuge, and the OD_{270} of the supernatant fluid was measured (21, 154). In *C. perfringens*, ~ 90% of the material absorbing at 270 nm comprises DPA (156).

5.4 Results

Germination of *C. perfringens* spores with Pi. In our previous study we observed that 25 mM sodium phosphate (pH 7.0) was able to induce little germination of *C. perfringens* spores (154). To evaluate if Pi indeed could induce spore germination we assayed germination of spores of SM101 (a FP isolate) and F4969 (a NFB isolate) with various concentration of Pi at various pH. Surprisingly, Pi (100 mM) was able to induce germination of SM101 spores between a pH range of 5.5 to 6.5, but not at below or higher than 5.0 and 7.0, respectively (Fig. 5.1A). Indeed, phase contrast microscopy indicates that > 90% of SM101 spores had become phase dark when germinated with Pi between a pH range of 5,5 – 6.5 (data not shown). However, Pi was unable to induce germination of F4969 spores at all tested pH (Fig. 5.1A,B), confirmed by phase contrast microscopy showing that 100% of F4969 spores germinated with 100 mM Pi (at various pH) remained phase bright (data not shown).

SM101 spores exhibited a maximum rate of germination with 250 mM Pi at pH 6.0 (Fig. 5.1C). Interestingly, SM101 spores were able to germinate significantly after 60 min with Pi concentrations as low as 20 mM (Fig. 5.1D), where phase contrast microscopy showed that ~ 85% of the spores had become phase dark. In contrast, F4969 spores showed no significant increase in their maximum rate of germination (Fig. 5.1C), and no significant extent of germination with the exception of a small but significant decrease in OD_{600} at 400 mM (Fig. 5.1D). Phase contrast microscopy of F4969 spores germinated with Pi concentrations lower than 250 mM showed that > 99% spores remained phase bright, however, with 400 mM ~ 10 % and ~ 30% of the spores had become phase dark and grey, respectively, after 60 min of incubation.

Collectively, these results suggest that Pi at pH 6.0 is a unique germinant for spores of SM101 but not for F4969.

To discriminate if the ability of SM101 spores to germinate with Pi (pH 6.0) is due to Pi or pH 6.0 in the absence of Pi, we evaluated various organic and inorganic buffers. As expected, SM101 spores, but not F4969 spores, germinated well with Pi (pH 6.0); however, little to insignificant germination was observed when SM101 spores were incubated with 100 mM of other buffers at pH 6.0 (Fig. 5.2). In addition, F4969 spores also exhibited no germination with any of the buffer tested (Fig. 5.2). Phase contrast microscopy also indicated that > 90% of SM101 and > 99% of F4969 spores remained phase bright after 60 min of incubation with tested organic and inorganic buffers without Pi (data not shown). These results confirmed that the germination phenotype of SM101 spores is uniquely induced by Pi (pH 6.0).

The Pi-induced germination of spores of SM101 is not due to domestication and manipulation of this laboratory strain, as spores of NCTC8798 (the parent strain of SM101) germinated similarly as SM101 spores with Pi (Fig. 5.3), suggesting that Pi (pH 6.0) might be a universal germinant for spores of *C. perfringens* FP isolates. To examine this hypothesis, germination experiments were extended to spores of 7 additional *C. perfringens* isolates including, four type A FP isolates (i.e., E13, NCTC10239, FD1041 and 6263), two NFB isolates (NB16 and B40) and one *cpe*-negative type C isolate (JGS1495). Strikingly, as observed with spores of SM101 (Fig. 5.1A-C) spores of all FP isolates with the exception of FD1041, were able to germinate well with Pi (pH 6.0) (Fig. 5.3). In contrast, spores of NFB isolates were unable to germinate with Pi (pH 6.0) when incubated up to 60 min (Fig. 5.3).

Although NFB spores required a lower heat activation temperature (75˚C, 10 min) (154), similar results were observed (i.e., no significant germination) with Pi-germination when NFB spores were heat activated at 75˚C for 10 min (data not shown). Interestingly, the negative Pi-germination phenotype is not confined to *cpe*-positive type A NFB human isolates, as spores of strain JGS1495 (a type C *cpe*-negative animal isolate) were also unable to germinate with Pi (pH 6.0) (Fig. 5.3). Collectively, these results clearly indicate that Pi (pH 6.0) is a unique germinant for spores of most FP isolates.

Role of germinant receptors on Pi germination of *C. perfringens* spores. To gain more insight into the mechanism of Pi-germination of *C. perfringens* spores, germination of spores of strains carrying mutations in germination receptor genes *gerKA-KC*, *gerKB* and *gerAA* (154, 157) were assayed using optimum (250 mM) Pi concentration (Fig. 5.1C). Although *gerKB* spores exhibited significantly ($p < 0.05$) less germination during the first 20 min than wild-type spores, their extent of germination after 60 min was similar (Fig. 5.4A). This was confirmed by phase contrast microscopy, where > 95% of wild-type and *gerKB* spores had become phase dark after 60 min of incubation with Pi (data not shown). In contrast, *gerAA* spores germinated slower and to a significantly ($p < 0.001$) lesser extent than wild-type and *gerKB* spores (Fig. 5.4A), as ~ 50% of *gerAA* spores had become phase dark after 60 min of incubation (data not shown). The germination defect in *gerKA-KC* spores was significantly ($p < 0.01$) more pronounced than in *gerAA* spores (Fig. 5.4A), with phase contrast microscopy results showing that ~ 35% of *gerKA-KC* spores had become

phase dark after 60 min of incubation with Pi (data not shown). *gerKA-KC* spores exhibited a maximum rate ~ 5-fold lower than that of wild-type spores and ~ 2-fold lower than that of *gerKB* and *gerAA* spores when germinated with Pi (Fig. 5.4B). However, *gerKB* and *gerAA* spores exhibited similar maximum rate, but this was ~ 2-fold lower when compared to that of wild-type spores (Fig. 5.4B). These results suggest that GerKA-KC and GerAA receptors are the major receptors involved in Pi-germination, and that GerKB is required for normal Pi-triggered germination.

In *Bacillus* and *Clostridium* spores, binding of germinants to specific receptors located in the spore's inner membrane triggers the release of the spore core's large depot of DPA as 1:1 chelete to Ca^{2+} (Ca-DPA) (154, 207). Consequently, to investigate if Pi also triggers DPA release through the Ger-receptors during *C. perfringens* spore germination, we measured DPA release during Pi germination. Consistent with the similarities observed in the extent of germination between *gerKB* and wild-type spores, *gerKB* spores also released almost similar level of DPA as wild-type spores (Fig. 5.4C). However, although *gerAA* spores released the majority of their DPA during Pi germination, the amount of DPA remaining in the spore core of *gerAA* spores was still slightly higher than that of wild-type spores, which is statistically significant ($p < 0.01$) (Fig. 5.4C). *gerKA-KC* spores released significantly ($p < 0.001$) less DPA than wild-type spores. The amount of DPA released by *gerKA-KC* spores was also significantly ($p < 0.05$) less than *gerKB* and *gerAA* spores. These results are consistent with above findings and confirmed that GerKA and/or GerKC and GerAA receptors plays roles in Pi-triggered germination, and with GerKB having an auxiliary role.

Role of antiporters on Pi germination of *C. perfringens* spores. Recently we have identified two putative antiporters (i.e., GerO and GerQ) involved in germination of *C. perfringens* spores (158); GerO, a putative Na^+/K^+-H^+ antiporter, is required for normal germination, while GerQ is required mainly in absence of GerO. When we assayed germination of *gerO* and *gerQ* spores in the presence of Pi (pH 6.0), *gerQ* spores exhibited a slightly slower and lesser extent of germination than that of wild-type spores (Fig. 5.5A). However, *gerO* spores germinated much slower and to a lesser extent than wild-type spores. Indeed, phase contrast microscopy indicated that only ~ 75% of *gerO* spores had become phase dark after 60 min of germination with Pi (pH 6.0) (data not shown). Interestingly, *gerO gerQ* spores had significantly ($p < 0.01$) slower germination than wild-type, *gerQ* and *gerO* spores (Fig. 5.5A), and phase contrast microscopy indicated that only ~ 30 % of *gerO gerQ* spores had become phase dark (data not shown). The *gerQ*, *gerO* and *gerO gerQ* spores exhibited respectively, 30, 70 and 80% lower maximum rate compared to that of wild-type (Fig. 5.5B). These results clearly suggest that GerO and GerQ, only in the absence of GerO, are essential for normal Pi-triggered germination.

Interestingly, *gerQ* spores were able to release similar amounts of DPA as wild-type spores after 60 min of incubation with Pi (Fig. 5.5C). To our surprise, *gerO* spores also released similar amounts of DPA as wild-type spores (Fig. 5.5C), although it is unclear why they germinated to a significantly lesser extent than wild-type spores (Fig. 5.5A and see discussions). However, *gerO gerQ* spores released significantly ($p < 0.001$) less DPA than wild-type, *gerO* and *gerQ* spores (Fig. 5.5C). These results

indicate that Pi-induced DPA release is affected only in the absence of both GerO and GerQ antiporters.

5.5 Discussion

The ability of spore of FP isolates to adapt in FP environments seems to be beyond the fact that spores of FP isolates exhibit ~ 60-fold higher heat resistance than spores of NFB isolates (193). The unique ability of FP isolates' spores to germinate with K^+ ions (154) might provide greater advantage over spores of NFB isolates present in meat products to germinate, outgrowth and proliferate. In this respect, and perhaps the major conclusion of this work is that Pi is a unique and novel germinant of spores of *C. perfringens* FP isolates. The Pi, together with K^+, which are intrinsic minerals found in meat and meat products, might provide spores of FP isolates significant advantages over spores of other *C. perfringens* isolates to germinate, outgrow and proliferate in meat products during inadequate processing of meat products, or subsequent warming. Several studies (82, 184) indicate that *C. perfringens* FP isolates belong to a different evolutionary lineage than all other *C. perfringens* isolates. Indeed, the differential germination phenotype of FP spores reinforces the theory that FP isolates have efficiently adapted to fit FP niches. However, it is unclear what genetic difference in the germination apparatus of FP versus NFB isolates might have evolved to produce this difference in K^+- and Pi-germination phenotype, since strain F4969 (a NFB isolate) has intact copies of all four germinant receptors' genes (*gerAA*, *gerKB*, and *gerKA-KC*), and showed > 95% identity to that of SM101 (130). One possibility that deserves further research is that

perhaps key residues within these receptor proteins might be essential for the unique Pi- and K^+-germination of FP isolates.

A second major conclusion is that Pi-germination of FP spores requires, although to different extents, all four Ger-receptors. The main germinant receptor proteins involved in Pi-germination are the GerKA and/or GerKC, which are also essential for L-asparagine germination and have a major role in KCl-germination (154). However, *gerKA-KC* spores are still able to germinate significantly with KCl (154), which is also the case with Pi-germination, suggesting that other Ger-proteins might have a role in Pi-germination. Indeed, GerAA has also a role in Pi-germination, with *gerAA* spores germinating much slower and to a lesser extent than wild-type spores (Fig. 5.4A) and this was not the case for KCl-germination (154). In contrast, GerKB, which was previously shown to have no role in nutrient-germination of FP spores (157), appears to have an important role for normal germination of FP spores with Pi. Clearly, the majority of *C. perfringens* Ger-receptors are involved in Pi germination suggesting the following scenarios. i) all *C. perfringens* Ger-receptors form a complex-receptor; or, ii) each Ger-receptor is capable of individually sensing Pi with different levels of sensitivity. Further biochemical studies on receptor proteins should help clarifying these scenarios.

A third notable conclusion is that the putative antiporters GerO and GerQ, only in the absence of GerO, are required for normal Pi-germination of *C. perfringens* spores. Our previous study showed that these antiportes are also essential for normal germination of *C. perfrinegns* spores with various germinants, and that their role precedes DPA release (158). Interestingly, GerO was also essential for normal Pi-

germination; however, in contrast to *gerO* spores releasing significantly less DPA than wild-type spores through K^+-germination (158), here, *gerO* spores are able to release the majority of their DPA during Pi-germination, and this seems likely because of the presence of GerQ. However, it is possible that the higher amount of DPA released by *gerO* spores in this study versus previous (158) could be due to the nature of the novel germinant (Pi), or due to the fact that we used 2-fold higher concentration of Pi, creating a higher ionic strength in the medium that could in some fashion stimulate more DPA release. Although the maximum rate of germination of *gerQ* spores was lower than that of wild-type, they germinated to a similar extent as wild-type spores, and also released the majority of their DPA. In addition, GerQ only became essential in *gerO gerQ* spores, which had significantly slower germination than *gerO*, *gerQ* and wild-type spores, and released ~ half of their DPA. These results reinforce the role of cation transporters in germination of *C. perfringens* spores with all known germinants including Pi (158). Although these putative antiporters are required for normal germination with all known nutrient germinants of *C. perfringens*, it is not yet clear whether the effect of *gerO* or *gerQ* mutation is directly on spore germination or spore formation. Further studies are currently being carried out to help decide between these two scenarios.

Fig. 5.1: Effect of pH and Pi concentration in germination of *C. perfringens* spores.

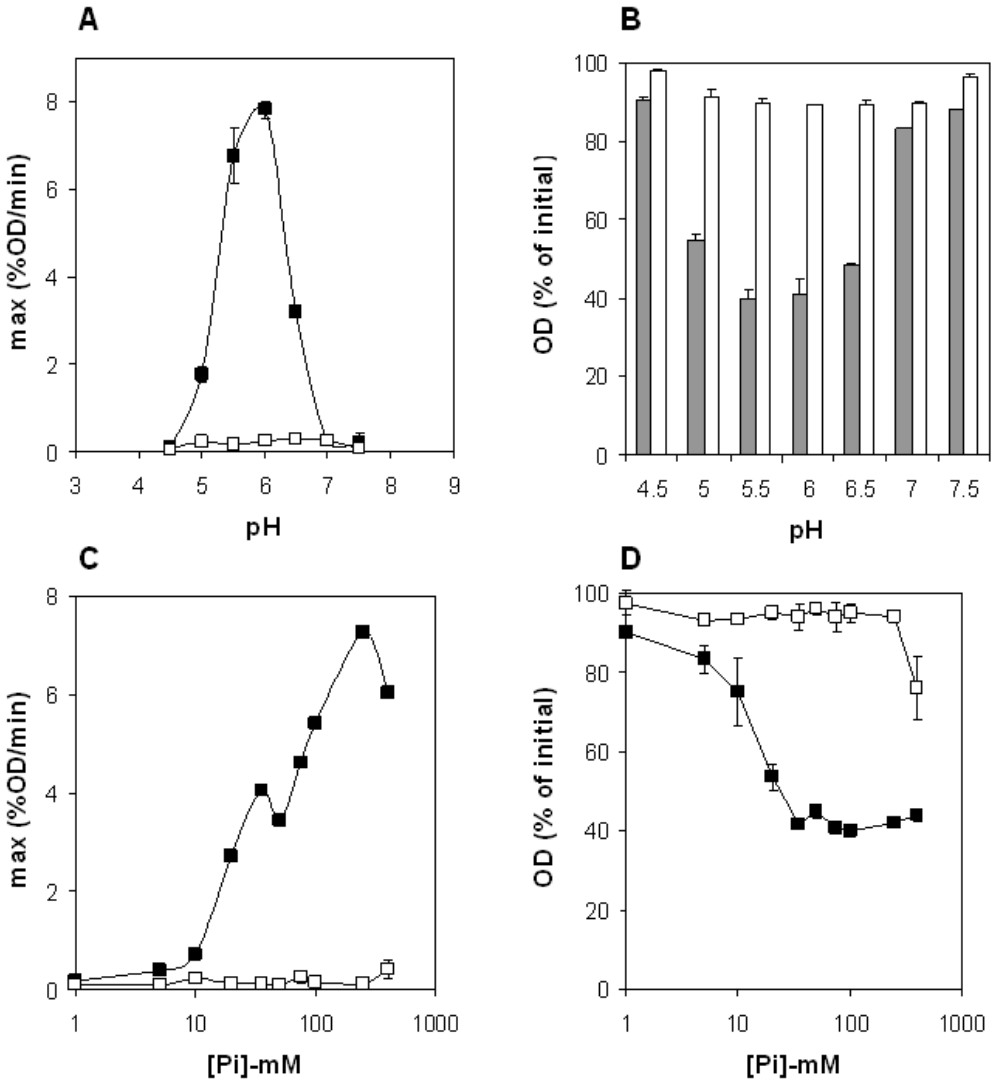


Fig. 5.1A-C. Effect of pH (A and B) and Pi concentration (C and D) in germination of *C. perfringens* spores. Heat-activated spores of strains SM101 (filled squares or grey bars) and F4969 (open squares or white bars) were incubated with: A,B) 100 mM sodium phosphate with various pH and the maximum rate (A), and extent of germination after 60 min (B) were calculated as described in Materials and Methods; C,D) sodium phosphate (pH 6.0) at various concentrations and the maximum rate (C), and

extent of germination after 60 min (D) were calculated as described in Materials and Methods. Error bars denote standard deviations.

Fig. 5.2: Germination of spores of *C. perfringens* in different buffers.

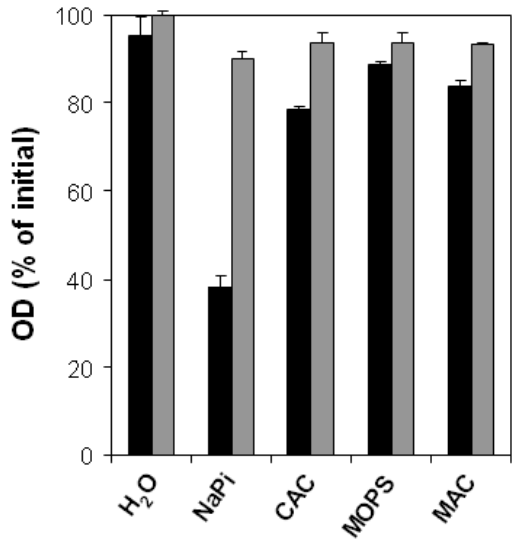


Fig. 5.2. Germination of spores of *C. perfringens* strains SM101 (black bars) and F4969 (grey bars) in different buffers. Heat activated spores were incubated at 40ºC and pH 6.0 in 100 mM of: NaPi, sodium phosphate; CAC, citric acid; MOPS, 3-morpholinopropanesulfonic acid; MAC, Maleic acid; and the OD_{600} was measured after 60 min of incubation. Error bars denote standard deviations.

Fig. 5.3: Germination of spores from various *C. perfringens* isolates with Pi.

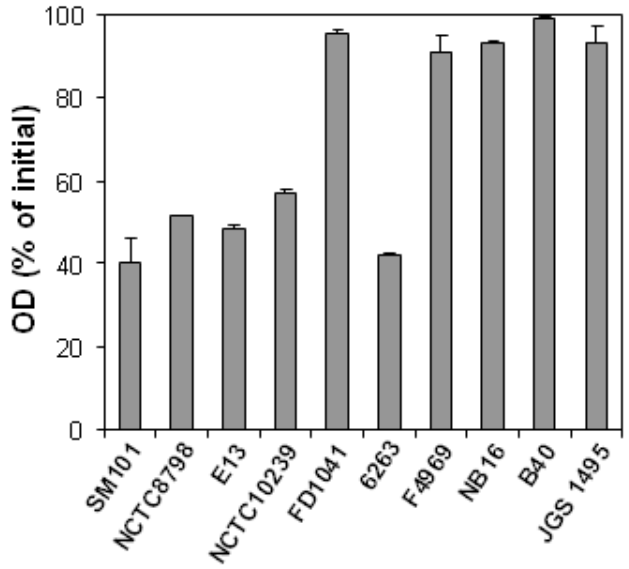


Fig. 5.3. Germination of spores from various *C. perfringens* isolates with Pi. Heat activated spores of FP type A (SM101, NCTC8798, E13, NCTC10239, FD1041 and 6263), NFB type A (F4969, NB16 and B40) and a *cpe*-negative type C (JGS1495) isolates were incubated with 100 mM sodium phosphate (pH 6.0) for 60 min and OD_{600} was measured as described in Materials and Methods.

Fig. 5.4: Germination of spores of *C. perfringens ger* receptor mutant strains with Pi.

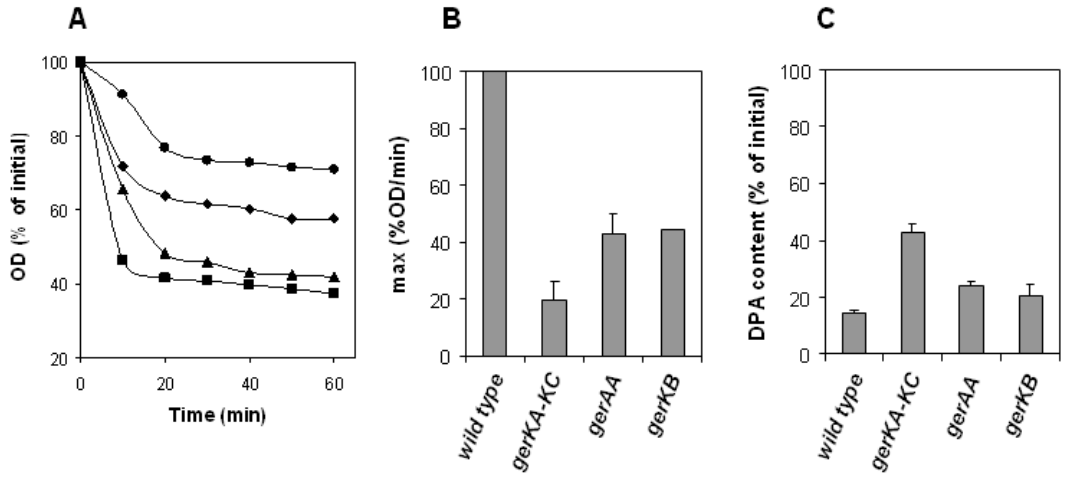


Fig. 5.4. Germination of spores of *C. perfringens ger* receptor mutant strains with Pi. A) Heat activated spores of *C. perfringens* strains SM101 (wild-type) (filled squares), DPS101 (*gerKA-KC*) (filled circles), DPS103 (*gerAA*) (filled diamonds), DPS108 (*gerKB*) (filled triangles) were incubated at 40ºC with 250 mM sodium phosphate (pH 6.0) and OD_{600} was measured as described in Materials and Methods. B), Heat activated spores of various *C. perfringens* strains were incubated at 40ºC with 250 mM sodium phosphate (pH 6.0) and maximum rates of spore germination were determined as % change in OD_{600}/min. All values are given relative to the value for SM101 spores, and this latter value was set at 100. C), DPA release during *C. perfringens* spore germination with Pi. Heat activated spores of *C. perfringens* were germinated with 250 mM sodium phosphate (pH 6.0) at 40ºC and after 60 min the DPA content was measured as described in Materials and Methods.

Fig. 5.5: Germination of spores of *C. perfringens* antiporter mutant strains with Pi.

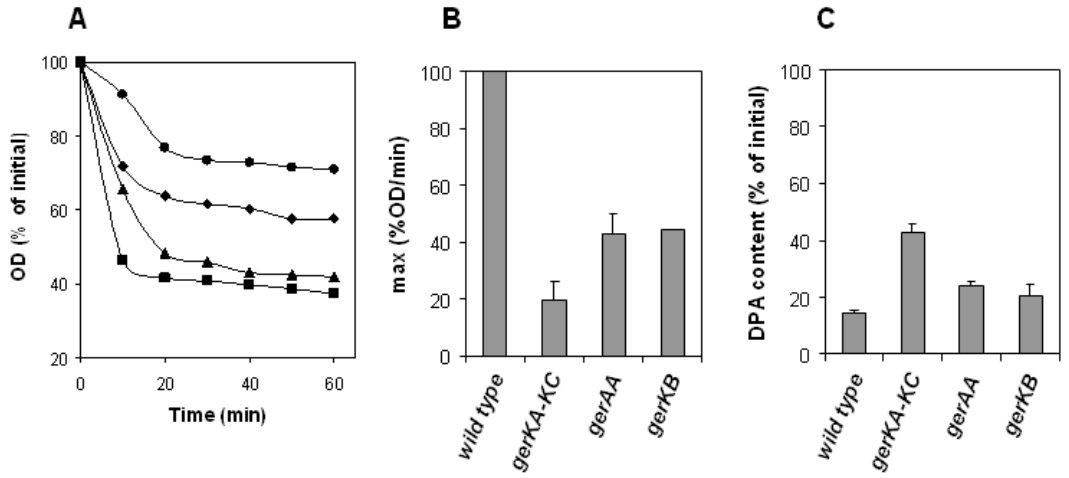


Fig. 5.5. Germination of spores of *C. perfringens* antiporter mutant strains with Pi. A) Heat activated spores of *C. perfringens* strains SM101 (wild-type) (filled squares), DPS113 (*gerQ*) (open triangles), DPS116 (*gerO*) (open diamonds), DPS108 (*gerO gerQ*) (open circles) were incubated at 40ºC with 250 mM sodium phosphate (pH 6.0) and OD_{600} was measured as described in Materials and Methods. B), Heat activated spores of various *C. perfringens* strains were incubated at 40ºC with 250 mM sodium phosphate (pH 6.0) and maximum rates of spore germination were determined as % change in OD_{600}/min. All values are given relative to the value for SM101 spores, and this latter value was set at 100. C), DPA release during *C. perfringens* spore germination with Pi. Heat activated spores of *C. perfringens* were germinated with 250 mM sodium phosphate (pH 6.0) at 40ºC and after 60 min the DPA content was measured as described in Materials and Methods.

Chapter 6

Characterization of *Clostridium perfringens* spores that lack SpoVA proteins and dipicolinic acid.

Daniel Paredes-Sabja, Barbara Setlow, Peter Setlow and Mahfuzur R. Sarker

Published in:

Journal of Bacteriology 2008 **190** (13): 4648-4659

6.1 Abstract

Spores of *Clostridium perfringens* possess high heat resistance, and when these spores germinate and return to active growth, they can cause gastrointestinal disease. Work with *Bacillus subtilis* has shown that the spore's dipicolinic acid (DPA) level can markedly influence both spore germination and resistance, and that the proteins encoded by the *spoVA* operon are essential for DPA uptake by the developing spore during sporulation. We now find that proteins encoded by the *spoVA* operon are also essential for the uptake of Ca^{2+} and DPA into the developing spore during *C. perfringens* sporulation. Spores of a *spoVA* mutant had little if any Ca^{2+} and DPA, and their core water content was ~2-fold higher than that of wild-type spores. These DPA-less spores did not germinate spontaneously, as do DPA-less *B. subtilis* spores. Indeed, wild-type and *spoVA C. perfringens* spores germinated similarly with a mixture of L-asparagine and KCl (AK), KCl alone or a 1:1 chelate of Ca^{2+} and DPA (Ca-DPA). However, the *C. perfringens spoVA* spores had 20-fold lower viability than wild-type spores. Decoated wild-type and *spoVA* spores exhibited little if any germination with AK, KCl, or exogenous Ca-DPA, and had 10^3- to 10^4-fold lower colony forming efficiency than intact spores. However, lysozyme treatment rescued these decoated spores. Although the level of DNA protective α/β-type small, acid-soluble spore proteins in *spoVA* spores was similar to that in wild-type spores, *spoVA* spores exhibited markedly lower resistance to moist heat, formaldehyde, HCl, hydrogen peroxide, nitrous acid and UV radiation. In sum, these results suggest that: (i) SpoVA proteins are essential for Ca-DPA uptake by developing spores during *C. perfringens* sporulation; (ii) SpoVA proteins and Ca-DPA release are not required for *C.*

perfringens spore germination; and (iii) a low core water content is essential for full resistance of *C. perfringens* spores to moist heat, UV radiation and chemicals.

6.2 Introduction

Clostridium perfringens food poisoning is caused mainly by enterotoxigenic type A isolates that have the ability to form metabolically dormant spores that are extremely resistant to heat, radiation, and toxic chemicals (151, 174, 176, 193). These highly resistant spores can survive traditional methods for cooking meat and poultry products as well as other processing treatments used in the food industry. The surviving spores can then go through germination and outgrowth generating the vegetative cells that cause disease (117).

Spores of *Bacillus* species also have the extreme resistance of *C. perfringens* spores. The factors involved in *B. subtilis* spore resistance include the: i) relatively impermeable spore inner membrane; ii) spore core's low water content; iii) high levels of pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)) in the spore core, and the type and amount of cations chelated by DPA; and iv) the saturation of spore DNA with α/β-type small, acid-soluble spore proteins (SASP) (208, 209). Studies have shown that α/β-type SASP are also an important factor in *C. perfringens* spore resistance to moist-heat, UV radiation and some chemicals (151, 174, 176), while small changes in these spore's core water content alter moist heat and nitrous acid resistance (152).

Spore germination has been well studied in *B. subtilis* (127, 207), and can be initiated by a variety of compounds (termed germinants) including amino acids and

some other nutrients and nutrient mixtures, a 1:1 chelate of Ca^{2+} and DPA, cationic surfactants and hydrostatic pressure (145, 148). The germinant receptors, located in the spore's inner membrane (69, 147), sense nutrients present in the environment and stimulate the release of monovalent cations (H^+, Na^+, and K^+), divalent cations (Ca^{2+}, Mg^{2+}, and Mn^{2+}), and the spore core's large depot (~25% of core dry wt) of DPA (207) present in a 1:1 chelate with divalent cations, predominantly Ca^{2+} (Ca-DPA) (128, 129, 204, 206). Ca-DPA release is crucial to spore progression from germination into outgrowth as: i) water replaces Ca-DPA, thus elevating the spore core water content; and ii) Ca-DPA triggers the initiation of hydrolysis of the spore's peptidoglycan (PG) cortex by the cortex-lytic enzyme (CLE) CwlJ that is probably directly activated by Ca-DPA (146). Cortex hydrolysis then allows the core to expand and take up even more water to the level found in vegetative cells. This latter event restores protein movement and enzyme activity in the spore core, and allows resumption of energy metabolism and macromolecular synthesis (34, 207).

Release of Ca-DPA during *B. subtilis* spore germination as well as DPA uptake during sporulation into the developing spore from the mother cell, the site of DPA synthesis, require the SpoVA proteins that may be components of some type of gated channel in the spore's inner membrane (242, 244). In *B. subtilis* the hexacistronic *spoVA* operon encodes the SpoVA proteins (93), and most if not all of these are likely to be membrane proteins as shown directly for SpoVAD (243). The *spoVA* operon is transcribed exclusively during *B. subtilis* sporulation in the developing forespore just prior to DPA uptake (236, 242). *B. subtilis* strains with deletions in the *spoVA* operon initiate sporulation, but the *spoVA* spores are extremely unstable and lyse during

sporulation, probably because of their lack of DPA (236). *B. subtilis* spores that lack DPA due to inactivation of the DPA synthase are also unstable and lyse during sporulation (236). The reason for the instability of DPA-less *B. subtilis* spores is not clear, but may be because the second of two redundant CLEs, SleB, is spontaneously activated in spores that lack DPA (236).

Clostridium species also contain a *spoVA* operon, although this is only tricistronic, containing *spoVAC*, *spoVAD* and *spoVAE* (14, 18, 133, 197), suggesting that not all the SpoVA proteins in spores of *Bacillus* species are essential for DPA movement during sporulation and spore germination in *Clostridium* species. To study the role of SpoVA proteins and DPA levels in the properties of spores of *Clostridium* species, *C. perfringens* was used to examine the expression of the *spoVA* operon during growth and sporulation, and to construct a *spoVA* deletion mutation. The properties of the resultant *spoVA* spores, in particular their Ca-DPA level, resistance and germination, were then examined to help elucidate the role of SpoVA proteins and DPA in *C. perfringens* spore properties. Surprisingly, while *spoVA C. perfringens* spores lacked DPA, these spores were stable, and germinated relatively normally. However, these *spoVA* spores had significantly higher core water content than wild-type spores, and exhibited markedly decreased resistance to moist heat, UV radiation and a number of chemicals.

6.3 Materials and Methods

Bacterial strains and plasmids. The *C. perfringens* strains and plasmids used in this study are described in Table 6.1.

Construction of *gusA*-fusion plasmids and β-glucuronidase assay. Expression of *C. perfringens spoVAC*, *spoVAD* and *spoVAE* was examined by fusing DNA upstream of each gene to *Escherichia coli gusA* in pMRS127, an *E. coli-C. perfringens* shuttle vector (174). Briefly, 300-400-bp DNA fragments upstream of *spoVAC*, *spoVAD* or *spoVAE* from *C. perfringens* SM101 were PCR-amplified using primers CPP274/CPP275, CPP382/CPP381 and CPP387/CPP384 (forward and reverse primers had SalI and PstI cleavage sites, respectively) (Table 6.2). These PCR-fragments were digested with SalI and PstI and cloned between the SalI and PstI cleavage sites in pMRS127 to create *spoVAC- spoVAD-*, *spoVAE-gusA* fusion constructs, giving plasmids pDP51, pDP79 and pDP80 (Table 6.1). These plasmids were introduced into *C. perfringens* SM101 by electroporation (35) and erythromycin-resistant (Em^r, 50 μg/ml) transformants were selected. The transformants carrying the plasmid with the *spoVAC- spoVAD-* or *spoVAE-gusA* fusions were grown vegetatively in TGY medium (3% trypticase soy, 2% glucose, 1% yeast extract, 0.1% L-cysteine) (91) and sporulated in Duncan-Strong (DS) (36) medium, and assayed for β-glucuronidase (GUS) activity as described (56, 174). GUS specific activity was expressed in Miller Units and calculated as described (81).

Construction of a *C. perfringens spoVA* deletion mutant. To isolate a derivative of *C. perfringens* SM101 with a deletion of the entire *spoVA* operon, a Δ*spoVA* suicide vector was constructed as follows: A 1001-bp DNA fragment carrying 270-bp from the N-terminal coding region and 731-bp upstream of *spoVAC* was PCR amplified

using primers CPP288/CPP289 (Table 6.2), which had KpnI and SpeI cleavage sites at the 5'-ends of the forward and reverse primers, respectively. A 1378-bp fragment carrying 324-bp from the C-terminal coding region and 1054-bp downstream of *spoVAE* was PCR amplified using primers CPP290/CPP291 (Table 6.2), which had PstI and XhoI cleavage site at the 5'-ends of the forward and reverse primers, respectively (Table 6.2). These PCR fragments were cloned into plasmid pCR-XL-TOPO giving plasmids pDP31 and pDP32. An ~1.0-kb KpnI-SpeI fragment from pDP31 was cloned into pDP1 (Table 6.1) giving plasmid pDP33, and an ~1.4-kb PstI-XhoI fragment from pDP32 was cloned in pDP33 giving pDP34. Next an ~3.2-kb EcoRI fragment carrying the tetracycline resistance gene (*tetM*) from pJIR1886 (Table 6.1) was cloned into the EcoRI site of pDP34, giving pDP35. Finally, an ~1.1 SmaI fragment carrying the erythromycin resistance gene (*ermB*) from PJIR599 (Table 6.1) was cloned into a unique SmaI site in pDP35, giving pDP45 which cannot replicate in *C. perfringens*. Plasmid pDP45 was introduced into *C. perfringens* SM101 by electroporation (35), and the *spoVA* deletion strain DPS104 was isolated by allelic exchange (192). The presence of the *spoVA* deletion in strain DPS104 (Fig. 6.1A) was confirmed by PCR and Southern blot analyses (data not shown).

Construction of *spoVA* complemented strain. An ~ 2.5 kb fragment containing *spoVAC*, *spoVAD* and *spoVAE* plus 393-bp upstream of *spoVAC* was PCR amplified using primers CPP341/CPP347 (Table 6.2), and cloned into pCR®-XL-TOPO (Invitrogen) giving pDP53. The ~ 2.5 kb *Kpn*I-*Sal*I fragments of pDP53 was cloned between the KpnI and SalI sites of plasmid pJIR750 (11) to create the *spoVA*-

complementing plasmid pDP54. Plasmid pDP54 was introduced into *C. perfringens* strain DPS104 by electroporation (35) and Cm^r transformants were selected. The presence of plasmid pDP54 in DPS104(pDP54) was confirmed by PCR (data not shown).

Spore preparation and purification. Starter cultures (10 ml) of *C. perfringens* isolates were prepared by overnight growth at 37°C in fluid thioglycollate broth (FTG) (Difco) as described (91). Sporulating cultures of *C. perfringens* were prepared by inoculating 0.2 ml of an FTG starter culture into 10 ml of DS sporulation medium (36), and this culture was incubated for 24 h at 37ºC to form spores as confirmed by phase-contrast microscopy. Large amounts of spores were prepared by scaling-up the latter procedure. Spore preparations were cleaned by repeated centrifugation and washing with sterile distilled water until spores were > 99% free of sporulating cells, cell debris and germinated spores, suspended in distilled water at a final optical density at 600 nm (OD_{600}) of ~ 6 and stored at -20ºC (154).

Measurement of spore properties. For most analyses of **s**pore Ca^{2+} and DPA content, spore coats were extracted to remove material that might interfere with the quantitation of either the amount of spores being assayed or of DPA. Spore coats were extracted from a spore suspension with an OD_{600} of ~ 20 in 1 ml of 50 mM Tris-HCl (pH 8.0)-8 M urea-1% (wt/vol) SDS-50 mM dithiothreitol for 90 min at 37ºC (Treatment 1) and the spores washed three times with 150 mM NaCl and twice with water (169). Note that this extraction procedure did not kill spores as determined on

plates containing lysozyme (see Results). An aliquot of decoated spores at an OD_{600} of 12 was incubated at 100ºC for 60 min, the sample was cooled on ice, centrifuged 5 min, and Ca^{2+} and DPA were measured in the supernatant fluid as described (186, 254). While most analyses used decoated spores, similar results were obtained when spores were not initially decoated.

For determination of spore core wet densities by equilibrium density gradient centrifugation (105), decoated spores were suspended in 100 µl of 30% Histodenz (Nycodenz) (Sigma, St. Louis, MO), incubated for 60 min on ice, loaded on the top of 2 ml step gradients of 51-70% (for *spoVA* spores) or 61-80% (for wild-type spores) Histodenz in ultraclear centrifuge tubes and the tubes centrifuged for 45 min at 14,000 rpm and 20°C in a swinging bucket rotor in a Beckman TL-100 ultracentrifuge (105).

Spore germination assays. Spore suspensions were heat activated at 80°C for 10 min, cooled in water at ambient temperature for 5 min, and subsequently incubated at 40°C for 10 min as described (154). Spore germination with nutrients or Ca-DPA (50 mM $CaCl_2$, 50 mM DPA adjusted to pH 8.0 with Tris-base) was routinely measured by monitoring the OD_{600} of spore cultures (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA), which falls ~ 60% upon complete germination of wild-type spores and levels of germination were confirmed by phase-contrast microscopy. However, upon complete germination of *spoVA* spores the OD_{600} fell only by ~ 40% due to the lower refractive index of *spoVA* spores. The extent of spore germination was calculated by measuring the decrease in OD_{600} and was expressed as a percentage of initial OD_{600}. All values reported are averages of two experiments performed on at

least two independent spore preparations, and individual values varied by less than 10% from average values shown.

Decoating treatments. Spores, 1 ml at an OD_{600} of 20 were decoated either by Treatment 1 as described above; or in 1 ml of 0.1 M sodium borate (pH 10)-2% 2-mercaptoethanol for 60 min at 37ºC (123) (Treatment 2). Decoated spores were washed at least 9 times with sterile distilled water before use.

DPA release. DPA release from untreated or decoated spores during nutrient germination was measured by heat activating a spore suspension (OD_{600} of 1.5) as described above, cooling and incubating with 100 mM AK (100 mM in both L-asparagine and KCl) in 25 mM sodium phosphate buffer (pH 7.0) at 40ºC for 60 min. For measuring DPA release during dodecylamine germination, untreated or decoated spores (OD_{600} of 1.5) were incubated at 60ºC with 1 mM dodecylamine in 25 mM Tris-HCl (pH 7.4). Aliquots (1 ml) of germinating cultures were centrifuged for 2 min at 8,000 rpm in a microcentrifuge and DPA in the supernatant fluid was measured by monitoring the OD_{270} as described (21, 154). The total DPA content of wild-type spores was measured by boiling an aliquot (1 ml) for 60 min, centrifuging at 8,000 rpm in a microcentrifuge for 15 min, and measuring the OD_{270} of the supernatant fluid as described (21, 154). In *B. subtilis* spores ≥85% of the material absorbing at 270 nm is DPA (6, 21).

Assessment of colony forming efficiency of spores. To assess the colony-forming efficiency of untreated and decoated spores, spores at an OD_{600} of 1 were heat activated at 80ºC for 10 min, aliquots of various dilutions were plated on Brain Heart Infusion (BHI) agar with or without lysozyme (1 μg/ml) in the plates, the plates incubated at 37ºC anaerobically for 24 h, and colonies were counted.

SASP extraction. Extraction of SASP from *C. perfringens* spores and their analysis by polyacrylamide gel electrophoresis (PAGE) at low pH was as described (174, 176). Briefly, SASP were extracted from 40 mg (dry wt) of disrupted spores with dilute acid, and the extracts were processed and lyophilized. The dry residue was dissolved in 30 μl of 8M urea, 5-μl aliquots run on PAGE at low pH, and the gels were stained with Coomassie Brilliant Blue (Bio-Rad Laboratories, Hercules, CA), all as described (176). Analysis of relative band intensities on stained gels by densitometry was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Spore resistance. The resistance of *C. perfringens* spores to moist heat was measured as described (174, 193). Briefly, 24-h grown DS medium cultures of *C. perfringens* strains were heat-treated at 75ºC for 20 min to kill vegetative cells. Initial Colony Forming Units (CFU) were determined by serially diluting heat-treated DS medium spore cultures in phosphate-buffered saline (140 mM NaCl-25 mM sodium phosphate buffer (pH 7.0)) (PBS), plating on BHI agar, and anaerobic incubation for 24 h at

37ºC. The 75ºC-treated DS medium spore cultures were then heated at 94 or 100ºC for various times, aliquots of appropriate dilutions were plated and incubated anaerobically for 24 h at 37ºC as described above. Plots of CFU/ml versus time at 94 or 100ºC were used to determine decimal reduction values (D_{94^o} or D_{100^o} values), which are the times cultures need to be kept at 94 or 100ºC to achieve 90% reduction in CFU/ml.

C. perfringens spore resistance to UV radiation was determined as described (169, 176). Briefly, purified spores at an OD_{600} of 2 were diluted 100-fold in 25 mM sodium phosphate buffer (pH 6.8) and UV irradiated at 254 nm with a UVGL-25 Mineralight lamp (UVP Inc., Upland, CA) for various times. Appropriate dilutions were spread onto BHI plates, and incubated as described above prior to assessment of colony formation.

The resistance of *C. perfringens* spores to chemicals was determined as described (151). Briefly, purified spores at an OD_{600} of ~1 were treated with: (i) 2M hydrogen peroxide (Mallinckrodt Baker Inc., Phillipsburg, NJ) at room temperature and aliquots were neutralized with catalase (Sigma) as described (199); (ii) 300 mM HCl at room temperature and aliquots were diluted 100-fold in 25 mM in sodium phosphate buffer (pH 7.0); (iii) 400 mM $NaNO_2$ - 400 mM Na acetate buffer (pH 4.5) at room temperature, and aliquots were diluted 10-fold in 500 mM sodium phosphate buffer (pH 8.5); (iv) 25 g/l formaldehyde (Sigma) at 30°C, and aliquots were diluted 10-fold in 400 mM glycine (pH 7.0) and incubated for 20 min at room temperature prior to analysis. Determination of spore killing was as described above.

6.4 Results

Identification of the putative *spoVA* operon in *C. perfringens*. The SpoVA proteins are encoded by six open reading frames (ORFs) in a hexacistronic operon in *B. subtilis* (93). In contrast, only three ORFs (CPR2017, CPR2018 and CPR2019) encoding proteins with high similarity (65 to 73%) to *B. subtilis* SpoVA proteins were found in the *C. perfringens* SM101 genome (130) (Fig. 6.1A). These ORFs are annotated as *spoVAC* (CPR2019), *spoVAD* (CPR2018) and *spoVAE* (CPR2017) and form a putative tricistronic operon. This organization of the *spoVA* operon is not unique to *C. perfringens* as it is conserved among *Clostridium* species (14, 18, 133, 197). The first gene in the putative tricistronic operon, *spoVAC* (CPR2019), is predicted to encode a 155-residue protein with 4 transmembrane alpha-helical segments (TMS). The *spoVAD* gene (CPR2018) is predicted to encode a protein of 339 amino acid residues with no TMS; perhaps this is a peripheral inner membrane protein that interacts with other inner membrane proteins. The *spoVAE* gene (CPR2017) is predicted to encode a 121-residue protein with 4 TMS.

Expression of *C. perfringens spoVA* genes. To assess whether the putative *C. perfringens spoVA* genes are expressed uniquely during sporulation and comprise a tricistronic operon, DNA upstream of each gene was fused to *E. coli gusA* and GUS activity was measured after introduction of the various fusions into *C. perfringens* SM101. No significant GUS activity was observed in vegetative cultures carrying plasmid pDP51 with a *spoVAC-gusA* fusion (data not shown). However, a sporulating culture carrying plasmid pDP51 (*spoVAC-gusA*) exhibited significant GUS activity

(Fig. 6.1B), indicating that a sporulation-specific promoter is located upstream of *spoVAC*. Expression of GUS from the *spoVAC-gusA* fusion began ~ 3 h after induction of sporulation and GUS specific activity reached a maximum 8-10 h after initiation of sporulation, and then fell somewhat (Fig. 6.1B). The decrease in GUS specific activity late in sporulation is consistent with *spoVA* being packaged into the spore where it cannot be easily assayed (113). Phase bright spores became visible ~ 6 h after induction of sporulation (data not shown), when ~ 50% of the spore's maximum DPA level had been accumulated, and DPA accumulation lagged ~ 1 h behind expression of the *spoVA* operon (Fig. 6.1B). Thus *spoVAC*, and probably *spoVA* operon transcription as well as accumulation of the SpoVA proteins precede DPA accumulation by the developing spore, as is also the case in *B. subtilis* (113).

In contrast to the results with the *spoVAC-gusA* fusion, there was no detectable GUS activity observed in either vegetative or sporulating cultures carrying plasmids pDP79 (*spoVAD-gusA*) or pDP80 (*spoVAE-gusA*) (Fig. 6.1B; and data not shown). These results indicate that no promoter is present in DNA 300-400 bp upstream of *spoVAD* or *spoVAE* and thus these genes almost certainly form a tricistronic operon in *C. perfringens* with *spoVAC* as the first gene in the operon, and further that this operon is expressed only during sporulation.

Effect of a *spoVA* mutation on *C. perfringens* spore properties. Previous studies with *B. subtilis* (38, 39, 44, 125, 236) have shown that sporulation of strains with null mutations in any of the first five cistrons of the *spoVA* operon gives immature forespores that lyse within the sporangium. Isolation of stable *spoVA* spores in *B.*

subtilis requires the elimination of either all three functional nutrient germinant receptors or the CLE, SleB (236). Surprisingly, spores of the *C. perfringens spoVA* mutant (strain DPS104) were stable, appeared bright in the phase-contrast microscope, and were easily purified to give clean spore preparations containing > 95% free spores. As expected, the Ca^{2+} and DPA contents of *C. perfringens spoVA* spores were negligible compared to those in wild-type spores (Table 6.3). Sporulation of strain DPS104 with DPA present in the sporulation medium at 100 μg/ml also gave spores that contained no DPA (data not shown), although this exogenous DPA level restores nearly wild-type DPA levels to spores of *B. subtilis* strains defective in DPA synthase (38, 39, 125, 144). However, attempts to correct the DPA-less phenotype of *spoVA* spores by complementation with the wild-type *spoVA* operon failed, as the DPA level in DPS104(pDP54) spores was similar to that of *spoVA* spores (data not shown). Together the latter findings indicate that SpoVA proteins are essential for Ca-DPA uptake during *C. perfringens* sporulation. Interestingly, the molar ratio of Ca^{2+} to DPA in *C. perfringens* wild-type spores was 0.7:1, indicating that DPA may also be forming complexes with other divalent cations such as Mg^{2+} and Mn^{2+} (Table 6.3) (54, 128, 129, 204, 206).

As noted above, the *spoVA C. perfringens* spores appeared bright when observed by phase-contrast microscopy, suggesting that these spores' core was less hydrated than the protoplast of growing cells. However, the *spoVA* spores were less bright than wild-type spores (data not shown), suggesting that these spores' core is not as dehydrated as that of wild-type spores. Indeed, the core wet density of *spoVA* spores was significantly lower than that of wild-type spores (Table 6.3). The latter

difference indicated that the *spoVA* spore core contains almost twice as much water/g wet wt than does the wild-type spore core (Table 6.3).

In addition to differences in Ca-DPA content and core wet density between wild-type and *spoVA* spores, *spoVA* spores had lower colony forming efficiency than wild-type spores. When heat activated (80ºC, 10 min) wild-type and *spoVA* spores were plated on BHI agar and incubated overnight at 37ºC under anaerobic conditions, the viability of *spoVA* spores was ~ 20-fold lower than that of wild-type spores (Table 6.4). The lower apparent viability of *spoVA* spores was not due to their killing during heat activation, as unheated *spoVA* spores actually gave 6-fold fewer colonies than heat-activated *spoVA* spores (data not shown). The viability of the *spoVA* spores was also not increased on plates containing lysozyme (Table 6.4).

Effect of a *spoVA* mutation on germination of *C. perfringens* spores. One possible explanation for the lower viability of the *C. perfringens spoVA* spores noted above is that Ca-DPA and/or SpoVA proteins are also essential for the germination of *C. perfringens* spores. If this is correct then *spoVA C. perfringens* spores should germinate to a much lower extent than wild-type spores. However, wild-type and *spoVA* spores germinated similarly in BHI broth through 60 min of incubation (Fig. 6.2A). Although the fall in OD_{600} of wild-type and *spoVA* spores incubated at 40°C in BHI broth for 18h aerobically to inhibit cell growth was 60 and 45%, respectively (data not shown), phase-contrast microscopy revealed that > 99% of both the *spoVA* and wild-type spores had become phase dark and thus had germinated fully. Similarly, while *spoVA* spores appeared to germinate to a lesser extent than wild-type spores

with AK or Ca-DPA after 60 min of incubation when spore germination was monitored by the OD_{600} of the cultures (Fig. 6.2B and C), phase-contrast microscopy indicated again that > 99 and ~ 90%% of both the wild-type and *spoVA* spores incubated with AK and Ca-DPA had germinated fully, respectively (data not shown). To more rigorously quantify this result, *spoVA* and wild-type spores were germinated with 100 mM AK (pH 7.0) at 40ºC for 80 min. While > 99% of the spores of both strains had become phase-dark as determined by phase contrast microscopy, the OD_{600} of the germinating wild-type and *spoVA* spore cultures had decreased by 62 and 42%, respectively (average of four independent experiments). These results indicate that: i) as suggested above, the refractive index of *spoVA* spores, and thus their OD_{600} is ~30% lower than that of wild-type spores, most likely due to the higher water content in the *spoVA* spore core; consequently the difference between the OD_{600} of a culture of dormant and fully germinated spores is ~30% greater for wild-type spores; and ii) DPA-less *C. perfringens* spores are able to germinate normally with either nutrients or exogenous Ca-DPA.

Germination of decoated *C. perfringens* spores. The results noted above suggested that cortex hydrolysis during *C. perfringens* spore germination can be independent of both SpoVA proteins and any signaling by Ca-DPA, and thus perhaps CLEs are not activated directly by Ca-DPA in *C. perfringens*. To begin to examine the roles of CLEs in *C. perfringens* spore germination, we decoated wild-type and *spoVA* spores with treatments that are reported to remove CLEs (123, 146), and examined the germination of the decoated spores. While it is still not clear which *C. perfringens*

CLEs identified by analyses of enzyme activity on spore cortex peptidoglycan in vitro are actually involved in cortex PG degradation during spore germination, it was notable that both wild-type and *spoVA* spores decoated by Treatment 1 were unable to germinate and remained phase bright when incubated for 60 min with BHI, AK or Ca-DPA (Fig. 6.2D, E, F; and data not shown). Even after an 18 h incubation, > 99% of these decoated spores remained phase bright (data not shown). These results are consistent with Treatment 1 removing or inactivating all CLEs in *C. perfringens* spores, including any putative CLE directly activated by Ca-DPA. Although wild-type and *spoVA* spores decoated with Treatment 2 exhibited little germination after incubation at 40°C for 60 min in BHI broth (Fig. 6.2D), ~ 20% of the spores had become phase dark after 18 h of incubation (data not shown). In addition, Treatment 2 decoated wild-type and *spoVA* spores did germinate at a very slow rate with AK or Ca-DPA (Fig. 6.2E, F), and ~ 30% of the spores were phase dark after 18 h of incubation with AK and Ca-DPA (data not shown), consistent with Treatment 2 completely removing the CLE SleC but only ~ 90% of SleM from *C. perfringens* spores (92). Interestingly, no DPA was released from the core of wild-type spores decoated with Treatment 1 when these spores were incubated with AK, yet nearly all the spore's DPA was released when these spores were incubated with dodecylamine (Fig. 6.3), suggesting that perhaps the spore's germinant receptors had been damaged by Treatment 1. In contrast, wild-type spores decoated with Treatment 2 did release DPA when incubated with AK or dodecylamine (Fig. 6.3). These results strongly suggest that *spoVA* spores need undamaged germinant receptors and at least one CLE to initiate cortex hydrolysis during spore germination.

Colony forming efficiency of decoated *C. perfringens* spores. The extremely poor germination of the decoated spores noted above suggested that decoated wild-type and *spoVA* spores might have much lower colony forming efficiency than intact spores. Indeed, when heat activated wild-type and *spoVA* spores were plated on BHI agar and incubated for 24 h at 37ºC under anaerobic conditions, the colony forming efficiencies of wild-type and *spoVA* spores decoated by Treatment 1 were ~10^4- and 10^3-fold lower, respectively, than those of the corresponding intact spores (Table 6.4). No additional colonies appeared when plates were incubated for up to 3 days. Wild-type and *spoVA* spores decoated with the milder Treatment 2 exhibited much smaller decreases in colony forming efficiencies (Table 6.4). Strikingly, when spores decoated with either treatment were plated on BHI agar containing 1 μg/ml lysozyme, the colony forming efficiencies were restored to those of the corresponding intact spores (Table 6.4). These results indicate that the decoating treatments had not simply killed the spores.

Levels of α/β-type SASP in wild-type and *spoVA* spores. In *B. subtilis*, α/β-type SASP are degraded during the first min of germination (190, 205) by a germination specific protease (GPR) specific for a highly conserved sequence found in all SASP (209, 228). *B. subtilis* GPR is synthesized as a zymogen with a molecular mass of ~ 46 kDa (termed P_{46}) beginning at approximately the third h of sporulation (191) and is autoprocessed to the ~ 41 kDa active protease (P_{41}) about 2 h later. This latter autoprocessing is triggered synergistically by Ca-DPA accumulation in the forespore,

and by decreases in the developing forespore's core pH and water content, conditions that preclude attack of P_{41} on its SASP substrates (73, 74, 191). *C. perfringens* also contains a *gpr* gene encoding a protein with high similarity to *B. subtilis* GPR, including the highly conserved $P_{46}P_{41}$ autoprocessing site (191). If P_{46} autoprocessing is regulated in *C. perfringens* as it is in *B. subtilis*, perhaps little, if any, GPR is autoprocessed to P_{41} during sporulation of strain DPS104 (*spoVA*). However, if P_{41} is present in *C. perfringens spoVA* spores, perhaps this protease can catalyze some SASP hydrolysis in the more hydrated core of *spoVA* spores. Given the important role played by α/β-type SASP in *C. perfringens* spore resistance (151, 174, 176), it was thus of interest to compare the levels of α/β-type SASP in *C. perfringens spoVA* and wild-type spores. PAGE at low pH of a SASP extract from wild-type and *spoVA* spores gave only a tight group of protein bands (Fig. 6.4), one of which was a major one, as seen previously (176). The intensities of these bands, and thus total α/β-type SASP levels, were similar in extracts from spores of these two strains (Fig. 6.4), as was confirmed by densitometric analysis of gels from three independent experiments (data not shown). These results suggest that either: i) little if any P_{46} is autoprocessed to P_{41} during sporulation of strain DPS104 (*spoVA*); ii) or the hydration level of the *spoVA* spore core is still low enough to preclude P_{41} activity on α/β-type SASP. It is also possible that the binding of α/β-type SASP to DNA in the *spoVA* spores helps protect these proteins against GPR digestion (73, 74, 191) (but see Discussion).

Moist heat, UV, and chemical resistance of *C. perfringens spoVA* spores. Previous work (174, 176) has shown that binding of α/β-type SASP to DNA is a major factor

in *C. perfringens* spore resistance to moist heat and UV radiation, and that small changes in spore core water content have significant effects on spore resistance to moist heat and nitrous acid but not UV radiation (152). As expected, *C. perfringens spoVA* spores were very sensitive to moist heat (Fig. 6.5A, B), with 70- to 80-fold lower $D_{94^{o}C}$ (5 min; results from three independent experiments) and $D_{100^{o}C}$ (0.5 min) values than wild-type spores ($D_{94^{o}C}$ 410 min; $D_{100^{o}C}$ 45 min). However, the heat resistance of *spoVA* spores could not be restored to wild-type levels by complementation with the wild-type *spoVA* operon, as the heat resistance of DPS104(pDP54) spores was similar to that of *spoVA* spores (data not shown). The *spoVA* spores were also significantly more sensitive to UV radiation than wild-type spores (Fig. 6.5C).

Previous studies (152) have also shown that the resistance of *C. perfringens* mutant spores with slightly higher core water content to hydrogen peroxide and HCl is essentially identical to that of wild-type spores. However, *C. perfringens spoVA* spores with ~ 2-fold higher water content than wild-type spores were more sensitive than wild-type spores not only to nitrous acid (Fig. 6.6A), but also to HCl (Fig. 6.6B), formaldehyde (Fig. 6.6C) and hydrogen peroxide (Fig. 6.6D).

6.5 Discussion

The work in this communication indicates that *C. perfringens spoVA* spores accumulate no Ca-DPA and do not take up exogenous DPA, and that the *spoVA* operon is expressed only during *C. perfringens* sporulation. These results are similar to those found with *B. subtilis* (39, 144, 236), and indicate that in *C. perfringens* the *spoVA* operon also encodes sporulation-specific genes essential for the uptake of Ca^{2+} and DPA during spore maturation. Unfortunately, we were unable to restore the DPA-less phenotype of *C. perfringens spoVA* spores by complementation with a wild-type *spoVA* operon, although the reason for this failure is not clear. Perhaps an alternative approach, such as introduction of the *C. perfringens spoVA* operon into a *B. subtilis spoVA* strain and vice versa could be successful. It was, however, most striking that sporulation of the *spoVA C. perfringens* strain gave stable Ca-DPA-less spores, since *B. subtilis spoVA* strains give only immature spores that lyse during sporulation, probably due to their germination within the sporangium (39, 236). Spores of *B. subtilis* strains that cannot synthesize DPA are also unstable and lyse during sporulation (39, 144). *B. subtilis* spores with significantly lower than normal DPA levels can be isolated, but exhibit relatively rapid spontaneous germination (55). Thus, *B. subtilis* spores with low or no DPA levels, for whatever reason, spontaneously and rapidly trigger subsequent events in the spore germination process, most notably the hydrolysis of cortex PG. Indeed, the activity of one of the two redundant CLEs, CwlJ, appears to be directly stimulated by Ca-DPA, while the second redundant CLE, SleB, somehow is activated in spores that lack or have low DPA, possibly responding to a change in the strain on cortex PG (146, 236). However, clearly the CLEs in *C.*

perfringens spores are not activated in spores that lack DPA. The reason for the stability of the DPA-less *C. perfringens spoVA* spores is unclear and may well reflect significant differences in the signaling mechanisms operating during germination of *B. subtilis* and *C. perfringens* spores, in particular the signaling mechanisms involving Ca-DPA. It obviously will be of great interest in future work to determine if *C. perfringens* spores also have a CLE whose activity responds directly to DPA or Ca-DPA, although work to date indicates that Ca-DPA triggers germination of these spores by acting through a nutrient germinant receptor (154).

In addition to the major general conclusions noted above, the current work also strongly indicates that the core water content plays a major role in the resistance of *C. perfringens* spores to moist heat. A recent study (152) found that wild-type *C. perfringens* spores made at 42°C had ~20% less core water than spores made at 26°C, and that D_{100} values for moist heat were ~4-fold lower in 26°C as compared to 42°C spores, both of which had essentially identical DPA levels. In the current work, an ~2-fold higher core water content in *spoVA* spores was accompanied by an 80-fold lower D_{100} value. While some of the effects on moist heat resistance of *spoVA* spores may be due to the loss of specific protective effects of Ca-DPA, most previous work (49), primarily with spores of *Bacillus* species, has found a strong inverse relationship between a spore's moist heat resistance and its core water content; this also seems to be true for *C. perfringens* spores over a wide range of core water contents. Presumably a low core water content is most effective in stabilizing essential proteins in the spore core, and thus a higher core water core results in an increased rate of inactivation of such proteins during moist heat treatment (30).

A notable observation made in this work was that *C. perfringens spoVA* spores were significantly more sensitive to formaldehyde, hydrogen peroxide and nitrous acid than were wild-type spores. Previous work has shown that *B. subtilis* spores with or without Ca-DPA have core water contents similar to those found in this work for the corresponding *C. perfringens* spores (144, 236). *B. subtilis* spores that lack DPA are also significantly more sensitive to formaldehyde and hydrogen peroxide than are wild-type spores, although nitrous acid resistance has not been tested (144). *B. subtilis* spore resistance to formaldehyde, hydrogen peroxide and nitrous acid is due in large part to the protection of spore DNA against these agents by DNA's saturation with α/β-type SASP (208). However, the levels of α/β-type SASP in DPA-less *B. subtilis* spores appear to be essentially identical to those in wild-type spores (144, 236), as was also the case with *C. perfringens* spores. Thus the decreased resistance of DPA-less *C. perfringens* spores to formaldehyde, hydrogen peroxide and nitrous acid is not due to decreased levels of α/β-type SASP. Possible explanations for this decreased resistance are thus that: i) the increased hydration of the DPA-less spores allows more rapid reaction of reactive chemicals with either DNA saturated with α/β-type SASP or other targets in the spore core such as proteins; or ii) while levels of α/β-type SASP in the DPA-less spores are normal, a significant amount of these proteins may not be bound to the DNA because a higher core water content promotes their dissociation, a process known to take place in fully germinated *B. subtilis* spores that have a core water content comparable to that in growing cells (190); this protein-free DNA would then be very sensitive to DNA damaging chemicals, as is the case in *B. subtilis* and *C. perfringens* spores (151, 174, 176, 208). Analysis of whether hydrogen peroxide kills

DPA-less *C. perfringens* spores by DNA damage might resolve this issue, as at least with *B. subtilis* spores, DNA is so well protected by α/β-type SASP that this agent does not kill these spores by DNA damage, although it does cause lethal DNA damage in *B. subtilis* spores that lack most α/β-type SASP (199, 208). However, there have as yet been no detailed studies of the mechanism of the killing of *C. perfringens* spores by hydrogen peroxide, or even any other agent. The DPA-less *C. perfringens spoVA* spores were also more sensitive to HCl than were wild-type spores. HCl resistance of DPA-less *B. subtilis* spores has not been tested, so this is a novel observation. Since the mechanism of *C. perfringens* spore killing by and resistance to HCl are not known, it is difficult to explain this observation, although at least in *B. subtilis* spores, the α/β-type SASP are not involved in HCl resistance (208).

A third notable observation made in this work is that the DPA-less *C. perfringens spoVA* spores were markedly more UV sensitive that wild-type spores. This was not seen previously with *C. perfringens* spores that had 15-20% higher core water contents but retained normal DPA levels, so perhaps sensitization to UV requires a much larger increase in core water content, as was also the case for sensitization to hydrogen peroxide (152). The major factor in the UV resistance of *B. subtilis* spores is the saturation of spore DNA with α/β-type SASP, and this is also important in the UV resistance of *C. perfringens* spores (174, 176, 205, 206, 208). Perhaps a significant amount of the *C. perfringens* α/β-type SASP are dissociated from the DNA in the DPA-less spores as suggested above thus causing their decreased UV resistance. This does not appear to occur in DPA-less *B. subtilis* spores that have almost identical UV resistance to wild-type spores (144, 236), but perhaps the *C.*

perfringens α/β-type SASP have significantly lower affinity for DNA than do the comparable *B. subtilis* proteins. While, this has not been studied directly, it has been shown that one *C. perfringens* α/β-type SASP is less effective in restoring resistance properties to α/β-type SASP-deficient *B. subtilis* spores than is a *B. subtilis* protein, although the reason for this difference has not been established (101). In addition, the *C. perfringens* and *B. subtilis* α/β-type SASP exhibit significant differences in primary sequence, in particular in the spacing between two highly conserved regions recently shown to be crucial for the binding to and protection of DNA by a *B. subtilis* protein (100, 209).

One of the more surprising findings in this work was that the colony forming efficiency of the *C. perfringens spoVA* spores was 20-fold lower than that of wild-type spores, although this is not the case with DPA-less *B. subtilis* spores (144, 236). The lower colony forming efficiency of the *C. perfringens spoVA* spores was not due to inefficient spore germination, as these spores germinated as well as wild-type spores and their viability was not increased by inclusion of lysozyme in plating medium. Thus the majority of these spores appear to be dead. While we are not certain why this is the case, if some of the chromosome in the DPA-less *C. perfringens* spores is indeed not saturated with α/β-type SASP, as suggested above, perhaps this naked DNA is particularly susceptible to damage caused by endogenous agents. Indeed, while *B. subtilis* spores that lack either DPA or most α/β-type SASP are fully viable, the viability of *B. subtilis* spores that lack both DPA and α/β-type SASP is drastically reduced, and these spores die during sporulation largely due to DNA damage (202). Perhaps analysis of surviving *C. perfringens spoVA* spores for evidence of DNA

damage, for example mutations, might clarify the reason that so many of these spores appear dead.

It was also notable that decoating of wild-type or *spoVA C. perfringens* spores caused a significant reduction in apparent spore viability, with this reduction being greatest for the harsher decoating regimen of Treatment 1. However, these decoated spores were not dead, since their viability could be completely restored by inclusion of lysozyme in the plating medium. This result strongly suggests that the decoating treatment has removed the CLEs that degrade the *C. perfringens* cortex PG during spore germination, although this same decoating treatment does not remove all CLEs from *B. subtilis* spores. This suggests that CLEs in *C. perfringens* spores are located only in or adjacent to the outer edge of the cortex. The CLEs SleC and SleM, one of which (SleC) is activated by proteolysis early in germination, have been identified in and purified from *C. perfringens* spores, and their enzymatic activity has been characterized (24, 92, 121, 137, 212, 239). However, the role of these proteins *in vivo* during spore germination has not been established, and they show little homology with the CLEs CwlJ and SleB that catalyze cortex hydrolysis during *B. subtilis* spore germination. Analysis of *C. perfringens* spores with mutations in the *sleC* and *sleM* genes should allow a better understanding of the role of the encoded proteins in spore germination, and how these CLEs interact with germinant receptors and Ca-DPA.

Fig. 6.1: Arrangement and expression of the *spoVA* operon in *C. perfringens*.

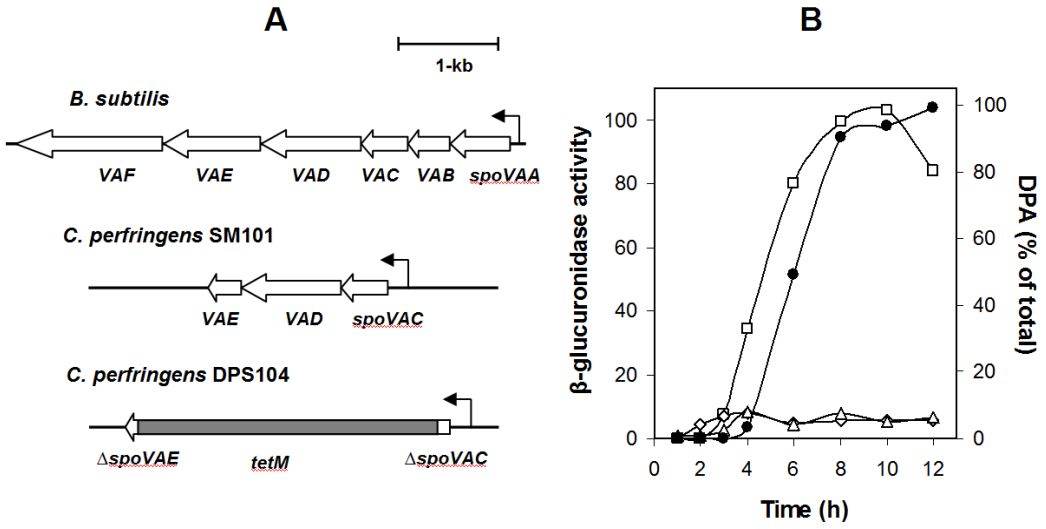


Fig. 6.1A,B. Arrangement of the *spoVA* operon in *B. subtilis* and in *C. perfringens* strains and expression of the *spoVA* operon during *C. perfringens* sporulation. A) The arrangement of the *spoVA* operons of the various organisms is shown with the location of the known (*B. subtilis*) and putative (*C. perfringens*) promoters indicated by the leftward pointing arrows. B) GUS specific activity from the *spoVAC-gusA* fusion (open squares), *spoVAD-gusA* fusion (open diamonds) or *spoVAE-gusA* fusion (open triangles) and level of DPA accumulated (filled circles) in *C. perfringens* SM101 grown in sporulation medium were determined as described in Materials and Methods.

Fig. 6.2: Germination of intact and decoated *C. perfringens* wild-type and *spoVA* spores with various germinants.

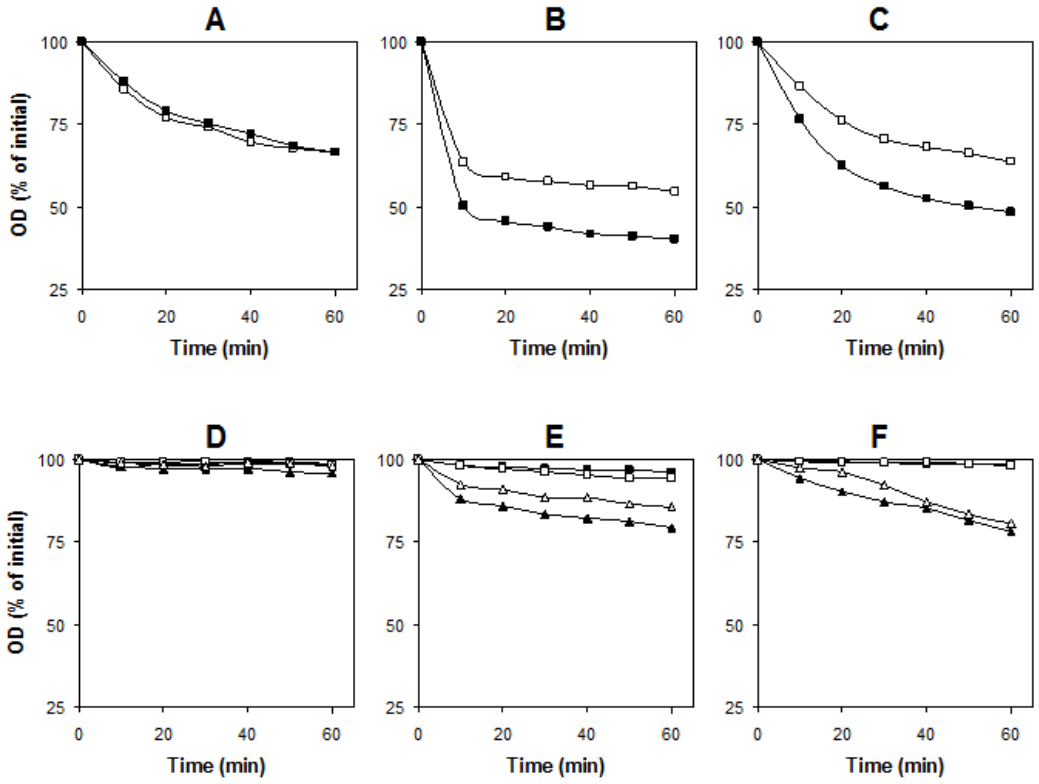


Fig. 6.2A-F. Germination of intact and decoated *C. perfringens* wild-type and *spoVA* spores with various germinants. A-C) Heat activated intact spores of strains SM101 (wild-type) (filled squares) and DPS104 (*spoVA*) (open squares) were incubated at 40ºC with BHI broth (A), 100 mM AK (B), or 50 mM Ca-DPA (C), and at various times, the OD_{600} was recorded as described in Materials and Methods. D-F) Spores were decoated by Treatment 1 (squares); or by Treatment 2 (triangles). Decoated spores of strains SM101 (wild-type) (filled symbols) and DPS104 (*spoVA*) (open symbols) were heat activated and incubated with BHI broth (D), 100 mM AK (E), or 50 mM Ca-DPA (F), and at various times, the OD_{600} was recorded as described in Materials and Methods. No significant germination of intact or decoated spores of strains SM101 or DPS104 was observed when heat activated spores were incubated in 25 mM sodium phosphate buffer at (pH 7.0) at 40ºC for 60 min.

Fig. 6.3: DPA release from decoated *C. perfringens* spores incubated with various germinants.

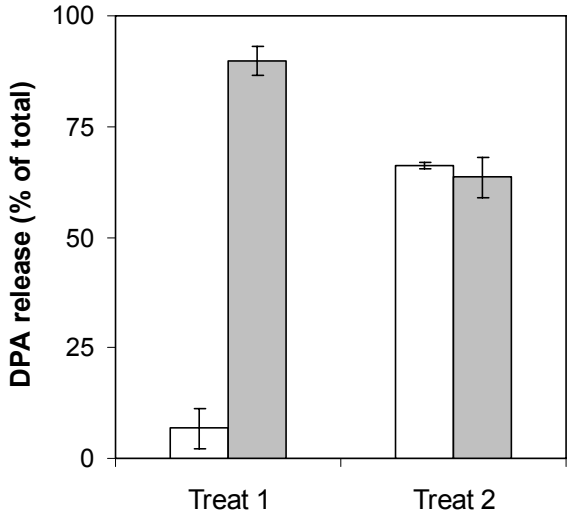


Fig. 6.3. DPA release from decoated *C. perfringens* spores incubated with various germinants. Spores of *C. perfringens* strain SM101 (wild-type) were decoated by: i) Treatment 1 (Treat 1); or ii) Treatment 2 (Treat 2). Decoated spores were germinated with 100 mM AK, 25 mM sodium phosphate buffer (pH 7.0) for 60 min at 37ºC (white bars), or with 1 mM dodecylamine, 25 mM Tris-HCl (pH 7.4) for 60 min at 60ºC (grey bars), and DPA release was measured as described in Materials and Methods.

Fig. 6.4: Levels of α/β-type SASP in *C. perfringens* wild-type and *spoVA* spores.

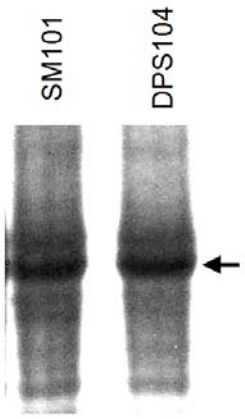


Fig. 6.4. Levels of α/β-type SASP in *C. perfringens* wild-type and *spoVA* spores. Spores of *C. perfringens* strains SM101 (wild-type) and DPS104 (*spoVA*) were dry ruptured, SASP extracted, extracts processed, lyophilized, the dry residue dissolved in 30 μl of 8M urea, 5-μl aliquots run on PAGE at low pH, and the gel was stained as described in Methods. The arrow indicates the major band reactive with antibody to the *B. subtilis* α/β-type SASP, SspC (176).

Fig. 6.5: Resistance of *C. perfringens* wild-type and *spoVA* spores to heat and UV radiation.

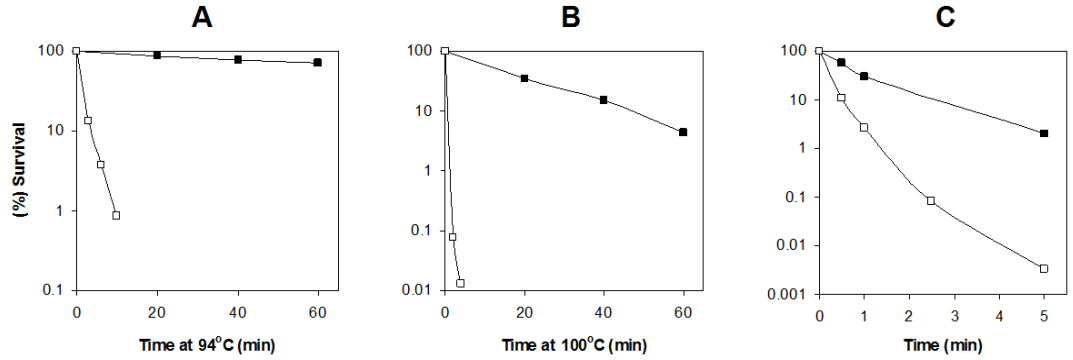


Fig. 6.5. Resistance of *C. perfringens* wild-type and *spoVA* spores to moist heat at 94ºC (A), or 100ºC (B), and to UV radiation (C). DS medium spore cultures or purified spores of *C. perfringens* strains SM101 (wild-type) (filled squares) and DPS104 (*spoVA*) (open squares) were used to measure survival upon various treatments as described in Materials and Methods. The variability of the survival values in these experiments was ± 20%.

Fig. 6.6: Resistance of *C. perfringens* wild-type and *spoVA* spores to various chemicals.

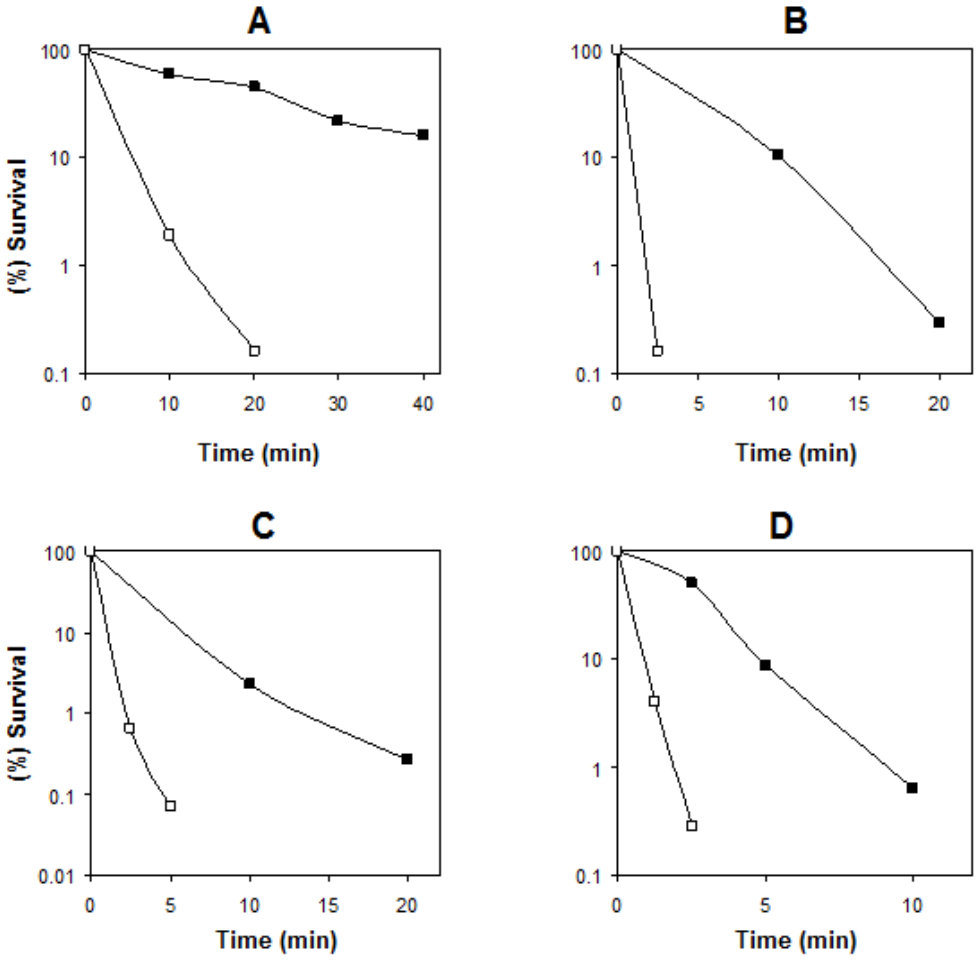


Fig. 6.6A-D. Resistance of *C. perfringens* wild-type and *spoVA* spores to nitrous acid (A), HCl (B), formaldehyde (C), and hydrogen peroxide (D). Spores of *C. perfringens* strains SM101 (wild-type) (filled squares) and DPS104 (*spoVA*) (open squares) were purified and their survival upon various treatments was measured as described in Materials and Methods. The variability of the survival values in these experiments was ± 15%.

Table 6.1. Bacterial strains and plasmids used

Strain or Plasmid	Relevant characteristic(s)	Reference or source
C. perfringens		
SM101	Electroporatable derivative of food poisoning type A isolate, NCTC8798; carries a chromosomal *cpe*	(256)
DPS104	*spoVA* mutant (*spoVA*::*tetM*) derivative of SM101	This study
SM101(pDP51)	Wild-type strain carrying *spoVA-gusA* fusion	This study
SM102(pDP79)	Wild-type strain carrying *spoVAD-gusA* fusion	This study
SM101(pDP80)	Wild-type strain carrying *spoVAE-gusA* fusion	This study
DP104(pDP54)	*spoVA* mutant complemented with wild-type *spoVA*	This study
Plasmids		
pJIR418	*C. perfringens/E. coli* shuttle vector; Cm^r and Em^r.	(219)
pMRS127	*C. perfringens/E. coli* shuttle vector carrying a promoterless *gusA*; Em^r	(174)
pJIR599	pBluescript II carrying erythromycin resistance gene (*ermB*), Em^r	(8)
pJIR750	*C. perfringens/E. coli* shuttle vector, Cm^r.	(11)
pJIR1886	pBluescript II carrying tetracycline resistance gene (*tetM*); Tet^r	J. I. Rood
pDP1	*gerAA* internal fragment (~ 800-bp) cloned in EcoRI site of pCR®-XL-TOPO®	D. Paredes-Sabja
pDP31	~ 1.0-kb PCR fragment containing 730-bp upstream and 270-bp N-terminal coding region of *spoVAC* in pCR®-XL-TOPO®	This study
pDP32	~ 1.4-kb PCR fragment containing 324-bp C-terminal coding region and 1054-bp downstream of *spoVAE* in pCR®-XL-TOPO®	This study
pDP33	~ 1.0-kb *Kpn*I-*Spe*I fragment from pDP31 cloned between KpnI/SpeI sites of pDP1	This study
pDP34	~ 1.4-kb *Pst*I-*Xho*I fragment from pDP32 cloned between PstI/XhoI sites of pDP33	This study
pDP35	~ 3.2-kb *Eco*RI fragment carrying *tetM* from pJIR1886 cloned in EcoRI site of pDP34	This study
pDP45	~ 1.1-kb *Sma*I fragment carrying *ermB* from pJIR599 cloned in SmaI site of pDP35	This study
pDP51	~ 393-bp PCR amplified upstream region of *spoVAC* cloned in pMRS127	This study
pDP53	~ 2.5-kb PCR fragment containing *spoVA* operon and upstream region cloned in pCR®-XL-TOPO®	This study
pDP54	~ 2.5-kb *Kpn*I-*Sal*I fragment containing, *spoVA* operon and upstream region from pDP53 in pJIR750	This study

pDP79	~ 378-bp PCR amplified upstream region of *spoVAD* cloned in pMRS127	This study
pDP80	~ 351-bp PCR amplified upstream region of *spoVAE* cloned in pMRS127	This study

Table 6.2. Primers used in this study

Primer name	Primer sequence[a]	Position[b]	Gene	Use[c]
CPP288	GGGTACCCCTATGAGGATATATTTCAGAT TGGG	-731 to -706	*spoVAC*	MP
CPP289	GACTAGTCTGCTCCAGCCTTAGCGGC	+252 to +270	*spoVAC*	MP
CPP290	GCTGCAGCGGTGCAATCTGTGTAATAGGA CAG	+42 to +66	*spoVAE*	MP
CPP291	CCTCGAGGCTT GTG CAG ACA GTC CTA AGC	+1399 to +1420	*spoVAE*	MP
CPP341	GGGTACCCGATGAAGGAACGCCGGTT	-393 to -375	*spoVAC*	CP
CPP347	GCGTCGACGTACAACTAAAAAAGCTGTA CCT	+521 to +544	*spoVAE*	CP
CPP274	GCGTCGACGATGAAGGAACGCCGGTT	-393 to -375	*spoVAC*	GUS
CPP275	GCTGCAGAGCTATATTAATCACCCTTTTCTAT T	-27 to -1	*spoVAC*	GUS
CPP382	GCAGCGTCGAC GTAGGTGGATTAATTTGTGTAATAGG	-371 to -346	*spoVAD*	GUS
CPP381	GACGCTGCAG TACTCATTCTTATCACCCCAC	-14 to +7	*spoVAD*	GUS
CPP387	GCAGCGTCGACAAAGAGTATGGATATGA TATTAGTAAGG	-335 to -307	*spoVAE*	GUS
CPP384	GACGCTGCAGCTTGCAAAATTCTCATCTC CTCATTATT	-12 to +16	*spoVAE*	GUS

[a] Restriction sites are marked by underlining

[b] The nucleotide position numbering begins from the first codon and refers to the relevant position within the respective gene sequence.

[c] MP, construction of mutator plasmid; CP, construction of complemented plasmid; GUS, construction of *gusA* fusion plasmid.

Table 6.3. Effects of *spoVA* mutation on *C. perfringens* spore properties

Strain (genotype)	DPA ($\mu g/ml/OD_{600}$)[a]	Ca^{2+} ($\mu g/ml/OD_{600}$)[a]	Core wet density (g/ml)[b]	Water content (g/100 g of protoplast [wet weight])[c]
SM101 (wild-type)	21.7	3.6	1.381	31
DPS104 (*spoVA*)	< 0.27	< 0.07	1.303	62

[a] Values are averages of determinations on three different spore preparations.

[b] Values are averages of determination of two different spore preparations that differed by ≤0.003 g/ml.

[c] Spore core water content was calculated according to formula y = -0.00254x + 1.460 (105), where y is the spore core wet density and x is the core water content in grams per 100 g of protoplast (core) weight (wet weight).

Table 6.4. Colony formation by spores of *C. perfringens* strains[a]

Treatment[b] and strain (genotype)	Spore titer (CFU/ml/OD_{600})[c]	
	BHI	BHI + Lyz[d]
No treatment		
SM101 (wild-type)	3.7×10^7	4.6×10^7
DPS104 (*spoVA*)	1.9×10^6	2.2×10^6
Treatment 1 [d]		
SM101 (wild-type)	3.3×10^3	9.8×10^7
DPS104 (*spoVA*)	2.2×10^3	1.7×10^6
Treatment 2		
SM101 (wild-type)	3.6×10^6	3.7×10^7
DPS104 (*spoVA*)	9.3×10^5	5.7×10^6

[a] Heat activated spores of various strains were plated on BHI agar, and colonies were counted after anaerobic incubation at 37ºC for 24 h.

[b] Spores were decoated by treatment 1 or treatment 2.

[c] The titers are the average from three experiments

[d] BHI plates containing 1μg/ml lysozyme...

Chapter 7

SleC is essential for cortex peptidoglycan hydrolysis during germination of spores of the pathogenic bacterium *Clostridium perfringens*.

Daniel Paredes-Sabja, Peter Setlow and Mahfuzur R. Sarker

Published in:

Journal of Bacteriology 2009 **191** (8): 2711-2720

7.1 Abstract

Clostridial spore germination requires degradation of the spore's peptidoglycan (PG) cortex by cortex-lytic enzymes (CLEs), and two *C. perfringens* CLEs, SleC and SleM, degrade cortex PG *in vitro*. We now find that only SleC is essential for cortex hydrolysis and viability of *C. perfringens* spores. *C. perfringens sleC* spores did not germinate completely with nutrients, KCl or a 1:1 chelate of Ca^{2+} and dipicolinic acid (DPA) (Ca-DPA), and the colony forming efficiency of *sleC* spores was 10^3-fold lower than wild-type spores. However, *sleC* spores incubated with various germinants released most of their DPA, although slower than wild-type or *sleM* spores, and DPA release from *sleC sleM* spores was very slow. In contrast, germination and viability of *sleM* spores were similar to that of wild-type spores, although *sleC sleM* spores had 10^5-fold lower viability. These results allow the following conclusions about *C. perfringens* spore germination: (i) SleC is essential for cortex hydrolysis; (ii) although SleM can degrade cortex PG *in vitro*, this enzyme is not essential; (iii) action of SleC alone or with SleM can accelerate DPA release; and (iv) Ca-DPA does not trigger spore germination by activation of CLEs.

7.2 Introduction

Clostridium perfringens is a gram-positive, spore-forming anaerobic bacterium and a significant cause of histotoxic and gastrointestinal (GI) diseases in humans and animals (117, 119). *C. perfringens* isolates are classified into 5 types, A through E, based on their ability to produce alpha-, beta-, epsilon- and iota-toxin (119). A small percentage (< 5%) of type A isolates produce the *C. perfringens* enterotoxin (CPE)

and cause type A food poisoning and non-food-borne GI diseases (117). *C. perfringens* isolates also can form metabolically dormant spores that are resistant to many stress factors, and can survive for long periods in the environment. Under favorable conditions these surviving spores can germinate, outgrow, return to vegetative growth and then release toxins and cause disease (119).

Bacterial spores initiate germination when they sense compounds termed germinants, including nutrients, a 1:1 chelate of Ca^{2+} and pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)) (Ca-DPA) and cationic surfactants (145, 154, 207). In spores of *Bacillus subtilis* and related species, nutrient germinants are sensed by receptors located in the spore's inner membrane, with subsequent initiation of biophysical events including release of monovalent cations (H^+, Na^+, and K^+), and the spore core's large depot of DPA present as a 1:1 chelate with divalent cations, predominantly Ca^{2+} (207). Hydrolysis of the spore's peptidoglycan (PG) cortex is a later event in germination, and in *B. subtilis* is triggered at least in part by Ca-DPA release (153, 207). Cortex PG hydrolysis is essential for completion of spore germination, since this removes a physical constraint allowing the core to expand and take up water to the level found in vegetative cells (171). This full hydration now allows enzyme activity in the spore core leading to initiation of energy metabolism, macromolecular synthesis and spore outgrowth (34, 207).

In *Bacillus* spores the PG cortex comprises ≥ 80% of spore PG, with the remainder in the nascent germ cell wall that becomes the cell wall of the outgrowing spore (120). Cortex PG generally has three structural differences from germ cell wall and vegetative cell PG, as studied best in *B. subtilis* (7, 170, 172). 1) While about one-

fourth of cortex N-acetyl-muramic acid (NAM) residues are substituted with short peptides, essentially all NAM residues in germ cell wall and vegetative cell PG carry these short peptides. As a consequence cortex PG is less highly cross-linked than germ cell wall or vegetative PG. 2) About one-fourth of NAM residues in cortex PG carry a single L-alanine residue, a modification not found in germ cell wall or vegetative cell PG, although this modification is not present in *C. perfringens* cortex PG (139). 3) Approximately every second muramic acid residue in cortex PG has been converted to muramic-δ-lactam (MAL), a modification again not found in germ cell wall or vegetative cell PG (247, 248). MAL appears to be the recognition element for cortex-lytic enzymes (CLEs) that hydrolyze the cortex during spore germination but do not act on germ cell wall PG (110, 207).

In *B. subtilis* and *B. megaterium* there are two redundant CLEs that degrade cortex PG during germination, SleB and CwlJ, as well as a third enzyme SleL (YaaH) that by itself is not sufficient for cortex hydrolysis during germination, although may contribute to the hydrolysis in some way (34, 98, 110, 207). SleB is synthesized in the developing forespore and is located primarily at the inner edge of the cortex in a mature form possessing lytic transglycosylase activity (16, 27), but the mechanism for regulation of SleB activity is unclear. In contrast to SleB, CwlJ is synthesized in the mother cell and is located primarily at the outer edge of the spore cortex (10, 27, 77). While CwlJ appears to be synthesized in an active form, the specificity of this enzyme has not been determined. However, CwlJ is activated during germination by Ca-DPA, either released from the spore core or supplied exogenously (110, 207).

Work with *C. perfringens* strain S40 has identified two CLEs, SleC and SleM, as potentially involved in cortex PG hydrolysis during spore germination. The *sleC* and *sleM* genes are present in the genomes of all sequenced *C. perfringens* strains (130, 213), and homologues are also present in other *Clostridium* species (18, 133, 197). SleC is synthesized in the mother cell compartment of the sporulating cell as a precursor with four domains, and the N-terminal pre-region and the C-terminal pro-region are removed during sporulation. SleC exists in the spore as the inactive zymogen, pro-SleC, consisting of an N-terminal pro-sequence and the mature active enzyme, and pro-SleC is converted to the active enzyme early in spore germination through removal of the pro-region by germination-specific-proteases (GSPs) (121, 137, 239). However, it is not clear what regulates GSPs activity. Active SleC is a bifunctional enzyme with lytic transglycosylase and N-acetylmuramoyl-L-alanine amidase activity on crosslinked peptide moieties in the cortex (92), and the purified enzyme is active on the cortex in decoated spores (92). SleM is also synthesized in the mother cell compartment during sporulation but as the mature enzyme, and has N-acetylmuramidase activity. SleM appears to degrade only SleC-modified cortex PG, and has no activity on decoated spores (24). Both SleC and SleM appear to be located on the outer edge of the spore cortex and are removed when spores are decoated (24).

While significant knowledge has been obtained on the properties of SleC and SleM *in vitro*, the actual role of these enzymes during *C. perfringens* spore germination has not been established. Consequently, in the current work we have constructed *sleC*, *sleM* and *sleC sleM* mutant strains of *C. perfringens* and used these strains to determine the roles of SleC and SleM during spore germination.

7.3 Materials and Methods

Bacterial strains and plasmids. The *C. perfringens* strains and the plasmids used in this study are described in Table S7.1.

Construction of *gusA*-fusion plasmids and β-glucuronidase assay. DNA fragments (300-400 bp) upstream of *sleC* and *sleM* from *C. perfringens* SM101, which included the 52 and 74-bp intergenic regions between *sleC* and *cspB,* and *sleM* and CPR1312, respectively, that most likely contain these genes' promoters, as shown for *C. perfringens* strain S40 (112), were PCR amplified using the primer pairs CPP399/CPP388 and CPP396/CPP398. The forward and reverse primers (Table S7.2) had SalI and PstI cleavage sites, respectively. These PCR-fragments were digested with SalI and PstI and cloned between SalI and PstI sites in pMRS127 to create *sleC*- and *sleM-gusA* fusions, giving plasmids pDP86 and pDP87 (Table S7.1). These plasmids were introduced by electroporation (35) into *C. perfringens* SM101 and erythromycin-resistant (Em^r; 50 μg/ml) transformants were selected. Transformants carrying plasmids with the *sleC-gusA* and *sleM-gusA* fusions were grown vegetatively in TGY medium (3% trypticase soy, 2% glucose, 1% yeast extract, 0.1% L-cysteine) (91) and in Duncan-Strong (DS) (36) sporulation medium, and assayed for GUS activity as described (256). GUS specific activity was expressed in Miller Units that were calculated as described (174).

Construction of a *C. perfringens sleC* deletion mutant. To isolate a derivative of *C. perfringens* SM101 with a deletion of *sleC*, a Δ*sleC* suicide vector was constructed as follows. A 2235-bp DNA fragment carrying 114-bp from the N-terminal coding region and 2121-bp upstream of *sleC* was PCR amplified using primers CPP357/CPP365 (forward and reverse primers (Table S7.2) had KpnI and BamHI cleavage sites at the 5'-ends, respectively). A 2087-bp DNA fragment carrying 242-bp from the C-terminal coding region and 1845-bp downstream of *sleC* was PCR amplified using primers CPP359/CPP364 (forward and reverse primers (Table S7.2) had PstI and XhoI cleavage sites at the 5'-ends, respectively). These PCR fragments were cloned into pCR-XL-TOPO (Invitrogen, Carsbad, CA) giving plasmids pDP60 and pDP62. An ~ 2.2-kb KpnI-BamHI fragment from pDP60 was cloned in pDP25 (Table S7.1) giving plasmid pDP61, and an ~2.1-kb PstI-XhoI fragment from pDP62 was cloned into pDP61 (Table S7.1) giving pDP63. Finally, an ~ 5.5-kb KpnI-XhoI fragment from pDP63 was cloned into pMRS104 (Table S7.1) giving pDP64 which cannot replicate in *C. perfringens*. Plasmid pDP64 was introduced into *C. perfringens* SM101 by electroporation, and a chloramphenicol resistant (Cm^r; 20 μg/ml) *sleC* mutant was isolated as described (192). The identity of the *sleC* strain DPS107 was confirmed by PCR and Southern blot analyses (data not shown).

Construction of a *C. perfringens sleM* deletion mutant. To isolate a derivative of *C. perfringens* SM101 with a deletion of *sleM*, a Δ*sleM* suicide vector was constructed as follows. A 1006-bp DNA fragment carrying 61-bp from the N-terminal coding region and 945-bp upstream of *sleM* was PCR amplified using primers CPP401/CPP403

(forward and reverse primers (Table S7.2) had KpnI and SpeI cleavage sites at the 5'-ends, respectively). A 1436-bp DNA fragment carrying 225-bp from the C-terminal coding region and 1211-bp downstream of *sleM* was PCR amplified using primers CPP404/CPP400 (forward and reverse primers (Table S7.2) had PstI and XhoI cleavage sites at the 5'-ends, respectively). These PCR fragments were cloned into pCR-XL-TOPO giving plasmids pDP91 and pDP92. An ~ 1.0-kb KpnI-SpeI fragment from pDP91 was cloned in pDP25 (Table S7.1) giving plasmid pDP93, and an ~1.4-kb PstI-XhoI fragment from pDP92 was cloned into pDP93 (Table S7.1) giving pDP94. Finally, an ~ 3.7-kb KpnI-XhoI fragment from pDP94 was cloned into pMRS104 (Table S7.1) giving pDP95 which cannot replicate in *C. perfringens*. Plasmid pDP95 was introduced into *C. perfringens* SM101 by electroporation, and a Cm^r *sleM* mutant was isolated as described (192). The identity of the *sleM* strain DPS109 was confirmed by PCR and Southern blot analyses (data not shown).

Construction of a *sleC sleM* double mutant. To isolate a derivative of *C. perfringens* SM101 with deletions of both *sleC* and *sleM*, a Δ*sleC* suicide vector encoding tetracycline resistance (Tet^r; 2 μg/ml) was constructed as follows: A 3.2-kb BamHI-PstI fragment carrying *tetM* from pDP35 was cloned into pDP64 (Table S7.1) giving plasmid pDP65. Next, a ~1.1 SmaI fragment carrying *ermB* from pJIR599 was cloned into pDP65 (Table S7.1) giving pDP66, which cannot replicate in *C. perfringens*. Plasmid pDP66 was introduced into *C. perfringens* DPS109 by electroporation, and a Cm^r Tet^r *sleC sleM* mutant was isolated as described (192). The identity of the *sleC*

sleM strain DPS110 was confirmed by PCR and Southern blot analyses (data not shown).

Construction of a Δ*sleC* strain complemented with *sleC*. To construct a *sleC* strain complemented with wild-type *sleC*, a suicide-complementing plasmid targeted to the *plc* locus was constructed as follows. A 1704-bp PCR fragment carrying 1590-bp upstream and 114-bp of the N-terminal coding region of *plc* was PCR-amplified using primers CPP507/CPP511 (forward and reverse primers (Table S7.2) had SacI and KpnI cleavage sites at the 5'-ends, respectively). A 1264-bp PCR fragment carrying 309-bp from the C-terminal coding region and 955-bp downstream of *plc* was PCR amplified using primers CPP516/CPP510 (forward and reverse primers (Table S7.2) had SalI and SphI cleavage sites at the 5'-ends, respectively). These PCR fragments were cloned into pCR-XL-TOPO (Invitrogen, Carsbad, CA) giving plasmids pDP126 and pDP127 (Table S7.1). An ~ 1.7-kb SacI-KpnI fragment from pDP126 was cloned into pMRS104 (Table S7.1) giving plasmid pDP128, and an ~1.3-kb SalI-SphI fragment from pDP127 was cloned into pDP128 (Table S7.1) giving pDP129. Next, an ~1.9-kb PCR fragment (including 437-bp upstream of *sleC* and the *sleC* coding region) was PCR amplified with Phusion™ High-Fidelity DNA Polymerase (New England BioLabs® Inc.) with primers CPP482/CPP488 (forward and reverse primers (Table S7.2) had KpnI and SalI cleavage sites at the 5'-ends, respectively), and cloned into Zero-Blunt®-TOPO® (Table S7.1) giving pDP115. As shown by assays of GUS activity (Fig. 7.1B), the 437-bp region upstream of *sleC* contained a strong promoter, as expected (Masayama *et al*., 2006). Finally, a ~ 1.9-kb KpnI-SalI fragment from

pDP115 was cloned into pDP129 giving pDP138 (Table S7.1) which cannot replicate in *C. perfringens*. Plasmid pDP138 was introduced into the *C. perfringens* Δ*sleC* strain DPS107 by electroporation (35), and Em^r Cm^r transformants were selected. The presence of plasmid pDP138 in DPS107(pDP138) was confirmed by PCR and Southern blot analyses (data not shown).

Spore preparation and purification. Starter cultures (10 ml) of *C. perfringens* isolates were prepared by overnight growth at 37ºC in fluid thioglycollate broth (FTG) (Difco) as described (91). *C. perfringens* sporulating cultures were prepared by inoculating 0.2 ml of an FTG starter culture into 10 ml of DS sporulation medium (36), incubated for 24 h at 37ºC and the presence of spores was confirmed by phase-contrast microscopy. Large amounts of spores were prepared by scaling-up the latter procedure, as described (154). Spore preparations were cleaned by repeated centrifugation and washing with sterile distilled water until spore suspensions were > 99% free of sporulating cells, cell debris and germinated spores, suspended in distilled water at a final optical density at 600 nm (OD_{600}) of ~ 6 and stored at -20ºC.

Decoating treatment. Spores at an OD_{600} of 20 were decoated in 1 ml of 50 mM Tris-HCl (pH 8.0)-8 M urea-1% (wt/vol) SDS-50 mM dithiothreitol for 90 min at 37ºC and the spores were washed three times with 150 mM NaCl and twice with water (169). This extraction procedure did not kill spores as determined on BHI agar plates (see below) containing 1 μg/ml of lysozyme.

Assessment of colony forming efficiency of spores. Untreated and decoated spores at an OD_{600} of 1.0 were heat activated at 80ºC for 10 min, aliquots of various dilutions plated on BHI agar with or without lysozyme (1 μg/ml) in the plates, the plates incubated at 37ºC anaerobically for 24 h, and colonies were counted and results expressed as CFU/ml/OD_{600}.

Spore germination. Spore suspensions were heat activated at 80°C for 10 min, cooled in water at ambient temperature for 5 min, and incubated at 40°C for 10 min as described (154). Germination of spores with an OD_{600} of 1 with BHI broth, Ca-DPA (50 mM $CaCl_2$, 50 mM DPA adjusted to pH 8.0 with Tris-HCl) or KCl (100 mM KCl-25 mM sodium phosphate buffer (pH 7.0)) (Paredes-Sabja *et al*., 2008b) was routinely measured by monitoring the OD_{600} of spore cultures (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA), which falls ~ 60% upon complete germination of wild-type spores; levels of germination were also confirmed by phase-contrast microscopy. All values reported are averages of two experiments performed on at least two independent spore preparations, and individual values varied by less than 15% from average values shown.

DPA release. DPA release from spores during nutrient and nonnutrient germination was measured by heat activating (80ºC, 10 min) a spore suspension (OD_{600} of 1.5), cooling and incubating in BHI broth or in 25 mM sodium phosphate buffer (pH 7.0) alone or with 100 mM KCl (pH 7.0) or in 25 mM Tris-HCl (pH 8.0) alone or with 50 mM Ca-DPA adjusted to pH 8.0 with Tris-HCl at 40ºC for 60 min and 18 h. A 1-ml

aliquot was centrifuged in a microcentrifuge (13,2000 rpm, 2 min), and the spore pellet was washed four times with 1 ml distilled water. Control experiments were done for each experiment and reveal that losses of spores due to these multiple centrifugations were less than 10% of the initial amount and appropriate corrections for such losses were made accordingly. The residual spore DPA content was determined by boiling samples for 60 min, cooling on ice for 5 min, centrifugation at 13,200 rpm in a microcentrifuge for 5 min, and measuring the OD_{270} of the supernantant fluid as described (21, 203). The DPA content of the initial dormant spores was measured by boiling an aliquot (1 ml) for 60 min, centrifugation at 13,200 rpm in a microcentrifuge for 5 min, and measuring the OD_{270} of the supernatant fluid as described (21, 154). In *B. subtilis* spores ≥85% of the material absorbing at 270 nm is DPA (6, 21). To confirm that the material from *C. perfringens* spores that absorbed at 270 nm was indeed DPA, total DPA and DPA released upon germination from *C. perfringens* spores were also measured by a colorimetric assay in parallel with control assays with pure DPA (186). Comparison of the measurements by OD_{270} and the colorimetric assay indicated that ~ 90% of the material absorbing at 270 nm was indeed DPA (data not shown).

For measuring DPA release during dodecylamine germination, spores (OD_{600} of 1.5) were incubated at 60ºC with 1 mM dodecylamine in 25 mM Tris-HCl (pH 7.4). Aliquots (1 ml) of germinating cultures were centrifuged for 3 min at 13,200 rpm in a microcentrifuge and DPA in the supernatant fluid was measured by monitoring the OD_{270} as described (21, 154). The initial DPA content in dormant spores was

measured similarly. No significant DPA release was observed when spores were incubated in 25 mM Tris-Hcl (pH 7.4) at 60ºC for 1 h (data not shown).

Hexosamine release. The release of hexosamine-containing fragments of cortex PG into the germination medium was measured by germinating heat activated spores at an OD_{600} of 25 in 100 mM KCl and 10 mM Tris-HCl (pH 7.4). After incubation for 2 h at 40ºC, samples (1 ml) were centrifuged, and analyses of hexosamine in the supernatant fluid were carried out as described (51, 168). Analyses of hexosamine-containing material in dormant spores were carried out similarly.

7.4 Results

Evaluation of expression of *sle* genes in *C. perfringens* SM101. When the *C. perfringens* SM101 (130) genome was subjected to BLASTP analyses to identify genes encoding CLEs, two ORFs (CPR2566 and CPR1311) encoding proteins with high identity (90-94%) to CLEs from *C. perfringens* strain S40 were identified. CPR2566 is predicted to encode a 438 aa protein with 90% identity to SleC from *C. perfringens* S40 (Fig. 7.1A). CPR1311 is predicted to encode a 200 aa protein with 94% identity to SleM from *C. perfringens* S40 (Fig. 7.1A). Both the *sleC* and *sleM* genes appear likely to be monocistronic (112).

To assess whether the *C. perfringens* SM101 *sle* genes are expressed during sporulation, DNA upstream of each *sle* gene's coding sequence including the intergenic regions between the *sle* genes and the preceding gene (Fig. 7.1A), that most likely contain these genes' promoters as shown for *C. perfringens* strain S40 (112),

was fused to *Escherichia coli gusA*, and β-glucuronidase (GUS) activity was measured after introducing the various fusions into *C. perfringens* SM101. No significant GUS activity was observed in vegetative cultures of strain SM101 carrying *sleC-gusA* or *sleM-gusA* (Fig. 7.1B,C). However, sporulating cultures of strains carrying these *gusA*-fusions exhibited significant GUS activity (Fig. 7.1B,C), indicating that a sporulation-specific promoter is located upstream of each *sle* gene. Expression of GUS from the *sleC-gusA* fusion began ~ 2 h after induction of sporulation and GUS specific activity increased until 12 h (Fig. 7.1B). Expression of GUS from the *sleM-gusA* fusion also began ~ 2 h after induction of sporulation and reached a maximum specific activity ~ 6 h after induction of sporulation (Fig. 7.1C). Collectively, these results agree with previous work on expression of the *sleC* and *sleM* genes in *C. perfringens* S40 (112), and indicate that SleC and SleM could be involved in cortex hydrolysis during *C. perfringens* spore germination.

Effect of *sleC* and *sleM* mutations on spore germination in BHI broth and colony formation of spores on BHI plates. Several *in vitro* studies (24, 92, 239) indicate that *C. perfringens* SleC and SleM can specifically hydrolyze intact spore cortex or cortex fragments. To establish the roles of these CLEs during *C. perfringens* spore germination, we constructed strains carrying deletions of *sleC* (strain DPS107), *sleM* (strain DPS109), and both *sleC* and *sleM* (strain DPS110) (Table S7.1, Fig. 7.2A), and examined the germination of spores of these strains in BHI broth (Fig. 7.2B). The fall in OD_{600} of *sleM* spores in BHI broth was significantly ($p < 0.001$) faster and reached a lower level than that of wild-type spores during the first 30 min of incubation for

reasons that are not clear, but spores of both strains germinated to a similar extent after 60 min (Fig. 7.2B). Phase contrast microscopy of these spores also found similar levels (~ 80%) of phase dark spores (indicative of completion of spore germination) after 60 min of incubation in BHI broth with this number rising to ≥ 99% after 18 h (data not shown). However, only ~10 and 20% of wild-type spores had become phase dark after incubation in phosphate buffer alone for 1 or 18 h, respectively (data not shown). In contrast to wild-type and *sleM* spores, *sleC* and *sleC sleM* spores exhibited only a small decrease in OD_{600} after 60 min of incubation in BHI broth (Fig. 7.2B) and this decrease was similar to that observed with and *sleC sleM* spores incubated in phosphate buffer alone (data not shown). These results strongly suggest that SleC, but not SleM, is essential for hydrolysis of the PG cortex during *C. perfringens* spore germination and thus the large fall in OD_{600} during germination.

Phase contrast microscopy further indicated that ~ 50 and 30% of *sleC* and *sleC sleM* spores, respectively, had become phase grey after 1 h of incubation in BHI broth, indicative of some decrease in the spore core's refractive index, while after 18 h, ~ 80 and 60% of *sleC* and *sleC sleM* spores, respectively, had become phase grey (data not shown). However, ~ 30 and 20% of *sleC* and *sleC sleM* spores, respectively, had become phase grey after 1 h of incubation in phosphate buffer alone and these values increased to ~ 60% for both *sleC* and *sleC sleM* spores after 18 h. These data suggest that SleM may be important in conjunction with SleC in some way in effecting a fall in the spore core's refractive index during germination, perhaps by facilitating DPA release (and see below).

The severe germination defects of *sleC* and *sleC sleM* spores suggested that the colony forming efficiency of these spores might be lower than that of wild-type spores, since cortex hydrolysis is essential for the resumption of vegetative growth. No significant difference in colony forming efficiency was observed between wild-type and *sleM* spores on BHI agar plates (Table 7.1). In contrast, *sleC* spores exhibited ~ 10^3-fold lower colony-formation efficiency than wild-type spores, and the colony forming efficiency of *sleC sleM* spores was ~ 10^5- and 10^2-fold lower than that of wild-type and *sleC* spores, respectively (Table 7.1). However, the colony forming efficiencies of the *sleC* and *sleC sleM* spores were restored to wild-type levels when spores were decoated and plated on BHI agar containing 1 μg/ml lysozyme. Thus the *sleC* and *sleC sleM* spores were completely viable, but just unable to complete cortex hydrolysis and germination. These results: i) further support the essential role of SleC in cortex hydrolysis during germination and outgrowth of *C. perfringens* spores; and ii) suggest that only in the absence of SleC does SleM have a role in the germination of *C. perfringens* spores, and even then has only a secondary role.

The appearance of at least the great majority of colonies from *sleC* and *sleC sleM* spores was not due to genetic reversion, as PCR-analyses did not detect wild-type *sleC* and *sleM* genes in colonies obtained from *sleC* and *sleC sleM* spores after 24 h (data not shown). To test if longer incubation of *sleC* and *sleC sleM* spores on BHI agar plates at 37ºC would lead to increased colony formation efficiency, plates were incubated for up to 7 d and colonies counted every 24 h. As expected, > 99% of total colonies from wild-type spores appeared after only 24 h (data not shown). Surprisingly, when plates with *sleC* and *sleC sleM* spores were incubated for longer

periods, 10- to 100-fold higher colony counts appeared (Fig. 7.3A). However, these additional colonies only appeared surrounding those colonies that arose during the first 24 h of incubation (Fig. 7.3B), and no new isolated colonies appeared (data not shown). While the cause of this behavior of the *sleC* and *sleC sleM* spores is not completely clear, it could be due to the diffusion of some cell wall hydrolase released by vegetative cells in the initial colony, with this hydrolase then degrading the cortex of a small population of *sleC* spores that have a defective coat. Indeed, the maximum number of colonies that appeared from *sleC* and *sleC sleM* spores during the 7 d incubation was 10-to 100-fold lower than from wild-type spores (data not shown), suggesting that only a small percentage of *C. perfringens* spore populations gave rise to these late appearing colonies. In addition, *sleC* spore viability went up only ~ 10-fold in 48 h, while the *sleC sleM* spores required ~ 5 days to reach their maximum level. Perhaps cortex hydrolysis by a cell wall hydrolase from vegetative cells on defective spores is accelerated by SleM.

DPA release by *C. perfringens* spores germinated with BHI broth. The germination and viability defects noted above with *sleC* and *sleC sleM* spores suggest that these spores cannot complete the germination process because they cannot degrade cortex PG. However, since many of the *sleC* and *sleC sleM* spores incubated in BHI broth became phase grey in the phase contrast microscope, this suggested that there was a decrease in these spores' core refractive index, perhaps because of DPA release and its replacement by water. As expected, wild-type and *sleM* spores released most of their DPA after 1 h of germination in BHI broth (Fig. 7.4), with slightly more

released from the *sleM* spores, consistent with the slightly more rapid germination of these spores as measured by the fall in OD_{600} (Fig. 7.2B), although after 18 h, both *sleM* and wild-type spores had released ≥ 90% of their DPA (Fig. 7.4). In contrast, *sleC* spores released only ~ 50 and 85% of their DPA after 1 and 18 h germination in BHI broth, respectively (Fig. 7.4). Although *sleC sleM* spores released DPA during incubation in BHI broth, the amount released was significantly less than from wild-type, *sleC* and *sleM* spores incubated similarly, and was only slightly higher than the amount of DPA released from *sleC sleM* spores incubated in buffer alone (Fig. 7.4). Collectively, these results suggest that DPA release during *C. perfringens* spore germination does take place during germination of *sleC* spores in BHI broth, but is significantly slowed in absence of cortex hydrolysis, especially in the absence of both SleC and SleM. These results further suggest that SleM might have some role in DPA release, but is clearly not sufficient for cortex hydrolysis.

Complementation of *sleC* mutant with wild-type *sleC*. To be certain that the phenotypes observed for *sleC* spores were due directly to deletion of *sleC* and not any possible secondary effects due to strain construction, we constructed a suicide plasmid pDP138, carrying a mutated allele of the alpha-toxin gene (*plc*) in which a wild-type copy of *sleC* plus its promoter region (see Materials and Methods and Fig. 7.1) was inserted. The *plc* locus was chosen for insertion of wild-type *sleC* into the chromosome of the *sleC* strain by a homologous single recombination event, assuming that *plc* disruption would not affect spore germination. Plasmid pDP138 was introduced into the *sleC* strain DPS107, and PCR with DNA from one

DPS107(pDP138) transformant yielded products from wild-type *plc*, Δ*sleC* and the Δ*plc*::*sleC* allele, consistent with wild-type *sleC* in pDP138 being integrated into the *plc* locus by a single cross-over event (data not shown). Spores of strain DPS107(pDP138) germinated like wild-type spores in BHI broth as assessed by either the fall in OD_{600} (Fig. 7.2B) or phase contrast microscopy (data not shown). The low colony formation efficiency of *sleC* spores was also restored to that of wild-type spores when the *sleC* strain was complemented with wild-type *sleC* [strain DPS107(pDP138)] (Fig. 7.2B, Table 7.1). The slow DPA release from *sleC* spores during germination in BHI broth was also restored to the faster rate seen with wild-type spores in the complemented strain (Fig. 7.4). These results thus confirm that the phenotypes of *sleC* spores are due exclusively to deletion of *sleC*.

Effect of *sleC* and *sleM* mutations on *C. perfringens* spore germination with KCl. To gain further understanding of the roles of SleC and SleM in *C. perfringens* spore germination, wild-type, *sleC*, *sleM* and *sleC sleM* spores were incubated with the germinant KCl (154). As expected, *sleM* spores germinated like wild-type spores with KCl, when germination was assessed by the fall in OD_{600} (Fig. 7.5A). In contrast, *sleC* and *sleC sleM* spores showed only a minimal fall in OD_{600} upon incubation with KCl (Fig. 7.5A). The small decrease in OD_{600} observed for the latter spores was even lower than that of wild-type spores incubated in sodium phosphate buffer (Fig. 7.5A). Phase contrast microscopy indicated that ~ 60 and ~ 30% of *sleC* and *sleC sleM* spores incubated in 100 mM KCl, respectively, had become phase grey after 1 h, while ~ 10% of wild-type spores incubated for 1 h in sodium phosphate buffer had become

phase dark. This suggest that while the OD_{600} decrease of wild-type spores incubated in buffer alone was because a small percentage of the spores germinated spontaneously thus becoming phase dark, the OD_{600} decrease of *sleC* and *sleC sleM* spores incubated in KCl was due to the release of DPA by a fraction of the spore population, thus slightly reducing these spores' refractive index.

As expected, there were no significant differences in DPA release during KCl germination between *sleM* and wild-type spores, as the majority of these spores' DPA was released in 1 h (Fig. 7.5B). The *sleC* spores also released DPA during incubation in KCl, and while this release was faster than that for spores incubated in buffer alone, it was significantly slower than for wild-type or *sleM* spores incubated in KCl (Fig. 7.4, 7.5B). DPA release from *sleC sleM* spores incubated in KCl was even slower, and no faster than from *sleC sleM* spores incubated in buffer alone (Fig. 7.4, 7.5B).

In addition to DPA release, a second major event in spore germination is the hydrolysis of the spore's PG cortex and release of PG fragments into the medium. The hydrolysis of the spore's PG cortex during germination can thus be monitored by measuring the release of hexosamine-containing material into the medium (171, 232). As expected, wild-type and *sleM* spores released similar amounts of hexosamine into the medium after 2 h of germination with KCl, 35-40% of total spore hexosamine (Fig. 7.5C). However, no detectable release of hexosamine-containing material was observed from *sleC* and *sleC sleM* spores after a similar incubation (Fig. 7.5C). These results are similar to those found with germinating wild-type and *cwlJ sleB* spores of *B. subtilis* and *B. megaterium* (200, 203).

Ca-DPA germination of *C. perfringens* spores. In addition to nutrients, spores of *Bacillus* and *Clostridium* species are germinated by exogenous Ca-DPA (154, 207). Ca-DPA triggers germination of *B. megaterium* and *B. subtilis* spores by activation of the CLE CwlJ (146, 203). To determine if a *C. perfringens* CLE is also activated by Ca-DPA, the Ca-DPA germination of wild-type, *sleC*, *sleM* and *sleC sleM* spores was examined (Fig. 7.6). As expected, *sleC* and *sleC sleM* spores did not exhibit the large fall in OD_{600} in Ca-DPA germination seen with wild-type and *sleM* spores (Fig. 7.6A), and no significant decrease in OD_{600} was observed when spores of these four strains were incubated in 25 mM Tris-HCl (pH 8.0) (data not shown). However, phase contrast microscopy found that after 1 h of incubation with Ca-DPA, ~ 50 to 30% of the *sleC* and *sleC sleM* spores, respectively, had become phase grey, while only ~ 20% of *sleC* and *sleC sleM* spores had become phase grey after 1 h of incubation with 25 mM Tris-HCl (pH 8.0) (data not shown); only ~ 10% of wild-type spores had become phase dark after 1 h with 25 mM Tris-HCl (pH 8.0) (data not shown).

Measurements of DPA release (Fig. 7.6B) showed that, as found with BHI broth and KCl incubations, wild-type and *sleM* spores incubated for 1 h with Ca-DPA released significantly more DPA that did *sleC* spores, although DPA release from the *sleC* spores was much greater than during incubation with buffer alone (Fig. 7.6B). However, the amount of DPA released from *sleC sleM* spores during incubation with Ca-DPA was only slightly higher than the amount released during incubation in buffer (Fig. 7.6B). Unfortunately, the precipitation of the exogenous Ca-DPA after 1 h precluded assessment of DPA release over longer times.

Dodecylamine germination of *C. perfringens* spores. In addition to Ca-DPA, KCl and nutrients, *C. perfringens* spores are also germinated by the cationic surfactant dodecylamine (154). While the precise mechanism of dodecylamine germination is not known, the absence of CLEs does not have any effect on DPA release during dodecylamine germination of spores of at least two *Bacillus* species (203, 207). Similarly, wild-type, *sleM*, and *sleC sleM C. perfringens* spores and spores of the *sleC* strain complemented with wild-type *sleC* exhibited relatively similar rates and extents of DPA release during dodecylamine germination, while *sleC* spores released their DPA even faster (Fig. 7.7). Thus cortex hydrolysis is also not essential for normal DPA release during dodecylamine germination of *C. perfringens* spores.

7.5 Discussion

Spores of *Bacillus* species contain three CLEs, with either of two of these enzymes being essential (24, 110, 203, 207). In contrast, SleC is the single essential CLE in *C. perfringens* spores, with SleM playing only an auxiliary role. However, SleM can contribute to cortex hydrolysis during spore germination, as shown by the lower viability of *sleC sleM* spores than *sleC* spores. SleC and SleM are active on cortex PG fragments *in vitro*, but SleM, unlike SleC, is inactive on the cortex in intact but decoated spores (24, 121). This suggests that SleM, unlike SleC, is normally inactive on cortex PG in intact spores that may be under strain because of the high osmotic pressure of the poorly hydrated spore core (207). By this reasoning, only SleC would be essential for cortex hydrolysis during spore germination. However, perhaps in a minority of *sleC* spores the cortex is under less stress, and SleM can hydrolyze

this “low-stress” cortex. Alternatively, SleM may act in a small percentage of *sleC* spores that have a defective cortex, perhaps due to partial cortex hydrolysis during spore formation by an enzyme that acts on sporulating cell PG to allow release of the dormant spore. Indeed, if a few *sleC* spores have defective coats, a small amount of hydrolytic enzyme action during sporulation on such defective spores might generate spores whose cortex is susceptible to hydrolysis by SleM alone.

While loss of SleC eliminated cortex hydrolysis during *C. perfringens* spore germination, *sleC* spores still released their DPA in response to germinants that act through the spore’s germinant receptors (154). Spores of *Bacillus* species lacking both essential CLEs also release their DPA during nutrient germination (203, 207). These observations suggest that full cortex hydrolysis is not essential for DPA release during spore germination, and that at least the initiation of DPA release likely precedes cortex hydrolysis. However, DPA release from *sleC* spores germinating with BHI broth or KCl was slower than from germinating wild-type or *sleM* spores, with the rate of DPA release from germinating *sleC sleM* spores being even slower and at most only slightly greater than from spores incubated in buffer. This further indicates that while cortex hydrolysis is not essential for the initiation of DPA release during spore germination, cortex hydrolysis can accelerate DPA release. This effect of cortex hydrolysis of the rate of DPA release during germinant receptor-mediated spore germination has also been seen with *B. megaterium* and *B. subtilis* spores in which loss of CwlJ but not SleB slows DPA release significantly during nutrient germination (77, 203). Unfortunately, why cortex hydrolysis should accelerate DPA release from spores

during germinant receptor-mediated germination and why SleM should contribute to this effect are not clear.

Another notable observation in this work concerns the effects of loss of CLEs on Ca-DPA germination of *C. perfringens* spores. With *B. megaterium* and *B. subtilis* spores, Ca-DPA triggers germination by activation of the CLE CwlJ, and bypasses the spore's germinant receptors (203, 207). In contrast, Ca-DPA appears to trigger *C. perfringens* spore germination through the GerK germinant receptor (154). This latter finding leads to the prediction that Ca-DPA will trigger DPA release from *C. perfringens* spores lacking SleC, and this is what was observed, even though DPA release during Ca-DPA germination of *sleC C. perfringens* spores was slower than from wild-type or *sleM* spores. The similar response of rates of Ca-DPA release during Ca-DPA, BHI broth and KCl germination to the absence of SleC and SleC plus SleM further suggests that DPA is released by the same mechanism during spore germination triggered by these three agents. Unfortunately, the mechanism for DPA release during spore germination is not known, although may involve the SpoVA proteins (153, 244).

The final notable finding was that DPA release by *C. perfringens* spores during dodecylamine germination was not slowed by the absence of SleC or SleC and SleM. Indeed, *sleC* spores released their DPA faster during dodecylamine germination than did wild-type spores. DPA release during dodecylamine germination of *B. megaterium* and *B. subtilis* spores is also not slowed by the absence of these spores' redundant CLEs (203, 207). The mechanism of spore germination by dodecylamine is not known, but the germinant receptors are likely not involved (154, 207). There is also

evidence with *B. subtilis* spores that DPA release during dodecylamine germination involves the SpoVA proteins thought to be involved in DPA release during nutrient germination (244). It has been suggested that dodecylamine may activate a DPA channel present in the spore's inner membrane (207, 244), but this has not been proven.

Hydrolysis of cortex PG is the culminating event in bacterial spore germination, and is essential for resumption of enzymatic activity in the spore core and eventual vegetative growth (207). As a consequence, cortex hydrolysis is essential for spores of pathogenic organisms such as *C. perfringens* to cause disease. The identification of SleC as the major essential CLE in *C. perfringens* spores thus makes this enzyme of interest for development of inhibitors, since such compounds would block spore germination and thus the ability of spores to cause disease. Cortex hydrolysis also makes the now fully germinated spore much less resistant to common decontamination procedures. Consequently, a drug that could rapidly activate SleC in spores would also be useful, as this would allow decontamination of germinated *C. perfringens* spores under less harsh conditions than needed for destruction of the more resistant dormant spores.

Fig. 7.1: Arrangement and expression of *sle* genes in *C. perfringens* SM101.

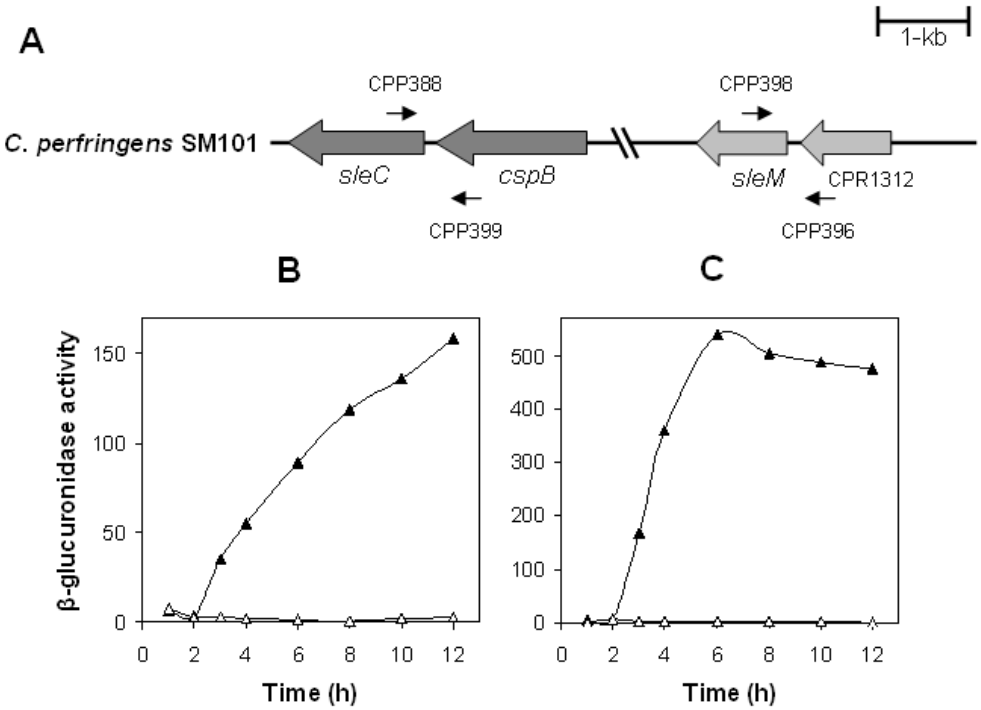


Fig. 7.1A-C. Arrangement and expression of *sle* genes in *C. perfringens* SM101. A) The arrangement of *sleC* and *sleM* in *C. perfringens* SM101 is shown and the locations of the primers used to amplify the upstream regions of each gene are indicated. The *sleC* and *sleM* promoters were predicted to be within the intergenic regions between *sleC* and *cspB*, and *sleM* and CPR1312, respectively. B, C) GUS specific activities from B) *sleC-gusA* and C) *sleM-gusA* fusions in *C. perfringens* SM101 grown in TGY vegetative (open triangles) and DS sporulation (filled triangles) media were determined as described in Materials and Methods. Data represent averages of three independent experiments, and time zero denotes the time of inoculation of cells into either TGY or DS medium.

Fig. 7.2: *C. perfringens sle* deletion mutants and germination of their spores in BHI broth.

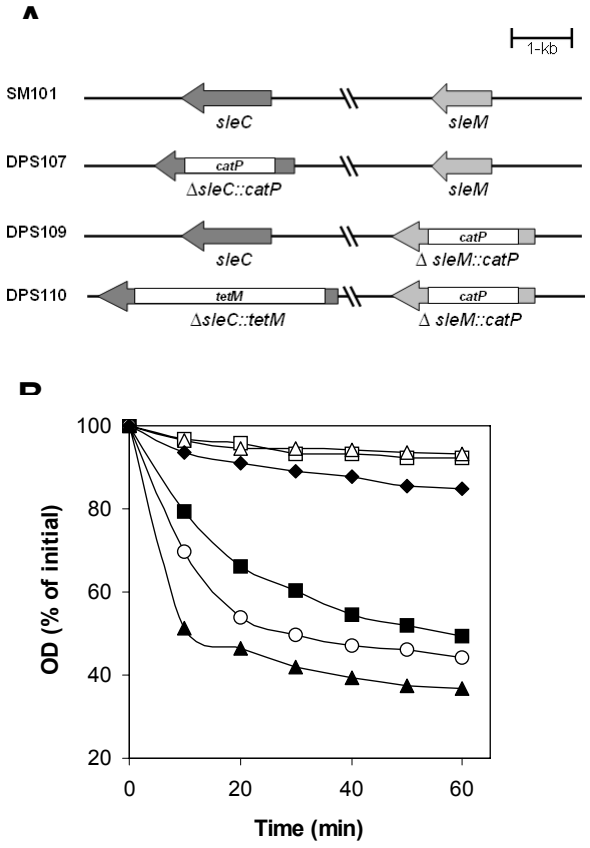


Fig. 7.2A,B. *C. perfringens sle* deletion mutants and germination of their spores in BHI broth. A) Arrangement of *sleC* and *sleM* in various *C. perfringens* deletion mutant strains. B) Spores of *C. perfringens* strains SM101 (wild-type) (filled squares), DPS107 (*sleC*) (open triangles), DPS109 (*sleM*) (open circles), DPS110 (*sleC sleM*) (open squares) and DPS107(pDP138) (*sleC* strain complemented with wild-type *sleC*) (filled triangles) were incubated at 40°C in BHI broth and the OD_{600} was measured as described in Materials and Methods. Spores of *C. perfringens* strains SM101 (wild-type) (filled diamonds) and DPS107 (*sleC*) were incubated at 40ºC in 25 mM sodium phosphate buffer (pH 7.0) and the OD_{600} was measured as described in Materials and Methods. There was essentially no decrease in OD_{600} for the *sleC* spores incubated in buffer alone (data not shown).

Fig. 7.3: Germination of *C. perfringens* spores over long periods on BHI agar plates.

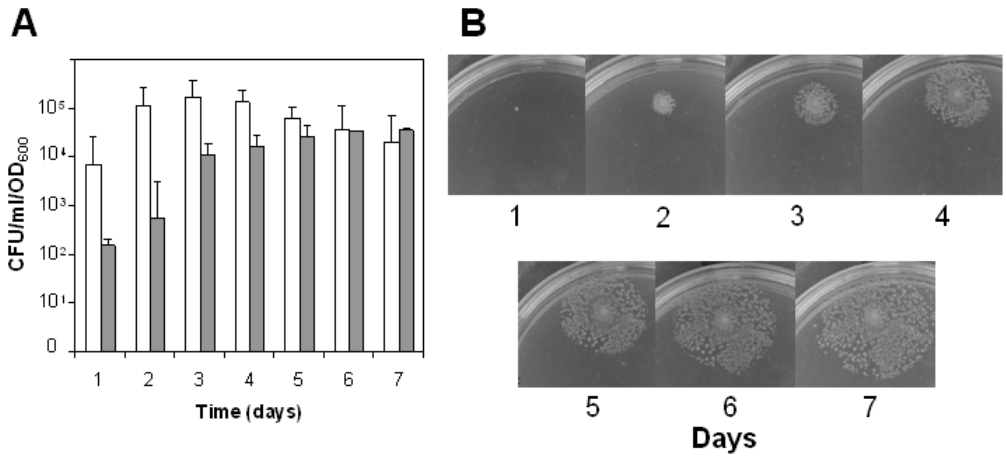


Fig. 7.3A,B. Germination of *C. perfringens* spores over long periods on BHI agar plates. A) Spores of *C. perfringens* strains DPS107 (*sleC*) (white bars) and DPS110 (*sleC sleM*) (grey bars) were applied to BHI plates that were incubated at 37ºC for 7 d and the total number of colonies were counted every 24 h and expressed as colony forming units (CFU). B) Plates described in (A) were photographed every 24 h. Similar results in the experiments in (A) and (B) were obtained with two different batches of spores, and error bars represent standard deviation.

Fig. 7.4: DPA release during germination of *C. perfringens* spores with BHI broth.

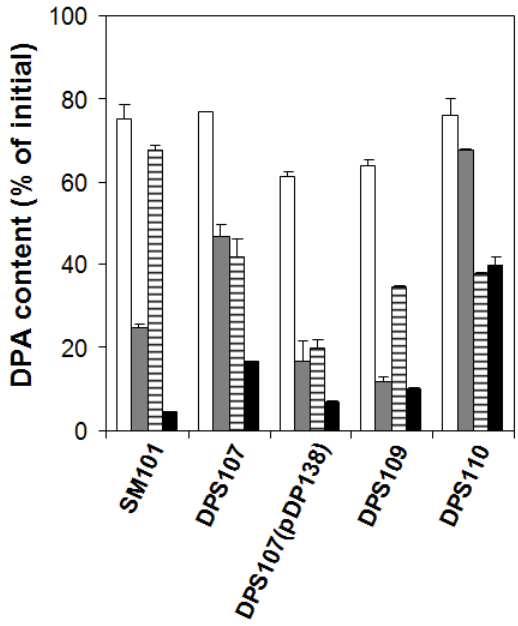


Fig. 7.4. DPA release during germination of *C. perfringens* spores with BHI broth. Heat-activated spores of strains SM101 (wild-type), DPS107 (*sleC*), DPS107(pDP138) (*sleC* strain complemented with wild-type *sleC*), DPS109 (*sleM*), and DPS110 (*sleC sleM*) were incubated at 40ºC in BHI broth, and after 1 h (grey bars) and 18 h (black bars), DPA release was measured as described in Materials and Methods. *C. perfringens* spores from various strains were also incubated in 25 mM sodium phosphate buffer (pH 7.0), and after 1 (white bars) and 18 h (horizontal-lines bars) DPA release was measured as described in Materials and Methods. Data represent the average of two independent experiments with two different spore preparations, and error bars are standard deviation.

Fig. 7.5: Germination of spores of *C. perfringens* strains with KCl.

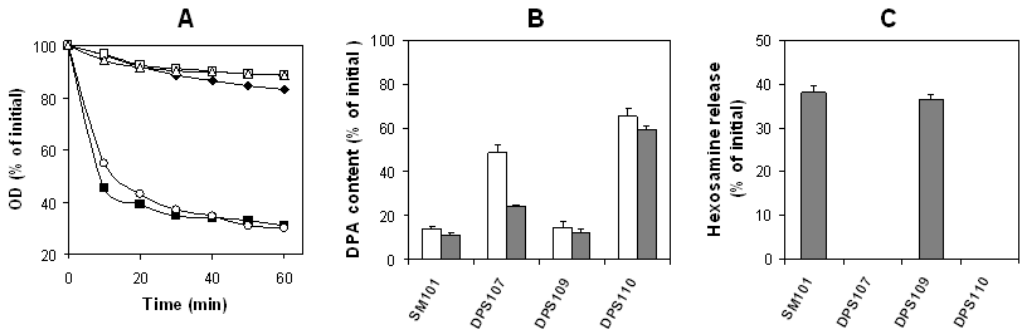


Fig. 7.5A-C. Germination of spores of *C. perfringens* strains with KCl. A) Heat activated *C. perfringens* spores of strains SM101(wild-type) (filled squares), DPS107 (*sleC*) (open triangles), DPS109 (*sleM*) (open circles), and DPS110 (*sleC sleM*) (open squares) were germinated with KCl and the OD_{600} was measured as described in Materials and Methods. Heat-activated spores of strain SM101 (wild-type) (filled diamonds) were also incubated in 25 mM sodium phosphate buffer (pH 7.0) alone and the OD_{600} measured. B) DPA-release during *C. perfringens* spore germination with KCl. Heat activated spores of *C. perfringens* strains were germinated with KCl and after 1 h (white bars) and 18 h (grey bars) the DPA content of the spores was measured as described in Materials and Methods. DPA release of *C. perfringens* spores from various strains in 25 mM sodium phosphate buffer (pH 7.0) was as shown in Fig. 4. C) Release of hexosamine-containing material during *C. perfringens* spore germination with KCl. Heat activated spores of *C. perfringens* strains were germinated with KCl and after 2 h the hexosamine-containing material released into the medium was measured as described in Methods. Values for hexosamine-containing material released are expressed relative to the amount of hexosamine in dormant spores that was defined as 100%. Data represent the average of two independent experiments with two different spore preparations, and error bars are standard deviation.

Fig. 7.6: Germination of *C. perfringens* spores with Ca-DPA.

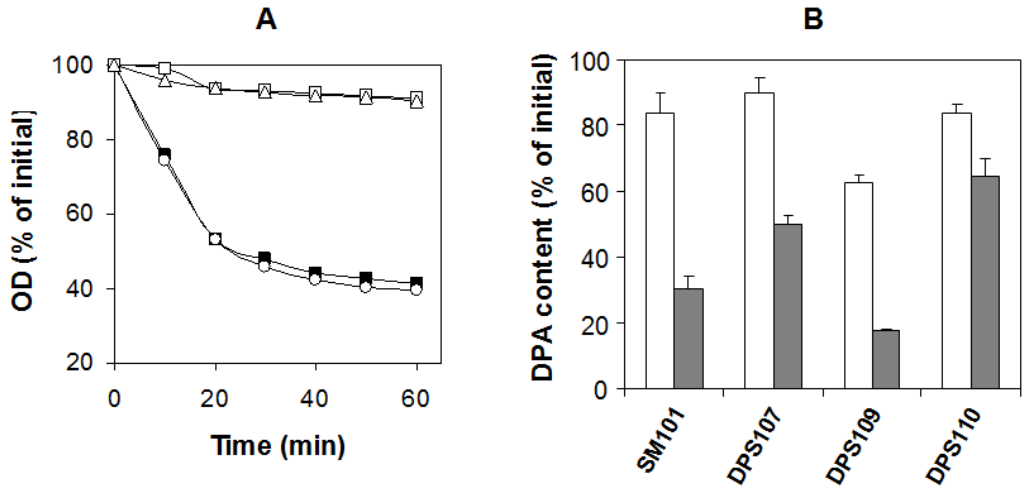


Fig. 7.6A,B. Germination of *C. perfringens* spores with Ca-DPA. A) Heat activated *C. perfringens* spores of strains SM101 (wild-type) (filled squares), DPS107 (*sleC*) (open triangles), DPS109 (*sleM*) (open circles), and DPS110 (*sleC sleM*) (open squares) were germinated with 50 mM Ca-DPA and the OD_{600} was measured as described in Materials and Methods. B) Heat activated spores of *C. perfringens* strains were germinated with 50 mM Ca-DPA (grey bars) and 25 mM Tris-HCl buffer (pH 8.0) (white bars) for 1 h and the DPA remaining in the spores was measured as described in Materials and Methods. Data represent the average of two independent experiments with two different spore preparations, and error bars are standard deviation.

Fig. 7.7: Dodecylamine germination of *C. perfringens* spores.

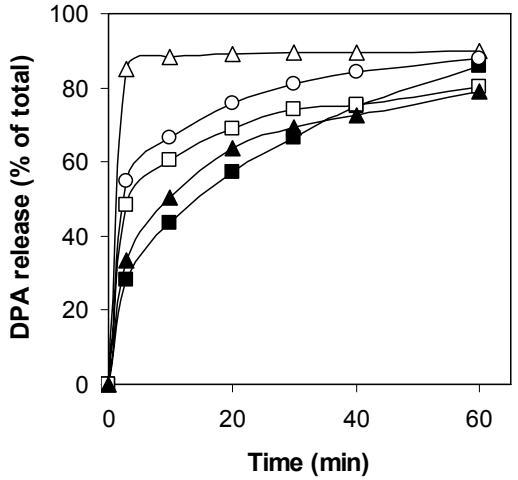


Fig. 7.7. Dodecylamine germination of *C. perfringens* spores. *C. perfringens* spores of strains SM101 (wild-type) (filled squares), DPS107 (*sleC*) (open triangles), DPS109 (*sleM*) (open circles), DPS110 (*sleC sleM*) (open squares) and DPS107(pDP138) (*sleC* strain complemented with wild-type *sleC*) (filled triangles) were germinated with dodecylamine, and at various times DPA release was measured as described in Materials and Methods.

Table 7.1. Colony formation by spores of *C. perfringens* strains[a]

Strain/genotype	Spore titer (CFU/ml/OD_{600})[b]	
	BHI	BHI + Lyz[c]
SM101/wild-type	4.0×10^7	8.9×10^7
DPS107/*sleC*	1.4×10^4	3.9×10^7
DPS107(pDP138)/*sleC* carrying wild-type *sleC*	2.7×10^7	ND
DPS109/*sleM*	3.4×10^7	ND
DPS110/*sleM sleC*	1.6×10^2	6.6×10^7

[a]Heat activated spores of various strains were plated on BHI agar, and colonies were counted after incubation at 37ºC for 24 h.

[b]Titers are the average of colony forming units (CFU)/ ml/OD_{600} determined in three experiments and the variance was less than 25%.

[c]Spores were decoated and plated on BHI plates containing 1μg/ml lysozyme. ND, not determined.

7.6 Supplementary information

Supplementary tables

Table S7.1. Bacterial strains and plasmids used

Strain or Plasmid	Relevant characteristics	Source (Reference)
C. perfringens		
SM101	Electroporatable derivative of food poisoning type A isolate, NCTC8798; carries a chromosomal *cpe*	(256)
SM101(pDP86)	Wild-type strain carrying *sleC-gusA* fusion	This study
SM101(pDP87)	Wild-type strain carrying *sleM-gusA* fusion	This study
DPS107	*sleC* (*sleC*::*catP*) mutant of SM101	This study
DPS109	*sleM* (*sleM*::*catP*) mutant of SM101	This study
DPS110	*sleC sleM* (*sleC*::*tetM sleM*::*catP*) mutant of SM101	This study
DPS107(pDP138)	*sleC* mutant (*sleC*::*catP*) complemented with wild-type *sleC* from strain SM101	This study
Plasmids		
pJIR418	*C. perfringens/E. coli* shuttle vector; Cm^r and Em^r.	(219)
pJIR751	*C. perfringens/E. coli* shuttle vector; Em^r.	(11)
pJIR599	pBluescript II carrying *ermB*; Em^r	(8)
pMRS104	Derivative of pJIR1418 carrying no origin of replication for *C. perfringens*; Em^r.	(67)
pMRS127	*C. perfringens/E. coli* shuttle vector carrying a promoterless *gusA*; Em^r	(174)
pDP25	~ 1.3-kb *Sma*I-*Nae*I fragment from pJIR418, containing *catP* between EcoRI sites of pCR®-XL-TOPO®	This study
pDP35	pCR®-XL-TOPO® carrying *tetM*; Tet^r	(153)
pDP60	~ 2.2-kb PCR fragment containing 2121-bp upstream and 114–bp N-terminal coding region of *sleC* in pCR®-XL-TOPO®	This study
pDP61	~ 2.2-kb *Kpn*I-*BamH*I fragment from pDP60 cloned between KpnI/BamHI sites of pDP25	This study
pDP62	~ 2.1-kb PCR fragment containing 242-bp C-terminal coding region and 1845-bp downstream of *sleC* in pCR®-XL-TOPO®	This study
pDP63	~ 2.1-kb *Pst*I-*Xho*I fragment from pDP62 cloned between PstI/XhoI sites of pDP61	This study
pDP64	~ 5.5-kb *Kpn*I-*Xho*I fragment from pDP63 cloned between KpnI/SalI sites of pMRS104	This study
pDP65	~ 3.2-kb *BamH*I-*Pst*I fragment carrying *tetM* from pDP35 cloned in BamHI/PstI of pDP63	This study
pDP66	~ 1.1-kb *Sma*I fragment carrying *ermB* from pJIR599 cloned into SmaI site of pDP65	This study
pDP86	437-bp PCR amplified upstream region of *sleC* cloned into pMRS127	This study
pDP87	495-bp PCR amplified upstream region of *sleM* cloned into pMRS127	This study

pDP91	~ 1.0-kb PCR fragment containing 945-bp upstream and 61-bp N-terminal coding region of *sleM* in pCR®-XL-TOPO®	This study
pDP92	~ 1.4-kb PCR fragment containing 225-bp C-terminal coding region and 1211-bp downstream of *sleM* in pCR®-XL-TOPO®	This study
pDP93	~ 1.0-kb *Kpn*I-*Spe*I fragment from pDP91 cloned between KpnI/SpeI sites of pDP25	This study
pDP94	~ 1.4-kb *Pst*I-*Xho*I fragment from pDP92 cloned between PstI/XhoI sites of pDP93	This study
pDP95	~ 3.7-kb *Kpn*I-*Xho*I fragment from pDP94 cloned between KpnI/SalI sites of pMRS104	This study
pDP115	~ 1.9-kb PCR fragment containing 437-bp upstream and coding region of *sleC* cloned into Zero-Blunt® TOPO®	This study
pDP126	1704-bp PCR fragment containing 1590-bp upstream and 114-bp N-terminal coding region of *plc* in pCR®-XL-TOPO®	This study
pDP127	1264-bp PCR fragment containing 309-bp C-terminal and 955-bp downstream coding region of plc in pCR®-XL-TOPO®	This study
pDP128	~ 1.7-kb SacI-KpnI fragment from pDP126 cloned between SacI/KpnI sites of pMRS104	This study
pDP129	~ 1.3-kb SalI-SphI fragment from pDP127 cloned between SalI/SphI sites of pDP128	This study
pDP138	~ 1.9-kb KpnI-SalI fragment from pDP115 cloned between KpnI/SalI sites of pDP129	This study

Table S7.2. Primers used in this study

Primer name	Primer sequence[a]	Position[b]	Gene	Use[c]
CPP399	GCAGCGTCGACTATCTACTGTTCCAGGACA AGGATT	-437 to -412	*sleC*	GUS
CPP388	GACGCTGCAGAAACATTGAACCTTCAATCT ACCTAATGT	+3 to +32	*sleC*	GUS
CPP396	GCAGCGTCGACCCTTAAAATATGATACAGA AGTAATGATTGAAG	-495 to -462	*sleM*	GUS
CPP398	GACGCTGCAGCTGGACCAAATCTACTTAAT GGATTTTTATC	+6 to +37	*sleM*	GUS
CPP357	GGGTACCCGTAGCATGAGAACAGAGACAG C	-2121 to -2100	*sleC*	MP
CPP365	CGCGGATCCGCGCCCTATAGCTATACCATC TTGTCC	+90 to +114	*sleC*	MP
CPP359	GCTGCAGCGGATATGGGTTTGTATGTTTAG AGAG	+1075 to +1097	*sleC*	MP
CPP364	CCTCGAGGATATCCACATTATACCCAATGT TTGG	+2856 to +2882	*sleC*	MP
CPP401	GCGGGTACCCCAGAAAGAGAGATATCAAT ACAAAGTGGT	-945 to -916	*sleM*	MP
CPP403	GGGACTAGTCCATCTCTACTAAATTCATTT AAATCTGGACC	+30 to +61	*sleM*	MP
CPP404	GACGCTGCAGCGTTACTTGGAGAGCTAATA AAGAAGATG	+729 to +758	*sleM*	MP
CPP400	GCACCTCGAGGAAACTGTAGCATCTTCACT TGGTCC	+2140 to +2165	*sleM*	MP
CPP507	GGAGCTCCTTACTTCTTCTTTTGGTCTATTT TCCTC	-1590 to -1561	*plc*	CP
CPP511	GGGTACCAGTTCCTGTTCCATCAATTTTTCC ATC	+87 to +114	*plc*	CP
CPP516	GGTCGACGATGCTGGAACAGATGACTACA TG	+888 to +912	*plc*	CP
CPP510	GGCATGCCCTTAAAACCTCACCATGCTTGT TG	+2127 to +2152	*plc*	CP
CPP482	GGGTACCC TATCTACTGTTCCAGGACAAGGATT	-437 to -412	*sleC*	CP
CPP488	GCGTCGACATTTGCAGTTTTAATAGTAGCA CTTCC	+1486 to +1513	*sleC*	CP

[a] Restriction sites are underlined

[b] The nucleotide position numbering begins from the first codon and refers to the relevant position within the respective gene sequence.

[c] MP, construction of mutator plasmid; GUS, construction of *gusA* fusion plasmid; CP, construction of complementing plasmid.

Chapter 8

The protease CspB is essential for initiation of cortex hydrolysis and DPA release during germination of spores of *Clostridium perfringens* type A food poisoning isolates.

Daniel Paredes-Sabja, Peter Setlow and Mahfuzur R. Sarker

Published in:

Microbiology: **Submitted for Publication**

8.1 Abstract

The genome of the *Clostridium perfringens* food poisoning (FP) isolate SM101 encodes a subtilisin-like protease, CspB, upstream of the *sleC* gene encoding the enzyme essential for degradation of the peptidoglycan cortex during spore germination. SleC is an inactive pro-SleC in dormant spores that is converted to active SleC during spore germination, and Csp proteases convert pro-SleC to the active enzyme *in vitro*. In current work, the germination and viability of spores of a *cspB* deletion mutant of strain SM101 have been studied. The *cspB* spores were unable to germinate significantly with either a rich nutrient medium, KCl, or a 1:1 chelate of Ca^{2+} and dipicolinic acid (DPA), and the viability of these spores was ~10^4 lower than that of wild-type spores, although *cspB* and wild-type spores had similar viability on plates containing lysozyme. Germination of *cspB* spores was blocked prior to DPA release and cortex hydrolysis, and germination and viability defects in these spores were complemented by an ectopic *cspB*. These results suggest that Csp proteases are essential to generate active SleC and allow cortex hydrolysis early in *C. perfringens* spore germination. However, Csp proteases likely play another role in spore germination, since *cspB* spores did not release DPA upon exposure to germinants, while *sleC* spores have been shown previously to release DPA, albeit slowly, upon exposure to germinants.

8.2 Introduction

Clostridium perfringens is an anaerobic, spore forming pathogenic bacterium, and many strains cause gastrointestinal (GI) diseases in humans and animals (117). *C.*

perfringens spores are metabolically dormant, resistant to many environmental insults and once conditions are favorable, these spores can germinate, outgrow, return to vegetative growth and then release toxins and cause disease (117, 119, 154).

C. perfringens spores initiate germination when they sense a variety of compounds termed germinants, which include L-asparagine, KCl, a 1:1 chelate of Ca^{2+} and pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)) (Ca-DPA) and a cationic surfactant (154). In spores of *Bacillus* species, nutrient germinants are sensed by specific germinant receptors located in the spore's inner membrane, each encoded by a tricistronic operon of the *gerA* family (207). Although there is no intact tricistronic *gerA*-type operon in *C. perfringens*, the products of the bicistronic *gerKAC* operon are essential for spore germination with either L-asparagine or Ca-DPA, and partially required for germination with KCl (154). Upon binding of the germinant to its cognate receptor, DPA is released from the spore core as a 1:1 chelate with divalent cations, predominantly Ca^{2+}. This event, at least in *Bacillus* spores triggers the initiation of cortex hydrolysis through the activation of the cortex-lytic enzyme (CLE) CwlJ that is present in dormant spores as the mature, potentially active enzyme (146). In *C. perfringens*, the CLE SleC alone is sufficient to degrade the spore's peptidoglycan (PG) cortex, and Ca-DPA does not appear to directly activate cortex hydrolysis (153, 154, 156). SleC is present in dormant *C. perfringens* spores, although not in the mature potentially active form, but rather as an inactive zymogen, pro-SleC, which is cleaved into active SleC early in spore germination (239). These findings suggest that signaling pathways differ in at least some aspects of germination of spores of *Bacillus* and *Clostridium* species. Ultimately, removal of the PG cortex eliminates a

physical constraint allowing the core to expand and hydrate to levels found in vegetative cells (171), thus restoring enzyme activity and metabolism and allowing spore outgrowth (34, 207).

Work with *C. perfringens* S40 has identified three germination-specific serine proteases (Csp proteases) that belong to the subtilisin subfamily (212, 216). The majority of subtilisins are synthesized as pre-pro-enzymes, subsequently secreted into an external medium in a pro-form following the removal of the pre-peptide (or signal peptide), and activated autocatalytically by cleavage of the pro-peptide (215). Mature subtilisins possess three conserved residues (asp, his, ser) that comprise these enzymes' catalytic triad (216), and the three Csp proteins in *C. perfringens* S40 possess this catalytic triad as well as the oxyanion binding hole (212). The Csp proteins are synthesized in the mother cell compartment of the sporulating cell and subsequently translocated into the PG cortex of the developing forespore, and during this period there is processing at both the N- and C-termini generating pro-SleC (112, 212). 26 *In vitro* studies have shown that a fraction from germinated *C. perfringens* S40 spore extracts containing the three Csp proteins can process pro-SleC to SleC, thus activating the enzyme for degradation of the cortex of decoated spores (92, 137, 212). Surprisingly, genome sequencing (130, 213) indicates that *C. perfringens* type A food poisoning (FP) isolates encode only one Csp, CspB, although non-FP isolates such as S40 carry a *cspABC* tricistronic operon encoding all three Csp proteins (Fig. 1A). However, the precise function of Csp proteins in *C. perfringens* spore germination is unclear. Consequently, in the current work we have constructed a *cspB*

mutant of a *C. perfringens* FP isolate to elucidate the role of CspB in spore germination.

8.3 Materials and Methods

Bacterial strains and plasmids. *C. perfringens*, and plasmids used in this study are described in Table S1.

Construction of *cspB-gusA* fusion plasmid and β–glucuronidase assay. A 441 bp DNA fragment upstream of *cspB* from *C. perfringens* SM101, which included the 170-bp intergenic region between *cspB* and CPR2568 that most likely contains the *cspB* gene's promoter, were PCR-amplified using primers CPP393/CPP397 (all primers used in this work are listed in Table S2). The forward and reverse primers had SalI and PstI sites, respectively. This PCR-fragment was digested with SalI and PstI and cloned between SalI and PstI sites in pMRS127 to create a *cspB-gusA* fusion, giving plasmid pDP85. This plasmid was introduced by electroporation (35) into *C. perfringens* SM101 and erythromycin-resistant (Em^r; 50 μg/ml) transformants were selected. Transformants carrying the *cspB-gusA* fusion plasmid pDP85 were grown in TGY vegetative medium (3% trypticase soy, 2% glucose, 1% yeast extract, 0.1% L-cysteine) (91) and in Duncan-Strong (DS) (36) sporulation medium, and assayed for GUS activity as described (256). GUS specific activity was expressed in Miller Units that were calculated as described (174).

Construction of *C. perfringens cspB* deletion mutant. To isolate a derivative of *C. perfringens* SM101 with a deletion of *cspB*, a Δ*cspB* suicide vector was constructed as follows. A 2016-bp DNA fragment carrying 112-bp from the N-terminal coding region and 1949-bp upstream of *cspB* was PCR amplified using primers CPP356/CPP360 (forward and reverse primers had KpnI and SpeI cleavage sites at the 5'-ends, respectively). A 1689-bp fragment carrying 54-bp from the C-terminal coding region and 1635-bp downstream of *cspB* was PCR amplified using primers CPP359/CPP364 (forward and reverse primers had PstI and XhoI cleavage sites at the 5'-ends, respectively). These PCR fragments were cloned into plasmid pCR-XL-TOPO® (Invitrogen, Carlsbad, CA) giving plasmids pDP55 and pDP57, respectively. An ~ 2.0-kb KpnI-SpeI fragment from pDP55 was cloned into pDP25 giving plasmid pDP56, and an ~ 1.7-kb PstI-XhoI fragment from pDP57 was cloned in pDP56, giving pDP58. Next, an ~ 5.0-kb KpnI-XhoI fragment from pDP58 was cloned between the KpnI and SalI sites of pMRS104, giving pDP59. Finally, a 3.2-kb EcoRI fragment carrying *tetM* was excised from pDP35, the ends filled and cloned into the HpaI site in *catP* of pDP59 giving plasmid pDP165 which cannot replicate in *C. perfringens*. Plasmid pDP165 was introduced into *C. perfringens* strain SM101 by electroporation (35), and the *cspB* deletion strain DPS117 was isolated by allelic exchange (192). The presence of the *cspB* deletion in strain DPS117 was confirmed by PCR and Southern blot analyses (data not shown).

Construction of a *cspB* mutant strain complemented with *cspB*. To construct a *cspB* strain complemented with wild-type *cspB*, a suicide-complementing plasmid

targeted to the *plc* locus was constructed as follows. An ~2.4-kb DNA fragment carrying 441-bp upstream and the coding region of *cspB* was PCR amplified with Phusion™ High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) using primers CPP484/CPP485 (forward and reverse primers had KpnI and SalI sites at their 5'-ends, respectively). As shown by assays of GUS activity (Fig. 1B), the 441-bp region upstream of *cspB* contains a sporulation-specific promoter. This PCR fragment was cloned into ZeroBlunt® TOPO® (Invitrogen) giving plasmid pDP121. Next, a 2.4-kb KpnI-SalI fragment from pDP121 was cloned into KpnI/SalI sites of pDP129 (a suicide plasmid containing ~ 1.7- and 1.3-kb upstream and downstream, respectively, of the *plc* locus (156)), giving plasmid pDP184 which cannot replicate in *C. perfringens*. Plasmid pDP184 was introduced into the *C. perfringens cspB* strain DPS117 by electroporation (35) and Em^r Tet^r transformants of strain DPS117(pDP184) were selected. The presence of both plasmid pDP184 and the original *cspB* deletion in the latter strain were confirmed by PCR and Southern blot analyses (data not shown).

Spore preparation and germination. Spores of various *C. perfringens* strains were prepared as previously described (153, 154, 156) and stored at -20ºC until use. All spore preparations used in this work were > 99% pure as determined by phase contrast microscopy. Spore germination was as described elsewhere (153, 154, 156). Briefly, spore suspensions with an OD_{600} = 6 were heat activated at 80ºC for 10 min, cooled in water at ambient temperature for 5 min, and incubated at 40ºC for 10 min. Germination of spores at an OD_{600} of 1 with brain heart infusion (BHI) broth, KCl

(100 mM KCl-25 mM sodium phosphate buffer (pH 7.0)), a mixture of 100 mM L-asparagine-100 mM KCl-25 mM sodium phosphate (pH 7.0) (AK) or Ca-DPA (50 mM $CaCl_2$, 50 mM DPA adjusted to pH 8.0 with Tris base) was routinely measured by monitoring the OD_{600} of spore cultures (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA), which falls ~ 60% upon complete germination of wild-type spores. Levels of germination were also confirmed by phase-contrast microscopy. All values reported are averages of two experiments performed on at least two independent spore preparations, and individual values varied by less than 10% from average values shown.

Spore decoating treatment and assessment of colony forming ability. Spores at an OD_{600} of 20 were decoated in 1 ml of 0.1 M sodium borate (pH 10)-2% 2-mercaptoethanol for 60 min at 37ºC (123, 153), and the decoated spores were washed 10 times with distilled water before use.

To assess spores' colony forming efficiency, untreated and decoated spores at an OD_{600} of 1 were heat activated (80ºC; 10 min), aliquots of various dilutions plated on BHI agar with or without lysozyme (1μg/ml), the plates incubated at 37ºC anaerobically for 24 h, and colonies were counted.

Analytical procedures. DPA remaining in spores during germination was measured as described (153, 154, 156). Briefly, heat activated spore suspensions (OD_{600} of 1.5) were cooled and incubated at 40ºC in 25 mM sodium phosphate buffer (pH 7.0) or in KCl as described above. After 1- or 24-h, aliquots (1 ml) were centrifuged in a

microcentrifuge (13,200 rpm; 3 min), the spore pellet was washed twice with 1 ml distilled water and suspended in 1 ml distilled water. The remaining spore DPA was determined by boiling samples for 60 min, cooling on ice for 5 min, centrifuging in a microcentrifuge for 5 min, and measuring the OD_{270} of the supernatant fluid as described (156). The initial DPA content of dormant spores was determined by boiling 1 ml aliquots for 60 min, centrifuging in a microcentrifuge (13,200 rpm; 5 min), and measuring the OD_{270} of the supernatant fluid as described (153, 154, 156). In *C. perfringens* spores ~ 90% of the material absorbing at 270 nm released from spores by boiling is DPA (156).

The release of fragments of cortex PG containing hexosamine during germination was measured by germinating heat activated spores at an OD_{600} of 25 in 100 mM KCl and 10 mM Tris-HCl (pH 7.4). After 2 h of incubation at 40ºC, samples (1 ml) were centrifuged (13,200 rpm; 5 min), and analyses of hexosamine in the supernatant fluid were carried out as described (51, 156). Analysis of total hexosamine-containing material in dormant spores was also carried out as described (51, 156).

8.4 Results

Arrangement of the *csp-sleC* locus and expression of *cspB* in *C. perfringens* SM101. Previous studies (112, 212) with *C. perfringens* S40 indicate that three Csp proteases (CspA, B and C) belonging to the subtilisin family of serine proteases are encoded by a tricistronic operon upstream of the *sleC* gene (156). However, in the FP isolate, SM101, there is only one *csp*, *cspB*, upstream of *sleC* (Fig. 1A). Amino acid

sequence alignments indicate that $CspB^{SM101}$ shares high homology (92% identity (I) and 96% similarity (S)) to $CspB^{S40}$, with much lower homology to $CspA^{S40}$ (32% I, 50% S) and $CspC^{S40}$ (31% I, 51% S) (Fig. S1).

To determine if *cspB* is expressed during sporulation in *C. perfringens* SM101, upstream DNA from *cspB*, including the intergenic region between *cspB* and the preceding gene (Fig. 1A) that most likely contains the *cspB* gene's promoter, was fused to *E. coli gusA* and GUS activity was measured after introducing the fusion into *C. perfringens* SM101. Vegetative cultures of SM101 carrying the *cspB-gusA* fusion had no significant GUS activity (Fig. 1B), and there was also no detectable GUS activity in sporulating cultures of strain SM101 without the *cspB-gusA* fusion (data not shown). However, there was significant GUS activity in sporulating cultures carrying the *cspB-gusA* fusion (Fig. 1B), indicating that a sporulation specific promoter is located upstream of *cspB*. GUS expression from this putative *cspB* promoter appeared ~ 4 h after the start of sporulation, and reached a maximum after 10 h (Fig. 1B). Although no putative sequence similar to those of mother cell's sporulation-specific promoters dependent on SigE and SigK were found in the upstream region of *cspB* from SM101, this region is similar to that of *cspA* from strain S40, thus it is most likely that, as shown previously with a *cspA-gfp* fusion in strain S40 (112), the *cspB* is also expressed only in the mother cell compartment of *C. perfringens* SM101 sporulating cell.

Effect of *cspB* deletion on SM101 spore germination and colony formation with BHI broth. Since *cspB* is expressed during sporulation, we hypothesized that *cspB* is

essential for pro-SleC processing and therefore for cortex hydrolysis during germination of spores of *C. perfringens* SM101. Consequently, we constructed a *cspB* deletion (strain DPS117) (Fig. 2A). As expected, wild-type spores germinated well in BHI broth as measured by changes in the OD_{600} of the germinating culture (Fig. 2B). However, *cspB* spores exhibited little if any germination in BHI broth, and their germination was even less than wild-type spores in phosphate buffer as measured by the fall in the OD_{600} (Fig. 2B). Phase contrast microscopy also showed that > 90% of wild-type spores had become phase dark after 60 min in BHI broth indicating that the great majority of these spores had completed germination, while *cspB* spores remained phase bright (data not shown), indicative of no germination. The germination defect of *cspB* spores was complemented by ectopic insertion of a wild-type *cspB* gene in the *cspB* strain, indicating that the germination defect in *cspB* spores is due to specific inactivation of *cspB* (Fig. 2B). These results suggest that *cspB* is essential for germination of spores of *C. perfringens* FP isolates.

The severity of the germination defect of *cspB* spores suggested that as found for *sleC* spores (156), the colony forming efficiency of *cspB* spores might be lower than that of wild-type spores, since Csp proteins appear to be required for activation of the major *C. perfringens* CLE, SleC (156, 212). Indeed, the colony forming efficiency of *cspB* spores was ~ 10^4-fold lower than that of wild-type spores (Table 1). However, this defect was eliminated when *cspB* spores were decoated and plated on BHI agar plates containing lysozyme (Table 1), indicating that *cspB* spores were viable, but probably incapable of degrading their PG cortex as noted previously for *sleC* spores (156). The latter result also suggest that active SleC might not be generated during

germination of *cspB* spores, since SleC alone is essential for cortex degradation during spore germination, and thus full spore viability (156). The absence of active SleC in *cspB* spores could be due to lack of conversion of pro-SleC to SleC during germination. Unfortunately, due to lack of SleC antibody we are unable to provide stronger evidence for this hypothesis. Indeed, since the low viability of *cspB* spores on plates without lysozyme was complemented by wild-type *cspB* inserted in the *plc* locus (Table 1), it appears most likely that pro-SleC is present in *cspB* spores but cannot be converted to active SleC during germination. Interestingly, incubation of *cspB* spores on BHI plates without lysozyme for long periods of time did slowly give rise to colonies in a manner similar to that previously reported for *sleC* spores (156), i.e., some colonies started to appear only surrounding those colonies that appeared within the first 24 h, suggesting that a cell wall hydrolase released from growing or sporulating cells was diffusing through the medium and germinating a small percentage of *cspB* spores that have a defective coat.

In *B. subtilis* and *B. megaterium*, Ca-DPA triggers germination by activation of the CLE CwlJ (146, 203). In contrast, recent work (154, 156) suggests that Ca-DPA triggers *C. perfringens* spore germination through the GerKA-KC receptor and not by activating either pro-SleC or SleC. Since *cspB* spores most likely possess pro-SleC as noted above, we sought to provide definitive evidence for this suggestion by examining the germination of wild-type, *cspB* and DPS117(pDP184) (*cspB* mutant complemented with wild-type *cspB*) spores with exogenous Ca-DPA. As expected, wild-type spores germinated well with exogenous Ca-DPA, while *cspB* spores exhibited little if any germination as assessed either by the decrease in OD_{600} of

germinating cultures or by phase contrast microscopy (Fig. 2C; and data not shown). However, the Ca-DPA germination defect in *cspB* spores was complemented by an ectopic copy of *cspB* (Fig. 2C; and data not shown). These results suggest that Ca-DPA does not initiate germination by activating pro-SleC, but rather presumably through the GerKA-KC receptor as suggested previously (154).

CspB is essential for initiation of cortex hydrolysis during germination of *C. perfringens* spores. Previously we reported that during *C. perfringens* spore germination *sleC* spores release their DPA slower than wild-type spores and are unable to degrade their PG cortex (156). To gain more insight into the role of CspB in *C. perfringens* spore germination, wild-type, *cspB* and DPS117(pDP184) spores were germinated with KCl (154). Wild-type spores germinated completely with KCl, while *cspB* spores showed a minimal fall in OD_{600} upon incubation with KCl, although this defect was eliminated in the *cspB* strain complemented with an ectopic copy of *cspB* (Fig. 3A). Phase contrast microscopy further showed that > 99% of wild-type and DPS117(pDP184) spores had become phase dark after 1 h of KCl germination, while the *cspB* spores remained phase bright (data not shown). The phase contrast microscopy results and the lack of fall in the OD_{600} of *cspB* spores suggested that little if any DPA was released during KCl-germination of these spores. To directly confirm this conclusion, we measured DPA remaining in KCl-germinated wild-type, *cspB* and DPS117(pDP184) spores. After 1 h of incubation in KCl, wild-type spores had released the majority of their DPA (Fig. 3B). However, the amount of DPA released from *cspB* spores after 1 h of incubation with KCl was minimal (Fig. 3B), and

comparable to that released from wild-type spores incubated for 1 h at 40°C in sodium phosphate buffer alone (data not shown), in which *C. perfringens* spores germinate minimally if at all. Incubation of *cspB* spores in KCl for 24 h led to only an insignificant increase in the amount of DPA released (Fig. 3B). However, DPA release during KCl germination was restored to *cspB* spores upon their complementation with wild-type *cspB* (Fig. 3B).

The culminating event in spore germination is the hydrolysis of the spore's PG cortex and release of PG fragments into the medium. Therefore, we monitored cortex hydrolysis by assaying the release of hexosamine-containing material into the medium (51, 156). As expected, wild-type spores released significant hexosamine-containing material into the medium after 2 h of germination with KCl (Fig. 3C). In contrast, hexosamine-containing material was not released by *cspB* spores during KCl germination, although this release was restored in *cspB* spores complemented with wild-type *cspB* (Fig. 3C). These results clearly indicate that CspB is essential in some fashion for cortex hydrolysis during *C. perfringens* spore germination.

8.5 Discussion

Results in this communication provide strong support for the hypothesis that Csp proteases are essential in germination of *C. perfringens* spores in order to activate pro-SleC and thus allow cortex PG degradation. This has been suggested previously based on results from *in vitro* studies of the effects of Csp proteins on pro-SleC and the activity of pro-SleC and SleC on cortex PG in decoated spores (137, 239). The current work shows that *C. perfringens* spores lacking their sole Csp protein, CspB,

cannot germinate with all germinants tested. In addition, these *cspB* spores had extremely low viability, most likely because of their poor germination. The fact that the low viability of *cspB* spores could be corrected if these spores were decoated and applied to plates containing lysozyme is consistent with the defect in the germination of *cspB* spores being in cortex degradation, and thus that the function of CspB is to convert inactive pro-SleC into the active enzyme SleC that can degrade cortex PG. It is, of course, possible that a *cspB* deletion somehow prevents *sleC* expression, and that *cspB* spores also lack even pro-SleC; indeed, this possibility is further suggested by the fact that *cspB* is just upstream of *sleC*. Thus a *cspB* deletion might interfere with *sleC* transcription somehow, perhaps even transcription from a promoter upstream of *cspB*. However, this seems an unlikely scenario for a variety of reasons including: i) an ectopic *sleC* with only upstream DNA from the intergenic region between *cspB* and *sleC* can completely complement the spore germination defects of a *sleC* deletion strain (156); ii) there is a very stable stem loop structure encoded in the intergenic region between *cspB* and *sleC*, and it appears likely that this is a strong terminator of *cspB* transcription with a predicted ΔG of -6.3 kcal/mol (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi); iii) there is a strong promoter in the intergenic region between *cspB* and *sleC* that drives sporulation-specific *sleC* expression (156); and iv) ectopic expression of *cspB* completely complemented the germination and viability defects in *cspB* spores, indicating that pro-SleC is almost certainly present in *cspB* spores.

Since *cspB* spores are likely to have normal levels of pro-SleC, it further seems likely that it is CspB that processes this zymogen and thus triggers cortex hydrolysis

during spore germination. The obvious question is then what triggers CspB activity during spore germination, as Csp proteins appear to be present in spores in a potentially active form. However, there is currently no information available on the regulation of the activity of CspB, or Csp proteases in general, and this is clearly an important matter for further study.

While it appears clear that CspB is essential for spore germination almost certainly by activating pro-SleC, it is also possible that CspB may have some other function and/or substrate. The evidence for this is that if CspB's sole function is to activate pro-SleC, then the germination phenotype of *cspB* spores should be identical to that of *sleC* spores. Indeed, the viability of *sleC* spores is 10^4-fold lower than wild-type spores and *sleC* spores germinate very poorly with all germinants tested (156), as are also the case for *cspB* spores. However, while *sleC* spores cannot complete germination very efficiently because they cannot degrade their PG cortex, they slowly release much of their Ca-DPA during germination with a number of germinants (156); in contrast *cspB* spores do not. Thus, *cspB* spores have a more severe germination phenotype than *sleC* spores, consistent with CspB having some germination-associated function in addition to processing pro-SleC. However, the nature of this function as well as other possible substrates in spores for CspB is not known, and this is also a matter for further work.

The involvement of CspB in pro-SleC activation and thus cortex hydrolysis is a further example of the differences in the signaling pathways in the germination of spores of *Clostridium* and *Bacillus* species. In *Bacillus* species, spores have two redundant CLEs, CwlJ and SleB, and both are present in spores in a mature form that

is activated during germination either by Ca-DPA released from the spore core (CwlJ), or perhaps by some drastic change in strain on the cortical PG following Ca-DPA release and its replacement by water in the spore core (SleB) (146, 203, 207). In further contrast to the situation in *Clostridium* spores, there is no known involvement of Csp proteases, or indeed any protease at all in the regulation of CLE activity in spores of *Bacillus* species.

The final conclusion from the current work concerns the difference in the number of Csp proteases in the FP and non-FP strains, one in the former and three in the latter. While it is formally possible that three Csp proteases are needed for full SleC processing, including removal of the N- and C-terminal pre-sequences (239), clearly a single Csp is sufficient to generate active SleC in strain SM101. Thus there appears to be no clear necessity for three Csp proteases for the germination of *C. perfringens* spores. However, it is certainly possible that the additional two Csp proteases in non-FP isolates have some additional roles in spore germination, and this too is a matter for further study.

Fig. 8.1: Genomic arrangement and expression of *cspB* in *C. perfringens* strain SM101.

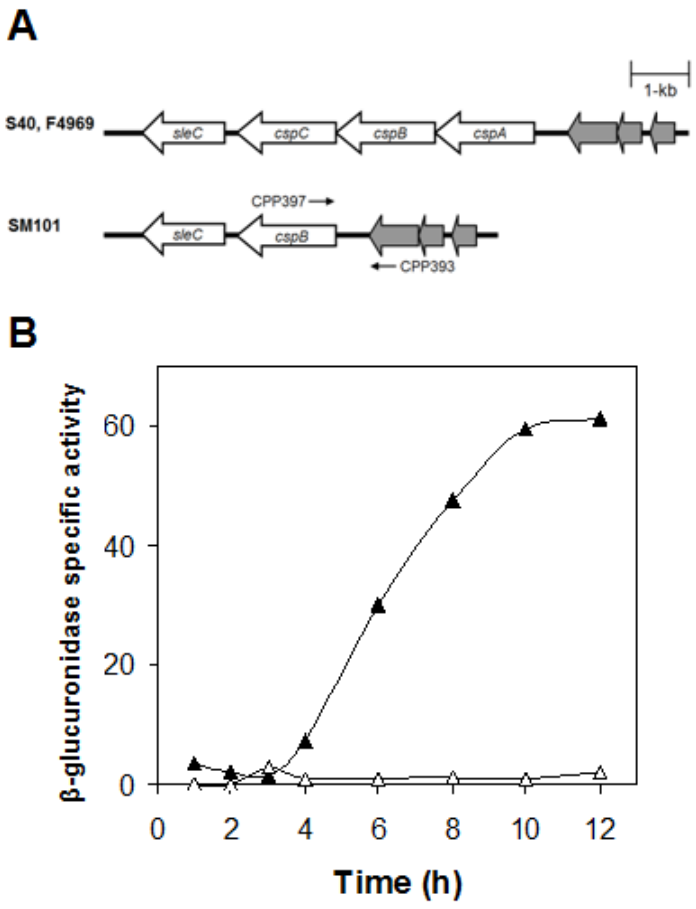


Fig. 8.1A,B. A) Genomic arrangement and B) expression of *cspB* in *C. perfringens* SM101. A) The arrangement of the *csp-sleC* locus in the *C. perfringens* FP strain SM101 and the non-FP strains F4969 and S40 and the location of primers used to amplify the upstream region of *cspB* from strain SM101 are indicated. B) GUS specific activity from the *cspB-gusA* fusion in *C. perfringens* wild-type SM101 grown in TGY vegetative (open symbols) and DS sporulation (filled symbols) media were determined as described in Materials and Methods. Data are the average of three independent experiments, and time zero denotes the time of inoculation of cells into either TGY or DS medium.

Fig. 8.2: Schematic representation of *cspB* deletion mutant in *C. perfringens* SM101 and germination of mutant spores in BHI broth.

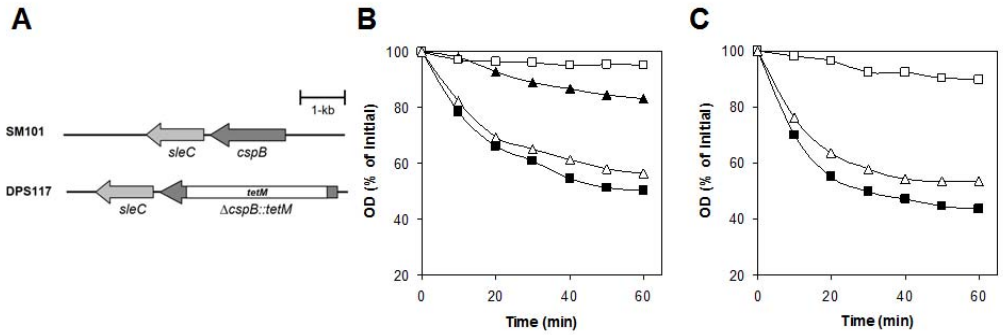


Fig. 8.2A-C. A) Schematic representation of *cspB* deletion mutant in *C. perfringens* SM101 and B,C) germination of mutant spores in BHI broth. A) Arrangement of *cspB* in the *C. perfringens* wild-type and *cspB* mutant. B,C) Heat activated spores of *C. perfringens* strains SM101 (wild-type) (filled squares), DPS117 (*cspB*) (open squares) and DPS117(pDP184) (*cspB* mutant complemented with wild-type *cspB*) (open triangles) were germinated at 40ºC in: B) BHI broth; or C) Ca-DPA and the OD_{600} was measured as described in Materials and Methods. Heat activated spores of SM101 (wild-type) (filled triangles) were germinated at 40ºC in: B) 25 mM sodium phosphate (pH 7.0).

Fig. 8.3: Germination of spores of *C. perfringens* strains with KCl.

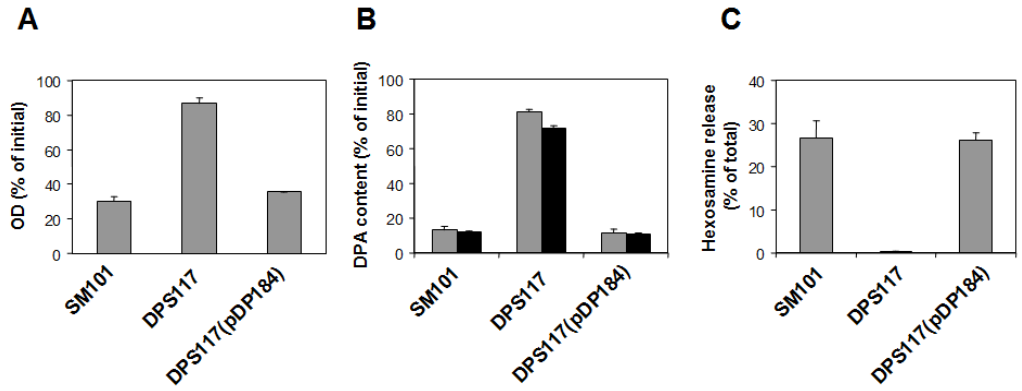


Fig. 8.3A-C. Germination of spores of *C. perfringens* strains with KCl. A) Heat activated *C. perfringens* spores of strains SM101 (wild-type), DPS117 (*cspB*), and DPS117(pDP184) (*cspB* mutant complemented with *cspB*) were germinated with KCl and the OD_{600} was measured after 1 h as described in Methods. B) DPA-release during *C. perfringens* spore germination with KCl. Heat activated spores of *C. perfringens* strains were germinated with KCl and after 1 h (grey bars) and 18 h (black bars) the DPA content of spores was measured as described in Methods. DPA release from spores of *C. perfringens* strains incubated at 40°C in 25 mM sodium phosphate buffer (pH 7.0) was less than 20%. C) Release of hexosamine-containing material during *C. perfringens* spore germination with KCl. Heat activated spores of *C. perfringens* strains were germinated with KCl for 2 h and the hexosamine-containing material released into the medium was measured as described in Methods. Values of hexosamine-containing material released are expressed relative to the amount of hexosamine in dormant spores that was defined as 100%. Data represent the average of three independent experiments with two different spore preparations, and error bars are standard deviation.

Table 8.1. Colony formation by spores of *C. perfringens* strains*

Strain/genotype	Spore titer (c.f.u./ml/OD_{600}) †	
	BHI	BHI + Lyz‡
SM101/wild-type	4.1×10^7	6.6×10^7
DPS117/*cspB*	3.8×10^3	6.0×10^7
DPS117(pDP184) *cspB* carrying wild-type *cspB*	5.3×10^7	ND§

* Heat activated spores of various strains were plated on BHI agar with or without lysozyme, and colonies were counted after incubation at 37ºC for 24 h.

† Titers are the average of colony forming units (c.f.u.)/ ml/OD_{600} determined in three experiments and the variance was less than 25%.

‡ Spores were decoated and plated on BHI plates containing 1μg/ml lysozyme.

§ ND - Not determined.

8.6 Supplementary Information

Supplementary Tables

Table S8.1. Bacterial strains and plasmids used

Strain or Plasmid	Relevant characteristic	Source/Reference
C. perfringens		
SM101	Electroporatable derivative of food poisoning type A isolate, NCTC8798; carries a chromosomal *cpe*	(256)
SM101(pDP85)	Wild-type strain carrying *cspB-gusA* fusion	This study
DPS117	*cspB* mutant (*cspB*::*tetM*)	This study
DPS117(pDP184)	*cspB* mutant (*cspB*::*tetM*) complemented with wild-type *cspB*	This study
Plasmids		
pMRS104	No origin of replication for *C. perfringens*; Em^r.	(67)
pMRS127	*C. perfringens/E. coli* shuttle vector carrying a promoterless *gusA*; Em^r	(174)
pDP25	*E. coli* shuttle vector; Cm^r, Kan^r.	(156)
pDP35	pCR-XL-TOPO carrying *tetM*; Tet^r	(153)
pDP55	~ 2.0-bp PCR fragment containing 112–bp of N-terminal-coding region and 1949–bp upstream of *cspB* in pCR®-XL-TOPO® (Invitrogen)	This study
pDP56	~ 2.0-kb KpnI-SpeI fragment from pDP55 cloned between KpnI/SpeI sites of pDP25	This study
pDP57	~ 1.7-kb PCR fragment containing 54–bp of C-terminal-coding region and 1635–bp downstream of *cspB* in pCR®-XL-TOPO®	This study
pDP58	~ 1.7-kb PstI-XhoI fragment from pDP57 cloned between PstI/XhoI sites of pDP56	This study
pDP59	~ 5.0-kb KpnI-XhoI fragment from pDP58 cloned between KpnI/SalI sites of pMRS104	This study
pDP85	441-bp PCR amplified upstream region of *cspB* cloned in pMRS127	This study
pDP121	~ 2.4-kb PCR fragment containing P_{cspB} and *cspB*, cloned into ZeroBlunt® TOPO® (Invitrogen)	This study
pDP129	Δ*plc* locus; no origin of replication for *C. perfringens*; Em^r.	(156)
pDP165	~ 3.2-kb EcoRI fragment carrying *tetM* from pDP35, cloned into HpaI site of pDP59.	This study
pDP184	~ 2.4-kb KpnI-SalI fragment from pDP121 cloned between KpnI/SalI sites of pDP129.	This study

Table S8.2. Primers used

Primer name	Primer sequence*	Position†	Gene	Use‡
CPP393	GCAGCGTCGACTGATATGACTGGATATAGAATTCAAGAGAATGA	-413 to -380	*cspB*	GUS
CPP397	GACGCTGCAGCTATTCCACCCTTAGCTTTATTCTCCAT	+1 to +28	*cspB*	GUS
CPP356	GGGTACCCGTTGAACTCTAGGTGGGCAAC	-1949 to -1928	*cspB*	MP
CPP360	GACTAGTCCAACTAATTCTATATCTTCATTATTAGGCG	+82 to +112	*cspB*	MP
CPP359	GCTGCAGCGGATATGGGTTTGTATGTTTAGAGAG	+1641 to +1667	*cspB*	MP
CPP364	CCTCGAGGATATCCACATTATACCCAATGTTTGG	+3304 to +3330	*cspB*	MP
CPP484	GGGTACCCTGATATGACTGGATATAGAATTCAAGAGAATGA	-413 to -380	*cspB*	CP
CPP485	GCGTCGACCCTATAGCTATACCATCTTGTCCAG	+1960 to +1985	*cspB*	CP

* Restriction sites are underlined..

† The nucleotide position numbering begins from the first codon and refers to the relevant position within the respective gene sequence.

‡ MP, construction of mutator plasmid; GUS, construction of *gusA* fusion plasmid; CP, construction of complementing plasmid.

Supplementary Figure

Fig. S8.1. Sequence alignment of Csp proteins of *C. perfringens* strains

Fig. S8.1. Sequence alignment of Csp proteins of *C. perfringens* strains. The alignment was generated by use of T-Coffee (134). The grey boxes show identical amino acid residues in 2-3 Csps, black boxes show identical amino acid residues in all four Csps, and red arrows denote Asp, His and Ser residues that likely comprise the catalytic triad of subtilisins. *C. perfringens* strains, proteins and accession numbers are: S40|CspA, *C. perfringens* S40 CspA, AB042154; S40|CspB, *C. perfringens* S40 CspB, AB042154; S40|CspC, *C. perfringens* S40 CspC, AB042154; SM101|CspB, *C. perfringens* SM101, YP_699814.

Chapter 9

Strategy to inactivate *Clostridium perfringens* spores in meat products.

Saeed Akhtar[*], Daniel Paredes-Sabja[*], J. Antonio Torres, and Mahfuzur R. Sarker

*First two authors contributed equally in this manuscript.

Published in:

Food Microbiology 2009 **26**: 272-277

9.1. Abstract

The current study aimed to develop an inactivation strategy for *Clostridium perfringens* spores in meat through a combination of spore activation at low pressure (100 to 200 MPa, 7 min) and elevated temperature (80ºC, 10 min); spore germination at high temperatures (55, 60 or 65ºC); and inactivation of germinated spores with elevated temperatures (80 and 90ºC, 10 and 20 min) and high pressure (586 MPa, at 23 and 73ºC, 10 min). Low pressures (100 to 200 MPa) were insufficient to efficiently activate *C. perfringens* spores for germination. However, *C. perfringens* spores were efficiently activated with elevated temperature (80ºC, 10 min), and germinated at temperatures lethal for vegetative cells (≥ 55ºC) when incubated for 60 min with a mixture of L-asparagine and KCl (AK) in phosphate buffer (pH 7) and in poultry meat. Inactivation of spores (~ 4 decimal reduction) in meat by elevated temperatures (80 to 90ºC for 20 min) required a long germination period (55ºC for 60 min). However, similar inactivation level was reached with shorter germination period (55ºC for 15 min) when spore contaminated-meat was treated with pressure-assisted thermal processing (586 MPa, 73ºC, 10 min). Therefore, the most efficient strategy to inactivate *C. perfringens* spores in poultry meat containing 50 mM AK consisted: i) a primary heat treatment (80ºC, 10 min) to pasteurize and denature the meat proteins and to activate *C. perfringens* spores for germination; ii) cooling of the product to 55ºC in about 20 min and further incubation at 55ºC for about 15 min for spore germination; and iii) inactivation of germinated spores by pressure-assisted thermal processing (586 MPa at 73ºC for 10 min). Collectively, this study demonstrates the

feasibility of an alternative and novel strategy to inactivate *C. perfringens* spores in meat products formulated with germinants specific for *C. perfringens*.

9.2. Introduction

Clostridium perfringens is an anaerobic endospore forming gram positive bacteria and a significant cause of histotoxic and gastrointestinal (GI) diseases in humans and animals (95, 117). *Clostridium perfringens* type A food poisoning currently ranks as the third most common food-borne illness (117, 138). The GI symptoms of this food-borne illness are caused by a small group (~5%) of type A isolates that produces *C. perfringens* enterotoxin (CPE) (117, 192).

C. perfringens spores of food poisoning isolates exhibit high resistance to various environmental stress factors including: i) moist heat; ii) osmotic, nitrite-, and pH-induced stress; iii) prolonged frozen storage; and iv) high hydrostatic pressure (102, 103, 150, 193). The resistance of *C. perfringens* food poisoning isolates might facilitate their survival in processed meat and poultry (117), products most commonly implicated in *C. perfringens* type A food poisoning outbreaks (234).

The food industry is developing alternative approaches to conventional processing technologies, including high pressure processing (HPP), to meet consumer expectations for increased food safety, extended shelf life and improved food quality (161, 162, 235). Although the application of 400 to 800 MPa inactivates most pathogenic and spoilage bacteria (235), the inactivation of bacterial spores has been a major challenge. For example, no significant inactivation of spores of 6 *Bacillus* species was obtained when these spores were treated with 980 MPa for 40 min at room

temperature (226). Therefore, new strategies are needed to increase spore sensitivity to HPP treatments.

Recent studies on *C. perfringens* spores (151-153, 174, 176) have shown that the factors involved in spore resistance include the: i) cortex peptidoglycan (PG) structure; ii) spore's core low water content; iii) high levels of pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)) in the spore core; and, iv) saturation of spore DNA with α/β-type small, acid soluble proteins (SASPs). However, it has been shown that during germination of *Bacillus subtilis* spores, these factors of resistance are quickly lost and germinated spores become sensitive to environmental stress (73, 207, 209). Interestingly, *C. perfringens* spores can complete their germination in less than 20 min in the presence of L-asparagine and K^+ ions (AK) (154). Since germinated spores are sensitive to heat and pressure, we hypothesized that formulating foods with certain germinants and then using HPP protocols that include a spore germination step may lead to an effective strategy to inactivate bacterial spores. Consequently, the objectives of this study were to develop: i) a germination-activation procedure for *C. perfringens* spores and ii) thermal and HPP protocols for the inactivation of germinated spores of *C. perfringens* in poultry meat.

9.3. Materials and Methods

Bacterial strains. The *C. perfringens* isolates used in this study consisted of five food poisoning isolates (SM101, NCTC10239, FD1041, E13 and NCTC8239) (193).

Spore preparations. Starter cultures (10 ml) of *C. perfringens* were prepared by inoculating cook meat stocks into tubes containing 10 ml of fluid thioglycollate broth (FTG) (Difco) and incubated overnight (18 h) at 37ºC (91). Sporulating cultures of *C. perfringens* were prepared by inoculating 0.2 ml of an FTG starter culture into 10 ml of Duncan-Strong (DS) sporulation medium (36). After incubation for 24 h at 37°C, sporulation was confirmed by phase-contrast microscopy. Spore preparations were cleaned by repeated centrifugation and washings with sterile distilled water until the spores were > 99% free of sporulating cells, cell debris and germinated spores. After suspension in distilled water at a final OD_{600} of ~ 6, they were stored at -20ºC until used (154).

High pressure and nutrient- germination of *C. perfringens* spores. Studies were conducted to determine the activation of *C. perfringens* spore germination by low hydrostatic pressure treatments at moderate temperature using a 2.2-L isostatic pressure vessel (Model 4-04486-1, Engineered Pressure Systems, Inc., Haverhill, MA, USA). The pressure-transmitting fluid was water containing 5% (v/v) Hydrolubric 120-B (Houghton, Intl. Inc., Valley Forge, Pa, U.S.A.). Spore suspensions (0.5 ml) of strain SM101 at an OD_{600} ~1 (~ 10^8 spores/ml) in 25 mM phosphate buffer (pH 7.0) were placed in sterile plastic pouches and thermally sealed with an impulse sealer (IPK-205HK, Impak Corp., Los Angeles, CA., USA). Samples were treated with 100, 150 and 200 MPa for 7 min. The pressure come-up time was 20 s, and the initial temperature of the pressure vessel was 50ºC. Adiabatic heating was estimated to be 3 and 6ºC for 100 and 200 MPa, respectively (150). Vessel decompression took less

than 5 s, and samples remained in the pressure vessel for 1 min each time during sample loading and unloading, respectively. After pressure treatment, spores were briefly sonicated (11 W, 3 s) to disrupt spore clumps formed during the pressure-treatment and then incubated at 40ºC. Germination was monitored by measuring the OD_{600} of the spore suspension (Smartspec^{TM} 3000 Spectrophotometer, Bio-Rad Laboratories, Hercules, CA, USA). Spore germination was confirmed by phase-contrast microscopy. The extent of spore germination was calculated by measuring the decrease in OD_{600} and expressed as percentage of initial OD_{600} noting that the OD_{600} reduction approached 67% for greater than 99% germination. Values reported are averages of at least two experiments.

Heat treatment of *C. perfringens* spores in phosphate buffer. An alternative approach to inactivate *C. perfringens* spores is using germination temperatures that allow germination but are lethal to vegetative cells. A previous study showed that heat-activated spores of a food poisoning *C. perfringens* isolate (strain SM101) can germinate at temperatures lethal to vegetative cells (i.e., higher than 55ºC) when incubated in 25 mM phosphate buffer (pH 7.0) with 100 mM L-asparagine (AK mixture) and 100 mM KCl, but not when incubated with KCl or L-asparagine alone (154). Therefore, spore suspensions at an OD_{600} ~1 of the five food poisoning isolates used in this study were heat-activated (80ºC, 10 min), cooled to room temperature in a water bath and then incubated with 100 mM L-asparagine and 100 mM KCl (AK) in 25 mM phosphate buffer (pH 7.0) at 55, 60 and 65ºC. Germination was monitored by measuring OD_{600} as previously described. Since a decrease in OD_{600} of ~ 67%

corresponds to more than 99% spore germination as confirmed by phase-contrast microscopy, the percentage decrease in OD_{600} was converted to percent of germination taking an OD_{600} decrease of 67 % as 100 % germination. All values reported are averages of at least two experimental values.

Heat treatment of *C. perfringens* spores in BHI broth. Preliminary experiments indicated that *C. perfringens* spore germination in BHI broth exhibited a similar extent of germination in the presence of 50 or 100 mM AK, thus 50 mM AK was used in this study. No difference was observed in the extent of spore germination with filter-sterilized and autoclaved (121ºC, 20 min) germinant solutions, thus germinant solutions were autoclaved. Aliquots (100 µl) of spore suspensions of NCTC8239 at an initial OD_{600} ~ 6 were transferred into 5 ml BHI tubes with 50 mM AK. Tubes were heat-treated (80ºC, 10 min) to activate *C. perfringens* spores and then transferred to a water bath at 55ºC. After 15, 30 and 60 min of incubation, aliquots (1 ml) were heat-treated (80 to 90ºC, 10 to 20 min) and cooled in an ice-water bath.

Heat and pressure treatments of *C. perfringens* spores in poultry meat. The resistance of bacteria in meat is generally higher than in buffers (50, 85) due to food matrix interactions with solutes reducing their bioavailability (149). Therefore, to evaluate the effect of poultry meat on the germination and inactivation of *C. perfringens* spores, and to validate results observed in laboratory media, experiments were conducted in a poultry meat model system (1) inoculated with a spore cocktail of the 5 food poisoning isolates used in this study. Poultry meat obtained from a local

supplier was ground in a sterile blender (Model 31BL46UL, Waring Products Division, Dynamics Corporation of America, New Hartford, CT, USA) inside a laminar flow hood (Labconco Corporation Biosafety Cabinet Model 36208/36209 Type A, Kansas City, MO, USA). The blender jar and lid were sanitized with 75% ethanol and then UV-sterilized. Ground poultry meat (10 g) was placed in 4"x4" UV-sterilized polyethylene bags (Gauge 0.03", JC Danzak Inc., Westfield, MA, USA) stored at -20ºC until use (1). Bags were thawed and 560 µl of a 1 M AK solution was added to reach a final concentration of 50 mM, and manually mixed under sterile conditions with 100 µl of a spore cocktail containing equal amounts of the five food poisoning isolates (strains SM101, NCTC1029, NCTC8239, FD1041 and E13). Bags where sealed and further mixed with the stomacher (Seward Stomacher, London, UK) to ensure equal distribution of the germinant and spores. Initial *C. perfringens* counts on meat samples were ~ 10^6 spores/g. Samples were mixed vigorously for 5 min using a stomacher (Seward Stomacher, London, UK) to ensure even distribution of AK and spores in the meat. Bags were compressed into a thin layer (approximately 0.3 cm thick) and heat-sealed (Model IPK-205HK, Impak Crop., Los Angeles, CA, USA).

For thermal inactivation of germinated spores in poultry meat, samples were heat-treated (80ºC, 10 min) to activate bacterial spores and then incubated for 15, 30 and 60 min in a water bath at 55ºC. Meat samples were then placed in a water bath at 80 or 90ºC for 10 and 20 min and then cooled on ice for 10 min. For pressure-assisted thermal inactivation of germinated spores, meat samples were heat treated (80ºC, 10 min), incubated in a water bath at 55ºC for 15 and 30 min. To inactivate germinated spores, meat samples were pressure-treated for 10 min at 586 MPa with an initial

pressure vessel temperature of 23ºC and 73ºC. Pressure come-up and release time took less than 90 and 20 s, respectively. Samples remained in the pressure vessel for 2 min before and after each pressure treatment, respectively, and after treatment were cooled for 5 min in an ice-water bath. Adiabatic heating of the samples in the high pressure vessel when pressurized at 586 MPa was estimated to increase meat temperature by about 15ºC (150). The variable temperature conditions shown here imposed the need for extreme care in following identical experimental steps to obtain reproducible data (i.e., initial sample temperature, loading time, vessel closing and opening time, compression and decompression time, and sample removal time).

Measurement of spore inactivation. To measure spore inactivation in BHI broth, aliquots (1 ml) obtained at various times were placed in an ice-water bath, serially diluted in 25 mM phosphate buffer (pH 7.4), plated on BHI agar plates, and incubated overnight at 37ºC. Results were expressed in terms of decimal reduction (DR, DR = $\log_{10}$[final count/ml BHI] - $\log_{10}$[initial count/ml BHI]), where initial counts refer to counts from the heat-activated sample prior to incubation for spore germination.

To measure spore inactivation in poultry meat, pouches were submerged in alcohol. The content was aseptically transferred into sterile 7”x12” filter stomacher bag (LABPLAS Inc., Quebec, Canada) and then 90 ml of sterile 0.1% buffered peptone water (BPW) was added and the mixture homogenized for 2 min in a stomacher (Lab Blender A400,Tekman Co., Cincinnati, OH, USA). The homogenate was serially diluted in BPW, plated on BHI agar plates (Difco, BD Diagnostic Systems, Sparks, MD, USA), and then incubated anaerobically overnight at 37ºC.

Results were expressed as DR values (DR = $\log_{10}$[final count/g meat] - $\log_{10}$[initial count/g meat]), where initial counts refer to counts from the heat-activated sample prior to the incubation for germination at 55ºC.

Statistical Analyses. All treatments were repeated once and those showing a variability coefficient higher than 20% were tested an additional time. Student's *t*-test was used for a specific contrast. The statistical package used was SPlus Version 6.2 (Insightful Corp, Seattle, WA, USA).

9.4. Results and discussions

HPP-induced germination of *C. perfringens* spores. A negligible extent of germination was observed when pressure-treated (at 100, 150 or 200 MPa for 7 min at 50ºC) *C. perfringens* spores were incubated at 40ºC for 60 min in 25 mM phosphate buffer (pH 7.0) (Fig. 1). Microscopy analysis was consistent with negligible germination, as only a small fraction of the pressure-treated spore population (~ 10%) had become phase dark (data not shown). This result is in contrast to the result obtained with *B. subtilis* spores, where a low pressure (~150 MPa) could induce spore germination by activating the GerA-type receptors (15, 148). In *B. subtilis* spores, the requirements for low-pressures activation of GerA-type receptors are similar to requirements for nutrient activation of these receptors (15). Although *C. perfringens* GerA-type receptors have not been fully characterized, they have striking differences with those of *B. subtilis* (154). Indeed *C. perfringens* GerKA and GerKC proteins are required for nutrient germination (i.e., L-asparagine) as *B. subtilis* GerA-type

receptors, yet they are also required for normal DPA release through chemical germination (i.e., Ca-DPA and dodecylamine) and spore outgrowth (154), suggesting differences on the germination apparatus between *C. perfringens* and *B. subtilis* spores. These differences might be related with the lack of low-pressure induced germination observed in *C. perfringens* spores.

Nutrient-induced germination of *C. perfringens* spores at elevated temperature. A small fraction of germinated spores (phase dark) was observed for all five food poisoning isolates when spore suspensions were incubated at 55, 60 and 65ºC in phosphate buffer containing L-asparagine or KCl (Table 1). However, a significant ($p < 0.01$) increase in the extent of germination was observed when spores from all five food poisoning isolates were incubated at 55, 60 or 65ºC with the AK mixture in the same buffer. The effect was most significant when spores were incubated at 55ºC, reaching an average of 76% germination for all food poisoning isolates with the exception of strain FD1041, for which only 40% of spores became phase dark (data not shown). The release of DPA, an early stage of spore germination, is barely detectable by phase microscopy (see below) and reduces spore heat resistance. These results suggest that all tested *C. perfringens* spores can initiate and complete germination at 55ºC when incubated with 100 mM of the AK mixture in 25 mM phosphate buffer at pH 7.0. The ability to germinate at high temperatures seems to be unique for *C. perfringens* spores, since spores of *Bacillus cereus* (12, 29) and *Clostridium botulinum* (167), germinate only up to 40ºC. *B. subtilis* spores (250) can germinate up to 50ºC.

Heat inactivation of germinated *C. perfringens* spores in BHI broth. The effect of a rich medium on *C. perfringens* spore germination at 55ºC with 50 mM AK and subsequent inactivation of the germinated spores at 55ºC, was examined in BHI broth with spores of strain NCTC8239, a high heat-resistant isolate (D_{100^o} ~ 120 min) (193). Less than a 0.5 decimal reduction (DR) was observed when NCTC8239 spores were incubated up to 60 min with BHI broth alone. However, NCTC8239 spores incubated for 60 min in BHI broth plus AK showed a 1.6 DR, suggesting that the AK-induced germinated spores were inactivated at 55ºC (Fig. 2). To test if a second heat treatment would increase spore inactivation, a heat inactivation treatment after various germination times was evaluated. Surprisingly, the addition of a second heat treatment (80ºC, 10 to 20 min) after various incubation times increased spore inactivation from 2.8 to 3.6 DR (Fig. 3). Increasing the temperature to 90ºC for 10 min produced no further effect on spore inactivation. However, when AK-induced germinated spores were treated at 90ºC for 20 min, the spore inactivation increased to ~ 5.3 DR (Fig. 3). These results indicate that at 55ºC, 50 mM AK induces germination of spores which then can be inactivated by a subsequent heat treatment.

Heat and pressure inactivation of germinated spores of *C. perfringens* in poultry meat. Interestingly, *C. perfringens* spore counts were reduced by 1.1-1.4 DR when spore contaminated-meat (spore-meat) was incubated at 55ºC for 15 to 60 min (Fig. 4), suggesting that *C. perfringens* spores were germinated and thus slightly inactivated in meat with no added germinants. Germination was significantly enhanced with the

addition of germinant (50 mM AK) to the meat, which then increased spore inactivation (Fig. 4). For example, 3.9 DR was observed when AK-induced germinated spores in meat were incubated for 60 min at 55ºC and treated at 90ºC for 20 min (Fig. 4). However, the inactivation of AK-induced *C. perfringens* spores observed in meat products was, in general, significantly lower ($p < 0.05$) than that in BHI broth. This suggests that the meat matrix provides some sort of protection to the germinated spores; or, that the bioavailability of AK to induce spore germination is significantly reduced by the meat matrix. Therefore, a maximum inactivation of *C. perfringens* spores can be achieved if meat containing 50 mM AK is incubated for at least 60 min at 55ºC and is subsequently treated at an elevated temperature.

Thermal processing of meat products with temperature shifts would consequently increase the length of the processing regime, increasing production costs and lowering product quality. However, HPP treatments posses the advantage of acting homogeneously and instantaneously through the entire product, quickly increasing the temperature of the product by adiabatic heating (235). Therefore, in an effort to shorten the germination step to less than 30 min, heat treatment was replaced with HPP treatments as the inactivation step of germinated spores**.** Spore-meat samples incubated with AK for 15 and 30 min at 55ºC, and subsequently treated with 586 MPa at 23ºC for 10 min, showed similar spore inactivation (~ 3DR) as meat samples incubated for a similar germination condition and treated at 80ºC for 20 min and 90ºC for 10 min (Fig. 4, 5). However, spore-meat samples incubated with AK for 15 to 30 min and treated with 586 MPa at 73ºC for 10 min showed a significantly ($p < 0.01$) higher spore inactivation (4 DR) which was comparable to that observed with

spore-meat samples incubated with AK for 60 min at 55ºC and subsequently subjected to 90ºC for 20 min (Fig. 4, 5). These results indicate that an HPP treatment at 73ºC reduced the germination step to 15 min while retaining a high degree of spore inactivation (4 DR).

9.5. Conclusions

The results from this study suggest a novel strategy for the inactivation of *C. perfringens* spores in meat products, which should consist of the following steps: i) A primary heat treatment (e.g., 80ºC for 10 min) to pasteurize and denature the meat proteins for adequate palatability and to activate *C. perfringens* spores for germination in the presence of AK; ii) Cooling of the product to 55ºC in about 20 min and incubation at this temperature for about next 15 min for spore germination; and iii) Inactivation of germinated spores by pressure-assisted thermal processing (e.g., 586 MPa at 73ºC for 10 min). However, the efficiency of this novel strategy requires the bioavailability of L-asparagine and KCl necessary for rapid spore germination (i.e., greater than 50 mM). The FDA authorizes the use of L-asparagine as a nutrient or dietary supplement, while KCl is an additive in the generally recognized as safe (GRAS) category.

Fig. 9.1: HPP-induced germination of *C. perfringens* spores.

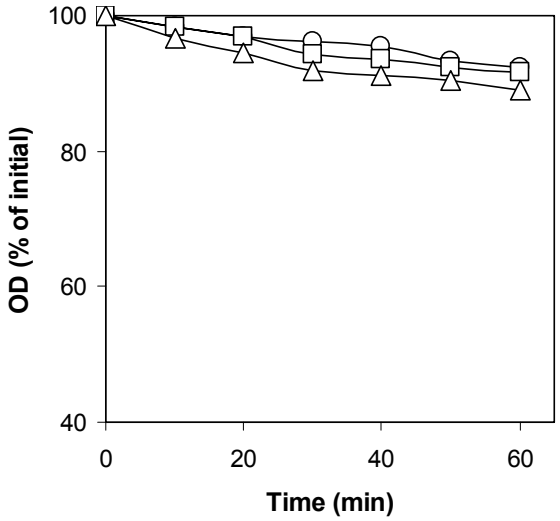


Fig. 9.1. HPP-induced germination of *C. perfringens* spores. Spores were treated at 50ºC for 7 min with: 100 MPa (circles); 150 MPa (squares); 200 MPa (triangles); and germination was assessed by measuring decrease in OD_{600} after incubating these pressure-treated spores at 40C for 60 min in 25 mM sodium phosphate buffer as described in Materials and Methods.

Fig. 9.2: Effect of incubation temperature on *C.perfringens* spore germination.

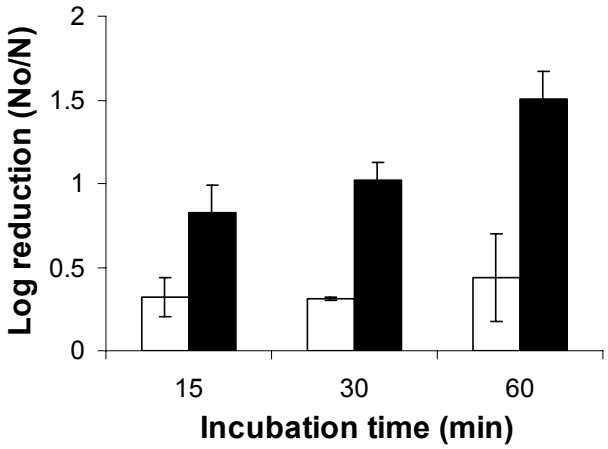


Fig. 9.2. Effect of incubation temperature on *C.perfringens* spore germination. Heat-activated spores of NCTC8239 were incubated with BHI broth plus 50 mM AK at 37ºC (open bar) or 55ºC (filled bar), and at various times aliquots plated onto BHI agar, survivals determined and DR value calculated as described in Materials and Methods.

Fig. 9.3: Heat inactivation of *C. perfringens* germinated spores in BHI broth.

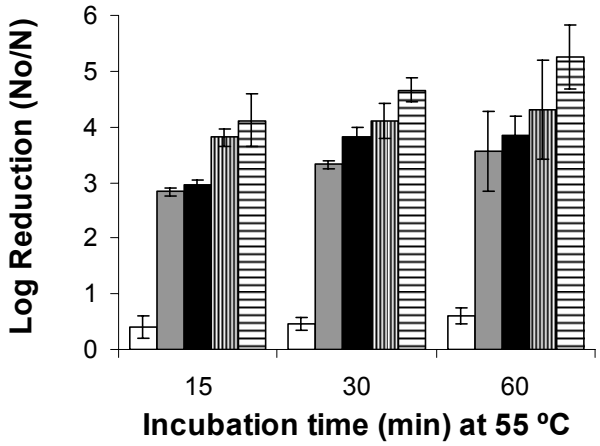


Fig. 9.3. Heat inactivation of *C. perfringens* germinated spores in BHI broth. Spores of strain NCTC8239 were incubated at 55ºC in BHI broth plus 50 mM AK (grey, black, vertical-lines and horizontal-lines bars) and with no germinant (white bars). At various times, aliquots were heat treated: at 80ºC for 10 min (grey bars) and 20 min (black bars); at 90ºC for 10 min (vertical-lines bars) and 20 min (white and horizontal-lines bars), and survivors were determined as described in Materials and Methods.

Fig. 9.4: Heat-inactivation of *C. perfringens* spores in poultry meat.

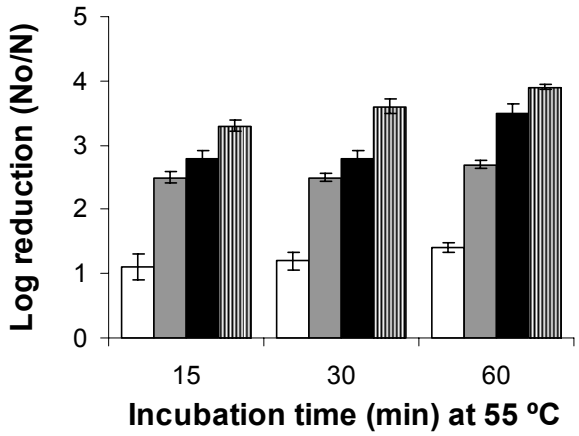


Fig. 9.4. Heat-inactivation of *C. perfringens* spores in poultry meat. Spores from a cocktail of 5 food poisoning isolates were incubated at 55ºC in poultry meat with 50 mM AK (grey, black and vertical-lines bars) and without germinants (white bars). At various times aliquots were heat treated at: 80ºC for 20 min (grey bars); 90ºC for 10 min (black bars); 90ºC for 20 min (white and black bars). Survivors were determined as described in Materials and Methods.

Fig. 9.5: HPP-inactivation of *C. perfringens* spores in poultry meat.

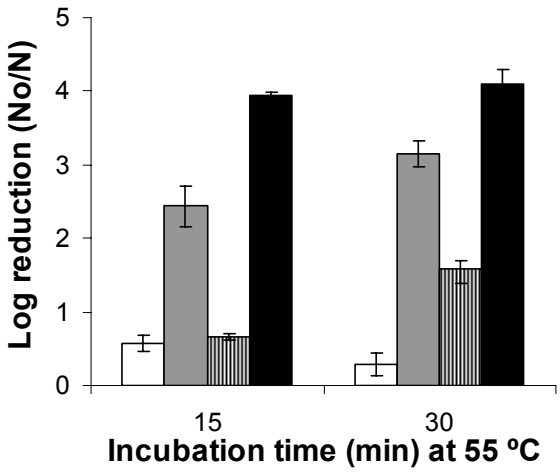


Fig. 9.5. HPP-inactivation of *C. perfringens* spores in poultry meat. Spores from a cocktail of 5 food poisoning isolates were incubated for 15 to 30 min at 55ºC in meat with no germinants (white and horizontal bars) and with 50 mM AK (grey and black bars) and pressurized for 10 min at 586 MPa at: 23ºC (white and grey bars); 73ºC (horizontal-lines and black bars). Survivors were determined as described in Materials and Methods.

Table 9.1. Germination of *C. perfringens* spores from food poisoning isolates at various temperatures.

Strain	Mean % germination in 60 min for[a]:								
	55ºC			60ºC			65ºC		
	Asn[b]	KCl[b]	AK[b]	Asn	KCl	AK	Asn	KCl	AK
SM101	21	30	100	24	19	66	13	19	69
E13	27	48	93	28	55	82	28	24	46
FD1041	30	40	43	22	19	25	15	18	18
NCTC8239	10	49	76	24	22	75	7	19	36
NCTC10239	36	48	66	22	22	39	12	13	57
Avg[b]	25	43	76	24	28	57	15	19	45

[a] All experiments were performed in phosphate buffer (25 mM phosphate buffer at pH 7.0) with 100 mM germinants. Results are the average of duplicate experiments. The variance in all cases was less than 10% of the mean.

[b] Asn indicates L-asparagine; KCl, potassium chloride; AK, a 1:1 mixture of L-asparagine and potassium chloride; Avg, average.

Chapter 10

Conclusions

Clostridium perfringens is an anaerobic, Gram positive, spore forming pathogen of both humans and animals, and can produce a wide array of toxins. *C. perfringens* spores are thought to be the infectious morphotype of all CPAD. To cause diseases, these spores must germinate through the sensing of specific germinants in the environment, release the nascent cell, outgrow, multiply to high number and release toxins causing diseases. Extensive research has been done in *B. subtilis* spore germination, however, there are very few studies in spore germination of *Clostridium* species. Unfortunately, due to significant differences in the components of the germination apparatus between *Bacillus* and *Clostridium* species, work in spore germination of *Bacillus* species is not sufficient to identify new strategies to inactivate *Clostridium* spores. In the current study, we performed molecular analyses of spore germination of *C. perfringens* and developed a strategy to inactivate spores in meat.

The first was to identify and characterize the germinants and the receptors involved in *C. perfringens* spore germination. Result from these studies found differential germination requirements between spores of FP and NFBGID isolates in that: (i) while a mixture of L-asparagine and KCl was a good germinant for spores of FP and NFBGID isolates, KCl and, to a lesser extent, L-asparagine triggered spore germination in FP isolates only; and ii) L-alanine and L-valine induced significant germination of spores of NFBGID but not FP isolates. In contrast to *B. subtilis*, *C. perfrinegns* genomes sequenced to date possess no tricistronic *gerA*-like operon, but

has a monocistronic *gerAA* that is far from a *gerK* locus. The *gerK* locus contains a bicistronic *gerKA-gerKC* operon and a monocistronic *gerKB* upstream and in the opposite orientation to *gerKA-gerKC*. Consequently, through the construction of mutations into strain SM101, a *C. perfringens* FP isolate, the role of *gerAA* and *gerKA* genes in *C. perfringens* spore germination were investigated. Spores of a *gerK* mutant, lacking the GerKA and GerKC proteins, germinated slower than wild-type with KCl, did not germinate with L-asparagine, and very poorly compared to wild-type spores with non-nutrients germinants dodecylamine and a 1:1 chelate of Ca^{2+} and dipicolinic acid. In contrast, *gerAA* spores, lacking the GerAA proteins, germinated similarly as wild-type spores with high concentrations of KCl, although slightly slower with low concentration of KCl. Furthermore, *gerK*, but not *gerAA* spores, exhibited significantly lower colony formation efficiency than wild-type spores. These results indicate that GerKA and/or GerKC is the main germinant receptor, while GerAA plays an auxiliary role in germination. In contrast to *B. subtilis*, where Ca-DPA acts through a CLE, CwlJ, in *C. perfringens* Ca-DPA seems to initiate germination through the GerKA and/or GerKC receptor. However, the relatively high colony formation efficiency and the significant germination of *gerK* spores, suggested that GerKB might also have some role in *C. perfringens* spore germination. Consequently, to explore this hypothesis, through the construction of a *gerKB* mutation, the role of GerKB on spore germination was investigated. Results from this study indicated that, similarly as *gerK* and *gerAA*, *gerKB* is likely to be expressed in the forespore, *gerKB* spores germinated similarly as wild-type spores with high concentration of nutrients but significantly slower with low nutrient concentration. Most notably of *gerKB* spores was the

significantly lower colony-forming efficiency and slower outgrowth than wild-type spores. The results of this study suggest that GerKB plays an auxiliary role in spore germination, and is required for normal spore viability.

Since *C. perfrinegns* spores are able to germinate with K^+ ions alone, and significant germination was observed in *C. perfringens* spores lacking the main germinant receptor(s) proteins, GerKA and/or GerKC, we raised the hypothesis that GrmA-like antiporters might also play some role in *C. perfringens* spore germination. Two putative GrmA-like antiporters (i.e., GerO and GerQ) are encoded in the genome of all *C. perfringens* genomes sequenced to date. *C. perfringens* strain SM101 carrying *gerO*- and *gerQ-gusA* fusions indicate that these genes are expressed uniquely during sporulation; while strain SM101 carrying *gerO*- and *gerQ-gfp* showed that these genes are expressed uniquely in the mother cell compartment of the sporulating cell. Complementation studies of K^+ uptake and Na^+ sensitive *E. coli* mutants with *C. perfringens* GerO and GerQ indicate that while GerO is capable of translocating K^+ and Na^+, GerQ is only capable of translocating to a small extent Na^+ ions. Construction of *gerO*, *gerQ* and *gerO gerQ* strains of *C. perfringens* revealed that spores lacking GerO had defective germination in rich medium, KCl, L-asparagine, and Ca-DPA, but not with dodecylamine, defect that might be prior to DPA release during germination. In contrast, loss of GerQ had a much smaller effect on spore germination. Homology modeled structure of GerO was similar to that of the *E. coli* Na^+/H^+ antiporter NhaA, and GerO, but not GerQ had two adjacent Asp residues important in this group of cation transporters, and replacement of these residues for Asn reduced the protein's ability to complement *gerO* spores. Although results from

this study indicate that putative antiporters have some role on *C. perfringens* spore germination, it is unclear whether their role is direct or during spore formation.

C. perfringens type A FP spores are capable of germinating with K^+ ions, a intrinsic mineral of meats commonly associated with FP. Inorganic phosphate (Pi) is also intrinsically found in meat products. Consequently we hypothesized that FP spores are capable of germinating in presence of Pi. Results from this study show that Pi was able to induce germination of the majority of FP, but not NFBGID isolates. Pi-induced germination of FP spores required the presence of GerKA and/or GerKC protein, while GerAA and to a much lesser extent, GerKB, showed auxiliary roles. Pi-induced germination also required the putative Na^+/K^+-H^+ antiporter, GerO, for normal germination. These results indicate that spores of FP isolates might be better adapted to food poisoning environments.

Since, *in B. subtilis* the main event after binding of the nutrient to its cognate receptor is the release of DPA, presumably through the SpoVA proteins, we investigated the role of the SpoVA proteins and Ca-DPA in *C. perfringens* spore germination. In *B. subtilis*, the SpoVA proteins have been associated with Ca-DPA uptake and subsequent release during sporulation and germination, respectively. In addition, Ca-DPA acts as a signal molecule for cortex hydrolysis, activating the cortex lytic enzyme (CLE) CwlJ. Consequently, the role of SpoVA proteins and Ca-DPA in *C. perfringens* spore germination, was investigated through the construction of a *spoVA* mutant. Surprisingly, the *spoVA* spores were stable and germinated like wild-type spores. In contrast, *B. subtilis spoVA* mutant, although is able to sporulate and produce spores, these spores lyse quickly during purification. These results suggest

major differences in the germination pathway of germination between *C. perfringens* and *B. subtilis*, also indicate that Ca-DPA is not needed for *C. perfringens* spore germination, and that CLEs are activated through a unknown mechanism.

Cortex hydolysis is the hallmark of spore germination, since it allows the release of the nascent cell to the environment. Two *C. perfringens* CLEs (i.e., SleC and SleM) degrade PG spore cortex hydrolysis *in vitro*, however, due to lack of genetic tools, their *in vivo* role in *C. perfringens* spore germination remains unclear. Consequently, through construction of *sleC*, *sleM* and *sleC sleM* mutants in strain SM101, their *in vivo* role was investigated. Results from this study show that SleC is essential for cortex hydrolysis and viability of *C. perfringens* spores. *C. perfringens sleC* spores were not able to germinate with nutrients and non-nutrient germinants, however long incubation periods allowed the release of most of their DPA, and this was much slower in *sleC sleM* spores. In contrast, *sleM* spores germinated similar as wild type in presence of nutrient and non-nutrient germinants. The results of this study indicate that SleC is essential for cortex hydrolysis, while SleM, although can degrade cortex PG in vitro, is not essential.

In vitro work shows that Csp proteins process the inactive proSleC into the mature enzyme, SleC. However, the *in vivo* role of the Csp proteins has not been established. In this study, through the construction of a *cspB* mutant in *C. perfringens* strain SM101, the *in vivo* role of CspB during spore germination was investigated. *C. perfringens cspB* spores exhibited significantly less viability than wild-type spores, and were unable to germinate with either rich medium or Ca-DPA. Germination of *cspB* spores was blocked prior to DPA release and cortex hydrolysis. Results from this

study indicate that CspB is essential to generate active SleC and allow cortex hydrolysis early in *C. perfringens* spore germination. In contrast to *B. subtilis*, Ca-DPA does not activate the CLEs during *C. perfringens* spore germination and supports previous results that Ca-DPA acts trough the GerKA and/or GerKC receptor. However, it is still unclear how CspB is activated early during *C. perfringens* spore germination.

The detail understanding of the molecular mechanism of *C. perfringens* spore germination allowed us to develop a strategy to inactivate *C. perfringens* spores in meat. *C. perfringens* spores posses high heat and pressure resistance, however, they loss their resistance properties during early stages of germination. This study aimed inactivate *C. perfringens* spores in meat through a combination of spore activation at low pressure (100-200 MPa, 7 min) and elevated temperature (80ºC, 10 min); spore germination at high temperature; and inactivation of germinated spores with elevated temperatures (80 and 90ºC, 10 and 20 min) and high pressure (586 MPa, at 23 and 73ºC for 10 min). In contrast to *B. subtilis* spores, germination of *C. perfringens* spores could not be triggered with low pressures. However, they germinated efficiently when heat activated in presence of L-asparagine and KCl at temperatures lethal for vegetative cells, and these germinated spores were efficiently inactivated by subsequent treatment with pressure assisted thermal processing (586 MPa at 73ºC for 10 min). This study shows the feasibility of a novel strategy to inactivate *C. perfringens* spores in meat products formulated with germinants.

In conclusion, the work done in this study increases our insight of the mechanism of spore germination of the pathogenic bacterium *C. perfringens*. We have

found that GerKA and/or GerKC as the main germinant receptors in *C. perfringens* spores; SpoVA proteins are essential for Ca-DPA uptake during spore formation; Ca-DPA is not required for initiation of cortex hydrolysis; SleC is essential for cortex hydrolysis; and CspB is essential for processing of proSleC into mature SleC and initiation of cortex hydrolysis. In addition, we developed a strategy to inactivate C. perfringens spores in meats. Collectively, these findings are essential for the identification of drug targets, and development of new strategies and treatments to block or induce *C. perfringens* spore germination.

Bibliography

1. **Akhtar, S., D. Paredes-Sabja, and M. R. Sarker.** 2008. Inhibitory effects of polyphosphates on *Clostridium perfringens* growth, sporulation and spore outgrowth. Food Microbiology **25:**802-808.
2. **Al-Sheikhly, F., and R. B. Truscott.** 1976. The pathology of necrotic enteritis of chickens following infusion of broth cultures of Clostridium perfringens into the duodenum. Avian Diseases **21:**230-240.
3. **Al-Sheikhly, F., and A. Al-Saieg.** 1979. Role of Coccidia in the occurence of necrotic enteritis in chickens. Avian Diseases **24:**324-333.
4. **Alberto, F., V. Broussolle, D. R. Mason, F. Carlin, and M. W. Peck.** 2003. Variability in spore germination response by strains of proteolytic *Clostridium botulinum* types A, B and F. Lett. Appl. Microbiol. **36:**41-45.
5. **Alouf, J. E., and C. Jolivet-Reynaud.** 1981. Purification and characterization of *Clostridium perfringens* delta-toxin. Infect Immun **31:**536-546.
6. **Atluri, S., K. Ragkousi, D. E. Cortezzo, and P. Setlow.** 2006. Cooperativity between different nutrient receptors in germination of spores of *Bacillus subtilis* and reduction of this cooperativity by alterations in the GerB receptor. J. Bacteriol. **188:**28-36.
7. **Atrih, A., P. Zollner, G. Allmaier, and S. J. Foster.** 1996. Structural analysis of *Bacillus subtilis* 168 endospore peptidoglycan and its role during differentiation. J Bacteriol **178:**6173-6183.
8. **Awad, M. M., A. E. Bryant, D. L. Stevens, and J. I. Rood.** 1995. Virulence studies on chromosomal alpha-toxin and theta-toxin mutants constructed by

allelic exchange provide genetic evidence for the essential role of alpha-toxin in *Clostridium perfringens*-mediated gas gangrene. Mol. Microbiol. **15:**191-202.

9. **Awad, M. M., D. M. Ellemor, A. E. Bryant, O. Matsushita, R. L. Boyd, D. L. Stevens, J. J. Emmins, and J. I. Rood.** 2000. Construction and virulence testing of a collagenase mutant of *Clostridium perfringens*. Microb Pathog **28:**107-117.

10. **Bagyan, I., and P. Setlow.** 2002. Localization of the cortex lytic enzyme CwlJ in spores of *Bacillus subtilis*. J Bacteriol **184:**1219-1224.

11. **Bannam, T. L., and J. I. Rood.** 1993. *Clostridium perfringens-Escherichia coli* shuttle vectors that carry single antibiotic resistance determinants. Plasmid **29:**233-235.

12. **Barlass, P. J., C. W. Houston, M. O. Clements, and A. Moir.** 2002. Germination of *Bacillus cereus* spores in response to L-alanine and to inosine: the roles of *gerL* and *gerQ* operons. Microbiology **148:**2089-2095.

13. **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.

14. **Bettegowda, C., X. Huang, J. Lin, I. Cheong, M. Kohli, S. A. Szabo, X. Zhang, L. A. Diaz, Jr., V. E. Velculescu, G. Parmigiani, K. W. Kinzler, B. Vogelstein, and S. Zhou.** 2006. The genome and transcriptomes of the anti-tumor agent *Clostridium novyi*-NT. Nat. Biotechnol. **24:**1573-1580.

15. **Black, E. P., K. Koziol-Dube, D. Guan, J. Wei, B. Setlow, D. E. Cortezzo, D. G. Hoover, and P. Setlow.** 2005. Factors influencing germination of *Bacillus subtilis* spores via activation of nutrient receptors by high pressure. Appl. Environ. Microbiol. **71:**5879-5887.

16. **Boland, F. M., A. Atrih, H. Chirakkal, S. J. Foster, and A. Moir.** 2000. Complete spore-cortex hydrolysis during germination of *Bacillus subtilis* 168 requires SleB and YpeB. Microbiology **146:**57-64.

17. **Broussolle, V., F. Alberto, C. A. Shearman, D. R. Mason, L. Botella, C. Nguyen-The, M. W. Peck, and F. Carlin.** 2002. Molecular and physiological characterization of spore germination in *Clostridium botulinum* and *C. sporogenes*. Anaerobe **8:**89-100.

18. **Bruggemann, H., S. Baumer, W. F. Fricke, A. Wiezer, H. Liesegang, I. Decker, C. Herzberg, R. Martinez-Arias, R. Merkl, A. Henne, and G. Gottschalk.** 2003. The genome sequence of *Clostridium tetani*, the causative agent of tetanus disease. Proc. Natl. Acad. Sci. U S A **100:**1316-1321.

19. **Bryson, K., L. J. McGuffin, R. L. Marsden, J. J. Ward, J. S. Sodhi, and D. T. Jones.** 2005. Protein structure prediction servers at University College London. Nucleic Acids Res. **33:**W36-38.

20. **Bujnicki, J. M., A. Elofsson, D. Fischer, and L. Rychlewski.** 2001. Structure prediction meta server. Bioinformatics **17:**750-751.

21. **Cabrera-Martinez, R. M., F. Tovar-Rojo, V. R. Vepachedu, and P. Setlow.** 2003. Effects of overexpression of nutrient receptors on germination of spores of *Bacillus subtilis*. J. Bacteriol. **185:**2457-2464.

22. **Carman, R. J.** 1997. *Clostridium perfringens* spontaneous and antibiotic associated diarrhoea of man and other animals. Rev Med Microbiol **8:**S43-S45.

23. **Cavaillon, J. M., C. Jolivet-Reynaud, C. Fitting, B. David, and J. E. Alouf.** 1986. Ganglioside identification on human monocyte membrane with *Clostridium perfringens* delta-toxin. J Leukoc Biol **40:**65-72.

24. **Chen, Y., S. Miyata, S. Makino, and R. Moriyama.** 1997. Molecular characterization of a germination-specific muramidase from *Clostridium perfringens* S40 spores and nucleotide sequence of the corresponding gene. J. Bacteriol. **179:**3181-3187.

25. **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:**7542-7547.

26. **Cheng, J., A. A. Guffanti, and T. A. Krulwich.** 1994. The chromosomal tetracycline resistance locus of *Bacillus subtilis* encodes a Na^+/H^+ antiporter that is physiologically important at elevated pH. J. Biol. Chem. **269:**27365-27371.

27. **Chirakkal, H., M. O'Rourke, A. Atrih, S. J. Foster, and A. Moir.** 2002. Analysis of spore cortex lytic enzymes and related proteins in *Bacillus subtilis* endospore germination. Microbiology **148:**2383-2392.

28. **Christie, G., and C. R. Lowe.** 2007. Role of chromosomal and plasmid-borne receptor homologues in the response of *Bacillus megaterium* QM B1551 spores to germinants. J. Bacteriol. **189:**4375-4383.

29. **Clements, M. O., and A. Moir.** 1998. Role of the *gerI* operon of *Bacillus cereus* 569 in the response of spores to germinants. J. Bacteriol. **180:**6729-6735.

30. **Coleman, W. H., D. Chen, Y. Q. Li, A. E. Cowan, and P. Setlow.** 2007. How moist heat kills spores of *Bacillus subtilis*. J. Bacteriol. **189:**8458-8466.

31. **Collie, R. E., J. F. Kokai-Kun, and B. A. McClane.** 1998. Phenotypic characterization of enterotoxigenic *Clostridium perfringens* isolates from non-foodborne human gastrointestinal diseases. Anaerobe **4:**69-79.

32. **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:**30-36.

33. **Cornillot, E., B. Saint-Joanis, G. Daube, S. Katayama, P. E. Granum, B. Canard, and S. T. Cole.** 1995. The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. Mol. Microbiol. **15:**639-647.

34. **Cowan, A. E., D. E. Koppel, B. Setlow, and P. Setlow.** 2003. A soluble protein is immobile in dormant spores of *Bacillus subtilis* but is mobile in germinated spores: implications for spore dormancy. Proc. Natl. Acad. Sci. USA **100:**4209-4214.

35. **Czeczulin, J. R., R. E. Collie, and B. A. McClane.** 1996. Regulated expression of *Clostridium perfringens* enterotoxin in naturally *cpe*-negative type A, B, and C isolates of *C. perfringens*. Infect. Immun. **64:**3301-3309.

36. **Duncan, C. L., and D. H. Strong.** 1968. Improved medium for sporulation of *Clostridium perfringens*. Appl. Microbiol. **16:**82-89.

37. **Duncan, C. L., D. H. Strong, and M. Sebald.** 1972. Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. J Bacteriol **110:**378-391.

38. **Errington, J., S. M. Cutting, and J. Mandelstam.** 1988. Branched pattern of regulatory interactions between late sporulation genes in *Bacillus subtilis*. J. Bacteriol. **170:**796-801.

39. **Errington, J.** 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. **57:**1-33.

40. **Feavers, I. M., J. Foulkes, B. Setlow, D. Sun, W. Nicholson, P. Setlow, and A. Moir.** 1990. The regulation of transcription of the *gerA* spore germination operon of *Bacillus subtilis*. Mol. Microbiol. **4:**275-282.

41. **Fernandez-Miyakawa, M. E., B. H. Jost, S. J. Billington, and F. A. Uzal.** 2008. Lethal effects of *Clostridium perfringens* epsilon toxin are potentiated by alpha and perfringolysin-O toxins in a mouse model. Vet Microbiol **127:**379-385.

42. **Fisher, D. J., K. Miyamoto, B. Harrison, S. Akimoto, M. R. Sarker, and B. A. McClane.** 2005. Association of beta2 toxin production with *Clostridium perfringens* type A human gastrointestinal disease isolates carrying a plasmid enterotoxin gene. Mol Microbiol **56:**747-762.

43. **Flanagan, J. J., R. K. Tweten, A. E. Johnson, and A. P. Heuck.** 2009. Cholesterol Exposure at the Membrane Surface Is Necessary and Sufficient to Trigger Perfringolysin O Binding. Biochemistry.

44. **Fort, P., and J. Errington.** 1985. Nucleotide sequence and complementation analysis of a polycistronic sporulation operon, *spoVA*, in *Bacillus subtilis*. J. Gen. Microbiol. **131:**1091-1105.

45. **Foster, S. J., and K. Johnstone.** 1987. Purification and properties of a germination-specific cortex-lytic enzyme from spores of *Bacillus megaterium* KM. Biochem. J. **242:**573-579.

46. **Fujita, K., J. Katahira, Y. Horiguchi, N. Sonoda, M. Furuse, and S. Tsukita.** 2000. *Clostridium perfringens* enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein. FEBS Lett **476:**258-261.

47. **Garmory, H. S., N. Chanter, N. P. French, D. Bueschel, J. G. Songer, and R. W. Titball.** 2000. Occurrence of *Clostridium perfringens* beta2-toxin amongst animals, determined using genotyping and subtyping PCR assays. Epidemiol Infect **124:**61-67.

48. **Gazdzinski, P., and R. J. Julian.** 1992. Necrotic enteritis in turkeys. Avian Dis **36:**792-798.

49. **Gerhardt, P., and R. E. Marquis.** 1989. Spore thermoresistance mechanisms, p. 43-63. *In* R. S. I. Smith, and P. Setlow (ed.), Regulation of prokaryotic development, 1st ed. American Society for Microbiology, Washington, D.C.

50. **Ghazala, S., D. Coxworthy, and T. Alkalani.** 1995. Thermal kientics of *Streptococcus faecium* in nutrient broth/sous vide products under pasteurized conditions. J. Food. Proc. Preserv. **19:**243-257.

51. **Ghuysen, J. M., D. J. Tipper, and J. L. Strominger.** 1966. Enzymes that degrade bacterial cell walls. Meth. Enzymol. **8:**685-699.

52. **Gibert, M., C. Jolivet-Reynaud, and M. R. Popoff.** 1997. Beta2 toxin, a novel toxin produced by *Clostridium perfringens*. Gene **203:**65-73.

53. **Ginalski, K., A. Elofsson, D. Fischer, and L. Rychlewski.** 2003. 3D-Jury: a simple approach to improve protein structure predictions. Bioinformatics **19:**1015-1018.

54. **Gould, G. W., and A. Hurst.** 1969. The bacterial spore. Academic Press, London, New York.

55. **Granger, A. C., and P. Setlow.** 2008. Unpublished results.

56. **Griffith, K. L., and J. Wolf, R.E.** 2002. Measuring beta-galactosidase activity in bacteria: cell growth, permeabilization, and enzyme assay in 96-well arrays. Biochem. Biophys. Res. Commun. **290:**397-402.

57. **Guffanti, A. A., J. Cheng, and T. A. Krulwich.** 1998. Electrogenic antiport activities of the Gram-positive Tet proteins include a $Na^+(K^+)/K^+$ mode that mediates net K^+ uptake. J. Biol. Chem. **273:**26447-26454.

58. **Harrison, B., D. Raju, H. S. Garmory, M. M. Brett, R. W. Titball, and M. R. Sarker.** 2005. Molecular characterization of *Clostridium perfringens* isolates from humans with sporadic diarrhea: evidence for transcriptional

regulation of the beta2-toxin-encoding gene. Appl Environ Microbiol **71:**8362-8370.

59. **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.

60. **Hatheway, C. L.** 1990. Toxigenic clostridia. Clin. Microbiol. Rev. **3:**66-98.

61. **Helmboldt, C. F., and E. S. Bryant.** 1971. The pathology of necrotic enteritis in domestic fowl. Avian Dis **15:**775-780.

62. **Herholz, C., R. Miserez, J. Nicolet, J. Frey, M. Popoff, M. Gibert, H. Gerber, and R. Straub.** 1999. Prevalence of beta2-toxigenic *Clostridium perfringens* in horses with intestinal disorders. J Clin Microbiol **37:**358-361.

63. **Heuck, A. P., C. G. Savva, A. Holzenburg, and A. E. Johnson.** 2007. Conformational changes that effect oligomerization and initiate pore formation are triggered throughout perfringolysin O upon binding to cholesterol. J Biol Chem **282:**22629-22637.

64. **Hoch, J. A., and J. L. Mathews.** 1973. Chromosomal location of pleiotropic negative sporulation mutations in *Bacillus subtilis*. Genetics **73:**215-228.

65. **Hornstra, L. M., Y. P. de Vries, M. H. Wells-Bennik, W. M. de Vos, and T. Abee.** 2006. Characterization of germination receptors of *Bacillus cereus* ATCC 14579. Appl. Environ. Microbiol. **72:**44-53.

66. **Hornstra, L. M., P. L. de Leeuw, R. Moezelaar, E. J. Wolbert, Y. P. de Vries, W. M. de Vos, and T. Abee.** 2007. Germination of *Bacillus cereus* spores adhered to stainless steel. Int J Food Microbiol **116:**367-371.

67. **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:**233-240.

68. **Huang, I. H., and M. R. Sarker.** 2006. Complementation of a *Clostridium perfringens spo0A* mutant with wild-type *spo0A* from other *Clostridium* species. Appl. Environ. Microbiol. **72:**6388-6393.

69. **Hudson, K. D., B. M. Corfe, E. H. Kemp, I. M. Feavers, P. J. Coote, and A. Moir.** 2001. Localization of GerAA and GerAC germination proteins in the *Bacillus subtilis* spore. J. Bacteriol. **183:**4317-4322.

70. **Hunte, C., E. Screpanti, M. Venturi, A. Rimon, E. Padan, and H. Michel.** 2005. Structure of a Na^+/H^+ antiporter and insights into mechanism of action and regulation by pH. Nature **435:**1197-1202.

71. **Igarashi, T., B. Setlow, M. Paidhungat, and P. Setlow.** 2004. Effects of a *gerF* (lgt) mutation on the germination of spores of *Bacillus subtilis*. J. Bacteriol. **186:**2984-2991.

72. **Igarashi, T., and P. Setlow.** 2005. Interaction between individual protein components of the GerA and GerB nutrient receptors that trigger germination of *Bacillus subtilis* spores. J. Bacteriol. **187:**2513-2518.

73. **Illades-Aguiar, B., and P. Setlow.** 1994. Studies of the processing of the protease which initiates degradation of small, acid-soluble proteins during germination of spores of *Bacillus* species. J. Bacteriol. **176:**2788-2795.

74. **Illades-Aguiar, B., and P. Setlow.** 1994. Autoprocessing of the protease that degrades small, acid-soluble proteins of spores of *Bacillus* species is triggered by low pH, dehydration, and dipicolinic acid. J. Bacteriol. **176:**7032-7037.

75. **Inoue, H., T. Noumi, T. Tsuchiya, and H. Kanazawa.** 1995. Essential aspartic acid residues, Asp-133, Asp-163 and Asp-164, in the transmembrane helices of a Na^+/H^+ antiporter (NhaA) from *Escherichia coli*. FEBS Lett. **363:**264-268.

76. **Ireland, J. A., and P. C. Hanna.** 2002. Amino acid- and purine ribonucleoside-induced germination of *Bacillus anthracis* DeltaSterne endospores: *gerS* mediates responses to aromatic ring structures. J. Bacteriol. **184:**1296-1303.

77. **Ishikawa, S., K. Yamane, and J. Sekiguchi.** 1998. Regulation and characterization of a newly deduced cell wall hydrolase gene (*cwlJ*) which affects germination of *Bacillus subtilis* spores. J. Bacteriol. **180:**1375-1380.

78. **Itodo, A. E., A. A. Adesiyun, J. O. Adekeye, and J. U. Umoh.** 1986. Toxin-types of *Clostridium perfringens* strains isolated from sheep, cattle and paddock soils in Nigeria. Vet Microbiol **12:**93-96.

79. **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.

80. **Jay, J. M., M. J. Loessner, and D. A. Golden.** 2005. Modern Food Microbiology, seventh ed. Springer Science + Business Media ,Inc, San Marcos, California.

81. **Jefferson, R. A., S. M. Burgess, and D. Hirsh.** 1986. beta-Glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc. Natl. Acad. Sci. USA **83:**8447-8451.

82. **Johansson, A., A. Aspan, E. Bagge, V. Baverud, B. E. Engstrom, and K. E. Johansson.** 2006. Genetic diversity of *Clostridium perfringens* type A isolates from animals, food poisoning outbreaks and sludge. BMC Microbiol **6:**47.

83. **Jolivet-Reynaud, C., J. M. Cavaillon, and J. E. Alouf.** 1982. Selective cytotoxicity of *Clostridium perfringens* delta toxin on rabbit leukocytes. Infect Immun **38:**860-864.

84. **Jones, D. T.** 1999. Protein secondary structure prediction based on position-specific scoring matrices. J. Mol. Biol. **292:**195-202.

85. **Juneja, V. K., B. S. Eblen, and G. M. Ranson.** 2001. Thermal inactivation of *Salmonella* spp. in chicken broth, beef, pork, turkey, and chicken: determination of D- and Z-values. J. Food Sci. **66:**146-152.

86. **Kabsch, W.** 1976. A solution for the best rotation to relate two sets of vectors. Acta Cryst **32:**922-923.

87. **Katahira, J., H. Sugiyama, N. Inoue, Y. Horiguchi, M. Matsuda, and N. Sugimoto.** 1997. *Clostridium perfringens* enterotoxin utilizes two structurally related membrane proteins as functional receptors in vivo. J Biol Chem **272:**26652-26658.

88. **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:**720-726.

89. **Keyburn, A. L., J. D. Boyce, P. Vaz, T. L. Bannam, M. E. Ford, D. Parker, A. Di Rubbo, J. I. Rood, and R. J. Moore.** 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathog **4:**e26.

90. **Klaasen, H. L., M. J. Molkenboer, J. Bakker, R. Miserez, H. Hani, J. Frey, M. R. Popoff, and J. F. van den Bosch.** 1999. Detection of the beta2 toxin gene of *Clostridium perfringens* in diarrhoeic piglets in The Netherlands and Switzerland. FEMS Immunol Med Microbiol **24:**325-332.

91. **Kokai-Kun, J. F., J. G. Songer, J. R. Czeczulin, F. Chen, and B. A. McClane.** 1994. Comparison of Western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens*. J. Clin. Microbiol. **32:**2533-2539.

92. **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:**884-892.

93. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin, and et al.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature **390:**249-256.

94. **Kuwabara, N., H. Inoue, Y. Tsuboi, K. Mitsui, M. Matsushita, and H. Kanazawa.** 2006. Structure-function relationship of the fifth transmembrane domain in the Na^+/H^+ antiporter of *Helicobacter pylori*: Topology and function of the residues, including two consecutive essential aspartate residues. Biochemistry **45:**14834-14842.

95. **Labbe, R.** 1989. *Clostridium perfringens*, p. 192-234. *In* M. P. Doyle (ed.), Food-borne bacterial pathogens. Marcel Dekker, New York.

96. **Lahti, P., A. Heikinheimo, T. Johansson, and H. Korkeala.** 2007. *Clostridium perfringens* type A isolates carrying the plasmid-borne enterotoxin gene (genotypes IS1151-*cpe* or IS1470-like-*cpe*) are a common cause of food poisonings. J. Clin. Microbiol.

97. **Lahti, P., A. Heikinheimo, T. Johansson, and H. Korkeala.** 2008. *Clostridium perfringens* type A strains carrying a plasmid-borne enterotoxin gene (genotype IS*1151*-*cpe* or IS*1470*-like-*cpe*) as a common cause of food poisoning. J Clin Microbiol **46:**371-373.

98. **Lambert, E. A., and D. L. Popham.** 2008. The *Bacillus anthracis* SleL (YaaH) protein is an N-acetylglucosaminidase involved in spore cortex depolymerization. J Bacteriol **190:**7601-7607.

99. **Lawrence, G., P. D. Walker, J. Garap, and M. Avusi.** 1979. The occurrence of *Clostridium welchii* type C in Papua New Guinea. P N G Med J **22:**69-73.

100. **Lee, K. S., D. Bumbaca, J. Kosman, P. Setlow, and M. J. Jedrzejas.** 2008. Structure of a protein-DNA complex essential for DNA protection in spores of *Bacillus* species. Proc. Natl. Acad. Sci. U S A **105:**2806-2811.

101. **Leyva-Illades, J. F., B. Setlow, M. R. Sarker, and P. Setlow.** 2007. Effect of a small, acid-soluble spore protein from *Clostridium perfringens* on the resistance properties of *Bacillus subtilis* spores. J. Bacteriol. **189:**7927-7931.

102. **Li, J., and B. A. McClane.** 2006. Comparative effects of osmotic, sodium nitrite-induced, and pH-induced stress on growth and survival of *Clostridium perfringens* type A isolates carrying chromosomal or plasmid-borne enterotoxin genes. Appl. Environ. Microbiol. **72:**7620-7625.

103. **Li, J., and B. A. McClane.** 2006. Further comparison of temperature effects on growth and survival of *Clostridium perfringens* type A isolates carrying a chromosomal or plasmid-borne enterotoxin gene. Appl. Environ. Microbiol. **72:**4561-4568.

104. **Li, J., and B. A. McClane.** 2008. A novel small acid soluble protein variant is important for spore resistance of most *Clostridium perfringens* food poisoning isolates. PLoS Pathog **4:**e1000056.

105. **Lindsay, J. A., T. C. Beaman, and P. Gerhardt.** 1985. Protoplast water content of bacterial spores determined by buoyant density sedimentation. J. Bacteriol. **163:**735-737.

106. **Liu, H., N. H. Bergman, B. Thomason, S. Shallom, A. Hazen, J. Crossno, D. A. Rasko, J. Ravel, T. D. Read, S. N. Peterson, J. Yates, 3rd, and P. C. Hanna.** 2004. Formation and composition of the *Bacillus anthracis* endospore. J Bacteriol **186:**164-178.

107. **Lovell, S. C., I. W. Davis, W. B. Arendall, P. I. W. de Bakker, J. M. Word, M. G. Prisant, J. S. Richardson, and D. C. Richardson.** 2002. Structure

validation by Calpha geometry: phi,psi and Cbeta deviation. Proteins **50:**437-450.

108. **Magge, A., A. C. Granger, P. G. Wahome, B. Setlow, V. R. Vepachedu, C. A. Loshon, L. Peng, D. Chen, Y. Q. Li, and P. Setlow.** 2008. Role of dipicolinic acid in the germination, stability, and viability of spores of *Bacillus subtilis*. J. Bacteriol. **190:**4798-4807.

109. **Makino, S., N. Ito, T. Inoue, S. Miyata, and R. Moriyama.** 1994. A spore-lytic enzyme released from *Bacillus cereus* spores during germination. Microbiology **140 (Pt 6):**1403-1410.

110. **Makino, S., and R. Moriyama.** 2002. Hydrolysis of cortex peptidoglycan during bacterial spore germination. Med. Sci. Monit. **8:**RA119-127.

111. **Martin, T. G., and J. A. Smyth.** 2009. Prevalence of *netB* among some clinical isolates of *Clostridium perfringens* from animals in the United States. Vet Microbiol **136:**202-205.

112. **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:**227-234.

113. **Mason, J. M., R. H. Hackett, and P. Setlow.** 1988. Regulation of expression of genes coding for small, acid-soluble proteins of *Bacillus subtilis* spores: studies using *lacZ* gene fusions. J. Bacteriol. **170:**239-244.

114. **Matsushita, O., K. Yoshihara, S. Katayama, J. Minami, and A. Okabe.** 1994. Purification and characterization of *Clostridium perfringens* 120-kilodalton collagenase and nucleotide sequence of the corresponding gene. J Bacteriol **176:**149-156.

115. **McClane, B. A.** 1994. *Clostridium perfringens* enterotoxin acts by producing small molecule permeability alterations in plasma membranes. Toxicology **87:**43-67.

116. **McClane, B. A.** 1996. An overview of *Clostridium perfringens* enterotoxin. Toxicon **34:**1335-1343.

117. **McClane, B. A.** 2007. *Clostridium perfringens*, p. 423-444. *In* M. P. Doyle and L. R. Beuchat (ed.), Food Microbiology: fundamentals and frontiers, 3rd ed. ASM Press, Washington, D.C.

118. **McDonel, J. L., and C. L. Duncan.** 1975. Histopathological effect of *Clostridium perfringens* enterotoxin in the rabbit ileum. Infect Immun **12:**1214-1218.

119. **McDonnell, J. L.** 1986. Toxins of *Clostridium perfringens* type A, B, C, D, and E, p. 477-517. *In* F. Dorner and J. Drews (ed.), Pharmacology of bacterial toxins. Pergamon Press, Oxford.

120. **Meador-Parton, J., and D. L. Popham.** 2000. Structural analysis of *Bacillus subtilis* spore peptidoglycan during sporulation. J. Bacteriol. **182:**4491-4499.

121. **Miyata, S., R. Moriyama, N. Miyahara, and S. Makino.** 1995. A gene (*sleC*) encoding a spore-cortex-lytic enzyme from *Clostridium perfringens* S40

spores; cloning, sequence analysis and molecular characterization. Microbiology **141:**2643-2650.

122. **Miyata, S., R. Moriyama, K. Sugimoto, and S. Makino.** 1995. Purification and partial characterization of a spore cortex-lytic enzyme of *Clostridium perfringens* S40 spores. Biosci. Biotechnol. Biochem. **59:**514-515.

123. **Miyata, S., S. Kozuka, Y. Yasuda, Y. Chen, R. Moriyama, K. Tochikubo, and S. Makino.** 1997. Localization of germination-specific spore-lytic enzymes in *Clostridium perfringens* S40 spores detected by immunoelectron microscopy. FEMS Microbiol. Lett. **152:**243-247.

124. **Moerman, F.** 2005. High hydrostatic pressure inactivation of vegetative microorganisms, aerobic and anaerobic spores in pork Marengo, a low acidic particulate food products. Meat Sci **69:**225-232.

125. **Moir, A., and D. A. Smith.** 1990. The genetics of bacterial spore germination. Annu. Rev. Microbiol. **44:**531-553.

126. **Moir, A., E. H. Kemp, C. Robinson, and B. M. Corfe.** 1994. The genetic analysis of bacterial spore germination. Soc. Appl. Bacteriol. Symp. Ser. **23:**9S-16S.

127. **Moir, A., B. M. Corfe, and J. Behravan.** 2002. Spore germination. Cell. Mol. Life Sci. **59:**403-409.

128. **Murrell, W. G., and A. D. Warth.** 1965. Composition and heat resistance of bacterial spores, p. 1-12. *In* L. L. Campbell and H. O. Halvorson (ed.), Spore III. American Society for Microbiology, Washington, D.C.

129. **Murrell, W. G.** 1967. The biochemistry of the bacterial endospore. Adv. Micriobiol. Physiol. **1:**133-251.

130. **Myers, G. S., D. A. Rasko, J. K. Cheung, J. Ravel, R. Seshadri, R. T. DeBoy, Q. Ren, J. Varga, M. M. Awad, L. M. Brinkac, S. C. Daugherty, D. H. Haft, R. J. Dodson, R. Madupu, W. C. Nelson, M. J. Rosovitz, S. A. Sullivan, H. Khouri, G. I. Dimitrov, K. L. Watkins, S. Mulligan, J. Benton, D. Radune, D. J. Fisher, H. S. Atkins, T. Hiscox, B. H. Jost, S. J. Billington, J. G. Songer, B. A. McClane, R. W. Titball, J. I. Rood, S. B. Melville, and I. T. Paulsen.** 2006. Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. Genome Res. **16:**1031-1040.

131. **Nagahama, M., S. Hayashi, S. Morimitsu, and J. Sakurai.** 2003. Biological activities and pore formation of *Clostridium perfringens* beta toxin in HL 60 cells. J Biol Chem **278:**36934-36941.

132. **Nicholson, W. L., and P. Setlow.** 1990. *Sporulation, germination and outgrowth*, p. 391-450. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley and Sons, Chichester.

133. **Nolling, J., G. Breton, M. V. Omelchenko, K. S. Makarova, Q. Zeng, R. Gibson, H. M. Lee, J. Dubois, D. Qiu, J. Hitti, Y. I. Wolf, R. L. Tatusov, F. Sabathe, L. Doucette-Stamm, P. Soucaille, M. J. Daly, G. N. Bennett, E. V. Koonin, and D. R. Smith.** 2001. Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. J. Bacteriol. **183:**4823-4838.

134. **Notredame, C., D. G. Higgins, and J. Heringa.** 2000. T-Coffee: A novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. **302:**205-217.

135. **Nozaki, K., K. Inaba, T. Kuroda, M. Tsuda, and T. Tsuchiya.** 1996. Cloning and sequencing of the gene for Na^+/H^+ antiporter of *Vibrio parahaemolyticus*. Biochem. Biophys. Res. Commun. **222:**774-779.

136. **Ohtani, K., S. K. Bhowmik, H. Hayashi, and T. Shimizu.** 2002. Identification of a novel locus that regulates expression of toxin genes in *Clostridium perfringens*. FEMS Microbiol Lett **209:**113-118.

137. **Okamura, S., K. Urakami, M. Kimata, T. Aoshima, S. Shimamoto, R. Moriyama, and S. Makino.** 2000. The N-terminal prepeptide is required for the production of spore cortex-lytic enzyme from its inactive precursor during germination of *Clostridium perfringens* S40 spores. Mol. Microbiol. **37:**821-827.

138. **Olsen, S. J., L. C. MacKinnon, J. S. Goulding, N. H. Bean, and L. Slutsker.** 2000. Surveillance for foodborne-disease outbreaks--United States, 1993-1997. MMWR. CDC Surveill. Summ. **49:**1-62.

139. **Orsburn, B., S. B. Melville, and D. L. Popham.** 2008. Factors contributing to heat resistance of *Clostridium perfringens* endospores. Appl Environ Microbiol **74:**3328-3335.

140. **Orsburn, B., K. Sucre, D. L. Popham, and S. B. Melville.** 2009. The SpmA/B and DacF proteins of *Clostridium perfringens* play important roles in spore heat resistance. FEMS Microbiol Lett **291:**188-194.

141. **P.R. Murray, K. S. R., G.S. Kobayashi, M.A. Pfaller.** 1998. Clostridium, Third edition ed. Mosby Inc, St. Louis, MO.

142. **Padan, E.** 2008. The enlightening encounter between structure and function in the NhaA Na^+-H^+ antiporter. Trends Biochem. Sci. **33:**435-443.

143. **Paidhungat, M., and P. Setlow.** 1999. Isolation and characterization of mutations in *Bacillus subtilis* that allow spore germination in the novel germinant D-alanine. J. Bacteriol. **181:**3341-3350.

144. **Paidhungat, M., B. Setlow, A. Driks, and P. Setlow.** 2000. Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. J. Bacteriol. **182:**5505-5512.

145. **Paidhungat, M., and P. Setlow.** 2000. Role of *ger* proteins in nutrient and nonnutrient triggering of spore germination in *Bacillus subtilis*. J. Bacteriol. **182:**2513-2519.

146. **Paidhungat, M., K. Ragkousi, and P. Setlow.** 2001. Genetic requirements for induction of germination of spores of *Bacillus subtilis* by Ca^{2+}-dipicolinate. J. Bacteriol. **183:**4886-4893.

147. **Paidhungat, M., and P. Setlow.** 2001. Localization of a germinant receptor protein (GerBA) to the inner membrane of *Bacillus subtilis* spores. J. Bacteriol. **183:**3982-3990.

148. **Paidhungat, M., B. Setlow, W. B. Daniels, D. Hoover, E. Papafragkou, and P. Setlow.** 2002. Mechanisms of induction of germination of *Bacillus subtilis* spores by high pressure. Appl. Environ. Microbiol. **68:**3172-3175.

149. **Parada, J., and J. M. Aguilera.** 2007. Food microstructure affects the bioavailability of several nutrients. J. Food. Sci. **72:**R21-32.

150. **Paredes-Sabja, D., M. Gonzalez, M. R. Sarker, and J. A. Torres.** 2007. Combined Effects of Hydrostatic Pressure, Temperature and pH on the Inactivation of Spores of *Clostridium perfringens* type A and *Clostridium sporogenes* in buffer solutions. J. Food Sci. **72:**M202-M206.

151. **Paredes-Sabja, D., D. Raju, J. A. Torres, and M. R. Sarker.** 2008. Role of small, acid-soluble spore proteins in the resistance of *Clostridium perfringens* spores to chemicals. Int. J. Food. Microbiol. **122:**333-335.

152. **Paredes-Sabja, D., N. Sarker, B. Setlow, P. Setlow, and M. R. Sarker.** 2008. Roles of DacB and Spm proteins in *Clostridium perfringens* spore resistance to moist heat, chemicals and UV radiation. Appl. Environ. Microbiol. **74:**3730-3738.

153. **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:**4648-4659.

154. **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:**1190-1201.

155. **Paredes-Sabja, D., and M. R. Sarker.** 2009. *Clostridium perfringens* sporulation and its relevance to pathogenesis. Fut Microbiol **In revision**.

156. **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:**2711-2720.

157. **Paredes-Sabja, D., P. Setlow, and M. R. Sarker.** 2009. Role of GerKB in germination and outgrowth of *Clostridium perfringens* spores. Appl. Environ. Microbiol. **doi:10.1128/AEM.00048-09**.

158. **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 **doi:10.1128/JB.00158-09**.

159. **Paredes-Sabja, D., P. Setlow, and M. R. Sarker.** 2009. CspB is essential for initiation of cortex hydrolysis during spore germination of *Clostridium perfringens* type A food poisoning isolates. J. Bacteriol. **Submitted for Publication**.

160. **Perelle, S., M. Gibert, P. Boquet, and M. R. Popoff.** 1993. Characterization of *Clostridium perfringens* iota-toxin genes and expression in *Escherichia coli*. Infect Immun **61:**5147-5156.

161. **Perez Lamela, C., and J. A. Torres.** 2008. Pressure processing of foods: Part 2. Microbial inactivation & chemical changes in pressure-assisted thermal processing (PATP). AgroFOOD Industry Hi-tech **19:**34-36.

162. **Perez Lamela, C., and J. A. Torres.** 2008. Pressure processing of foods: Part 1. An alternative for high flavor quality foods retaining health enhancing factors. AgroFOOD Industry Hi-tech **19:**60-62.

163. **Petit, L., M. Gibert, D. Gillet, C. Laurent-Winter, P. Boquet, and M. R. Popoff.** 1997. *Clostridium perfringens* epsilon-toxin acts on MDCK cells by forming a large membrane complex. J Bacteriol **179:**6480-6487.

164. **Petit, L., M. Gibert, and M. R. Popoff.** 1999. *Clostridium perfringens*: toxinotype and genotype. Trends Microbiol **7:**104-110.

165. **Petit, L., E. Maier, M. Gibert, M. R. Popoff, and R. Benz.** 2001. *Clostridium perfringens* epsilon toxin induces a rapid change of cell membrane permeability to ions and forms channels in artificial lipid bilayers. J Biol Chem **276:**15736-15740.

166. **Petit, L., M. Gibert, A. Gourch, M. Bens, A. Vandewalle, and M. R. Popoff.** 2003. *Clostridium perfringens* epsilon toxin rapidly decreases membrane barrier permeability of polarized MDCK cells. Cell Microbiol **5:**155-164.

167. **Plowman, J., and M. W. Peck.** 2002. Use of a novel method to characterize the response of spores of non-proteolytic *Clostridium botulinum* types B, E and F to a wide range of germinants and conditions. J. Appl. Microbiol. **92:**681-694.

168. **Popham, D. L., B. Illades-Aguiar, and P. Setlow.** 1995. The *Bacillus subtilis dacB* gene, encoding penicillin-binding protein 5*, is part of a three-gene operon required for proper spore cortex synthesis and spore core dehydration. J. Bacteriol. **177:**4721-4729.

169. **Popham, D. L., S. Sengupta, and P. Setlow.** 1995. Heat, hydrogen peroxide, and UV resistance of *Bacillus subtilis* spores with increased core water content

and with or without major DNA-binding proteins. Appl. Environ. Microbiol. **61:**3633-3638.

170. **Popham, D. L., J. Helin, C. E. Costello, and P. Setlow.** 1996. Analysis of the peptidoglycan structure of *Bacillus subtilis* endospores. J. Bacteriol. **178:**6451-6458.

171. **Popham, D. L., J. Helin, C. E. Costello, and P. Setlow.** 1996. Muramic lactam in peptidoglycan of *Bacillus subtilis* spores is required for spore outgrowth but not for spore dehydration or heat resistance. Proc. Natl. Acad. Sci. USA **93:**15405-15410.

172. **Popham, D. L.** 2002. Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. Cell Mol Life Sci **59:**426-433.

173. **Raju, D., and M. R. Sarker.** 2005. Comparison of the levels of heat resistance of wild-type, *cpe* knockout, and *cpe* plasmid-cured *Clostridium perfringens* type A strains. Appl. Environ. Microbiol. **71:**7618-7620.

174. **Raju, D., M. Waters, P. Setlow, and M. R. Sarker.** 2006. Investigating the role of small, acid-soluble spore proteins (SASPs) in the resistance of *Clostridium perfringens* spores to heat. BMC Microbiol. **6:**50.

175. **Raju, D., and M. R. Sarker.** 2007. Production of small, acid-soluble spore proteins in *Clostridium perfringens* nonfoodborne gastrointestinal disease isolates. Can. J. Microbiol. **53:**514-518.

176. **Raju, D., P. Setlow, and M. R. Sarker.** 2007. Antisense-RNA-mediated decreased synthesis of small, acid-soluble spore proteins leads to decreased

resistance of *Clostridium perfringens* spores to moist heat and UV radiation. Appl. Environ. Microbiol. **73:**2048-2053.

177. **Raso, J., M. M. Gongora-Nieto, G. V. Barbosa-Canovas, and B. G. Swanson.** 1998. Influence of several environmental factors on the initiation of germination and inactivation of *Bacillus cereus* by high hydrostatic pressure. Int. J. Food Microbiol. **44:**125-132.

178. **Riddell, C., and X. M. Kong.** 1992. The influence of diet on necrotic enteritis in broiler chickens. Avian Dis **36:**499-503.

179. **Riemann, H., and Z. J. Ordal.** 1961. Germination of bacterial endospores with calcium and dipicolinic acid. Science **133:**1703-1704.

180. **Riesenman, P. J., and W. L. Nicholson.** 2000. Role of the spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar UV radiation. Appl. Environ. Microbiol. **66:**620-626.

181. **Rode, L. J., and J. W. Foster.** 1962. Ionic germination of spores of *Bacillus megaterium* QM B 1551. Arch. Mikrobiol. **43:**183-200.

182. **Rood, J. I., and S. T. Cole.** 1991. Molecular genetics and pathogenesis of *Clostridium perfringens*. Microbiol. Rev. **55:**621-648.

183. **Rood, J. I.** 1998. Virulence genes of *Clostridium perfringens*. Annu Rev Microbiol **52:**333-360.

184. **Rooney, A. P., J. L. Swezey, R. Friedman, D. W. Hecht, and C. W. Maddox.** 2006. Analysis of core housekeeping and virulence genes reveals cryptic lineages of *Clostridium perfringens* that are associated with distinct disease presentations. Genetics **172:**2081-2092.

185. **Rosskopf-Streicher, U., P. Volkers, K. Noeske, and E. Werner.** 2004. Quality assurance of *Clostridium perfringens* epsilon toxoid vaccines--ELISA versus mouse neutralisation test. Altex **21:**65-69.

186. **Rotman, Y., and M. L. Fields.** 1967. A modified reagent for dipicolinic acid analysis. Anal. Biochem. **22:**168.

187. **Saier, M. H., Jr., B. H. Eng, S. Fard, J. Garg, D. A. Haggerty, W. J. Hutchinson, D. L. Jack, E. C. Lai, H. J. Liu, D. P. Nusinew, A. M. Omar, S. S. Pao, I. T. Paulsen, J. A. Quan, M. Sliwinski, T. T. Tseng, S. Wachi, and G. B. Young.** 1999. Phylogenetic characterization of novel transport protein families revealed by genome analyses. Biochim. Biophys. Acta. **1422:**1-56.

188. **Sakurai, J., M. Nagahama, and M. Oda.** 2004. *Clostridium perfringens* alpha-toxin: characterization and mode of action. J Biochem **136:**569-574.

189. **Sali, A., and T. L. Blundell.** 1993. Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol. **234:**779-815.

190. **Sanchez-Salas, J. L., M. L. Santiago-Lara, B. Setlow, M. D. Sussman, and P. Setlow.** 1992. Properties of *Bacillus megaterium* and *Bacillus subtilis* mutants which lack the protease that degrades small, acid-soluble proteins during spore germination. J. Bacteriol. **174:**807-814.

191. **Sanchez-Salas, J. L., and P. Setlow.** 1993. Proteolytic processing of the protease which initiates degradation of small, acid-soluble proteins during germination of *Bacillus subtilis* spores. J. Bacteriol. **175:**2568-2577.

192. **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:**946-958.

193. **Sarker, M. R., R. P. Shivers, S. G. Sparks, V. K. Juneja, and B. A. McClane.** 2000. Comparative experiments to examine the effects of heating on vegetative cells and spores of *Clostridium perfringens* isolates carrying plasmid genes versus chromosomal enterotoxin genes. Appl. Environ. Microbiol. **66:**3234-3240.

194. **Sayeed, S., F. A. Uzal, D. J. Fisher, J. Saputo, J. E. Vidal, Y. Chen, P. Gupta, J. I. Rood, and B. A. McClane.** 2008. Beta toxin is essential for the intestinal virulence of *Clostridium perfringens* type C disease isolate CN3685 in a rabbit ileal loop model. Mol Microbiol **67:**15-30.

195. **Schaeffer, P., J. Millet, and J. P. Aubert.** 1965. Catabolic repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA **54:**704-711.

196. **Screpanti, E., and C. Hunte.** 2007. Discontinuous membrane helices in transport proteins and their correlation with function. J. Struct. Biol. **159:**261-267.

197. **Sebaihia, M., B. W. Wren, P. Mullany, N. F. Fairweather, N. Minton, R. Stabler, N. R. Thomson, A. P. Roberts, A. M. Cerdeno-Tarraga, H. Wang, M. T. Holden, A. Wright, C. Churcher, M. A. Quail, S. Baker, N. Bason, K. Brooks, T. Chillingworth, A. Cronin, P. Davis, L. Dowd, A. Fraser, T. Feltwell, Z. Hance, S. Holroyd, K. Jagels, S. Moule, K. Mungall, C. Price,**

E. Rabbinowitsch, S. Sharp, M. Simmonds, K. Stevens, L. Unwin, S. Whithead, B. Dupuy, G. Dougan, B. Barrell, and J. Parkhill. 2006. The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. Nat. Genet. **38:**779-786.

198. **Senior, A., and A. Moir.** 2008. The *Bacillus cereus* GerN and GerT protein homologs have distinct roles, in spore germination and outgrowth respectively. J. Bacteriol. **190:**6148-6152.

199. **Setlow, B., and P. Setlow.** 1993. Binding of small, acid-soluble spore proteins to DNA plays a significant role in the resistance of *Bacillus subtilis* spores to hydrogen peroxide. Appl. Environ. Microbiol. **59:**3418-3423.

200. **Setlow, B., E. Melly, and P. Setlow.** 2001. Properties of spores of *Bacillus subtilis* blocked at an intermediate stage in spore germination. J. Bacteriol. **183:**4894-4899.

201. **Setlow, B., A. E. Cowan, and P. Setlow.** 2003. Germination of spores of *Bacillus subtilis* with dodecylamine. J. Appl. Microbiol. **95:**637-648.

202. **Setlow, B., S. Atluri, R. Kitchel, K. Koziol-Dube, and P. Setlow.** 2006. Role of dipicolinic acid in resistance and stability of spores of *Bacillus subtilis* with or without DNA-protective alpha/beta-type small acid-soluble proteins. J. Bacteriol. **188:**3740-3747.

203. **Setlow, B., L. Peng, C. A. Loshon, Y.-Q. Li, G. Christie, and P. Setlow.** 2009. Characterization of the germination of *Bacillus megaterium* spores lacking enzymes that degrade the spore cortex. J. Appl. Microbiol. **doi: 10.1111/j.1365-2672.2009.04210**.

204. **Setlow, P.** 1983. Germination and outgrowth, p. 211-254. *In* A. Hurst and G. W. Gould (ed.), The bacterial spore, vol. II. Academic Press, London, United Kingdom.

205. **Setlow, P.** 1988. Small, acid-soluble spore proteins of *Bacillus* species: structure, synthesis, genetics, function, and degradation. Annu. Rev. Microbiol. **42:**319-338.

206. **Setlow, P.** 1994. Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. Soc. Appl. Bacteriol. Symp. Ser. **23:**49S-60S.

207. **Setlow, P.** 2003. Spore germination. Curr. Opin. Microbiol. **6:**550-556.

208. **Setlow, P.** 2006. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. J. Appl. Microbiol. **101:**514-525.

209. **Setlow, P.** 2007. I will survive: DNA protection in bacterial spores. Trends. Microbiol. **15:**172-180.

210. **Setlow, P., and E. A. Johnson.** 2007. *Spores and their significance*, p. 35-68. *In* M. P. Doyle and L. R. Beuchat (ed.), Food Microbiology: fundamentals and frontiers, 3rd ed. American Society for Microbiology, Washington, D.C.

211. **Shah, I. M., M. H. Laaberki, D. L. Popham, and J. Dworkin.** 2008. A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. Cell **135:**486-496.

212. **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:**3742-3751.

213. **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. USA **99:**996-1001.

214. **Shimizu, T., H. Yaguchi, K. Ohtani, S. Banu, and H. Hayashi.** 2002. Clostridial VirR/VirS regulon involves a regulatory RNA molecule for expression of toxins. Mol Microbiol **43:**257-265.

215. **Shinde, U., and M. Inouye.** 1996. Propeptide-mediated folding in subtilisin: the intramolecular chaperone concept. Adv. Exp. Med. Biol. **379:**147-154.

216. **Siezen, R. J., and J. A. Leunissen.** 1997. Subtilases: the superfamily of subtilisin-like serine proteases. Protein Sci. **6:**501-523.

217. **Simpson, L. L., B. G. Stiles, H. Zepeda, and T. D. Wilkins.** 1989. Production by *Clostridium spiroforme* of an iotalike toxin that possesses mono(ADP-ribosyl)transferase activity: identification of a novel class of ADP-ribosyltransferases. Infect Immun **57:**255-261.

218. **Singh, U., C. M. Van Itallie, L. L. Mitic, J. M. Anderson, and B. A. McClane.** 2000. CaCo-2 cells treated with *Clostridium perfringens* enterotoxin form multiple large complex species, one of which contains the tight junction protein occludin. J Biol Chem **275:**18407-18417.

219. **Sloan, J., T. A. Warner, P. T. Scott, T. L. Bannam, D. I. Berryman, and J. I. Rood.** 1992. Construction of a sequenced *Clostridium perfringens*-*Escherichia coli* shuttle plasmid. Plasmid **27:**207-219.

220. **Smelt, J. P., J. C. Hellemons, P. C. Wouters, and S. J. van Gerwen.** 2002. Physiological and mathematical aspects in setting criteria for decontamination of foods by physical means. Int J Food Microbiol **78:**57-77.

221. **Soding, J.** 2005. Protein homology detection by HMM-HMM comparison. Bioinformatics **21:**951-960.

222. **Songer, J. G.** 1996. *Clostridial* enteric diseases of domestic animals. Clin Microbiol Rev **9:**216-234.

223. **Songer, J. G., and F. A. Uzal.** 2005. Clostridial enteric infections in pigs. J Vet Diagn Invest **17:**528-536.

224. **Southworth, T. W., A. A. Guffanti, A. Moir, and T. A. Krulwich.** 2001. GerN, an endospore germination protein of *Bacillus cereus*, is an Na^+/H^+-K^+ antiporter. J. Bacteriol. **183:**5896-5903.

225. **Sparks, S. G., R. J. Carman, M. R. Sarker, and B. A. McClane.** 2001. Genotyping of enterotoxigenic *Clostridium perfringens* fecal isolates associated with antibiotic-associated diarrhea and food poisoning in North America. J. Clin. Microbiol. **39:**883-888.

226. **Stewart, C. M., C. P. Dunne, A. Sikes, and D. G. Hoover.** 2000. Sensitivity of spores of *Bacillus subtilis* and *Clostridium sporogenes* PA 3679 to combinations of high hydrostatic pressure and other processing parameters. Innov Sci Emerg Technol **1:**49-56.

227. **Stragier, P., and R. Losick.** 1996. Molecular genetics of sporulation in *Bacillus subtilis*. Annu Rev Genet **30:**297-241.

228. **Sussman, M. D., and P. Setlow.** 1991. Cloning, nucleotide sequence, and regulation of the *Bacillus subtilis gpr* gene, which codes for the protease that initiates degradation of small, acid-soluble proteins during spore germination. J. Bacteriol. **173:**291-300.

229. **Swerdlow, B. M., B. Setlow, and P. Setlow.** 1981. Levels of H^+ and other monovalent cations in dormant and germinating spores of *Bacillus megaterium*. J. Bacteriol. **148:**20-29.

230. **Takeshi, K., Ando, Y., Oguma, K.** 1988. Germination of spores of *Clostridium botulinum* type G. J. Food Prot. **51:**37-38.

231. **Tani, K., T. Watanabe, H. Matsuda, M. Nasu, and M. Kondo.** 1996. Cloning and sequencing of the spore germination gene of *Bacillus megaterium* ATCC 12872: similarities to the NaH-antiporter gene of *Enterococcus hirae*. Microbiol. Immunol. **40:**99-105.

232. **Tennen, R., B. Setlow, K. L. Davis, C. A. Loshon, and P. Setlow.** 2000. Mechanisms of killing of spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. J. Appl. Microbiol. **89:**330-338.

233. **Thackray, P. D., J. Behravan, T. W. Southworth, and A. Moir.** 2001. GerN, an antiporter homologue important in germination of *Bacillus cereus* endospores. J. Bacteriol. **183:**476-482.

234. **Todd, E. C. D., Guzewich, J.J., and Bryan, F.L.** 1997. Surveillance of foodborne disease IV. Dissemination and uses of surveillance data. J. Food Prot. **60:**714-723.

235. **Torres, J. A., and G. Velazquez.** 2008. *Hydrostatic pressure processing of foods*. *In* J. Irudayaraj and S. Jin (ed.), Food Processing Operations and Modeling. CRC Press Inc.

236. **Tovar-Rojo, F., M. Chander, B. Setlow, and P. Setlow.** 2002. The products of the *spoVA* operon are involved in dipicolinic acid uptake into developing spores of *Bacillus subtilis*. J. Bacteriol. **184:**584-587.

237. **Tovar-Rojo, F., R. M. Cabrera-Martinez, B. Setlow, and P. Setlow.** 2003. Studies on the mechanism of the osmoresistance of spores of *Bacillus subtilis*. J. Appl. Microbiol. **95:**167-179.

238. **Tweten, R. K.** 1988. Cloning and expression in *Escherichia coli* of the perfringolysin O (theta-toxin) gene from *Clostridium perfringens* and characterization of the gene product. Infect Immun **56:**3228-3234.

239. **Urakami, K., S. Miyata, R. Moriyama, K. Sugimoto, and S. Makino.** 1999. Germination-specific cortex-lytic enzymes from *Clostridium perfringens* S40 spores: time of synthesis, precursor structure and regulation of enzymatic activity. FEMS Microbiol. Lett. **173:**467-473.

240. **Van der Sluis, W.** 2000. Clostridial enteritis is an often underestimated problem. World Poult **16:**42-43.

241. **Van Immerseel, F., J. I. Rood, R. J. Moore, and R. W. Titball.** 2009. Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens. Trends Microbiol **17:**32-36.

242. **Vepachedu, V. R., and P. Setlow.** 2004. Analysis of the germination of spores of *Bacillus subtilis* with temperature sensitive spo mutations in the *spoVA* operon. FEMS Microbiol. Lett. **239:**71-77.

243. **Vepachedu, V. R., and P. Setlow.** 2005. Localization of SpoVAD to the inner membrane of spores of *Bacillus subtilis*. J. Bacteriol. **187:**5677-5682.

244. **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:**1565-1572.

245. **Vidal, J. E., B. A. McClane, J. Saputo, J. Parker, and F. A. Uzal.** 2008. Effects of *Clostridium perfringens* beta-toxin on the rabbit small intestine and colon. Infect Immun **76:**4396-4404.

246. **Waites, W. M., and L. R. Wyatt.** 1974. The effect of pH, germinants and temperature on the germination of spores of *Clostridium bifermentans*. J. Gen. Microbiol. **80:**253-258.

247. **Warth, A. D., and J. L. Strominger.** 1969. Structure of the peptidoglycan of bacterial spores: occurrence of the lactam of muramic acid. Proc. Natl. Acad. Sci. USA. **64:**528-535.

248. **Warth, A. D., and J. L. Strominger.** 1972. Structure of the peptidoglycan from spores of *Bacillus subtilis*. Biochemistry **11:**1389-1396.

249. **Waters, M., A. Savoie, H. S. Garmory, D. Bueschel, M. R. Popoff, J. G. Songer, R. W. Titball, B. A. McClane, and M. R. Sarker.** 2003. Genotyping and phenotyping of beta2-toxigenic *Clostridium perfringens* fecal isolates associated with gastrointestinal diseases in piglets. J. Clin. Microbiol. **41:**3584-3591.

250. **Wax, R., E. Freese, and M. Cashel.** 1967. Separation of two functional roles of L-alanine in the initiation of *Bacillus subtilis* spore germination. J. Bacteriol. **94:**522-529.

251. **Wieckowski, E. U., A. P. Wnek, and B. A. McClane.** 1994. Evidence that an approximately 50-kDa mammalian plasma membrane protein with receptor-like properties mediates the amphiphilicity of specifically bound *Clostridium perfringens* enterotoxin. J Biol Chem **269:**10838-10848.

252. **Wijewanta, E. A., and P. Senevirtna.** 1971. Bacteriological studies of fatal Clostridium perfringens type-A infection in chickens. Avian Dis **15:**654-661.

253. **Willardsen, R. R., F.F. Busta, and C.E. Allen.** 1979. Growth of *Clostridium perfringens* in three different beef media and fluid thioglycollate medium at static and constantly rising temperatures. J. Food Prot. **42:**144-148.

254. **Yingst, D. R., and J. F. Hoffman.** 1983. Intracellular free Ca and Mg of human red blood cell ghosts measured with entrapped arsenazo III. Anal. Biochem. **132:**431-448.

255. **Zhang, Y., and J. Skolnick.** 2004. Scoring function for automated assessment of protein structure template quality. Proteins **57:**702-710.

256. **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:**136-142.

257. **Zuberi, A. R., A. Moir, and I. M. Feavers.** 1987. The nucleotide sequence and gene organization of the *gerA* spore germination operon of *Bacillus subtilis* 168. Gene **51:**1-11.

Appendices

Appendix A: Scientific work published or in preparation during Mr. Daniel Paredes-Sabja's Ph.D. degree.

1. Udompijitkul, P., **Paredes-Sabja, D.**, and M.R., Sarker. GerH, a histidine kinase, regulates expression of GerQ during sporulation of *Clostridium perfringens* **Manuscript in preparation.**
2. **Paredes-Sabja, D.**, Setlow, P., and M.R. Sarker, 2009. The protease CspB is essential for initiation of cortex hydrolysis and DPA release during germination of spores of *Clostridium perfringens* type A food poisonng isolates. **Microbiology. In Revision.**
3. **Paredes-Sabja, D.***, Udompijitkul, P.*, and M.R. Sarker. 2009. Inorganic phosphate induces germination of spores of *Clostridium perfringens* type A food poisoning isolates. **Applied and Environmental Microbiology. In Revision.**
4. **Paredes-Sabja, D.***, Alnoman, M.*, and Sarker, M. 2009. Comparative studies of germination of spores of *Clostridium perfringens* type A food-poisoning versus non-food borne isolates. **Archives of Microbiology. In Revision**.
5. Li, J., **Paredes-Sabja, D.**, M.R. Sarker and McClane, B.A. Further characterization of *Clostridium perfringens* small acid soluble protein-4 (Ssp4) proterties and expresion. **PloS ONE. In Press.**
6. **Paredes-Sabja, D.,** & Torres, J.A. 2009. Modeling of the Germination of Spores from *Clostridium perfringens* Food Poisoning Isolates. **Journal of Food Process Engineering**. **In Press.**
7. **Paredes-Sabja, D.,** and M.R. Sarker. 2009. *Clostridium perfringens* sporulation, and its relevance to pathogenesis. **Future Microbiology.** 4 (5): 519-525**.**
8. **Paredes-Sabja. D.**, Setlow, P. and M.R. Sarker. 2009. GerO, a putative Na^+/K^+-H^+ antiporter, is essential for normal germination of *Clostridium perfringens* spores. **Journal of Bacteriology.** 191 (12): 38222-3831.
9. **Paredes-Sabja, D.**, Setlow, P., and M.R. Sarker. 2009. Role of GerKB in germination and outgrowth of *Clostridium perfringens* spores. **Applied and Environmental Microbiology.** 75 (11): 3813-3817.
10. **Paredes-Sabja, D.**, Setlow, P., and M.R. Sarker. 2009. SleC is essential for cortex peptidoglycan hydrolysis during germination of spores of the pathogenic bacterium *Clostridium perfringens*. **Journal of Bacteriology**. 191 (8): 2711-2720.
11. Akhtar, S.*, **Paredes-Sabja, D.***, Torres, J.A., and M.R. Sarker. 2009. Strategy to inactivate *Clostridium perfringens* spores in meats. **Food Microbiology**. 26 (3): 272-277.
12. **Paredes-Sabja, D.**, Setlow, B., Setlow, P., and M.R. Sarker. 2008. Characterization of *Clostridium perfringens* spores that lack SpoVA proteins and dipicolinic acid. **Journal of Bacteriology**. 190 (13): 4648-4659.
13. **Paredes-Sabja, D.***, Bond, C.*, Setlow, P., Carman, R.B., and M.R. Sarker. 2008. Germination of Spores of *Clostridium difficile* Isolates from a Hospital Outbreak of Clostridium difficile-associated disease (CDAD). **Microbiology**. 154 (8): 2241-2250.
14. **Paredes-Sabja, D.,** Sarker, N., Setlow, B., Setlow, P., & M.R. Sarker. 2008. Roles of DacB and Spm proteins in *Clostridium perfringens* spore resistance to moist heat,

chemicals and UV radiation. **Applied and Environmental Microbiology**. 74 (12): 3730-3738.

15. Akhtar, S.*, **Paredes-Sabja, D.***, M.R. Sarker. 2008. Inhibitory effects of polyphosphates on *Clostridium perfringens* growth, sporulation and spore outgrowth. **Food Microbiology**. 25 (6): 802-808.
16. **Paredes-Sabja, D.**, Raju, D., Torres, J.A., & M.R., Sarker. 2008. Role of Small, Acid-soluble Spore Proteins in the Resistance of *Clostridium perfringens* Spores to Chemicals. **International Journal of Food Microbiology**. 122: 333-335.
17. **Paredes-Sabja, D.,** Torres, J.A., Setlow, P., & M.R., Sarker. 2008 *Clostridium perfringens* spore germination: characterization of germinants and their receptors. **Journal of Bacteriology**. 190 (4): 1190-1201.
18. **Paredes-Sabja, D.,** Gonzalez, M., Sarker, M R., J. A. Torres. 2007. Combined effects of Hydrostatic Pressure, Temperature and pH on the Inactivation of Spores of *Clostridium perfringens* type A and *Clostridium sporogenes* in buffer solutions. **Journal of Food Science**. Vol 72 (6), M202-206**.**
19. Huang, I.H., Raju, D., **Paredes-Sabja, D.**, and Sarker, M.R. 2007. *Clostridium perfringens*: Sporulation, Spore Resistance and Germination. **Bangladesh Journal of Microbiology**. Vol 24 (1), 1-8.
20. Tirado, J., **Paredes, D**., Velazquez, G., Torres, J.A. 2005. Microbial growth in refrigerated meat products. **Ciencia y Tecnologia Alimentaria** (Spain) 5: 66-76.
21. Tirado, J., **Paredes, D.**, Velazquez, G., Torres, J.A. 2006. Control de la cadena de frío para productos cárnicos refrigerados (Cold chain control for refrigerated products). **Industria Alimentaria** 28(2): 22-26
22. Tirado, J., **Paredes, D.**, Velazquez, G., Torres, J.A. 2005. Optimice la cadena de frío para reducir riesgos microbiológicos y extender la vida útil de productos cárnicos refrigerados (Improve your cold chain to reduce microbial risks and extend shelf life of refrigerated meat products). **CARTANETIF**, Publicación Oficial de la Asociación Nacional de Establecimientos Tipo Inspección Federal, A.C. 4, No. 17 (Septiembre-Octubre).

***** These authors contributed equally in this work

Book chapters:

23. Chapter 12 Crecimiento microbiano durante la comercialización de alimentos refrigerados**.** *In*, *Microbial growth during the commercial distribution of refrigerated foods* 2008. **Daniel G. Paredes-Sabja**, Elton Morales-Blancas, Kong Shun Ah-Hen, Gonzalo Velazquez, y J. Antonio Torres