

AN ABSTRACT OF THE DISSERTATION OF

Pathima Udompijitkul for the degree of Doctor of Philosophy in Food Science and Technology presented on April 15, 2013

Title: *Clostridium perfringens* Spores: Inactivation, Germination, and Formation

Abstract approved:

Mahfuzur R. Sarker

The enterotoxin-producing *Clostridium perfringens* type A isolates are responsible for the third most common foodborne illness in the United States and can also cause non-foodborne human gastrointestinal (GI) diseases such as antibiotic-associated and sporadic diarrheas. Three important factors contribute to the ability of *C. perfringens* to cause GI diseases, including its extremely rapid growth rate, its ubiquitous distribution in foods and environments, and its capability to form highly resistant endospores.

In the first study, the antimicrobial peptide nisin was evaluated for its antimicrobial effect against enterotoxigenic *C. perfringens* food poisoning (FP) and non-foodborne (NFB) GI disease isolates. Nisin did not affect spore germination, whereas germinated spores were very susceptible to low concentration of nisin and thus spores outgrowth were arrested. Nisin also exerted its inhibitory effect against vegetative growth of *C. perfringens* FP and NFB isolates in rich medium; however, FP

cells were less resistant to nisin than NFB cells. Nevertheless, nisin was not effective in controlling germination and outgrowth of *C. perfringens* spores in cooked meat products during storage at abusive temperature, even at ~ 4 times elevated concentration than the regulatory approved level. Strikingly, spores of NFB isolates also exhibited higher resistance to nisin than that of FP isolates in both laboratory medium as well as in meat systems. Collectively, despite its effectiveness in controlling spore outgrowth and vegetative cell growth in laboratory conditions, nisin showed no antimicrobial activity against *C. perfringens* spores inoculated into meat model systems.

The main focus of the second study was to develop an effective spore inactivation strategy on food contact surfaces by inducing spore germination prior to inactivation of the more susceptible spores with commonly used surface disinfecting agents. The mixture of L-asparagine and KCl (AK) was the most effective germinant for spores of enterotoxigenic *C. perfringens* type A. Germination temperature had a significant influence on the germination extent and subsequent inactivation by variety of surface disinfectants. Implementation of germination step significantly increased the inhibitory effect of all tested disinfecting agents against spores of *C. perfringens* FP strain SM101 with lower efficacy against the spores of NFB strain NB16. Furthermore, spores of *C. perfringens* FP isolates could germinate with AK upon their adhesion onto stainless steel chips and were subsequently inactivated with disinfectant agents by i.e. 1.53 to 2.70 log reductions of colony forming units per chip. Overall,

AK-induced germination followed by treatment with iodophore represents a promising strategy to inactivate spores of *C. perfringens* FP isolates on food contact surfaces.

Spore germination is initiated upon sensing a variety of compounds, termed germinants, via the cognate germinant receptors. In the third study, we identified sodium ions and inorganic phosphate (NaPi) at pH ~ 6.0 as a novel germinant for spores of enterotoxin-producing *C. perfringens* FP isolates. The spores lacking germination proteins GerAA and GerKA-KC were severely impaired in their ability to germinate with NaPi, whereas GerKB-negative spores germinated to a similar extent as wild type spores with NaPi, but their initial rate of germination was lesser. Spores lacking GerO or GerO GerQ germinated to a lower extent and with a significantly slower rate than wild type spores. In contrast, *gerQ* spores exhibited only a slightly slower and lesser extent of germination with NaPi than its parent strains. Therefore, the germinant receptor proteins GerKA-KC, GerAA, and the putative antiporter GerO are essential for normal germination of *C. perfringens* spores with NaPi.

In the fourth study, we demonstrated that polar, uncharged amino acids at pH 6.0 could efficiently trigger germination of spores of enterotoxigenic *C. perfringens*. While L-glutamine is a unique nutrient germinant for spores of *C. perfringens* FP isolates, L-asparagine, L-cysteine, L-serine, and L-threonine can induce germination of both FP and NFB spores. The germinant receptor GerKC is the major receptor involved in cysteine- and glutamine-induced germination and release of dipicolinic acid (DPA) from the spore's core, whereas less pronounced germination defects were observed in *gerAA* and *gerKB* spores. GerKC also has a key role in L-asparagine

germination. For serine and threonine (pH 6.0)-induced germination, GerKA is the dominant receptor and GerKC and GerKB are also required for efficient germination of FP spores.

The objectives of the fifth study were to identify and characterize the putative sensor histidine kinases of *C. perfringens*. We identified six genes encoding putative sporulation-associated sensor histidine kinases in the genome of *C. perfringens* SM101. These putative kinase genes were highly expressed under sporulation-stimulating conditions. Two genes encoding putative orphan sensor histidine kinases, *cpr1728* and *cpr1055*, were inactivated and roles of each putative kinase on various aspects in the life cycle of *C. perfringens* had been characterized. Inactivation of *cpr1728* and *cpr1055* significantly lowered *C. perfringens* sporulation capacity in two sporulation-inducing conditions. Moreover, sporulation delayed phenotype was also observed in strain lacking CPR1055. Inactivation of either *cpr1728* or *cpr1055* led to a marked defect in *C. perfringens* spore germination with all known germinants. Spores of two kinase mutants also exhibited slower outgrowth than their parental strain; however, no difference in colony forming efficiency was observed among tested strains. Additionally, mutations in *cpr1728* and *cpr1055* did not affect vegetative growth; however, both mutants grew at higher rate under sporulation-inducing conditions.

In conclusion, this dissertation reports the experimental results that are relevant to various aspects of *C. perfringens* spores. These include the development of spore inactivation strategies in food products as well as on food contact surfaces, the

identification of compounds triggering germination of spores of CPE-producing *C. perfringens*, and the insights into the roles of putative sensor histidine kinases in the process of spore formation and spore germination under a variety of conditions.

©Copyright by Pathima Udompijikul
April 15, 2013
All Rights Reserved

Clostridium perfringens Spores: Inactivation, Germination, and Formation

by

Pathima Udompijtkul

A DISSERTATION

Submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented April 15, 2013
Commencement June 2013

Doctor of Philosophy dissertation of Pathima Udompijitkul on April 15, 2013

APPROVED:

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 part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Pathima Udompijitkul, Author

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my major professor, Dr. Mahfuzur R. Sarker, who first accepted me to work in his laboratory and introduced me to the world of spore-forming bacteria, our beloved *Clostridium perfringens*. His wide knowledge and logical thinking have always been of great value to me. Thanks to his guidance, I have had a great opportunity to have hands-on experiences on many areas of my academic interests that provided me confidence and knowledge for my future path as a faculty member at Kasetsart University. Besides my mentor, many appreciations are for my thesis committee, Dr. Luiz Bermudez, Dr. Dan Rockey, Dr. Alan Bakalinsky, and Dr. Manoj Pастey.

Much gratefulness to the Office of Higher Education Commission (Thailand) for giving me an opportunity to fulfill my interest in food safety and funding me throughout my Ph.D. study in the United States. I would like to thank Dr. Michael Freitag, Dr. Lanelle Connolly and Dr. Angel Dago for a tremendous support and many valuable suggestions for the kinase study. I am also very grateful to Veltek Associates, Inc. for providing chemical disinfectant samples used in the surface decontamination project.

Many thanks to Dr. Sarker's lab members, all graduate students and department staffs in the Department of Food Science and Technology and Biomedical Sciences for giving me encouragement and friendship throughout my study at OSU. Last but not least, I would like to give my special gratitude to my uncle, aunts, brother,

and especially my beloved parents for their unconditional love, caring, encouragements, sympathy, understanding, and support.

CONTRIBUTION OF AUTHORS

Chapter 3. Dr. Daniel Paredes-Sabja was involved with manuscript preparation.

Chapter 4. Maryam Alnoman assisted in *Clostridium perfringens* spore preparation and Dr. Daniel Paredes-Sabja was involved with experimental design.

Chapter 5. Dr. Daniel Paredes-Sabja assisted with experimental design, data collection for germination receptor mutants' germination, and manuscript preparation.

Chapter 6. Maryam Alnoman assisted with data collection and spore preparation. Saeed Banawas provided *gerKC* and *gerKA* mutant strains as well as was involved in preparation of spores all germination receptor mutants. Dr. Daniel Paredes-Sabja constructed germination receptor mutants used in this study.

Chapter 7. Dr. Daniel Paredes-Sabja provided technical support for the construction of kinase mutants and β -glucuronidase plasmids.

TABLE OF CONTENTS

	<u>Page</u>
1. CHAPTER1 Introduction	1
2. CHAPTER 2 Literature Review	
2.1. Characteristics of the bacterium	8
2.2. <i>Clostridium perfringens</i> enterotoxin (CPE)	10
2.3. CPE-associated GI diseases	13
2.3.1. <i>C. perfringens</i> type A FP	14
2.3.2. Antibiotic-associated diarrhea and sporadic diarrhea	16
2.4. Stages of spore formation	17
2.5. Initiation of sporulation	18
2.6. Establishment of cell-specific transcription: a cascade of sigma factors in <i>B. subtilis</i>	20
2.6.1. SigF (σ^F)	21
2.6.2. SigE (σ^E)	21
2.6.3. SigG (σ^G)	22
2.6.4. SigK (σ^K)	23
2.7. Bacterial spore germination	25
2.8. Germinants and germination receptors of <i>C. perfringens</i>	27

TABLE OF CONTENTS (Continued)

	<u>Page</u>
2.9. Spore inactivation	29
2.10. Nisin	29
2.11. Chemical disinfecting agents	32
2.11.1. Alcohol	32
2.11.2. Quaternary ammonium compounds (Quats)	34
2.11.3. Iodophores	35
2.11.4. Hydrogen peroxide (HP)	36
2.11.5. Peroxyacetic acid or peracetic acid (PAA)	38
3. CHAPTER 3 Inhibitory Effects of Nisin Against <i>Clostridium perfringens</i> Food Poisoning and Non-food-borne Isolates	51
3.1. Introduction	53
3.2. Materials and methods	55
3.3. Results and discussions	60
3.4. Conclusions	65
4. CHAPTER 4 Inactivation Strategy for <i>Clostridium perfringens</i> Spores Adhered to Food Contact Surfaces	74
4.1. Introduction	77
4.2. Materials and methods	79
4.3. Results and discussions	86
4.4. Conclusions	95

TABLE OF CONTENTS (Continued)

	<u>Page</u>
5. CHAPTER 5 Inorganic phosphate and Sodium Ions are Co-Germinants for Spores of <i>Clostridium perfringens</i> type A Food Poisoning-Related Isolates	110
5.1. Introduction	112
5.2. Materials and methods	115
5.3. Results	118
5.4. Discussions	125
CHAPTER 6 Identification and Characterization of New Amino Acid Germinants for Spores of the Enterotoxigenic <i>Clostridium perfringens</i> Type A Isolates	138
6.1. Introduction	140
6.2. Materials and methods	143
6.3. Results	148
6.4. Discussions	160
CHAPTER 7 Roles of Putative Histidine Kinases in <i>Clostridium perfringens</i> Sporulation and Germination	183
7.1. Introduction	185
7.2. Materials and methods	188
7.3. Results	196
7.4. Discussions	212
CHAPTER 8 General Conclusion	241
Bibliography	248

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
3.1. Effect of nisin on <i>C. perfringens</i> spore germination	70
3.2. Effect of nisin on <i>C. perfringens</i> spore outgrowth	71
3.3. Effect of nisin on vegetative growth of <i>C. perfringens</i> isolates	72
3.4. Inhibitory effect of nisin on growth of <i>C. perfringens</i> spores in cooked meat products	73
4.1. Germination of spores of representative <i>C. perfringens</i> FP strain SM101 and NFB strain NB16 with AK	104
4.2. Effect of ethanol on inactivation of <i>C. perfringens</i> spores	105
4.3. Effect of Quats on inactivating <i>C. perfringens</i> spores	106
4.4. Effect of iodophores on inactivation of <i>C. perfringens</i> spores	107
4.5. Effect of DECON-SPORE 200 PLUS on inactivating <i>C. perfringens</i> spores	108
5.1. Effect of pH and NaPi concentration in germination of <i>C. perfringens</i> spores	132
5.2. Germination of spores of <i>C. perfringens</i> strain SM101 in different buffers	133
5.3. Germination of spores of <i>C. perfringens</i> strain SM101 with Na ⁺ and K ⁺	134
5.4. Germination of spores from various <i>C. perfringens</i> isolates with NaPi	135
5.5. Germination of spores of <i>C. perfringens ger</i> receptor mutant strains with NaPi	136

LIST OF FIGURES (Continued)

<u>Figure</u>	<u>Page</u>
5.6. Germination of spores of <i>C. perfringens</i> antiporter mutant strains with NaPi	137
6.1. Effect of cysteine and glutamine pHs on germination of <i>C. perfringens</i> spores	176
6.2. Effect of cysteine and glutamine concentrations on germination of <i>C. perfringens</i> spores	177
6.3. Germination of spores of <i>C. perfringens</i> various isolates with cysteine and glutamine	178
6.4. Germination of spores of <i>C. perfringens</i> various isolates with amino acids in polar, uncharged group including asparagine, serine, and threonine	179
6.5. Germination of spores of <i>C. perfringens</i> GR mutant strains with cysteine	180
6.6. Germination of spores of <i>C. perfringens</i> GR mutant strains with glutamine	181
6.7. Germination of spores of <i>C. perfringens</i> GR mutant strains with amino acids in polar, uncharged group	182
7.1. Alignment of amino acid sequences of the <i>B. subtilis</i> sporulation-associated sensor histidine kinases and the putative orphan histidine kinases of <i>C. perfringens</i>	231
7.2. Expression profiles of putative sensor histidine kinase genes in <i>C. perfringens</i> strains	233
7.3. Growth characteristics of <i>C. perfringens</i> strains under vegetative and sporulation conditions	234
7.4. Sporulation capability of <i>C. perfringens</i> strains	235

LIST OF FIGURES (Continued)

<u>Figure</u>	<u>Page</u>
7.5. Expression profiles of the <i>spo0A</i> and <i>spoIIIG</i> operon in various <i>C. perfringens</i> strains	236
7.6. Germination of <i>C. perfringens</i> spores with various germinants	237
7.7. Outgrowth of spores of <i>C. perfringens</i> strains	239
7.8. Germination of <i>C. perfringens</i> spores in BHI broth	240

LIST OF TABLES

<u>Table</u>	<u>Page</u>
2.1. Toxin typing of <i>C. perfringens</i>	9
4.1. Germination of <i>C. perfringens</i> spores from FP and NFB isolates with various germinants at 40 °C	102
4.2. Germination of <i>C. perfringens</i> spores from FP and NFB isolates with various germinants at RT	103
4.3. Inactivation of spores of <i>C. perfringens</i> FP isolates on SS chips ..	109
6.1. Germination of spores of <i>C. perfringens</i> representative FP and NFB stains with 20 amino acids at pH 7.0	175
7.1. <i>C. perfringens</i> strains and plasmids used in this study	224
7.2. Primers used in this study	229
7.3. Percentage amino acid sequences similarity between sporulation-associated sensor histidine kinases in <i>B. subtilis</i> and putative orphan kinases in <i>C. perfringens</i>	232
7.4. Maximum germination rates of spores of various <i>C. perfringens</i> strains with known germinants	238

Clostridium perfringens Spores: Inactivation, Germination, and Formation

CHAPTER 1

Introduction

Clostridium perfringens is a Gram-positive, anaerobic, endospore-forming rod shaped bacterium. *C. perfringens* type A isolates producing *C. perfringens* enterotoxin (CPE) are a causative agent of *C. perfringens* type A food poisoning (FP), which currently ranks as the third most commonly reported food-borne disease in the United States and accounts for almost 1,000,000 cases annually. Economic loss due to this single pathogen was estimated to reach \$12.5 billion per year (Buzby and Roberts, 1997; Lynch et al., 2006; McClane, 2007a; Scallan et al., 2011b). This pathogenic bacterium can also cause *C. perfringens*-associated non-food-borne (NFB) human gastrointestinal (GI) diseases, such as antibiotic-associated and sporadic diarrheas (Borriello et al., 1984; Collie and McClane, 1998; Lindström et al., 2011; Mpamugo et al., 1995). *C. perfringens* is a major concern to food manufacturers owing to its ability to form metabolically dormant spores that are highly resistant to various treatments such as moist heat, osmotic, nitrite, pH induced stress, prolonged frozen storage, and high pressure processing (Li and McClane, 2006a, 2006b; Paredes-Sabja et al., 2007; Sarker et al., 2000). These spore-resistant properties facilitate the survival of *C. perfringens* against various preservative measures generally applied to food industries

and make an effort to eliminate or at least reduce the contaminated spores very challenging.

C. perfringens sporulation provides two important contributions in the occurrence of *C. perfringens* FP and NFB human GI diseases: (i) the formation of highly resistant spores facilitates bacterial survival in a variety of food-associated and environmental stresses; (ii) CPE, the major virulence factor for the *C. perfringens*-related GI diseases, is only synthesized during sporulation (Huang et al., 2004; Li and McClane, 2010; McClane, 2007a). The initiation of sporulation is governed by the activity of multiple sensor histidine kinases that are hypothesized to sense the environmental stimuli and trigger the developmental process of spore formation via the activation of the master regulator for entry into sporulation, Spo0A (Hoch, 1993b; Huang et al., 2004; Jiang et al., 2000; Stephenson and Hoch, 2002). Although the regulation of sporulation has been extensively studied in *B. subtilis* (Errington, 2003; Hoch, 1993a; Piggot and Hilbert, 2004), such information is lacking in *C. perfringens* and the function of the putative sensor histidine kinases in this pathogen has never been reported. In addition to sporulation, the roles of two putative kinases in various aspects in the life cycle of *C. perfringens* were characterized including growth, germination, spore outgrowth and viability.

Given the importance of spores as an infectious morphotype of *C. perfringens* and in view of the necessity of spore germination to eventually exert its deleterious effects (Paredes-Sabja et al., 2008c), understanding factors influencing spore germination as well as germination mechanism may aid in designing germination

inhibitors or developing artificial germinants that facilitate spore killing under mild conditions. Recent progress has been made towards understanding the mechanisms of spore germination in *C. perfringens* (Paredes-Sabja et al., 2008b; Paredes-Sabja et al., 2009d; Paredes-Sabja et al., 2011; Xiao et al., 2011); however, compounds and conditions influencing the germination responses of this pathogenic bacterium are still lacking. Spore germination can be induced by a variety of compounds, termed germinants, which include amino acids, sugars, and purine nucleosides (Setlow, 2003). Each bacterial species has different germinant requirement or sometimes the differences are observed even between various strains (Abee et al., 2011; Alberto et al., 2003; Broussolle et al., 2008; Paredes-Sabja et al., 2008c; Paredes-Sabja et al., 2009e; Van Der Voort et al., 2010). Even though factors affecting germination have been well studied in the genus *Bacillus*, such information is much less available in *Clostridium* spp. Therefore, it is of our interest to identify and characterize new germinants for CPE-producing *C. perfringens* spores. In this dissertation, two independent studies focused on investigation of ionic and nutrient germinants for spores of *C. perfringens* FP and NFB isolates and the germinant receptor proteins responsible for sensing these newly identified germinants were identified.

It is well recognized that bacterial spores lose their resistant properties readily upon germination (Indest et al., 2009; Setlow, 2003). This notion is the basis of the development of a potential strategy toward eliminating threat posed by spore-forming bacteria, which is based on inducing spore germination, and then inactivating those less resistant germinated spores (Akhtar et al., 2009; Gould et al., 1968; Hornstra et

al., 2007; Løvdal et al., 2011; Nerandzic and Donskey, 2010). The cross-contamination of bacteria from contaminated food contact surfaces into finished food products is considered as one of the leading causes of food-related GI illnesses and it can occur in food processing plant, during product handling or food preparation (Ryu and Beuchat, 2005). Once attached to surfaces, bacterial cells and spores are more resistant to the effect of various commonly used disinfectants and this retention of *C. perfringens* on the food contact surfaces could serve as a continuous source of product contamination affecting their quality, shelf-life, and safety of the consumer (Hornstra et al., 2007). Indeed, the contaminated equipment accounted for 15% of the total cases of *C. perfringens* type A FP (Mcclane, 2007a). Based on the implementation of spore germination with a universal germinants and subsequent inactivation of germinated spores with commonly used surface disinfecting agents, we successfully developed a strategy to decontaminate spores of *C. perfringens* FP isolates adhered onto stainless steel chips as a model of food contact surfaces.

An increase in the retail market of minimally-processed, ready-to-eat food products free from added synthetic preservatives provides an exploitable environment suitable for survival and growth of an opportunistic pathogen such as *C. perfringens* (Novak and Juneja, 2002; Song et al., 2009). Moreover, *C. perfringens* is predominantly, but not exclusively, found in meat and poultry-containing products due to its auxotrophic requirement for 13 different amino acids, which the microorganisms cannot synthesize. This correlates well with the fact that meat dishes that are stored at abusive temperature are the most common food vehicle for *C. perfringens* type A FP

(Brynstad and Granum, 2002; McClane, 2007a; Novak and Juneja, 2002). Therefore, there is a need for effective measures to control germination and outgrowth of *C. perfringens* spores in cooked meat products, especially if the proper cooling rate and storage temperature cannot be achieved. A more novel and attractive antimicrobial agent is nisin, a 34-amino acid polypeptide produced by certain strains of *Lactococcus lactis* subsp. *lactis* (Bhatti et al., 2004). Nisin is in the Generally Recognized As Safe (GRAS) category by the United States Food and Drug Administration (FDA) (Thomas and Delves-Broughton, 2005). It is nontoxic, heat stable, readily degraded by digestive enzymes upon human consumption, and does not contribute to off-flavors or off-odors (Delves-Broughton, 2005; Jay et al., 2005). According to those positive attributes, nisin has gained much of interest from both academic and industrial and has been successfully used a food preservative for various types of products (Thomas and Delves-Broughton, 2005). The inhibitory effect of nisin against *C. perfringens* had been previously reported although with a limited laboratory or environmental strains (Banerjee and Sarkar, 2004; Eastoe and Long, 1959; Guerlava et al., 1998; Paik et al., 2006; Scannell et al., 2000). Therefore, a detailed study of nisin activity on growth, spore germination and outgrowth against a collection of *C. perfringens* FP and NFB clinical isolates was conducted. Additionally, the potential use of nisin to control germination and outgrowth of spores of *C. perfringens* in cooked meat products during storage at abusive temperature had been evaluated as well.

Objectives of this study

This dissertation consists of five individual studies with the following objectives.

- (1) In the first study, the objectives were to evaluate (i) the inhibitory effect of nisin against a collection of clinical isolates of *C. perfringens* FP and NFB GI diseases, and (ii) the efficacy of nisin as an antimicrobial agent in food model system contaminated with *C. perfringens* spores during storage at abusive temperature.
- (2) The second study was aimed to develop spore decontamination strategy by (i) optimizing the universal germinant for spores of *C. perfringens* FP and NFB isolates; (ii) evaluating the spore inactivation strategy by implementation of spore germination prior to killing by commonly used disinfectants; and (iii) verifying the developed decontamination approach in stainless steel coupons as a model for food contact surfaces.
- (3) The objectives of the third study were to (i) determine the ability of the inorganic phosphate and sodium ions (NaPi) to induce germination of CPE-producing *C. perfringens* spores; and (ii) characterize the germinant receptor and putative $\text{Na}^+/\text{H}^+-\text{K}^+$ antiporter proteins involved in NaPi-induced germination
- (4) The fourth study was aimed to (i) identify and optimize amino acids as nutrient germinants for spores of enterotoxigenic *C. perfringens*; (ii) Compare the germinant selectivity between *C. perfringens* FP and NFB isolates; and (iii)

characterize germinant receptors in *C. perfringens* spore that are responsible for sensing the newly identified amino acid germinants.

- (5) For the fifth study, the objectives were as follows: (i) to identify putative sensor histidine kinases of *C. perfringens*; (ii) to examine the expression profiles of the *C. perfringens* putative sensor histidine kinase genes; and (iii) to characterize the roles of putative sensor histidine kinases in various aspects in the life cycle of *C. perfringens*.

CHAPTER 2

Literature Review

2.1. Characteristics of the bacterium

Clostridium perfringens is a Gram-positive, rod-shaped, nonmotile, spore-forming bacterium. *C. perfringens* is ubiquitously found in virtually all environments including soil, water, dust, milk, sewage and the intestinal tracts of humans and animals. It is considered to be an anaerobe since no colony was produced on agar plate after continuous exposure to oxygen. However, *C. perfringens* is moderately aerotolerant (Brynestad and Granum, 2002; Mcclane, 2007a). The optimal growth temperature for vegetative cells of *C. perfringens* ranges from 43 to 45 °C; although growth can also occur between 15 to 50 °C (Novak et al., 2005). This pathogenic bacterium does not grow at 6 °C; nevertheless, its spore form is cold resistant. Thus, the viable spores in refrigerated and frozen food products can germinate, and then multiply to cause *C. perfringens* type A FP once the contaminated foods are warmed for serving (Mcclane, 2007a). Under near optimal conditions for other environmental factors, the minimum water activity for growth of *C. perfringens* is 0.93 (Mcclane, 2007a). pH also significantly affect growth of *C. perfringens* with the optimum pH of pH 6 to 7. This organism grows very poorly at pH ≤ 5 and ≥ 8.3 (Labbe, 1989). The

precise oxidation-reduction potential for growth of *C. perfringens* is dependent on other environmental factors. It was suggested that *C. perfringens* can grow over a wide range of oxidation reduction potentials and growth between -125 and +350 mV (millivolts) had been reported (Wrigley, 2001).

C. perfringens is a prolific toxin producer with an ability to produce at least 14 different toxins. However, an individual *C. perfringens* cell only carries a sub-set of these toxin genes. This property has been used as a toxin-based typing system, which classified *C. perfringens* isolates into five types (A-E) based on the production of four major toxins (alpha, beta, epsilon, and iota) (Table 2.1).

Table 2.1. Toxin typing of *C. perfringens* (Mcclane, 2007a; Petit et al., 1999)

Type ^a	Toxin produced ^b			
	Alpha	Beta	Epsilon	Iota
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

^a *C. perfringens* type

^b +, produced; -, not produced

C. perfringens is an important causative agent for human GI disease due to its ability to produce GI-active toxins (e.g. *C. perfringens* enterotoxin (CPE) and beta (β) toxin). Two forms of food-borne illnesses caused by *C. perfringens* are related to two distinct *C. perfringens* toxinotypes (Mcclane, 2007a; Mcclane et al., 2006). First, the life-threatening necrotic enteritis or also known as pig-bel disease, is caused by *C. perfringens* type C (producing β -toxin), which causes necrosis of the intestine.

Symptoms of pig-bel disease include diarrhea and abdominal pain while stools are bloody and laden with mucous. This disease is more severe than *C. perfringens* type A FP and death due to enteritis had been reported (Mcclane et al., 2000; Wrigley, 2001). The other form of food related disease having *C. perfringens* type A as an etiological agent is known as *C. perfringens* type A FP. Even though the symptoms are generally mild including diarrhea and abdominal pain, the incidences of outbreaks of *C. perfringens* type A FP are very common in developed countries (Brynstad and Granum, 2002; Lindström et al., 2011). The disease symptoms are caused by the activity of enterotoxin produced during *C. perfringens* sporulation in the small intestine of victims (Brynstad and Granum, 2002; Mcclane, 2007a)

2.2. *Clostridium perfringens* enterotoxin (CPE)

CPE is a 35-kDa polypeptide with heat-labile and pH-sensitive properties (Mcclane, 2007a; Mcclane et al., 2006). It is primarily produced by *C. perfringens* type A in which about 1% to 5% of *C. perfringens* type A isolates were reported to produce this medically important toxin (Mcclane, 2007a). The production of CPE is highly dependent on bacterial sporulation. Several lines of evidences are clearly proven that CPE is present in *C. perfringens* cells grown under sporulation-inducing condition about the same time with the formation of heat-resistant spores, but no CPE was detected in vegetative growth cultures. Moreover, CPE could be detected in the culture media after mother cell lysis to release mature spores (Duncan et al., 1972a; Labbe, 1980). Large amount of CPE is often produced, representing ~ 20% of the total

protein inside the sporulating cell (Collie et al., 1998; Czeczulin et al., 1993). Only small populations of *C. perfringens* isolates carry *cpe* with mostly belong to type A *C. perfringens* (Mcclane, 2007a; Mcclane et al., 2006). CPE is essential for the pathogenesis of *C. perfringens* type A FP, CPE-associated non-foodborne human GI diseases, and certain veterinary GI diseases (Mcclane et al., 2006).

Even though several lines of evidences strongly suggested that CPE production is dependent on *C. perfringens* sporulation, the mechanism(s) of sporulation-regulated CPE production at the molecular level is not fully understood. Previous studies demonstrated that Spo0A, the master regulator for sporulation initiation in *C. perfringens*, had essential roles for both heat-resistant endospore formation as well as CPE production because *spo0A* knockout mutant cannot produce spores and no CPE was detected by western blot analysis (Huang et al., 2004). The homologues of alternative sigma factors that regulate sporulation in *Bacillus subtilis* are also encoded by *C. perfringens* (Myers et al., 2006) and recent studies suggested that all four sigma factor SigF, SigE, SigG, and SigK are necessary for the formation of spores. In contrast, only SigF, SigE, and SigK are needed for CPE synthesis (Harry et al., 2009; Li and Mcclane, 2010).

CPE is considered an enterotoxin as it causes fluid and electrolyte losses from the GI tract of many mammalian species (Mcclane, 2007a). The model of CPE action is as follows: at pathophysiologic concentrations, CPE binds via its C-terminal portion to the protein receptor(s) of certain claudin, which serve a structural role in epithelial tight junctions, resulting in the formation of a small complex of 90 kDa (Fujita et al.,

2000; McClane et al., 2006). CPE is localized on the membrane surface. Then, this small complex interacts with other proteins to form ~ 155 kDa large complex that induces plasma membrane permeability alterations, including Ca^{2+} influx, because this large complex is a pore or portion of a pore. Concentrations of CPE had significant impact on the cell death pathway triggered by alterations in membrane permeability (Chakrabarti et al., 2003). At low dose, there is moderate Ca^{2+} influx resulting in apoptosis, whereas higher concentration of CPE causes a massive influx of Ca^{2+} and oncosis is triggered. Either apoptotic or oncotic cell death pathways cause morphological damage, thereby permitting CPE to reach receptors on the basolateral surface of the CPE-treated cell and adjacent cells. Following this event, additional formation of the 155-kDa large complex and ~200-kDa complex consisting of occludin takes place which subsequently lead to the internalization of tight junction protein initiating tight junction damage. Eventually, the paracellular permeability alters and contributes to CPE-induced diarrhea (McClane, 2007a; McClane et al., 2006)

Previous studies found that removing the first ~ 45 N-terminal amino acid residues activated CPE toxicity (Kokai-Kun and McClane, 1997). Proteases such as trypsin and chymotrypsin can activate CPE *in vitro*; thus, it was hypothesized that similar proteolytic activation by the action of intestinal protease may occur in the intestines during GI disease (Brynstad and Granum, 2002; McClane et al., 2006).

2.3. CPE-associated GI diseases

C. perfringens strains producing CPE are the important causative agent for several human GI diseases such as *C. perfringens* type A FP, antibiotic-associated diarrhea (AAD), and sporadic diarrhea (SD). Interestingly, CPE-encoding *cpe*, can be located either on the chromosome or on a large plasmid of *C. perfringens* type A (Collie and McClane, 1998; Cornillot et al., 1995). The majority of FP disease was caused by *C. perfringens* strains carrying *cpe* on their chromosome (*C-cpe*), whereas NFB human GI diseases including AAD and SD are closely related to plasmid-borne *cpe* strains (*P-cpe*) (Brynstad et al., 1997; Collie and McClane, 1998; Lindström et al., 2011; Sarker et al., 2000). However, recent investigations also indicated that *P-cpe* isolates had been linked to FP outbreaks in Japan and Europe (Lahti et al., 2008; Nakamura et al., 2004; Tanaka et al., 2003).

Vegetative cells and spores of *C-cpe* isolates possess dramatically higher resistance properties against heat, cold, nitrite-and osmotic-induced stresses (Li and McClane, 2006a, 2006b; Sarker et al., 1999). Furthermore, *C-cpe* strains typically grow faster at optimum temperature and are able to grow at wider ranges of temperature than those of *P-cpe* isolates. These distinct phenotypes could favor the growth and survival of *C. perfringens* *C-cpe* isolates in food processing environments and, at least in part, explain the strong association between this isolate type with *C. perfringens* type A FP. The recent genetic characterization of type A enterotoxigenic *C. perfringens* clearly suggested that regardless of date of isolation, geographic origin,

or isolation source; *C-cpe* isolates form a distinct cluster from other *C. perfringens* isolates (Deguchi et al., 2009; Xiao et al., 2012).

2.3.1. *C. perfringens* type A FP

C. perfringens type A toxicoinfection currently ranks as the third common cause of foodborne outbreaks in the United States and accounts for almost million cases annually (Scallan et al., 2011a). The economic costs due to *C. perfringens* type A FP was estimated to reach \$12.5 billion (Buzby and Roberts, 1997). Apart from CPE synthesis, there are three important factors contributing to its ability to cause foodborne illness (Mcclane, 2007a). First, *C. perfringens* is one of the fastest growing bacteria with the doubling time less than 10 min under optimum conditions; thus, this allows the organism to rapidly multiply in foods (Labbe and Huang, 1995; McClane, 2007a). Second, its spore-forming capability makes this pathogenic bacterium withstands to a variety of environmental insults, such as radiation, heat, low temperature, high hydrostatic pressure, chemical preservatives, and desiccation (Li and McClane, 2006a, 2006b; McClane, 2007a; Paredes-Sabja et al., 2007; Sarker et al., 2000). Third, *C. perfringens* spores are able to survive in incompletely cooked and inadequately reheated food products (Mcclane, 2007a).

C. perfringens type A FP outbreaks are typically associated with meat and poultry-containing dishes. Since spores of *C. perfringens* are widely distributed in soil and water, they often contaminate raw meat and poultry during slaughter operations (Juneja and Thippareddi, 2004). Initially, thermal treatment applied by the meat processing industry could activate the highly resistant *C. perfringens* spores

(Thippareddi et al., 2003). These heat-activated spores could survive cooking and rapidly germinate and outgrow in cooked products especially during improper cooling rate and abusive temperature storage to high cell numbers. When these heavily contaminated food products ($\sim 10^6$ bacteria/g of food) are ingested, some vegetative cells survive passage through the stomach and remain viable upon entering the small intestine. In the small intestine of the victim, *C. perfringens* initially multiply, and then sporulate, concurrently with CPE formation in sporulating cells. CPE is released together with the mature spores when the mother cells lyse. Then, CPE binds to intestinal epithelial cells causing damage and initiates fluid loss, i.e., diarrhea. *C. perfringens* type A FP is usually mild and self-limiting within 12 to 24 hours. The characteristic symptoms include diarrhea and severe abdominal cramps, but vomiting and fever are rare (Mcclane, 2007a; Mcclane et al., 2000; Mcclane et al., 2006).

Owing to the high heat resistance of *C. perfringens* spores, inactivation of spores is always a challenge. Elimination of spore contamination in foods and preventing contamination during processing and handling could be achieved via good hygiene and high-quality food sources. Proper cooling of prepared food and adequate re-heating finished products before consumption are also an effective measure aid in prevention. Those strategies can prevent the occurrence of *C. perfringens* type A FP (Wrigley, 2001).

2.3.2. Antibiotic-associated diarrhea and sporadic diarrhea

The CPE-producing *C. perfringens* has also been reported to cause approximately 5 -20% of AAD and SD cases in humans (Mpamugo et al., 1995; Vaishnavi and Kaur, 2008) and these were not related to food consumption (Collie et al., 1998). AAD is generally developed after antibiotic treatment, while SD is considered to develop independently of any antibiotic treatment. It was proposed that AADs and SDs are caused by a small inoculum of P-*cpe* cells, and subsequently *cpe* plasmid is conjugatively transferred to the *cpe*-negative normal gut population *C. perfringens* that have been already adapted to the gut environments (Heikinheimo et al., 2006; Sparks et al., 2001). Nevertheless, the route of P-*cpe* transmission is unclear. According to current knowledge that *C. perfringens* P-*cpe* strains could be isolated from food products as well as the reported outbreaks of FP caused by *C. perfringens* carrying plasmid-borne *cpe* (Lahti et al., 2008; Miki et al., 2008; Nakamura et al., 2004; Tanaka et al., 2003). This suggests that the occurrence of AAD and SD in some cases might also transmit via foods, and thus consider as FP (Lindström et al., 2011). AAD and SD are more prevalent in elderly as well as people taking antibiotics and often occur in the nosocomial settings where they might be transmitted person-to-person. The symptomatic therapy to restore fluid/electrolyte balance is necessary for most cases of AAD and SD (Mcclane et al., 2006).

2.4. Stages of spore formation

Although the process of spore formation has been most studied in *Bacillus* spp., especially *Bacillus subtilis* (Errington, 2003; Piggot and Hilbert, 2004; Sonenshein, 2000), it was reported to be identical in the *Clostridium* species (Dürre and Hollergschwandner, 2004). This process can be basically divided into seven stages (0 to VII) (Hitchins and Slepecky, 1969; Piggot and Coote, 1976; Ryter, 1965). The actively growing vegetative cells are defined as stage 0. Stage I and II are characterized by the presentation of cell DNA as an axial filament and the asymmetric cell division, forming two compartments separated by a septum within a cell, which are the larger mother cell compartment and the smaller prespore compartment. Initially, only one-third of chromosome is present in the prespore after asymmetric division; however, the remaining two-thirds is rapidly pumped into the prespore compartment via the action of the DNA translocase protein SpoIIIE. Thus, two unequally sized cells have identical genomes. During stage III, the prespore is engulfed by the mother cell forming the forespore as a free-floating protoplast surrounded by inner and outer forespore membranes. Stage IV is the step in which the synthesis of two peptidoglycan layers occur, the primordial germ cell wall and the cortex in the space between inner and outer membranes surrounding the forespore. The successive event in stage V is the formation of the complex structure of proteins outside surface of forespore known as spore coat. The spore maturation in stage VI leads to the spore's resistance properties to UV and radiation and heat, although there is no apparent morphological change during this stage. Finally, mother cell lyses to

liberate the mature spore into the environment in stage VII. The structure of mature spore protects the dormant microorganism from a variety of environmental insults until spore find itself in more favorable conditions for vegetative growth. The dormant spore then undergoes germination and outgrowth, followed by the resumption of vegetative growth cycle (De Jong et al., 2010; Errington, 2003; Hilbert and Piggot, 2004; Leggett et al., 2012).

2.5. Initiation of sporulation

The initiation of sporulation can only occur at a certain time period of cell's life cycle. In *B. subtilis*, at least two extracellular environmental signals such as nutrient deprivation and high cell density lead to the developmental process of spore formation. On the other hand, the intracellular environments including DNA damage and blocking of either initiation or progression of DNA replication prevent the initiation of sporulation (Hilbert and Piggot, 2004; Piggot and Hilbert, 2004; Sonenshein, 2000). Spo0A is the master regulator for entry into sporulation in both *Bacillus* and *Clostridium* spp. (Dürre and Hollergschwandner, 2004; Harris et al., 2002; Hoch, 1993b; Huang and Sarker, 2006; Huang et al., 2004; Underwood et al., 2009). In *B. subtilis*, Spo0A is activated by the phosphorelay, a more complex variant of two-component signal transduction system (Burbulys et al., 1991). Its phosphorylated form, Spo0A~P acts as both transcriptional activator and repressor of various genes involved in stationary-phase events and sporulation initiation (Fujita et al., 2005; Hilbert and Piggot, 2004). At least five sensor histidine kinases are

associated with the phosphorelay in *B. subtilis*, KinA (Antoniewski et al., 1990; Perego et al., 1989), KinB (Trach and Hoch, 1993), KinC (Ledeaux and Grossman, 1995), KinD, and KinE (Jiang et al., 2000). It was suggested that each of the kinases are responsible for specific stimuli (Jiang et al., 2000; Stephenson and Hoch, 2002). Among these, KinA and KinB are the primary sensor histidine kinases feeding phosphate into phosphorelay and initiate sporulation (Jiang et al., 2000; Ledeaux et al., 1995). In response to the unidentified signal(s), kinases autophosphorylate and donate a phosphoryl group to the response regulator Spo0F giving Spo0F~P, which in turn, transfers the phosphoryl group to phosphotransferase Spo0B. Eventually, Spo0B~P transfer the phosphoryl group to Spo0A. This sequential transfer of phosphate is termed a phosphorelay (Burbulys et al., 1991). Once activated, Spo0A~P can either activate or repress transcription by binding to a consensus sequence, TGNCGAA, in or near promoters recognized by RNA polymerase vegetative sigma factor SigA and an alternative sigma factor SigH. The binding of Spo0A~P leads to changes in the expression of more than 500 genes, which correspond to approximately one-eighth of the total genes in *B. subtilis* (Fawcett et al., 2000). Previous study identified 121 genes are under the direct control of Spo0A and 25 of these regulated genes are transcription regulators themselves; therefore, Spo0A~P causes direct and indirect transcriptional changes (Hilbert and Piggot, 2004; Molle et al., 2003).

The other key positive regulator for sporulation initiation is the alternative sigma factor SigH. SigH is the transcription factor of various phosphorelay components such as Spo0A, Spo0F, KinA, and KinE (Errington, 2003; Hoch, 1993a).

In turn, Spo0A~P indirectly regulates transcription of *spo0H* encoding for SigH by suppressing the synthesis of AbrB, which is a transition state repressor for *spo0H* and other sporulation-specific genes (Hoch, 1993a). Thus, relieving the repression effect of AbrB and allows the expression of stationary phase and sporulation genes under its control (Perego and Hoch, 2002; Phillips and Strauch, 2002). For *B. subtilis* sporulation, the key players in the regulation of gene expression are Spo0A, SigH, SigF, SigE, SigG, and SigK (Piggot and Hilbert, 2004).

2.6. Establishment of cell-specific transcription: a cascade of sigma factors in *B. subtilis*

After stage II, the sporulating cell is divided into two compartments of unequal size: the forespore and the mother cell, by the formation of asymmetric septum (Errington, 2003; Phillips and Strauch, 2002). At this stage cells are committed to sporulate since prior to this point cells are still able to revert back to vegetative growth or undergo other alternative survival strategies depending on the appropriate environmental signals that had been received (Phillips and Strauch, 2002). Upon completion of asymmetric division, the sporulation-associated sigma factor SigF and SigG regulate gene expression in the forespore compartment, whereas SigE and SigK are responsible for transcriptional control in the mother cell compartment (Haldenwang, 1995; Kroos et al., 1999). In *B. subtilis*, the synthesis and activation of these sporulation-specific sigma factors occur in an sequential cascade in which SigF appears first, and then followed by SigE, SigG, and SigK (Kroos et al., 1999).

2.6.1. *SigF* (σ^F)

The RNA polymerase sigma factor SigF, encoded by *spoIIAC*, is synthesized prior to the formation of polar septum, but is held inactive until septation is completed. In the pre-divisional cell, the binding of anti-sigma factor SpoIIAB maintains SigF in an inactive stage; however, this inhibition is relieved by the anti-anti-sigma factor SpoIIAA. SpoIIAA functionality depends on its phosphorylation stage in which SpoIIAB (a kinase and anti-sigma factor) phosphorylate SpoIIAA rendering it inactive. On the other hand, SpoIIE (a phosphatase) dephosphorylate and activate SpoIIAA. The non-phosphorylated SpoIIAA interacts with the SpoIIAB-SigF complex to displace the SigF thereby the latter become active and direct gene expression in the early-phase of sporulation in the prespore compartment (Errington, 2003; Phillips and Strauch, 2002). It was suggested that the key functions of SigF are to couple prespore and mother cell specific gene expression via the control of various genes whose products are required for the activation of mother cell-specific sigma factors as well as to direct synthesis of the late forespore transcription factor SigG (Hilbert and Piggot, 2004).

2.6.2. *Sig E* (σ^E)

Like SigF, SigE is also synthesized before the polar septum formed as an inactive precursor, pro-SigE, which is then activated by proteolytic processing to active SigE upon the completeness of septum formation by the action of SpoIIGA, a putative serine protease (Kroos et al., 1999; Piggot and Hilbert, 2004). The activation of SpoIIGA requires the SigF-controlled SpoIIR protein synthesized in the prespore;

therefore, the appearance of functional SigE is tied to SigF-directed gene transcription in the forespore by an intercellular signal transduction pathway (Errington, 2003; Hofmeister et al., 1995; Piggot and Losick, 2002). Expression of *spoIIGB* encoding for pro-SigE is considerably enhanced in the mother cell compartment by the activity of Spo0A that becomes a mother cell-specific transcription following asymmetric division. This results in the confined activity of SigE in the mother cell compartment (Errington, 2003; Fujita and Losick, 2003; Piggot and Losick, 2002). The main function of SigE is to regulate an early mother cell-specific gene expression to prevent asymmetric division in the mother cell, to trigger engulfment of the prespore, to initiate spore coat assembly, and to direct synthesis of the late mother cell-specific sigma factor SigK (Hilbert and Piggot, 2004).

2.6.3. SigG (σ^G)

SigG is made in the pre-engulfment prespore, but is not activated until the complete of engulfment process (Stage III). Transcription of *spoIIIG*, which encodes SigG is sporulation specific as it is dependent on RNA polymerase containing SigF (Errington, 2003; Piggot and Hilbert, 2004). The products of *spoIIIA* and *spoIIIJ* are needed for releasing SigG from inhibition in the forespore compartment. *spoIIIA* is selectively expressed in the mother cell under SigE control. Previous findings showed that by switching its expression site to forespore causes defective in sporulation. On the other hand, SpoIIIJ is synthesized in the forespore. However, the mechanism of SpoIIIA proteins activate SigG in the forespore is not fully understood. Perhaps, they transmitted signals to SpoIIIJ in the forespore that leads to activation of SigG although

additional unidentified factors are likely to exist (Piggot and Hilbert, 2004). The major genes under SigG control have roles in sporulation, germination, and protection of spore from DNA damage. Thus, SigG has three main following functions: to couple late forespore and mother cell gene expressions, to protect bacterial spores from environmental hazards, and to prepare the spore for the germination once the condition for growth are resumed (Hilbert and Piggot, 2004).

2.6.4. *SigK* (σ^K)

SigK is the late mother cell specific sigma factor. In *B. subtilis*, *sigK* gene encoding a full length of sigma factor SigK, is interrupted by the *skin* (sigma K intervening) element. SpoIVCA is responsible for excising *skin* element to generate an uninterrupted *sigK*. The expression of *sigK* and *spoIVCA* is confined to the mother cell under the control of SigE and the SigE-directed regulatory protein SpoIID (Hilbert and Piggot, 2004; Phillips and Strauch, 2002). Regulation of SigK is similar to SigE as it is synthesized as an inactive precursor, pro-SigK. The pro-SigK required proteolysis to become active SigK upon receiving signals generated in the forespore compartment (Hilbert and Piggot, 2004). Processing pro-SigK required SpoIVFB, a protease that cleaves the prosequence from pro-SigK, which located in the mother cell membrane. Two additional mother cell proteins, SpoIVFA and BofA, function as negative regulators for the action of SpoIVFB. The signal produced by the SigG-controlled forespore specific protein SpoIVB is recruited to reach SpoIVFA-SpoIVFB-BofA complex and overcome the inhibitory effects of SpoIVFA and BofA thereby triggering pro-SigK processing. Hence, the activation of SigK occurs soon

after the completion of engulfment and appearance of SigG in the forespore (Hilbert and Piggot, 2004; Phillips and Strauch, 2002). The key functions of SigK are to regulate transcription of genes whose products are involved in formation of spore coat, spore maturation, and control of SigK-dependent transcriptional regulator GerE (Hilbert and Piggot, 2004).

Unlike *B. subtilis*, the regulation of endospore formation in *C. perfringens* has not been well studied. Recent investigations revealed similarities and differences in the sporulation mechanisms between these two bacterial species. In respect to the similarity, both *B. subtilis* and *C. perfringens* utilize Spo0A to initiate sporulation and SigF is the first sporulation-associated sigma factor that present and regulates the production of other sporulation-specific sigma factor including SigE, SigG, and SigK (Huang et al., 2004; Li and McClane, 2010; Piggot and Hilbert, 2004) although the mechanisms responsible for synthesis and activation of these sporulation specific sigma factors in *C. perfringens* has not yet been identified. Like *B. subtilis*, all four alternative sigma factor are absolutely required for the formation of *C. perfringens* spores (Harry et al., 2009; Li and McClane, 2010). The obvious difference in sporulation between *B. subtilis* and *C. perfringens* is the lack of phosphorelay signal transduction pathway in the latter. Since *C. perfringens* and other *Clostridium* spp. do not have homologues of Spo0F and Spo0B, the initial signaling cascade that results in Spo0A phosphorylation, and then trigger sporulation must be distinct (Dürre and Hollergschwandner, 2004; Paredes et al., 2005; Steiner et al., 2011; Stephenson and Hoch, 2002; Underwood et al., 2009). It is most likely that *C. perfringens* Spo0A

becomes phosphorylated and activated via the direct interaction with the putative sensor histidine kinases acting in response to unknown sporulation stimulatory signal(s). Indeed, recent studies demonstrated that several sensor histidine kinases in *Clostridium difficile* and *Clostridium acetobutylicum* can transfer phosphoryl group directly to Spo0A protein in vitro. These results support the hypothesis that initiation of sporulation in *Clostridium* spp. is controlled by two-component signal transduction system rather than the multicomponent phosphorelay (Steiner et al., 2011; Underwood et al., 2009).

2.7. Bacterial spore germination

Bacterial spores are formed from vegetative cells in the process of sporulation when cells encounter unfavorable growth conditions (Piggot and Hilbert, 2004). Spores are metabolically dormant and highly resistant to a variety of environmental stresses (Setlow, 2006). In spite of their dormancy, spores monitor their surrounding environments and respond rapidly to the favorable growth conditions by initiating the process of spore germination, followed by outgrowth and ultimately return to actively growing cells (Moir, 2006; Setlow, 2003; Setlow and Johnson, 2007). The process of spore germination is initiated by the presence of nutrients, termed germinants, including amino acids, sugars, and purine nucleocides (Setlow, 2003). Furthermore, the combination of nutrients could also trigger spore germination. For example, a mixture of asparagine, glucose, fructose, and K^+ (AGFK) induces germination of spores of *Bacillus* and *Clostridium* spp. (Paredes-Sabja et al., 2008c; Setlow, 2003;

Wax and Freese, 1968). Germination of spores proceeds in two stages, I and II (Paidhungat and Setlow, 2002; Setlow, 2003). Permeation proteins facilitate movement of nutrient germinant through spore's outer layer structure. Then, germinant molecules interact with their cognate germinant receptor proteins located in the spore's inner membrane (Hudson et al., 2001; Paidhungat and Setlow, 2001). After spores exposed to nutrient germinants, H^+ , monovalent cations, and Zn^{2+} were released from spore core. This leads to spore core's pH raise ~ 1 pH unit that is an essential change for the spore metabolism once core's water content is sufficient for enzyme action. Then, the spore core's large depot of pyridine-2,6-dicarboxylic acid (dipicolinic acid; DPA) and its associated divalent cations, primarily Ca^{2+} are also released. DPA release from the core is replaced with water leading to an increase in spore core water content resulting in decreased moist heat resistance. At this stage; nevertheless, the core hydration level is not high enough for allowing either protein motion or enzyme action (Cowan et al., 2003; Setlow et al., 2001). All these early events in spore germination are considered as stage I. Stage II of germination begins with the hydrolysis of spore's peptidoglycan cortex. The swelling of spore core through more water uptake and expansion of germ cell wall accompany this event; thus, completing the germination process. Upon full hydration of spore core, the protein mobility resume thereby allowing enzyme activity and metabolism followed by macromolecular synthesis in the subsequent developmental process of spore outgrowth that converts the germinated spores into actively growing cells (Paidhungat and Setlow, 2002; Setlow, 2003).

2.8. Germinants and germination receptors of *C. perfringens*

Sensing the presence of nutrient in the surrounding environments via spore's germinant receptors is the initial step for triggering spore germination process (Moir, 2006; Moir et al., 2002; Setlow, 2003). Compounds triggering germination of *C. perfringens* spores are not well characterized. Limited studies with few strains of *C. perfringens* reported that lysozyme is required for germination of the heat-damaged *cpe*-negative NCTC3624 spores (Cassier and Sebald, 1969), and 50 to 100 mM KCl at pH ranges of 5.0 to 9.0 was a strong germinants for spores of FP strain NCTC8238. The recent study is consistent with the latter in which 100 to 200 mM KCl can induce germination of spores of most *C. perfringens* FP isolates. Like *B. subtilis*, AGFK is a potent germinant for *C. perfringens* SM101 spores; however, much of the stimulatory effect of AGFK is owing to the presence of K⁺. Additionally, the mixture of L-asparagine and KCl (AK) could efficiently induce germination of spores of both *C. perfringens* FP and NFB isolates (Paredes-Sabja et al., 2008c).

A variety of compounds, individually or in combination, are capable of initiating germination of bacterial spores. Previous studies suggested that multiple germination receptors work in concert to initiate the germination cascade (Atluri et al., 2006; Christie and Lowe, 2007). In their environment, spores are perhaps exposed to various germination stimuli simultaneously. Since multiple germination receptors seem to interact synergistically (Atluri et al., 2006; Wolgamott and Durham, 1971), it is plausible that these multiple interlocking pathways would enhance the binding

affinity of each cooperating receptor for their cognate ligand (Ross and Abel-Santos, 2010).

In the genome of *C. perfringens* SM101, four ORFs (CPR0614, CPR0615, CPR0616, and CPR1053) encoding proteins with significant similarity to the GerA-type receptor proteins of *B. subtilis* (50-55%) were identified and characterized in the previous investigations (Paredes-Sabja et al., 2009c; Paredes-Sabja et al., 2008c). Owing to the high similarity with the “A” subunit of GerA-type of *B. subtilis* receptor proteins, ORF CPR1053 was termed GerAA. While the *gerK* locus in *C. perfringens* SM101 consists of three ORFs, CPR0614, CPR0615, and CPR0616 were designated as *gerKB*, *gerKA*, and *gerKC* with 39 to 56% similarity to the respectively orthologues in *B. subtilis*. Among these, *gerKA* and *gerKC* are organized in a bicistronic operon, whereas *gerKB* is transcribed in an opposite direction from the *gerKA-KC* (Paredes-Sabja et al., 2008c).

GerKA and/or GerKC are required for germination of spores of *C. perfringens* SM101 with L-asparagine, while they showed a partial role in spore germination with AK and KCl compared with spores of the parental strain (Paredes-Sabja et al., 2008c). These results suggested that products of *gerK* operon are involved in nutrient germination. It was notable that GerKA-KC also has a major role in *C. perfringens* spore germination with exogenous Ca-DPA. On the other hand, *gerAA* mutant spores germinated similarly to the wild type strain with all known germinants at high concentration, suggesting an auxiliary role in initiating germination. Furthermore, GerKB is required for normal germination of *C. perfringens* SM101 spores under sub-

optimal concentration of KCl, AK, and L-asparagine, but it does not have a role in Ca-DPA germination as was observed for spores lacking GerAA (Paredes-Sabja et al., 2009c; Paredes-Sabja et al., 2008c).

2.9. Spore inactivation

C. perfringens remains concern to food industry due to its ability to produce metabolically dormant spores that are highly resistant to various stresses related to food preservation approaches such as moist heat, extreme pH, osmotic, nitrite, prolong low temperature storage, and high pressure processing (Li and McClane, 2006a, 2006b; Paredes-Sabja et al., 2007; Sarker et al., 2000). If the conditions are favorable, these spores can germinate, outgrow and rapidly proliferate in contaminated foods that are temperature-abused during cooling and storage (McClane, 2007a). Alternative technologies to the conventional heat processing treatment for spore inactivation by utilizing the natural antimicrobial compounds in foods has gained interest recently in order to meet consumer expectations for increased food safety, extended shelf life and improved food quality with low chemical preservatives (Novak and Juneja, 2002; Thomas and Delves-Broughton, 2005).

2.10. Nisin

Nisin is a 34-residue-long polypeptide that produced by certain strains of food grade lactic acid bacterium *Lactococcus lactis* subsp. *lactis* (Delves-Broughton, 2005; Thomas and Delves-Broughton, 2005). It belongs to the family of lantibiotics (lanthionine-containing antibiotics) and is overall positive charged (+4) with the structure possesses amphipathic character; the N-terminal contains various

hydrophobic residues, whereas the C-terminal is more hydrophilic (Breukink and De Kruijff, 1999; Thomas and Delves-Broughton, 2005). Nisin is ribosomally synthesized as a linear precursor peptide and then post-translationally modified in the way that serine and threonine residues are dehydrated giving dehydroalanine and dehydrobutyryne, respectively. Subsequently, five dehydrated residues formed the characteristic lanthionine or β -methyllanthionine rings by coupling to cysteine residues (Breukink and De Kruijff, 1999; Chatterjee et al., 2005).

Nisin has broad-spectrum antimicrobial activity toward Gram-positive bacteria by exerting two distinct mechanisms (Breukink and De Kruijff, 2006). First, it forms pores in the cell membranes, and dissipates the proton motive force. This inhibits amino acid uptake as well as promotes rapid efflux of small metabolites, ions, or cytoplasmic solute (Jung and Sahl, 1991). Second, nisin inhibits the biosynthesis of bacterial cell wall by binding to and mislocalization of lipid II, an essential intermediate for cell wall biosynthesis (Breukink and De Kruijff, 2006). These two-edged sword mode of action are primarily explained for the rare emergence of microbial resistance to nisin although it has been widely used as a food preservative for a long period of time (Gut et al., 2011). Besides its activity against vegetative cells, nisin can also inhibit bacterial spores (Eastoe and Long, 1959; Gut et al., 2008; Montville et al., 2006; Rayman et al., 1981; Scott and Taylor, 1981). Spores of *Bacillus* and *Clostridium* spp. were reported to be very sensitive to nisin and sensitivity is increased by acidic condition and spore-damaging heat treatments (Thomas and Delves-Broughton, 2005). However, the mode of action of nisin against

spores has much less been studied. Recent findings in *Bacillus anthracis* indicated that membrane disruption by nisin prevents the establishment of membrane potential and oxidative metabolism leading to the inhibition of spore outgrowth (Gut et al., 2011; Gut et al., 2008).

Nisin has been approved for use as an antimicrobial agent in foods since 1969 by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (Delves-Broughton, 2005). In the United States, nisin was approved as Generally-Recognized-As-Safe (GRAS) substance by FDA in 1988 and currently has been permitted for use in over 50 countries on a variety of products such as processed cheese, cereal and starch-based dessert, pasteurized vegetables, ready-to-eat soup and broth, and liquid egg products (Delves-Broughton, 2005; Thomas and Delves-Broughton, 2005). The added levels are dependent on types of food, heat processing, pH, storage conditions, initial bacterial load, and the required shelf life. It is effective over a wide range of pHs (pH 3.5 to 8.0) (Rayman et al., 1981; Thomas and Delves-Broughton, 2005). Nisin possesses numerous positive attributes as a food preservative including broad-spectrum antimicrobial activity, long history of successful usage, no apparent cross-resistance in bacteria that may affect antibiotic therapeutics, is quickly digested, and has no off-flavors and off-odors contribution to food products (Jay et al., 2005; Thomas and Delves-Broughton, 2005)

2.11. Chemical disinfecting agents

The objective of utilizing chemical sanitizers is to sanitize the food contact surfaces in order to eliminate or at least reduce the potentially harmful bacteria. A variety of disinfecting agents that vary in compositions and activity are available for use in food processing, foodservice operations and households, depending on the use pur. Furthermore, several factors affect the efficiency of chemical sanitizers such as exposure time, temperature, pH, concentration, equipment cleanliness, water hardness, microbial load, and surface properties of bacterial cells and equipment (Cords et al., 2005; Husmark and Rönner, 1992; Marriott, 1999; Parkar et al., 2001). Therefore, the characteristics of each sanitizing agent must be understood in order that the most appropriate sanitizer for a specific application as well as the optimal usage conditions could be selected properly.

2.11.1. Alcohol

Several investigators suggested that alcohol lacks sporicidal activity in general; thus, it is categorized as sporostatic agent although at appropriate concentration, ethanol is rapidly lethal against numerous of vegetative bacteria and fungi (Ali et al., 2001; Hared et al., 1963; Heuzenroeder and Johnson, 1958; McDonnell and Russell, 1999; Russell, 1990). By contrast, some previous studies exhibited the sporicidal property of ethanol with extended exposure time. For example, *B. anthracis* spores were killed by ethanol concentrations between 42% and 100% within 48 hours and the survivals of anaerobic bacteria were reduced after treatment with 40% to 80% ethanol for 4 weeks (Ali et al., 2001). Alcohol is considered to be non-specific antimicrobial

owing to its multiplicity of toxic effect mechanisms. For vegetative cells, the principle modes of action are protein coagulation/denaturation. Furthermore, mechanisms of alcohol killing by associated disruptions of cytoplasmic integrity, cell lysis, and interference of cellular metabolism were reported as well (Ali et al., 2001; McDonnell and Russell, 1999).

Alcohol is known to inhibit bacterial spore germination at concentrations similar to those that inhibit the growth of vegetative cells; nevertheless, the inhibitory effect of spore germination might be reversible (Trujillo and Laible, 1970). This phenomenon might be described by a fairly loose binding of an inhibitor to its site(s) of action on spore surface because mere washing is adequate to dislodge the inhibitor (Ali et al., 2001; Trujillo and Laible, 1970). Alcohol was reported to inhibit L-alanine-triggered germination of *B. subtilis* spores and this inhibition is attributed to an interaction of a hydrophobic region in or near the receptor site(s) for L-alanine on the spores with the hydrophobic group on the alcohol (Yasuda-Yasaki et al., 1978).

Alcohol-based products are typically applied for mid-shift cleaning and disinfecting in high-risk area during production to limit water usage as a control measure to prevent growth and spread of pathogenic bacteria. It is not tolerant to the presence of organic matter; therefore, surfaces need to be thoroughly cleaned, and then alcohol reapplied. The most effective concentrations range from 60% to 70% (Holah, 2003).

2.11.2. Quaternary Ammonium Compounds (Quats)

The Quats are derivatives of ammonium salts (NH_4X) which hydrogen atoms are replaced by alkyl groups (R1 to R4). The sum of carbon atoms in the four R group is more than 10, and at least one R group must contain a chain length in the range of C_8 to C_{18} (Russell, 1990). These make the Quats family diverse and variations in antimicrobial properties have been previously reported (Cords et al., 2005).

Quats have antimicrobial activity against bacteria, yeasts, molds, protozoa, and viruses. They are sporostatic but not sporicidal (Cords et al., 2005; Russell, 1990). Unlike alcohol, Quats do not inhibit spore germination. They rather affect spore outgrowth, albeit via an unknown mechanism (McDonnell and Russell, 1999; Russell, 1990; Russell et al., 1985). It is likely that Quats are able to bind strongly to the spore coat, but they cannot penetrate into the spore. This might account for the lack of sporicidal property of this disinfectant (Rosenberg et al., 1985). Mode of action of Quats is mainly involved with cell membrane damage, but their effects on denaturation of essential proteins and release of nitrogenous and phosphorous-containing cell constituents are established as well (Baker et al., 1941; Cords et al., 2005; Hotchkiss, 1946). Various environmental factors affect an antimicrobial activity of Quats such as pH, temperature, organic soil, and water hardness. Generally, the biocidal efficacy of Quats is enhanced with increasing temperature (Cords et al., 2005). The effectiveness of Quats is dramatically decreased in the presence of organic matters; however, this phenomenon depends on the type of soil and the type of Quats involved (Holah, 2003).

Many positive aspects contribute to used of Quats as a disinfectant. They are colorless, relatively odorless, non-corrosive to metals, non-irritating to the skin, and relatively low toxicity to most common surface material equipment (Cords et al., 2005; Holah, 2003). Quats are frequently used on floors, walls, furnishing, and equipment. Owing to their good penetrant property, they are suitable for porous surfaces (Marriott, 1999).

2.11.3. Iodophores

Iodophores are the combination of iodine and surface-active agents that act as carriers and solubilizers for the iodine. It was discovered in 1949 that iodine can solubilize in polyvinylpyrrolidone (PVP) and surface-active agent and these formed complexes that retain the biocidal property of iodine and reduce the undesirable property of iodine. These iodophore preparations were able to solubilize nearly 30% of their weight of iodine and release an available iodine upon dilution of the concentrated solution (Davis, 1962). The complex of iodine and carrier serves three important functions: (i) to increase solubility of iodine, (ii) to provide a sustained-release reservoir of iodine, and (iii) to reduce the equilibrium concentration of free molecular iodine (Gottardi, 2001).

The concentration of free iodine in iodophore is the critical factor determining its bactericidal and sporicidal activity and spore-killing efficiency of iodophore is strongly dependent on pH and concentration. The acidic pH and higher than normal general sanitation of iodophore solution were required for inactivation of bacterial spores (Russell, 1990). The molecular iodine can penetrate the cell wall of

microorganisms rapidly and this lead to the fundamental of the mode of action of iodine-based antimicrobial agent which cause denaturation of DNA, cell wall damage and loss of intracellular materials (Gottardi, 2001). In *B. subtilis*, killing of spores by iodine-based disinfectant is not due to DNA damage. This agent rather inactivates, either directly or indirectly, one or more of spore cortex lytic enzymes or it might modify the cortex itself in the way that the latter will not be the substrate for the endogenous corticolytic enzyme (Tennen et al., 2000).

Various factors influence the efficacy of iodophore as a germicide. At typical used concentration, iodophores are most effective at pH 2.5 to 3.5; nevertheless, iodophores are less sensitive to pH alterations than chlorine-containing compounds. At concentrated form, iodophores have a long shelf life at room temperature; however, excessive loss of iodine occurs at temperature above 50 °C. Under normal application condition in food industry, the vaporization of iodine is minimal (Cords et al., 2005; Holah, 2003; Marriott, 1999). Hard water does not affect the germicidal activity of iodophore; however, the presence of excessive organic matters could result in loss of iodine in the iodophore solution. Because of their amber color, this provides the visible evidence of the tentative presence of disinfectant (Marriott, 1999). Iodophores are widely used in food processing plants for sanitizing food-handling equipment as well as for hand sanitizing since iodophores are not skin-irritation (Marriott, 1999).

2.11.4. Hydrogen peroxide (HP)

HP (H_2O_2) is effective against a wide range of microorganisms including bacteria, yeasts, molds, viruses, and endospore-forming bacteria (McDonnell and

Russell, 1999). Gram-positive bacteria were more susceptible to the action of HP than Gram-negative bacteria and high concentration of HP (10% to 30%), rise in solution temperature, and long contact time were required for its sporicidal property (Block, 2001; McDonnell and Russell, 1999). The anaerobic bacteria was reported to be more sensitive because they cannot produce catalase to break down HP (Block, 2001). However, HP can be deactivated by catalase and peroxidases (Russell, 1990). For HP, the destruction of microorganisms involved the formation of the hydroxyl radical from the reaction of HP with superoxide ion. The hydroxyl radicals are responsible for bacterial killing by attacking membrane lipids, DNA, and other essential cell components (Block, 2001; Keyer et al., 1995). The presence of hydroxyl radicals is also essential for the sporicidal activity of HP against spores of *C. perfringens* causing the protoplast lysis (Ando and Tsuzuki, 1986a, 1986b).

HP had been approved by FDA for sterilization of equipment and containers in aseptic packaging of foods and beverages (Cords et al., 2005). Furthermore, HP can be applied on all types of surfaces, equipment, floors, walls, steel mesh gloves, belts, and other areas where contamination exists (Marriott, 1999). A major benefits of HP as a disinfectant is its low toxicity at the used concentrations and its safe decomposition product of water and oxygen (Cords et al., 2005). Several factors affect the efficiency of HP for its antimicrobial property. The greater activity of HP was achieved at acid pH ranges and higher operating temperature. As with other antimicrobial agents, increasing HP concentrations leads to shorten of the exposure time and fasten the rate of kill of microorganisms (Cords et al., 2005).

2.11.5. Peroxyacetic acid or peracetic acid (PAA)

PAA (CH_3COOOH) is strong oxidizing agent with a broad spectrum of antimicrobial activity. It is bactericidal as well as sporicidal at higher concentration (Milojkovic, 1969). By comparison to HP, PAA is more potent germicidal agent that is effective at cooler temperatures and lower concentration. Furthermore, PAA has higher tolerance to the presence of organic materials and is unaffected by catalase and peroxidases (Block, 2001; Cords et al., 2005; Russell, 1990). Similar to HP, PAA may denature proteins and enzymes, and increase cell wall permeability via the disruption of sulhydryl (-SH) and sulfur (S-S) bonds (Baldry and Fraser, 1988). It was established that hydroxyl radicals are the lethal species involved in bactericidal activity of PAA and HP (Clapp et al., 1994). Additionally, small, acid-soluble protein that bind spore's DNA might be reacted with HP and PAA to leave the DNA unprotected, thereby being susceptible to attack by these peroxygens (Setlow and Setlow, 1993). Nevertheless, it is notable that PAA and HP display synergism in their antimicrobial activity (Alasri et al., 1993).

In 1986, FDA approved PAA to be applied as an indirect food additive in a sanitizing solution on food contact surfaces in food processing plants (Block, 2001; Cords et al., 2005). It is stable under an ambient temperature for several days, but depleted more rapidly at elevated temperatures. At the typically used concentration, PAA has little odor, non-corrosive to stainless steel and aluminum as well as good tolerance to hard water. Moreover, it decomposes into HP, water, oxygen and acetic acid, which are non-toxic when present in food or other environments (Cords et al.,

2005). PAA has been used worldwide in food processing and beverage industries including meat and poultry processing plants, canneries, dairies, breweries, wineries, and soft-drink plants (Block, 2001).

References

- Abee, T., Groot, M.N., Tempelarrs, M., Zwietering, M., Moezelaar, R., & Van de Voort, M. (2011). Germination and outgrowth of spores of *Bacillus cereus* group members: Diversity and role of germinant receptors. *Food Microbiology*, *28*, 199-208.
- Akhtar, S., Paredes-Sabja, D., Torres, J.A., & Sarker, M.R. (2009). Strategy to inactivate *Clostridium perfringens* spores in meat products. *Food Microbiology*, *26*, 272-277.
- Alasri, A., Valverde, M., Rogues, C., Michel, G., Cabassud, C., & Aptel, P. (1993). Sporicidal properties of peracetic acid and hydrogen peroxide, alone and in combination, in comparison with chlorine and formaldehyde for ultrafiltration membrane disinfection. *Canadian Journal of Microbiology*, *39*, 52-60.
- Alberto, F., Broussolle, V., Mason, D.R., Carlin, F., & Peck, M.W. (2003). Variability in spore germination response by strains of proteolytic *Clostridium botulinum* types A, B and F. *Letters in Applied Microbiology*, *36*, 41-45.
- Ali, Y., Dolan, M.J., Fendler, E.J., & Larson, E.L. (2001). Alcohols. In: Block, S.S. (Ed.), *Disinfection, sterilization, and preservation* (pp. 229-254). Philadelphia: Lippincott Williams & Wilkins.
- Ando, Y., & Tsuzuki, T. (1986a). Changes in decoated spores of *Clostridium perfringens* caused by treatment with some enzymatic and non-enzymatic systems. *Letters of Applied Microbiology*, *3*, 61-64.
- Ando, Y., & Tsuzuki, T. (1986b). The effect of hydrogen peroxide on spores of *Clostridium perfringens*. *Letters in Applied Microbiology*, *2*, 65-68.
- Antoniewski, C., Savelli, B., & Stragier, P. (1990). The *SpoIIJ* gene, which regulates early development steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. *Journal of Bacteriology*, *172*, 86-93.
- Atluri, S., Ragkousi, K., Cortezzo, D.E., & Setlow, P. (2006). Cooperativity between different nutrient receptors in germination of spores of *Bacillus subtilis* and reduction of this cooperativity by alterations in the GerB receptor. *Journal of Bacteriology*, *188*, 28-36.
- Baker, Z., Harrison, R.W., & Miller, B.F. (1941). Action of synthetic detergents on metabolism of bacteria. *Journal of Experimental Medicine*, *73*, 249.

- Baldry, M.G.C., & Fraser, J.A.L. (1988). Disinfection with peroxygens. *Critical Reports in Applied Chemistry* 22, 91-116.
- Banerjee, M., & Sarkar, P.K. (2004). Antibiotic resistance and susceptibility to some food preservative measures of spoilage and pathogenic micro-organisms from spices. *Food Microbiology*, 21, 335-342.
- Bhatti, M.A., Veeramachaneni, A., & Shelef, L.A. (2004). Factors affecting the antilisterial effects of nisin in milk. *International Journal of Food Microbiology*, 97, 215-219.
- Block, S.S. (2001). Peroxygen compounds. In: Block, S.S. (Ed.), *Disinfection, sterilization, and preservation* (pp. 185-204). Philadelphia: Lippincott Williams & Wilkins.
- Borriello, S.P., Larson, H.E., Welch, A.R., Barclay, F., Stringer, M.F., & Bartholomew, B.A. (1984). Enterotoxigenic *Clostridium perfringens*: a possible cause of antibiotic-associated diarrhea. *Lancet*, 1, 305-307.
- Breukink, E., & de Kruijff, B. (1999). The lantibiotic nisin, a special case or not? *Biochimica et Biophysica Acta*, 1462, 223-234.
- Breukink, E., & de Kruijff, B. (2006). Lipid II as a target for antibiotics. *Nature Reviews Drug Discovery*, 5, 321-332.
- Broussolle, V., Gauillard, F., Nguyen-The, C., & Carlin, F. (2008). Diversity of spore germination in response to inosine and L-alanine and its interaction with NaCl and pH in the *Bacillus cereus* group. *Journal of Applied Microbiology*, 105, 1081-1090.
- Brynstad, S., & Granum, P.E. (2002). *Clostridium perfringens* and foodborne infections. *International Journal of Food Microbiology*, 74, 195-202.
- Brynstad, S., Synstad, B., & Granum, P.E. (1997). The *Clostridium perfringens* enterotoxin gene is on a transposable element in type A human food poisoning strains. *Microbiology*, 143, 2109-2115.
- Burbulys, D., Trach, K.A., & Hoch, J.A. (1991). Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. *Cell*, 64, 545-552.
- Buzby, J.C., & Roberts, T. (1997). Economic costs and trade impacts of microbial foodborne illness. *World Health Statistics Quarterly*, 50, 57-66.
- Cassier, M., & Sebald, M. (1969). Germination lysozyme-dépendante des spores de *Clostridium perfringens* ATCC3624 après traitement thermique. *Ann Institut Pasteur Actualit*, 117, 312-324.
- Chakrabarti, G., Zhou, X., & McClane, B.A. (2003). Death pathways activated in CaCo-2 cells by *Clostridium perfringens* enterotoxin. *Infection and Immunity*, 71, 4260-4270.
- Chatterjee, C., Paul, M., Xie, L., & van der Donk, W.A. (2005). Biosynthesis and mode of action of lantibiotics. *Chemical Reviews*, 105, 633-683.
- Christie, G., & Lowe, C.R. (2007). Role of chromosomal and plasmid-borne receptor homologues in the response of *Bacillus megaterium* QM B1551 spores to germinants. *Journal of Bacteriology*, 189, 4375-4383.

- Clapp, P.A., Davies, M.J., French, M.S., & Gilbert, B.C. (1994). The bactericidal action of peroxides; on E.P.R. spin-trapping study. *Free Radical Research*, *21*, 147-167.
- Collie, R.E., Kokai-Kun, J.F., & McClane, B.A. (1998). Phenotypic characterization of enterotoxigenic *Clostridium perfringens* isolates from non-foodborne human gastrointestinal diseases. *Anaerobe*, *4*, 69-79.
- Collie, R.E., & McClane, B.A. (1998). Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. *Journal of Clinical Microbiology*, *36*, 30-36.
- Cords, B.R., Burnett, S.L., Hilgren, J., Finley, M., & Magnusen, J. (2005). Sanitizers: Halogens, surface - active agents, and peroxides. In: Davidson, P.M., Sofos, J.N., Branen, A.L. (Eds.), *Antimicrobials in food* (pp. 507-572). Boca Raton: CRC Press.
- Cornillot, E., Saint-Joanis, B., Daube, G., Katayama, S., Granum, P.E., Canard, B., & Cole, S.T. (1995). The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. *Molecular Microbiology*, *15*, 639-647.
- Cowan, A.E., Koppel, D.E., Setiawan, B., & Setlow, P. (2003). A cytoplasmic protein is immobile in the cytoplasm of dormant spores of *Bacillus subtilis*: implications of spore dormancy. *Proceedings of the National Academy of Sciences* *100*, 4209-4214.
- Czczulin, J.R., Hanna, P.C., & McClane, B.A. (1993). Cloning, nucleotide sequencing, and expression of the *Clostridium perfringens* enterotoxin gene in *Escherichia coli*. *Infection and Immunity*, *61*, 3429-3439.
- Davis, J.G. (1962). Iodophores as detergent-sterilizers *Journal of Applied Bacteriology*, *25*, 195.
- de Jong, I.G., Veening, J.-W., & Kuipers, O.P. (2010). Heterochronic phosphorelay gene expression as source of heterogeneity in *Bacillus subtilis* spore formation. *Journal of Bacteriology*, *192*, 2053-2067.
- Deguchi, A., Miyamoto, K., Kuwahara, T., Miki, Y., Kaneko, I., Li, J., McClane, B.A., & Akimoto, S. (2009). Genetic characterization of type A enterotoxigenic *Clostridium perfringens* strains. *PLoS ONE*, *4*, e5598.
- Delves-Broughton, J. (2005). Nisin as a food preservative. *Food Australia*, *57*, 525-527.
- Duncan, C.L., Strong, D.H., & Sebald, M. (1972). Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. *Journal of Bacteriology*, *110*, 378-391.
- Dürre, P., & Hollergschwandner, C. (2004). Initiation of endospore formation in *Clostridium acetobutylicum*. *Anaerobe*, *10*, 69-74.
- Eastoe, J.E., & Long, J.E. (1959). The effect of nisin on the growth of cells and spores of *Clostridium welchii* in gelatine. *Journal of Applied Bacteriology*, *22*, 1-7.
- Errington, J. (2003). Regulation of endospore formation in *Bacillus subtilis*. *Nature Reviews Microbiology*, *1*, 117-126.

- Fawcett, P., Eichenberger, P., Losick, R., & Youngman, P. (2000). The transcriptional profile of early to middle sporulation in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences* 97, 8063-8068.
- Fujita, K., Katahira, J., Horiguchi, Y., Sonoda, N., Furuse, M., & Tsukita, S. (2000). *Clostridium perfringens* enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein. *FEBS Letters*, 476, 258-261.
- Fujita, M., González-Pastor, J.E., & Losick, R. (2005). High- and low- threshold genes in the Spo0A regulon of *Bacillus subtilis*. *Journal of Bacteriology*, 187, 1357-1368.
- Fujita, M., & Losick, R. (2003). The master regulator for entry into sporulation in *Bacillus subtilis* becomes a cell-specific transcription factor after asymmetric division. *Genes & Development*, 1166-1174.
- Gottardi, W. (2001). Iodine and iodine compounds. In: Block, S.S. (Ed.), *Disinfection, sterilization, and preservation* (pp. 159-184). Philadelphia: Lippincott Williams & Wilkins.
- Gould, G.W., Jones, A., & Wrighton, C. (1968). Limitations of the initiation of germination of bacterial spores as a spore control procedure. *Journal of Applied Bacteriology*, 31, 357-366.
- Guerlava, P., Nolf, S., & Tholozan, J.L. (1998). Rapid cooling, moderate heat treatment and nisin addition influence cell homeostasis of *Clostridium perfringens* type A. *International Journal of Food Microbiology*, 39, 195-203.
- Gut, I.M., Blanke, S.R., & van der Donk, W.A. (2011). Mechanism of inhibition of *Bacillus anthracis* spore outgrowth by the lantibiotic nisin. *ACS Chemical Biology*, 6, 744-752.
- Gut, I.M., Prouty, A.M., Ballard, J.D., van der Donk, W.A., & Blanke, S.R. (2008). Inhibition of *Bacillus anthracis* spore outgrowth by nisin. *Antimicrobial Agents and Chemotherapy*, 52, 4281-4288.
- Haldenwang, W.G. (1995). The sigma factors of *Bacillus subtilis*. *Microbiology and Molecular Biology Reviews*, 59, 1-30.
- Hared, R., Baik, E., & Gash, S. (1963). Efficiency of antiseptic when acting on dried organisms. *British Medical Journal*, 1.
- Harris, L.M., Welker, N.E., & Papoutsakis, E.T. (2002). Northern, morphological, and fermentation analysis of *spo0A* inactivation and overexpression in *Clostridium acetobutylicum* ATCC824. *Journal of Bacteriology*, 184, 3586-3597.
- Harry, K.H., Zhou, R., Kroos, L., & Melville, S.B. (2009). Sporulation and enterotoxin (CPE) synthesis are controlled by the sporulation-specific sigma factors SigE and SigK in *Clostridium perfringens*. *Journal of Bacteriology*, 191, 2728-2742.
- Heikinheimo, A., Lindström, M., Granum, P.E., & Korkeala, H. (2006). Humans as reservoir for enterotoxin gene-carrying *Clostridium perfringens* type A. *Emerging Infectious Diseases*, 12, 1724-1729.

- Heuzenroeder, M., & Johnson, K.D. (1958). Sterilization of chemical agents. *Journal of Pharmaceutical Sciences*, *40*, 944-948.
- Hilbert, D.W., & Piggot, P.J. (2004). Compartmentalization of gene expression during *Bacillus subtilis* spore formation. *Microbiology and Molecular Biology Reviews*, *68*, 234-262.
- Hitchins, A.D., & Slepecky, R.A. (1969). Bacterial sporulation as a modified procaryotic cell division. *Nature*, *223*, 804-807.
- Hoch, J.A. (1993a). The phosphorelay signal transduction pathway in the initiation of *Bacillus subtilis* sporulation. *Journal of Cellular Biochemistry*, *51*, 55-61.
- Hoch, J.A. (1993b). Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis*. *Annual Review of Microbiology*, *47*, 441-465.
- Hofmeister, A.E., Londono-Vallejo, A., Harry, E., Stragier, P., & Losick, R. (1995). Extracellular signal protein triggering the proteolytic activation of a developmental transcription factor in *Bacillus subtilis*. *Cell*, *83*, 219-226.
- Holah, J.T. (2003). Cleaning and disinfection. In: Lelieveld, H.L.M., Mostert, M.A., Holah, J.White, B. (Eds.), *Hygiene in food processing* (pp. 235-278). Boca Raton, FL: CRC Press.
- Hornstra, L.M., de Leeuw, P.L.A., Moezelaar, R., Wolbert, E.J., de Vries, Y.P., de Vos, W.M., & Abee, T. (2007). Germination of *Bacillus cereus* spores adhered to stainless steel. *International Journal of Food Microbiology*, *116*, 367-371.
- Hotchkiss, R.D. (1946). The nature of bactericidal action of surface active agents. *Annals of the New York Academy of Sciences*, *46*, 479.
- Huang, I.H., & Sarker, M.R. (2006). Complementation of a *Clostridium perfringens* *spo0A* mutant with wild-type *spo0A* from other *Clostridium* species. *Applied Environmental Microbiology*, *72*, 6388-6393.
- Huang, I.H., Waters, M., Grau, R.R., & Sarker, M.R. (2004). Disruption of the gene (*spo0A*) encoding sporulation transcription factor blocks endospore formation and enterotoxin production in enterotoxigenic *Clostridium perfringens* type A. *FEMS Microbiology Letters*, *233*, 233-240.
- Hudson, K.D., Corfe, B.M., Kemp, E.H., Feavers, I.M., Coote, P.J., & Moir, A. (2001). Localization of GerAA and GerAC germination proteins in the *Bacillus subtilis* spore. *Journal of Bacteriology*, *183*, 4317-4322.
- Husmark, U., & Rönner, U. (1992). The influence of hydrophobic, electrostatic, and morphologic properties on the adhesion of *Bacillus* spores. *Biofouling*, *5*, 335-344.
- Indest, K.J., Buchholz, W.G., Faeder, J.R., & Setlow, P. (2009). Workshop report: Modelling the molecular mechanism of bacterial spore germination and elucidating reasons for germination heterogeneity. *Journal of Food Science*, *74*, R73-R78.
- Jay, J.M., Loessner, M.J., & Golden, D.A., 2005. *Modern Food Microbiology*. Springer Science + Business Media ,Inc, San Marcos, California.

- Jiang, M., Shao, W., Perego, M., & Hoch, J.A. (2000). Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. *Molecular Microbiology*, *38*, 535-542.
- Juneja, V.K., & Thippareddi, H. (2004). Inhibitory effects of organic acid salts on growth of *Clostridium perfringens* from spore inocula during chilling of marinated ground turkey breast. *International Journal of Food Microbiology*, *93*, 155-163.
- Jung, G., & Sahl, H.G., 1991. *Nisin and novel lantibiotics*. Escom, Leiden, The Netherlands.
- Keyer, K., Gort, A.S., & Imlay, J.A. (1995). Superoxide and the production of oxidative DNA damage. *Journal of Bacteriology*, *177*, 6782-6790.
- Kokai-Kun, J.F., & McClane, B.A. (1997). Deletion analysis of the *Clostridium perfringens* enterotoxin. *Infection and Immunity*, *65*, 1014-1022.
- Kroos, L., Zhang, B., Ichikawa, H., & Yu, Y.-T.N. (1999). Control of σ factor activity during *Bacillus subtilis* sporulation. *Molecular Microbiology*, *31*, 1285-1294.
- Labbe, R. (1980). Relationship between sporulation and enterotoxin production in *Clostridium perfringens* type A. *Food Technology*, *34*, 88-90.
- Labbe, R., & Huang, T.H. (1995). Generation times and modeling of enterotoxin-positive and enterotoxin-negative strains of *Clostridium perfringens* in laboratory media and ground beef. *Journal of Food Protection*, *58*, 1303-1306.
- Labbe, R.G. (1989). *Clostridium perfringens*. In: Doyle, M.P. (Ed.), *Food-borne bacterial pathogens* (pp. 192-234). New York: Marcel Dekker.
- Lahti, P., Heikinheimo, A., Johansson, T., & Korkeala, H. (2008). *Clostridium perfringens* type A strains carrying a plasmid-borne enterotoxin gene (genotype IS1151-cpe or IS1470-like-cpe) as a common cause of food poisoning. *Journal of Clinical Microbiology*, *46*, 371-373.
- LeDeaux, J.R., & Grossman, A.D. (1995). Isolation and characterization of *kinC*, a gene that encodes a sensor histidine kinase homologous to the sporulation sensor kinases KinA and KinB in *Bacillus subtilis*. *Journal of Bacteriology*, *177*, 166-175.
- LeDeaux, J.R., Yu, N., & Grossman, A.D. (1995). Different roles for KinA, KinB, and KinC in the initiation of sporulation in *Bacillus subtilis*. *Journal of Bacteriology*, *177*, 861-863.
- Leggett, M.J., McDonnell, G., Denyer, S.P., Setlow, P., & Maillard, J.-Y. (2012). Bacterial spore structures and their protective role in biocide resistance. *Journal of Applied Microbiology*, *113*, 485-498.
- Li, J., & McClane, B.A. (2006a). 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. *Applied and Environmental Microbiology*, *72*, 7620-7625.
- Li, J., & McClane, B.A. (2006b). Further comparison of temperature effects on growth and survival of *Clostridium perfringens* type A isolates carrying a

- chromosomal or plasmid-borne enterotoxin gene. *Applied and Environmental Microbiology*, 72, 4561-4568.
- Li, J., & McClane, B.A. (2010). Evaluating the involvement of alternative sigma factor SigF and SigG in *Clostridium perfringens* sporulation and enterotoxin synthesis. *Infection and Immunity*, 78, 4286-4293.
- Lindström, M., Heikinheimo, A., Lahti, P., & Korkeala, H. (2011). Novel insights into the epidemiology of *Clostridium perfringens* type A food poisoning. *Food Microbiology*, 28, 192-198.
- Løvdal, I.S., Hovda, M.B., Granum, P.E., & Rosnes, J.T. (2011). Promoting *Bacillus cereus* spore germination for subsequent inactivation by mild heat treatment. *Journal of Food Protection*, 74, 2079-2089.
- Lynch, M., Painter, J., Woodruff, R., & Braden, C. (2006). Surveillance for foodborne-disease outbreaks - United States, 1998-2002. *Morbidity and Mortality Weekly Report*, 55, 1-42.
- Marriott, N.G. (1999). Sanitizers. In: Marriott, N.G. (Ed.), *Principles of food sanitation* (pp. 139-157). Gaithersburg: Aspen Publishers, Inc.
- McClane, B.A. (2007). *Clostridium perfringens*. In: Doyle, M.P. Beuchat, L.R. (Eds.), *Food Microbiology: fundamentals and frontiers* (pp. 423-444). Washington, D.C.: ASM Press.
- McClane, B.A., Lyerly, D.J., Moncrief, J.S., & Wilkins, T. (2000). Enterotoxigenic Clostridia. In: Fischetti, V.A., Novick, R., Farretti, J., Potney, D.Rood, J.I. (Eds.), *Gram-positive pathogens*. Washington D.C.: ASM Press.
- McClane, B.A., Uzal, F.A., Miyakawa, M.E.F., Lyerly, D., & Wilkins, T. (2006). The enterotoxigenic clostridia. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. Stackebrandt, E. (Eds.), *The Prokaryotes* (pp. 698-752). New York: Springer New York.
- McDonnell, G., & Russell, A.D. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clinical Microbiology Reviews*, 12, 147-179.
- Miki, Y., Miyamoto, K., Kaneko-Hirano, I., Fujiuchi, K., & Akimoto, S. (2008). Prevalence and characterization of enterotoxin gene-carrying *Clostridium perfringens* isolates from retail meat products in Japan. *Applied and Environmental Microbiology*, 74, 5366-5372.
- Milojkovic, V. (1969). Disinfectant properties of peracetic acid. *Mikrobioljija*, 6, 145-149.
- Moir, A. (2006). How do spores germinate? *Journal of Applied Microbiology*, 101, 526-530.
- Moir, A., Corfe, B.M., & Behravan, J. (2002). Spore germination. *Cellular and Molecular Life Sciences*, 59, 403-409.
- Molle, V., Fujita, M., Jensen, S.T., Eichenberger, P., Gonzalez-Pastor, J.E., Liu, J.S., & Losick, R. (2003). The Spo0A regulon of *Bacillus subtilis*. *Molecular Microbiology*, 50, 1683-1701.
- Montville, T.J., De Siano, T., Nock, A., Padhi, S., & Wade, D. (2006). Inhibition of *Bacillus anthracis* and potential surrogate bacilli growth from spore inocula by

- nisin and other antimicrobial peptides. *Journal of Food Protection*, *69*, 2529-2533.
- Mpamugo, O., Donovan, T., & Brett, M.M. (1995). Enterotoxigenic *Clostridium perfringens* as a cause of sporadic diarrhoea. *Journal of Medical Microbiology*, *43*, 442-445.
- Myers, G.S., Rasko, D.A., Cheung, J.K., Ravel, J., Seshadri, R., DeBoy, R.T., Ren, Q., Varga, J., Awad, M.M., Brinkac, L.M., Daugherty, S.C., Haft, D.H., Dodson, R.J., Madupu, R., Nelson, W.C., Rosovitz, M.J., Sullivan, S.A., Khouri, H., Dimitrov, G.I., Watkins, K.L., Mulligan, S., Benton, J., Radune, D., Fisher, D.J., Atkins, H.S., Hiscox, T., Jost, B.H., Billington, S.J., Songer, J.G., McClane, B.A., Titball, R.W., Rood, J.I., Melville, S.B., & Paulsen, I.T. (2006). Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. *Genome Research*, *16*, 1031-1040.
- Nakamura, M., Kato, A., Tanaka, D., Gyobu, Y., Higaki, S., Karasawa, T., & Yamagishi, T. (2004). PCR identification of the plasmid-borne enterotoxin gene (cpe) in *Clostridium perfringens* strains isolates from food poisoning outbreaks. *International Journal of Food Microbiology*, *294*, 261-265.
- Nerandzic, M.M., & Donskey, C.J. (2010). Triggering germination represents a novel strategy to enhance killing of *Clostridium difficile* spores. *PLoS ONE*, *5*, 1-8.
- Novak, J.S., & Juneja, V. (2002). *Clostridium perfringens*: hazards in new generation foods. *Innovative Food Science & Emerging Technology*, *3*, 127-132.
- Novak, J.S., Peck, M.W., Juneja, V.K., & Johnson, E.A. (2005). *Clostridium botulinum* and *Clostridium perfringens*. In: Fratamico, P.M., Bhunia, A.K., Smith, J.L. (Eds.), *Foodborne pathogen: Microbiology and molecular biology* (pp. 383-408). Norfolk, UK: Caister Academic Press.
- Paidhungat, M., & Setlow, P. (2001). Localization of a germinant receptor protein (GerBA) to the inner membrane of *Bacillus subtilis* spores. *Journal of Bacteriology*, *183*, 3982-3990.
- Paidhungat, M., & Setlow, P. (2002). Spore germination and outgrowth. In: Hoch, J.A., Losick, R., Sonenshein, A.L. (Eds.), *Bacillus subtilis and its relatives: From Genes to cells* (pp. 537-548). Washington, DC: ASM Press.
- Paik, H.D., Kim, H.J., Nam, K.J., Kim, C.J., Lee, S.E., & Lee, D.S. (2006). Effect of nisin on the storage of *sous vide* processed Korean seasoned beef. *Food Control*, *17*, 994-1000.
- Paredes - Sabja, D., Setlow, P., & Sarker, M.R. (2011). Germination of spores of *Bacillales* and *Clostridiales* species: mechanisms and protein involved. *Trends in Microbiology*, *19*, 85-94.
- Paredes, C.J., Alsaker, K.V., & Papoutsakis, E.T. (2005). A comparative genomic view of clostridial sporulation and physiology. *Nature Review Microbiology*, *3*, 969-978.
- Paredes-Sabja, D., Gonzalez, M., Sarker, M.R., & Torres, J.A. (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*, 72, M202-M206.
- Paredes-Sabja, D., Setlow, B., Setlow, P., & Sarker, M.R. (2008a). Characterization of *Clostridium perfringens* spores that lack SpoVA proteins and dipicolinic acid. *Journal of Bacteriology*, 190, 4648-4659.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009a). Role of GerKB in germination and outgrowth of *Clostridium perfringens* spores. *Applied and Environmental Microbiology*, 75, 3813-3817.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009b). SleC is essential for cortex peptidoglycan hydrolysis during germination of spores of the pathogenic bacterium *Clostridium perfringens*. *Journal of Bacteriology*, 191, 2711-2720.
- Paredes-Sabja, D., Torres, J.A., Setlow, P., & Sarker, M.R. (2008b). *Clostridium perfringens* spore germination: characterization of germinants and their receptors. *Journal of Bacteriology*, 190, 1190-1201.
- Paredes-Sabja, D., Udombijitkul, P., & Sarker, M.R. (2009c). Inorganic phosphate and sodium ions are cogerminants for spores of *Clostridium perfringens* type A food poisoning - related isolates *Applied and Environmental Microbiology*, 75, 6299-6305.
- Parkar, S.G., Flint, S.H., Palmer, J.S., & Brooks, J.D. (2001). Factors influencing attachment of thermophilic bacilli to stainless steel. *Journal of Applied Microbiology*, 90, 901-908.
- Perego, M., Cole, S.P., Burbulys, D., Trach, K.A., & Hoch, J.A. (1989). Characterization of the gene for a protein kinase which phosphorylates the sporulation-regulatory proteins Spo0A and Spo0F of *Bacillus subtilis*. *Journal of Bacteriology*, 171, 6187-6196.
- Perego, M., & Hoch, J.A. (2002). Two-component systems, phosphorelays, and regulation of their activities by phosphatase. In: Sonenshein, A.L., Hoch, J.A., Losick, R. (Eds.), *Bacillus subtilis and its closet relatives: From genes to cells* (pp. 473-482). Washington, DC: ASM Press.
- Petit, L., Gilbert, M., & Popoff, M.R. (1999). *Clostridium perfringens*: toxinotype and genotype. *Trends in Microbiology*, 7, 104-110.
- Phillips, A.D., & Strauch, M.A. (2002). *Bacillus subtilis* sporulation and stationary phase gene expression. *Cellular and Molecular Life Sciences*, 59, 392-402.
- Piggot, P.J., & Coote, J.G. (1976). Genetic aspects of bacterial endospore formation. *Bacteriological Reviews*, 40, 908-962.
- Piggot, P.J., & Hilbert, D.W. (2004). Sporulation of *Bacillus subtilis*. *Current Opinion in Microbiology*, 7, 579-586.
- Piggot, P.J., & Losick, R. (2002). Sporulation genes and intercompartmental regulation. In: Sonenshein, A.L., Hoch, J.A., Losick, R. (Eds.), *Bacillus subtilis and its closet relatives* (pp. 483-517). Washington, D.C.: ASM Press.
- Rayman, M.K., Aris, B., & Hurst, A. (1981). Nisin: A possible alternative or adjunct to nitrite in the preservation of meats. *Applied and Environmental Microbiology*, 41, 375-380.

- Rosenberg, E., Brown, D.R., & Demain, A.L. (1985). The influence of gramicidin S on hydrophobicity of germinating *Bacillus brevis* spores. *Archives of Microbiology*, *142*, 51-54.
- Ross, C., & Abel-Santos, E. (2010). The Ger receptor family from sporulating bacteria. *Current Issues in Molecular Biology*, *12*, 147-158.
- Russell, A.D. (1990). Bacterial spores and chemical sporicidal agents. *Clinical Microbiology Reviews*, *3*, 99-119.
- Russell, A.D., Jones, B.D., & Milburn, P. (1985). Reversal of the inhibition of bacterial spore germination and outgrowth by antibacterial agents. *International Journal of Pharmaceutics*, *25*, 105-112.
- Ryter, A. (1965). Etude morphologique de la sporulation de *Bacillus subtilis*. *Ann Institut Pasteur Actuali*, *108*, 40-60.
- Ryu, J.H., & Beuchat, L.R. (2005). Biofilm formation and sporulation by *Bacillus cereus* on a stainless steel surface and subsequent resistance of vegetative cells. *Journal of Food Protection*, *68*, 2614-2622.
- Sarker, M.R., Carman, R.J., & McClane, B.A. (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. *Molecular Microbiology*, *33*, 946-958.
- Sarker, M.R., Shivers, R.P., Sparks, S.G., Juneja, V.K., & McClane, B.A. (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. *Applied and Environmental Microbiology*, *66*, 3234-3240.
- Scallan, E., Griffin, P.M., Angulo, F.J., Tauxe, R.V., & Hoekstra, R.M. (2011a). Foodborne illness acquired in the United States - unspecified agents. *Emerging Infectious Diseases*, *17*, 16-22.
- Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.-A., Roy, S.L., Jones, J.L., & Griffin, P.M. (2011b). Foodborne illness acquired in the United States - major pathogens. *Emerging Infectious Diseases*, *17*, 7-15.
- Scannell, A.G.M., Ross, R.P., Hill, C., & Arendt, E.K. (2000). An effective lacticin biotreservative in fresh pork sausage. *Journal of Food Protection*, *63*, 370-375.
- Scott, V.N., & Taylor, S.L. (1981). Effect of nisin on the outgrowth of *Clostridium botulinum* spores. *Journal of Food Science*, *46*, 117-126.
- Setlow, B., Melly, E., & Setlow, P. (2001). Properties of spores of *Bacillus subtilis* blocked at an intermediate stage in spore germination. *Journal of Bacteriology*, *183*, 4894-4899.
- Setlow, B., & Setlow, P. (1993). Binding of small, acid-soluble spore proteins to DNA plays a significant role in the resistance of *Bacillus subtilis* spores to hydrogen peroxide. *Applied and Environmental Microbiology*, *59*, 3418-3423.
- Setlow, P. (2003). Spore germination. *Current Opinion in Microbiology*, *6*, 550-556.
- Setlow, P. (2006). Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology*, *101*, 514-525.

- Setlow, P., & Johnson, E.A. (2007). *Spores and their significance*. In: Doyle, M.P. Beuchat, L.R. (Eds.), *Food Microbiology: fundamentals and frontiers* (pp. 35-68). Washington, D.C.: American Society for Microbiology.
- Sonenshein, A.L. (2000). Control of sporulation initiation in *Bacillus subtilis*. *Current Opinion in Microbiology*, 3, 561-565.
- Song, M.S., Kim, C.J., Cho, W.I., & Sunwoo, H.H. (2009). Growth inhibition of *Clostridium perfringens* vegetative cells and spores using chicken immunoglobulin Y. *Journal of Food Safety*, 29, 511-520.
- Sparks, S.G., Carman, R.J., Sarker, M.R., & McClane, B.A. (2001). Genotyping of enterotoxigenic *Clostridium perfringens* fecal isolates associated with antibiotic-associated diarrhea and food poisoning in North America. *Journal of Clinical Microbiology*, 39, 883-888.
- Steiner, E., Dago, A.E., Young, D.I., Heap, J.T., Minton, N.P., Hoch, J.A., & Young, M. (2011). Multiple orphan histidine kinases interact directly with Spo0A to control the initiation of endospore formation in *Clostridium acetobutylicum*. *Molecular Microbiology*, 80, 641-654.
- Stephenson, K., & Hoch, J.A. (2002). Evolution of signalling in the sporulation phosphorelay. *Molecular Microbiology*, 46, 297-304.
- Tanaka, D., Isobe, J., Hosorogi, S., Kimata, K., Shimizu, M., Katori, K., Gyobu, Y., Nagai, Y., Yamagishi, T., Karasawa, T., & Nakamura, S. (2003). An outbreak of foodborne gastroenteritis caused by *Clostridium perfringens* carrying the *cpe* gene on a plasmid. *Japanese Journal of Infectious Diseases*, 56, 137-139.
- Tennen, R., Setlow, B., Davis, K.L., Loshon, C.A., & Setlow, P. (2000). Mechanisms of killing of spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. *Journal of Applied Microbiology*, 89, 330-338.
- Thippareddi, H., Juneja, V.K., Phebus, R.K., Marsden, J.L., & Kastner, C.L. (2003). Control of *Clostridium perfringens* germination and outgrowth by buffered sodium citrate during chilling of roast beef and injected pork. *Journal of Food Protection*, 66, 376-381.
- Thomas, L.V., & Delves-Broughton, J. (2005). Nisin. In: Davidsson, P.M., Sofos, J.N., Branen, A.L. (Eds.), *Antimicrobials in food* (pp. 237-274). Boca Raton: CRC Press.
- Trach, K.A., & Hoch, J.A. (1993). Multisensory activation of the phosphorelay initiating sporulation in *Bacillus subtilis*: identification and sequence of the protein kinase of the alternate pathway. *Molecular Microbiology*, 8, 69-79.
- Trujillo, P., & Laible, N. (1970). Reversible inhibition of spore germination by alcohols. *Applied Microbiology*, 20, 620-623.
- Underwood, S., Guan, S., Vijayasubhash, V., Baines, S.D., Graham, L., Lewis, R.J., Wilcox, M.H., & Stephenson, K. (2009). Characterization of the sporulation initiation pathway of *Clostridium difficile* and its role in toxin production. *Journal of Bacteriology*, 191, 7296-7305.

- Vaishnavi, C., & Kaur, S. (2008). *Clostridium perfringens* enterotoxin in antibiotic-associated diarrhea. *Indian Journal of Pathology and Microbiology*, 51, 198-199.
- van der Voort, M., Garcí, D., Moezelaar, R., & Abee, T. (2010). Germination receptor diversity and germination responses of four strains of the *Bacillus cereus* group. *International Journal of Food Microbiology*, 139, 108-115.
- Wax, R., & Freese, E. (1968). Initiation of the germination of *Bacillus subtilis* spores by a combination of compounds in place of L-alanine. *Journal of Bacteriology*, 95, 433-438.
- Wolgamott, G.D., & Durham, N.N. (1971). Initiation of spore germination in *Bacillus cereus*: a proposed allosteric receptor. *Canadian Journal of Microbiology*, 17, 1043-1048.
- Wrigley, D.M. (2001). *Clostridium perfringens*. In: Hui, Y.H., Pierson, M.D., Gorham, J.R. (Eds.), *Foodborne disease handbook* (pp. 139-168). New York: Marcel Dekker, Inc.
- Xiao, Y., Francke, C., Abee, T., & Wells-Bennik, M.H. (2011). Clostridial spore germination versus bacilli: Genome mining and current insights. *Food Microbiology*, 28, 266-274.
- Xiao, Y., Wagendorp, A., Moezelaar, R., Abee, T., & Wells-Bennik, M.H. (2012). A wide variety of *Clostridium perfringens* type A food-borne isolates that carry a chromosomal cpe gene belong to one multilocus sequence typing cluster. *Applied and Environmental Microbiology*, 78, 7060-7068.
- Yasuda-Yasaki, K., Namiki-Kanie, S., & Hachisuka, Y. (1978). Inhibition of *Bacillus subtilis* spore germination by various hydrophobic compounds: demonstration of hydrophobic character of the L-alanine receptor site. *Journal of Bacteriology*, 136, 484-490.

CHAPTER 3**Inhibitory Effects of Nisin Against *Clostridium perfringens* Food Poisoning and Non-food-borne Isolates**

Pathima Udompijtkul, Daniel Paredes-Sabja, and Mahfuzur R. Sarker

Published in:

Journal of Food Science (2012) 77(1): p. M51-M56

ABSTRACT

The enterotoxigenic *Clostridium perfringens* type A is the causative agent of *C. perfringens* type A food poisoning (FP) and non-food-borne (NFB) human gastrointestinal diseases. Due to its ability to form highly resistant endospores, it has become a great concern to the meat industry to produce meat free of *C. perfringens*. In this study, we evaluated the antimicrobial effect of nisin against *C. perfringens* FP and NFB isolates. No inhibitory effect of nisin was observed against germination of spores of both FP and NFB isolates in laboratory medium. However, nisin effectively arrested outgrowth of germinated spores of *C. perfringens* in rich medium. Interestingly, germinated spores of NFB isolates possessed higher resistance to nisin than that of FP isolates. Furthermore, nisin exhibited inhibitory effect against vegetative growth of both FP and NFB isolates in laboratory medium, with vegetative cells of NFB isolates showed higher resistance than that of FP isolates. However, the antimicrobial activity of nisin against *C. perfringens* was significantly decreased in a meat model system. In conclusion, although nisin showed inhibitory effect against spore outgrowth and vegetative cells of *C. perfringens* FP and NFB isolates in laboratory conditions, no such effect was observed against *C. perfringens* spores inoculated into a meat model system.

3.1. Introduction

Clostridium perfringens is a Gram-positive, rod-shaped, spore-forming bacterium with a prolific toxin-producing ability. A small group (~ 5%) of *C. perfringens* type A isolates produce the *C. perfringens* enterotoxin (CPE). The CPE is the major cause of *C. perfringens* type A food poisoning (FP) as well as non-food-borne (NFB) human gastrointestinal (GI) illnesses such as antibiotic-associated diarrhea and sporadic diarrhea (Sarker et al., 1999). Previous studies have shown that the CPE-encoding gene (*cpe*) can be located either on the chromosome or on a large plasmid; however, *C. perfringens* isolates associated with FP typically carry the *cpe* on their chromosome, whereas those isolates associated to NFB GI diseases carry the *cpe* on a large plasmid (Collie and McClane, 1998; Sarker et al., 2000). In developed countries, *C. perfringens* type A FP is one of the most common reported food-borne outbreaks (Lindström et al., 2011).

In the food industry, thermal treatments typically applied during meat processing are sufficient to kill *C. perfringens* vegetative cells; however, its spores may survive and become highly committed to germinate, outgrow, and multiply to hazardous levels (McClane, 2007a; Sarker et al., 2000). These heat-activated spores are a major concern to food processors, especially during improper chilling after cooking or stored under abusive temperature, conditions that allow a rapid proliferation of *C. perfringens* (Reddy Velugoti et al., 2007; Thippareddi et al., 2003).

Recently, the consumers' demand for safe, minimally-processed, convenient, and ready-to eat food products has increased, posing a major challenge to food

manufacturers for the search of alternative food additives to fulfill consumers' requirements (Cotter et al., 2005). These trends are likely to have an important impact on the epidemiology of *C. perfringens* FP, as milder processing conditions might allow the less resistant spores of *C. perfringens* NFB isolates to survive and subsequently propagate (Li and McClane, 2006b; Sarker et al., 2000). Indeed, several reports have provided evidence that this scenario is likely to occur (Lahti et al., 2008; Lindström et al., 2011). In this context, nisin, a 34-amino acid polypeptide produced by *Lactococcus lactis* subsp. *lactis* is an attractive option due to its many positive attributes (Cotter et al., 2005; Davies and Delves-Broughton, 2000). Nisin exerts its antimicrobial properties against several Gram-positive organisms (Banerjee and Sarkar, 2004; Bozianis and Adams, 1999; Cotter et al., 2005; Delves-Broughton, 2005; Eastoe and Long, 1959) via two different mechanisms: i) pore formation in the lipid membranes (Ruhr and Sahl, 1985); and ii) cell wall synthesis inhibition by binding and mislocalization of lipid II (Hasper et al., 2006; Wiedemann et al., 2001). Importantly, nisin has been successfully used as a food preservative in a variety of food products (Thomas and Delves-Broughton, 2005).

Although a couple of studies have been conducted on the antimicrobial activity of nisin against *C. perfringens* (Banerjee and Sarkar, 2004; Eastoe and Long, 1959; Guerlava et al., 1998; Paik et al., 2006; Scannell et al., 2000), each of those studies was limited to a single laboratory or environmental strains of *C. perfringens* type A. Therefore, a detail study on the antimicrobial effect of nisin against a collection of enterotoxigenic *C. perfringens* type A FP and NFB clinical isolates both

in laboratory conditions and in meat products is warranted. Consequently, the objectives of this study were to evaluate i) the inhibitory effects of nisin against germination and outgrowth of spores of FP and NFB isolates; and ii) the potential use of nisin as an antimicrobial agent in controlling germination and outgrowth of *C. perfringens* spores inoculated into a meat model system stored under abusive storage conditions.

3.2. Materials and methods

3.2.1. Bacterial strains

The enterotoxigenic *C. perfringens* type A isolates used in this study consist of: 6 FP isolates (SM101, NCTC10239, FD1041, E13, NCTC8239, and NCTC8798) and 3 NFB isolates (NB16, B40, and F4969). The origin of these *C. perfringens* strains has been described previously (Sarker et al., 2000). All isolates were maintained as cooked meat (Difco, BD Diagnostic Systems, Sparks, Md., U.S.A.) stock cultures stored at -20 °C and bacterial growth was retrieved with fluid thioglycollate medium (FTG) (Difco).

3.2.2. Spore preparation and purification

Spores of *C. perfringens* were prepared and purified as described previously (Paredes-Sabja et al., 2008c). Briefly, *C. perfringens* were inoculated into 10 ml FTG and incubated overnight at 37 °C. Next, the overnight cultures were transferred into fresh 10 ml FTG and grown for 8 h at 37 °C, and 0.4 ml aliquots were transferred into 10 ml DS sporulation medium (1.5% proteose peptone, 0.4% yeast extract, 0.1%

sodium thioglycolate, 0.5% sodium phosphate dibasic (Na_2HPO_4) (anhydrous), 0.4% soluble starch) (Duncan and Strong, 1968), and incubated overnight at 37 °C. Spore suspensions were obtained by scaling up the latter procedure and purifying by repeated washing with sterile distilled water until spore suspensions were > 99% free spores. Spore suspensions were stored at -80 °C until use.

3.2.3. Nisin solution preparation

Stock solutions of nisin (Sigma, St. Louis, Mo., U.S.A. consisting 2.5% pure nisin and an estimated potency of 10^6 IU/g) were prepared at concentrations of 1.0 mM (equivalent to 3,354 IU/ml) and 4.0 mM (equivalent to 13,416 IU/ml) in 0.02 N HCl. Solutions were filter-sterilized (0.45 μm , Millipore, Bedford, Mass., U.S.A.) and stored at 4 °C. Nisin stock solution was used within one week after preparation.

3.2.4. Germination of C. perfringens spores in brain heart infusion (BHI) broth supplemented with nisin

Spore suspensions were heat-activated at 80 °C for 10 min for FP spores and at 75 °C for 10 min for NFB spores, cooled in water bath at room temperature for 5 min, and then at 40 °C for 10 min. Spores of *C. perfringens* FP and NFB isolates were heat activated at different temperatures as previous study demonstrated that spores of FP isolates germinated better when heat activated at 80 °C for 10 min, whereas NFB spores germinated better with activation at 75 °C for 10 min (Paredes-Sabja et al., 2008c) . These heat-activated spores (to a final concentration at OD_{600} of ~ 1.0) were mixed with pre-warmed 0.5 ml BHI broth (Difco) supplemented with various concentrations of nisin and spore germination was routinely measured by monitoring the decrease in OD_{600} (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories,

Hercules, CA, U.S.A.) due to loss of spore's refractility upon germination. Results were expressed as percentage of OD₆₀₀ loss relative to the initial value at 10 and 60 min after adding to BHI (Paredes-Sabja et al., 2008c).

3.2.5. Outgrowth of *C. perfringens* spores in the presence of nisin

The ability of spores to outgrow was examined in vegetative growth medium TGY (3% Trypticase, 2% Glucose, 1% Yeast extract, and 0.1% L-cysteine). Briefly, aliquots (200 µl) of spore suspensions at an OD₆₀₀ of ~ 6.0 were heat activated (at 80 °C for 10 min for FP spores and at 75 °C for 10 min for NFB spores), and then inoculated into the pre-warmed 10 ml TGY supplemented with various concentrations of nisin. Cultures were incubated at 37 °C and the bacterial growth was monitored by measuring OD₆₀₀ over time intervals up to 180 min. The results were expressed as percentage of increase in OD₆₀₀.

3.2.6. Vegetative growth of *C. perfringens* isolates in the presence of nisin

Aliquot of 0.4 ml of an overnight FTG growth culture of each *C. perfringens* isolate was inoculated into 10 ml of TGY and grown for 3 h at 37 °C. A 0.4 ml aliquot of 3-h TGY culture was then inoculated into 10 ml of TGY supplemented with various concentrations of nisin. The vegetative growth was monitored by measuring OD₆₀₀ at various time points.

3.2.7. Germination and outgrowth of *C. perfringens* spores in meat in presence of nisin

To evaluate the effect of nisin on growth of *C. perfringens* in meat samples, the 3-strain spore cocktails of FP (SM101, NCTC10239, and FD1041) and NFB

(NB16, B40, and F4969) isolates were prepared by combining equal number of spores containing $\sim 10^8$ heat-resistant spores per ml and stored at $-20\text{ }^\circ\text{C}$ until use.

Meat samples were purchased from local supermarket in Corvallis, OR., U.S.A. and used immediately. Meat samples were ground in a sterile stainless steel blender (Waring products Corporation blender model 700B, Winsted, Conn., U.S.A.). Ground meat samples (10 g/bag) were weighed and placed into the UV-sterilized plastic bags (5.5" W \times 6"L) (SealaMeal vacuum storage bag, Sunmeam Products, Inc., Boca Raton, Fla., U.S.A.), sealed, and autoclaved and stored at $-20\text{ }^\circ\text{C}$ until use.

Thawed meat sample (10g) were inoculated with 100 μl of the spore cocktail of *C. perfringens* FP or NFB isolates prepared as described above to give a final inoculum level of approximately 6 log colony forming unit (CFU)/g, and then mixing with nisin to obtain a final concentration of 66 μM , 100 μM , and 250 μM (equivalent to 221, 335, and 839 IU/g, respectively). Bags were resealed and massaged manually for 1 min to ensure uniform distribution of spores and nisin and cooked at $80\text{ }^\circ\text{C}$ for 13 min as previously described (Akhtar et al., 2008) in order to simulate cooking process and activation of spores. For initial bacterial counts, meat samples were aseptically transferred into a stomacher bag and mixed with 90 ml of sterile 0.1% peptone water (pH 7.0) (Difco) and serially diluted in 0.1% peptone water, plated onto BHI agar, incubated anaerobically at $37\text{ }^\circ\text{C}$ for 24 h and colonies counted.

C. perfringens spore-contaminated meats were incubated under abusive storage conditions by storing in anaerobic condition at $37\text{ }^\circ\text{C}$. After 12 h, samples were mixed with 90 ml of sterile 0.1% peptone water, serially diluted, plated onto BHI agar,

incubated anaerobically at 37 °C for 24 h and colonies counted. Results were expressed as log CFU/g.

3.2.8. Statistical analysis

Data were analyzed by the Analysis of Variance Procedure (PROC ANOVA) using the statistical software SAS version 9.2 (SAS Inst. Inc., Cary, N.C., USA). Analysis of variance (ANOVA) among treatments was performed and comparisons of mean values were established by Duncan's New Multiple Range Test at the significant level of 0.05. In all figures, error bars represent the standard error from the mean. All experiments were done at least in triplicate.

3.3. Results and Discussion

3.3.1. Nisin does not inhibit *C. perfringens* spore germination in BHI broth.

Since *C. perfringens* spores must germinate before developing into actively growing vegetative cells, the effect of nisin on germination was first assessed. Initial control experiments, where *C. perfringens* spores were incubated with nisin in buffer (25 mM Na₂HPO₄, pH 7.0) (Paredes-Sabja et al., 2008c), showed that nisin did not trigger germination of spores of both FP and NFB isolates after 60 min of incubation (data not shown). In BHI broth, no detectable difference in the initiation of germination of spores of FP isolates SM101 and NFB isolate NB16 was observed at any nisin concentration tested during the first 10 min of incubation (Fig. 3.1A, C). Similarly, nisin had no effect on the extent of germination of SM101 and NB16 spores after 60 min of incubation (Fig. 3.1A, C). The effect of nisin on spore germination was verified with two additional enterotoxigenic *C. perfringens* type A isolates (the FP isolate NCTC10239 and the NFB isolate B40) with essentially similar results (Fig. 3.1B, D). Furthermore, the germination kinetics of FP and NFB spores were similar in BHI broth supplemented with or without nisin (data not shown). Collectively, these results indicate that the germination of spores of *C. perfringens* FP and NFB isolates in BHI broth is not inhibited in the presence of nisin.

3.3.2. Nisin blocks *C. perfringens* spore outgrowth.

The lack of effect of nisin on germination of *C. perfringens* spores led us to hypothesize that nisin exerts its inhibitory effect during the stage of spore outgrowth, as previously reported in the case of *Bacillus anthracis* and *Clostridium butyricum*

(Gut et al., 2008; Ramseier, 1960). In the absence of nisin, spores of all tested isolates were able to initiate outgrowth after ~ 80 min of inoculation into the rich medium (Fig. 3.2A, B, C, and D). However, the presence of 0.1 μM nisin (0.34 IU/ml) was sufficient to inhibit outgrowth of spores of FP isolates significantly ($p < 0.05$) as compared to control; although outgrowth of SM101 spores blocked completely (Fig. 3.2A), a slight outgrowth was observed with NCTC10239 spores after ~180 min of inoculation (Fig. 3.2B). In contrast, spores of NFB isolates were able to initiate outgrowth after ~110 min of inoculation into rich medium supplemented with 0.1 μM nisin (Fig. 3.2C, D). However, nisin at a concentration of 1 μM (3.4 IU/ml) completely inhibited outgrowth of spores of all isolates tested (Fig. 3.2A, B, C, D). These results suggest that nisin effectively arrests growth of germinated spores of *C. perfringens*, and spores of NFB isolates exhibit higher resistance to nisin than spores of FP isolates.

3.3.3. Nisin inhibits growth of C. perfringens vegetative cells in laboratory media.

When we examined vegetative growth of representative FP (SM101) and NFB (NB16) strains in the presence of nisin, we observed a lengthening of lag phase for both strains with all nisin concentrations tested (Fig. 3.3A1 and 3.3A2). In case of SM101, 1 μM nisin (3.4 IU/ml) arrested growth for 6 h and then resumed to comparable level as control within 10 h. However, 5 μM nisin (17.7 IU/ml) significantly ($p < 0.05$) inhibited the growth of SM101 over 24 h time period (Fig. 3.3A1). In contrast, growth of NB16 cells was arrested for up to 8 h with both 5 and 10 μM (17.7 and 33.5 IU/ml, respectively) nisin and the growth was resumed to control

level in 24 h. Similar lengthening of lag phase were also observed in previous studies with *C. perfringens* strains NCTC8798 (Guerlava et al., 1998) and DSM756 (Scannell et al., 2000). It was suggested that the growth delay in presence of nisin could result from the physiological state of cells and the partial death of initial populations; thus, an observed growth could result from the multiplication of the survivors of the resistant bacteria (Guerlava et al., 1998).

Next, we extended our experiments on additional FP and NFB isolates to evaluate whether the inhibitory effect of nisin against vegetative growth of enterotoxigenic *C. perfringens* is strain-specific. The SM101 growth-inhibition trend was also observed in 5 additional FP isolates, as 1 μM nisin reduced growth during 6 h and resumed to control level within 24 h (Fig. 3.3B, C and data not shown). In the case of NFB isolates, 1 μM nisin had a slight inhibitory effect on growth of B40, but a significant ($p < 0.05$) growth-inhibition on F4969 after 6 h. However, increasing nisin concentration to 10 μM delayed the growth of B40 but completely inhibited growth of F4969 after 24 h of incubation (Fig. 3.3D, E). Finally, complete inhibition of vegetative growth of all tested FP and NFB isolates was observed with nisin concentrations of 10 and 20 μM (33.5 and 67 IU/ml, respectively), respectively. Collectively, these results suggest that: i) concentrations higher than 10 μM is required to inhibit vegetative growth of *C. perfringens*, and ii) vegetative cultures of *C. perfringens* NFB isolates, with the exception of F4969, exhibit higher resistance to nisin than FP isolates.

3.3.4. Nisin does not inhibit germination and outgrowth of C. perfringens spores in cooked beef and poultry meats.

To validate our results during *in vivo* conditions as well as to evaluate the practical application of nisin in real food products, we tested the effect of nisin on a meat model system stored under abusive conditions (Akhtar et al., 2008). None of the nisin concentrations that shown to inhibit outgrowth of spores of *C. perfringens* in laboratory medium was able to inhibit germination and outgrowth of FP and NFB spores in our meat model system during abusive storage (data not shown). According to the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS), the maximum level of nisin allowed to use in cooked ready-to-eat meat and poultry products is 2.5 mg per pound (equivalent to 5.5 mg/kg) as active nisin in the finished product (Usda and Fsis, 2010). The nisin used in this study consists of 2.5% pure nisin. Therefore, the nisin concentrations selected to apply into meat samples were based on the allowable level of USDA-FSIS which is equivalent to 66 μM (5.5 mg/kg of pure nisin or 221 IU/g), and two other elevated concentrations, 100 μM (8.4 mg/kg pure nisin or 335 IU/g) and 250 μM (20.8 mg/kg pure nisin or 839 IU/g). No decrease in initial CFU/g for FP or NFB spores were observed in all nisin concentrations tested (Fig. 3.4A, B). Instead, increased CFU/g was observed after 12 h anaerobic incubation of spores with meat plus nisin (Fig. 3.4A, B). In case of FP spores, CFU/g increase with 100 or 250 μM nisin was significantly lower compared to control and samples with 66 μM nisin ($p < 0.05$) (Fig. 3.4A). Strikingly, NFB spores were able to reach similar CFU/g levels after 12 h anaerobic incubation with meat

supplemented with all tested nisin concentrations ($p > 0.05$) (Fig. 3.4B). These results suggest that while some inhibition of growth of FP spores in meat occurs at concentrations of nisin of ~ 4 -fold higher than the allowable level by the USDA-FSIS, such concentrations are not effective against NFB spores. Similar results were observed in a meat model system using poultry meat (data not shown). The lack of inhibitory effect of nisin against *C. perfringens* spores in meats could not be explained by the influence of high level (6 log CFU/g) *C. perfringens* spore contamination, as nisin (even at 250 μ M) was also failed to control *C. perfringens* growth in beef samples contaminated with 3 log CFU/g (data not shown).

The lack of nisin's antimicrobial activity against *C. perfringens* in meats could be due to the presence of glutathione which has been shown to inactivate nisin, but heating promotes the binding of glutathione with meat proteins rendering its unavailability to conjugate with nisin thereby removing its ability to inhibit nisin activity (Stergiou et al., 2006). The meat samples used in our study were thoroughly cooked before the addition of *C. perfringens* spores and nisin, so it is unlikely that nisin would be inactivated by glutathione. However, it is possible that the relatively high pH of the meats (pH ~ 5.8) and the thermal processing step used in this work could have induced some loss of nisin's antimicrobial activity as previously suggested (Caserio et al., 1979; Fowler, 1979; Fowler and Mccann, 1971; Rayman et al., 1981).

Generally *C. perfringens* type A FP outbreaks are caused by FP isolates; however, recent studies demonstrated that the NFB isolates can also be considered as the causative agent for food-related illnesses (Lahti et al., 2008). Typically, spores and

vegetative cells of FP isolates possess higher resistance to various food preservation approaches such as heat, low temperature, osmotic stress, and nitrite than NFB isolates (Li and McClane, 2006a, 2006b; Sarker et al., 2000). However, no difference in resistance to high hydrostatic pressure in absence of heat and to polyphosphates was observed between these two groups of isolates (Akhtar et al., 2008; Paredes-Sabja et al., 2007). In the current study, we now found that even in the different growth environments (laboratory media and meat model systems), NFB isolates exhibited a higher resistance to nisin than FP isolates. Collectively, these findings suggest that NFB isolates might well be adapted to mild thermal treatments and food grade antimicrobial preservatives and might become potential to cause food-related GI diseases. To date, most of the meat inoculation studies with *C. perfringens* have been conducted only with FP isolates and our work, for the first time, is being conducted to examine the fate of NFB isolates in meat and poultry products, especially during prolong storage time and abusive storage conditions.

3.4. Conclusions

In summary, current work demonstrated the following important findings: 1) nisin showed inhibitory effect against spore outgrowth and vegetative cells of *C. perfringens* FP and NFB isolates in laboratory conditions; 2) NFB isolates exhibited higher resistance to nisin than FP isolates; and 3) no inhibitory effect of nisin was observed against *C. perfringens* spores inoculated into a meat model system. Despite the fact that NFB isolates exhibit lower resistance to various food processing and

preservatives than FP isolates, their similar resistance to high hydrostatic pressure and polyphosphates, and higher resistance to nisin suggests that under the current food processing trend of milder thermal treatments coupled with various preservatives, NFB isolates might well be suited to survive food environments and cause FP GI diseases as previously suggested. Finally, since current study found no inhibitory effect of nisin against *C. perfringens* in meat model system, caution should be taken when formulating meat products with nisin as a preservative, at least against *C. perfringens*.

Acknowledgements

This work was supported by a grant from the Agricultural Research Foundation of Oregon State University and by a Department of Defense Multi-disciplinary University Research Initiative (MURI) award through the U.S. Army Research Laboratory and the U. S. Army Research Office under contract number W911NF-09-1-0286 (all to MRS). P.U. was supported by a fellowship from the Office of the Higher Education Commission (Thailand).

References

- Akhtar, S., Paredes-Sabja, D., & Sarker, M.R. (2008). Inhibitory effects of polyphosphates on *Clostridium perfringens* growth, sporulation and spore outgrowth. *Food Microbiology*, *25*, 802-808.
- Banerjee, M., & Sarkar, P.K. (2004). Antibiotic resistance and susceptibility to some food preservative measures of spoilage and pathogenic micro-organisms from spices. *Food Microbiology*, *21*, 335-342.
- Boziaris, I.S., & Adams, M.R. (1999). Effect of chelators and nisin produced in situ on inhibition and inactivation of Gram negatives. *International Journal of Food Microbiology*, *53*, 105-113.
- Caserio, G., Stecchini, M., Pastore, M., & Genari, M. (1979). The individual and combined effect of nisin and nitrite on the spore germination of *Clostridium perfringens* in meat mixtures subjected to fermentation. *Ind Aliment*, *18*, 894-898.
- Collie, R.E., & McClane, B.A. (1998). Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. *Journal of Clinical Microbiology*, *36*, 30-36.
- Cotter, P.D., Hill, C., & Ross, R.P. (2005). Bacteriocins: developing innate immunity for food. *Nature*, *3*, 777-788.
- Davies, E.A., & Delves-Broughton, J. (2000). Nisin. In: Batt, C.A. Patel, P.D. (Eds.), *Encyclopedia of food microbiology* (pp. 191-198). London: Academic Press.
- Delves-Broughton, J. (2005). Nisin as a food preservative. *Food Australia*, *57*, 525-527.
- Duncan, C.L., & Strong, D.H. (1968). Improved medium for sporulation of *Clostridium perfringens*. *Applied Microbiology*, *16*, 82-89.
- Eastoe, J.E., & Long, J.E. (1959). The effect of nisin on the growth of cells and spores of *Clostridium welchii* in gelatine. *Journal of Applied Bacteriology*, *22*, 1-7.
- Fowler, G.G. (1979). The potential of nisin. *Food Manuf*, *54*, 57-59.
- Fowler, G.G., & McCann, B. (1971). The growing use of nisin in the dairy industry. *Australian Journal of Dairy Technology*, *26*, 44-46.
- Guerlava, P., Nolf, S., & Tholozan, J.L. (1998). Rapid cooling, moderate heat treatment and nisin addition influence cell homeostasis of *Clostridium perfringens* type A. *International Journal of Food Microbiology*, *39*, 195-203.
- Gut, I.M., Prouty, A.M., Ballard, J.D., van der Donk, W.A., & Blanke, S.R. (2008). Inhibition of *Bacillus anthracis* spore outgrowth by nisin. *Antimicrobial Agents and Chemotherapy*, *52*, 4281-4288.
- Hasper, H.E., Kramer, N.E., Smith, J.L., Hillman, J.D., Zachariah, C., Kuipers, O.P., de Kruijff, B., & Breukink, E. (2006). An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. *Science*, *313*, 1636-1637.
- Lahti, P., Heikinheimo, A., Johansson, T., & Korkeala, H. (2008). *Clostridium perfringens* type A strains carrying a plasmid-borne enterotoxin gene (genotype IS1151-cpe or IS1470-like-cpe) as a common cause of food poisoning. *Journal of Clinical Microbiology*, *46*, 371-373.

- Li, J., & McClane, B.A. (2006a). 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. *Applied and Environmental Microbiology*, 72, 7620-7625.
- Li, J., & McClane, B.A. (2006b). Further comparison of temperature effects on growth and survival of *Clostridium perfringens* type A isolates carrying a chromosomal or plasmid-borne enterotoxin gene. *Applied and Environmental Microbiology*, 72, 4561-4568.
- Lindström, M., Heikinheimo, A., Lahti, P., & Korkeala, H. (2011). Novel insights into the epidemiology of *Clostridium perfringens* type A food poisoning. *Food Microbiology*, 28, 192-198.
- McClane, B.A. (2007). *Clostridium perfringens*. In: Doyle, M.P. Beuchat, L.R. (Eds.), *Food Microbiology: fundamentals and frontiers* (pp. 423-444). Washington, D.C.: ASM Press.
- Paik, H.D., Kim, H.J., Nam, K.J., Kim, C.J., Lee, S.E., & Lee, D.S. (2006). Effect of nisin on the storage of *sous vide* processed Korean seasoned beef. *Food Control*, 17, 994-1000.
- Paredes-Sabja, D., Gonzalez, M., Sarker, M.R., & Torres, J.A. (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*, 72, M202-M206.
- Paredes-Sabja, D., Torres, J.A., Setlow, P., & Sarker, M.R. (2008). *Clostridium perfringens* spore germination: characterization of germinants and their receptors. *Journal of Bacteriology*, 190, 1190-1201.
- Ramseier, H.R. (1960). The influence of nisin on *Clostridium butyricum*. *Archives of Microbiology*, 37, 57-94.
- Rayman, M.K., Aris, B., & Hurst, A. (1981). Nisin: A possible alternative or adjunct to nitrite in the preservation of meats. *Applied and Environmental Microbiology*, 41, 375-380.
- Reddy Velugoti, P., Rajagopal, L., Juneja, V., & Thippareddi, H. (2007). Use of calcium, potassium, and sodium lactates to control germination and outgrowth of *Clostridium perfringens* spores during chilling of injected pork. *Food Microbiology*, 24, 687-694.
- Ruhr, E., & Sahl, H.G. (1985). Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. *Antimicrobial Agents and Chemotherapy*, 27, 841-845.
- Sarker, M.R., Carman, R.J., & McClane, B.A. (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. *Molecular Microbiology*, 33, 946-958.
- Sarker, M.R., Shivers, R.P., Sparks, S.G., Juneja, V.K., & McClane, B.A. (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. *Applied and Environmental Microbiology*, 66, 3234-3240.
- Scannell, A.G.M., Ross, R.P., Hill, C., & Arendt, E.K. (2000). An effective lacticin biotreservative in fresh pork sausage. *Journal of Food Protection*, 63, 370-375.
- Stergiou, V.A., Thomas, L.V., & Adams, M.R. (2006). Interactions of nisin with glutathione in model protein system and meat. *Journal of Food Protection*, 69, 951-956.
- Thippareddi, H., Juneja, V.K., Phebus, R.K., Marsden, J.L., & Kastner, C.L. (2003). Control of *Clostridium perfringens* germination and outgrowth by buffered sodium citrate during chilling of roast beef and injected pork. *Journal of Food Protection*, 66, 376-381.
- Thomas, L.V., & Delves-Broughton, J. (2005). Nisin. In: Davidsson, P.M., Sofos, J.N., Branen, A.L. (Eds.), *Antimicrobials in food* (pp. 237-274). Boca Raton: CRC Press.
- USDA, & FSIS, 2010. Safe and Suitable Ingredients Used in the Production of meat, poultry, and egg products, FSIS Directive 7120.1 Revision 2, Washington, DC.
- Wiedemann, I., Breukink, E., Van Kraaij, C., Kuipers, O.P., Bierbaum, G., De Kruijff, B., & Sahl, H.G. (2001). Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. *Journal of Biological Chemistry*, 276, 1772-1779.

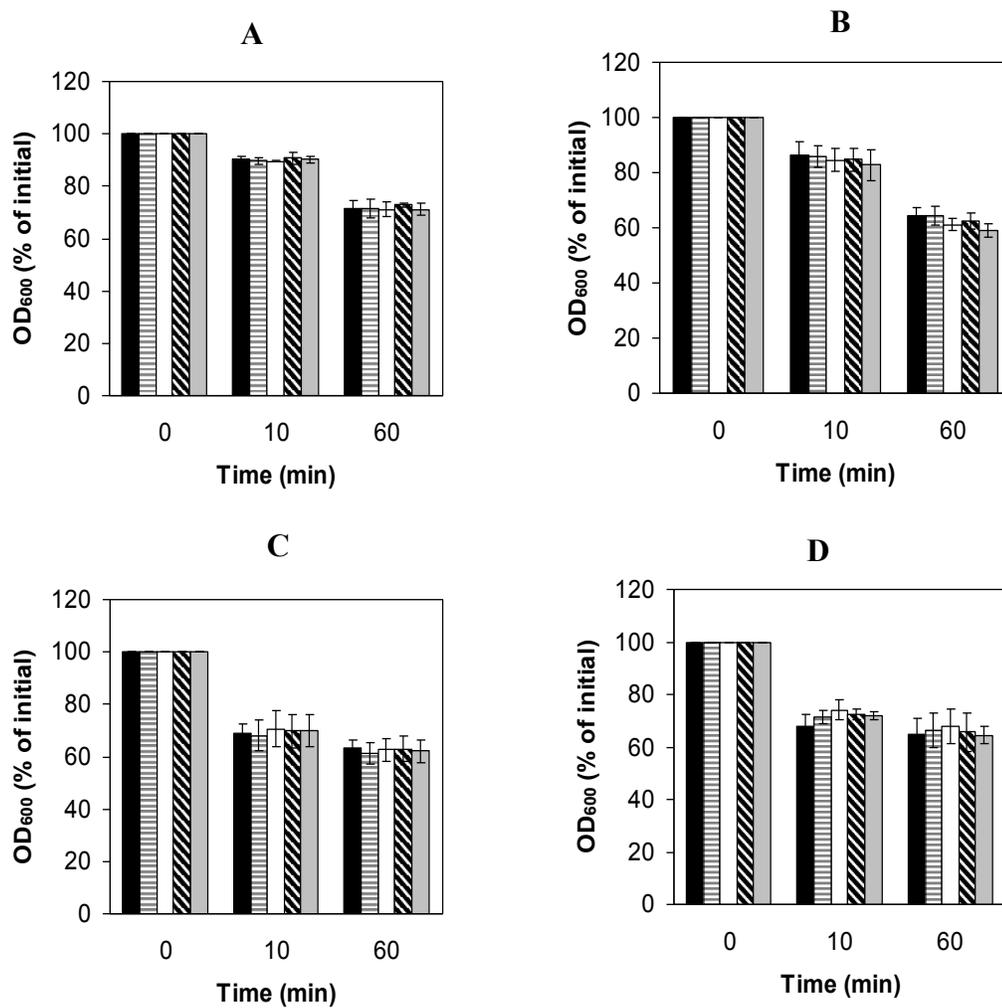


Fig. 3.1A-D. Effect of nisin on *C. perfringens* spore germination. Spores of *C. perfringens* strain SM101 (A), NCTC10239 (B), NB16 (C) and B40 (D) were heat-activated, inoculated into BHI broth containing various concentrations of nisin: 0 μM (black bars); 0.1 μM (white bars with gray stripes); 1 μM (white bars); 10 μM (black bars with white diagonal stripes), and 100 μM (grey bars), and germination was monitored by measuring OD₆₀₀ decrease.

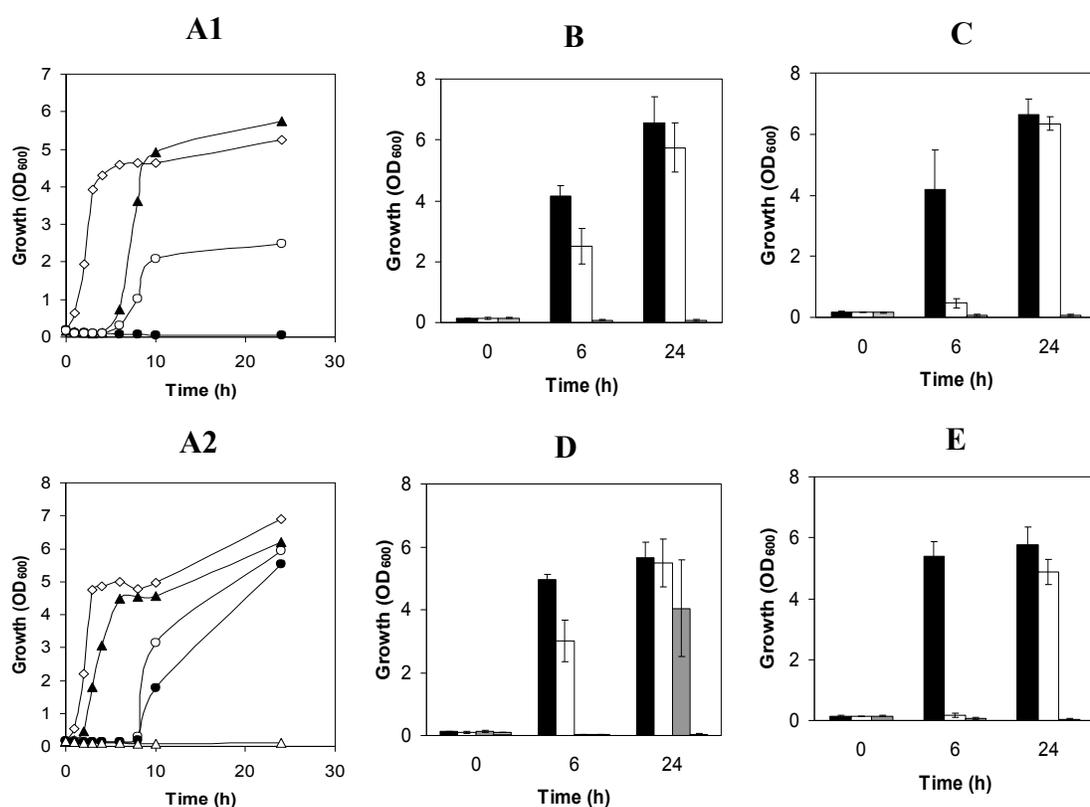


Fig. 3.3A-E. Effect of nisin on vegetative growth of *C. perfringens* isolates. (A) *C. perfringens* FP isolate SM101 (A1) and NFB isolate NB16 (A2) were inoculated into TGY vegetative medium containing nisin concentrations: 0 μM (\diamond), 1 μM (\blacktriangle), 5 μM (\circ), 10 μM (\bullet), and 20 μM (Δ). Growth was monitored by measuring OD₆₀₀ at hourly intervals. (B-E) Vegetatively growing cells of strains: NCTC10239 (B), FD1041 (C), B40 (D) and F4969 (E) were inoculated into TGY medium containing nisin concentrations: 0 μM (black bars); 1 μM (white bars), 10 μM (grey bars), and 20 μM (white bars with black strips). Growth was monitored by measuring OD₆₀₀ at 6 and 24 h post-inoculation.

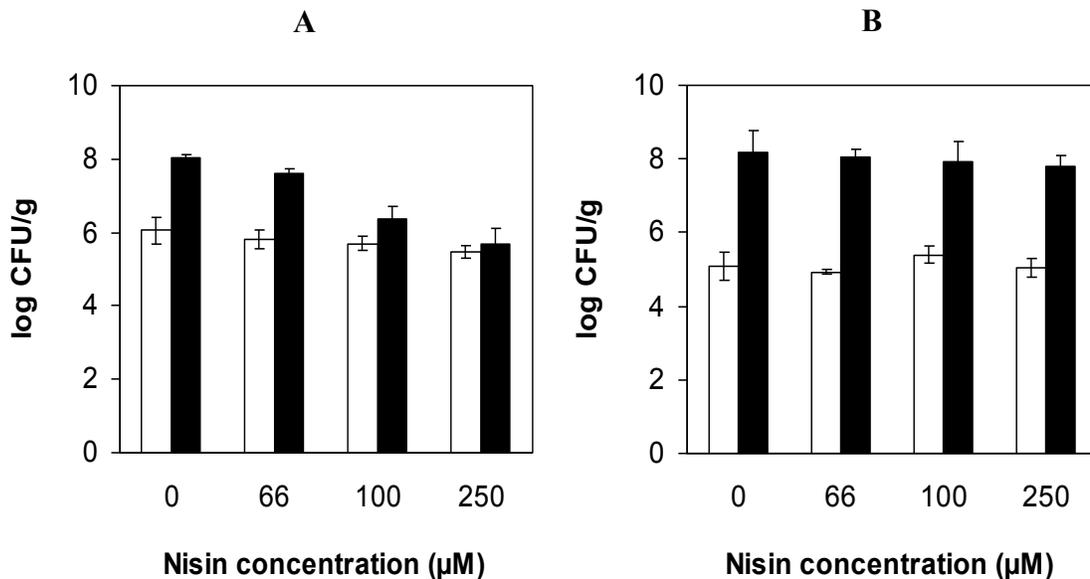


Fig. 3.4A-B. Inhibitory effect of nisin on growth of *C. perfringens* spores in cooked meat products. Germination and outgrowth of spores of 3-strain cocktail of *C. perfringens* FP (A) or NFB (B) isolates in cooked beef samples containing various concentrations of nisin. Initial viable counts (white bars) and viable counts after 12 h of anaerobic incubation at 37 °C (black bars) were determined by plating onto BHI agar and incubated anaerobically at 37 °C for 24 h.

CHAPTER 4

Inactivation Strategy for *Clostridium perfringens* Spores Adhered to Food Contact Surfaces

Pathima Udombijitkul, Maryam Alnoman, Daniel Paredes-Sabja, and
Mahfuzur R. Sarker

Published in:

Food Microbiology (2013) 34(2): p. 328-336

Abstract

The contamination of enterotoxigenic *Clostridium perfringens* spores on food contact surfaces poses a serious concern to food industry due to their high resistance to various preservation methods typically applied to control food-borne pathogens. In this study, we aimed to develop an strategy to inactivate *C. perfringens* spores on stainless steel (SS) surfaces by inducing spore germination and killing of germinated spores with commonly used disinfectants. The mixture of L-asparagine and KCl (AK) induced maximum spore germination for all tested *C. perfringens* food poisoning (FP) and non-food-borne (NFB) isolates. Incubation temperature had a major impact on *C. perfringens* spore germination, with 40 °C induced higher germination than room temperature (RT) (20 ± 2 °C). In spore suspension, the implementation of AK-induced germination step prior to treatment with disinfectants significantly ($p < 0.05$) enhanced the inactivation of spores of FP strain SM101. However, under similar conditions, no significant spore inactivation was observed with NFB strain NB16. Interestingly, while the spores of FP isolates were able to germinate with AK upon their adhesion to SS chips, no significant germination was observed with spores of NFB isolates. Consequently, the incorporation of AK-induced germination step prior to decontamination of SS chips with disinfectants significantly ($p < 0.05$) inactivated the spores of FP isolates. Collectively, our current results showed that triggering spore germination considerably increased sporicidal activity of the commonly used disinfectants against *C. perfringens* FP spores attached to SS chips. These findings

should help in developing an effective strategy to inactivate *C. perfringens* spores adhered to food contact surfaces.

4.1 Introduction

Clostridium perfringens is a Gram-positive, anaerobic, rod-shaped, spore-forming bacterium, which can be classified into 5 types, A-E (Mcclane, 2007a). The small group (less than 5%) of *C. perfringens* type A isolates produces *C. perfringens* enterotoxin (CPE), which is responsible for most symptoms of *C. perfringens*-associated gastrointestinal (GI) diseases (Lindström et al., 2011; Mcclane, 2007a; Sarker et al., 1999). CPE encoding gene (*cpe*) can be located either on the chromosome or on large plasmids in *C. perfringens* (Brynstad et al., 1997; Cornillot et al., 1995). The chromosomal *cpe*-positive strains (*C-cpe*) are generally linked to food poisoning (FP) due to its higher resistance to heat, low temperature, NaCl, and nitrite than the plasmid-borne *cpe* carrying strains (*P-cpe*), while the *P-cpe* isolates are associated with non-foodborne (NFB) GI diseases (Collie and Mcclane, 1998; Li and Mcclane, 2006a, 2006b; Lindström et al., 2011; Sarker et al., 2000; Sparks et al., 2001). Nevertheless, the recent investigations suggested that *P-cpe* isolates could be a causative agent for *C. perfringens* type A FP (Lahti et al., 2008; Tanaka et al., 2003). Since *C. perfringens* spores are much more resistant than their vegetative counterparts to a variety of lethal factors such as heat, prolonged refrigeration and frozen temperatures, chemicals, and high hydrostatic pressure (Li and Mcclane, 2006a, 2006b; Paredes-Sabja et al., 2007; Sarker et al., 2000), they can survive thermal processing and sanitizing treatments employed in the food industry. Also, due to the spore's high hydrophobic characteristics, spore elimination is usually more difficult when attached to food contact surfaces (Blatchley et al., 2005). The contaminated

food-contact surfaces could be the potential source of pathogen transmission to food products in the food processing, catering, and domestic environments (Bae and Lee, 2012; Kusumaningrum et al., 2003).

It is well recognized that germinated spores are more susceptible to various stress factors than their dormant form (Akhtar et al., 2009; Clouston and Wills, 1969; Nerandzic and Donskey, 2010); therefore, inducing spore germination prior to disinfection step could be a potential strategy to improve elimination or reduction of *C. perfringens* spores from food contact surfaces. Previous studies demonstrated the use of this strategy in reducing *C. perfringens* spores in poultry products, as well as in increasing sensitivity of *Bacillus subtilis*, *Bacillus coagulans*, *Bacillus cereus*, *Clostridium difficile*, and *Clostridium botulinum* spores to subsequent inactivation by heat, radiation, and chemicals (Akhtar et al., 2009; Durban et al., 1970; Gould et al., 1968; Løvdal et al., 2011; Munakata, 1974; Nerandzic and Donskey, 2010; Stuy, 1956). In the current study, we aimed to develop an inactivation strategy for *C. perfringens* spores attached to stainless steel (SS) surfaces by inducing spore germination followed by subsequent killing of germinated spores with disinfectants typically used in food processing facilities and domestic kitchens. Our results showed that inducing germination significantly increased sporicidal activity of commonly used disinfectants against spores of *C. perfringens* FP isolates on SS surfaces.

4.2. Material and methods

4.2.1. Bacterial strains and growth conditions

The enterotoxigenic *C. perfringens* type A isolates used in this study consists of 5 FP isolates (SM101, NCTC8239, NCTC10239, E13, and 6263) and 4 NFB isolates (B40, NB16, F4969, and F5603) (Harrison et al., 2005a; Sarker et al., 2000). The stock culture of each isolate had been maintained in cooked meat medium (Difco, Becton Dickinson, Spark, MD) at -20 °C. Bacterial growth was revived by inoculating 0.1 ml cooked meat culture into 10 ml fluid thioglycollate (FTG) medium (Difco) and incubating overnight (18 h) at 37 °C.

4.2.2. Spore preparation and purification

Sporulating cultures of *C. perfringens* were prepared as described previously (Paredes-Sabja et al., 2008c). Briefly, 0.4-ml aliquots of an actively growing FTG cultures were inoculated into 10-ml freshly prepared Duncan Strong (DS) sporulation medium (1.5% protease peptone, 0.4% yeast extract, 0.1% sodium thioglycolate, 0.5% sodium phosphate dibasic [Na_2HPO_4 ; anhydrous], 0.4% soluble starch) (Duncan and Strong, 1968) and incubated for 24 h at 37 °C. A large number of *C. perfringens* spores was prepared by scaling up the aforementioned procedure and spores were purified by repeated washing with cold sterile distilled water until the spore suspensions were > 99% free of sporulating cells, cell debris, and germinating spores as observed under a phase contrast microscope. Free spores were suspended in sterile distilled water to obtain a final optical density at 600 nm (OD_{600}) of ~ 6 and stored at -20 °C until used (Paredes-Sabja et al., 2008c).

4.2.3. Spore germination

The germinants used in this study consisted of potassium chloride (KCl; Fisher Scientific, Fair Lawn, NJ), L-asparagine (Sigma-Aldrich, Co., St. Louis, MO), L-glutamine (Sigma), and L-cysteine hydrochloride, monohydrate (J.T. Baker, Mallinckrodt Baker, Inc. Philipsburg, NJ) that was prepared in single or in various combinations of ingredients. All germinant solutions were prepared with 25 mM Na₂HPO₄ buffer adjusted to pH 7.0 and autoclaved (121 °C, 20 min). Germination assay was performed as previously described with some modifications. Briefly, spore suspensions were heat activated at 80 °C, 10 min for FP isolates or 75 °C, 10 min for NFB isolates (Paredes-Sabja et al., 2008c). Then, 0.1 ml of heat activated spores (to the final concentration of OD₆₀₀ ~1.0) were cooled in water bath at ambient temperature for 5 min before mixing with 0.5 ml of the pre-warmed germinant equilibrated at 40 °C or at room temperature (RT) (20 ± 2 °C). Spore germination was routinely monitored by measuring the decrease in OD₆₀₀ (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA) in every 10 min intervals, as spores loss their refractility upon germination. Spore germination was also confirmed by phase contrast microscopy, as germinating spores become phase dark, whereas dormant spores remain phase bright under microscope. Results were expressed as percentage decrease of OD₆₀₀ relative to the initial values (Paredes-Sabja et al., 2008c).

4.2.4. Inactivation of spores in suspension

In all inactivation assays, 0.1 ml of *C. perfringens* spores ($\sim 10^8$ CFU/ml) was induced to germinate with 0.5 ml of selected pre-warmed germinant or 25 mM Na₂HPO₄ buffer (pH 7.0) (Control) as described in section 4.2.3. Spore germination was performed at 40 °C or at RT for 30 min before mixing with disinfectants. All disinfectants were prepared with sterile distilled water to the desired concentrations and used within 30 min after preparation. Inactivation experiments of the dormant (germinated with Na₂HPO₄ buffer) and germinating (germinated with AK) spores were performed in parallel for every disinfectant at ambient temperature. In all experiments, at least 100-fold dilution of the reaction mixtures were made immediately in 25 mM Na₂HPO₄ buffer (pH 7.5) in order to terminate the action of tested disinfectants. Preliminary results indicated that this neutralization method was effective and resulted in no residual inhibitory effect in the recovery medium (data not shown). To verify the germination abilities of different spore batches, 0.1 ml spore suspensions were heat activated and germinated with 0.5 ml of buffer or germinant solutions for 30 min at both tested temperature and decrease in OD₆₀₀ measured. There was no difference in the germination of spores prepared in different batches.

Ethanol is generally used for disinfecting the high – risk areas in food processing to limit water usage in order to prevent bacterial growth and spread of pathogens (Holah, 2003). The effect of initiation of germination on sensitivity to 70% (v/v) ethanol was assessed by inoculating 0.15 ml of germinated spores into 0.35 ml of absolute ethanol (100%) to give the final concentration of 70% (v/v) ethanol. After 5

min exposure at RT, 0.1 ml samples were serially diluted in 25 mM Na₂HPO₄ buffer (pH 7.5) and plated onto BHI agar to enumerate the number of viable cells.

Iodophore, the iodine – based sanitizer, have been reported to have a broad sporicidal activity against various spore-forming bacteria (Cords et al., 2005). In this study, the sporicidal activity of iodophore sanitizer against spores of *C. perfringens* isolates had been evaluated at 2 concentrations recommended by the manufacturer. Iodophore solution was prepared by diluting the appropriate amount of commercial iodophore sanitizer containing 1.6% titratable iodine (BTF[®] iodophore sanitizer; National Chemicals, Inc., Winona, MN) with sterile distilled water. The 0.1 ml of germinated spores were mixed with 0.5 ml of 30 ppm (mg/L) or 15 ppm iodophore to give a final concentrations of 25 ppm or 12.5 ppm titratable iodine, respectively. Spores were exposed to sanitizer for 2, 5, and 10 min and 0.1 ml were serially diluted in phosphate buffer and plated for microbial analysis.

Quaternary Ammonium Compounds (Quats) has been broadly used to disinfect food contact surfaces and food processing equipment due to its non-corrosive, environmental and user-friendly characteristics (Holah, 2003). Quats (SANI - 512 containing 10% (v/v) active ingredient, CANI Inc., Lansdale, PA) was prepared by diluting appropriate amount of Quats yielding the concentration of 240 ppm. The 0.1 ml of germinated spores were mixed with 0.5 ml of Quats solution to give a final concentration of 200 ppm. After 1 min treatment (as recommended by manufacturer), 0.1 ml were diluted and plated for microbial analysis.

DECON-SPORE 200 PLUS[®] (DeconSpore) (Veltek Associates, Inc., Malvern, PA) (Active ingredients: Hydrogen peroxide (H₂O₂), 27.50% and Peroxyacetic acid 5.80%; Inert ingredients 66.70%) is a certified sporicidal agent and has been claimed to have a spore – killing activity against *B. subtilis* ATCC 6633 and *Clostridium sporogenes* ATCC 11437 according to manufacturer's technical data file. DeconSpore was prepared at 6% (v/v) concentration solution, and then, 0.1 ml of the germinated spore suspension was mixed with 0.5 ml of 6% DeconSpore yielding a final concentration of 5% (v/v) as recommended by the manufacturer. Various contact times were evaluated for this particular sanitizer ranging from 5 to 60 min. After treatment, 0.1 ml sample was serially diluted and plated for microbial enumeration.

4.2.5. Stainless steel (SS) surface preparation and inactivation of spores on SS

SS chips 300 series, no. 4 finish (The Home Depot, Corvallis, OR) were used in this study. The SS were cut into 2 x 3 inches pieces and each chip was washed with 1% (w/v) Alconox[®] (VWR International, West Chester, PA), rinsed with distilled water, dried and wrapped individually with aluminum foil. SS chips were autoclaved at 121 °C for 20 min and stored at RT until used. The spores cocktail from 3 *C. perfringens* FP isolates (SM101, NCTC8239 and NCTC10239) was prepared by combining 40 µl of purified spore suspension (OD₆₀₀ ~ 6.0) of each strain to give a final volume of 0.12 ml of the cocktail spores. The cocktail spores of FP isolates were then heat activated at 80 °C for 10 min, and cooled in water bath at room temperature for 5 min. The 0.1 ml of the heat-activated spore cocktail was inoculated onto each sterile SS chips and spread with a sterile bent glass rod. The contaminated SS chips

were dried under Class II laminar flow hood for 60 min. In each set of experiment with each sanitizer, 6 SS chips were incorporated as follows: 1) One chip as negative control (no spore contamination; dry for 1 h); 2) One chip as initial spore population count (spore inoculation; dry for 1 h); 3) Two chips for spore germination with control buffer (spore inoculation; dry for 1 h, and germinate with 25 mM Na₂HPO₄ buffer); and 4) Two chips for spore germination with AK (spore inoculation, dry for 1 h, and germinate with AK). For triggering spore germination on SS chips, the contaminated chips were aseptically transferred to sterile petri dishes (100 mm x 20 mm, Kimble Chase, Vineland, NJ) containing 50 ml of the pre-warmed germinants to cover the entire surface of SS chips. Spore germination was performed at 40 °C for 30 min. The SS chips samples were transferred with the sterile forceps to the sterile beakers containing 250 ml of tested disinfectant and left them for a pre-determined period. The SS chips samples were then removed and placed into another sterile beakers containing 250 ml of sterile distilled water for 1 min to neutralize and remove excess sanitizers on SS surfaces before transferring them to a clean area and evaluating for bacterial survivors.

4.2.6. Microbiological analysis

For suspension test in section 4.4, the numbers of *C. perfringens* spores were determined at several points including the inoculum level after heat activation of spores (referred as initial number), after germination induction for 30 min (referred as 0 min), and the number of bacterial survivals after exposing to a variety of disinfectants for different time periods. All serial dilutions were made with 25 mM

Na₂HPO₄ buffer (pH 7.5) and 0.1 ml portion were surface plated onto BHI agar and incubated anaerobically with gas pack (GasPak™ EZ Anaerobe Container system, Becton, Dickson and Company, Sparks, MD) at 37 °C for 24 h.

For monitoring killing of spores on food contact surfaces in section 4.5, populations of *C. perfringens* spores on SS chips were determined before and after triggering germination and also after treatment with tested disinfectants. The entire surface of individual chip was swabbed with 4 sterile cotton swabs (Puritan Medical Products Company LLC, Guilford, ME) until the surface was completely dried. All the swabs were then placed in a test tube containing 10 ml of 25 mM Na₂HPO₄ buffer (pH 7.5) and mixed vigorously with Vortex mixer (Vortex Genie2, Model G-560, Scientific Industries Inc., NY) for 1 min to elute bacterial cells from swabs. The number of viable *C. perfringens* cells was determined by serially diluted aliquots from swabs, plated onto BHI agar, incubated anaerobically at 37 °C for 24 h and then colonies counted. The populations of non-germinated spores on SS chips were determined by heating collected samples from SS at 75 °C for 20 min and subsequently enumerated on BHI agar plates.

4.2.7. Statistical analysis

All experiments were performed at least in 3 independent replications with 2 different spore preparations. Results of the microbial analysis were transformed into log₁₀ values and data were analyzed by Analysis of Variance Procedure (PROC ANOVA) using the statistical software SAS version 9.2 (SAS Inst. Inc., Cary, NC). Analysis of variance (ANOVA) among treatments was performed and multiple

comparisons of mean values were analyzed with Tukey's test at the significant level of 0.05.

4.3. Results and discussions

4.3.1. Optimizing C. perfringens spores' germination condition

We were able to identify a variety of novel germinants for *C. perfringens* spores such as L-cysteine, formula 1 (mixture of L-asparagine, KCl, and L-cysteine) and formula 2 (mixture of L-asparagine, KCl, L-cysteine, and L-glutamine) (Table 4.1 and 4.2). The extent of spore germination was dependent on various factors including source of strains, germination temperatures, and types of germinants, with most FP isolates were able to germinate to a higher extent with variety of germinants than NFB isolates (Tables 4.1, 4.2 and data not shown). These results are in agreement with the findings from spores of *Bacillus* spp. and proteolytic *C. botulinum*, where germination characteristic of one strain cannot be used to predict responses of other strains of the same isolate type (Alberto et al., 2003; Foerster and Foster, 1966; Wolf and Thorley, 1957). Therefore, we included a total of 9 *C. perfringens* strains (5 FP and 4 NFB) in our current study to obtain the more reliable results on selecting the best "all – purpose" germinant for *C. perfringens* type A spores.

We have found that spores of most FP isolates could germinate significantly both at 40 °C and at RT (20 ± 2 °C); however, the extent of germination at 40 °C was higher than at RT with most germinants tested (Tables 4.1 and 4.2). In contrast, the germination of spores of NFB isolates was highly affected by germination

temperature, as evidenced by a negligible extent of germination of spores of all 4 NFB isolates was observed at RT with all tested germinants (Table 4.2). Results from phase contrast microscopy were consistent with majority of spores remained phase bright (data not shown). Longer incubation (90 min or even 24 h) at RT did not induce significant increase in germination of spores of NFB isolates (data not shown). These results, in conjunction with our previous findings with FP isolates (Akhtar et al., 2009), support that incubation temperature played a critical role in inducing *C. perfringens* spore germination. The differences in germination temperature-response between FP versus NFB isolates could be due to their adaptation to environmental niches related to food industry (Li and McClane, 2006a, 2006b; Miki et al., 2008; Sarker et al., 2000; Wen and McClane, 2004). Similar temperature effect on germination was observed with spores of other spore-forming *Bacillus* and *Clostridium* species, where sub-optimal germination temperature resulted in lower rate and extent of germination (Leuschner and Lillford, 1999; Levinson and Hyatt, 1970a, 1970b; Ramirez and Abel-Santos, 2010; Van Opstal et al., 2004; Vary and Halvorson, 1965).

Our current results are in agreement with the previous finding that the mixture of AK served as a universal germinant for spores of both FP and NFB isolates (Fig. 4.1 and Tables 4.1, 4.2) (Paredes-Sabja et al., 2008c). L-cysteine and formula 1 could induce germination of spores of FP and NFB isolates in a lower extent compared to AK at 40 °C. However, formula 1 could overall induce slightly higher extent of germination than AK at RT, the former could not trigger germination of NFB spores at

this temperature and the difference in extent of germination was insignificant (Table 4.2). Also, the germination kinetics of most strains tested was constant at 30 min post – inoculation for all germinants at both 40 °C and RT. Therefore, in subsequent experiments, we have selected AK as a universal germinant to trigger *C. perfringens* spore germination and 30 min period was chosen for inducing spore germination before subsequent inactivation (Fig. 4.1 and data not shown).

4.3.2. Inducing germination and inactivation of *C. perfringens* spores in suspension

It has long been recognized that spores loss their resistance immediately upon germination (Hornstra et al., 2007). Therefore, we initially assessed the working hypothesis that stimulating spore germination can improve subsequent inactivation of germinated spores by commonly used disinfectants for food contact surfaces. Our results showed that the spore populations did not significantly change after germination with buffer or AK for 30 min at RT or 40 °C ($p > 0.05$) as compared to the number of heat-activated spore inocula implying that Na_2HPO_4 buffer and AK did not inactivate spores of *C. perfringens* during germination induction (data not shown). Therefore, the population of germination-induced cells was referred as 0 min treatment count in all experiments.

Ethanol and Quats had no effect on dormant spores of *C. perfringens* FP strain SM101 and NFB strain NB16 at both 40 °C and RT ($p > 0.05$) (Fig. 4.2A-B and Fig. 4.3A-B). However, germination of SM101 and NB16 spores with AK at 40 °C followed by ethanol (70%) treatment resulted in reduction of 2.14 and 0.72 log CFU/ml, respectively (Fig. 4.2A-B). While RT-germination led to a significant ($p <$

0.05) killing of SM101 spores with ethanol, this effect was not observed with NB16 spores, as they were unable to germinate with AK at RT (Fig. 4.2B). Extending treatment time up to 15 min did not improve the killing efficacy of 70% ethanol against *C. perfringens* spores (data not shown). Although a reduction of ~ 2 logs of *B. subtilis* spores upon incubation with 70% ethanol at 65 °C for 2 h had been previously reported (Setlow et al., 2002); and 90% ethanol was ineffective against *C. difficile* spores at RT (Nerandzic and Donskey, 2010), our current results suggest that spore germination at 40 °C can accelerate *C. perfringens* spore inactivation by ethanol at relatively lower temperature and concentration as compared to aforementioned studies with other spore-forming bacteria.

The survivals of SM101 spores were reduced by 2.66 and 2.90 log CFU/ml when 200 ppm Quats were applied for 1 min to spores germinated with AK at RT and 40 °C (Fig 4.3A), respectively. In contrast, no significant reduction in survival of NB16 spores was observed after inducing AK germination at 40 °C followed by 1 min treatment with 200 ppm Quats (Fig. 4.3B). These results suggest that spores of NFB isolates exhibit higher resistance to Quats than that of FP isolates as was observed in the case of nisin resistance (Udompijtkul et al., 2012). These differences might be due to strain-specific susceptibility to Quats (Dawson et al., 2011). Like alcohol inactivation experiment, increasing treatment time up to 10 min did not show higher killing of germinated spores with 200 ppm Quats (data not shown). Although ethanol and Quats are classified as sporostatic compounds that lack abilities to kill bacterial spores even at longer exposure period (Russell, 1990), our current results

demonstrated that initiating spore germination prior to inactivation with these sporostatic agents could enhance their spore – killing abilities, especially against spores of *C. perfringens* FP isolates.

Treatment with 25 ppm iodophore for 5 min was required to get a significant ($p < 0.05$) level of sporicidal activity against dormant spores of both FP strain SM101 and NFB strain NB16 after germinating with control buffer at RT and 40 °C (Fig. 4C-D; black and grey bars), whereas 12.5 ppm iodophore was ineffective against dormant spores even with the longer treatment time (10 min) (Fig. 4A-B; black and grey bars). The requirement for longer treatment time, compared to manufacturer recommended time (5 min versus 2 min, respectively), to inactivate *C. perfringens* spores is in agreement with previous studies where 100-500 ppm iodophore showed only limited sporicidal activity against *Bacillus* spores with the exposure time of 12 - 240 min (Cords et al., 2005; Cousins and Allan, 1967). Regardless of germination temperature, AK germinated SM101 spores were killed significantly ($p < 0.05$) after exposing to 25 ppm iodophore for 2 min ($> 5 \log$ CFU/ml reduction) (Fig. 4.4 C). However, under similar conditions, a lesser sporicidal activity of iodophore was observed against germinated spores of NFB strain NB16 (Fig. 4.4D). Germination temperature played a major role in enhancing the spore – killing efficacy of 12.5 ppm iodophore; germination induction especially at 40 °C facilitate inactivation of spores of FP strain SM101 (Fig. 4.4A).

As shown in Fig. 4.5, DeconSpore (the mixture of H₂O₂ and peroxyacetic acid) exhibited sporicidal activity against *C. perfringens* dormant spores with the extended

exposure time (30 – 60 min). However, germination of SM101 spores with AK at either 40 °C or RT caused a significant reduction ($p < 0.05$) in spore survival after 5 min treatment with DeconSpore. Stimulation of spore germination also shortened the treatment time (10 min versus 60 min for dormant spores) to achieve ~ 4 – 5 log spore reductions (Fig. 4.5A); however, this magnitude of killing was not observed in case of NFB strain NB16 for both germinated and non-germinated spores (Fig. 4.5B). This differential sporicidal activity of DeconSpore could be due to the variability of effectiveness of peroxyacetic acid or H₂O₂. For example, the peroxyacetic acid-based sanitizer showed a strong sporicidal effect against *Clostridium estertheticum* spores (Broda, 2007); however, this was ineffective against *B. cereus* and *Bacillus thuringiensis* spores attached to stainless steel and apples (Kreske et al., 2006). Also, exposure planktonic *Bacillus* spp. spores to 100,000 ppm H₂O₂ for 1 min at RT showed a limited sporicidal action (Khadre and Yousef, 2001).

4.3.3. Inactivation of *C. perfringens* spores on SS chips

Next, we evaluated whether the inactivation strategy developed above for spores in suspension could be utilized for inactivation of spores attached to food contact surface materials typically used in food industries. SS was selected as a model of food contact surfaces due to its widely use in both food industries and domestic kitchens (Holah and Thorpe, 1990; Liu et al., 2006). After inoculating the SS chip with spores (approximately 10⁸ CFU/ml) cocktail from three *C. perfringens* FP isolates, the initial adhesion of spores on chip was approximately 5 log CFU/chip (Count A, Table 4.3). Increasing the concentration of spores and drying period during

adhesion did not increase spores adherence to SS chips (data not shown). This suggests that the spore population of 10^8 CFU/ml was already excessive for bacterial adhesion. Because the suspension tests indicated that inducing germination at 40 °C with AK before inactivation gave a greater spore killing effect than at RT for both FP and NFB isolates, the surface decontamination experiments were conducted only with spores germinated at 40 °C.

Table 4.3 results clearly showed that AK could induce significant ($p < 0.05$) germination of *C. perfringens* FP spores attached onto SS chips (Count C); therefore, there is a potential to develop inactivation strategy for *C. perfringens* spores by implementing germination step prior to sanitization. Previous study with *B. cereus* spores germinated with the mixture of L-alanine and inosine at 30 °C for 60 min showed the identical spore germination efficiencies in suspension and on SS chips (Hornstra et al., 2007). However, we have found contradictory results with NFB isolates' spores; AK could induce germination of spores of NFB strains only in suspensions, but not on SS chips (Table 4.1 and data not shown). The initial number of NFB spores attached to SS surfaces after drying was approximately the same (5 log CFU/chip) as FP spores (data not shown); thus, NFB spores remained viable, but lost their ability to germinate with AK. It is unclear whether this could be due to the long exposure of NFB spores to oxygen during drying period (1 h) prior to germination induction. Therefore, we examined spore inactivation on SS chips using only spores of FP isolates. In contrast to the suspension experiments, we observed ~ 1 log CFU/chip reduction of viable spores upon germination with AK compared to initial counts and

spores exposed to control buffer. Thus, the decrease of *C. perfringens* spores after AK germination on SS chips might be attributed to the lower adherence capacities of germinated spores compared to the non-germinated spores and they detached from the surface during germination process.

Based on the data in the suspension tests, the most effective and practical exposure time for each disinfectant had been selected to apply onto SS chips. Our results demonstrated the significant reduction in the populations of *C. perfringens* spores after treatment with all tested disinfectants (Table 4.3, Count D) compared to respective control and AK – induced germination samples ($p < 0.05$). Under dormant condition (shown in the control experiments), only iodophore showed a minor sporicidal activity with 0.33 - 0.42 log reductions when compared with the viable counts for the untreated spores. The insignificant increase in Count D of control samples compared with Count A was probably due to the slightly variations of spore inoculum size into each SS chip. Furthermore, after treatment, SS chips became wet with the disinfectant solution and neutralized water that could facilitate the removal of adhered spores from the surfaces compared to the detachment of cells from dry surfaces with wet cotton swabs. Germinating spores were much more susceptible to the antimicrobial effect of disinfectants and resulted in the decrease of 1.53 – 2.70 log CFU/chip. Exposing germinated spores with 25 ppm iodophore for 5 min being the most effective treatment, which was comparable to treatment with 12.5 ppm iodophore for 10 min. Ethanol, Quats and DeconSpore had similar sporicidal efficiency against

C. perfringens spores adhered onto SS surfaces under conditions in these experiments (Table 4.3).

The sporicidal activities of all tested disinfectants were greatly lower when spores attached to surfaces (Table 4.3), especially considering the initial lower spore number deposited on the SS chips ($\sim 5 \log$ CFU/coupon). This might be attributed to the limited access of disinfectants to spores embedded on crevices on SS surfaces or spores that forming clumps. Bacterial spores adhered onto SS surfaces were more resistant to disinfectants than spores in suspensions, which is in agreement with previous findings (André et al., 2012; Kreske et al., 2006). It was suggested that bacterial spores respond differently to the environmental signals upon their adhesion to the surfaces and surface properties were believed to play an important role in adhesion and retention of bacterial spores on particular surfaces (Faille et al., 2001; Faille et al., 2002; Simmonds et al., 2003).

Owing to their high hydrophobic nature, bacterial spores exhibit stronger attachment to SS surfaces than vegetative counterparts (Parker et al., 2001). This poses the problem in an attempt to remove spores from surfaces by conventional sanitizing protocols that are effective against vegetative cells. Therefore, retention of spores on surfaces can be a continuous source of cross - contamination into food products affecting product's shelf life and safety of consumer. The germination-induction step prior to decontamination of SS surfaces is expected to reduce the number of spores in the processing lines. However, our results indicated that not all the spores were germinated at the same time or even within the longest time period set in this study

(data not shown). This heterogeneity of *C. perfringens* spore germination has been demonstrated in our previous study (Wang et al., 2011a). The condition(s) that could trigger germination of these superdormant spores is currently unknown; however, it is plausible to assume that these spores will remain dormant in the food industry environments (Hornstra et al., 2007). Since the major cause of *C. perfringens* type A FP is the consumption of food products contaminated with the spores that could germinate and outgrowth during storage at abusive temperature (Mcclane, 2007a), the contamination of the highly dormant spores that are unable to germinate in food processing environments and foodstuffs should not pose any threat for safety. It was suggested that only bacterial spores that could germinate instantly with germination – inducing compounds are potentially being spores that respond promptly to the nutrient – rich condition of food products (Hornstra et al., 2007).

4.4. Conclusions

Our current study demonstrated the following important findings: 1) The mixture of AK (100 mM, pH 7.0) was the most effective universal germinant for spores of the enterotoxigenic *C. perfringens* type A; 2) Germination temperature influenced on extent of spore germination and subsequent inactivation by commonly used disinfectants; 3) Initiating AK-germination enhanced sporicidal effects of all tested disinfectants against spores of FP isolates; and finally 4) Inducing spore germination on SS surfaces with AK for 30 min at 40 °C followed by iodophore treatment (25 ppm for 5 min) represented a promising strategy to inactivate spores of

C. perfringens FP isolates. Collectively, these results suggested that germination stimulation should have various benefits, such as increasing the efficacies of disinfectants, shortening the exposure time to disinfectants, and inactivating spores at lower concentration and temperature of sanitizers which might have a potential in reducing cost of production and length of sanitation regime. However, further studies are necessary to improve germination conditions for higher extent of germination, to evaluate this decontamination protocol on other types of food contact surfaces, and finally to validate the effectiveness of the invented strategy under the practical conditions of food industry environments.

Acknowledgements

The authors would like to thank Kevan Acker and Vathani Logendarn for helping in *C. perfringens* spore preparation. We are grateful to Veltek Associates, Inc. for providing DECON-SPORE 200 PLUS[®] in this study. This work was supported by a grant from the Agricultural Research Foundation of Oregon State University, and by a Department of Defense Multidisciplinary University Research Initiative (MURI) award through the U.S. Army Research Laboratory and the U. S. Army Research Office under contract number W911NF-09-1-0286 (all to MRS). PU and MA were supported by fellowships from the Office of Higher Education Commission (Thailand) and from the Ministry of Higher Education (Saudi Arabia), respectively.

References

- Akhtar, S., Paredes-Sabja, D., Torres, J.A., & Sarker, M.R. (2009). Strategy to inactivate *Clostridium perfringens* spores in meat products. *Food Microbiology*, *26*, 272-277.
- Alberto, F., Broussolle, V., Mason, D.R., Carlin, F., & Peck, M.W. (2003). Variability in spore germination response by strains of proteolytic *Clostridium botulinum* types A, B and F. *Letters in Applied Microbiology*, *36*, 41-45.
- André, S., Hédin, S., Remize, F., & Zuber, F. (2012). Evaluation of peracetic acid sanitizers efficiency against spores isolated from spoiled can in suspension and on stainless steel surfaces. *Journal of Food Protection*, *75*, 371-375.
- Bae, Y.M., & Lee, S.Y. (2012). Inhibitory effects of UV treatment and a combination of UV and dry heat against pathogens on stainless steel and polypropylene surfaces. *Journal of Food Science*, *71*, M61-M64.
- Blatchley, E.R., Meusen, A., Aronson, A.I., & Brewster, L. (2005). Inactivation of *Bacillus* spores by ultraviolet or gamma radiation. *Journal of Environmental Engineering*, *131*, 1245-1252.
- Broda, D.M. (2007). The effect of peroxyacetic acid - based sanitizer, heat and ultrasonic waves on the survival of *Clostridium estertheticum* spores *in vitro*. *Letters in Applied Microbiology*, *45*, 336-341.
- Brynstad, S., Synstad, B., & Granum, P.E. (1997). The *Clostridium perfringens* enterotoxin gene is on a transposable element in type A human food poisoning strains. *Microbiology*, *143*, 2109-2115.
- Clouston, J.G., & Wills, P.A. (1969). Initiation of germination and inactivation of *Bacillus pumilus* spores by hydrostatic pressure. *Journal of Bacteriology*, *97*, 684-690.
- Collie, R.E., & McClane, B.A. (1998). Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. *Journal of Clinical Microbiology*, *36*, 30-36.
- Cords, B.R., Burnett, S.L., Hilgren, J., Finley, M., & Magnusen, J. (2005). Sanitizers: Halogens, surface - active agents, and peroxides. In: Davidson, P.M., Sofos, J.N., Branan, A.L. (Eds.), *Antimicrobials in food* (pp. 507-572). Boca Raton: CRC Press.
- Cornillot, E., Saint-Joanis, B., Daube, G., Katayama, S., Granum, P.E., Canard, B., & Cole, S.T. (1995). The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. *Molecular Microbiology*, *15*, 639-647.
- Cousins, C.M., & Allan, C.D. (1967). Sporicidal properties of some halogens. *Journal of Applied Bacteriology*, *30*, 168-174.
- Dawson, L.F., Valiente, E., Donahue, E.H., Birchenough, G., & Wren, B.W. (2011). Hypervirulent *Clostridium difficile* PCR-ribotypes exhibit resistance to widely used disinfectants. *PLoS ONE*, *6*, e25754.
- Duncan, C.L., & Strong, D.H. (1968). Improved medium for sporulation of *Clostridium perfringens*. *Applied Microbiology*, *16*, 82-89.

- Durban, E., Goodnow, R., & Grecz, N. (1970). Changes in resistance to radiation and heat during sporulation and germination of *Clostridium botulinum* 33A. *Journal of Bacteriology*, *102*, 590-592.
- Faille, C., Fontaine, F., & Benezech, T. (2001). Potential occurrence of adhering living *Bacillus* spores in milk product processing lines. *Journal of Applied Microbiology*, *90*, 892-900.
- Faille, C., Jullien, C., Fontaine, F., Bellon-Fontaine, M.-N., Slomianny, C., & Benezech, T. (2002). Adhesion of *Bacillus* spores and *Escherichia coli* cells to inert surfaces: role of surface hydrophobicity. *Canadian Journal of Microbiology*, *48*, 728-738.
- Foerster, H.F., & Foster, J.W. (1966). Response of *Bacillus* spores to combinations of germinative compounds. *Journal of Bacteriology*, *91*, 1168-1177.
- Gould, G.W., Jones, A., & Wrighton, C. (1968). Limitations of the initiation of germination of bacterial spores as a spore control procedure. *Journal of Applied Bacteriology*, *31*, 357-366.
- Harrison, B., Raju, D., Garmory, H.S., Brett, M.M., Titball, R.W., & Sarker, M.R. (2005). Molecular characterization of *Clostridium perfringens* isolates from humans with sporadic diarrhea: evidence for transcriptional regulation of the beta2-toxin-encoding gene. *Applied and Environmental Microbiology*, *71*, 8362-8370.
- Holah, J.T. (2003). Cleaning and disinfection. In: Lelieveld, H.L.M., Mostert, M.A., Holah, J.White, B. (Eds.), *Hygiene in food processing* (pp. 235-278). Boca Raton, FL: CRC Press.
- Holah, J.T., & Thorpe, R.H. (1990). Cleanability in relation to bacterial retention on unused and abraded domestic sink materials. *Journal of Applied Bacteriology*, *69*, 599-608.
- Hornstra, L.M., de Leeuw, P.L.A., Moezelaar, R., Wolbert, E.J., de Vries, Y.P., de Vos, W.M., & Abee, T. (2007). Germination of *Bacillus cereus* spores adhered to stainless steel. *International Journal of Food Microbiology*, *116*, 367-371.
- Khadre, M.A., & Yousef, A.E. (2001). Sporicidal action of ozone and hydrogen peroxide: a comparative study. *International Journal of Food Microbiology*, *71*, 131-138.
- Kreske, A.C., Ryu, J.H., & Beuchat, L.R. (2006). Evaluation of chlorine, chlorine dioxide, and peroxyacetic acid-based sanitizer for effectiveness in killing *Bacillus cereus* and *Bacillus thuringiensis* spores in suspensions, on the surface of stainless steel, and on apples. *Journal of Food Protection*, *69*, 1892-1903.
- Kusumaningrum, H.D., Riboldi, G., Hazeleger, W.C., & Beumer, R.R. (2003). Survival of foodborne pathogens on stainless steel surfaces and cross-contamination to foods. *International Journal of Food Microbiology*, *85*, 227-236.
- Lahti, P., Heikinheimo, A., Johansson, T., & Korkeala, H. (2008). *Clostridium perfringens* type A strains carrying a plasmid-borne enterotoxin gene

- (genotype IS1151-*cpe* or IS1470-like-*cpe*) as a common cause of food poisoning. *Journal of Clinical Microbiology*, 46, 371-373.
- Leuschner, R.G.K., & Lillford, P.J. (1999). Effect of temperature and heat activation on germination of individual spores of *Bacillus subtilis*. *Letters in Applied Microbiology*, 29, 228-232.
- Levinson, H.S., & Hyatt, M.T. (1970a). Activation energy for glucose-induced germination of *Bacillus megaterium* spores. *Journal of Bacteriology*, 103, 270-271.
- Levinson, H.S., & Hyatt, M.T. (1970b). Effects of temperature on activation, germination, and outgrowth of *Bacillus megaterium* spores. *Journal of Bacteriology*, 101, 58-64.
- Li, J., & McClane, B.A. (2006a). 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. *Applied and Environmental Microbiology*, 72, 7620-7625.
- Li, J., & McClane, B.A. (2006b). Further comparison of temperature effects on growth and survival of *Clostridium perfringens* type A isolates carrying a chromosomal or plasmid-borne enterotoxin gene. *Applied and Environmental Microbiology*, 72, 4561-4568.
- Lindström, M., Heikinheimo, A., Lahti, P., & Korkeala, H. (2011). Novel insights into the epidemiology of *Clostridium perfringens* type A food poisoning. *Food Microbiology*, 28, 192-198.
- Liu, C., Duan, J., & Su, Y.C. (2006). Effects of electrolyzed oxidizing water on reducing *Listeria monocytogenes* contamination on seafood processing surfaces. *International Journal of Food Microbiology*, 106, 248-253.
- Løvdal, I.S., Hovda, M.B., Granum, P.E., & Rosnes, J.T. (2011). Promoting *Bacillus cereus* spore germination for subsequent inactivation by mild heat treatment. *Journal of Food Protection*, 74, 2079-2089.
- McClane, B.A. (2007). *Clostridium perfringens*. In: Doyle, M.P. Beuchat, L.R. (Eds.), *Food Microbiology: fundamentals and frontiers* (pp. 423-444). Washington, D.C.: ASM Press.
- Miki, Y., Miyamoto, K., Kaneko-Hirano, I., Fujiuchi, K., & Akimoto, S. (2008). Prevalence and characterization of enterotoxin gene-carrying *Clostridium perfringens* isolates from retail meat products in Japan. *Applied and Environmental Microbiology*, 74, 5366-5372.
- Munakata, N. (1974). Ultraviolet sensitivity of *Bacillus subtilis* spores upon germination and outgrowth. *Journal of Bacteriology*, 120, 59-65.
- Nerandzic, M.M., & Donskey, C.J. (2010). Triggering germination represents a novel strategy to enhance killing of *Clostridium difficile* spores. *PLoS ONE*, 5, 1-8.
- Paredes-Sabja, D., Gonzalez, M., Sarker, M.R., & Torres, J.A. (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*, 72, M202-M206.

- Paredes-Sabja, D., Torres, J.A., Setlow, P., & Sarker, M.R. (2008). *Clostridium perfringens* spore germination: characterization of germinants and their receptors. *Journal of Bacteriology*, *190*, 1190-1201.
- Parkar, S.G., Flint, S.H., Palmer, J.S., & Brooks, J.D. (2001). Factors influencing attachment of thermophilic bacilli to stainless steel. *Journal of Applied Microbiology*, *90*, 901-908.
- Ramirez, N., & Abel-Santos, E. (2010). Requirements for germination of *Clostridium sordellii* spores *in vitro*. *Journal of Bacteriology*, *192*, 418-425.
- Russell, A.D. (1990). Bacterial spores and chemical sporicidal agents. *Clinical Microbiology Reviews*, *3*, 99-119.
- Sarker, M.R., Carman, R.J., & McClane, B.A. (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. *Molecular Microbiology*, *33*, 946-958.
- Sarker, M.R., Shivers, R.P., Sparks, S.G., Juneja, V.K., & McClane, B.A. (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. *Applied and Environmental Microbiology*, *66*, 3234-3240.
- Setlow, B., Loshon, C.A., Genest, P.C., Cowan, A.E., Setlow, C., & Setlow, P. (2002). Mechanisms of killing spores of *Bacillus subtilis* by acid, alkali and ethanol. *Journal of Applied Microbiology*, *92*, 362-375.
- Simmonds, P., Mossel, B.L., Intaraphan, T., & Deeth, H.C. (2003). Heat resistance of *Bacillus* spores when adhered to stainless steel and its relationship to spore hydrophobicity. *Journal of Food Protection*, *66*, 2070-2075.
- Sparks, S.G., Carman, R.J., Sarker, M.R., & McClane, B.A. (2001). Genotyping of enterotoxigenic *Clostridium perfringens* fecal isolates associated with antibiotic-associated diarrhea and food poisoning in North America. *Journal of Clinical Microbiology*, *39*, 883-888.
- Stuy, J.H. (1956). Studies on the mechanism of radiation in activation of microorganisms. III. Inactivation of germinating spores of *Bacillus cereus*. *Biochimica et Biophysica Acta*, *22*, 241-246.
- Tanaka, D., Isobe, J., Hosorogi, S., Kimata, K., Shimizu, M., Katori, K., Gyobu, Y., Nagai, Y., Yamagishi, T., Karasawa, T., & Nakamura, S. (2003). An outbreak of foodborne gastroenteritis caused by *Clostridium perfringens* carrying the *cpe* gene on a plasmid. *Japanese Journal of Infectious Diseases*, *56*, 137-139.
- Udompijitkul, P., Paredes - Sabja, D., & Sarker, M.R. (2012). Inhibitory effects of nisin against *Clostridium perfringens* food poisoning and nonfood-borne isolates. *Journal of Food Science*, *77*, M51-56.
- Van Opstal, I., Bagamboula, C.F., Vanmuysen, S.C.M., Wuytack, E.Y., & Michiels, C.W. (2004). Inactivation of *Bacillus cereus* spores in milk by mild pressure and heat treatments. *International Journal of Food Microbiology*, *92*, 227-234.
- Vary, J.C., & Halvorson, H.O. (1965). Kinetics of Germination of *Bacillus* Spores. *Journal of Bacteriology*, *89*, 1340-1347.

- Wang, G., Zhang, P., Paredes - Sabja, D., Green, C., Setlow, P., Sarker, M.R., & Li, Y.Q. (2011). Analysis of the germination of individual *Clostridium perfringens* spores and its heterogeneity. *Journal of Applied Microbiology*, *111*, 1212-1223.
- Wen, Q., & McClane, B.A. (2004). Detection of enterotoxigenic *Clostridium perfringens* type A isolates in American retail foods. *Applied and Environmental Microbiology*, *70*, 2685-2691.
- Wolf, J., & Thorley, C.M. (1957). The effects of various germination agents on the spores of some strains of *Bacillus subtilis*. *Journal of Applied Bacteriology*, *20*, 384-389.

Table 4.1. Germination of *C. perfringens* spores from FP and NFB isolates with various germinants at 40 °C

Strains	Decrease in OD ₆₀₀ (%Mean ± SD) after germination at 40 °C for 30 min ^a with:					
	AK ^b	L-Gln	L-Cys	Formula 1 ^c	Formula 2 ^d	Control ^e
FP isolates						
SM101	52.96 ± 1.18	16.48 ± 1.20	47.13 ± 2.34	48.80 ± 0.47	43.47 ± 7.92	4.89 ± 2.74
NCTC8239	46.67 ± 0.19	6.54 ± 2.66	3.87 ± 0.34	40.51 ± 0.92	40.50 ± 0.37	5.34 ± 0.75
NCTC10239	54.57 ± 0.44	29.64 ± 1.64	38.37 ± 1.09	35.67 ± 0.99	37.38 ± 1.16	9.65 ± 1.43
E13	59.63 ± 1.05	30.98 ± 1.32	36.11 ± 0.59	34.88 ± 0.52	34.43 ± 0.54	12.66 ± 1.13
6263	47.18 ± 0.35	21.25 ± 2.88	18.82 ± 1.13	22.42 ± 2.24	21.47 ± 1.50	8.93 ± 0.82
NFB isolates						
F5603	53.95 ± 1.13	12.14 ± 1.49	14.57 ± 0.53	14.93 ± 1.09	13.01 ± 0.79	7.19 ± 1.50
NB16	45.30 ± 8.98	5.04 ± 1.73	37.99 ± 3.37	24.65 ± 6.32	8.65 ± 0.77	5.91 ± 5.87
B40	27.34 ± 1.67	3.59 ± 1.20	46.71 ± 1.42	11.59 ± 5.20	7.56 ± 0.81	3.66 ± 1.26
F4969	33.39 ± 5.43	3.81 ± 3.06	57.50 ± 0.44	15.18 ± 1.93	6.98 ± 2.39	6.88 ± 2.25

^a Results were average ± SD of triplicate replications.

^b AK is the mixture of 100 mM L-asparagine and 100 mM KCl adjusted to pH 7.0.

^c Formula 1 is the mixture of 100 mM L-asparagine, 100 mM KCl, and 100 mM L-cysteine adjusted to pH 7.0.

^d Formula 2 is the mixture of 100 mM L-asparagine, 100 mM KCl, 100 mM L-cysteine, and 100 mM L-glutamine adjusted to pH 7.0.

^e Control is 25 mM Na₂HPO₄ adjusted to pH 7.0.

All germinants were tested at 100 mM and prepared with 25 mM Na₂HPO₄ buffer and adjusted to pH 7.0.

Table 4.2. Germination of *C. perfringens* spores from FP and NFB isolates with various germinants at RT

Strains	Decrease in OD ₆₀₀ (%Mean ± SD) after germination at RT for 30 min ^a with:					
	AK ^b	L-Gln	L-Cys	Formula 1 ^c	Formula 2 ^d	Control ^e
FP isolates						
SM101	47.46 ± 2.74	4.44 ± 2.23	14.36 ± 1.45	43.14 ± 1.70	43.96 ± 1.76	10.70 ± 3.93
NCTC8239	28.80 ± 8.98	4.54 ± 1.49	11.66 ± 2.30	32.35 ± 2.64	17.63 ± 3.12	3.43 ± 1.32
NCTC10239	41.88 ± 1.32	7.62 ± 1.21	12.11 ± 0.17	33.89 ± 2.08	24.58 ± 4.72	4.76 ± 2.60
E13	23.14 ± 1.08	11.11 ± 1.51	29.41 ± 0.34	24.85 ± 3.84	24.01 ± 3.24	7.85 ± 1.24
6263	6.59 ± 0.15	5.32 ± 1.57	7.73 ± 0.20	7.73 ± 0.20	10.71 ± 1.31	5.19 ± 0.77
NFB isolates						
F5603	7.83 ± 0.06	5.99 ± 3.66	8.71 ± 1.18	14.49 ± 1.42	6.88 ± 0.97	7.78 ± 2.16
NB16	2.40 ± 0.51	3.52 ± 2.33	2.36 ± 1.81	7.34 ± 1.32	4.28 ± 0.83	2.63 ± 1.69
B40	3.74 ± 3.16	2.14 ± 1.73	4.68 ± 0.62	5.28 ± 1.40	8.30 ± 3.31	3.17 ± 0.64
F4969	5.50 ± 2.38	7.01 ± 1.61	4.07 ± 0.11	5.65 ± 1.41	4.14 ± 0.86	2.84 ± 2.17

^a Results were average ± SD of triplicate replications.

^b AK is the mixture of 100 mM L-asparagine and 100 mM KCl adjusted to pH 7.0.

^c Formula 1 is the mixture of 100 mM L-asparagine, 100 mM KCl, and 100 mM L-cysteine adjusted to pH 7.0.

^d Formula 2 is the mixture of 100 mM L-asparagine, 100 mM KCl, 100 mM L-cysteine, and 100 mM L-glutamine adjusted to pH 7.0.

^e Control is 25 mM Na₂HPO₄ adjusted to pH 7.0.

All germinants were prepared with 25 mM Na₂HPO₄ buffer and adjusted to pH 7.0.

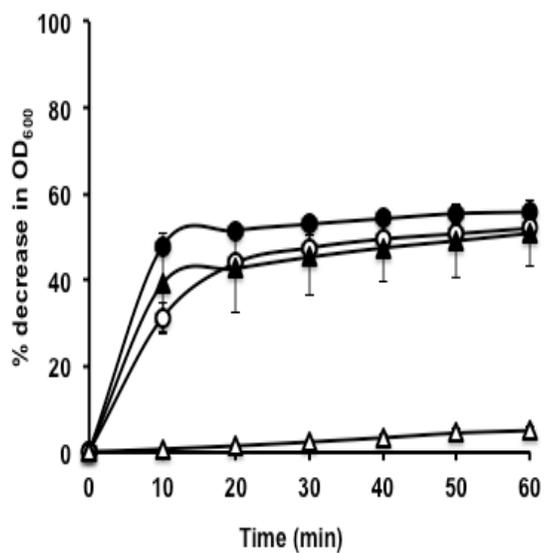


Fig. 4.1. Germination of spores of representative *C. perfringens* FP strain SM101 and NFB strain NB16 with AK. Heat-activated spores of SM101 (circle) and NB16 (triangle) were incubated at 40 °C (Filled symbols) and RT (20 ± 2 °C) (Empty symbols) with 100 mM AK adjusted to pH 7.0, and the OD₆₀₀ was measured as described in Material and Methods. Results were expressed as percentage of OD₆₀₀ decrease over 60 min. Data were means of triplicate experiments.

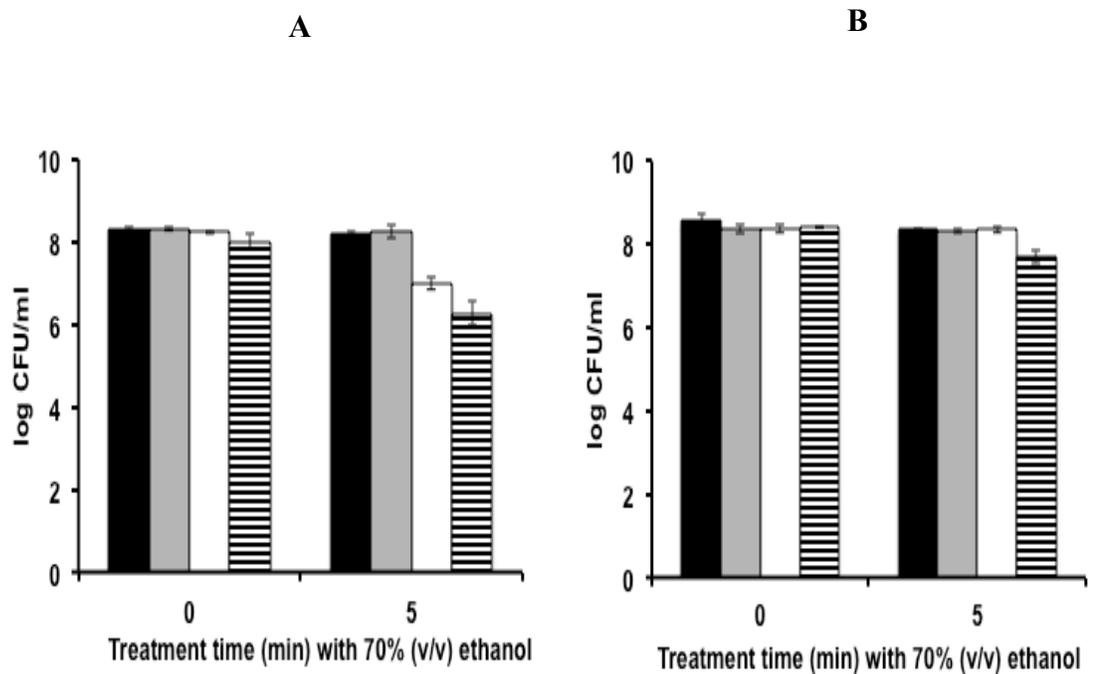


Fig. 4.2A-B. Effect of ethanol on inactivation of *C. perfringens* spores. Heat-activated spores of SM101 (A) and NB16 (B) were germinated with 25 mM Na₂HPO₄ buffer (pH 7.0) (black, grey bars) or with 100 mM AK (white, horizontal line bars) at RT (black, white bars) or 40 °C (grey, horizontal line bars) for 30 min; germinated spores were treated with 70% (v/v) ethanol; and spore survival was monitored by plating onto BHI agar and colony counted. Data were means of triplicate experiments.

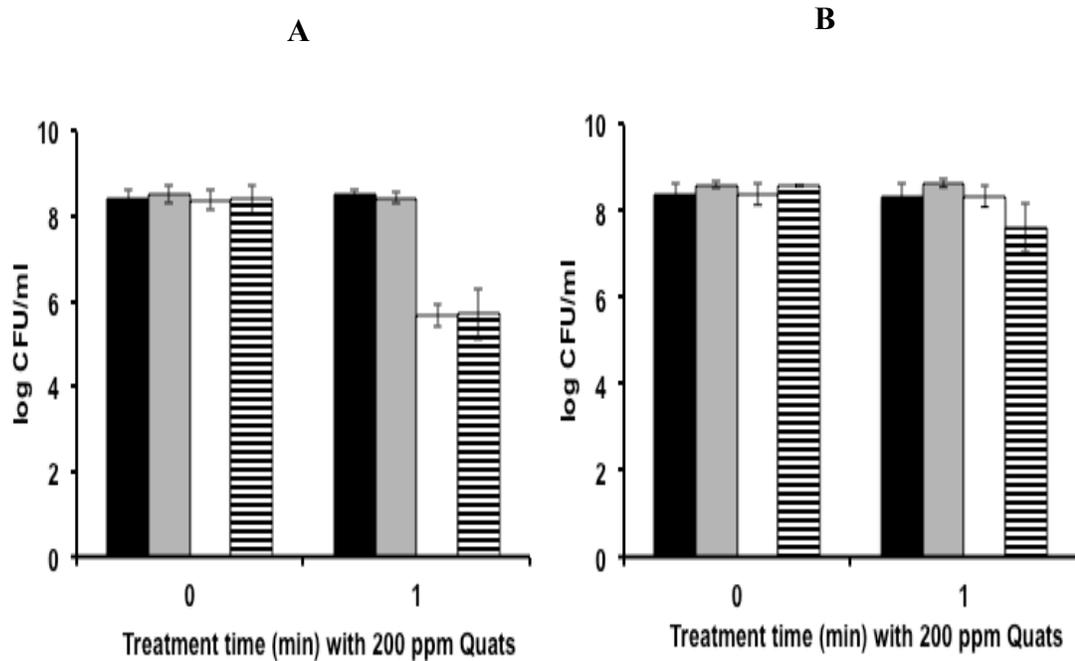


Fig. 4.3A-B. Effect of Quats on inactivating *C. perfringens* spores. Heat-activated spores of SM101 (A) and NB16 (B) were germinated with 25 mM Na₂HPO₄ buffer (pH 7.0) (black, grey bars) or with 100 mM AK (white, horizontal line bars) at RT (black, white bars) or 40 °C (grey, horizontal line bars) for 30 min; germinated spores were treated with 200 ppm Quats; and spore survival was monitored by plating onto BHI agar and colony counted. Data were means of triplicate experiments.

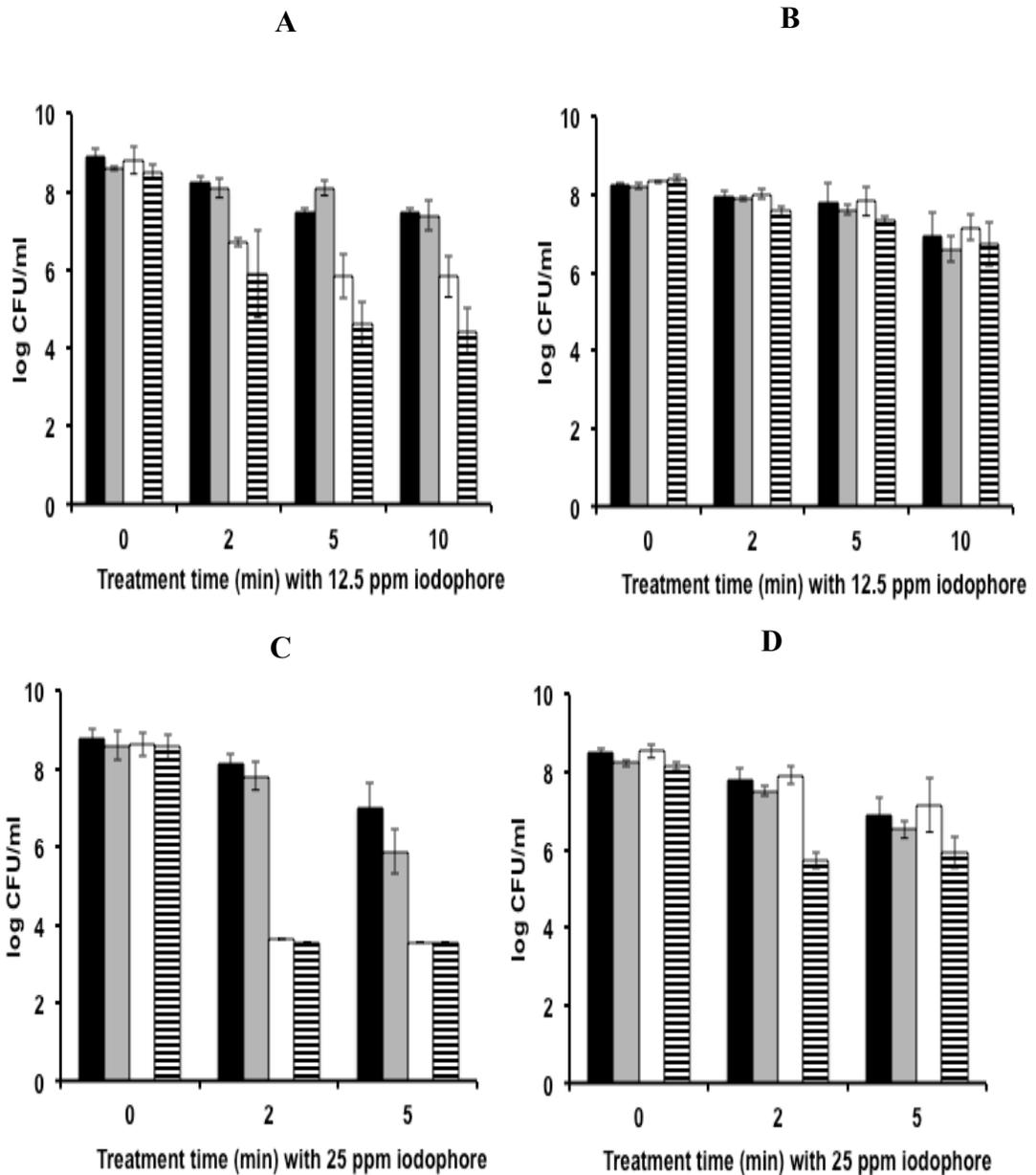


Fig. 4.4A-D. Effect of iodophores on inactivation of *C. perfringens* spores. Heat-activated spores of SM101 (A, C) and NB16 (B, D) were germinated with 25 mM Na₂HPO₄ buffer (pH 7.0) (black, grey bars) or with 100 mM AK (white, horizontal line bars) at RT (black, white bars) or 40 °C (grey, horizontal line bars) for 30 min; germinated spores were treated with 12.5 ppm (A, B) and 25 ppm iodophore (C, D) for indicated time periods; and spore survival was monitored by plating onto BHI agar and colony counted. Data were means of triplicate experiments.

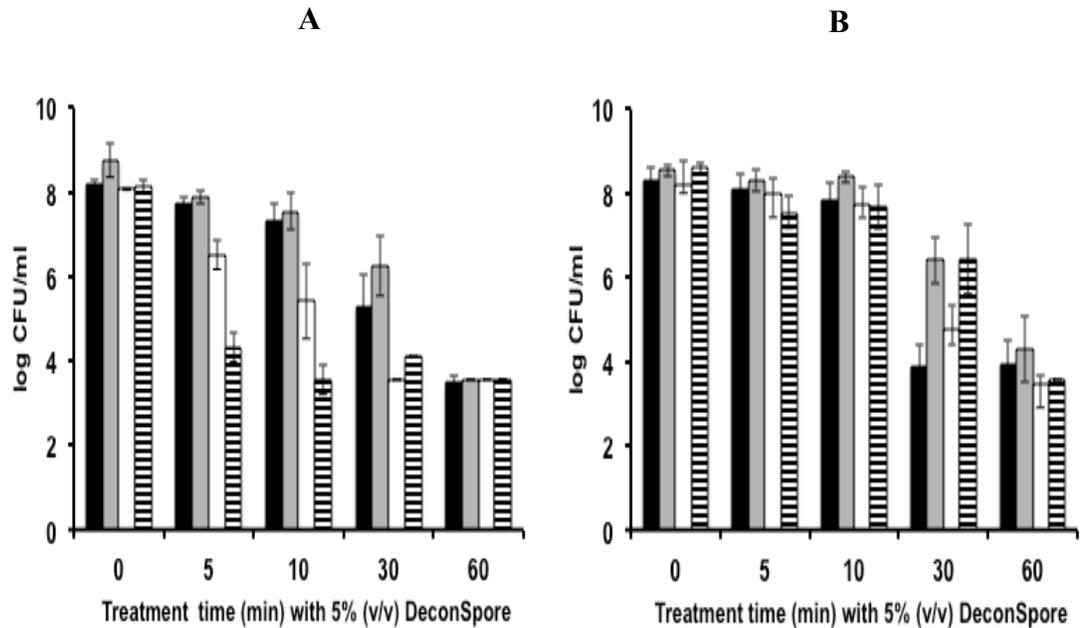


Fig. 4.5A-B. Effect of DECON-SPORE 200 PLUS on inactivating *C. perfringens* spores. Heat-activated spores of SM101 (A) and NB16 (B) were germinated with 25 mM Na₂HPO₄ buffer (pH 7.0) (black, grey bars) or with 100 mM AK (white, horizontal line bars) at RT (black, white bars) or 40 ° C (grey, horizontal line bars) for 30 min; germinated spores were treated with 5% (v/v) DECON-SPORE 200 PLUS for indicated time periods; and spore survival was monitored by plating onto BHI agar and colony counted. Data were means of triplicate experiments.

Table 4.3. Inactivation of spores of *C. perfringens* FP isolates on SS chips

Disinfectant/ Concentration	Treatment time (min)	Germinant	Initial	Germination (Total)	Germination (Dormant spores)	After treatment	Log reduc tion ^e
			Count A ^a	Count B ^b	Count C ^c	Count D ^d	
Ethanol 70% (v/v)	5	Control	5.40 ± 0.10	5.58 ± 0.12	5.27 ± 0.09	5.63 ± 0.36	- 0.23
		AK		4.49 ± 0.18	3.42 ± 0.97	3.67 ± 0.42	1.73
Iodophore 12.5 ppm	10	Control	5.62 ± 0.16	5.15 ± 0.16	5.03 ± 0.31	5.20 ± 0.08	0.42
		AK		5.06 ± 0.52	3.26 ± 0.48	2.94 ± 0.39	2.68
Iodophore 25 ppm	5	Control	5.53 ± 0.18	5.36 ± 0.12	5.12 ± 0.10	5.19 ± 0.15	0.33
		AK		4.03 ± 0.10	3.26 ± 0.14	2.83 ± 0.46	2.70
Quats 200 ppm	1	Control	5.39 ± 0.38	5.08 ± 0.11	4.85 ± 0.09	5.65 ± 0.38	- 0.27
		AK		4.08 ± 0.29	3.23 ± 0.07	3.86 ± 0.21	1.53
DeconSpore 5% (v/v)	10	Control	5.93 ± 0.24	5.85 ± 0.21	5.78 ± 0.26	5.97 ± 0.15	- 0.04
		AK		4.83 ± 0.40	3.61 ± 0.38	4.25 ± 0.32	1.68

^a Count A values were means ± SD in log CFU/chip of the number of the heat-activated FP spores that had been inoculated and dried on SS chips for 1 h. The populations of total viable cells were determined immediately as described in Material and Methods.

^b Count B values were means ± SD in log CFU/chip of the number of the heat-activated and dried FP spores on SS chips that had been induced to germinate with corresponding germinants for 30 min at 40 °C and populations of total viable cells in swabbing samples were determined as described in Material and Methods.

^c Count C values were means ± SD in log CFU/chip of the number of the heat-activated and dried FP spores on SS chips (the same SS chips as Count B) that had been induced to germinate with corresponding germinants for 30 min at 40 °C and populations of non-germinated (dormant) spores were determined with heat treatment of the germinating spores at 75 °C for 20 min as described in Material and Methods.

^d Count D values were means ± SD in log CFU/chip of the number of total viable cells remaining on SS chips after inducing to germinate and decontaminate with the particular disinfectants for indicated contact time as described in Material and Methods.

^e Log reduction values were the reduction of total viable cells in log CFU/chip after treatment with tested disinfectants and calculated as the values of Count A – Count D.

CHAPTER 5**Inorganic phosphate and Sodium Ions are Co-Germinants for Spores of
Clostridium perfringens type A Food Poisoning-Related Isolates**

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

^{*}First two authors contributed equally to this work.

Published in:

Applied and Environmental Microbiology (2009) 75(19): p. 6299-6305

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 the co-germinants Na^+ and inorganic phosphate (NaPi) at ~pH 6.0. Spores of *gerKA-KC* and *gerAA* mutant germinated to a lesser extent and released less DPA than wild-type spores with NaPi. Although *gerKB* spores germinated to a similar extent as wild-type spores with NaPi, their rate of germination was lower. Similarly, *gerO* and *gerO gerQ* mutant spores germinated slower and released less DPA than wild-type spores with NaPi. In contrast, *gerQ* spores germinated to a slightly lesser extent than wild-type spores, but released all of their DPA during NaPi germination. In sum, this study identified NaPi 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 NaPi-induced spore germination.

5.1.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 (Mcclane, 2007a; McDonnell, 1986). 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 (Mcclane, 2007a). Mostly type A isolates that produce the *C. perfringens* enterotoxin (CPE) have been associated with *C. perfringens* GI-illnesses (Mcclane, 2007a). *C. perfringens* *cpe*-positive isolates can carry the *cpe* gene on either the chromosome or a plasmid (Collie and Mcclane, 1998; Cornillot et al., 1995). 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* (Collie and Mcclane, 1998; Cornillot et al., 1995; Lahti et al., 2008; Sparks et al., 2001). 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 (100°C) (Sarker et al., 2000), cold (4°C) and freezing (-20°C) temperatures (Li and Mcclane, 2006b), and chemicals used in food industry settings (Li and Mcclane, 2006a); making FP spores more suited for FP environments than NFB spores. However, these dormant spores, under favorable environmental conditions, germinate to return to active growth,

proliferate to high numbers and then produce toxins to cause disease (Mcclane, 2007a).

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 (Paidhungat and Setlow, 2001). 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) (Paredes-Sabja et al., 2008c). 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 (Paredes-Sabja et al., 2009c; Paredes-Sabja et al., 2008c). 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 (Setlow, 2003). In *Bacillus subtilis*, release of Ca-DPA acts as signal for activation of the cortex-lytic enzyme (CLE) CwlJ (Paidhungat et al., 2001). In contrast, Ca-DPA release from the spore core has no role in triggering cortex hydrolysis during *C. perfringens* spore germination (Paredes-Sabja et al., 2008b; Paredes-Sabja et al., 2009b; Paredes-Sabja et al., 2009d); instead, Ca-DPA induces germination via GerKA and/or GerKC receptor(s) (Paredes-Sabja et al., 2009c; Paredes-Sabja et al., 2008c). 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 (Paredes-Sabja et al., 2009d; Setlow, 2003).

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 (Paredes-Sabja et al., 2008c), 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/>). Similarly, sodium ions are also present in meats (~ 30 mM), and especially in processed meat products (~ 300 - 400 mM) (USDA; <http://www.nal.usda.gov/fnic/food-comp/search/>). Consequently, in this study we found that Na⁺ and Pi at ~ 100 mM and pH 6.0 are unique co-germinants for 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.

5.2. Materials and methods

5.2.1. 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) (Zhao and Melville, 1998), NCTC8798 (Duncan et al., 1972b), NCTC10239, E13, FD1041 (Collie and McClane, 1998) and 6263 (Harrison et al., 2005b); Three NFB isolates carrying a plasmid copy of the *cpe* gene: F4969, NB16 and B40 (Collie and McClane, 1998); One type C *cpe*-negative isolate: JGS1495 (from diarrheic pig) (Garmory et al., 2000); *C. perfringens* SM101 *ger* mutants: DPS101 (*gerKA-KC*) and DPS103 (*gerAA*) (Paredes-Sabja et al., 2008c), DPS108 (*gerKB*) (Paredes-Sabja et al., 2009c); and *C. perfringens* SM101 antiporter mutants: DPS113 (*gerQ*), DPS116 (*gerO*) and DPS115 (*gerO gerQ*) (Paredes-Sabja et al., 2009a).

5.2.2. 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 (Kokai-Kun et al., 1994). 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 (Duncan and Strong, 1968); this culture was incubated for 24 h at 37°C 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 OD₆₀₀ of ~ 6 and stored at -20°C (Paredes-Sabja et al., 2008c).

5.2.3. Germination assays

Spores at an OD₆₀₀ of ~ 1.0 were routinely heat shocked (80°C; 10 min) and cooled in water at ambient temperature and germinated at 40°C and pH 6.0 with 100 mM of each of sodium phosphate (NaPi), citric acid, sodium citrate, 3-morpholinopropanesulfonic acid, maleic acid, acetic acid, or sodium acetate as previously described (Paredes-Sabja et al., 2008c). Spore germination was routinely measured by monitoring the OD₆₀₀ 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. To measure the rate at which the spore population decreases its optical density, the maximum rates of spore germination were determined by measuring the OD₆₀₀ of germinating cultures every 2.5 min, the maximum slopes calculated and maximum rates were expressed as the maximum rate of loss in OD₆₀₀ of the spore suspension relative to the initial OD₆₀₀ of the culture.

5.2.4. DPA release

DPA release during NaPi-germination was measured by incubating a heat activated spore suspension of an OD₆₀₀ of 1.0 at 40°C with 250 mM NaPi (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 OD₂₇₀ as described previously (Cabrera-Martinez et al., 2003; Paredes-Sabja et al., 2008c). 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 OD₂₇₀ of the supernatant fluid was measured (Cabrera-Martinez et al., 2003; Paredes-Sabja et al., 2008c). In *C. perfringens*, ~ 90% of the material absorbing at 270 nm comprises DPA (Paredes-Sabja et al., 2009d).

5.2.5. Statistical analyses

Students' *t* test was used for specific comparisons. All experiments were repeated four times with two independent spore preparations.

5.3. Results

5.3.1. Germination of *C. perfringens* spores with sodium phosphate (NaPi)

In our previous study we observed that 25 mM NaPi (pH 7.0) was able to induce little, but significant, germination of *C. perfringens* spores (Paredes-Sabja et al., 2008c). To evaluate if NaPi indeed could induce spore germination we assayed germination of spores of SM101 (a FP isolate) and F4969 (a NFB isolate) with various concentration of NaPi at various pH. Surprisingly, NaPi (100 mM) was able to induce germination of SM101 spores between a pH range of 5.0 - 6.5, but not below pH 5.0 and higher than pH 7.0 (Fig. 5.1A). Although the rates of germination at pH 5.0 and 6.5 were much slower than that of pH 6.0, SM101 spores were able to germinate to a similar extent between a pH range of 5.0 - 6.5 (Fig. 5.1B). Indeed, phase contrast microscopy indicates that > 90% of SM101 spores had become phase dark when germinated with NaPi between a pH range of 5.0 – 6.5 (data not shown). However, NaPi was unable to induce germination of F4969 spores at all tested pH (Fig. 5.1A,B). Phase contrast microscopy showed that 100% of F4969 spores incubated with 100 mM NaPi (at various pH) remained phase bright, indicating no germination (data not shown).

SM101 spores exhibited a maximum rate of germination with 250 mM NaPi at pH 6.0 (Fig. 5.1C). Interestingly, SM101 spores were able to germinate significantly after 60 min with NaPi 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 rate of germination (Fig.

5.1C), and no significant extent of germination with the exception of a small but significant decrease in OD₆₀₀ at 400 mM NaPi (Fig. 5.1D). Phase contrast microscopy of F4969 spores with NaPi concentrations lower than 250 mM showed that > 99% spores remained phase bright, however, with 400 mM ~ 10 % and ~ 30% of the spores became phase dark and grey, respectively, after 60 min of incubation. Collectively, these results suggest that NaPi at pH 6.0 is a unique germinant for spores of SM101 but not for F4969.

5.3.2. Na⁺ and Pi are co-germinants

To discriminate if the ability of SM101 spores to germinate with NaPi (pH 6.0) is due to NaPi or pH 6.0 in the absence of NaPi, we evaluated various organic and inorganic buffers. As expected, SM101 spores, but not F4969 spores, germinated well with NaPi (pH 6.0) (Fig. 5.2 and data not shown); however, little to insignificant germination was observed when SM101 spores were incubated with 100 mM of other buffers (i.e., MOPS, maleic acid) at pH 6.0 (Fig. 5.2). In addition, F4969 spores also exhibited no germination with any of the buffer tested (data not shown). 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 NaPi (data not shown), suggesting that the effect is uniquely due to presence of sodium and/or Pi. Since previous study (Paredes-Sabja et al., 2008c) indicated that K⁺ can trigger germination of *C. perfringens* spores, it is possible that Na⁺ is acting as a co-germinant with Pi. Indeed, 100 mM sodium citrate (pH 6.0) and 100 mM sodium acetate (pH 6.0) induced a slight increase in the extent of germination

of SM101 spores compared to that with citric acid and acetic acid, respectively (Fig. 5.2). To evaluate further the role of Na^+ in spore germination, germination assays were conducted with various concentrations of NaCl (in 25 mM NaPi to adjust pH) at pH 6.0 and 7.0 (Fig. 5.3). While SM101 spores exhibited little extent of germination at concentrations < 25 mM NaCl at pH 7.0, a significant level of germination was observed when SM101 spores were incubated at concentrations > 50 mM NaCl at pH 7.0. However, when the pH was reduced to 6.0, addition of as little as 1 mM Na^+ from NaCl, in addition to the 25 mM from the NaPi buffer, was able to induce significant level of germination (Fig. 5.3). As expected, the extent of germination of SM101 spores increased when Na^+ was substituted by K^+ (Fig. 5.3). The K^+ ions were able to induce higher extents of germination of SM101 spores at pH 7.0 than at pH 6.0 (Fig. 5.3). It was somewhat surprising that at a concentration of 250 mM of either Na^+ or K^+ induced similar extent of germination of SM101 spores at both pH 6.0 and 7.0 (Fig. 5.3). However, these results contradict with results from previous studies (Ando, 1974; Paredes-Sabja et al., 2008c) where the pH of 50 mM NaCl was not reported (Ando, 1974) and the germination assays were carried out at 30°C (Paredes-Sabja et al., 2008c). Indeed, in this study, no significant extent of germination was observed when SM101 spores were incubated with 100 mM NaCl (pH 7.0, adjusted with 25 mM NaPi) at 30°C (data not shown). Collectively, these results suggest that Na^+ acts as co-germinant with Pi.

5.3.3. NaPi triggers germination of spores of most food poisoning isolates

NaPi-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 NaPi (Fig. 5.4), suggesting that NaPi (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 NaPi (pH 6.0) (Fig. 5.4). In contrast, spores of NFB isolates were unable to germinate with NaPi (pH 6.0) when incubated up to 60 min (Fig. 5.4). Although NFB spores required a lower heat activation temperature (Paredes-Sabja et al., 2008c), similar results were observed (i.e., no significant germination) with NaPi-germination when NFB spores were heat activated at 75°C for 10 min (data not shown). Interestingly, the negative NaPi-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 NaPi (pH 6.0) (Fig. 5.4). Collectively, these results clearly indicate that NaPi (pH 6.0) is a unique germinant for spores of most FP isolates.

5.3.4. Role of germinant receptors in NaPi germination of *C. perfringens* spores

To gain insight into the mechanism of NaPi-germination of *C. perfringens* spores, germination of spores of strains carrying mutations in germination receptor genes *gerKA-KC*, *gerKB* and *gerAA* (Paredes-Sabja et al., 2009c; Paredes-Sabja et al., 2008c) were assayed using optimum (250 mM) NaPi 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. 5.5A). 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 NaPi (data not shown). In contrast, *gerAA* spores exhibited significantly ($p < 0.001$) lesser extent of germination than that of wild-type and *gerKB* spores (Fig. 5.5A), as $\sim 50\%$ 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. 5A), with phase contrast microscopy results showing that $\sim 35\%$ of *gerKA-KC* spores had become phase dark after 60 min of incubation with NaPi (data not shown). These results suggest that GerKA-KC and GerAA receptors are the major receptors involved in NaPi-germination, and that GerKB may play an auxiliary role.

The rate of germination was measured to determine the role of the Ger-receptors in the initial triggering of germination by NaPi. Interestingly, *gerKA-KC* spores exhibited a rate of germination ~ 5 -fold lower than that of wild-type spores and ~ 2 -fold lower than that of *gerKB* and *gerAA* spores when germinated with NaPi (Fig.

5.5B). However, *gerKB* and *gerAA* spores exhibited similar rate of germination, but this was ~ 2-fold lower when compared to that of wild-type spores (Fig. 5.5B). These results suggest that GerKA-KC and GerAA receptors are the major receptors involved in NaPi-germination, and that GerKB is required for normal NaPi-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 chelate to Ca^{2+} (Ca-DPA) (Paredes-Sabja et al., 2008c; Setlow, 2003). Consequently, to investigate the role of each *ger*-receptor in DPA-release during NaPi-induced germination, we measured DPA release during NaPi 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. 5C). However, although *gerAA* spores released the majority of their DPA during NaPi germination, the amount of DPA remaining in the core of *gerAA* spores was still slightly higher than that of wild-type spores, which is statistically significant ($p < 0.01$) (Fig. 5.5C). *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 suggest that GerKA and/or GerKC are mainly involved in DPA-release during NaPi-triggered germination, and with GerAA and GerKB having an auxiliary role.

5.3.5. Role of antiporters in NaPi germination of *C. perfringens* spores

Recently we have identified two putative antiporters (i.e., GerO and GerQ) involved in germination of *C. perfringens* spores (Paredes-Sabja et al., 2009a); GerO, a putative Na⁺/K⁺-H⁺ antiporter, is required for normal germination with all known germinants for *C. perfringens*, while GerQ is required mainly in absence of GerO. To evaluate their roles in NaPi-triggered germination, spores of antiporter mutants were assayed for germination in the presence of NaPi (pH 6.0). *gerQ* spores exhibited a slightly slower and lesser extent of germination than that of wild-type spores (Fig. 5.6A); *gerO* spores germinated even more lesser extent. Indeed, phase contrast microscopy indicated that only ~ 75% of *gerO* spores had become phase dark after 60 min of germination with NaPi (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. 5.6A), 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 rate of germination than that of wild-type (Fig. 6B). These results clearly suggest that GerO and GerQ, only in the absence of GerO, are essential for normal NaPi-triggered germination.

Interestingly, *gerQ* spores were able to release similar amounts of DPA as wild-type spores after 60 min of incubation with NaPi (Fig. 5.6C), consistent with the majority of the spores completing germination. To our surprise, *gerO* spores also released similar amounts of DPA as wild-type spores (Fig. 5.6C), although it is unclear why the extent of germination was significantly less than wild-type spores

(Fig. 5.6A and see discussions). However, *gerO gerQ* spores released significantly ($p < 0.001$) less DPA than wild-type, *gerO* and *gerQ* spores (Fig. 5.6C). These results indicate that NaPi-induced DPA release is affected only in the absence of both GerO and GerQ antiporters, similarly as in the case of K^+ -induced germination (Paredes-Sabja et al., 2009a).

5.4. 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 (Sarker et al., 2000). The unique ability of FP isolates' spores to germinate with K^+ ions (Paredes-Sabja et al., 2008c) might provide greater advantage over spores of NFB isolates present in meat products to germinate, outgrow and proliferate. In this respect and perhaps the major conclusion of this work is that Na^+ and Pi are novel co-germinants of spores of *C. perfringens* FP isolates. Na^+ and Pi, together with K^+ , are intrinsic minerals found in meat and processed meat products and 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. For example, in non-processed meat products, where Na^+ concentration is ~ 30 mM, Na^+ and Pi might act as co-germinants together with the major germinant K^+ triggering germination of FP spores during ripening of the meats. Contrarily, in processed meat products with Na^+ concentrations of ~ 300-400 mM, it is likely that Na^+ might become a major germinant

together with K^+ triggering germination of FP spores. Results from this study also suggest that spores of FP isolates might be sensing these ions through an evolved ionic germination mechanism not present in spores of NFB isolates. This concept has been previously proposed for some strains of *B. megaterium* and *C. perfringens* strain NCTC8238 (Ando, 1974; Rode and Foster, 1962). Several studies (Johansson et al., 2006; Rooney et al., 2006), 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 NaPi-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 (Myers et al., 2006). One possibility that deserves further research is that perhaps key residues within these receptor proteins might be essential for the unique NaPi and K^+ -germination of FP isolates.

A second major conclusion is that NaPi-germination of FP spores requires, although to different extents, all four Ger-receptors. The main germinant receptor proteins involved in NaPi-germination are the GerKA and/or GerKC, which are also essential for L-asparagine germination and have a major role in KCl-germination (Paredes-Sabja et al., 2008c). However, *gerKA-KC* spores are still able to germinate significantly with KCl (Paredes-Sabja et al., 2008c) as well as with NaPi (this study),

suggesting that other Ger-proteins might have a role in DPA release during NaPi-germination. Indeed, in contrast to KCl-germination (Paredes-Sabja et al., 2008c), GerAA also has a role in NaPi-germination, with *gerAA* spores germinating at a much slower rate and to a lesser extent than wild-type spores (Fig. 5.5A,B). In contrast, GerKB, which was previously shown to have no role in nutrient-germination of FP spores (Paredes-Sabja et al., 2009c), appears to play a role in normal germination of FP spores with NaPi. Clearly, the majority of *C. perfringens* Ger-receptors are required for initial triggering of germination with NaPi, suggesting that all *C. perfringens* Ger-receptors form a complex-receptor and that absence of GerKA-KC, GerAA or GerKB have major, medium and little effect on the complex-receptor's function, respectively. Further biochemical studies on receptor proteins should help clarify this issue.

A third notable conclusion is that the putative antiporters GerO and GerQ, only in the absence of GerO, are required for normal NaPi-germination of *C. perfringens* spores. Our previous study showed that these antiporters are also essential for normal germination of *C. perfringens* spores with various germinants, and that their role precedes DPA release (Paredes-Sabja et al., 2009a). Interestingly, GerO was also essential for normal NaPi-germination; however, in contrast to *gerO* spores releasing significantly less DPA than wild-type spores through K⁺-germination (Paredes-Sabja et al., 2009a), here, *gerO* spores are able to release the majority of their DPA during NaPi-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 (Paredes-Sabja et al., 2009a) could be due to the nature of the novel germinant (NaPi), or the fact that we used 2-fold higher concentration of NaPi, creating a higher ionic strength in the medium that could in some fashion stimulate more DPA release. Although the rate of germination of *gerQ* spores was lower than that of wild-type indicating reduced initial triggering of germination, 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 lower initial triggering of germination and germinated to a lesser extent 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 (Paredes-Sabja et al., 2009a) including NaPi (this study). However, 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.

Acknowledgements

This work was supported by a grant from the N. L. Tartar Foundation of Oregon State University, by a grant from the Agricultural Research Foundation of Oregon State University, and by a grant from the Army Research Office (all to MRS), and a fellowship from Office of the Higher Education Commission (Thailand) to PU.

References

- Ando, Y. (1974). Ionic germination of spores of *Clostridium perfringens* type A. *Japanese Journal of Microbiology*, *18*, 433-439.
- Cabrera-Martinez, R.M., Tovar-Rojo, F., Vepachedu, V.R., & Setlow, P. (2003). Effects of overexpression of nutrient receptors on germination of spores of *Bacillus subtilis*. *Journal of Bacteriology*, *185*, 2457-2464.
- Collie, R.E., & McClane, B.A. (1998). Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. *Journal of Clinical Microbiology*, *36*, 30-36.
- Cornillot, E., Saint-Joanis, B., Daube, G., Katayama, S., Granum, P.E., Canard, B., & Cole, S.T. (1995). The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. *Molecular Microbiology*, *15*, 639-647.
- Duncan, C.L., & Strong, D.H. (1968). Improved medium for sporulation of *Clostridium perfringens*. *Applied Microbiology*, *16*, 82-89.
- Duncan, C.L., Strong, D.H., & Sebald, M. (1972). Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. *Journal of Bacteriology*, *110*, 378-391.
- Garmory, H.S., Chanter, N., French, N.P., Bueschel, D., Songer, J.G., & Titball, R.W. (2000). Occurrence of *Clostridium perfringens* beta2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiology & Infection*, *124*, 61-67.
- Harrison, B., Raju, D., Garmory, H.S., Brett, M.M., Titball, R.W., & Sarker, M.R. (2005). Molecular characterization of *Clostridium perfringens* isolates from humans with sporadic diarrhea: evidence for transcriptional regulation of the beta2-toxin-encoding gene. *Applied and Environmental Microbiology*, *71*, 8362-8370.
- Johansson, A., Aspan, A., Bagge, E., Baverud, V., Engstrom, B.E., & Johansson, K.E. (2006). Genetic diversity of *Clostridium perfringens* type A isolates from animals, food poisoning outbreaks and sludge. *BMC Microbiology*, *6*, 47.
- Kokai-Kun, J.F., Songer, J.G., Czczulin, J.R., Chen, F., & McClane, B.A. (1994). Comparison of Western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens*. *Journal of Clinical Microbiology*, *32*, 2533-2539.
- Lahti, P., Heikinheimo, A., Johansson, T., & Korkeala, H. (2008). *Clostridium perfringens* type A strains carrying a plasmid-borne enterotoxin gene (genotype IS1151-*cpe* or IS1470-like-*cpe*) as a common cause of food poisoning. *Journal of Clinical Microbiology*, *46*, 371-373.
- Li, J., & McClane, B.A. (2006a). 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. *Applied and Environmental Microbiology*, *72*, 7620-7625.
- Li, J., & McClane, B.A. (2006b). Further comparison of temperature effects on growth and survival of *Clostridium perfringens* type A isolates carrying a

- chromosomal or plasmid-borne enterotoxin gene. *Applied and Environmental Microbiology*, *72*, 4561-4568.
- McClane, B.A. (2007). *Clostridium perfringens*. In: Doyle, M.P. Beuchat, L.R. (Eds.), *Food Microbiology: fundamentals and frontiers* (pp. 423-444). Washington, D.C.: ASM Press.
- McDonnell, J.L. (1986). Toxins of *Clostridium perfringens* type A, B, C, D, and E. In: Dorner, F. Drews, J. (Eds.), *Pharmacology of bacterial toxins* (pp. 477-517). Oxford: Pergamon Press.
- Myers, G.S., Rasko, D.A., Cheung, J.K., Ravel, J., Seshadri, R., DeBoy, R.T., Ren, Q., Varga, J., Awad, M.M., Brinkac, L.M., Daugherty, S.C., Haft, D.H., Dodson, R.J., Madupu, R., Nelson, W.C., Rosovitz, M.J., Sullivan, S.A., Khouri, H., Dimitrov, G.I., Watkins, K.L., Mulligan, S., Benton, J., Radune, D., Fisher, D.J., Atkins, H.S., Hiscox, T., Jost, B.H., Billington, S.J., Songer, J.G., McClane, B.A., Titball, R.W., Rood, J.I., Melville, S.B., & Paulsen, I.T. (2006). Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. *Genome Research*, *16*, 1031-1040.
- Paidhungat, M., Ragkousi, K., & Setlow, P. (2001). Genetic requirements for induction of germination of spores of *Bacillus subtilis* by Ca²⁺-dipicolinate. *Journal of Bacteriology*, *183*, 4886-4893.
- Paidhungat, M., & Setlow, P. (2001). Localization of a germinant receptor protein (GerBA) to the inner membrane of *Bacillus subtilis* spores. *Journal of Bacteriology*, *183*, 3982-3990.
- Paredes-Sabja, D., Setlow, B., Setlow, P., & Sarker, M.R. (2008a). Characterization of *Clostridium perfringens* spores that lack SpoVA proteins and dipicolinic acid. *Journal of Bacteriology*, *190*, 4648-4659.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009a). GerO, a putative Na⁺/H⁺-K⁺ antiporter, is essential for normal germination of spores of the pathogenic bacterium *Clostridium perfringens*. *Journal of Bacteriology*, *191*, 3822-3831.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009b). The protease CspB is essential for initiation of cortex hydrolysis and dipicolinic acid (DPA) release during germination of spores of *Clostridium perfringens* type A food poisoning isolates *Microbiology*, *155*, 3464-3472.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009c). Role of GerKB in germination and outgrowth of *Clostridium perfringens* spores. *Applied and Environmental Microbiology*, *75*, 3813-3817.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009d). SleC is essential for cortex peptidoglycan hydrolysis during germination of spores of the pathogenic bacterium *Clostridium perfringens*. *Journal of Bacteriology*, *191*, 2711-2720.
- Paredes-Sabja, D., Torres, J.A., Setlow, P., & Sarker, M.R. (2008b). *Clostridium perfringens* spore germination: characterization of germinants and their receptors. *Journal of Bacteriology*, *190*, 1190-1201.
- Rode, L.J., & Foster, J.W. (1962). Ionic germination of spores of *Bacillus megaterium* QM B 1551. *Arch Mikrobiol*, *43*, 183-200.

- Rooney, A.P., Swezey, J.L., Friedman, R., Hecht, D.W., & Maddox, C.W. (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.
- Sarker, M.R., Shivers, R.P., Sparks, S.G., Juneja, V.K., & McClane, B.A. (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. *Applied and Environmental Microbiology*, *66*, 3234-3240.
- Setlow, P. (2003). Spore germination. *Current Opinion in Microbiology*, *6*, 550-556.
- Sparks, S.G., Carman, R.J., Sarker, M.R., & McClane, B.A. (2001). Genotyping of enterotoxigenic *Clostridium perfringens* fecal isolates associated with antibiotic-associated diarrhea and food poisoning in North America. *Journal of Clinical Microbiology*, *39*, 883-888.
- Zhao, Y., & Melville, S.B. (1998). Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (*cpe*) of *Clostridium perfringens*. *Journal of Bacteriology*, *180*, 136-142.

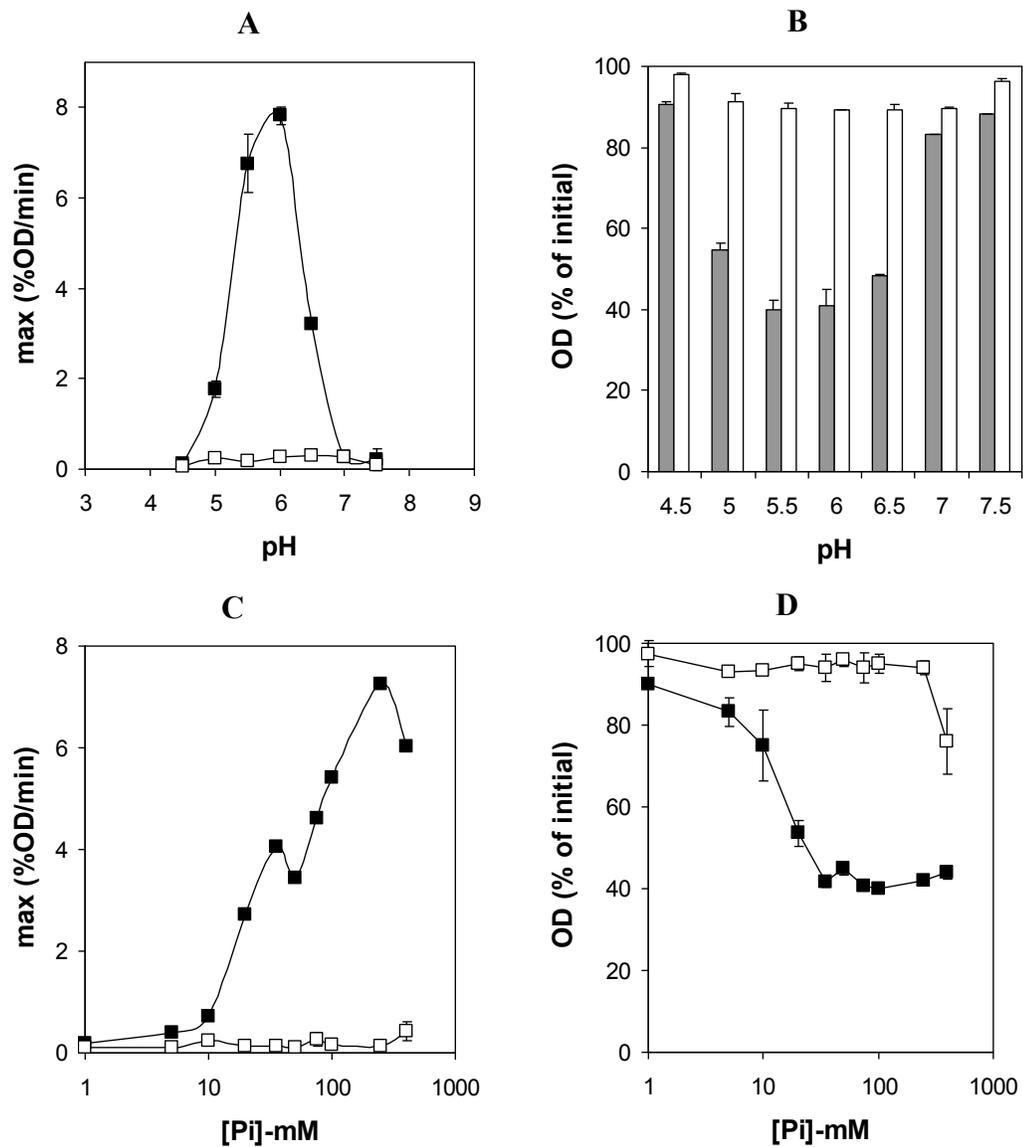


Fig. 5.1A-D. Effect of pH (A and B) and NaPi concentration (C and D) in germination of *C. perfringens* spores. Heat-activated spores of strains SM101 (filled squares or grey bars) and F4969 (open squares or white bars) were incubated with: A,B) 100 mM NaPi with various pH and the maximum rate (A), and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

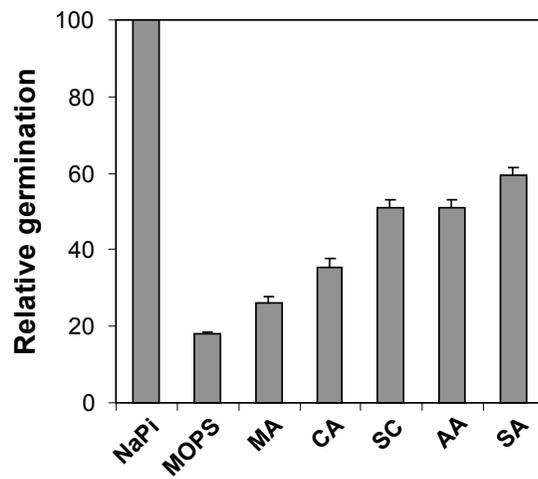


Fig. 5.2. Germination of spores of *C. perfringens* strain SM101 in different buffers. Heat activated spores were incubated at 40 °C and pH 6.0 in 100 mM of: NaPi, sodium phosphate; MOPS, 3-morpholinopropanesulfonic acid; MA, maleic acid; CA, citric acid; SC, sodium citrate; AA, acetic acid; SA, sodium acetate; and the OD₆₀₀ was measured after 60 min of incubation. All values are given relative to the value of NaPi, and this latter value was set at 100. Error bars denote standard deviations.

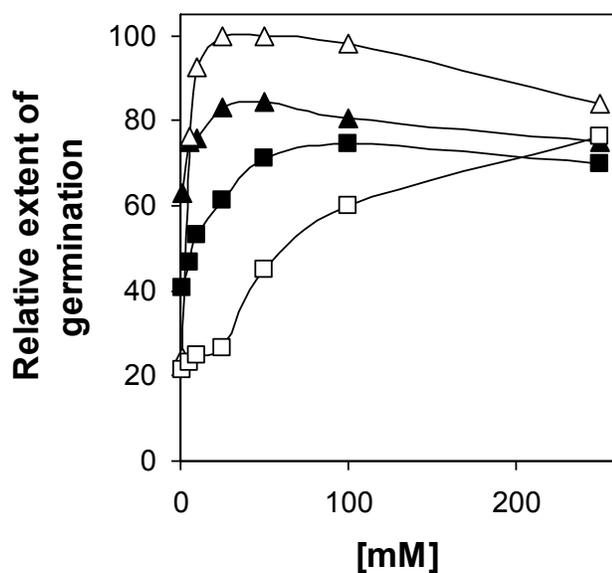


Fig. 5.3. Germination of spores of *C. perfringens* strain SM101 with Na^+ and K^+ . Heat activated spores of SM101 were incubated with various concentrations of NaCl (squares) and KCl (triangles) in 25 mM NaPi buffer at a pH 6.0 (filled symbols) and pH 7.0 (open symbols) at 40 °C and OD_{600} was measured after 60 min of incubation. All values are given relative to 50 mM KCl (pH 7.0), and this latter was set at 100. Standard deviation was less than 4%.

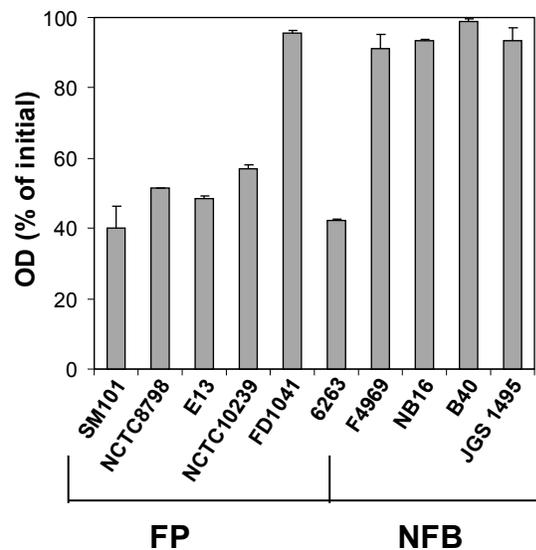


Fig. 5.4. Germination of spores from various *C. perfringens* isolates with NaPi. Heat activated spores of FP type A (SM101, NCTC8798, E13, NCTC10239, FD1041 and 6263), NFB type A (F4969, NB16 and B40) and a *cpe*-negative type C (JGS1495) isolates were incubated with 100 mM NaPi (pH 6.0) for 60 min and OD₆₀₀ was measured as described in Materials and methods.

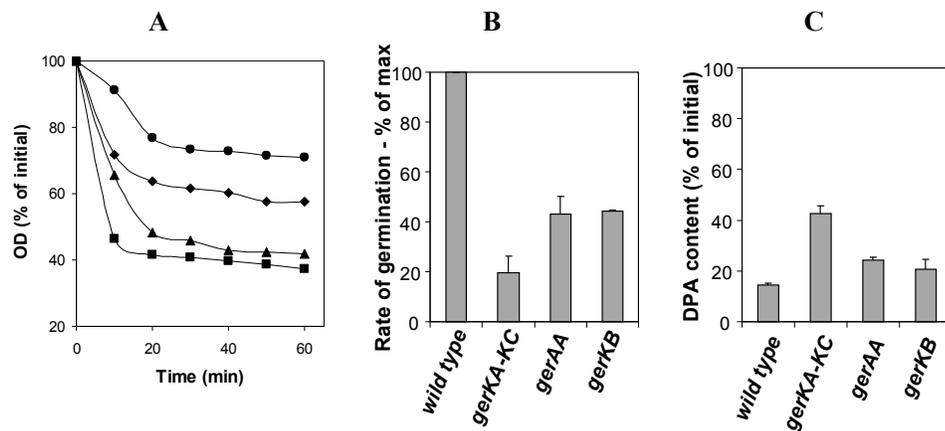


Fig. 5.5A-C. Germination of spores of *C. perfringens* *ger* receptor mutant strains with NaPi. A) Heat activated spores of *C. perfringens* strains SM101 (wild-type) (filled squares), DPS101 (*gerKA-KC*) (filled circles), DPS103 (*gerAA*) (filled diamonds), DPS108 (*gerKB*) (filled triangles) were incubated at 40 °C with 250 mM NaPi (pH 6.0) and OD₆₀₀ was measured as described in Materials and methods. B), Heat activated spores of various *C. perfringens* strains were incubated at 40°C with 250 mM NaPi (pH 6.0) and maximum rates of spore germination were determined as % change in OD₆₀₀/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 NaPi. Heat activated spores of *C. perfringens* were germinated with 250 mM NaPi (pH 6.0) at 40 °C and after 60 min the DPA content was measured as described in Materials and methods.

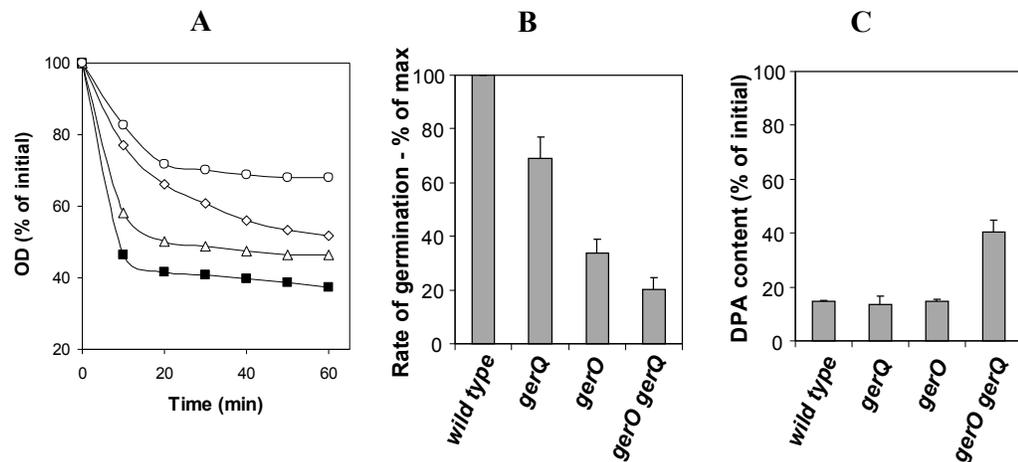


Fig. 5.6A-C. Germination of spores of *C. perfringens* antiporter mutant strains with NaPi. A) Heat activated spores of *C. perfringens* strains SM101 (wild-type) (filled squares), DPS113 (*gerQ*) (open triangles), DPS116 (*gerO*) (open diamonds), DPS108 (*gerO gerQ*) (open circles) were incubated at 40 °C with 250 mM NaPi (pH 6.0) and OD₆₀₀ was measured as described in Materials and methods. B), Heat activated spores of various *C. perfringens* strains were incubated at 40°C with 250 mM NaPi (pH 6.0) and maximum rates of spore germination were determined as % change in OD₆₀₀/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 NaPi. Heat activated spores of *C. perfringens* were germinated with 250 mM NaPi (pH 6.0) at 40 °C and after 60 min. the DPA content was measured as described in Materials and methods.

CHAPTER 6

Identification and Characterization of New Amino Acid Germinants for Spores of the Enterotoxigenic *Clostridium perfringens* Type A Isolates

Pathima Udompijtkul, Maryam Alnoman, Saeed Banawas, Daniel Paredes-Sabja, and
Mahfuzur R. Sarker

Manuscript will be submitted to *Applied and Environmental Microbiology*

Abstract

Clostridium perfringens spore germination plays a critical role in the pathogenesis of *C. perfringens*-associated food poisoning (FP) and non-food-borne (NFB) gastrointestinal diseases. Germination is initiated when bacterial spores sense the specific nutrient germinants (such as amino acids) through germinant receptors (GRs). In this study, we aimed to identify and characterize amino acid germinants for spores of enterotoxigenic *C. perfringens* type A. The polar, uncharged amino acids at pH 6.0 efficiently induced germination of *C. perfringens* spores. L-asparagine, L-cysteine, L-serine, and L-threonine triggered germination of spores in most FP and NFB isolates; whereas, L-glutamine was a unique germinant for FP spores. Comparative experiments demonstrated that cysteine is the superior germinant for spores of NFB isolates. For cysteine- or glutamine-induced germination, the GR-mutant *gerKC* spores germinated to a significantly lower extent and released less DPA than wild type spores; however, fewer pronounced germination defects were observed in *gerAA* and *gerKB* spores. The *gerKA*, *gerKC*, and *gerKB* spores germinated significantly slower with serine and threonine than their parental strain, suggesting the requirement for these GR proteins for normal germination in *C. perfringens*. In sum, the polar, uncharged amino acids at pH 6.0 were identified as novel nutrient germinants for spores of *C. perfringens* type A. All GR proteins seemed to have a role in germination, depending on the types of germinant, while the extent of spore germination was variable.

6.1. Introduction

Clostridium perfringens is a Gram-positive, anaerobic, rod-shaped, non-motile, endospore-forming bacteria that can cause a variety of human and veterinary diseases, due to its prolific toxin-producing capability (Mcclane, 2007a; Mcclane et al., 2006). It can be classified into five toxinotypes (A to E) based on production of four major toxins (α , β , ϵ , and ι toxins) (Mcclane, 2007b). Another important toxin produced by *C. perfringens* type A is *C. perfringens* enterotoxin (CPE), which is the major virulence factor responsible for most of *C. perfringens*-associated gastrointestinal (GI) diseases, including *C. perfringens* type A food poisoning (FP), antibiotic-associated diarrhea (AAD), sporadic diarrhea (SD) and nosocomial diarrhea diseases (Abraham et al., 2001; Asha and Wilcox, 2002; Borriello et al., 1984; Kobayashi et al., 2008; Mpamugo et al., 1995). Even though only a small group (less than 5%) of the global population of *C. perfringens* can produce CPE, *C. perfringens* type A FP still currently ranks as the third most commonly reported of the foodborne disease outbreaks in the United States and has been estimated to cause \$12.5 billion in economic losses annually (Brown, 2000; Buzby and Roberts, 1997; Lindström et al., 2011; Lynch et al., 2006; Mcclane, 2007a; Scallan et al., 2011a; Xiao et al., 2012). The CPE-producing *C. perfringens* is also implicated in ~ 5 to 20% of the total cases of AAD and SD (Mpamugo et al., 1995; Vaishnavi and Kaur, 2008). Previous evidence demonstrated that the *cpe* gene encoding for CPE was located either on the chromosome or on a plasmid (Collie and Mcclane, 1998; Cornillot et al., 1995). The chromosomal *cpe* isolates (C-*cpe*) are generally linked to *C. perfringens* type A FP

(referred to in this study as FP isolate), while the plasmid-borne *cpe* isolate (P-*cpe*) was identified as a causative agent for the non-foodborne (NFB) human GI diseases (referred to in this study as NFB isolate) (Collie and McClane, 1998; Cornillot et al., 1995; Sarker et al., 2000). Nevertheless, recent findings suggested that isolates carrying *cpe* on a plasmid could also be a causative agent for the emerging food-related *C. perfringens* type A FP (Lahti et al., 2008; Tanaka et al., 2003).

Bacterial spores are one of the most resistant living organisms and they can remain in a metabolically dormant stage for a long period of times (Carlin, 2011). They can; however, return to actively growing cells immediately once the conditions are favorable for growth. Bacterial spore germination typically initiates by bacteria sensing the presence of specific nutrient germinants in their surrounding environments via their GR (Paredes-Sabja et al., 2011; Setlow, 2003). In *C. perfringens* SM101 (an FP isolate) spores, four GRs encoded by a monocistronic *gerAA*, a *gerK* locus containing a bicistronic *gerKA-KC* operon, and an upstream oppositely-oriented *gerKB* were identified and their respective roles in germination were recently studied (Paredes - Sabja et al., 2009; Paredes-Sabja et al., 2008c). The ability of *C. perfringens* spores to germinate plays a critical role in the onset of *C. perfringens* type A FP. The contaminated spores in meat- and poultry-containing products must germinate, outgrow, and subsequently proliferate to a high cell population, especially after cooking and storage at improper temperatures. Symptoms of *C. perfringens* type A FP typically result from the ingestion of a large number of vegetative cells in these contaminated food products (McClane, 2007a). Despite its significance, detailed

studies on factors influencing germination, as well as molecules capable of triggering germination in enterotoxigenic *C. perfringens* spores, have not been conducted. The identification of specific germinants for limited strains of *C. perfringens* spores showed that lysozyme is required for germination of the heat-damaged *cpe*-negative NCTC3624 spores (Cassier and Sebald, 1969), and KCl at 50 to 100 mM, pH ranging from 5.0 to 9.0, was a good germinant for spores of the FP strain NCTC8238 (Ando, 1974). Our previous findings revealed the differences in the germination requirements among strains of CPE-positive *C. perfringens* FP and NFB isolates. The KCl, L-asparagine, and mixture of L-asparagine and KCl (AK), all at pH 7.0, and a mixture of sodium and inorganic phosphate (NaPi) at pH 6.0, can induce germination of various strains FP isolates; whereas, L-alanine and L-valine at pH 7.0 can initiate germination of some *C. perfringens* NFB isolates, and AK was a universal germinant for both isolates (Paredes-Sabja et al., 2008c; Paredes-Sabja et al., 2009e). The ability of all 20 naturally occurring standard amino acids to trigger the germination of spores of CPE-producing *C. perfringens* type A had never been examined. Thus, the aims of this study were i) to screen all 20 amino acids to identify potential germinants for spores of *C. perfringens* type A; and ii) to identify GRs, which were responsible for recognizing the new amino acid germinants. In this study, we found that cysteine at pH 6.0 was a universal germinant for both FP and NFB spores, and glutamine at pH 6.0 was a unique germination initiator only for FP isolates. Results also demonstrated that other amino acids in polar, uncharged groups including asparagine, serine, and threonine, all at 100 mM and pH 6.0, could efficiently trigger germination of various strains of FP

and NFB spores. Finally, most of GRs were required for spore germination with the polar, uncharged amino acids at pH 6.0, although to different extents. Understanding the nutrient-specific germination requirement and germination characteristics of collections of *cpe*-positive *C. perfringens* isolates would be beneficial in the development of an effective strategy to prevent spore germination and to control or eliminate *C. perfringens* spore contamination in food products and food processing environments.

6.2. Materials and methods

6.2.1. Bacterial strains

C. perfringens strains used in this study consist of seven strains of type A FP isolates carrying a chromosomal *cpe* gene (*C-cpe*), including SM101 (an electroporable derivative of the type A FP isolate NCTC8798) (Zhao and Melville, 1998), NCTC8798 (Duncan et al., 1972c), E13 (Sarker et al., 2000), NCTC8239, NCTC10239, FD1041 (Collie et al., 1998), and 6263 (Harrison et al., 2005a). Four strains of type A NFB isolates carry the plasmid-borne *cpe* gene (*P-cpe*), including NB16, B40, F4969, and F5603 (Sarker et al., 2000), and one poultry isolate, *cpb2*⁺, *tpeL*⁺, *netB*⁺ (JGS4125) (This study). The GR-mutants of *C. perfringens* SM101 in this study were constructed and characterized in previous studies, including DPS101 (*gerKA-KC*) (Paredes-Sabja et al., 2008c), DPS103 (*gerAA*) (Paredes-Sabja et al., 2008c), DPS108 (*gerKB*) (Paredes - Sabja et al., 2009), DPS119 (*gerKA*) (unpublished data), and DPS122 (*gerKC*) (unpublished data). All of the strains were maintained in

cooked-meat medium (Difco, Becton Dickinson, Spark, MD) and stored at -20 °C. Growth of *C. perfringens* was revived in 10 ml of fluid thioglycolate broth (FTG) (Difco) and grown for 16 h at 37 °C.

6.2.2. Spore preparation and purification

The sporulating cultures of *C. perfringens* were prepared by a previously described method (Paredes-Sabja et al., 2009e; Udompijittkul et al., 2012). Briefly, 0.4 ml aliquots of overnight FTG culture were transferred into new 10 ml FTG and incubated at 37 °C for ~ 8 h. Then, 0.4 ml of actively growing cells were transferred into 10 ml of freshly prepared Duncan-Strong (DS) sporulation medium (Duncan and Strong, 1968) and incubated overnight at 37 °C. The presence of spores was confirmed by phase-contrast microscopy. A large number of bacterial spores was obtained by scaling up the aforementioned procedure in 800 ml DS, and spores were purified by repeated washing with cold sterile distilled water until spore suspensions were > 99% free of cell debris, sporulating and germinating cells, as determined by phase-contrast microscopy. The spore suspensions were adjusted to a final optical density at 600 nm (OD_{600}) ~ 6.0 with sterile distilled water and stored at -80 °C until used.

6.2.3. Preparation of germinant solutions

Twenty standard amino acids in L-isomer were evaluated for their abilities to induce germination of CPE-producing *C. perfringens* spores. All tested solutions were prepared with 25 mM Na_2HPO_4 buffer (pH 7.0) at a final concentration of 100 mM and adjusted to a final pH of 7.0 with 1 M HCl or 1 M NaOH. The tested compounds

were categorized into five groups, according to their side chain (R group) (Table 6.1) (Berg et al., 2006). All amino acids in this study were purchased from Sigma (Sigma-Aldrich, Co., St. Louis, MO), except L-cysteine (J.T. Baker, Mallinckrodt Baker, Inc. Philipsburg, NJ).

To determine the effect of pH, cysteine and glutamine were prepared at 100 mM with 25 mM Na₂HPO₄ buffer (pH 7.0) and adjusted to various pHs ranging from pH 4.0 to 9.5 in 0.5-unit increments. The concentration effects of cysteine and glutamine were assessed at various ranges from 1 – 400 mM, with solutions prepared with 25 mM Na₂HPO₄ buffer (pH 7.0) and adjusted to the final pH of 6.0. The other polar, uncharged amino acid solutions (pH 6.0 at 100 mM), including serine, asparagine, and threonine, were also assayed.

To compare the efficacy of a variety of the known germinants for *C. perfringens* type A spores according to our previous study (Paredes-Sabja et al., 2008c; Paredes-Sabja et al., 2009e), all germinants were prepared at 100 mM with 25 mM Na₂HPO₄ buffer (pH 7.0) and adjusted to a final pH of 6.0 (inorganic phosphate (NaPi)) or pH of 7.0 (KCl, L-asparagine, and the combination of KCl and L-asparagine (AK)). The novel germinants, including cysteine and glutamine (pH 6.0, 100 mM), were prepared .

6.2.4. Spore germination measurement

Spore suspensions were heat activated at 80 °C for 10 min for the FP strain, while NFB spores were treated at 75 °C for 10 min, and then cooled in a water bath at room temperature for 5 min, before being equilibrated at 40 °C for 10 min. These two

isolates were heat activated at different temperatures, since our previous study demonstrated that FP spores germinated better when heat activated at 80 °C for 10 min, and spores of NFB isolates germinated better with activation at 75 °C for 10 min (Paredes-Sabja et al., 2008c). These heat-activated spores (to a final concentration at OD₆₀₀ of ~ 1.0) were mixed with 0.5-ml pre-warmed germinant solution, and spore germination was routinely measured by monitoring the change in OD₆₀₀ (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA), which decreases ~ 60% upon complete germination. The germination levels were also confirmed by phase-contrast microscopy at 60 min post-inoculation. The maximum rate of spore germination was expressed as a percentage of maximal loss of OD₆₀₀ per minute, relative to the initial value of spore suspension. Rate was determined by measuring the OD₆₀₀ of germinating cultures every 2.5 min, and the maximum slopes were calculated as previously described (Paredes-Sabja et al., 2009e). To compare the extent of *C. perfringens* spore germination, the germination assays were performed, as stated, and the decrease of OD₆₀₀ was monitored after 60 min of incubation. Results were expressed as the percentage of decrease in OD₆₀₀ relative to initial values.

The germination of GR-mutant strains of *C. perfringens* SM101 was assessed with 250 mM cysteine or glutamine adjusted to pH 6.0 or 100 mM of other polar, uncharged amino acid solution (pH 6.0). The germination of heat-activated spores of OD₆₀₀ ~ 1.0 was routinely monitored at 40 °C in a 96-well plate in a total volume of spore suspension and germinant of 0.2 ml, by using SynergyTM MX multi-mode microplate reader (BioTek[®] Instruments, Inc., Winooski, VT). The microplate was

vigorously shaken before OD₆₀₀ measurement, at every 2.5 min interval. and the maximum rate and extent of germination were expressed as stated.

6.2.5. DPA release measurement

The release of DPA during nutrient germination was performed as previously described (Paredes-Sabja et al., 2008c; Paredes-Sabja et al., 2009e). Briefly, spores of SM101 (parental strain) and GR mutant strains (OD₆₀₀ of 1.5) were heat activated at 80 °C for 10 min and then incubated with 250 mM cysteine or glutamine (pH 6.0) at 40 °C. The 1 ml aliquots of germinating cultures were taken at various time periods and centrifuged for 3 min at 13,2000 rpm in a microcentrifuge tube, and DPA in the supernatant fluid was determined by measuring absorbance at 270 nm (A₂₇₀), as described previously (Cabrera-Martinez et al., 2003; Paredes-Sabja et al., 2008c). The total spore DPA content was evaluated by boiling 1ml aliquot of germinating spores for 60 min and centrifuging for 5 min in a microcentrifuge. The A₂₇₀ of the supernatant fluid was measured. Our previous study indicated that ~ 90% of the material absorbing at 270 nm contained DPA in *C. perfringens* (Paredes-Sabja et al., 2009d)

6.2.6. Statistical analyses

All experiments, except the DPA-release assay, were performed at least in triplicate with two different batches of spore preparations. DPA-release measurement was performed in duplicate with two different spore preparations. Data were analyzed by analysis of variance procedures (PROC ANOVA), using the statistical software

SAS version 9.2 (SAS Inst. Inc., Cary, NC), and multiple comparisons among mean values were analyzed with Tukey's test at the significant level of 0.05.

6.3. Results

6.3.1. Ability of amino acids to trigger germination of *C. perfringens* spores

Although the specific germinants triggering the germination of bacterial spores varies among species, they are often nutrients required for vegetative growth and cell division (Gould, 2006). According to a previous study (Boyd et al., 1948), 13 different amino acids were demanded for vegetative growth of *C. perfringens*, due to its inability to synthesize. Therefore, we initially assessed for the ability of 20 amino acids to induce germination of the representative strains of *C. perfringens* FP (SM101) and NFB (F4969) isolates (Myers et al., 2006). All potential germinants were tested at a single pH (pH 7.0), since this is in the optimum pH for growth of *C. perfringens* (pH 6.0 – 7.0) (Mcclane, 2007a), and it was the most effective pH to induce maximum germination of *cpe*-positive *C. perfringens* spores with KCl and AK (Paredes-Sabja et al., 2008c).

Table 6.1 shows the percent of decrease in OD₆₀₀ after incubation for 60 min with 100 mM amino acid solutions adjusted to pH 7.0. Negligible spore germination was observed in 25 mM Na₂HPO₄ buffer (pH 7.0) used for preparing all germinant solutions in the current study (~ 10 – 15% decrease in OD₆₀₀ after 60 min), and more than 90% of spores remained phase bright when analyzed with microscopy, indicating no significant germination (data not shown). Thus, in this study, we defined

significant germination as at least 20% reduction in OD₆₀₀ compared to the initial value.

By adopting the aforementioned criteria, several amino acids appeared to be the potential germinants for *cpe*-positive *C. perfringens* spores. This included glycine, alanine, isoleucine, serine, cysteine, asparagine, glutamine, lysine, arginine, aspartic acid, and glutamic acid for SM101 spores. For F4969 spores, the potential germinants are cysteine, glutamine, and lysine. Interestingly, various amino acids in the polar, uncharged group had abilities to induce germination for both FP and NFB isolates (Table 6.1). Furthermore, asparagine (pH 7.0) was identified in our earlier finding as a good germinant for spores of various FP strains (Paredes-Sabja et al., 2008c).

6.3.2. Effect of pH and concentrations of cysteine and glutamine on spore germination

The previous studies suggested that the pH of germinative compounds plays an important role on the germination of *C. perfringens* spores (Paredes-Sabja et al., 2008c; Paredes-Sabja et al., 2009e). In order to define optimum pH levels and concentrations of cysteine and glutamine, NB16 was selected as a representative strain for the NFB isolate, due to its higher sporulation efficiency and germination capability than strain F4969 (data not shown), while SM101 was used as a representative strain for the FP isolate. The pH of 100 mM cysteine or glutamine solution was varied between pH 4.0 - 9.5, which covered the pH range for growth of *C. perfringens* (pH 5.0 – 9.0) (Novak et al., 2005). Both amino acid solutions were prepared with 25 mM sodium phosphate buffer to adjust the pH.

Interestingly, cysteine induced germination of spores of both SM101 and NB16 at a pH range of 5.0 to 7.0. The extent of germination was much lower at pH 7.5 to 8.0, and no significant germination was observed below pH 5.0 or above pH 8.0 (Fig. 6.1B). In contrast, glutamine can only trigger germination of SM101 spores at pH 5.5 to 6.5; it was unable to induce germination of NB16 spores in all tested pHs (Fig. 6.1C - D). The maximum rate of germination of NB16 spores with cysteine was observed at pH 6.0 (Fig. 6.1A). Although the rate of germination of SM101 spores with cysteine at pH 5.5 was slightly higher than that of pH 6.0, the difference was not significant ($p > 0.05$), and this strain was able to germinate to similar extents after 60 min with cysteine at a pH range of 5.0 to 7.0 (Fig.6.1B). Similarly, the most rapid rate of germination of SM101 spores with glutamine was at pH 6.0 (Fig. 6.1C).

To optimize the most effective concentrations to induce germination of *C. perfringens* spores, various concentrations of cysteine or glutamine adjusted to pH 6.0 (Fig. 6.2A – D) were evaluated. Since our previous study indicated that 100 mM Na⁺ and inorganic phosphate at pH 6.0 acted as a co-germinant for spores of FP isolates, the effect of 25 mM sodium phosphate buffer used for preparing amino acid solutions and adjusted to pH 6.0 was examined. The extent of SM101 and NB16 spores germinated with this buffer was minor and reflected ~ 17% and 11%, respectively, of OD₆₀₀ decrease, after 60 min of incubation at 40 °C. Furthermore, maximum rates of *C. perfringens* spore germination with this buffer was negligible (data not shown). The germination experiments were also performed with amino acid solutions prepared with 25 mM Tris-HCl and adjusted to a final pH 6.0, and comparable germination

responses were obtained (data not shown); therefore, germination results of *C. perfringens* spores with amino acids prepared with 25 mM Na₂HPO₄ buffer were reported. In addition to 25 mM sodium phosphate buffer, it is notable from Fig. 6.2B and 6.2D that cysteine (pH 6.0) can trigger significant germination at a concentration as low as 10 mM for both isolates, and 35 mM glutamine (pH 6.0) was required to obtain a similar extent of SM101 spore germination after 60 min. The SM101 spores exhibited maximum rates of germination with 250 mM cysteine or glutamine solutions (pH 6.0) (Fig. 6.2 A, C). For cysteine germination, the highest rate of germination was not significantly different ($p > 0.05$) at concentration ranges from 100 to 400 mM. In contrast to SM101 spores, NB16 spores showed the fastest germination rates with cysteine (pH 6.0) at 50 to 100 mM, but the rate of germination was dramatically ($p < 0.05$) decreased when the concentration of cysteine was raised to 250 and 400 mM (Fig. 6.2A). The extents of germination of both strains were correlated with the germination rates where no significant germination was observed after 60 min for NB16, at any concentration of glutamine tested; thus, the inability of glutamine to induce germination of NB16 spores was not due to sub-optimum concentration (Fig. 6.2D). The SM101 spores germinated well with 250 and 400 mM glutamine, and approximately 90% of the spores changed to phase dark under the phase contrast microscope (Fig. 6.2D and data not shown). For cysteine (pH 6.0)-triggered germination, the germination levels were comparable for NB16 and SM101 spores in the concentration range of 50 – 100 mM and 100 – 400 mM, respectively (Fig. 6.2B). Collectively, these results indicated that cysteine at pH 6.0 is an effective germinant

for spores of both FP strain SM101 and NFB strain NB16, while glutamine at pH 6.0 is a unique germinant for spores of SM101, but not for those of NB16.

6.3.3. Germination of various FP and NFB isolates with cysteine and glutamine at pH 6.0

Next, we evaluated whether the abilities of cysteine (pH 6.0) to induce germination of both isolates and glutamine (pH 6.0) to trigger germination of the FP isolate were only specific to SM101 and NB16 strains. Therefore, a total of twelve strains consisting of seven FP isolates (SM101, NCTC8798, E13, 6263, NCTC10239, NCTC8239, and FD1041), four NFB isolates (NB16, B40, F4969, and F5603), and one veterinary clinical isolate (JGS4125) were assayed with 100 mM cysteine or glutamine adjusted to pH 6.0, for their germination. Results shown in Fig. 6.3A revealed that cysteine (pH 6.0) could act as a universal germinant for spores of CPE-producing *C. perfringens* type A strains, regardless of the sources of isolation. Spores of all FP isolates, except 6263, germinated well in the presence of cysteine, while the extents of germination of NFB spores, upon incubation with cysteine, were even higher than FP spores. The ability of cysteine to induce spore germination was not restricted only to CPE-associated human GI disease isolates, as spores of JGS4125, a clinical isolate from poultry, were capable of significantly germinating with cysteine at pH 6.0. Similar to results observed with NB16 spores (Fig. 6.1 C-D, and Fig. 6.2 C-D), glutamine (pH 6.0) was unable to induce germination of spores of any tested human NFB isolates, as well as the animal isolate (Fig. 6.3B). However, spores of most FP strains (with the exception of strain FD1041) were able to germinate significantly with glutamine (pH 6.0) within 60 min. Overall, these data clearly

demonstrated that cysteine, at pH 6.0, acted as a universal germinant triggering germination of spores of both FP and NFB *C. perfringens* isolates; whereas, glutamine (pH 6.0) was a unique germinant for spores of most FP isolates.

6.3.4. Comparison of *C. perfringens* spore germination with various identified germinants

To test that cysteine at pH 6.0 is a superior germinant for spores of CPE-producing *C. perfringens*, we compared the maximum rate and extent of germination of various representative strains with previously known germinants for *C. perfringens* spores, including KCl (pH 7.0), asparagine (pH 7.0), AK (pH 7.0) (Paredes-Sabja et al., 2008c), NaPi (pH 6.0) (Paredes-Sabja et al., 2009e), cysteine (pH 6.0), and glutamine (pH 6.0). All germinants were evaluated at the same concentration at 100 mM. As consistent with our previous findings (Paredes-Sabja et al., 2008c; Paredes-Sabja et al., 2009e), spores of FP isolates (SM101 and NCTC8239) germinated well with KCl, asparagine, AK, and NaPi, but only AK could trigger germination of NFB spores (NB16 and F4969). Under the phase contrast microscope, the extent of spore germination after 60 min was comparable and reflected ~ 90% of phase dark germinating spores among strains of the same isolate types with most germinants, except the NCTC8239 spores germinated with glutamine (pH 6.0) and asparagine (pH 7.0) showed limited germination (data not shown). This could be due to a strain-specific characteristic and is consistent with the observation shown in Fig. 6.3B. It was notable that the extent of germination of spores of NFB strains (NB16 and F4969) with cysteine (pH 6.0) was much higher than that of AK-induced germination ($p < 0.05$) (data not shown). For SM101 spores, the rates of germination, as determined by

the percent of loss of OD₆₀₀ per min, were comparable for all tested germinants (~ 6% decrease in OD₆₀₀/min). The most rapid germination of NCTC8239 spores was detected with AK, with glutamine (pH 6.0) being the least effective germinant for this strain, as mentioned. Interestingly, as consistent with the greater germination extent for cysteine-induced NFB spore germination as compared to AK induction, the maximum rates of germination with cysteine (pH 6.0) were nearly two times faster than with AK. Even though the response of *C. perfringens* spores to identified germinants was influenced by strain, these results obviously demonstrate that cysteine at pH 6.0 is a superior germinant for spores of *C. perfringens* NFB isolates.

6.3.5. Germination of C. perfringens spores with other polar, uncharged amino acids adjusted at pH 6.0

To this point, our results suggested that cysteine and glutamine at pH 6.0 were effective germinants for spores of the enterotoxigenic *C. perfringens* type A. We know that a variety of polar, uncharged amino acids at pH 7.0 could be potential germinants for *C. perfringens* type A spores (Table 6.1) and cysteine and glutamine at pH ~ 6.0 gave the higher rate and extent of germination than at pH 7.0. This raised the question of whether this pH effect could also be achieved in other amino acids in the same group. Therefore, germination of seven representative strains of FP and NFB isolates was examined with 100 mM solution of asparagine, serine, and threonine adjusted to pH 6.0. The extent of germination as the percent of decrease in OD₆₀₀ after 60 min of incubation at 40 °C was depicted in Fig. 6.4A – C. Strikingly, asparagine (pH 6.0) induces significant germination of spores of both FP and NFB strains, except one NFB strain F4969 (Fig. 6.4A), which is in contrast to our previous finding that asparagine at

pH 7.0 was a unique germinant for FP isolates (Paredes-Sabja et al., 2008c). Serine and threonine at pH 6.0 also induced significant germination of spores of both FP and NFB isolates, but spores of FP strains NCTC8239 and 6263 did not respond well to these two amino acids (Fig. 6.4B and C). However, the level of spore germination with serine or threonine was lower than that with cysteine or asparagine at pH 6.0 (Fig. 6.3A and 4A). Collectively, these results suggested that all tested polar, uncharged amino acids, except glutamine, could be nutrient germinants triggering germination of spores of both FP and NFB isolates, when they are adjusted to pH 6.0. Thus, these data emphasize the important role of pH of amino acids in inducing germination of *C. perfringens* spores.

6.3.6. Role of GRs in polar, uncharged amino acid-triggered germination

In order to gain insight into the mechanism of cysteine- or glutamine-inducing germination, the germination of spores of *C. perfringens* SM101 and its GR mutants, including *gerKA*, *gerKB*, *gerKC*, and *gerAA*, were assayed by using the optimum concentrations (250 mM) of cysteine and glutamine (pH 6.0), as described (Fig. 6.2A – D). Although *gerKA* spores germinated significantly less than wild type spores did during the first 10 min ($p < 0.05$), the former spores germinated to almost identical levels as the wild type spores after 60 min. This suggests that GerKA might have a minor role, if at all, for cysteine (pH 6.0) germination (Fig. 6.5A). In contrast, *gerAA* and *gerKB* spores exhibited significantly less germination than those of the wild type strain ($p < 0.05$), and more pronounced defective germination, which was significantly lower than wild type and other GR mutant spores, was observed with *gerKC* spores.

These results suggest that GerKC is the main receptor for recognizing the cysteine (pH 6.0) germinant, while GerAA and GerKB could have some roles for normal germination of *C. perfringens* spores with this specific germinant. Similar observations were found for *C. perfringens* spore germination with 250 mM glutamine at pH 6.0; *gerKA* and wild type spores germinated similarly. The *gerKB* and *gerAA* spores; however, germinated slightly but significantly slower than those of *gerKA* and wild type spores after 60 min. Thus, GerAA and GerKB might have partial roles in spore germination with glutamine (pH 6.0). On the other hand, spores lacking GerKC rarely germinated with glutamine and ~ 85% of spores remained phase bright under the phase contrast microscope after 60 min of incubation with glutamine (pH 6.0) (Fig. 6.6A and data not shown). Therefore, GerKC is also a key receptor involved in glutamine-initiated germination.

The rate of germination was measured to determine the role of each GR to trigger germination in the initial stage with cysteine or glutamine at pH 6.0 (Fig. 6.5B and 6.6B). All strains carrying mutations in GR genes had a significantly slower rate of germination ($p < 0.05$), as compared to spores of wild type strains with 250 mM cysteine (pH 6.0). While spores of *gerAA*, *gerKA*, and *gerKB* exhibited similar rates of germination, these were ~ 2-fold lower than that of wild type spores. However, *gerKC* spores had a dramatically reduced (~ 16-fold) germination rate than that of wild type spores and ~ 7-fold lower than those of the other GR mutants (*gerAA*, *gerKA*, and *gerKB* spores) (Fig. 6.5B), implying the critical role of the GerKC receptor in initiating germination with cysteine (pH 6.0). A similar phenomenon was observed

when spores were germinated with 250 mM glutamine at pH 6.0, where spores of *gerAA*, *gerKA*, and *gerKB* had ~ 2-fold lower rate of germination compared to the wild type strain. These GR mutants showed a comparable rate of germination ($p > 0.05$) to each other. (Fig. 6.6B). Conversely, *gerKC* spores exhibited ~ 15-fold and ~ 7-fold reduction in their rate of germination, compared to the wild type and other GR mutants (*gerAA*, *gerKA*, and *gerKB*), respectively. Therefore, GerKC is the key receptor involved in glutamine (pH 6.0)- initiated germination of *C. perfringens* spores, as well.

Upon binding of nutrient germinants to their cognate germination receptor located in the inner membrane of *Bacillus* and *Clostridium* spores, a spore's large depot of pyridine-2,6-dicarboxylic acid (dipicolonic acid; DPA) is released as a 1:1 chelate with the divalent cations, mainly Ca^{2+} (Ca-DPA) (Paredes-Sabja et al., 2008c; Setlow, 2003). Therefore, the role of each GR for DPA released in cysteine or glutamine (pH 6.0) germination was examined (Fig. 6.5C and 6.6C). Similar extents of DPA release ($p > 0.05$) were observed with wild type and *gerKA* spores with 250 mM cysteine (pH 6.0) after 60 min (Fig. 6.5C), which was in agreement with the similar extents of germination between these two strains (Fig. 6.5A). With this germinant, *gerKB* spores released slightly higher DPA during 60 min than *gerAA* spores, but the amount of DPA remaining in the core of *gerKB* and *gerAA* spores was significantly greater than wild type and *gerKA* spores ($p < 0.05$). Consistent with the lowest germination extent of spores lacking GerKC with cysteine (pH 6.0), *gerKC* spores released significantly less DPA than wild type and other GR mutant spores ($p < 0.05$).

For glutamine-induced germination, wild type and *gerKA* spores also released a similar amount of DPA and at slightly lower levels for spores of *gerKB* and *gerAA* strains. Strikingly, the DPA content retained in the core of *gerKC* spores was significantly higher than other tested strains ($p < 0.05$) (Fig. 6.6C). These results suggest that GerKC is mainly involved in DPA release during *C. perfringens* spore germination with cysteine or glutamine, while GerKB and GerAA has some auxiliary roles.

Since we have demonstrated that other polar, uncharged amino acids at pH 6.0 can efficiently induce germination of *C. perfringens* spores, it is interesting to determine which GR is responsible for sensing these novel germinants. Asparagine, serine, or threonine was adjusted to pH 6.0 and assayed at 100 mM. In agreement with our previous finding that GerKA-KC played an important role in asparagine (pH 7.0) germination, we found in the current work that *gerKC* spores exhibited significantly less germination than spores of wild type and other GR mutants ($p < 0.05$), indicating that GerKC was absolutely required for normal germination of *C. perfringens* spores with asparagine (pH 6.0) (Fig. 6.7A). Although the impact of *gerKB* and *gerAA* deletions on spore germination was not as pronounced as in the *gerKC* mutant, extents of germination of *gerKB* and *gerAA* spores were also significantly lower than wild type after 60 min of incubation with asparagine (pH 6.0). Nevertheless, *gerKA* spores germinated similarly as did wild type spores. These results suggest that GerKC is the main GR involved in germination with asparagine (pH 6.0), with GerAA and GerKB possibly playing an auxiliary role.

Surprisingly, various GRs might be able to detect serine at pH 6.0 and initiate germination, as evidenced by spores lacking GerKA, GerKB, or GerKC showing considerably lower germination extents than wild type and *gerAA* spores (Fig. 6.7B). Among these three GRs, the magnitude of defectiveness in spore germination was highest in *gerKA* mutation, followed by *gerKC* and *gerKB*, respectively. The initial rate of germination triggering by serine (pH 6.0) was also significantly lowest in *gerKA* spores relative to other strains ($p < 0.05$) (data not shown). The *gerAA* spores, on the other hand, germinated at slightly higher levels than wild type spores; however, ~95% spores of these two strains lost refractility after 60 min (Fig. 6.7B and data not shown). Collectively, the sensing of serine as a nutrient germinant might be a unique and required action of several GRs simultaneously for efficient germination. Similar to what has been observed for serine (pH 6.0) germination, the defective germination phenotype, albeit at lower magnitude, was observed for *C. perfringens* spores germinated with 100 mM threonine at pH 6.0 (Fig. 6.7C). However, extents of germination of *gerAA* spores were indifferent from those of wild type spores over a 60-min time period ($p > 0.05$), suggesting that GerAA had no role in threonine germination. While *gerKB* and *gerKC* spores exhibited slightly-to-significantly reduced germination than wild type and *gerAA* spores, they had significantly higher rate and extent of germination than *gerKA* spores (Fig. 6.7C and data not shown). Thus, mutation in *gerKA* type receptor gave the most pronounced defect on spore germination with threonine (pH 6.0) and GerKB and GerKC might have some minor and partial role in germination with this particular germinant, respectively. In order to

verify if GerKA and GerKC were the two most important receptors involved in serine or threonine (pH 6.0) germination, spores of *gerKA-KC* double mutant were incubated with 100 mM serine or threonine for 60 min. As expected, spores lacking both GerKA and GerKC exhibited the most defective germination among all strains tested (data not shown) and indicated GerKA and GerKC are absolutely required for normal *C. perfringens* spore germination, using either serine or threonine.

6.4. Discussion

The current consumer demand for natural, minimally-processed, ready-to eat food products has urged food manufacturers to develop the mild preservative approaches to replace thermal processing and chemical preservations. This could have given the advantage to highly resistant spores of pathogenic bacteria, such as *C. perfringens*, to survive and then germinate, outgrow, and proliferate to highly infective dose levels in finished food products, causing *C. perfringens* type A FP outbreaks upon ingestion of the highly contaminated foods (Anderson et al., 2004; McClane, 2007a; Thippareddi et al., 2003). The spore germination plays a critical role in the pathogenesis of this foodborne illness (McClane, 2007a). Acquiring knowledge about factors triggering spore germination would be beneficial to develop effective strategies to control or eliminate *C. perfringens* spores and eventually to reduce the incidence of *C. perfringens* type A FP outbreaks.

In the current study, we have shown that a variety of polar, uncharged amino acids at pH ~ 6.0 can efficiently induce germination of spores of CPE-producing *C.*

perfringens type A. Even though the effect of this group of amino acid has never been reported for *C. perfringens* spores, besides asparagine at pH 7.0 for spores of FP isolates (Paredes-Sabja et al., 2008c), several polar, uncharged amino acids were strong germinants for other spore-forming bacteria. Cysteine has been reported to act as a co-germinant with L-lactate to induce germination of nonproteolytic *Clostridium botulinum* spores (Plowman and Peck, 2002). Cysteine, together with NaHCO₃, enhanced the germination capacity of *Clostridium frigidicarnis* (Adam et al., 2011). Moreover, cysteine, by itself, is a good germinant for spores of *Bacillus cereus* ATCC14579, *C. botulinum* strain B-aphis and 62A, and *Clostridium sporogenes* 3679h (Hornstra et al., 2006; Montville, 1984; Montville et al., 1985; Rowley and Feeherry, 1970; Tang and Frank, 1972). For *C. perfringens*, the availability of cysteine has a crucial role for the expression of genes involved in several key functions for the cellular physiology of this pathogen, such as toxin production (theta toxins and hyaluronidase), maintenance of cell redox status, and biogenesis of iron-sulfur clusters (André et al., 2010). Cysteine is also one of the absolutely required amino acids for vegetative growth of various strains of *C. perfringens* (Boyd et al., 1948). In addition, glutamine has been reported as a single germinant for *B. cereus* ATCC14579 (Hornstra et al., 2006) and as a co-germinant with glucose, fructose, and K⁺ (GFK) to induce germination of *Bacillus subtilis* spores (Wax and Freese, 1968). Besides glutamine, other polar, uncharged amino acids, such as asparagine plus GFK, were strong germinants for *B. subtilis* and, to a lesser extent, the combinations of cysteine or serine with GFK (Wax and Freese, 1968) and *B. cereus* ATCC14579 could germinate

with threonine only (Hornstra et al., 2006) Like cysteine, serine and threonine are necessary for vegetative cell growth of *C. perfringens*, but the effect of asparagine and glutamine was not evaluated in this study (Boyd et al., 1948).

In food-product environments, bacterial spores can encounter diverse conditions of varying pHs and temperatures (Van Der Voort et al., 2010). The pH of germinants seems to play an important role in initiating germination of *cpe*-positive *C. perfringens* spores, as our previous finding showed that *C. perfringens* SM101 spores can only germinate with 100 mM NaPi at the pH ranges of 5.0 to 6.5 (Paredes-Sabja et al., 2009e). In this study, cysteine and glutamine at the pH ranges of ~ 5.5 to 6.5 efficiently induced germination of *C. perfringens* spores, with pH ~ 6.0 being optimum. Furthermore, 100 mM of asparagine, serine, and threonine at pH 6.0 were strong germinants, as well, and it is noticeable that the extents of germination for representative strains (SM101 and F4969) increase for all polar, uncharged amino acids upon adjusting pH to 6.0, compared to pH 7.0. The fact that *C. perfringens* spores prefer slightly acidic pH for germination could be of benefit for the contaminated spores to germinate and grow in meat- and poultry-containing products because the pH of broiler chicken meat, lamb, and beef were 5.5 to 6.4, 6.3 to 6.6, and ~ 5.8, respectively (Carse and Locker, 1974; Luciano et al., 2008; Thomson, 1978; Udompijitkul et al., 2012), all of which fall within the optimum germination pH for *C. perfringens* spores. We showed in previous work that a variety of 100 mM organic or inorganic buffers, all adjusted to pH 6.0, could not induce germination of *C. perfringens* SM101 and F4969 spores without the presence of known germinants;

thus, significant germination of *C. perfringens* spores upon incubation with polar, uncharged amino acids adjusted to pH 6.0, as demonstrated in the current study, was not solely due to pH 6.0. However, these phenomena could be attributed to the synergistic effect between the presence of germinative molecules and a pH ~ 6.0. These strictly narrow pH ranges for *C. perfringens* spore germination suggest the bacterial adaptation to its environmental niches of meats and correlate well with the fact that the most common food vehicles for *C. perfringens* type A FP are cooked meat- and poultry-containing products (Mcclane, 2007a).

Meats are excellent sources of amino acids and, to a lesser extent, of certain minerals and vitamins required for growth of *C. perfringens* (Boyd et al., 1948; Lawrie, 1991). Therefore, nutrient molecules in food products might initiate spore germination under suitable conditions. The free amino acid content of raw meat and finished meat products varied considerably and are influenced by various factors, such as types of meat, specific muscle locations, breed, animal age, storage conditions, and other constituents in the finished products. According to several reports, glutamine was usually the most abundant amino acids present in fresh meat and meat-containing products. Asparagine, cysteine, serine and threonine are also established in a significant proportion of the total amino acid composition in meats (Aristoy and Toldrá, 1998; Lawrie, 1991; Løvdal et al., 2011; Thomson, 1978). Furthermore, our preliminary results showed that, upon adjusting the pH to ~ 6.0, many amino acids improved their germination-inducing capacity, compared to pH 7.0 (data not shown). Thus, it is expected that the combinations of various amino acids at the natural pH of

raw and prepared meat products could act synergistically to induce germination of *C. perfringens* spores; however, further study is required to test this hypothesis and to relate these to the amino acid contents present in specific types of food products. Moreover, amino acids are stable during cooking (Greenwood et al., 1951; Schweigert et al., 1949), and heat actually activates spores to promote rapid germination and outgrowth in the protein-rich environments of food products. We recently demonstrated that various strains of *C. perfringens* FP and NFB isolates were able to germinate and outgrow (by ~ 2 to 3 log CFU/g increase) in cooked beef and poultry during storage at improper temperatures for 12 h (Udompijtkul et al., 2012).

In this study, we focused on identifying and characterizing novel germinants. We found a diversity in the germination requirements between spores of *C. perfringens* FP and NFB isolates. The amino acids in the polar, uncharged group, including asparagine, cysteine, serine, and threonine at pH 6.0, are the universal germinant for spores of both types of *C. perfringens* isolates. Nevertheless, glutamine at pH 6.0 was a unique germinant for only FP strains. This variation in the germinant selectivity between FP and NFB isolates was intriguing because genome sequences of both *C. perfringens* strain SM101 (FP isolate) and F4969 (NFB isolate) contain intact copies of all four GR genes (*gerAA*, *gerKB*, *gerKA*, and *gerKC*) that show > 95% identity to each other (Myers et al., 2006; Paredes-Sabja et al., 2009e). One plausible explanation for this inter-strain difference could be due to the variations of key residues in the binding site of GRs between FP and NFB isolates. Previous findings in *B. subtilis* supported this hypothesis where the molecule-triggered germination

changed from L-alanine to D-alanine upon alterations of a few amino acids of GerBA and GerBB receptors (Paidhungat and Setlow, 1999). The diversity in germination response among bacterial strains could also be attributed to a variety of factors including the differential expression, and/or the absence of some germination apparatus involved in the response to a particular germinant, depending on the GR's functionality and sensitivity (Abee et al., 2011; Broussolle et al., 2008). The differential germination phenotypes between FP and NFB isolates could also come from the adaptation to fit the ecological niches specialized for each isolate in order to increase the possibility of surviving in such environments as were proposed in our previous studies with ionic germination of *C. perfringens* spores (Paredes-Sabja et al., 2008c; Paredes-Sabja et al., 2009e; Van Der Voort et al., 2010).

It was established that the nutrient-induced germination is mediated through the interactions between specific nutrients and their cognate GRs located in the spore's inner membrane (Abee et al., 2011; Hudson et al., 2001; Paidhungat and Setlow, 2001; Setlow, 2003). Almost all the genomes of spore-forming bacteria contain several GRs, and they are likely to respond to structurally diverse compounds (Abee et al., 2011; Moir, 2006; Ross and Abel-Santos, 2010). Likewise, the genome of *C. perfringens* SM101 harbors four germinant receptors encoding by *gerAA*, *gerKB*, *gerKA*, and *gerKC*. The functional studies of these GR-mutants showed that not all the GR proteins were required for the receptor functionality with certain germinative molecules (Paredes - Sabja et al., 2009; Paredes-Sabja et al., 2008c). These phenomena were also observed in the germination of spores of *C. perfringens* SM101

with cysteine and glutamine at pH 6.0. It was evident that three germinant receptor proteins, GerKB, GerAA, and GerKC, were required, although at a different priority for normal spore germination. Our previous studies revealed that, under optimal concentration (100 mM) of L-asparagine (pH 7.0), GerAA and GerKB were not essential, while GerKA and/or GerKC showed prominent activity for the *C. perfringens* SM101 spore germinated with this particular amino acid (Paredes - Sabja et al., 2009; Paredes-Sabja et al., 2008c). Although the 100 mM asparagine at pH 6.0 induced a higher response than at pH 7.0 for SM101 spores, both of them were considered a strong germinant. For the current work, it is now elucidated that the insignificant response of *gerKA-KC* spores with asparagine (pH 7.0) is primarily attributed to a defect in the GerKC receptor. Unlike asparagine (pH 7.0)-mediated germination in our earlier findings, GerKB and GerAA receptors had some contributing roles in normal spore germination with asparagine at pH 6.0, and their activity might explain the relatively small but significant germination response observed in spores lacking GerKC. In contrast to the situation with *B. subtilis*, where the loss of any cistron of the tricistronic receptor operon abolishes the receptor functionality (Atluri et al., 2006; Moir et al., 2002), we discovered that the disruption of *gerKA* had no effect on GerKC-mediated germination with cysteine, glutamine, or asparagine, all at pH 6.0.

For threonine- and serine-induced germination of SM101 spores, a different pathway might exist. The GerKA, GerKC, and GerKB were required for spore germination with these two amino acids, although to a different extent. One possibility

is that GerKA, GerKC, and GerKB receptors are functionally redundant for interacting with serine or threonine. If this hypothesis is true, loss of one receptor protein should not abolish germination response to these two amino acids. However, this seems not to be the case for *C. perfringens* spore germination with serine and threonine (pH 6.0), as loss of each individual GR protein provided distinct levels of germination defect. Alternatively, these results suggest that *C. perfringens* GerKA, GerKC, and GerKB form a receptor complex, and the contributed functions of GerKA, GerKC, and GerKB to the complex are as follows: major, intermediate, and minor, respectively, based on reduced germination responses observed in strains lacking those GR proteins. Further biochemical analysis of GRs should aid in clarifying this hypothesis.

Since spores of *C. perfringens* are highly resistant to various stress factors and preservative technologies typically applied in food manufacturing, such as heat, refrigerated and frozen temperature, high hydrostatic pressure, and chemical preservations (Li and McClane, 2006a, 2006b; Paredes-Sabja et al., 2007; Sarker et al., 2000; Udompijitkul et al., 2012). Therefore, the removal and elimination *C. perfringens* spores from foods and food processing environments are always very challenging. One potential approach is to induce *C. perfringens* spores to germinate before inactivating the less resistant germinating spores using milder treatments; however, the major obstacle of this strategy is the heterogeneity of spore germination (Wang et al., 2011b). To overcome this trouble, a better understanding of specific germinants and spore germination in *C. perfringens* is necessary. This would enable the development of a powerful germinant or mixtures of germinants that are effective

on a wide range of isolates, as well as yield a strong and more uniform germination pattern of *C. perfringens* spores.

In conclusions, the important findings of the current work are the following: (i) cysteine and other polar, uncharged amino acids including asparagine, serine, and threonine at pH 6.0 are universal germinants for spores of CPE-producing *C. perfringens* FP and NFB isolates, with cysteine being the strongest germinant for NFB spores; (ii) glutamine at pH 6.0 is a unique germinant for spores of FP isolates; and (iii) a different set of spore-specific GR might act cooperatively to recognize these novel identified nutrient germinants. GerKC is the major receptors involved in cysteine, glutamine, and asparagine (pH 6.0) germination, while GerKB and GerAA play auxiliary roles. For serine- and threonine (pH 6.0)-induced germination, GerKA is the dominant receptor and GerKC and GerKB are also required for efficient germination of FP spores.

Acknowledgements

This work was supported by a grant from the Agricultural Research Foundation of Oregon State University, and by a Department of Defense Multidisciplinary University Research Initiative (MURI) award through the U.S. Army Research Laboratory and the U. S. Army Research Office under contract number W911NF-09-1-0286 (all to MRS). PU was supported by fellowships from the Office of Higher Education Commission (Thailand). MA and SB were supported by

the Ministry of Higher Education (Saudi Arabia). We thank Denny Weber for help editing the manuscript.

References

- Abee, T., Groot, M.N., Tempelarrs, M., Zwietering, M., Moezelaar, R., & Van de Voort, M. (2011). Germination and outgrowth of spores of *Bacillus cereus* group members: Diversity and role of germinant receptors. *Food Microbiology*, *28*, 199-208.
- Abrahamo, C., Carman, R.J., Hahn, H., & Liesenfeld, O. (2001). Similar frequency of detection of *Clostridium perfringens* and *Clostridium difficile* toxins in patients with antibiotic-associated diarrhea. *European Journal of Clinical Microbiology and Infectious Diseases*, *20*, 676-677.
- Adam, K.H., Brunt, J., Brightwell, G., Flint, S.H., & Peck, M.W. (2011). Spore germination of the psychrotolerant, red meat spoiler, *Clostridium frigidicarnis*. *Letters in Applied Microbiology*, *53*, 92-97.
- Anderson, K.G., Hansen, T.B., & Knøchel, S. (2004). Growth of heat treated enterotoxin-positive *Clostridium perfringens* and the implications for safe cooling rates. *Journal of Food Protection*, *67*, 83-89.
- Ando, Y. (1974). Ionic germination of spores of *Clostridium perfringens* type A. *Japanese Journal of Microbiology*, *18*, 433-439.
- André, G., Haudecoeur, E., Monot, M., Ohtani, K., Shimizu, T., Dupuy, B., & Martin-Verstraete, I. (2010). Global regulation of gene expression in response to cysteine availability in *Clostridium perfringens*. *BMC Microbiology*, *10*, 234.
- Aristoy, M.-C., & Toldrá, F. (1998). Concentration of free amino acids and dipeptides in porcine skeletal muscles with different oxidative patterns. *Meat Science*, *50*, 327-332.
- Asha, N.J., & Wilcox, M.H. (2002). Laboratory diagnosis of *Clostridium perfringens* antibiotic-associated diarrhoea. *Journal of Medical Microbiology*, *51*, 891-894.
- Atluri, S., Ragkousi, K., Cortezzo, D.E., & Setlow, P. (2006). Cooperativity between different nutrient receptors in germination of spores of *Bacillus subtilis* and reduction of this cooperativity by alterations in the GerB receptor. *Journal of Bacteriology*, *188*, 28-36.
- Berg, J.M., Tymoczko, J.L., & Stryer, L. (2006). Protein composition and structure. In: Berg, J.M., Tymoczko, J.L., Stryer, L. (Eds.), *Biochemistry* (pp. 25-64). New York: W.H. Freeman and Company.

- Borriello, S.P., Larson, H.E., Welch, A.R., Barclay, F., Stringer, M.F., & Bartholomew, B.A. (1984). Enterotoxigenic *Clostridium perfringens*: a possible cause of antibiotic-associated diarrhea. *Lancet*, *1*, 305-307.
- Boyd, M.J., Logan, M.A., & Tytell, A.A. (1948). The growth requirements of *Clostridium perfringens* (welchii) BP6K. *The Journal of Biological Chemistry*, *174*, 1013-1025.
- Broussolle, V., Gauillard, F., Nguyen-The, C., & Carlin, F. (2008). Diversity of spore germination in response to inosine and L-alanine and its interaction with NaCl and pH in the *Bacillus cereus* group. *Journal of Applied Microbiology*, *105*, 1081-1090.
- Brown, K.L. (2000). Control of bacterial spores. *British Medical Bulletin*, *56*, 158-171.
- Buzby, J.C., & Roberts, T. (1997). Economic costs and trade impacts of microbial foodborne illness. *World Health Statistics Quarterly*, *50*, 57-66.
- Cabrera-Martinez, R.M., Tovar-Rojo, F., Vepachedu, V.R., & Setlow, P. (2003). Effects of overexpression of nutrient receptors on germination of spores of *Bacillus subtilis*. *Journal of Bacteriology*, *185*, 2457-2464.
- Carlin, F. (2011). Origin of bacterial spores contaminating foods. *Food Microbiology*, *28*, 177-182.
- Carse, W.A., & Locker, R.H. (1974). A survey of pH values at the surface of beef and lamb carcasses, stored in a chiller. *Journal of the Science of Food and Agriculture*, *25*, 1529-1535.
- Cassier, M., & Sebald, M. (1969). Germination lysozyme-dépendante des spores de *Clostridium perfringens* ATCC3624 après traitement thermique. *Ann Inst Pasteur*, *117*, 312-324.
- Collie, R.E., Kokai-Kun, J.F., & McClane, B.A. (1998). Phenotypic characterization of enterotoxigenic *Clostridium perfringens* isolates from non-foodborne human gastrointestinal diseases. *Anaerobe*, *4*, 69-79.
- Collie, R.E., & McClane, B.A. (1998). Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. *Journal of Clinical Microbiology*, *36*, 30-36.
- Cornillot, E., Saint-Joanis, B., Daube, G., Katayama, S., Granum, P.E., Canard, B., & Cole, S.T. (1995). The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. *Molecular Microbiology*, *15*, 639-647.
- Duncan, C.L., & Strong, D.H. (1968). Improved medium for sporulation of *Clostridium perfringens*. *Applied Microbiology*, *16*, 82-89.
- Duncan, C.L., Strong, D.H., & Sebald, M. (1972). Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. *Journal of Bacteriology*, *110*, 378-391.
- Gould, G.W. (2006). History of science-spores: Lewis B Perry Memorial lecture 2005. *Journal of Applied Microbiology*, *101*, 507-513.
- Greenwood, D.A., Kraybill, H.R., & Schweigert, B.S. (1951). Amino acid composition of fresh and cooked beef cuts. *The Journal of Biological Chemistry*, *193*, 23-28.

- Harrison, B., Raju, D., Garmory, H.S., Brett, M.M., Titball, R.W., & Sarker, M.R. (2005). Molecular characterization of *Clostridium perfringens* isolates from humans with sporadic diarrhea: evidence for transcriptional regulation of the beta2-toxin-encoding gene. *Applied and Environmental Microbiology*, 71, 8362-8370.
- Hornstra, L.M., de Vries, Y.P., Wells-Bennik, M.H., de Vos, W.M., & Abee, T. (2006). Characterization of germination receptors of *Bacillus cereus* ATCC 14579. *Applied and Environmental Microbiology*, 72, 44-53.
- Hudson, K.D., Corfe, B.M., Kemp, E.H., Feavers, I.M., Coote, P.J., & Moir, A. (2001). Localization of GerAA and GerAC germination proteins in the *Bacillus subtilis* spore. *Journal of Bacteriology*, 183, 4317-4322.
- Kobayashi, S., Wada, A., Shibasaki, S., Annaka, M., Higuchi, H., Adachi, K., Mori, N., Ishigawa, T., Masuda, Y., Watanabe, H., Yamamoto, N., Yamaoka, S., & Inamatsu, T. (2008). Spread of a large plasmid carrying the cpe gene and the tcp locus amongst *Clostridium perfringens* isolates from nosocomial outbreaks and sporadic cases of gastroenteritis in a geriatric hospital. *Epidemiology & Infection*, 137, 108-113.
- Lahti, P., Heikinheimo, A., Johansson, T., & Korkeala, H. (2008). *Clostridium perfringens* type A strains carrying a plasmid-borne enterotoxin gene (genotype IS1151-cpe or IS1470-like-cpe) as a common cause of food poisoning. *Journal of Clinical Microbiology*, 46, 371-373.
- Lawrie, R.A. (1991). Meat and human nutrition. In: Lawrie, R.A. (Ed.), *Meat science* (pp. 225-234). Oxford, England: Pergamon Press.
- Li, J., & McClane, B.A. (2006a). 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. *Applied and Environmental Microbiology*, 72, 7620-7625.
- Li, J., & McClane, B.A. (2006b). Further comparison of temperature effects on growth and survival of *Clostridium perfringens* type A isolates carrying a chromosomal or plasmid-borne enterotoxin gene. *Applied and Environmental Microbiology*, 72, 4561-4568.
- Lindström, M., Heikinheimo, A., Lahti, P., & Korkeala, H. (2011). Novel insights into the epidemiology of *Clostridium perfringens* type A food poisoning. *Food Microbiology*, 28, 192-198.
- Løvdal, I.S., Hovda, M.B., Granum, P.E., & Rosnes, J.T. (2011). Promoting *Bacillus cereus* spore germination for subsequent inactivation by mild heat treatment. *Journal of Food Protection*, 74, 2079-2089.
- Luciano, F.B., Hossinian, F.S., Beta, T., & Holly, R.A. (2008). Effect of free-SH containing compounds on allyl isothiocyanate antimicrobial activity against *Escherichia coli* O157:H7. *Journal of Food Science*, 73, M214-M220S.
- Lynch, M., Painter, J., Woodruff, R., & Braden, C. (2006). Surveillance for foodborne-disease outbreaks - United States, 1998-2002. *Morbidity and Mortality Weekly Report*, 55, 1-42.

- McClane, B., Uzal, F.A., Miyakawa, M.E.F., Lyerly, D., & Wilkins, T. (2006). The enterotoxigenic clostridia. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), *The Prokaryotes* (pp. 698-752). New York: Springer New York.
- McClane, B.A. (2007a). *Clostridium perfringens*. In: Doyle, M.P., Beuchat, L.R. (Eds.), *Food Microbiology: fundamentals and frontiers* (pp. 423-444). Washington, D.C.: ASM Press.
- McClane, B.A. (2007b). *Clostridium perfringens*. In: Doyle, M.P., Beuchat, L.R. (Eds.), *Food Microbiology Fundamentals and Frontiers* (pp. 423-444). Washington, D.C.: ASM Press.
- Moir, A. (2006). How do spores germinate? *Journal of Applied Microbiology*, *101*, 526-530.
- Moir, A., Corfe, B.M., & Behravan, J. (2002). Spore germination. *Cellular and Molecular Life Sciences*, *59*, 403-409.
- Montville, T.J. (1984). Characterization of a halo-acid-tolerant subpopulation from *Clostridium botulinum*. *Applied and Environmental Microbiology*, *46*, 311-316.
- Montville, T.J., Jones, S.B., Conway, L.K., & Sapers, G.M. (1985). Germination of spores from *Clostridium botulinum* B-aphis and Ba410. *Applied and Environmental Microbiology*, *50*, 795-800.
- Mpamugo, O., Donovan, T., & Brett, M.M. (1995). Enterotoxigenic *Clostridium perfringens* as a cause of sporadic diarrhoea. *Journal of Medical Microbiology*, *43*, 442-445.
- Myers, G.S., Rasko, D.A., Cheung, J.K., Ravel, J., Seshadri, R., DeBoy, R.T., Ren, Q., Varga, J., Awad, M.M., Brinkac, L.M., Daugherty, S.C., Haft, D.H., Dodson, R.J., Madupu, R., Nelson, W.C., Rosovitz, M.J., Sullivan, S.A., Khouri, H., Dimitrov, G.I., Watkins, K.L., Mulligan, S., Benton, J., Radune, D., Fisher, D.J., Atkins, H.S., Hiscox, T., Jost, B.H., Billington, S.J., Songer, J.G., McClane, B.A., Titball, R.W., Rood, J.I., Melville, S.B., & Paulsen, I.T. (2006). Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. *Genome Research*, *16*, 1031-1040.
- Novak, J.S., Peck, M.W., Juneja, V.K., & Johnson, E.A. (2005). *Clostridium botulinum* and *Clostridium perfringens*. In: Fratamico, P.M., Bhunia, A.K., Smith, J.L. (Eds.), *Foodborne pathogen: Microbiology and molecular biology* (pp. 383-408). Norfolk, UK: Caister Academic Press.
- Paidhungat, M., & Setlow, P. (1999). Isolation and characterization of mutations in *Bacillus subtilis* that allow spore germination in the novel germinant D-alanine. *Journal of Bacteriology*, *181*, 3341-3350.
- Paidhungat, M., & Setlow, P. (2001). Localization of a germinant receptor protein (GerBA) to the inner membrane of *Bacillus subtilis* spores. *Journal of Bacteriology*, *183*, 3982-3990.
- Paredes - Sabja, D., Setlow, P., & Sarker, M.R. (2009). Role of GerKB in germination and outgrowth of *Clostridium perfringens* spores. *Applied and Environmental Microbiology*, *75*, 3813-3817.

- Paredes - Sabja, D., Setlow, P., & Sarker, M.R. (2011). Germination of spores of *Bacillales* and *Clostridiales* species: mechanisms and protein involved. *Trends in Microbiology*, *19*, 85-94.
- Paredes-Sabja, D., Gonzalez, M., Sarker, M.R., & Torres, J.A. (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*, *72*, M202-M206.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009a). SleC is essential for cortex peptidoglycan hydrolysis during germination of spores of the pathogenic bacterium *Clostridium perfringens*. *Journal of Bacteriology*, *191*, 2711-2720.
- Paredes-Sabja, D., Torres, J.A., Setlow, P., & Sarker, M.R. (2008). *Clostridium perfringens* spore germination: characterization of germinants and their receptors. *Journal of Bacteriology*, *190*, 1190-1201.
- Paredes-Sabja, D., Udombijitkul, P., & Sarker, M.R. (2009b). Inorganic phosphate and sodium ions are cogermnants for spores of *Clostridium perfringens* type A food poisoning - related isolates *Applied and Environmental Microbiology*, *75*, 6299-6305.
- Plowman, J., & Peck, M.W. (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. *Journal of Applied Microbiology*, *92*, 681-694.
- Ross, C., & Abel-Santos, E. (2010). The Ger receptor family from sporulating bacteria. *Current Issues in Molecular Biology*, *12*, 147-158.
- Rowley, D.B., & Feeherry, F. (1970). Conditions affecting germination of *Clostridium botulinum* 62A spores in chemically defined medium. *Journal of Bacteriology*, *104*, 1151-1157.
- Sarker, M.R., Shivers, R.P., Sparks, S.G., Juneja, V.K., & McClane, B.A. (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. *Applied and Environmental Microbiology*, *66*, 3234-3240.
- Scallan, E., Griffin, P.M., Angulo, F.J., Tauxe, R.V., & Hoekstra, R.M. (2011). Foodborne illness acquired in the United States - unspecified agents. *Emerging Infectious Diseases*, *17*, 16-22.
- Schweigert, B.S., Guthneck, B.T., Kraybill, H.R., & Greenwood, D.A. (1949). The amino acid composition of pork and lamb cuts. *The Journal of Biological Chemistry*, *180*, 1077-1083.
- Setlow, P. (2003). Spore germination. *Current Opinion in Microbiology*, *6*, 550-556.
- Tanaka, D., Isobe, J., Hosorogi, S., Kimata, K., Shimizu, M., Katori, K., Gyobu, Y., Nagai, Y., Yamagishi, T., Karasawa, T., & Nakamura, S. (2003). An outbreak of foodborne gastroenteritis caused by *Clostridium perfringens* carrying the *cpe* gene on a plasmid. *Japanese Journal of Infectious Diseases*, *56*, 137-139.
- Tang, T., & Frank, H.A. (1972). Cysteine-induced germination and emergence of putrefactive anaerobe 3679h spores. In: Halvorson, H.O., Hanson,

- R. Campebell, L.L. (Eds.), *Spore V* (pp. 378-383). Washington, D.C.: American Society for Microbiology.
- Thippareddi, H., Juneja, V.K., Phebus, R.K., Marsden, J.L., & Kastner, C.L. (2003). Control of *Clostridium perfringens* germination and outgrowth by buffered sodium citrate during chilling of roast beef and injected pork. *Journal of Food Protection*, 66, 376-381.
- Thomson, J.E. (1978). Free amino acids in broiler chicken meat in relation to initial pH. *Poultry Science*, 57, 1468-1470.
- Udompijitkul, P., Paredes - Sabja, D., & Sarker, M.R. (2012). Inhibitory effects of nisin against *Clostridium perfringens* food poisoning and nonfood-borne isolates. *Journal of Food Science*, 77, M51-56.
- Vaishnavi, C., & Kaur, S. (2008). *Clostridium perfringens* enterotoxin in antibiotic-associated diarrhea. *Indian Journal of Pathology and Microbiology*, 51, 198-199.
- van der Voort, M., Garcí, D., Moezelaar, R., & Abee, T. (2010). Germination receptor diversity and germination responses of four strains of the *Bacillus cereus* group. *International Journal of Food Microbiology*, 139, 108-115.
- Wang, G., Zhang, P., Paredes - Sabja, D., Green, C., Setlow, P., Sarker, M.R., & Li, Y.-Q. (2011). Analysis of the germination of individual *Clostridium perfringens* spores and its heterogeneity. *Journal of Applied Microbiology*, 1-12.
- Wax, R., & Freese, E. (1968). Initiation of the germination of *Bacillus subtilis* spores by a combination of compounds in place of L-alanine. *Journal of Bacteriology*, 95, 433-438.
- Xiao, Y., Wagendorp, A., Moezelaar, R., Abee, T., & Wells-Bennik, M.H. (2012). A wide variety of *Clostridium perfringens* type A food-borne isolates that carry a chromosomal cpe gene belong to one multilocus sequence typing cluster. *Applied and Environmental Microbiology*, 78, 7060-7068.
- Zhao, Y., & Melville, S.B. (1998). Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (*cpe*) of *Clostridium perfringens*. *Journal of Bacteriology*, 180, 136-142.

Table 6.1. Germination of spores of *C. perfringens* representative FP and NFB strains with 20 amino acids at pH 7.0^a

Germinants	Decrease in OD ₆₀₀ (% Mean ± SD) in 60 min with:	
	SM101	F4969
Nonpolar, aliphatic		
Gly	23 ± 2.7	11 ± 2.4
Ala	22 ± 0.7	5 ± 0.3
Val	11 ± 0.4	4 ± 1.5
Leu	10 ± 2.3	10 ± 3.7
Met	15 ± 5.2	9 ± 0.9
Ile	20 ± 6.6	9 ± 0.4
Pro	4 ± 1.5	6 ± 4.0
Aromatic		
Phe	4 ± 2.4	9 ± 1.4
Tyr ^b	9 ± 0.1	6 ± 1.5
Trp ^b	18 ± 0.6	15 ± 2.1
Polar, uncharged		
Ser	25 ± 8.3	7 ± 1.1
Thr	15 ± 3.2	13 ± 3.4
Cys	51 ± 2.3	60 ± 1.0
Asn	45 ± 1.0	5 ± 2.2
Gln	43 ± 2.3	27 ± 9.7
Positively charged		
Lys	25 ± 0.3	20 ± 0.2
Arg	24 ± 0.8	5 ± 2.2
His	13 ± 0.1	7 ± 0.0
Negatively charged		
Asp	37 ± 5.4	8 ± 0.8
Glu	21 ± 11.2	7 ± 0.2

^a All amino acids, except for tyrosine and tryptophan, were used at 100 mM in 25 mM Na₂HPO₄ buffer and adjusted to pH 7.0.

^b Tyr and Trp were used at 3 mM in 25 mM Na₂HPO₄ buffer and adjusted to pH 7.0.

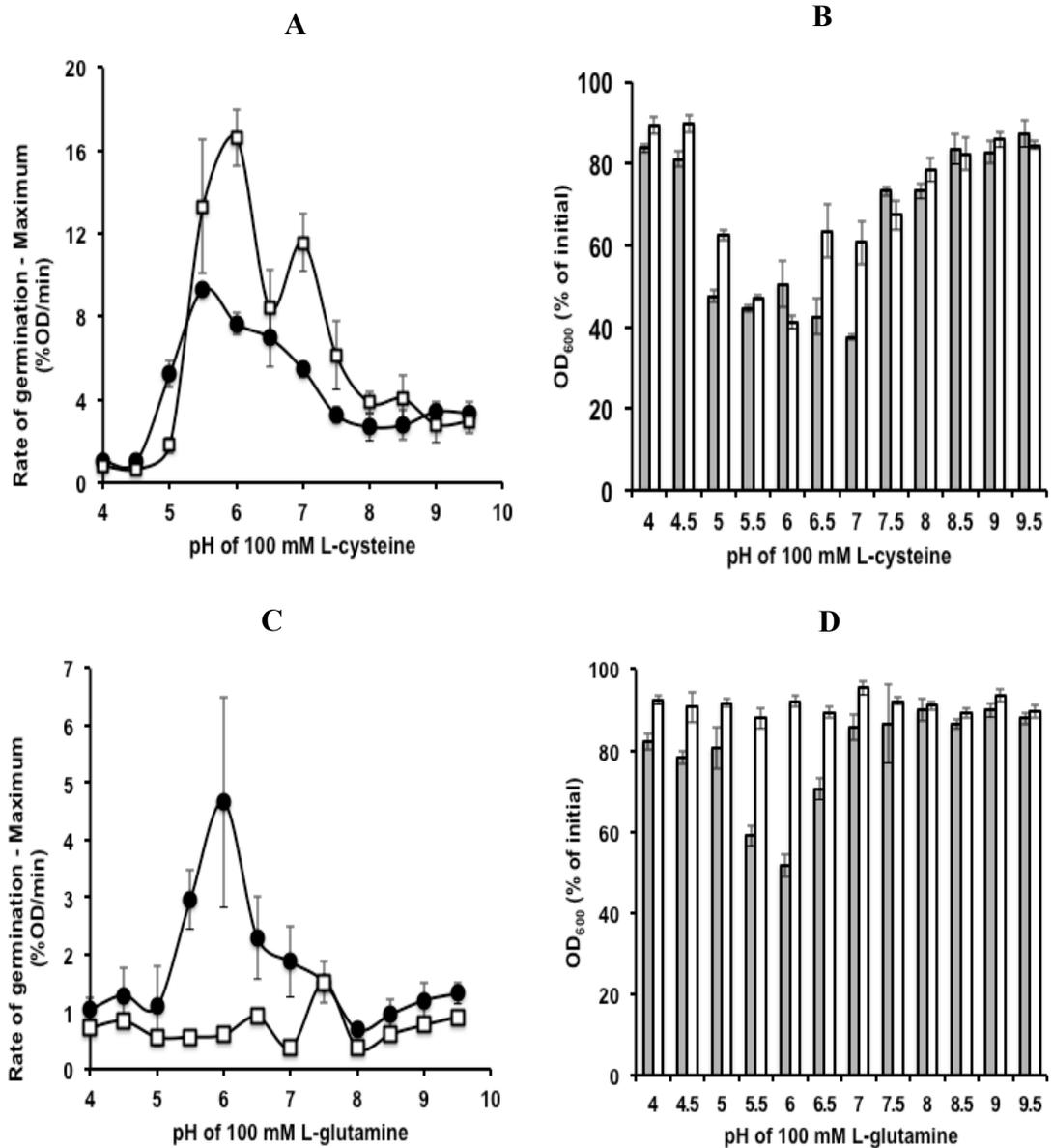


Fig. 6.1A-D. Effect of cysteine (A and B) and glutamine (C and D) pHs on germination of *C. perfringens* spores. Heat-activated SM101 (filled circles and grey bars) and NB16 (open squares and white bars) spores were incubated with 100 mM cysteine or glutamine adjusted to various pHs. Maximum rate (A and C) and extents of germination after 60 min (B and D) were calculated, as described in Materials and methods. Error bars represent standard deviations.

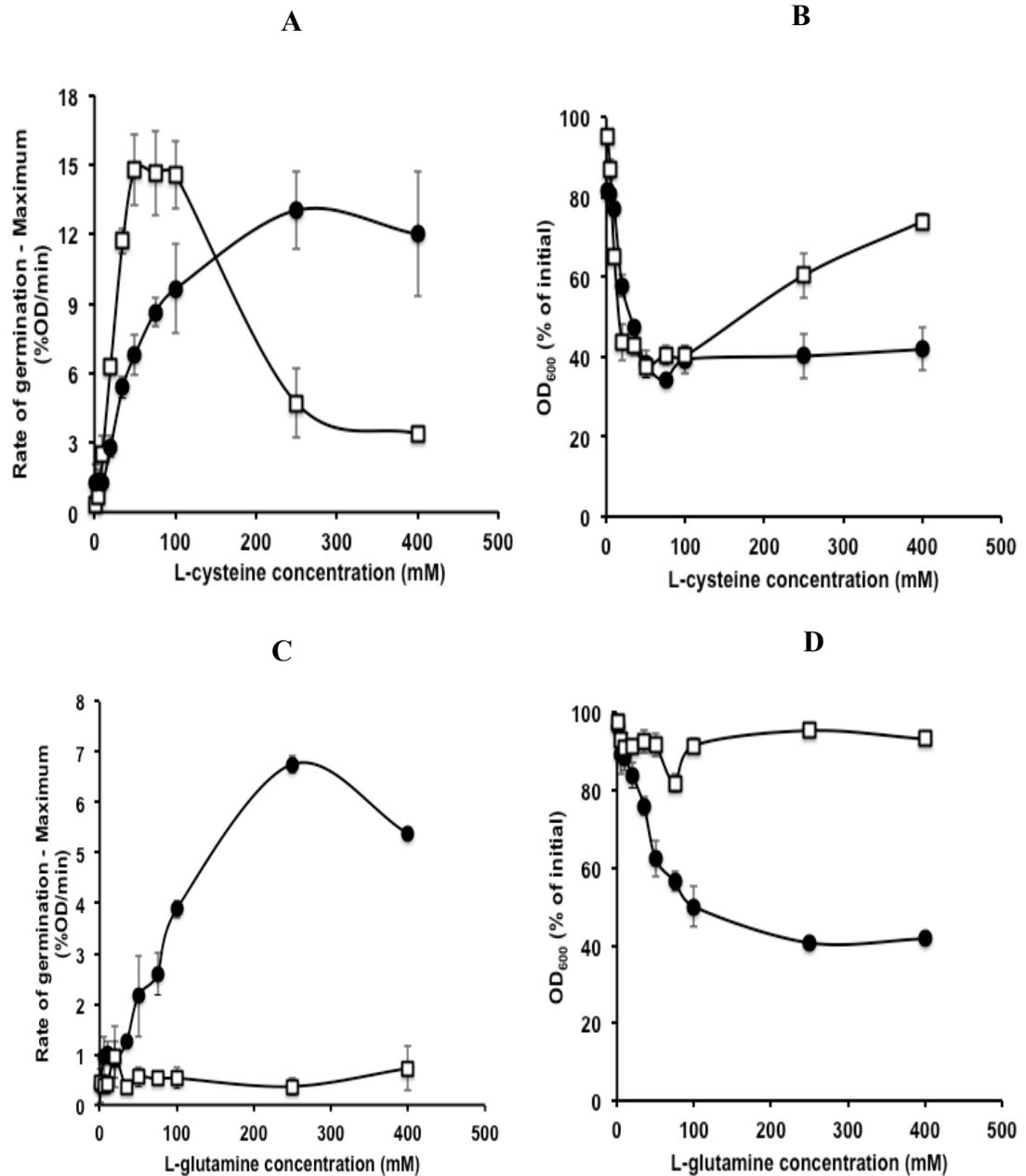


Fig. 6.2A-D. Effect of cysteine (A and B) and glutamine (C and D) concentrations on germination of *C. perfringens* spores. Heat-activated spores of strain SM101 (filled circles) and NB16 (open squares) were incubated with various concentrations of cysteine or glutamine (pH 6.0) and maximum rate (A and C) and extents of germination after 60 min (B and D) were calculated, as described in Materials and methods. Error bars represent standard deviations.

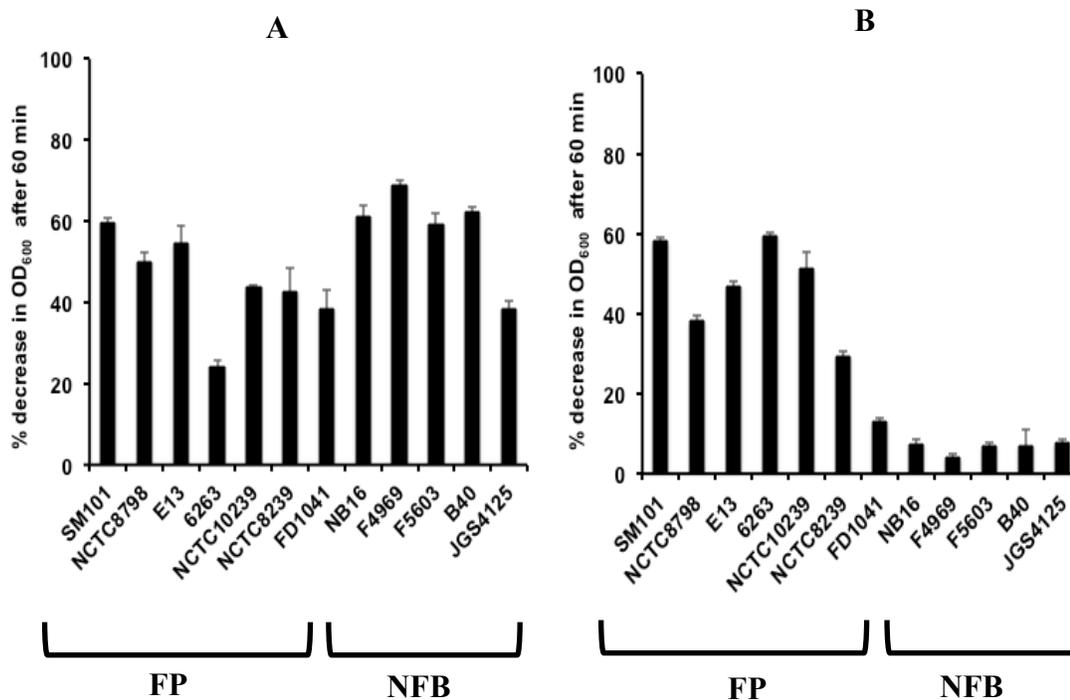


Fig. 6.3A-B. Germination of spores of *C. perfringens* various isolates with cysteine (A) and glutamine (B). Both germinants were tested at 100 mM (pH 6.0). Heat-activated spores of FP type A isolates (SM101, NCTC8798, E13, 6263, NCTC10239, NCTC8239, and FD1041), NFB type A isolates (NB16, F4969, F5603, and B40), and type A animal isolate (JGS1425) were incubated with germinants at 40 °C for 60 min, and OD₆₀₀ was measured, as described in Materials and methods. Data represent percentage decrease in OD₆₀₀ relative to the initial values and error bars indicate standard deviations.

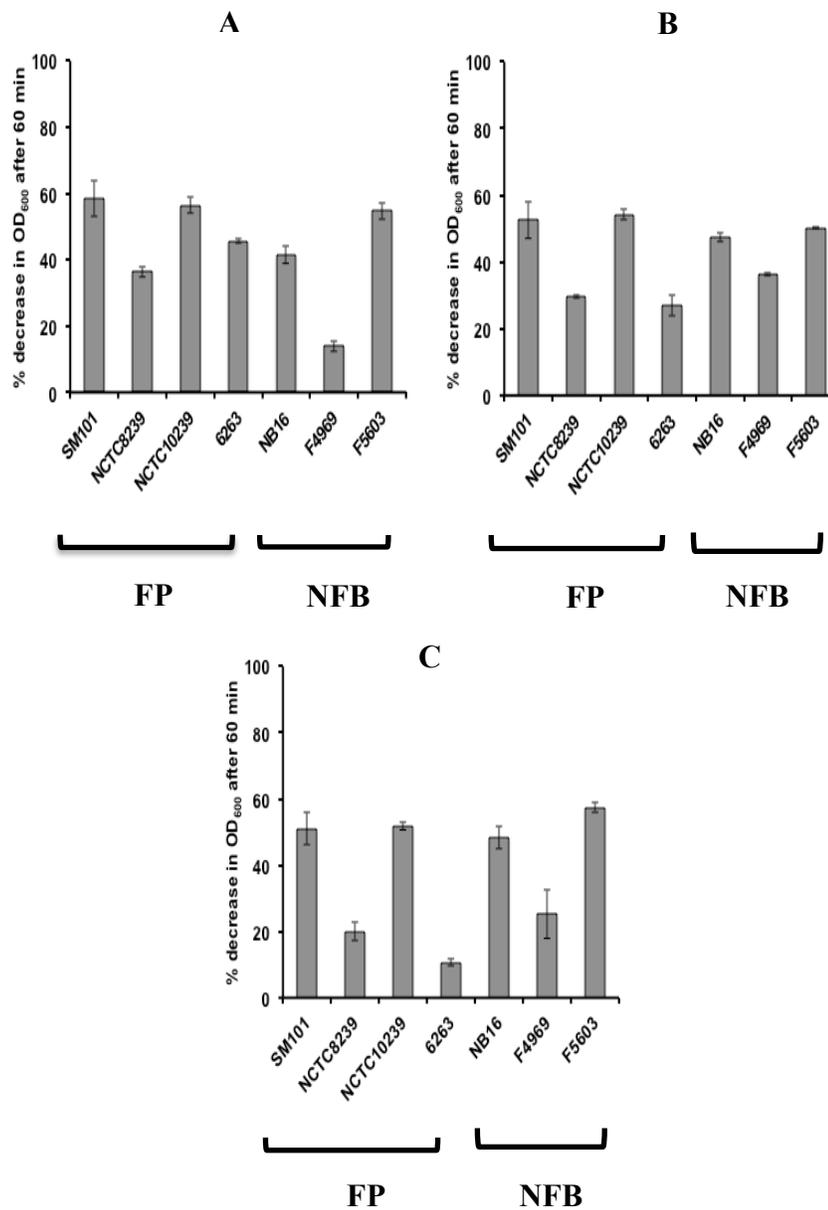


Fig. 6.4A-C. Germination of spores of *C. perfringens* various isolates with amino acids in polar, uncharged group including asparagine (A), serine (B), and threonine (C). All amino acid solutions were tested at 100 mM and adjusted to pH 6.0. Heat-activated type A FP isolates (SM101, NCTC8239, NCTC10239, and 6263) and type A NFB isolates (NB16, F4969, and F5603) were incubated with amino acid solutions at 40 °C for 60 min. OD₆₀₀ was measured and results were expressed as the percentage decrease in OD₆₀₀ relative to the initial values. Error bars indicate standard deviations.

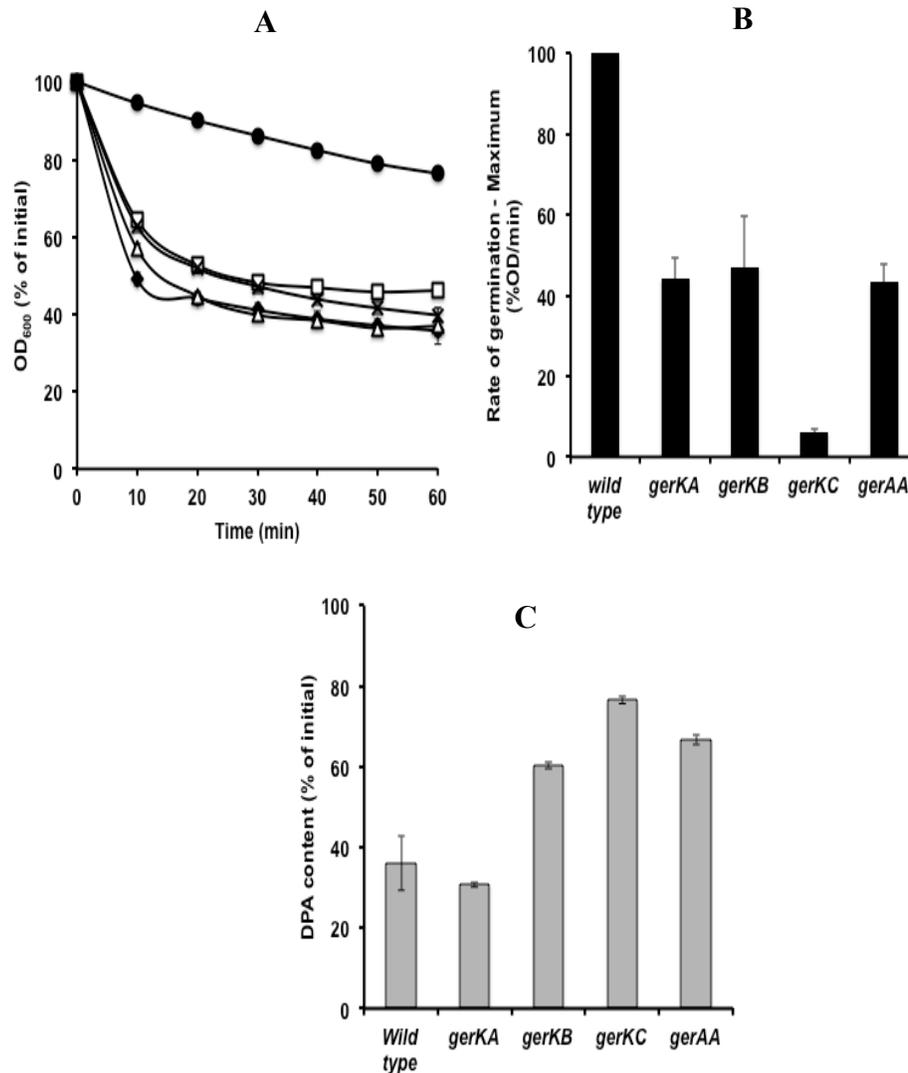


Fig. 6.5A-C. Germination of spores of *C. perfringens* GR-mutant strains with cysteine. (A) Heat-activated spores of strain SM101 (wild type) (filled diamonds), DPS103 (*gerAA* mutant) (open squares), DPS108 (*gerKB* mutant) (cross marks), DPS119 (*gerKA* mutant) (open triangles), and DPS122 (*gerKC* mutant) (filled circles) were incubated with 250 mM cysteine (pH 6.0) at 40 °C, and OD₆₀₀ was measured. (B) Heat-activated spores of SM101 and GR-derivative strains were incubated with 250 mM cysteine (pH 6.0), and maximum rates of spore germination were determined as % change of OD₆₀₀ per min relative to value for wild type strain (SM101), which was set at 100. (C) DPA release during *C. perfringens* spore germination with cysteine. Heat-activated spores of various strains were induced to germinate with 250 mM cysteine (pH 6.0) at 40 °C. The DPA content was measured, as described in Materials and methods after 60 min of incubation. Error bars indicate standard deviations.

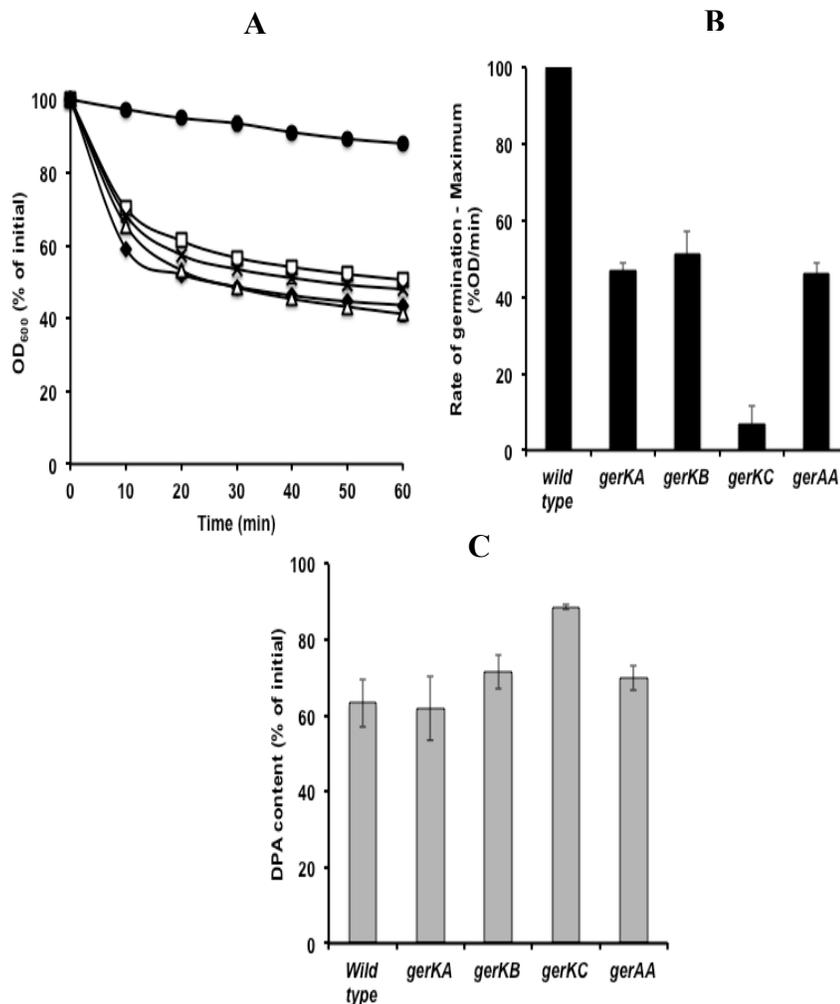


Fig. 6.6A-C. Germination of spores of *C. perfringens* GR mutant strains with glutamine. (A) Heat-activated spores of strain SM101 (wild type) (filled diamonds), DPS103 (*gerAA* mutant) (open squares), DPS108 (*gerKB* mutant) (cross marks), DPS119 (*gerKA* mutant) (open triangles), and DPS122 (*gerKC* mutant) (filled circles) were incubated with 250 mM glutamine (pH 6.0) at 40 °C, and OD₆₀₀ was measured. (B) Heat-activated spores of SM101 and GR-derivative strains were incubated with 250 mM glutamine (pH 6.0), and maximum rates of spore germination were determined as % change of OD₆₀₀ per min relative to value for wild type strain (SM101), which was set at 100. (C) DPA release during *C. perfringens* spore germination with glutamine. Heat-activated spores of various strains were induced to germinate with 250 mM glutamine (pH 6.0) at 40 °C. The DPA content was measured as described in Materials and methods after 60 min of incubation. Error bars indicate standard deviations.

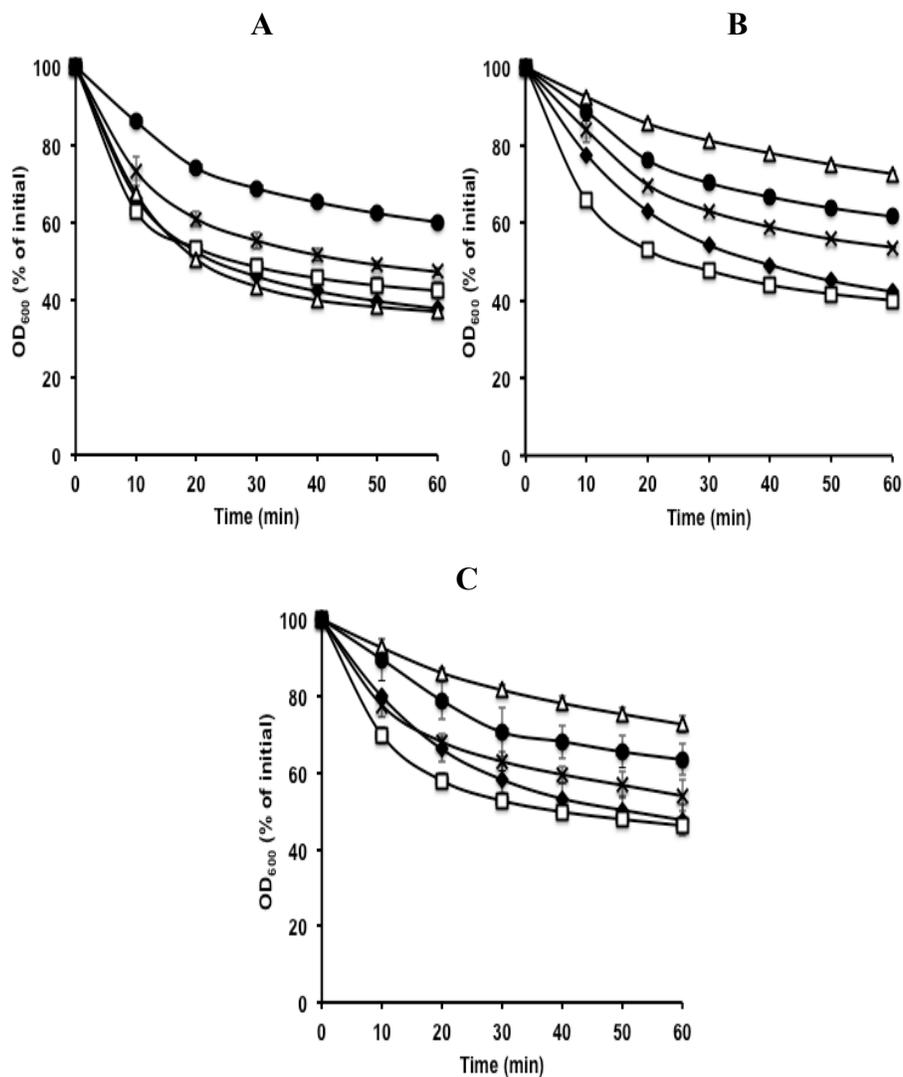


Fig. 6.7A-C. Germination of spores of *C. perfringens* GR mutant strains with amino acids in polar, uncharged group. All amino acid solutions were tested at 100 mM and adjusted to pH 6.0. Heat-activated spores of strain SM101 (wild type) (filled diamonds), DPS103 (*gerAA* mutant) (open squares), DPS108 (*gerKB* mutant) (cross marks), DPS119 (*gerKA* mutant) (open triangles), and DPS122 (*gerKC* mutant) (filled circles) were incubated at 40 °C with asparagine (A), serine (B), and threonine (C). Changes in OD₆₀₀ were measured as described in Materials and methods. Error bars denote standard deviations.

CHAPTER 7

Roles of Putative Histidine Kinases in *Clostridium perfringens* Sporulation and Germination

Pathima Udompijtkul, Daniel Paredes-Sabja, and Mahfuzur R. Sarker

Manuscript will be submitted to *Anaerobe*

Abstract

Clostridium perfringens sporulation and germination are important steps in the life cycle of this anaerobic pathogenic bacterium, not only to produce highly resistant endospores but also to contribute to the pathogenesis of *C. perfringens* type A-related gastrointestinal diseases. In this study, the roles of the putative sensor histidine kinases, CPR1728 and CPR1055, in *C. perfringens* growth, sporulation, spore germination, and outgrowth were characterized. Results showed that the expressions of *cpr1728* and *cpr1055* were highly induced under sporulation-inducing conditions and were dependent on a master regulator of sporulation, Spo0A. Inactivation of *cpr1728* and *cpr1055* genes did not affect vegetative growth; however, both mutants grew at a higher rate under sporulation-inducing condition. The strain lacking CPR1728 sporulated at approximately 3% of the frequency of the wild type, and the *cpr1055* mutant strain showed a delayed sporulation phenotype and eventually sporulated at 10% of the wild type level in Duncan-Strong sporulation medium. Furthermore, *cpr1728* and *cpr1055* spores germinate significantly slower with various nutrient and non-nutrient germinants than did wild type spores. Both CPR1728 and CPR1055 are also required for normal spore outgrowth in rich medium. In sum, this study demonstrates that *cpr1728* and *cpr1055* play roles in *C. perfringens* sporulation, spore germination and outgrowth.

7.1. Introduction

Clostridium perfringens is a Gram-positive, rod-shaped, nonmotile bacterium with a prolific toxin-producing ability, causing a wide variety of human and veterinary diseases (Mcclane, 2001). It is commonly classified into 5 toxinotypes, A to E, based on the production of four major toxins, α , β , ϵ , and ι . Some strains of *C. perfringens* type A can also produce another important toxin, *C. perfringens* enterotoxin (CPE), which is the major virulence factor associated with most symptoms of *C. perfringens*-related gastrointestinal (GI) diseases (Hatheway, 1990; Petit et al., 1999). The *C. perfringens* type A isolates producing *C. perfringens* enterotoxin (CPE) are a causative agent of *C. perfringens* type A food poisoning (FP), which currently ranks as the second most reported outbreak of bacterial foodborne diseases in the United States and accounts for almost a million cases per year in the U.S. alone (Scallan et al., 2011b). The economic loss associated with *C. perfringens*-incurred foodborne illnesses has been estimated to reach \$12.5 billion annually (Buzby and Roberts, 1997).

C. perfringens is a major concern to the food industry, due to its ability to form endospores that are resistant to various preservation technologies typically applied by food manufacturers (Li and Mcclane, 2006a, 2006b; Mcclane, 2001; Mcclane, 2007a; Paredes-Sabja et al., 2007; Paredes-Sabja et al., 2008a; Sarker et al., 2000). These technologies could be facilitating the pathogen's survival in incompletely cooked or inadequately warmed food products (Mcclane, 2007a; Novak and Juneja, 2002; Thippareddi et al., 2003). The *C. perfringens* type A FP is generally caused by

consumption of heavily contaminated meat- and poultry-containing products. Initially, as a result of storage at improper temperatures, *C. perfringens* spores that survived thermal processing can rapidly germinate, outgrow, and proliferate to high cell numbers in food products. After consumption of these highly contaminated foods, some vegetative cells survive passage through the stomach and remain viable upon entering the small intestine, where they multiply and sporulate. During *C. perfringens* sporulation, CPE is produced in the cytoplasm of the mother cell, and upon the lysis of mother cell, is released together with mature spores, causing the symptoms of *C. perfringens* FP (Duncan et al., 1972a; McClane, 2001). The CPE production is tightly regulated by bacterial sporulation (Duncan et al., 1972a; Huang et al., 2004; Philippe et al., 2006); therefore, the sporulation and germination capabilities of *C. perfringens* play critical roles, not only for the survival of the bacterium in the environment but also for the pathogenesis of *C. perfringens* type A FP.

The sporulation mechanism is well studied mainly in the soil bacterium *Bacillus subtilis*, in which it undergoes a highly organized differentiation program to initiate sporulation as a response to nutritional deprivation. This phenomenon only occurs when the effort to maintain vegetative growth has failed (Fujita et al., 2005; Underwood et al., 2009). Phosphorelay is an expanded variant of a two-component signal transduction system consisting of multiple sensor histidine kinases, the response regulator Spo0F, the phosphotransferase Spo0B, and response regulator/transcription factor Spo0A. When cells encounter nutrient starvation, they utilize the phosphorelay signal transduction system to initiate the process of spore formation. The ultimate goal

of this system is to phosphorylate and activate the Spo0A, the master transcription regulator of sporulation producing the active form of Spo0A, Spo0A~P (Burbulys et al., 1991; Hoch, 1995; Stephenson and Hoch, 2004). The phosphorylation of Spo0A promotes binding to a specific target sequence, the “0A box,” located in or near the promoters of the gene under direct Spo0A control, leading to gene activation or repression (Molle et al., 2003; Spiegelman et al., 1995). A previous study showed that Spo0A has an essential role for *C. perfringens* spore formation, as well as for CPE synthesis (Huang et al., 2004). Important components of this signal transduction system, the sensor histidine kinases, were hypothesized to be responsible for sensing the stimulatory signals and thereby transferring them to their specific cognate response regulator to initiate endospore formation (Jiang et al., 2000; Stephenson and Hoch, 2002). Even though the function of sensor histidine kinases governing initiation of spore formation was previously characterized in other spore-forming bacteria, including *B. subtilis*, *Bacillus anthracis*, *Clostridium difficile*, *Clostridium botulinum*, and *Clostridium acetobutylicum* (Antoniewski et al., 1990; Brunsing et al., 2005; Ledeaux and Grossman, 1995; Perego et al., 1989; Steiner et al., 2011; Trach and Hoch, 1993; Underwood et al., 2009; Wörner et al., 2006), no such information has been available for the pathogenic bacterium *C. perfringens*.

Spore germination is a prerequisite for the metabolically dormant spores to return to their actively growing cells (Ireland and Hanna, 2002; Paidhungat and Setlow, 2002). Germination occurs when bacterial spores sense a specific compound, the germinant, in their environments and then initiate the highly organized sequential

events of germination process (Paredes-Sabja et al., 2011; Setlow, 2003). Upon completion of germination, enzyme action and protein movement in the spore core resume allowing initiation of spore metabolism, followed by synthesis of macromolecules in the spore outgrowth process that ultimately converts the germinated spore into growing cells (Paidhungat and Setlow, 2002). It appears that the histidine kinase-associated bacterial spore germination has only been reported in Gram-negative bacterium *Myxococcus xanthus* (Shi et al., 2008).

In this study, we identified genes encoding putative sensor histidine kinases in *C. perfringens* SM101 and examined their expression profiles. Through the construction of mutations in two of these putative sensor histidine kinase genes, *cpr1728* and *cpr1055*, we were able to characterize their functions in various aspects in the life cycle of the pathogenic bacterium *C. perfringens*.

7.2. Materials and methods

7.2.1. Bacterial strains and plasmids

The *C. perfringens* strains and plasmids used in this study are listed in Table 7.1.

7.2.2. Construction of β -glucuronidase gene (gusA) fusion plasmids and β -glucuronidase assay

DNA fragments (300 to 500 bp) upstream of each gene in *C. perfringens* SM101 were expected to contain the promoter of the tested genes. The PCR fragment was amplified using the primer pairs described in Table 7.2. Those forward and

reverse primers had Sall and PstI cleavage sites at the 5' ends, respectively. The resulting PCR products were digested with Sall and PstI, and then cloned between the similar sites in plasmid pMRS127 in *Escherichia coli* DH5 α , which was used as a host for all plasmid constructions, as described previously (Paredes-Sabja et al., 2008b; Raju et al., 2006), in order to create the gene of interest-*gusA* fusion constructs as listed in Table 7.1 . These plasmids were then introduced into *C. perfringens* SM101 separately via electroporation (Czeczulin et al., 1996), and the erythromycin-resistant (Em^r) transformants were selected. In some experiments, the *gusA* fusion plasmid was introduced into *C. perfringens* IH101, which is the *spo0A* deletion strain (Huang et al., 2004), and the transformants were selected by monitoring erythromycin and chloramphenicol resistance (Em^r Cm^r). In the experiment to assess β -glucuronidase (Buchanan and Gustafson) activities, the transformants carrying gene of interest-*gusA* fusion plasmids were grown in TGY vegetative medium (3% trypticase soy broth, 2% glucose, 1% yeast extract, and 0.1% L-cysteine) (Kokai-Kun et al., 1994) and in Duncan-Strong (DS) sporulation medium [1.5% proteose peptone, 0.4% yeast extract, 0.1% sodium thioglycolate, 0.5% sodium phosphate dibasic (Na₂HPO₄:anhydrous), 0.4% soluble starch] (Duncan and Strong, 1968). Cells were collected at various times and assayed for GUS activities, as previously described (Zhao and Melville, 1998). The GUS-specific activity was expressed in Miller units, calculated as previously described (Raju et al., 2006).

7.2.3. Construction of a *cpr1728* mutant

In order to isolate a derivative strain of *C. perfringens* SM101 in the *cpr1728* locus, we inserted *catP* into the locus, which yielded the chloramphenicol resistance (Cm^r ; 20 $\mu\text{g/ml}$). The procedure utilized to construct the $\Delta\textit{cpr1728}$ mutator plasmid follows. A 2,038-bp DNA fragment containing a 775 bp from the N-terminal coding region and 1,263 bp upstream of *cpr1728* was PCR amplified with primer pairs CPP439/PPP471 having KpnI and SpeI cleavage sites at the 5' ends of the forward and reverse primers, respectively. A 1,615-bp DNA fragment, containing 50 bp from the C-terminal coding region and 1,565 bp downstream of *cpr1728*, was PCR amplified using primer pairs CPP435/PPP436 having PstI and XhoI cleavage sites at the 5' ends of the forward and reverse primers, respectively. These resulting PCR fragments were cloned into plasmid pCR-XL-TOPO (Invitrogen, Carlsbad, CA) in *E. coli*, giving plasmid pPU14 and pPU8, respectively. An $\sim 1.6\text{-kb}$ PstI-XhoI fragment from pPU8 was cloned in PstI-XhoI sites of plasmid PDP25, giving plasmid pPU10, and $\sim 2.0\text{-kb}$ -KpnI-SpeI fragment from plasmid pPU14 was cloned into the same sites of pPU10, giving plasmid pPU11. Then, $\sim 4.5\text{-kb}$ KpnI-XhoI-fragment carrying $\Delta\textit{cpr1728}::\textit{catP}$ from plasmid pPU11 was cloned into plasmid pMRS104 that cannot replicate in *C. perfringens* (Huang et al., 2004), yielding plasmid pPU15. Plasmid pPU15 was then introduced into *C. perfringens* SM101 via electroporation (Czeczulin et al., 1996), and the *cpr1728* deletion strain was selected by allelic exchange, as previously described (Sarker et al., 1999). The deletion of wild type *cpr1728* and

replacement with *catP* were confirmed by PCR analysis (data not shown) with specific internal detection primers CPP466/ CPP467. This mutant strain was named PU101.

7.2.4. Construction of a *cpr1055* mutant

For the *cpr1055* knockout mutant, a mutator plasmid was constructed as follows: A 1,700-bp DNA fragment containing 939 bp upstream of *cpr1055* and 761 bp N-terminal region of *cpr1055* coding sequence was PCR amplified by using primer pair CPP587/ CPP472 (The forward and reverse primers had KpnI and SpeI cleavage sites at 5' ends, respectively). A 1,279-bp containing 1,191 bp downstream of *cpr1055* and 88 bp from the C-terminal region of *cpr1055* was PCR amplified using primers CPP429/ CPP431. (The forward and reverse primers had PstI and XhoI cleavage site at their 5' ends, respectively). The resulting PCR products were then cloned into plasmid pCR-XL-TOPO, yielding plasmid pPU22 and pPU13, respectively. After the removal of a KpnI-SpeI upstream region of *cpr1728*, ~ 1.7-kb KpnI-SpeI fragment from pPU22 was cloned into similar sites in pPU10, giving plasmid pPU24. Next, after removal of a PstI-XhoI downstream fragment of *cpr1728*, ~ 1.3-kb PstI-XhoI fragment from pPU13 was cloned into pPU24, yielding pPU25. Then, an ~ 4.4-kb KpnI-XhoI fragment of pPU25 carrying $\Delta cpr1055::catP$ was cloned between the KpnI-SalI sites of a suicide vector pMRS104 to create the mutator plasmid pPU26, which cannot replicate in *C. perfringens* (Huang et al., 2004). This mutator plasmid pPU26 was then introduced into *C. perfringens* strain SM101 by electroporation, and Em^r Cm^r transformants were selected. The *cpr1055* knockout mutant was isolated by allelic exchange as previously described (Huang et al., 2004; Sarker et al., 1999). The

presence of *cpr1055* deletion was confirmed by PCR analysis with specific internal detection primers CPP468/ CPP469 (data not shown). The *cpr1055* mutant was designated as PU102.

7.2.5. Measurement of vegetative and sporulation growth

Growth of *C. perfringens* SM101, PU101, and PU102 were evaluated in vegetative and sporulation growing conditions. For inocula, *C. perfringens* strains were grown from cooked meat cultures in fluid thioglycolate broth (FTG; Difco) at 37 °C overnight with mutant strains grown in the presence of 20 µg/ml chloramphenicol. The 0.4 ml overnight cultures were inoculated into 10 ml TGY and incubated at 37 °C for 3 h, and the optical density at 600 nm (OD₆₀₀) was measured to adjust the volume of cell cultures to the same OD₆₀₀. These cells of the 3-h culture cells (early exponential phase) of *C. perfringens* SM101 and mutant strains were inoculated into 40 ml TGY vegetative medium without adding selective antibiotic. For growth assessment in sporulation-inducing condition, the overnight FTG cultures were transferred into 10 ml fresh FTG containing 20 µg/ml chloramphenicol and incubated at 37 °C for 8 h. The 8-h FTG cultures of SM101 and kinase mutants were then inoculated into 40 ml DS sporulation medium with no selective antibiotic addition. The presence of sporulating cells was confirmed under the phase contrast microscope at 6 h to 8 h post-inoculation. Changes in OD₆₀₀ of cell cultures were routinely monitored at various times up to 24 h after inoculation. The results were expressed as changes in OD₆₀₀ during 24-h growth period.

To examine the ability of the *C. perfringens* wild type, PU101, and the PU102 strains to form heat-resistant endospores, a 0.2 ml of cooked meat stock culture was inoculated into 10 ml FTG and incubated at 37 °C overnight before transferring 0.4 ml aliquots into 10 ml DS sporulating cultures and grown for 24 h. The sporulating cultures were then transferred into a new FTG medium and heat-shocked at 75 °C for 20 min before incubating at 37 °C for 8 h. The 8-h actively growing cells were transferred into a freshly prepared 10 ml DS medium or 10 ml modified sporulation medium (MSM; 1.5% peptone, 3% Trypticase, 0.4% starch, 0.5% NaCl, 0.02% MgSO₄, and 0.1% sodium thioglycolate) (Kim et al., 1967). The number of total viable cells and heat-resistant spores were assessed after various time periods of incubation at 37 °C. To determine the total viable cell counts in DS and MSM cultures, aliquots were serially diluted in 25 mM sodium phosphate buffer (Na₂HPO₄) (pH 7.5) and surface-plated onto Brain Heart Infusion (BHI) agar (Difco, BD Diagnostic Systems). The number of heat-resistant spores was estimated by heating the DS or MSM cultures at 75 °C for 20 min, in order to inactivate the non-sporulating cells. Aliquots of appropriate dilutions were then plated onto BHI (Difco) agar. After anaerobic incubation at 37 °C for 24 h, colonies were counted.

7.2.6. Spore preparation and purification

Spores of *C. perfringens* were prepared and purified as previously described (Paredes-Sabja et al., 2008c; Paredes-Sabja et al., 2009e). In brief, a 0.4 ml aliquot of 16-h to 18-h FTG culture was transferred to new 10 ml FTG and incubated at 37 °C for ~ 8 h. The 0.4 ml of actively growing cells were then transferred into 10 ml freshly

prepared DS sporulation medium (Duncan and Strong, 1968) and incubated overnight at 37 °C. The presence of spores was confirmed by phase-contrast microscopy. The large number of bacterial spores was obtained by scaling up this procedure in 600 ml DS, and spores were purified by repeated washing with cold sterile distilled water until spore suspensions were > 99% free of cell debris, sporulating and germinating cells, as determined by phase-contrast microscopy. The spore suspensions were adjusted to a final OD₆₀₀ ~ 6.0 with sterile distilled water and stored at - 80 °C until used. Spores of PU101 and PU102 were prepared and purified by the same procedure as for the wild type *C. perfringens* strain.

7.2.7. Spore germination assay

The spore germination assay was performed as described in the previous study (Paredes-Sabja et al., 2008c). Briefly, the purified spore suspensions were heat activated at 80 °C for 10 min, and then cooled in water at room temperature for 5 min, and incubated at 40 °C for 10 min before adding into the pre-warmed control buffer or germinant solutions at 40 °C. Spore germination was routinely measured by monitoring the reduction of OD₆₀₀ of spore suspensions (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA), in which the falling of an OD₆₀₀ by ~ 60% reflects complete spore germination. Levels of spore germination were also confirmed by phase-contrast microscopy. In addition, maximum rates of spore germination were determined by measuring the OD₆₀₀ of germinating cultures every 2.5 min. The maximum slopes were calculated, and results were expressed as

the percentage of loss in OD₆₀₀ of the spore suspension per min relative to an initial OD₆₀₀ for the cultures (Paredes-Sabja et al., 2009a).

7.2.8. Spore outgrowth assay

The ability of spores to outgrowth was examined in TGY vegetative medium, as it allows faster growth and maintains better anaerobiosis; the assay was performed as previously described (Paredes-Sabja et al., 2009a). Briefly, a 0.3 ml of spore suspension of an OD₆₀₀ of ~ 6.0 were heat-activated at 80 °C for 10 min, and then cooled in water at room temperature before inoculating into the pre-warmed 10 ml TGY medium. Cultures were incubated at 37 °C, and the OD₆₀₀ was measured over time intervals up to 180 min. Results were expressed as the percent increase in an OD₆₀₀ relative to the initial value of the relevant strains.

7.2.9. Assessment of colony-forming efficiencies

In order to assess the colony-forming ability of *C. perfringens* spores of wild type and mutant strains, purified spores at an OD₆₀₀ of ~1.0 (~ 10⁸ spores/ml) were heat-activated at 80 °C for 10 min. After cooling, aliquots were serially diluted in 25 mM Na₂HPO₄ buffer (pH 7.5), and appropriate dilutions were surface-plated onto BHI agar plates. The number of colonies was counted after 24 h incubation at 37 °C under anaerobic conditions (Paredes-Sabja et al., 2008c).

7.3. Results

7.3.1. Identification of putative sensor histidine kinases in *C. perfringens*

The initiation of sporulation in *Bacillus* and *Clostridium* species is regulated by the phosphorylation of the master regulator of sporulation, Spo0A, by a group of orphan sensor histidine kinases (Brunsing et al., 2005; Huang et al., 2004; Steiner et al., 2011; Underwood et al., 2009). To identify genes encoding for putative sensor histidine kinases homologues with the potential of being functional in the sporulation process of *C. perfringens*, two criteria were employed: (i) *B. subtilis* sporulation-associated histidine kinases are chromosomally located as orphans, i.e. unlinked to their response regulator (Fabret et al., 1999; Stephenson and Hoch, 2002) and (ii) all known sporulation-specific sensor kinases of *B. subtilis* share a high degree of sequence conservation in the region surrounding their active site histidine residue (Stephenson and Hoch, 2002). According to these predetermined criteria, the *C. perfringens* SM101 genome sequence (Myers et al., 2006) was subjected to BLASTP analyses. Six ORFs (CPR0195, CPR1055, CPR1316, CPR1493, CPR1728, and CPR1953), encoding proteins with high similarity (44 to 54 %) to all known *B. subtilis* sporulation-associated sensor histidine kinases, were identified (Fig. 7.1 and Table 7.3). All putative sensor histidine kinases in *C. perfringens* SM101 had a conserved sequence surrounding the predicted phosphorylatable histidine residue similar to the *B. subtilis* groups IIIB sensor kinases (Fig. 7.1) (Stephenson and Hoch, 2002). The CPR0195 encodes 791 amino acid residues and it is predicted to have eight transmembrane helices by using the TMHMM Server version 2.0

(<http://www.cbs.dtu.dk/services/TMHMM/>) (Sonnhammer et al., 1998). The CPR1055 encodes 558 amino acid residues with no predictive transmembrane segment. The CPR1316 encodes 787 amino acid residues with seven predicted transmembrane helices; CPR1493 encoding 1,086 amino acid residues is predicted to have two transmembrane helices. The CPR1728 encodes 571 amino acid residues with two predicted transmembrane helices. Lastly, CPR1953 encodes 678 amino acid residues with eight predicted transmembrane helices. Due to the greatest similarities between CPR1728 and CPR1055 to all known sporulation-associated sensor histidine kinases in *B. subtilis*, as well as their active expression during *C. perfringens* sporulation (Table 7.3, Fig. 7.2A-B, and data not shown), this study has focused on characterizing these two putative sensor histidine kinases homologues.

7.3.2. Expression profiles of cpr1728 and cpr1055

The *cpr1728* is the second gene in a putative bicistronic operon, preceded by a gene encoding hypothetical protein (*cpr1729*) of unknown function with stop codon located 13 bp upstream of the translational start site of *cpr1728*. No transcriptional terminator was identified in the intergenic region of *cpr1729* and *cpr1728*, but there is a predicted stable stem-loop structure encoded downstream of the *cpr1728* stop codon (data not shown) [determined using mfold (<http://mfold.rna.albany.edu>)]. Strikingly, the putative consensus sequence of the RNA polymerase sigma factor σ^H (Haldenwang, 1995) was found at positions -133 and -112 relative to the *cpr1728* start codon, raising the possibility that *cpr1728* might have an independent sporulation-specific promoter but is also co-transcribed with *cpr1729*. Consequently, we

constructed *cpr1728-gusA* and *cpr1729-gusA* fusions to assay whether *cpr1728* had an independent sporulation-specific promoter. Results indicated that no significant β -glucuronidase-specific activity (GUS activity) of *C. perfringens* SM101 carrying *cpr1728-gusA* was detected in cultures grown in TGY vegetative medium (Fig. 7.2A), but a low level of *cpr1729* expression was observed during the first 2 h of vegetative growth, and then it leveled off. In contrast, significant levels of GUS-specific activities were observed in sporulating cultures of the strain carrying the *cpr1728-gusA* fusion at much greater levels than in the strain harboring *cpr1729-gusA* (Fig. 7.2A). Moreover, the expression of *cpr1728* elevated at 4 h and peaked during the stationary phase of growth; whereas, the expression of *cpr1729* under the same experimental conditions was consistently low, but slightly increased after 6 h. According to the difference in expression profile and the presence of σ^H -like consensus sequence upstream of *cpr1728*, the *cpr1728* might transcribe from its independent sporulation-specific promoter; however, we cannot rule out the possibility that *cpr1728* could co-transcribe with its immediate upstream open reading frame from the weaker promoter, upon growing in the sporulation-induced condition. No GUS activity of *cpr1728-gusA* could be detected in IH101 (*spo0A* mutant) background; this implied that Spo0A was directly or indirectly necessary for the promoter activity of *cpr1728* in *C. perfringens*.

Unlike *cpr1728*, the GUS-specific activity of *C. perfringens* wild type strain harboring *cpr1055-gusA* could be detected under both vegetative and sporulative growth conditions. Consistent with the predicted role to initiate spore formation, the expression of *cpr1055* was highly induced, by approximately 3-fold in DS cultures

when the culture entered the stationary phase, and the expression remained almost constant afterward until it declined at 12 h post-inoculation (Fig. 7.2B). It is interesting to note that *cpr1055* has a peak of expression about the same time as its predicted cognate response regulator Spo0A (see below), which occurred at 4 h after *C. perfringens* cultures were inoculated into medium promoting sporulation. In support by this notion, the expression of *cpr1055* was downregulated in the *spo0A* mutant shortly after cultures entered a stationary phase and then rapidly dropped off to the same level as the wild type strain grown under nutrient-rich conditions.

Furthermore, the promoter activity for the remaining sensor histidine kinases identified in the previous section was assayed by introducing *gusA* fusion, containing a promoter of each putative kinase ORF into *C. perfringens* strain SM101 and IH101 (Spo0A mutant). Very weak promoter activities were obtained with wild type SM101 strains harboring *cpr0195-gusA* and *cpr1493-gusA* grown under vegetative and sporulation conditions. No significant GUS activity was observed with *cpr1953-gusA* either. However, a very high expression level could be detected when *cpr1954-gusA* construct was evaluated under sporulation conditions. The *cpr1954* is an immediate upstream ORF of *cpr1953*, and it was also annotated as a putative sensor histidine kinase; thus, presumably, *cpr1954* and *cpr1953* are in the same operon, and may transcribed from the sporulation-specific promoter located upstream of *cpr1954* (data not shown). Similar to *cpr1055* and *cpr1954* expression profiles, *cpr1316* expression was most active during the transition from exponential to stationary phase of growth when sporulation was induced, and then subsided. Nevertheless, expressions of

cpr1316 and *cpr1954* were only slightly reduced in IH101 background compared with the wild type strain under the same experimental conditions.

7.2.3. Inactivation of *cpr1728* and *cpr1055* affected sporulation, but not vegetative growth of *C. perfringens*

To characterize the phenotypes caused by inactivation of *cpr1728* or *cpr1055*, growth of *C. perfringens* wild type and the kinase mutant strains was cultivated in TGY and DS, respectively, for examining vegetative and sporulation growth. For both proliferation conditions, bacterial growth was determined by measuring OD₆₀₀ without adding selective antibiotic, in order to eliminate delayed growth of the mutant strains. The genotypic integrity of each strain was verified by performing PCR reactions with specific internal detection primers (Table 7.2) after 24 h of incubation (data not shown). All strains grew well in TGY. No differences were found between the parental SM101 and PU101 cells in growth rate, viability and gross cell morphology; however, *cpr1055* mutant (strain PU102) showed a slightly lower growing level than other strains over 24 h period (Fig. 7.3A and data not shown).

In contrast, inactivation of *cpr1728* and *cpr1055* in *C. perfringens* resulted in significantly different growth characteristics under sporulation-inducing conditions. Both PU101 and PU102 mutant cultures had higher growth rate than that of wild type. Moreover, mutant cultures continued exponentially growing for a couple of hours before reaching the stationary phase, as compared to wild type cultures grown under the same condition (Fig. 7.3B). Strikingly, growth was affected significantly during sporulation, when PU101 and PU102 cells, reaching higher OD₆₀₀ ($p < 0.05$) than that of their parental strain; the PU102 yielded the highest growth extent (Fig. 7.3B). The

sporulating cells of all tested strains were observed under the phase contrast microscope within 6 to 8 h post-incubation (data not shown). One notable observation was that PU101 and PU102 cells grown under sporulation-inducing condition appeared to be longer and thinner than their parental strain (data not shown).

7.3.4. C. perfringens PU101 (cpr1728 mutant) and PU102 (cpr1055 mutant) are partially deficient in their ability to sporulate

In other spore-forming bacteria, mutations in sporulation-associated sensor histidine kinases caused variations in the severity of sporulation defect, depending on the kinase activities and sporulation conditions (Antoniewski et al., 1990; Brunsing et al., 2005; Jiang et al., 2000; Ledeaux and Grossman, 1995; Trach and Hoch, 1993; Underwood et al., 2009). To test if the putative kinases CPR1728 and CPR1055 have some roles in *C. perfringens* spore formation, sporulation capacities of various strains were measured after growing in DS sporulation medium for 24 h. As expected, *C. perfringens* strains PU101 and PU102 produced significantly less ($p < 0.05$) heat-resistant spores than observed in wild type strain, with the frequencies of approximately 3% and 10% of the wild type level, respectively (Fig. 7.4A). However, the spore-forming efficiency determined as the ratio between heat-resistant spores per ml and total viable cells per ml from the same culture (Huang and Sarker, 2006) was not reported in this study, as mutant strains consistently yielded a higher number of total viable cell counts (vegetative cell plus endospores) than their parental strain. Therefore, the calculated sporulation efficiencies of PU101 and PU102 mutants were several hundred times lower relative to the level in wild type. Actually, the higher total cell counts observed in PU101 and PU102 were in complete agreement with the

significantly higher OD₆₀₀ observed for these strains grown in DS during growth determination characteristics (Fig. 7.3B).

In *B. subtilis*, mutation in *kinA* caused a delay in spore formation under some conditions (Perego et al., 1989); therefore, we evaluated whether this phenomenon could also happen in *C. perfringens* putative kinase mutants. Figure 7.4A shows that loss of CPR1055 (strain PU102) resulted in a severely delayed spore formation, and a less pronounced effect was obtained from PU101 at 6 h post-inoculation into DS medium. The number of heat-stable spores produced by PU102 was significantly less ($p < 0.05$) than those of wild type and PU101 by 3.64 and 2.20 log CFU/ml, respectively. The PU101 and PU102 sporulated at a frequency of ~ 4% and 0.02% of the wild type level, respectively, at this time period. After 12 and 24 h of incubation, PU102 was able to resume spore-forming capacity to reach ~ 6 log CFU/ml (~ 10% of the wild type level; see above) (Fig. 7.4A and data not shown). In order to eliminate the possibility that mutant strains produced additional spores upon longer incubation, *C. perfringens* SM101 and its putative kinases derivatives were grown in DS medium for a total of 48 h at 37 °C, and the number of heat-resistant spores and total viable counts were determined. For each strain, the differences in the spore level between 24 and 48 h of incubation were minor. The PU101 and PU102 constantly produced significantly ($p < 0.05$) fewer heat-stable spores than wild type even after a prolonged incubation period. We also found that total viable counts of 48-h cultures were slightly lower than the 24-h cultures for all strains. These might be attributed to the die-off of non-sporulating cells after a long period of incubation (data not shown). Furthermore,

similar levels of free spores in 24-h and 48-h DS cultures were observed under the phase contrast microscope and are consistent with the quantitative plating experiments (data not shown). Therefore, *C. perfringens* SM101 completed the sporulation process and released free mature spores within 24 h of growing in DS medium under conditions in the current study.

Compositions of sporulation media had a major influence on selecting which kinases to function in initiating sporulation in *B. subtilis* (Ledeaux et al., 1995). Therefore, the roles of CPR1728 and CPR1055 in *C. perfringens* spore formation were also evaluated in MSM broth. After 24 h of incubation at 37 °C, PU101 and PU102 showed moderately to slightly deficient sporulation, compared to wild type, with sporulation frequency of approximately 6% and 51%, respectively, relative to the wild type level, (Fig. 7.4B). Therefore, CPR1728 appears to be the major putative kinase responsible for formation of heat-resistant spores; whereas, CPR1055 had little impact on sporulation in MSM medium. Unlike in the DS medium, the total viable cell counts were equivalent for all tested strains in MSM broth (Fig. 7.4B). Collectively, these results strongly suggest that CPR1728 and CPR1055 are not absolutely essential for sporulation initiation of *C. perfringens* SM101, since the mutant strains were still able to sporulate significantly. It is rather that both putative kinases are required for efficient sporulation under at least two sporulation media tested. The partial defection in spore formation of the single knockout mutant of kinase genes has been demonstrated in other spore-forming bacteria (Antoniewski et al., 1990; Ledeaux and Grossman, 1995; Ledeaux et al., 1995; Perego et al., 1989; Trach and Hoch, 1993) and

indicated that several histidine kinases could share a similar role in spore formation, although to a different efficiency.

7.3.5. The CPR1055 is required for the expression of *spo0A* and *spoIIIG* operon

We also examined the expression profile of *C. perfringens spo0A* by introducing *spo0A-gusA* fusion plasmid into *C. perfringens* strains SM101, PU101, and PU102 to determine whether the putative orphan kinases have any ability to modulate the expression level of *spo0A*, as had been previously reported in *C. acetobutylicum* (Steiner et al., 2011). The expression of *spo0A-gusA* was monitored under vegetative and sporulation growing conditions (Fig. 7.5A). The *spo0A* was constitutively expressed in a low level in vegetative cells. On the other hand, the expression of *spo0A-gusA* began to rise sharply when the cell reached exponential growth and peaked upon entering the stationary phase, which is consistent with its role as a master regulator for entry into sporulation. Surprisingly, the expression of *spo0A-gusA* in *C. perfringens* SM101 was strongly dependent upon CPR1055 and, to a lesser extent, on CPR1728 under sporulation-inducing conditions. The expression level of *spo0A-gusA* in PU102 (*cpr1055* mutant) background was downregulated to a comparable level as found in vegetatively growing cells, which might, at least in part, explain the delayed spore formation by PU102. Inactivation of *cpr1728* (PU101 strain) resulted in a slight decrease in the expression level of *spo0A-gusA*, especially during the transition from the exponential to stationary phase of growth.

Upon the observation of the sporulating cells of PU102 under the phase contrast microscope, we consistently found that ~ 5 to 10% of the sporulating cultures

appeared as disporic cells, containing two developing forespores in one cell, which is the unique characteristic of the *sigE* mutant grown under sporulation conditions, reported for *B. subtilis* and *C. perfringens* (Errington, 2003; Harry et al., 2009). Therefore, it is possible that CPR1055 putative kinase might have some effect on modulating the expression of the *spoIIG* operon encoding SpoIIGA-SigE in *C. perfringens*. Indeed, for *B. subtilis*, a mutation in *kinA* caused a dramatic decrease in transcription of the *spoIIG* operon encoding for the RNA polymerase mother cell-specific sigma factor σ^E under sporulation conditions (Antoniewski et al., 1990). To test whether this is also true for *C. perfringens*, the upstream DNA likely to contain the promoter of *spoIIGA* was fused to *E. coli gusA*, and GUS activity was measured after this fusion was introduced into *C. perfringens* SM101, IH101, and PU102. We first assessed whether the *C. perfringens spoIIG* operon is expressed during sporulation (Fig. 7.5B). The *C. perfringens* SM101, carrying *spoIIGA-gusA* fusion, was grown in TGY vegetative medium and DS sporulation medium. Significant GUS activity was only observed in cultures grown under sporulation conditions and was most active during early sporulation induction. No significant expression level was detected in the vegetative cultures of *C. perfringens* SM101 carrying *spoIIGA-gusA*. These results indicate that a sporulation-specific promoter is located upstream of *spoIIGA*. The expression of *spoIIGA* began at ~ 2 h after sporulation induction and increased during the exponential growth phase. This result is in agreement with the early exponential *sigE* transcript accumulation in the same strain of *C. perfringens* background in the previous study (Harry et al., 2009). A barely detectable extent of

GUS activity was obtained when *spoIIGA-gusA* fusion was introduced into an asporogenous *spo0A* strain (Huang et al., 2004) (strain IH101) and grown in DS sporulation medium. This strengthens the sporulation-associated expression of *sigE* in *C. perfringens* and suggests that the transcription of the *spoIIG* operon might be under the direct control of Spo0A, as in *B. subtilis* (Haldenwang, 1995; Satola et al., 1992). Moreover, the lack of CPR1055 also led to a severe decrease in GUS-specific activity under sporulation conditions. This phenomenon could, at least in part, explain the disporic phenotype and the delay in the spore formation of PU102 in DS medium (Fig. 7.4B). However, it is not conclusive from these results whether the reduction in expression level of *spoIIGA-sigE* in PU102 was due to the regulatory role of the putative kinase by an unknown mechanism(s) or by the reduced *spo0A* expression level in the strain lacking CPR1055, as shown above (Fig. 7.5A).

7.3.6. Effect of inactivation of *cpr1728* and *cpr1055* on germination of *C. perfringens* spores

Besides growth and sporulation phenotypes, we also attempted to define the physiological role(s) of the putative histidine kinases, CPR1728 and CPR1055, in the life cycle of *C. perfringens*. The metabolically dormant spores typically monitor their surrounding environments and initiate the process of germination to return to actively growing cells upon sensing the presence of specific nutrients (germinants) (Moir, 2006; Paredes-Sabja et al., 2011; Setlow, 2003). Spores of *C. perfringens* SM101, PU101, and PU102 were heat-activated and then incubated at 40 °C with various known nutrient germinants for spores of *C. perfringens*. Surprisingly, using a variety of germinants, spores lacking CPR1728 (strain PU101) and CPR1055 (strain PU102)

demonstrated germination defects, as compared to the parental strain. The PU101 spores germinated significantly less than either wild type or PU102 spores ($p < 0.05$) with 100 mM L-asparagine (pH 7.0), while the latter two had similar germination response to this particular amino acid germinant (Fig. 7.6A). The germination levels of PU101 and PU102 spores with KCl (pH 7.0) and NaPi (pH 6.0) were also significantly lower than the wild type spores, with disruption in *cpr1055* (strain PU102) having the most pronounced effect (Fig. 7.6B-C). However, all strains germinated with similar kinetics and to similar extents with AK, and the majority of spores became phase dark under the microscope within 60 min, indicating that spores had completed germination (data not shown). The newly identified nutrient germinants L-cysteine and L-glutamine, adjusted to pH 6.0, were assessed for their ability to initiate germination of *C. perfringens* wild type, PU101, and PU102 spores. Both amino acids were tested at 250 mM, as our previous finding indicated that this concentration gave the highest germination rate for spores of *C. perfringens* SM101 (wild type strain). Wild type spores germinated very rapidly with L-cysteine and slower with L-glutamine; however, most of the wild type spores became phase dark, indicating complete germination after 60 min. In contrast, PU101 spores germinated very poorly with L-cysteine, and PU102 spores had a less pronounced germination defect with this germinant. (Fig. 7.6D). For L-glutamine, PU102 spores exhibited much poorer germination than wild type spores, and PU101 spores also germinated significantly less than its parental strain ($p < 0.05$) (Fig. 7.6E). Overall, CPR1728 and CPR1055

both have a significant role for nutrient-inducing *C. perfringens* spore germination, although to different extents depending on types of germinants.

The exogenous Ca-DPA has a role in spore germination via the promotion of cortex hydrolysis by activation of a spore cortex lytic enzyme CwlJ in *B. subtilis* and *Bacillus megaterium* (Paidhungat et al., 2001; Setlow et al., 2009). Our recent study exhibited that Ca-DPA triggers *C. perfringens* spores to germinate through the germinant receptor GerKA-KC and does not activate either pro-SleC or SleC, the only spore cortex lytic enzyme in *C. perfringens* SM101 (Paredes-Sabja et al., 2009d). When heat-activated spores of wild type, PU101, and PU102 strains were induced to germinate with 50 mM Ca-DPA, the PU101 and PU102 spores demonstrated a significantly slower germination compared to their parental strain (Fig. 6F), both mutants with similar germination kinetics; but the germination extent of PU102 was lower than PU101 after 60 min of incubation ($p < 0.05$). Furthermore, the lesser extents of mutant spore germination were further confirmed by phase-contrast microscopy, which revealed that 59% and 32% of PU101 and PU102 spores, respectively, became phase dark, and 74% of wild type spores became phase dark upon exposure to exogeneous Ca-DPA for 60 min at 40 °C. Therefore, CPR1728 and CPR1055 are both required for efficient germination of *C. perfringens* spores with the non-nutrient Ca-DPA.

To gain a better understanding of the effect of *cpr1728* and *cpr1055* deletions on the initial stage of germination of *C. perfringens* spores, the maximum rate of spore germination was measured as the maximum loss of OD₆₀₀ per min. With all

germinants tested, results clearly indicate that spores lacking CPR1728 had a much lower rate of spore germination than the wild type spores. A similar trend was observed for CPR1055 deletion spores with a less significant effect compared to PU101 (Table 7.4). Collectively, these results indicate that the putative sensor histidine kinases encoded by CPR1728 and CPR1055 are required for normal germination of *C. perfringens* spores with a variety of nutrient and non-nutrient germinants.

7.3.7. Effect of cpr1728 and cpr1055 inactivations on C. perfringens spore outgrowth and colony-forming efficiency

Because the germination responses of mutant strains were similar to wild type spores in TGY vegetative medium (data not shown), it was possible to assess the role of these genes in spore outgrowth. In this study, spores of PU101 and PU102 exhibited a significantly ($p < 0.05$) slower outgrowth in TGY medium, compared to the wild type spores (Fig. 7.7), while PU101 and PU102 spores outgrew at similar rates. Spores of the wild type strain lost their refractivity in the first 60 min, and then these spores started to outgrow rapidly. On the other hand, the lag periods before mutant spores began to outgrow were 150 min and 110 min for PU101 and PU102 spores, respectively. The clear defect of spore outgrowth capacity was observed for both mutant strains within the period of the experiment. At 3 h post-inoculation, the actively growing vegetative cells, as well as the replicating cells, were observed under the microscope for the wild type strain (data not shown). Since our results indicated that vegetative cells of both mutants grew about the same rate as the wild type in TGY (Fig. 7.3A) and germination extents were equivalent, the different responses observed

in this rich medium are, therefore, limited to the outgrowth stage. Collectively, these data indicate that CPR1728 and CPR1055 are required for normal *C. perfringens* spore outgrowth in rich medium.

The PU101 and PU102 spores germinated slightly but significantly less than their parent strain with BHI broth over the 60-min period of the germination assay (Fig. 7.8); this might be reflected in the colony-forming efficiency of these mutant strains in the rich medium, reported previously for *C. perfringens* strains with the defective germination phenotype (Paredes - Sabja et al., 2009; Paredes-Sabja et al., 2009a; Paredes-Sabja et al., 2009d; Paredes-Sabja et al., 2008c). However, no significant difference in colony-forming efficiency was observed among strains of wild type (3.3×10^8 CFU/ml/OD₆₀₀), PU101 (2.8×10^8 CFU/ml/OD₆₀₀), or PU102 (2.3×10^8 CFU/ml/OD₆₀₀) ($p > 0.05$). Thus, the germination defect of PU101 and PU102 manifested in BHI broth could have been due to the loss of CPR1728 and CPR1055 functions causing delayed germination response of some spore populations, rather than their inability to germinate with rich medium. To verify this hypothesis, the germination and outgrowth assay was performed in BHI broth. As expected, spores of PU101 and PU102 strains exhibited slower germination and outgrowth compared to wild type spores; nevertheless, within 6 h, the OD₆₀₀ increased to a level, similar to the parental strain (data not shown). These results implied that, once mutant spores completed germination and the outgrowth process, they rapidly proliferate in BHI and eventually yield similar titers within 24 h of incubation at 37 °C. Therefore, CPR1728

and CPR1055 are not necessary for *C. perfringens* spores to form colonies on rich medium.

7.4. Discussion

In the current study, we identified six putative orphan sensor histidine kinases of *C. perfringens* SM101 having the potential to initiate sporulation. Among these, we were able to construct two knockout mutant strains of *cpr1728* and *cpr1055* and to characterize the roles of these putative kinases in many aspects during the life cycle of the pathogenic bacterium *C. perfringens*.

First, we found that all tested putative histidine kinases of *C. perfringens* are highly expressed during sporulation. The promoters of *cpr1055*, *cpr1316* and *cpr1954* were highly active under sporulation-stimulating condition with the maximal expression observed at 2 h to 3 h post-sporulation induction. In contrast, the expression of *cpr1728* began ~ 2 h later than other active kinase genes and with the peak of expression observed during the stationary phase. It is unclear what the factor affecting the pattern of *cpr1728* expression is; however, in *B. subtilis*, previous studies suggest that the levels and time of expression are not correlated with the selection of which kinase functions in the initiation of sporulation. The most important determinant of *in vivo* kinase activity related to sporulation is the presence or absence of signal ligand(s) unique to each kinase to activate their function(s) (Jiang et al., 2000). Perhaps the effector(s) for *cpr1728* might be present only during the later phase of growth and thereby influence its activity at that period.

We also found that the promoter activities of *cpr1728* and *cpr1055* are under the control of the master regulator Spo0A; however, no obvious “0A box” (TGNCGAA), to which the phosphorylated Spo0A binds and regulates transcription

(Huang et al., 2004; Molle et al., 2003), could be found in the 5' regulatory region. Hence, it is likely that these two genes are under the indirect control of Spo0A. This dependency of the expression of sporulation kinases on Spo0A was previously reported for *B. subtilis* and *C. acetobutylicum* (Hoch, 1995; Molle et al., 2003; Steiner et al., 2011). On the other hand, the expression of *spo0A* was markedly influenced by CPR1055 and might provide a strategy to prevent *spo0A* hyper-expression and eventually lead to initiation of sporulation under inappropriate conditions. This mechanism for controlling synthesis and activity of Spo0A was recently demonstrated for the CAC0903 orphan kinase and Spo0A in *C. acetobutylicum* (Dürre and Hollergschwandner, 2004; Steiner et al., 2011).

In *C. difficile* and *C. acetobutylicum*, the sporulation sensor histidine kinases phosphorylate Spo0A directly *in vitro* (Steiner et al., 2011; Underwood et al., 2009) and thereby initiate spore formation. The histidine phosphotransferase domain, HisKA, of the six orphan kinases in *C. perfringens* showed approximately 43% and more than 50% amino acid identity, respectively, to that of *C. difficile* and *C. acetobutylicum*. Moreover, Spo0A is highly conserved among *Clostridium* species, as indicated in previous studies (Brown et al., 1994; Huang and Sarker, 2006). Therefore, it can be speculated that *C. perfringens* Spo0A could interact directly with the orphan sporulation-associated histidine kinases to control the sporulation initiation pathway.

Inactivation of *cpr1728* and *cpr1055* did not impair vegetative growth. However, mutant strains grown under sporulation-inducing condition exhibited various phenotypic differences from their parental strain. Moreover, cultures of mutant

strains grown in DS sporulation medium continued an exponential phase of growth for 2 – 3 h before reaching the stationary phase, in comparison to the parental SM101 strain grown under the same conditions. These sporulation growth characteristics seemed to be unique and owing to the loss of CPR1728 or CPR1055 activities. These characteristics are not the typical of asporogenous cells because *spo0A* mutant shows a similar growth rate and viability, compared to the wild type strain, except the presence of sporulating cells (data not shown). The unnoticeable growth rate, viability, and gross cell morphology was also been reported for the *C. difficile* strains lacking CD2492, the sporulation sensor histidine kinase, but no such information was available for sporulation growth of this particular kinase mutant strain (Underwood et al., 2009). The observations in this study that these sporulation growth phenotypes, as well as the gene expression profiles, strongly suggest the *cpr1728* and *cpr1055* are sporulation-specific genes.

We also found that deletion of *cpr1728* or *cpr1055* resulted in a partial defect in spore formation. The reduction in spore formation by both *C. perfringens* putative kinase mutants was comparable to the *B. subtilis* strain lacking KinA, the most important sporulation sensor histidine kinase, which sporulated at the frequency of 5% to 30% of the wild type level (Antoniewski et al., 1990; Jiang et al., 2000). These genetic evidences strongly suggested that CPR1728 and CPR1055 have a significant role in promoting spore formation. Furthermore, the deficiency in sporulation, especially in strain lacking CPR1728, is not only restricted to DS sporulation medium but is also found in MSM sporulation medium. Collectively, three important

conclusions can be drawn from these results. First, CPR1728 is the major sporulation-associated sensor histidine kinase, under laboratory conditions, typically used to induce *C. perfringens* sporulation. Second, CPR1728 and CPR1055 seemed to initiate sporulation from two independent pathways, with the CPR1728 pathway more active because it produces a more serious sporulation defect. Nevertheless, the previous study suggested that the activity of sporulation histidine kinases was influenced by the presence or absence of specific signals activating each kinase (Jiang et al., 2000). Thus, it is possible that the two sporulation systems, especially MSM medium, evaluated in this study lack the signal ligand recognized by CPR1055, which accounts for the small role of CPR1055 in triggering *C. perfringens* sporulation. Third, the residual spore formation observed in the kinase mutant strains indicated that, at least in laboratory culture, sporulation in *C. perfringens* SM101 resulted from the activity of more than one putative kinase. The other uncharacterized putative kinases might also have a role, but these are yet to be demonstrated. Unfortunately, an intensive effort to construct single- and double-knockout mutants of other putative sensor histidine kinases has not succeeded. This hypothesis is supported by the previous findings in *B. subtilis*, *Bacillus anthracis*, and *C. acetobutylicum* in which initiation of spore formation is attributed to the activity of multiple sensor histidine kinases to sense sporulation signal(s) and to activate the cell developmental process (Brunsing et al., 2005; Perego et al., 1989; Steiner et al., 2011; Trach and Hoch, 1993).

To our knowledge, the association of sensor histidine kinase by an unknown mechanism to bacterial spore germination has only been reported for the Gram-

negative bacterium *Myxococcus xanthus* (Shi et al., 2008). However, another class of kinase, the eukaryotic-like Ser/Thr kinase PrkC, is required for the germination response of *B. subtilis* and *B. anthracis* spores via the novel germination pathway having a peptidoglycan fragment from a variety of growing bacteria as a germinant (Shah et al., 2008; Squeglia et al., 2011). It was proposed that the extracellular region of PrkC could bind to peptidoglycan and induce *B. subtilis* spore germination, and this process is independent of the nutrient germinant receptors (Shah et al., 2008). However, we found in the current study that two of the putative sensor histidine kinase, CPR1728 and CPR1055, in *C. perfringens* SM101 are involved in the nutrient and chemical germination of spores of this pathogenic bacterium. It was hypothesized that the amino terminal signal input domain of sensor histidine kinases was responsible for sensing and recognizing the environmental and/or cellular signals to initiate a variety of cell developmental pathways (Hoch, 2000; Stephenson and Hoch, 2002). This signal transduction system typically requires the second component, a cognate response regulator, to exert the appropriate output responses corresponding to the specific stimuli (Hoch, 2000; Stephenson and Hoch, 2002). It is not known, however, whether that kind of putative response regulator protein exists; or, if it does exist, the question arises whether it has any role in germination initiation. Owing to the signal sensing nature of this class of protein, we hypothesized that CPR1728 and CPR1055 could act as a receptor to recognize the germinant and initiate *C. perfringens* spore germination via an alternate pathway bypassing the well characterized germination receptors (Paredes-Sabja et al., 2009c; Paredes-Sabja et al., 2008c).

Further studies are necessary to verify this notion and to unveil the germination mechanism involved with these two putative histidine kinases. It would also be interesting to assess if other putative sporulation kinases identified in the current work affect the germination capacity of *C. perfringens* once the kinase deletion strains become available. The differences in the contributing role of the CPR1728 and CPR1055 for each tested germinant are correlated well with the presence of several putative sensor histidine kinases, since it was suggested that kinases might respond to different stimuli unique to the individual protein and eventually elicit the same output response (Dürre, 2011; Jiang et al., 2000; Stephenson and Hoch, 2002).

As mentioned earlier, *C. perfringens* sporulation and spore germination have a critical role in the pathogenesis of *C. perfringens* type A FP and *C. perfringens*-related GI diseases. In this study, it is clearly shown that two of the characterized putative sensor histidine kinases, CPR1728 and CPR1055, have many important aspects in the life and disease cycle of *C. perfringens*, including sporulation, spore germination, and outgrowth. Understanding the histidine kinase-associated mechanism(s) of these bacterial developmental processes could be useful in developing inhibitors that selectively target kinases or other proteins, which are influenced by the kinases activity, such as Spo0A and SpoIIG. These antimicrobial interventions against *C. perfringens* would certainly be beneficial in preventing toxin production, developing a strategy to inhibit or delay spore germination and outgrowth in the environments and food processing facilities, and acquiring therapeutic approaches. Together, these could

eventually lead to the reduction in the incidence of and the therapy for GI illnesses caused by *C. perfringens*.

Acknowledgements

This work was supported by a grant from the the Agricultural Research Foundation of Oregon State University, and by a Department of Defense Multidisciplinary University Research Initiative (MURI) award through the U.S. Army Research Laboratory and the U. S. Army Research Office under contract number W911NF-09-1-0286 (all to MRS). PU was supported by fellowships from the Office of Higher Education Commission (Thailand). We thank Denny Weber for help editing the manuscript.

References

- Antoniewski, C., Savelli, B., & Stragier, P. (1990). The *SpoIIIJ* gene, which regulates early development steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. *Journal of Bacteriology*, *172*, 86-93.
- Brown, D.P., Ganova-Raeva, L., Green, B.D., Wilkinson, S.R., Young, M., & Youngman, P. (1994). Characterization of *spo0A* homologues in diverse *Bacillus* and *Clostridium* species identifies a probable DNA-binding domain. *Molecular Microbiology*, *14*, 411-426.
- Brunsing, R.L., La Clair, C., Tang, S., Chiang, C., Hancock, L.E., Perego, M., & Hoch, J.A. (2005). Characterization of sporulation histidine kinases of *Bacillus anthracis*. *Journal of Bacteriology*, *187*, 6972-6981.
- Burbulys, D., Trach, K.A., & Hoch, J.A. (1991). Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. *Cell*, *64*, 545-552.
- Buzby, J.C., & Roberts, T. (1997). Economic costs and trade impacts of microbial foodborne illness. *World Health Statistics Quarterly*, *50*, 57-66.
- Czeczulin, J.R., Collie, R.E., & McClane, B.A. (1996). Regulated expression of *Clostridium perfringens* enterotoxin in naturally *cpe*-negative type A, B, and C isolates of *C. perfringens*. *Infection and Immunity*, *64*, 3301-3309.

- Duncan, C.L., & Strong, D.H. (1968). Improved medium for sporulation of *Clostridium perfringens*. *Applied Microbiology*, *16*, 82-89.
- Duncan, C.L., Strong, D.H., & Sebald, M. (1972). Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. *Journal of Bacteriology*, *110*, 378-391.
- Dürre, P. (2011). Ancestral sporulation initiation. *Molecular Microbiology*, *80*, 584-587.
- Dürre, P., & Hollergschwandner, C. (2004). Initiation of endospore formation in *Clostridium acetobutylicum*. *Anaerobe*, *10*, 69-74.
- Errington, J. (2003). Regulation of endospore formation in *Bacillus subtilis*. *Nature Reviews Microbiology*, *1*, 117-126.
- Fabret, C., Feher, V.A., & Hoch, J.A. (1999). Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. *Journal of Bacteriology*, *181*, 1975-1983.
- Fujita, M., González-Pastor, J.E., & Losick, R. (2005). High- and low- threshold genes in the Spo0A regulon of *Bacillus subtilis*. *Journal of Bacteriology*, *187*, 1357-1368.
- Haldenwang, W.G. (1995). The sigma factors of *Bacillus subtilis*. *Microbiology and Molecular Biology Reviews*, *59*, 1-30.
- Harry, K.H., Zhou, R., Kroos, L., & Melville, S.B. (2009). Sporulation and enterotoxin (CPE) synthesis are controlled by the sporulation-specific sigma factors SigE and SigK in *Clostridium perfringens*. *Journal of Bacteriology*, *191*, 2728-2742.
- Hatheway, C.L. (1990). Toxigenic clostridia. *Clinical Microbiology Reviews*, *3*, 66-98.
- Hoch, J.A. (1995). Control of cellular development in sporulating bacteria by the phosphorelay two-component signal transduction system. In: Hoch, J.A. Silhavy, T.J. (Eds.), *Two-component signal transduction* (pp. 129-144). Washington, DC: American Society for Microbiology.
- Hoch, J.A. (2000). Two-component and phosphorelay signal transduction. *Current Opinion in Microbiology*, *3*, 165-170.
- Huang, I.H., & Sarker, M.R. (2006). Complementation of a *Clostridium perfringens spo0A* mutant with wild-type *spo0A* from other *Clostridium* species. *Applied and Environmental Microbiology*, *72*, 6388-6393.
- Huang, I.H., Waters, M., Grau, R.R., & Sarker, M.R. (2004). Disruption of the gene (*spo0A*) encoding sporulation transcription factor blocks endospore formation and enterotoxin production in enterotoxigenic *Clostridium perfringens* type A. *FEMS Microbiology Letters*, *233*, 233-240.
- Ireland, J.A., & Hanna, P.C. (2002). Amino acid- and purine ribonucleoside-induced germination of *Bacillus anthracis* DeltaSterne endospores: *gerS* mediates responses to aromatic ring structures. *Journal of Bacteriology*, *184*, 1296-1303.
- Jiang, M., Shao, W., Perego, M., & Hoch, J.A. (2000). Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. *Molecular Microbiology*, *38*, 535-542.

- Kim, C.H., Cheney, R., & Woodburn, M. (1967). Sporulation of *Clostridium perfringens* in a modified medium and selected foods. *Applied Microbiology*, *15*, 871-876.
- Kokai-Kun, J.F., Songer, J.G., Czczulin, J.R., Chen, F., & McClane, B.A. (1994). Comparison of Western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens*. *Journal of Clinical Microbiology*, *32*, 2533-2539.
- LeDeaux, J.R., & Grossman, A.D. (1995). Isolation and characterization of *kinC*, a gene that encodes a sensor histidine kinase homologous to the sporulation sensor kinases KinA and KinB in *Bacillus subtilis*. *Journal of Bacteriology*, *177*, 166-175.
- LeDeaux, J.R., Yu, N., & Grossman, A.D. (1995). Different roles for KinA, KinB, and KinC in the initiation of sporulation in *Bacillus subtilis*. *Journal of Bacteriology*, *177*, 861-863.
- Li, J., & McClane, B.A. (2006a). 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. *Applied and Environmental Microbiology*, *72*, 7620-7625.
- Li, J., & McClane, B.A. (2006b). Further comparison of temperature effects on growth and survival of *Clostridium perfringens* type A isolates carrying a chromosomal or plasmid-borne enterotoxin gene. *Applied and Environmental Microbiology*, *72*, 4561-4568.
- McClane, B.A. (2001). *Clostridium perfringens*. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), *Food Microbiology: fundamentals and frontiers* (pp. 351-372). Washington, D.C.: ASM Press.
- McClane, B.A. (2007). *Clostridium perfringens*. In: Doyle, M.P., Beuchat, L.R. (Eds.), *Food Microbiology: fundamentals and frontiers* (pp. 423-444). Washington, D.C.: ASM Press.
- Moir, A. (2006). How do spores germinate? *Journal of Applied Microbiology*, *101*, 526-530.
- Molle, V., Fujita, M., Jensen, S.T., Eichenberger, P., Gonzalez-Pastor, J.E., Liu, J.S., & Losick, R. (2003). The Spo0A regulon of *Bacillus subtilis*. *Molecular Microbiology*, *50*, 1683-1701.
- Myers, G.S., Rasko, D.A., Cheung, J.K., Ravel, J., Seshadri, R., DeBoy, R.T., Ren, Q., Varga, J., Awad, M.M., Brinkac, L.M., Daugherty, S.C., Haft, D.H., Dodson, R.J., Madupu, R., Nelson, W.C., Rosovitz, M.J., Sullivan, S.A., Khouri, H., Dimitrov, G.I., Watkins, K.L., Mulligan, S., Benton, J., Radune, D., Fisher, D.J., Atkins, H.S., Hiscox, T., Jost, B.H., Billington, S.J., Songer, J.G., McClane, B.A., Titball, R.W., Rood, J.I., Melville, S.B., & Paulsen, I.T. (2006). Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. *Genome Research*, *16*, 1031-1040.
- Novak, J.S., & Juneja, V. (2002). *Clostridium perfringens*: hazards in new generation foods. *Innovative Food Science and Emerging Technologies*, *3*, 127-132.

- Paidhungat, M., Ragkousi, K., & Setlow, P. (2001). Genetic requirements for induction of germination of spores of *Bacillus subtilis* by Ca^{2+} -dipicolinate. *Journal of Bacteriology*, *183*, 4886-4893.
- Paidhungat, M., & Setlow, P. (2002). Spore germination and outgrowth. In: Hoch, J.A., Losick, R., Sonenshein, A.L. (Eds.), *Bacillus subtilis and its relatives: From Genes to cells* (pp. 537-548). Washington, DC: ASM Press.
- Paredes - Sabja, D., Setlow, P., & Sarker, M.R. (2009). Role of GerKB in germination and outgrowth of *Clostridium perfringens* spores. *Applied and Environmental Microbiology*, *75*, 3813-3817.
- Paredes - Sabja, D., Setlow, P., & Sarker, M.R. (2011). Germination of spores of *Bacillales* and *Clostridiales* species: mechanisms and protein involved. *Trends in Microbiology*, *19*, 85-94.
- Paredes-Sabja, D., Gonzalez, M., Sarker, M.R., & Torres, J.A. (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*, *72*, M202-M206.
- Paredes-Sabja, D., Raju, D., Torres, J.A., & Sarker, M.R. (2008a). Role of small, acid-soluble spore proteins in the resistance of *Clostridium perfringens* spores to chemicals. *International Journal of Food Microbiology*, *122*, 333-335.
- Paredes-Sabja, D., Setlow, B., Setlow, P., & Sarker, M.R. (2008b). Characterization of *Clostridium perfringens* spores that lack SpoVA proteins and dipicolinic acid. *Journal of Bacteriology*, *190*, 4648-4659.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009a). GerO, a putative $\text{Na}^+/\text{H}^+-\text{K}^+$ antiporter, is essential for normal germination of spores of the pathogenic bacterium *Clostridium perfringens*. *Journal of Bacteriology*, *191*, 3822-3831.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009b). Role of GerKB in germination and outgrowth of *Clostridium perfringens* spores. *Applied and Environmental Microbiology*, *75*, 3813-3817.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009c). SleC is essential for cortex peptidoglycan hydrolysis during germination of spores of the pathogenic bacterium *Clostridium perfringens*. *Journal of Bacteriology*, *191*, 2711-2720.
- Paredes-Sabja, D., Torres, J.A., Setlow, P., & Sarker, M.R. (2008c). *Clostridium perfringens* spore germination: characterization of germinants and their receptors. *Journal of Bacteriology*, *190*, 1190-1201.
- Paredes-Sabja, D., Udompijitkul, P., & Sarker, M.R. (2009d). Inorganic phosphate and sodium ions are cogerminants for spores of *Clostridium perfringens* type A food poisoning - related isolates *Applied and Environmental Microbiology*, *75*, 6299-6305.
- Perego, M., Cole, S.P., Burbulys, D., Trach, K.A., & Hoch, J.A. (1989). Characterization of the gene for a protein kinase which phosphorylates the sporulation-regulatory proteins Spo0A and Spo0F of *Bacillus subtilis*. *Journal of Bacteriology*, *171*, 6187-6196.
- Perego, M., & Hoch, J.A. (2002). Two-component systems, phosphorelays, and regulation of their activities by phosphatase. In: Sonenshein, A.L., Hoch,

- J.A.Losick, R. (Eds.), *Bacillus subtilis and its closet relatives: From genes to cells* (pp. 473-482). Washington, DC: ASM Press.
- Petit, L., Gilbert, M., & Popoff, M.R. (1999). *Clostridium perfringens*: toxinotype and genotype. *Trends in Microbiology*, 7, 104-110.
- Philippe, V.A., Mendez, M.B., Huang, I.H., Orsaria, L.M., Sarker, M.R., & Grau, R.R. (2006). Inorganic phosphate induces spore morphogenesis and enterotoxin production in the intestinal pathogen *Clostridium perfringens*. *Infection and Immunity*, 74, 3651-3656.
- Raju, D., Waters, M., Setlow, P., & Sarker, M.R. (2006). Investigating the role of small, acid-soluble spore proteins (SASPs) in the resistance of *Clostridium perfringens* spores to heat. *BMC Microbiology*, 6, 50.
- Sarker, M.R., Carman, R.J., & McClane, B.A. (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. *Molecular Microbiology*, 33, 946-958.
- Sarker, M.R., Shivers, R.P., Sparks, S.G., Juneja, V.K., & McClane, B.A. (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. *Applied and Environmental Microbiology*, 66, 3234-3240.
- Satola, S., Baldus, J.M., & Moran, C.P. (1992). Binding of Spo0A stimulates spoIIIG promoter activity in *Bacillus subtilis*. *Journal of Bacteriology*, 174, 1448-1453.
- Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.-A., Roy, S.L., Jones, J.L., & Griffin, P.M. (2011). Foodborne illness acquired in the United States - major pathogens. *Emerging Infectious Diseases*, 17, 7-15.
- Setlow, B., Peng, L., Loshon, C.A., Li, Y.-Q., Christie, G., & Setlow, P. (2009). Characterization of the germination of *Bacillus megaterium* spores lacking enzymes that degrade the spore cortex. *Journal of Applied Microbiology*, 107, 318-328.
- Setlow, P. (2003). Spore germination. *Current Opinion in Microbiology*, 6, 550-556.
- Shah, I.M., Laaberki, M.H., Popham, D.L., & Dworkin, J. (2008). A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. *Cell*, 135, 486-496.
- Shi, X., Wegener-Feldbrügge, S., Huntley, S., Hamann, N., Hedderich, R., & Søgaard-Andersen, L. (2008). Bioinformatics and experimental analysis of proteins of two-component systems in *Myxococcus xanthus*. *Journal of Bacteriology*, 190, 613-624.
- Sonnhammer, E.L., von Heijne, G., & Krogh, A. (1998). A hidden Markov model for predicting transmembrane helices in protein sequences. *Proceedings of the 6th International Conference on Intelligent Systems for Molecular Biology*, 6, 175-182.
- Spiegelman, G.B., Bird, T.H., & Voon, V. (1995). Transcription regulation by the *Bacillus subtilis* response regulator Spo0A. In: Hoch, J.A. Silhavy, T.J. (Eds.),

- Two-component signal transduction* (pp. 159-179). Washington, DC: ASM Press.
- Squeglia, F., Marchetti, R., Ruggiero, A., Lanzetta, R., Marasco, D., Dworkin, J., Petoukhov, M., Molinaro, A., Berisio, R., & Silipo, A. (2011). Chemical basis of peptidoglycan discrimination by PrkC, a key kinase involved in bacterial resuscitation from dormancy. *Journal of the American Chemical Society*, *133*, 20676-20679.
- Steiner, E., Dago, A.E., Young, D.I., Heap, J.T., Minton, N.P., Hoch, J.A., & Young, M. (2011). Multiple orphan histidine kinases interact directly with Spo0A to control the initiation of endospore formation in *Clostridium acetobutylicum*. *Molecular Microbiology*, *80*, 641-654.
- Stephenson, K., & Hoch, J.A. (2002). Evolution of signalling in the sporulation phosphorelay. *Molecular Microbiology*, *46*, 297-304.
- Stephenson, K., & Hoch, J.A. (2004). Developing inhibitors to selectively target two-component and phosphorelay signal transduction systems of pathogenic microorganisms. *Current Medicinal Chemistry*, *11*, 765-773.
- Thippareddi, H., Juneja, V.K., Phebus, R.K., Marsden, J.L., & Kastner, C.L. (2003). Control of *Clostridium perfringens* germination and outgrowth by buffered sodium citrate during chilling of roast beef and injected pork. *Journal of Food Protection*, *66*, 376-381.
- Trach, K.A., & Hoch, J.A. (1993). Multisensory activation of the phosphorelay initiating sporulation in *Bacillus subtilis*: identification and sequence of the protein kinase of the alternate pathway. *Molecular Microbiology*, *8*, 69-79.
- Underwood, S., Guan, S., Vijayasubhash, V., Baines, S.D., Graham, L., Lewis, R.J., Wilcox, M.H., & Stephenson, K. (2009). Characterization of the sporulation initiation pathway of *Clostridium difficile* and its role in toxin production. *Journal of Bacteriology*, *191*, 7296-7305.
- Wörner, K., Szurmant, H., Chiang, C., & Hoch, J.A. (2006). Phosphorylation and functional analysis of the sporulation initiation factor Spo0A from *Clostridium botulinum*. *Molecular Microbiology*, *59*, 1000-1012.
- Zhao, Y., & Melville, S.B. (1998). Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (*cpe*) of *Clostridium perfringens*. *Journal of Bacteriology*, *180*, 136-142.

Table 7.1. *C. perfringens* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source/reference
Strain		
SM101	Electroporatable derivative of food poisoning type A isolate NCTC8798; carries a chromosomal <i>cpe</i> gene	(Zhao and Melville, 1998)
IH101	$\Delta spo0A::catP$	(Huang et al., 2004)
PU101	$\Delta cpr1728::catP$	
PU102	$\Delta cpr1055::catP$	This study
SM101(pPU2)	Wild type strain carrying <i>cpr0195-gusA</i> fusion	This study
IH101(pPU2)	<i>spo0A</i> mutant strain carrying <i>cpr0195-gusA</i> fusion	This study
SM101(pPU4)	Wild type strain carrying <i>cpr1055-gusA</i> fusion	This study
IH101(pPU4)	<i>spo0A</i> mutant strain carrying <i>cpr1055-gusA</i> fusion	This study
SM101(pPU6)	Wild type strain carrying <i>cpr1728-gusA</i> fusion	This study
IH101(pPU6)	<i>spo0A</i> mutant strain carrying <i>cpr1728-gusA</i> fusion	This study
SM101(pPU28)	Wild type strain carrying <i>cpr1316-gusA</i> fusion	This study
IH101(pPU28)	<i>spo0A</i> mutant strain carrying <i>cpr1316-gusA</i> fusion	This study
SM101(pPU30)	Wild type strain carrying <i>cpr1493-gusA</i> fusion	This study

Table 7.1. (Continued) *C. perfringens* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source/Reference
Strain		
IH101(pPU30)	<i>spo0A</i> mutant strain carrying <i>cpr1493-gusA</i> fusion	This study
SM101(pPU31)	Wild type strain carrying <i>cpr1953-gusA</i> fusion	This study
IH101(pPU31)	<i>spo0A</i> mutant strain carrying <i>cpr1493-gusA</i> fusion	This study
SM101(pPU32)	Wild type strain carrying <i>cpr1954-gusA</i> fusion	This study
IH101(pPU32)	<i>spo0A</i> mutant strain carrying <i>cpr1954-gusA</i> fusion	This study
SM101(pPU40)	Wild type strain carrying <i>spo0A-gusA</i> fusion	This study
PU101(pPU40)	<i>cpr1728</i> mutant strain carrying <i>spo0A-gusA</i> fusion	This study
PU102(pPU40)	<i>cpr1055</i> mutant strain carrying <i>spo0A-gusA</i> fusion	This study
SM101(pPU41)	Wild type strain carrying <i>cpr1729-gusA</i> fusion	This study

Table 1. (Continued) *C. perfringens* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source/Reference
SM101(pMW132)	Wild type strain carrying <i>spoIIIGA-gusA</i> fusion	This study
IH101(pMW132)	<i>spo0A</i> mutant strain carrying <i>spoIIIGA-gusA</i> fusion	This study
PU102(pMW132)	<i>cpr1055</i> mutant strain carrying <i>spoIIIGA-gusA</i> fusion	This study
Plasmid		
pMRS104	No origin of replication for <i>C. perfringens</i> ; Em ^r	(Huang et al., 2004)
pMRS127	<i>C. perfringens</i> / <i>E. coli</i> shuttle vector carrying promoterless <i>E. coli gusA</i> ; Erythromycin resistance (Em ^r)	(Raju et al., 2006)
pDP25	pCR-XL-TOPO carrying chloramphenicol resistance gene (<i>catP</i>); Cm ^r	(Paredes-Sabja et al., 2009d)
pPU2	415-bp Sali-PstI fragment carrying <i>cpr0195</i> promoter region cloned into pMRS127 to create a <i>cpr0195-gusA</i> fusion construct	This study
pPU4	415-bp Sali-PstI fragment carrying <i>cpr1055</i> promoter region cloned into pMRS127 to create a <i>cpr1055-gusA</i> fusion construct	This study
pPU6	441-bp Sali-PstI fragment carrying <i>cpr1728</i> promoter region cloned into pMRS127 to create a <i>cpr1728-gusA</i> fusion construct	This study
pPU8	1,615-bp PCR fragment containing 1,565-bp downstream and 50-bp of the C-terminal-coding region of <i>cpr1728</i> in pCR-XL-TOPO	This study

Table 1. (Continued) *C. perfringens* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source/Reference
pPU10	~1.6-kb PstI-XhoI fragment from pPU8 cloned between PstI/XhoI sites of pDP25	This study
pPU11	~4.5-kb KpnI-XhoI fragment from pPU10 cloned between KpnI/SalI sites of pMRS104	This study
pPU13	1,279-bp PCR fragment containing 1,191-bp downstream and 88-bp of the C-terminal coding region of <i>cpr1055</i> in pCR-XL-TOPO	This study
pPU14	2,038-bp PCR fragment containing 1,263-bp upstream and 775-bp of the N-terminal-coding region of <i>cpr1728</i> in pCR-XL-TOPO	This study
pPU15	~2.0-kb KpnI-SpeI fragment from pPU14 cloned between KpnI/SpeI sites of pPU11	This study
pPU22	1,700-bp PCR fragment containing 939-bp upstream and 761-bp of the N-terminal coding region of <i>cpr1055</i> in pCR-XL-TOPO	This study
pPU24	~ 1.7-kb KpnI-SpeI fragment from pPU22 cloned between KpnI/SpeI digested sites of pPU10	This study
pPU25	~1.3-kb PstI-XhoI fragment from pPU13 cloned between the PstI/XhoI sites of pPU24	This study
pPU26	~ 4.4-kb KpnI-XhoI fragment from pPU25 cloned between KpnI/SalI sites of pMRS104	This study
pPU28	462-bp SalI-PstI fragment carrying <i>cpr1316</i> promoter region cloned into pMRS127 to create a <i>cpr1316-gusA</i> fusion construct	This study
pPU30	427-bp SalI-PstI fragment carrying <i>cpr1493</i> promoter region cloned into pMRS127 to create a <i>cpr1493-gusA</i> fusion construct	This study

Table 1. (Continued) *C. perfringens* strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source/Reference
pPU31	406-bp Sall-PstI fragment carrying <i>cpr1953</i> promoter region cloned into pMRS127 to create a <i>cpr1953-gusA</i> fusion construct	This study
pPU32	496-bp Sall-PstI fragment carrying <i>cpr1954</i> promoter region cloned into pMRS127	This study
pPU40	368-bp Sall-PstI fragment carrying <i>spo0A</i> promoter region cloned into pMRS127 to create a <i>spo0A-gusA</i> fusion construct	This study
pPU41	555-bp Sall-PstI fragment carrying <i>scpr1729</i> promoter region cloned into pMRS127 to create a <i>spo0A-gusA</i> fusion construct	This study
pMW132	A Sall-PstI fragment carrying <i>spoIIIGA</i> promoter region cloned into pMRS127 to create a <i>spoIIIGA-gusA</i> fusion construction	This study

Table 7.2 Primers used in this study

Primer name	Primer sequence (5' – 3') ^a	Gene	Nucleotide position ^b	Use
CPP418	<u>GACGCTGCAGCT</u> AAGTACATAGAAGTCATTA TAGCTA G	<i>cpr1728</i>	+48 to +76	GUS
CPP421	<u>GCAGCGTCGAC</u> TCATAGTGGATGGAAAGGAACATAC	<i>cpr1728</i>	-374 to -349	GUS
CPP435	<u>GACGCTGCAGCA</u> ACTAGGAGTAGGGACTAAAA TAG	<i>cpr1728</i>	+1666 to +1690	MP
CPP436	<u>GCACCTCGAGG</u> TGCTGTCAAATCATCTAACAT TATTC	<i>cpr1728</i>	+3255 to +3281	MP
CPP439	<u>GCGGGTACCCCA</u> ATTAGAAGAAGAAGAGAAT GTTTAAAG	<i>cpr1728</i>	-1263 to -1235	MP
CPP466	GGTCATAGCTTTATTGTTAGAAAATG	<i>cpr1728</i>	+134 to +160	MP
CPP467	CTATTTTAGTCCCTACTCCTAGTT	<i>cpr1728</i>	+1666 to +1690	MP
CPP471	<u>GCGACTAGTCT</u> GTCAA AAGCTATTACTCCAC TAC	<i>cpr1728</i>	+751 to +775	MP
CPP834	<u>GCAGCGTCGAC</u> ATACACA ACTTACGAAAAGGT AGAAG	<i>cpr1729</i>	-505 to -479	GUS
CPP835	<u>GACGCTGCAGC</u> CATCGTTGAAA ACTATAAAT TCTTTG	<i>cpr1729</i>	+23 to +50	GUS
CPP423	<u>GCAGCGTCGAC</u> T TTTTCTTTTACTCATTTT TAC CTCC	<i>cpr1055</i>	-374 to -346	GUS
CPP419	<u>GACGCTGCAGG</u> ACATGACTCTATTATTTC TC	<i>cpr1055</i>	+15 to +38	GUS
CPP587	<u>GCGGGTACCC</u> GCCAGAAGCTAGGAATATTGG	<i>cpr1055</i>	-939 to -918	MP
CPP472	<u>GCGACTAGTC</u> GCTCTAATAATCCTTGCTTCT GC	<i>cpr1055</i>	+738 to +761	MP
CPP429	<u>GACGCTGCAGC</u> AGTTTTAGATGAGATAGCCTA ACATC	<i>cpr1055</i>	+1586 to +1613	MP

^a Restriction sites are marked by underlining.

^b Nucleotide numbering begins from the first base of the translation start codon and refers to the relevant position within the particular coding sequence.

^c GUS, construction of *gusA* fusion plasmid; MP, construction of the mutator plasmid.

Table 7.2 (Continued) Primers used in this study

Primer name	Primer sequence (5' – 3') ^a	Gene	Nucleotide position ^b	Use
CPP431	GCACCTCGAGGAAATATTAAGTACCCTATCAT ACC AGC	<i>cpr1055</i>	+2838 to +2865	MP
CPP468	GGTGATGATTATTTTGATTTATTTGTGC	<i>cpr1055</i>	+69 to +97	MP
CPP469	GAT GTTAAGGCTATCTCATCTAAACT	<i>cpr1055</i>	+1586 to +1613	MP
CPP883	GCAGCGTCGACGGTGGAGTTGTTCAAGGA ATG AG	<i>spo0A</i>	-320 to -297	GUS
CPP884	GACGCTGCAGCTCTTTGTTATCATCAGCAAT TAATACAG	<i>spo0A</i>	+19 to +48	GUS
CPP _{Spol} IGA-P-F	GCAGCGTCGAC CATTACAAGAATAGACATG	<i>spoIIIGA</i>	-183 to 155	GUS
CPP _{Spol} IGA-P-R	GACGCTGCAGGAGTGAGTTGATTTATGACTG	<i>spoIIIGA</i>	-14 to + 6	GUS
CPP420	GCAGCGTCGACAAGTAGAAAAGAAATTA GAGGAAAG	<i>cpr0195</i>	-404 to -375	GUS
CPP422	GACGCTGCAGGAATTATCCATGTTTTAAATC TACTACTC	<i>cpr0195</i>	-18 to +11	GUS
CPP470	GCAGCGTCGACCCTTCTGTCCAAAAGTACGG	<i>cpr1316</i>	-392 to -371	GUS
CPP473	GACGCTGCAGCTGTGACTATTGCTATTCTTT TTAATG	<i>cpr1316</i>	+43 to +70	GUS
CPP498	GCAGCGTCGACGTTAAAGACTAGGGATAA GTACTC	<i>cpr1493</i>	-376 to -352	GUS
CPP486	GACGCTGCAGTAGTATTAAGGAGAGAAAAGC ATATC	<i>cpr1493</i>	+25 to +51	GUS
CPP491	GCAGCGTCGACGATACAGATGAAGAAGAG TTATATG	<i>cpr1953</i>	-367 to -342	GUS
CPP487	GACGCTGCAGTTGCAATCTAGGATTTTCATA TTCC	<i>cpr1953</i>	+14 to +39	GUS
CPP963	GCAGCGTCGACGGAAGAAAATAATATAGAAAA GGGAATAG	<i>cpr1954</i>	-395 to -366	GUS
CPP964	GCAGCGTCGACAATGCTGTAAGTGGTCTA AAAAAATATAC	<i>cpr1954</i>	+69 to +98	GUS

^a Restriction sites are marked by underlining.

^b Nucleotide numbering begins from the first base of the translation start codon and refers to the relevant position within the particular coding sequence.

^c GUS, construction of *gusA* fusion plasmid; MP, construction of the mutator plasmid.

Active site regions of histidine kinases

B. subtilis orphan histidine

KinA	QLAAGIAHEIRNPLT
kinE	ELAAGIAHEIRNPMT
KinC	ELAAGIAHEVRNPLT
KinB	ELAASVAHEVRNPLT
KinD	TLAASTAHEIRNPLT
	.:***:*

C. perfringens SM101 putative orphan

histidine kinase

CPR1316	EFFANLSHELRTPIN
CPR1953	EFFTNICHELRTPIN
CPR1493	DYFVNLSHELRTPLN
CPR0195	EFFANISHELRTPLN
CPR1055	EFFANISHEFKTFVN
CPR1728	QFVADISHELKTPLT
	:.....**:**:..

Fig 7.1. Alignment of amino acid sequences of the *B. subtilis* sporulation-associated sensor histidine kinases and the putative orphan histidine kinases of *C. perfringens*. The sequence surrounding the phosphorylatable histidine of the *B. subtilis* group IIIB sensor kinases (Perego and Hoch, 2002; Stephenson and Hoch, 2002) and the putative orphan histidine kinases in *C. perfringens* SM101 are depicted. Active site histidines are highlighted in gray boxes. Asterisks represented identical residues, and colon indicates conserved residues. The alignment was generated by the program ClustalW.

Table 7.3. Percentage of amino acid sequence similarity between sporulation-associated sensor histidine kinases in *B. subtilis* and putative orphan kinases in *C. perfringens*

	Amino acid sequence similarity (%) to ^a				
	KinA	KinB	KinC	KinD	KinE
<i>C. perfringens</i> SM101 ^b					
CPR0195	48	52	45	48	49
CPR1055	49	48	49	51	46
CPR1316	46	51	45	50	46
CPR1493	49	49	50	49	54
CPR1728	51	52	51	53	47
CPR1953	44	47	47	47	45

^a The sporulation-specific sensor histidine kinases in *B. subtilis* strain 168.

^b The putative orphan sensor histidine kinases in genome of *C. perfringens* SM101 were identified by using BLASTP analyses.

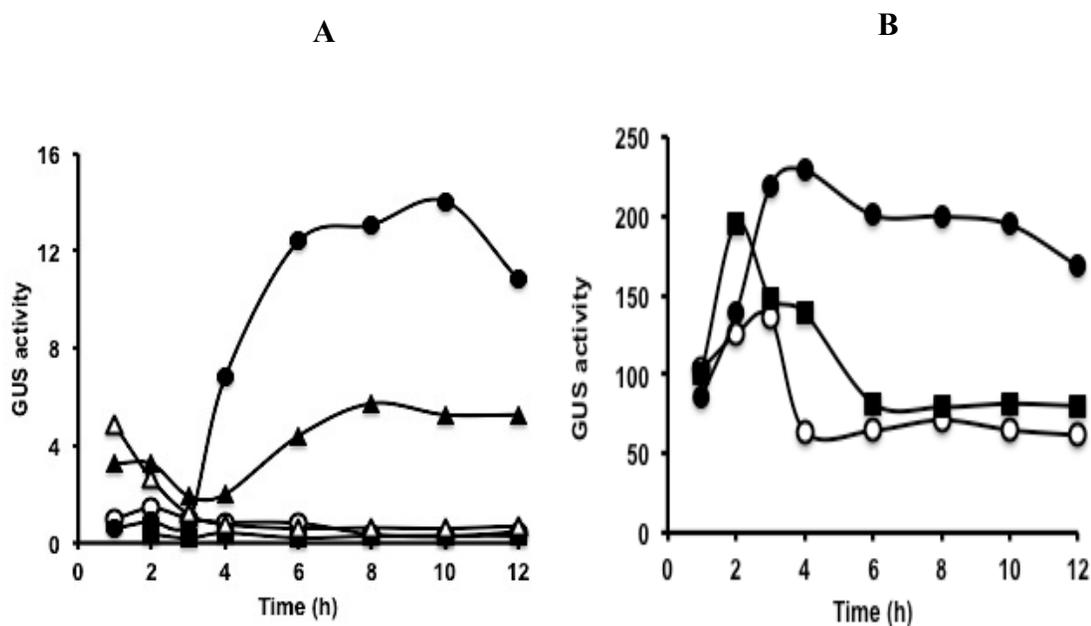


Fig. 7.2A – B. Expression profiles of putative sensor histidine kinase genes in *C. perfringens* strains. GUS-specific activities in Miller unit (Raju et al., 2006) from *cpr1728-gusA* and *cpr1729-gusA* (A) and *cpr1055-gusA* (B) fusions in *C. perfringens* various strains grown in TGY vegetative (empty symbols) or DS sporulation (filled symbols) media were determined as described in Materials and methods. Symbols: (A) GUS-specific activities of *cpr1728-gusA* in *C. perfringens* SM101 (circles) or IH101 (squares) and *cpr1729-gusA* in SM101 (triangles). (B) GUS-specific activity of *cpr1055-gusA* in *C. perfringens* SM101 (circle) and IH101 (squares). Time zero designated as the inoculation time of cultures into either TGY or DS media. Results are averages of three independent experiments.

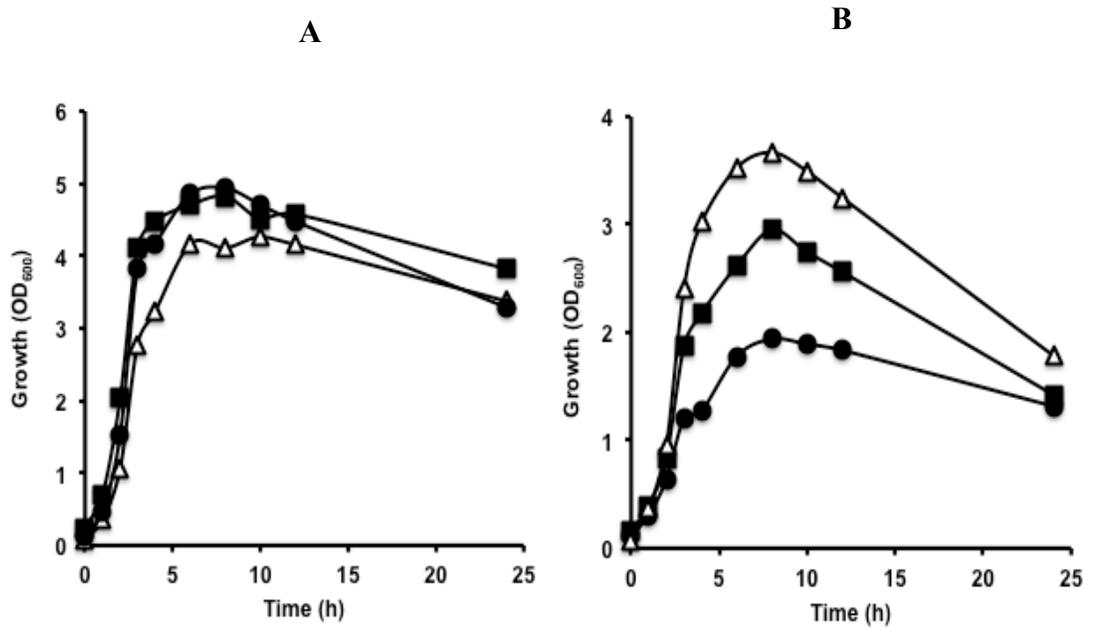


Fig. 7.3A – B. Growth characteristics of *C. perfringens* strains under vegetative and sporulation conditions. Growth of *C. perfringens* various strains in TGY vegetative medium (A) and DS sporulation (B) was evaluated. The actively growing strains SM101 (wild type) (filled circles), PU101 (filled squares), and PU102 (empty triangles) were inoculated into growth media and OD₆₀₀ was measured as described in Materials and methods. Time zero denoted as time of cells inoculated into either TGY or DS media. Data are means of three experiments.

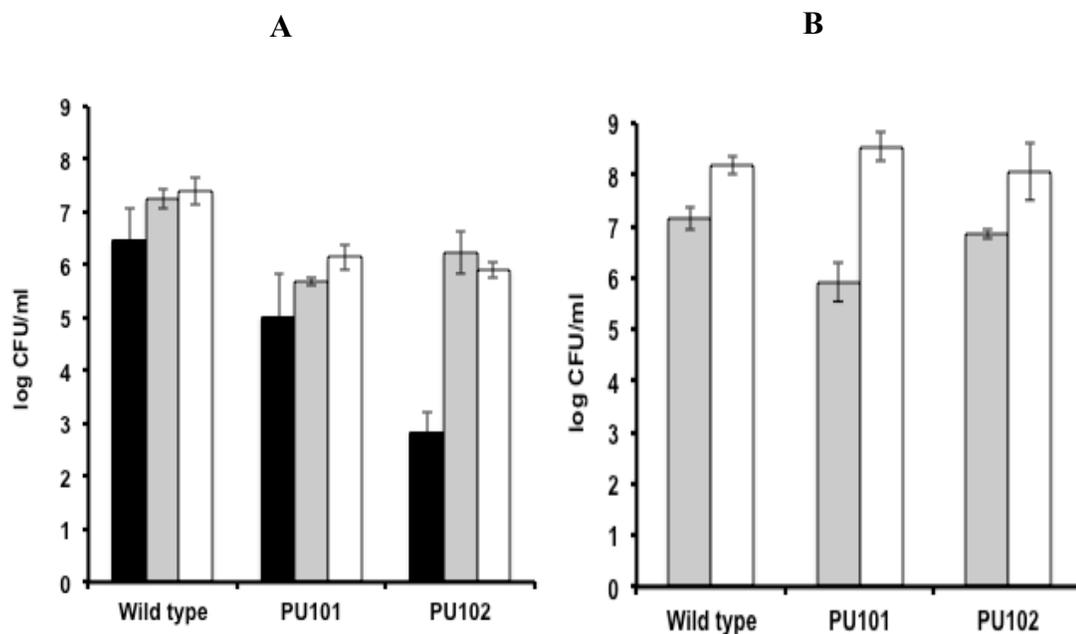


Fig 7.4A – B. Sporulation capability of *C. perfringens* strains. (A) The number of heat-resistant spores in DS medium was quantified after 6 h (black bars), 24 h (gray bars), and 48 h (white bars) post-inoculation as described in Materials and methods. (B) The populations of total viable cells (white bars) and heat-resistant spores (grey bars) of *C. perfringens* various strains grown in MSM medium for 24 h were determined both without and with heat treatment at 75 °C for 20 min, respectively. All values are averages of at least two independent experiments.

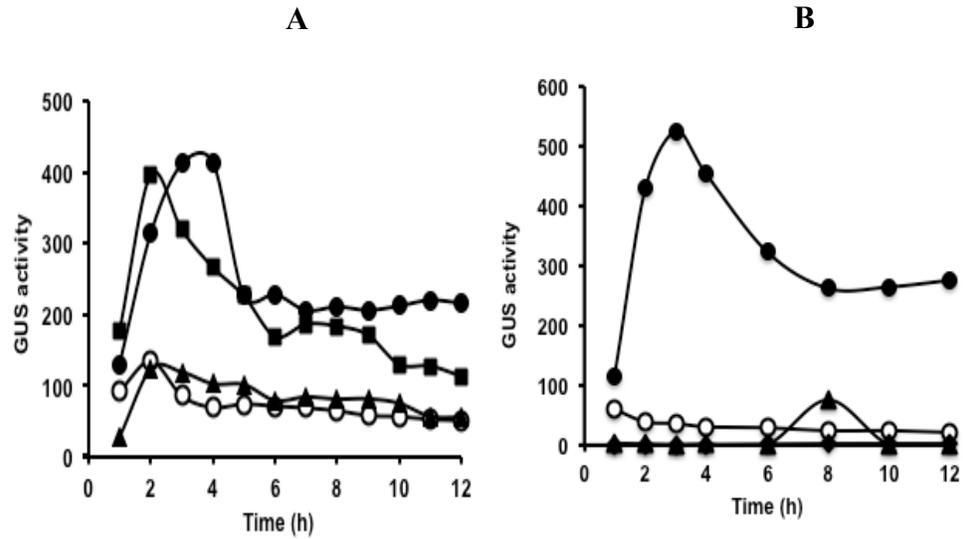


Fig. 7.5A – B. Expression profiles of the *spo0A* and the *spoIIG* operon in various *C. perfringens* strains. GUS-specific activity in Miller unit from *spo0A-gusA* (A) and *spoIIG-gusA* (B) fusions in *C. perfringens* strain SM101 (wild type) grown in TGY (empty circles) or DS (filled circles), IH101 grown in DS (filled diamond), PU101 grown in DS (filled squares), and PU102 grown in DS (filled triangles) were determined as described in Materials and methods. Data are averages of three independent experiments.

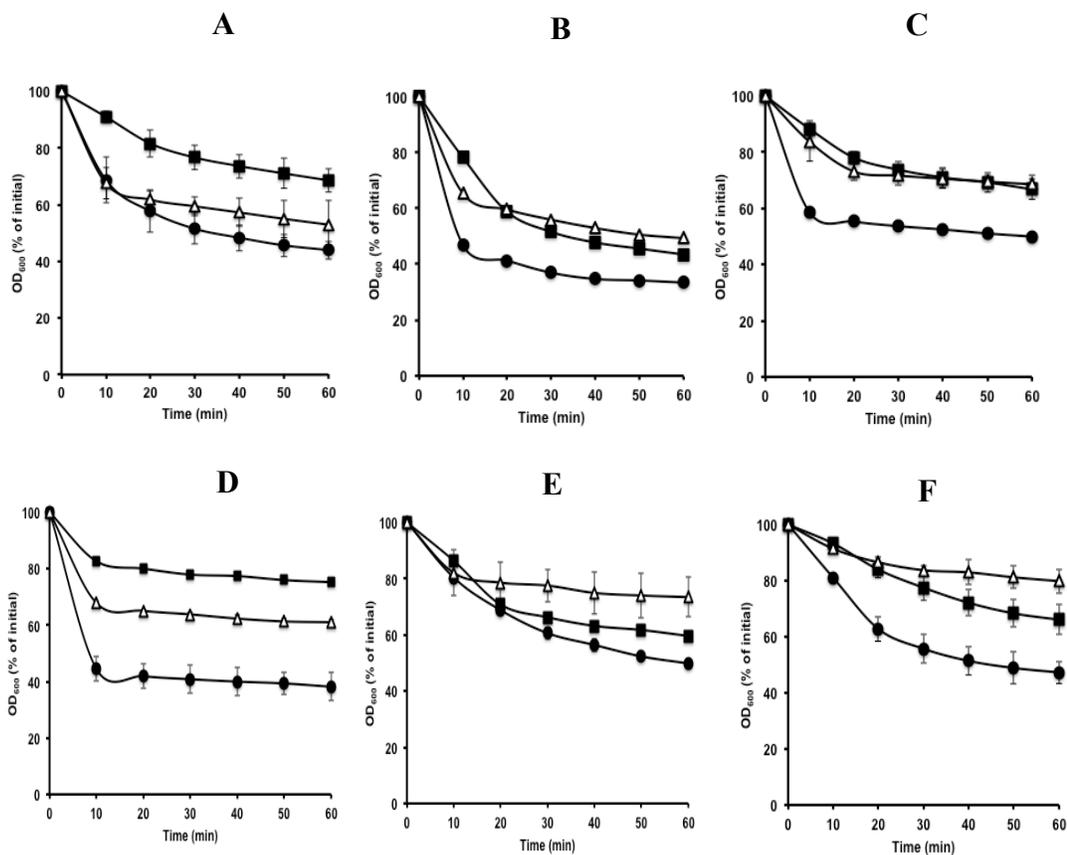


Fig. 7.6A – F. Germination of *C. perfringens* spores with various germinants. Heat-activated spores of *C. perfringens* SM101 (wild type) (filled circles), PU101 (filled squares), and PU102 (empty triangles) were germinated at 40 °C with (A) 100 mM L-asparagine (pH 7.0), (B) 100 mM KCl (pH 7.0), (C) 100 mM NaPi (pH 6.0), (D) 250 mM L-cysteine (pH 6.0), (E) 250 mM L-glutamine (pH 6.0), and (F) 50 mM Ca-DPA made to pH 8.0 with Tris base. All germinants, except where indicated, were prepared with 25 mM Na₂HPO₄ buffer (pH 7.0) and adjusted to the final desired pHs. *C. perfringens* SM101, PU101, and PU102 incubated with 25 mM Na₂HPO₄ buffer pH 6.0 and 7.0 as a control exhibited less than 17% decrease in an initial OD₆₀₀ after 60 min of incubation (data not shown). All data are averages of at least three experiments with at least two different spore preparations.

Table 7.4. Maximum germination rates of spores of various *C. perfringens* strains with known germinants

Germinants	Maximum rate of spores of indicated strains germination at 40 ° C with ^e :		
	SM101 (wild type)	PU101 (<i>cpr1728</i>)	PU102 (<i>cpr1055</i>)
L-Asn ^a	100	65	70
L-Cys ^b	100	17	55
L-Gln ^b	100	42	80
BHI ^c	100	24	90
KCl ^a	100	54	60
AK ^a	100	38	98
NaPi ^a	100	24	56
Ca-DPA ^d	100	40	49

^a Spores were germinated with KCl, L-Asn, the combination of L-Asn and KCl (AK) adjusted to a final pH of 7.0, and NaPi adjusted to a final pH 6.0. Each germinant was tested at 100 mM and prepared with 25 mM Na₂HPO₄ buffer (pH 7.0).

^b Spores were germinated with L-Cys and L-Gln adjusted to a final pH of 6.0. Each germinant was tested at 250 mM and prepared with 25 mM Na₂HPO₄ buffer (pH 7.0).

^c Spores were germinated with Brain Heart Infusion broth.

^d Spores were germinated with 50 mM Ca-DPA adjusted to a final pH of 8.0 with Tris base.

^e Maximum rates of spore germination were expressed as the percent change in OD₆₀₀/min. All given values are relative to the value of SM101 spores (set at 100) for the respective germinant.

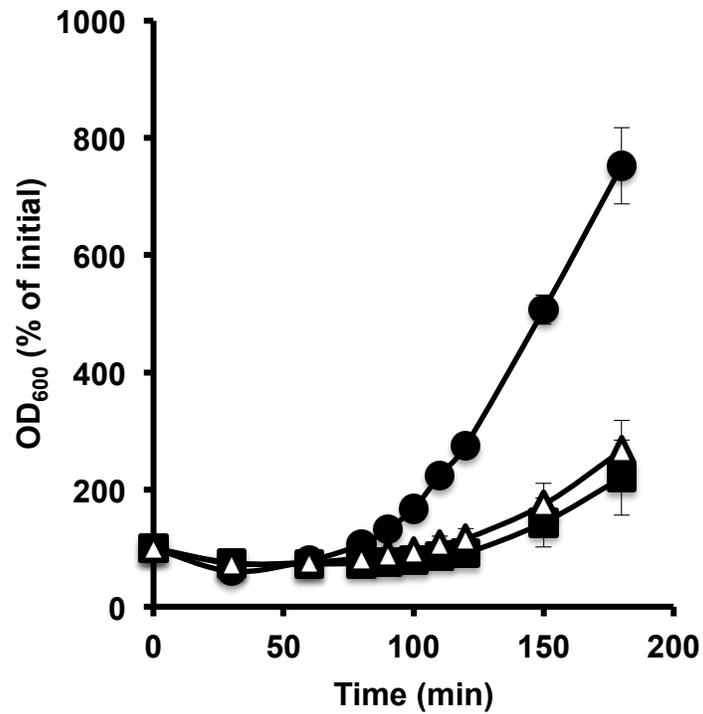


Fig. 7.7. Outgrowth of spores of *C. perfringens* strains. Heat-activated spores of *C. perfringens* SM101 (wild type) (filled circles), PU101 (filled squares), and PU102 (empty triangles) were incubated anaerobically in TGY vegetative medium at 37 °C, and the OD₆₀₀ was monitored as described in Materials and methods. Data are averages of three experiments with two different spore preparations.

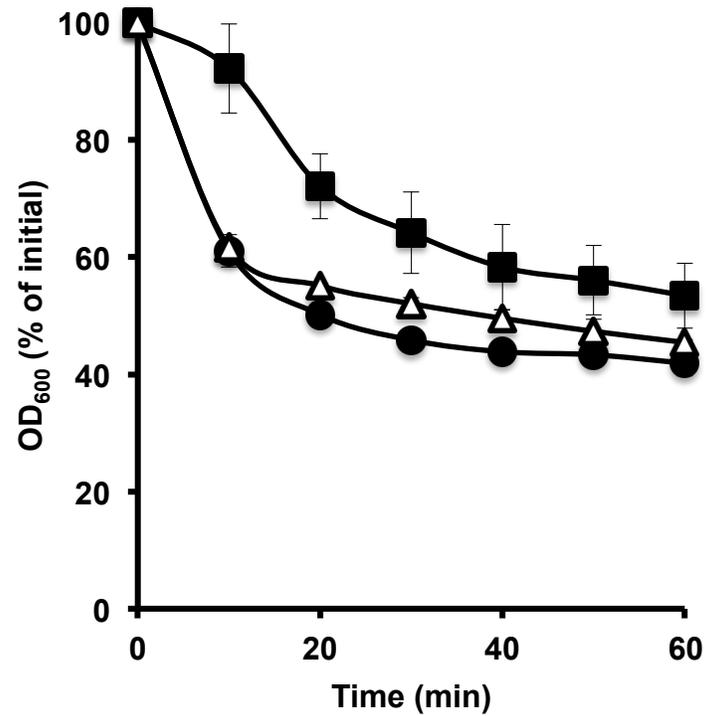


Fig. 7.8. Germination of *C. perfringens* spores in BHI broth. Heat-activated spores of *C. perfringens* SM101 (filled circles), PU101 (filled squares), and PU102 (empty triangles) were incubated at 40 °C with BHI broth, and the OD₆₀₀ was measured as described in Materials and methods. All values are averages of three experiments.

CHAPTER 8

General Conclusion

The enterotoxigenic *Clostridium perfringens* is an important human GI pathogen causing *C. perfringens* type A FP and NFB human GI diseases. The ability of this pathogenic bacterium to form highly resistant spores to a variety of environmental insults makes an effort to reduce or eliminate contaminated spores from food products and food processing environments to be very difficult. Due to the fact that dormant *C. perfringens* spores could return to actively growing cells in a short period of time via the process of spore germination and outgrowth. This couples with the fast growing rate of *C. perfringens* allowing the organism to rapidly multiply to the infectious dose. In an attempt to overcome these problems, five studies were performed to develop the spore inactivation strategies in meat products as well as on food contact surfaces, to examine compounds influencing germination of CPE-producing *C. perfringens* spores, and to identify and characterize the roles of putative sensor histidine kinases in the sporulation and germination processes of *C. perfringens*.

In the first study, we examined the inhibitory effect of nisin against the CPE-producing *C. perfringens* FP and NFB GI disease isolates, which carry *cpe* gene on a chromosome and on a large plasmid, respectively. Also, an interest is paid on evaluating the potential of nisin as antimicrobial agent in cooked meat stored under abusive condition. *C. perfringens* spore germination in rich medium was not affected

by the presence of nisin. In contrast, the germinating spores of *C. perfringens* were highly susceptible to nisin during outgrowth in which spores of NFB isolates possess higher resistance property to nisin than FP isolates. Moreover, nisin at concentration higher than 10 μ M was required to inhibit vegetative growth of *C. perfringens* in laboratory medium and vegetative cultures of NFB isolates except for strain F4969 exhibited a higher resistance to nisin than vegetative cultures of six FP isolates. However, the antimicrobial efficacy of nisin was dramatically decreased when incorporated into meat model systems. Only slightly inhibition of FP spores was observed in contaminated beef samples supplemented with nisin at \sim 4 folds concentration higher than maximum allowable level after 12 h abusive storage. Strikingly, NFB spores were not inactivated and reached similar log CFU/g as the control sample without nisin after 12-h anaerobic incubation with all tested nisin concentrations. Experiments in poultry meat samples gave similar results. Since nisin lacks the ability to control germination and outgrowth of *C. perfringens* spores in meat model system, caution should be considered carefully when utilizing nisin as a food preservative in meat containing products, at least against *C. perfringens*.

The second study was focused on the development of an inactivation strategy against spores of CPE-producing *C. perfringens* type A adhered to the stainless steel (SS) coupons as a model for food contact surfaces. This strategy is based on inducing spore to germinate before inactivating with a variety of commonly used surface disinfectants including 70% (v/v) ethanol, 200 ppm Quaternary Ammonium compounds (Quats), 12.5 and 25 ppm iodophores, and 5% (v/v) DECON-SPORE 200

PLUS[®]. We first optimized condition to trigger maximal germination of spores of *C. perfringens* FP and NFB isolates at 40 °C and 20 ± 2 °C (room temperature; RT). The combination of 100 mM L-asparagine and KCl at pH 7.0 (AK) is the most effective universal germinant for spores of both isolate types. Interestingly, germination temperature had significant impact on *C. perfringens* spore germination, with 40 °C induced higher germination than RT; and NFB spores could not germinate at RT with all tested germination-triggering compounds. Inducing spore germination with AK for 30 min prior to killing germinated spores significantly enhanced the sporicidal activity of all tested disinfecting agents against *C. perfringens* FP strain SM101. Nevertheless, significantly less inhibitory effect was observed against germinated spores of NFB strain NB16. Once spores were attached on SS surfaces, FP spores could survive and germinate with AK, whereas NFB spores remained viable but could not initiate significant germination under the same experimental conditions. Inducing adhered *C. perfringens* FP spores on SS chips for 30 min at 40 °C followed by decontamination with disinfectant significantly lowered the FP spores by 1.53 to 2.70 log CFU/chip. Collectively, triggering spore germination considerably increased sporicidal activity of the commonly used disinfectants against *C. perfringens* FP spores attached to SS chips. Results in this study should aid in developing an effective strategy to reduce the risk of *C. perfringens* spore cross-contamination from food contact surfaces.

Spore germination is a prerequisite step for *C. perfringens* to cause diseases. Thus, understanding factors influencing spore germination is an important basis for developing spore control measures. In the third study, the co-germinant sodium ion

and inorganic phosphate (NaPi) was evaluated for its ability to induce germination of spores of enterotoxigenic *C. perfringens* type A. Initially, results demonstrated that 100 mM NaPi at pH ranges of 5.0 to 6.5 could trigger *C. perfringens* SM101 (FP isolate) spore germination with maximal rate of germination was achieved at pH 6.0. On the contrary, 100 mM NaPi was unable to induce germination of *C. perfringens* F4969 (NFB isolate) spores at all tested pHs. Moreover, NaPi at pH 6.0 is the universal germinant for spores of most *C. perfringens* FP isolates, but none of the tested NFB isolate spores germinated with NaPi. The role of germinant receptors involving NaPi-induced germination of *C. perfringens* SM101 spores was determined. Spores of strain mutation in *gerKB* germinated with significantly slower rate, but the extent of germination was similar to wild type spores. Severe germination defect was observed with spores lacking GerKA-KC and GerAA proteins. Moreover, *gerKA-KC* and *gerAA* spores released significantly less DPA during NaPi-triggered germination than wild type spores suggesting the major role of GerKA-KC and GerAA in NaPi germination, while GerKB may play an auxiliary role. The putative antiporters GerO and GerQ had been evaluated for their function in NaPi germination of *C. perfringens* SM101 spores. Mutations in these antiporter proteins resulted in less germination than the parental spores with more pronounced effect observed in spores lacking GerO. Moreover, strain mutated in both *gerO* and *gerQ* showed the most severe germination defect and release the least amount of DPA upon germination with NaPi (pH 6.0). In sum, this study provided the evidence that NaPi at pH 6.0 is a novel germinant for spores of *C. perfringens* FP isolates and GerKA-KC, GerAA, and GerO are essential

for NaPi-induced spore germination. These could imply the adaptability of *C. perfringens* FP isolates to fit niches of FP environment.

To further improve spore decontamination strategy based on inducing spore germination prior to inactivation by mild treatments, investigations were conducted, in our fourth study, to identify compounds contributing to maximal germination of spores of CPE-producing *C. perfringens* type A. Additionally, GR proteins responsible for sensing these newly identified nutrient germinant were characterized as well. We found that the polar, uncharged amino acids at pH 6.0 could efficiently induce germination of *C. perfringens* spores. While L-asparagine, L-cysteine, L-serine, and L-threonine can efficiently induce germination of spores of most *C. perfringens* FP and NFB isolates; and L-glutamine acted as a unique germinant for FP spores. Furthermore, L-cysteine (pH 6.0) was the strongest germinant for NFB spores. The majority of *C. perfringens* GRs seemed to be required for initial triggering of germination with polar, uncharged amino acids (pH 6.0). For cysteine- or glutamine-induced germination, the germinant receptor mutant *gerKC* spores germinated to a significantly lower extent and released less DPA than wild type spores; however, less pronounced germination defects were observed in *gerAA* and *gerKB* spores. Spores lacking GerKA had severely inferior in germination response to serine and threonine followed by the GerKC-, and GerKB-deficient strains, respectively. Collectively, these results indicates that like the previously known germinants for *C. perfringens* spores, most of GR proteins seemed to be necessary for normal germination of spores with all polar, uncharged amino acids.

Lastly, the fifth study was conducted to identify and characterize putative sensor histidine kinases in *C. perfringens*. We identified six ORFs as putative sporulation-associated sensor histidine kinases in the genome of *C. perfringens* SM101 in which all contain the unique domains specified to the family of histidine kinase protein. The expressions of these putative kinases were highly induced under sporulation condition. Two knock out mutants of sensor histidine kinase genes, *cpr1728* and *cpr1055*, were constructed to determine their roles in various aspects in the life cycle of *C. perfringens*. Deletion of *cpr1728* or *cpr1055* did not affect vegetative growth of *C. perfringens*; however, mutant cells grown under sporulation condition showed a morphological difference as well as higher growth rate than their parental strain. Furthermore, the spore forming capability of strains lacking CPR1728 and CPR1055 were severely hampered in DS sporulation medium. Sporulation defect was also observed with *cpr1728*-deletion strain in modified sporulation medium and mutation in *cpr1055* yielded a delayed sporulation phenotype. Surprisingly, the putative kinases CPR1728 and CPR1055 also had a role during *C. perfringens* spore germination with nutrient and non-nutrient germinants. Both CPR1728 and CPR1055 were also required for normal spore outgrowth in rich medium. This current study provides the information on the functions of putative kinases involved in the sporulation and spore germination processes of *C. perfringens*. As sporulation and germination have a major role in the pathogenesis of *C. perfringens*-associated GI diseases, understanding the histidine kinase-associated mechanism(s) of these bacterial

developmental processes could aid in developing therapeutic intervention and control measures to reduce the occurrence of diseases caused by *C. perfringens*.

Bibliography

- Abee, T., Groot, M.N., Tempelarrs, M., Zwietering, M., Moezelaar, R., & Van de Voort, M. (2011). Germination and outgrowth of spores of *Bacillus cereus* group members: Diversity and role of germinant receptors. *Food Microbiology*, *28*, 199-208.
- Abraham, C., Carman, R.J., Hahn, H., & Liesenfeld, O. (2001). Similar frequency of detection of *Clostridium perfringens* and *Clostridium difficile* toxins in patients with antibiotic-associated diarrhea. *European Journal of Clinical Microbiology & Infectious Diseases*, *20*, 676-677.
- Adam, K.H., Brunt, J., Brightwell, G., Flint, S.H., & Peck, M.W. (2011). Spore germination of the psychrotolerant, red meat spoiler, *Clostridium frigidicarnis*. *Letters in Applied Microbiology*, *53*, 92-97.
- Akhtar, S., Paredes-Sabja, D., & Sarker, M.R. (2008). Inhibitory effects of polyphosphates on *Clostridium perfringens* growth, sporulation and spore outgrowth. *Food Microbiology*, *25*, 802-808.
- Akhtar, S., Paredes-Sabja, D., Torres, J.A., & Sarker, M.R. (2009). Strategy to inactivate *Clostridium perfringens* spores in meat products. *Food Microbiology*, *26*, 272-277.
- Alasri, A., Valverde, M., Rogues, C., Michel, G., Cabassud, C., & Aptel, P. (1993). Sporicidal properties of peracetic acid and hydrogen peroxide, alone and in combination, in comparison with chlorine and formaldehyde for ultrafiltration membrane disinfection. *Canadian Journal of Microbiology*, *39*, 52-60.
- Alberto, F., Broussolle, V., Mason, D.R., Carlin, F., & Peck, M.W. (2003). Variability in spore germination response by strains of proteolytic *Clostridium botulinum* types A, B and F. *Letters in Applied Microbiology*, *36*, 41-45.
- Ali, Y., Dolan, M.J., Fendler, E.J., & Larson, E.L. (2001). Alcohols. In: Block, S.S. (Ed.), *Disinfection, sterilization, and preservation* (pp. 229-254). Philadelphia: Lippincott Williams & Wilkins.
- Anderson, K.G., Hansen, T.B., & Knøchel, S. (2004). Growth of heat treated enterotoxin-positive *Clostridium perfringens* and the implications for safe cooling rates. *Journal of Food Protection*, *67*, 83-89.
- Ando, Y. (1974). Ionic germination of spores of *Clostridium perfringens* type A. *Japanese Journal of Microbiology*, *18*, 433-439.
- Ando, Y., & Tsuzuki, T. (1986a). Changes in decoated spores of *Clostridium perfringens* caused by treatment with some enzymatic and non-enzymatic systems. *Letters in Applied Microbiology*, *3*, 61-64.
- Ando, Y., & Tsuzuki, T. (1986b). The effect of hydrogen peroxide on spores of *Clostridium perfringens*. *Letters in Applied Microbiology*, *2*, 65-68.
- André, G., Haudecoeur, E., Monot, M., Ohtani, K., Shimizu, T., Dupuy, B., & Martin-Verstraete, I. (2010). Global regulation of gene expression in response to cysteine availability in *Clostridium perfringens*. *BMC Microbiology*, *10*, 234.

- André, S., Hédin, S., Remize, F., & Zuber, F. (2012). Evaluation of peracetic acid sanitizers efficiency against spores isolated from spoiled can in suspension and on stainless steel surfaces. *Journal of Food Protection*, *75*, 371-375.
- Antoniewski, C., Savelli, B., & Stragier, P. (1990). The *SpoIIJ* gene, which regulates early development steps in *Bacillus subtilis*, belongs to a class of environmentally responsive genes. *Journal of Bacteriology*, *172*, 86-93.
- Aristoy, M.-C., & Toldrá, F. (1998). Concentration of free amino acids and dipeptides in porcine skeletal muscles with different oxidative patterns. *Meat Science*, *50*, 327-332.
- Asha, N.J., & Wilcox, M.H. (2002). Laboratory diagnosis of *Clostridium perfringens* antibiotic-associated diarrhoea. *Journal of Medical Microbiology*, *51*, 891-894.
- Atluri, S., Ragkousi, K., Cortezzo, D.E., & Setlow, P. (2006). Cooperativity between different nutrient receptors in germination of spores of *Bacillus subtilis* and reduction of this cooperativity by alterations in the GerB receptor. *Journal of Bacteriology*, *188*, 28-36.
- Bae, Y.M., & Lee, S.Y. (2012). Inhibitory effects of UV treatment and a combination of UV and dry heat against pathogens on stainless steel and polypropylene surfaces. *Journal of Food Science*, *71*, M61-M64.
- Baker, Z., Harrison, R.W., & Miller, B.F. (1941). Action of synthetic detergents on metabolism of bacteria. *Journal of Experimental Medicine*, *73*, 249.
- Baldry, M.G.C., & Fraser, J.A.L. (1988). Disinfection with peroxygens. *Critical Reports in Applied Chemistry* *22*, 91-116.
- Banerjee, M., & Sarkar, P.K. (2004). Antibiotic resistance and susceptibility to some food preservative measures of spoilage and pathogenic micro-organisms from spices. *Food Microbiology*, *21*, 335-342.
- Berg, J.M., Tymoczko, J.L., & Stryer, L. (2006). Protein composition and structure. In: Berg, J.M., Tymoczko, J.L., Stryer, L. (Eds.), *Biochemistry* (pp. 25-64). New York: W.H. Freeman and Company.
- Bhatti, M.A., Veeramachaneni, A., & Shelef, L.A. (2004). Factors affecting the antilisterial effects of nisin in milk. *International Journal of Food Microbiology*, *97*, 215-219.
- Blatchley, E.R., Meusen, A., Aronson, A.I., & Brewster, L. (2005). Inactivation of *Bacillus* spores by ultraviolet or gamma radiation. *Journal of Environmental Engineering*, *131*, 1245-1252.
- Block, S.S. (2001). Peroxygen compounds. In: Block, S.S. (Ed.), *Disinfection, sterilization, and preservation* (pp. 185-204). Philadelphia: Lippincott Williams & Wilkins.
- Borriello, S.P., Larson, H.E., Welch, A.R., Barclay, F., Stringer, M.F., & Bartholomew, B.A. (1984). Enterotoxigenic *Clostridium perfringens*: a possible cause of antibiotic-associated diarrhea. *Lancet*, *1*, 305-307.
- Boyd, M.J., Logan, M.A., & Tytell, A.A. (1948). The growth requirements of *Clostridium perfringens* (welchii) BP6K. *Journal of Biological Chemistry*, *174*, 1013-1025.

- Boziaris, I.S., & Adams, M.R. (1999). Effect of chelators and nisin produced in situ on inhibition and inactivation of Gram negatives. *International Journal of Food Microbiology*, 53, 105-113.
- Breukink, E., & de Kruijff, B. (1999). The lantibiotic nisin, a special case or not? *Biochimica et Biophysica Acta*, 1462, 223-234.
- Breukink, E., & de Kruijff, B. (2006). Lipid II as a target for antibiotics. *Nature Reviews Drug Discovery*, 5, 321-332.
- Broda, D.M. (2007). The effect of peroxyacetic acid - based sanitizer, heat and ultrasonic waves on the survival of *Clostridium estertheticum* spores *in vitro*. *Letters in Applied Microbiology*, 45, 336-341.
- Broussolle, V., Gauillard, F., Nguyen-The, C., & Carlin, F. (2008). Diversity of spore germination in response to inosine and L-alanine and its interaction with NaCl and pH in the *Bacillus cereus* group. *Journal of Applied Microbiology*, 105, 1081-1090.
- Brown, D.P., Ganova-Raeva, L., Green, B.D., Wilkinson, S.R., Young, M., & Youngman, P. (1994). Characterization of spo0A homologues in diverse *Bacillus* and *Clostridium* species identifies a probable DNA-binding domain. *Molecular Microbiology*, 14, 411-426.
- Brown, K.L. (2000). Control of bacterial spores. *British Medical Bulletin*, 56, 158-171.
- Brunsing, R.L., La Clair, C., Tang, S., Chiang, C., Hancock, L.E., Perego, M., & Hoch, J.A. (2005). Characterization of sporulation histidine kinases of *Bacillus anthracis*. *Journal of Bacteriology*, 187, 6972-6981.
- Brynstad, S., & Granum, P.E. (2002). *Clostridium perfringens* and foodborne infections. *International Journal of Food Microbiology*, 74, 195-202.
- Brynstad, S., Synstad, B., & Granum, P.E. (1997). The *Clostridium perfringens* enterotoxin gene is on a transposable element in type A human food poisoning strains. *Microbiology*, 143, 2109-2115.
- Buchanan, C.E., & Gustafson, A. (1992). Mutagenesis and mapping of the gene for a sporulation-specific penicillin-binding protein in *Bacillus subtilis*. *J Bacteriol*, 174, 5430-5435.
- Burbulys, D., Trach, K.A., & Hoch, J.A. (1991). Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. *Cell*, 64, 545-552.
- Buzby, J.C., & Roberts, T. (1997). Economic costs and trade impacts of microbial foodborne illness. *World Health Statistics Quarterly*, 50, 57-66.
- Cabrera-Martinez, R.M., Tovar-Rojo, F., Vepachedu, V.R., & Setlow, P. (2003). Effects of overexpression of nutrient receptors on germination of spores of *Bacillus subtilis*. *Journal of Bacteriology*, 185, 2457-2464.
- Carlin, F. (2011). Origin of bacterial spores contaminating foods. *Food Microbiology*, 28, 177-182.
- Carse, W.A., & Locker, R.H. (1974). A survey of pH values at the surface of beef and lamb carcasses, stored in a chiller. *Journal of the Science of Food and Agriculture*, 25, 1529-1535.

- Caserio, G., Stecchini, M., Pastore, M., & Genari, M. (1979). The individual and combined effect of nisin and nitrite on the spore germination of *Clostridium perfringens* in meat mixtures subjected to fermentation. *Ind Aliment*, 18, 894-898.
- Cassier, M., & Sebald, M. (1969). Germination lysozyme-dépendante des spores de *Clostridium perfringens* ATCC3624 après traitement thermique. *Ann Institut Pasteur Actualit*, 117, 312-324.
- Chakrabarti, G., Zhou, X., & McClane, B.A. (2003). Death pathways activated in CaCo-2 cells by *Clostridium perfringens* enterotoxin. *Infection and Immunity*, 71, 4260-4270.
- Chatterjee, C., Paul, M., Xie, L., & van der Donk, W.A. (2005). Biosynthesis and mode of action of lantibiotics. *Chemical Reviews*, 105, 633-683.
- Christie, G., & Lowe, C.R. (2007). Role of chromosomal and plasmid-borne receptor homologues in the response of *Bacillus megaterium* QM B1551 spores to germinants. *Journal of Bacteriology*, 189, 4375-4383.
- Clapp, P.A., Davies, M.J., French, M.S., & Gilbert, B.C. (1994). The bactericidal action of peroxides; on E.P.R. spin-trapping study. *Free Radical Research*, 21, 147-167.
- Clouston, J.G., & Wills, P.A. (1969). Initiation of germination and inactivation of *Bacillus pumilus* spores by hydrostatic pressure. *Journal of Bacteriology*, 97, 684-690.
- Collie, R.E., Kokai-Kun, J.F., & McClane, B.A. (1998). Phenotypic characterization of enterotoxigenic *Clostridium perfringens* isolates from non-foodborne human gastrointestinal diseases. *Anaerobe*, 4, 69-79.
- Collie, R.E., & McClane, B.A. (1998). Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. *Journal of Clinical Microbiology*, 36, 30-36.
- Cords, B.R., Burnett, S.L., Hilgren, J., Finley, M., & Magnusen, J. (2005). Sanitizers: Halogens, surface - active agents, and peroxides. In: Davidson, P.M., Sofos, J.N., Branen, A.L. (Eds.), *Antimicrobials in food* (pp. 507-572). Boca Raton: CRC Press.
- Cornillot, E., Saint-Joanis, B., Daube, G., Katayama, S., Granum, P.E., Canard, B., & Cole, S.T. (1995). The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. *Molecular Microbiology*, 15, 639-647.
- Cotter, P.D., Hill, C., & Ross, R.P. (2005). Bacteriocins: developing innate immunity for food. *Nature*, 3, 777-788.
- Cousins, C.M., & Allan, C.D. (1967). Sporicidal properties of some halogens. *Journal of Applied Bacteriology*, 30, 168-174.
- Cowan, A.E., Koppel, D.E., Setiawan, B., & Setlow, P. (2003). A cytoplasmic protein is immobile in the cytoplasm of dormant spores of *Bacillus subtilis*: implications of spore dormancy. *Proceedings of the National Academy of Sciences* 100, 4209-4214.

- Czczulin, J.R., Collie, R.E., & McClane, B.A. (1996). Regulated expression of *Clostridium perfringens* enterotoxin in naturally *cpe*-negative type A, B, and C isolates of *Clostridium perfringens*. *Infection and Immunity*, *64*, 3301-3309.
- Czczulin, J.R., Hanna, P.C., & McClane, B.A. (1993). Cloning, nucleotide sequencing, and expression of the *Clostridium perfringens* enterotoxin gene in *Escherichia coli*. *Infection and Immunity*, *61*, 3429-3439.
- Davies, E.A., & Delves-Broughton, J. (2000). Nisin. In: Batt, C.A. Patel, P.D. (Eds.), *Encyclopedia of food microbiology* (pp. 191-198). London: Academic Press.
- Davis, J.G. (1962). Iodophores as detergent-sterilizers *Journal of Applied Bacteriology*, *25*, 195.
- Dawson, L.F., Valiente, E., Donahue, E.H., Birchenough, G., & Wren, B.W. (2011). Hypervirulent *Clostridium difficile* PCR-ribotypes exhibit resistance to widely used disinfectants. *PLoS ONE*, *6*, e25754.
- de Jong, I.G., Veening, J.-W., & Kuipers, O.P. (2010). Heterochronic phosphorelay gene expression as source of heterogeneity in *Bacillus subtilis* spore formation. *Journal of Bacteriology*, *192*, 2053-2067.
- Deguchi, A., Miyamoto, K., Kuwahara, T., Miki, Y., Kaneko, I., Li, J., McClane, B.A., & Akimoto, S. (2009). Genetic characterization of type A enterotoxigenic *Clostridium perfringens* strains. *PLoS ONE*, *4*, e5598.
- Delves-Broughton, J. (2005). Nisin as a food preservative. *Food Australia*, *57*, 525-527.
- Duncan, C.L., & Strong, D.H. (1968). Improved medium for sporulation of *Clostridium perfringens*. *Applied Microbiology*, *16*, 82-89.
- Duncan, C.L., Strong, D.H., & Sebald, M. (1972a). Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. *Journal of Bacteriology*, *110*, 378-391.
- Duncan, C.L., Strong, D.H., & Sebald, M. (1972b). Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. *Journal of Bacteriology*, *110*, 378-391.
- Duncan, C.L., Strong, D.H., & Sebald, M. (1972c). Sporulation and enterotoxin production by mutants of *Clostridium perfringens*. *Journal of Bacteriology*, *110*, 378-391.
- Durban, E., Goodnow, R., & Grecz, N. (1970). Changes in resistance to radiation and heat during sporulation and germination of *Clostridium botulinum* 33A. *Journal of Bacteriology*, *102*, 590-592.
- Dürre, P. (2011). Ancestral sporulation initiation. *Molecular Microbiology*, *80*, 584-587.
- Dürre, P., & Hollerschwandner, C. (2004). Initiation of endospore formation in *Clostridium acetobutylicum*. *Anaerobe*, *10*, 69-74.
- Eastoe, J.E., & Long, J.E. (1959). The effect of nisin on the growth of cells and spores of *Clostridium welchii* in gelatine. *Journal of Applied Bacteriology*, *22*, 1-7.
- Errington, J. (2003). Regulation of endospore formation in *Bacillus subtilis*. *Nature Reviews Microbiology*, *1*, 117-126.

- Fabret, C., Feher, V.A., & Hoch, J.A. (1999). Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. *Journal of Bacteriology*, *181*, 1975-1983.
- Faille, C., Fontaine, F., & Benezech, T. (2001). Potential occurrence of adhering living *Bacillus* spores in milk product processing lines. *Journal of Applied Microbiology*, *90*, 892-900.
- Faille, C., Jullien, C., Fontaine, F., Bellon-Fontaine, M.-N., Slomianny, C., & Benezech, T. (2002). Adhesion of *Bacillus* spores and *Escherichia coli* cells to inert surfaces: role of surface hydrophobicity. *Canadian Journal of Microbiology*, *48*, 728-738.
- Fawcett, P., Eichenberger, P., Losick, R., & Youngman, P. (2000). The transcriptional profile of early to middle sporulation in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences* *97*, 8063-8068.
- Foerster, H.F., & Foster, J.W. (1966). Response of *Bacillus* spores to combinations of germinative compounds. *Journal of Bacteriology*, *91*, 1168-1177.
- Fowler, G.G. (1979). The potential of nisin. *Food Manuf*, *54*, 57-59.
- Fowler, G.G., & McCann, B. (1971). The growing use of nisin in the dairy industry. *Australian Journal of Dairy Technology*, *26*, 44-46.
- Fujita, K., Katahira, J., Horiguchi, Y., Sonoda, N., Furuse, M., & Tsukita, S. (2000). *Clostridium perfringens* enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein. *FEBS Letters*, *476*, 258-261.
- Fujita, M., González-Pastor, J.E., & Losick, R. (2005). High- and low- threshold genes in the Spo0A regulon of *Bacillus subtilis*. *Journal of Bacteriology*, *187*, 1357-1368.
- Fujita, M., & Losick, R. (2003). The master regulator for entry into sporulation in *Bacillus subtilis* becomes a cell-specific transcription factor after asymmetric division. *Genes & Development*, 1166-1174.
- Garmory, H.S., Chanter, N., French, N.P., Bueschel, D., Songer, J.G., & Titball, R.W. (2000). Occurrence of *Clostridium perfringens* beta2-toxin amongst animals, determined using genotyping and subtyping PCR assays. *Epidemiology & Infection*, *124*, 61-67.
- Gottardi, W. (2001). Iodine and iodine compounds. In: Block, S.S. (Ed.), *Disinfection, sterilization, and preservation* (pp. 159-184). Philadelphia: Lippincott Williams & Wilkins.
- Gould, G.W. (2006). History of science-spores: Lewis B Perry Memorial lecture 2005. *Journal of Applied Microbiology*, *101*, 507-513.
- Gould, G.W., Jones, A., & Wrighton, C. (1968). Limitations of the initiation of germination of bacterial spores as a spore control procedure. *Journal of Applied Bacteriology*, *31*, 357-366.
- Greenwood, D.A., Kraybill, H.R., & Schweigert, B.S. (1951). Amino acid composition of fresh and cooked beef cuts. *Journal of Biological Chemistry*, *193*, 23-28.

- Guerlava, P., Nolf, S., & Tholozan, J.L. (1998). Rapid cooling, moderate heat treatment and nisin addition influence cell homeostasis of *Clostridium perfringens* type A. *International Journal of Food Microbiology*, *39*, 195-203.
- Gut, I.M., Blanke, S.R., & van der Donk, W.A. (2011). Mechanism of inhibition of *Bacillus anthracis* spore outgrowth by the lantibiotic nisin. *ACS Chemical Biology*, *6*, 744-752.
- Gut, I.M., Prouty, A.M., Ballard, J.D., van der Donk, W.A., & Blanke, S.R. (2008). Inhibition of *Bacillus anthracis* spore outgrowth by nisin. *Antimicrobial Agents and Chemotherapy*, *52*, 4281-4288.
- Haldenwang, W.G. (1995). The sigma factors of *Bacillus subtilis*. *Microbiology and Molecular Biology Reviews*, *59*, 1-30.
- Hared, R., Baik, E., & Gash, S. (1963). Efficiency of antiseptic when acting on dried organisms. *British Medical Journal*, *1*.
- Harris, L.M., Welker, N.E., & Papoutsakis, E.T. (2002). Northern, morphological, and fermentation analysis of *spo0A* inactivation and overexpression in *Clostridium acetobutylicum* ATCC824. *Journal of Bacteriology*, *184*, 3586-3597.
- Harrison, B., Raju, D., Garmory, H.S., Brett, M.M., Titball, R.W., & Sarker, M.R. (2005a). Molecular characterization of *Clostridium perfringens* isolates from humans with sporadic diarrhea: evidence for transcriptional regulation of the beta2-toxin-encoding gene. *Applied and Environmental Microbiology*, *71*, 8362-8370.
- Harry, K.H., Zhou, R., Kroos, L., & Melville, S.B. (2009). Sporulation and enterotoxin (CPE) synthesis are controlled by the sporulation-specific sigma factors SigE and SigK in *Clostridium perfringens*. *Journal of Bacteriology*, *191*, 2728-2742.
- Hasper, H.E., Kramer, N.E., Smith, J.L., Hillman, J.D., Zachariah, C., Kuipers, O.P., de Kruijff, B., & Breukink, E. (2006). An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. *Science*, *313*, 1636-1637.
- Hatheway, C.L. (1990). Toxigenic clostridia. *Clinical Microbiology Reviews*, *3*, 66-98.
- Heikinheimo, A., Lindström, M., Granum, P.E., & Korkeala, H. (2006). Humans as reservoir for enterotoxin gene-carrying *Clostridium perfringens* type A. *Emerging Infectious Diseases*, *12*, 1724-1729.
- Heuzenroeder, M., & Johnson, K.D. (1958). Sterilization of chemical agents. *Journal of Pharmaceutical Sciences*, *40*, 944-948.
- Hilbert, D.W., & Piggot, P.J. (2004). Compartmentalization of gene expression during *Bacillus subtilis* spore formation. *Microbiology and Molecular Biology Reviews*, *68*, 234-262.
- Hitchins, A.D., & Slepecky, R.A. (1969). Bacterial sporulation as a modified procaryotic cell division. *Nature*, *223*, 804-807.
- Hoch, J.A. (1993a). The phosphorelay signal transduction pathway in the initiation of *Bacillus subtilis* sporulation. *Journal of Cellular Biochemistry*, *51*, 55-61.
- Hoch, J.A. (1993b). Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis*. *Annual Review of Microbiology*, *47*, 441-465.

- Hoch, J.A. (1995). Control of cellular development in sporulating bacteria by the phosphorelay two-component signal transduction system. In: Hoch, J.A. Silhavy, T.J. (Eds.), *Two-component signal transduction* (pp. 129-144). Washington, DC: American Society for Microbiology.
- Hoch, J.A. (2000). Two-component and phosphorelay signal transduction. *Current Opinion in Microbiology*, 3, 165-170.
- Hofmeister, A.E., Londono-Vallejo, A., Harry, E., Stragier, P., & Losick, R. (1995). Extracellular signal protein triggering the proteolytic activation of a developmental transcription factor in *Bacillus subtilis*. *Cell*, 83, 219-226.
- Holah, J.T. (2003). Cleaning and disinfection. In: Lelieveld, H.L.M., Mostert, M.A., Holah, J.White, B. (Eds.), *Hygiene in food processing* (pp. 235-278). Boca Raton, FL: CRC Press.
- Holah, J.T., & Thorpe, R.H. (1990). Cleanability in relation to bacterial retention on unused and abraded domestic sink materials. *Journal of Applied Bacteriology*, 69, 599-608.
- Hornstra, L.M., de Leeuw, P.L.A., Moezelaar, R., Wolbert, E.J., de Vries, Y.P., de Vos, W.M., & Abee, T. (2007). Germination of *Bacillus cereus* spores adhered to stainless steel. *International Journal of Food Microbiology*, 116, 367-371.
- Hornstra, L.M., de Vries, Y.P., Wells-Bennik, M.H., de Vos, W.M., & Abee, T. (2006). Characterization of germination receptors of *Bacillus cereus* ATCC 14579. *Applied and Environmental Microbiology*, 72, 44-53.
- Hotchkiss, R.D. (1946). The nature of bactericidal action of surface active agents. *Annals of the New York Academy of Sciences*, 46, 479.
- Huang, I.H., & Sarker, M.R. (2006). Complementation of a *Clostridium perfringens* *spo0A* mutant with wild-type *spo0A* from other *Clostridium* species. *Applied Environmental Microbiology*, 72, 6388-6393.
- Huang, I.H., Waters, M., Grau, R.R., & Sarker, M.R. (2004). Disruption of the gene (*spo0A*) encoding sporulation transcription factor blocks endospore formation and enterotoxin production in enterotoxigenic *Clostridium perfringens* type A. *FEMS Microbiology Letters*, 233, 233-240.
- Hudson, K.D., Corfe, B.M., Kemp, E.H., Feavers, I.M., Coote, P.J., & Moir, A. (2001). Localization of GerAA and GerAC germination proteins in the *Bacillus subtilis* spore. *Journal of Bacteriology*, 183, 4317-4322.
- Husmark, U., & Rönner, U. (1992). The influence of hydrophobic, electrostatic, and morphologic properties on the adhesion of *Bacillus* spores. *Biofouling*, 5, 335-344.
- Indest, K.J., Buchholz, W.G., Faeder, J.R., & Setlow, P. (2009). Workshop report: Modelling the molecular mechanism of bacterial spore germination and elucidating reasons for germination heterogeneity. *Journal of Food Science*, 74, R73-R78.
- Ireland, J.A., & Hanna, P.C. (2002). Amino acid- and purine ribonucleoside-induced germination of *Bacillus anthracis* DeltaSterne endospores: *gerS* mediates responses to aromatic ring structures. *Journal of Bacteriology*, 184, 1296-1303.

- Jay, J.M., Loessner, M.J., & Golden, D.A., 2005. *Modern Food Microbiology*. Springer Science + Business Media ,Inc, San Marcos, California.
- Jiang, M., Shao, W., Perego, M., & Hoch, J.A. (2000). Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. *Molecular Microbiology*, *38*, 535-542.
- Johansson, A., Aspan, A., Bagge, E., Baverud, V., Engstrom, B.E., & Johansson, K.E. (2006). Genetic diversity of *Clostridium perfringens* type A isolates from animals, food poisoning outbreaks and sludge. *BMC Microbiology*, *6*, 47.
- Juneja, V.K., & Thippareddi, H. (2004). Inhibitory effects of organic acid salts on growth of *Clostridium perfringens* from spore inocula during chilling of marinated ground turkey breast. *International Journal of Food Microbiology*, *93*, 155-163.
- Jung, G., & Sahl, H.G., 1991. *Nisin and novel lantibiotics*. Escom, Leiden, The Netherland.
- Keyer, K., Gort, A.S., & Imlay, J.A. (1995). Superoxide and the production of oxidative DNA damage. *Journal of Bacteriology*, *177*, 6782-6790.
- Khadre, M.A., & Yousef, A.E. (2001). Sporicidal action of ozone and hydrogen peroxide: a comparative study. *International Journal of Food Microbiology*, *71*, 131-138.
- Kim, C.H., Cheney, R., & Woodburn, M. (1967). Sporulation of *Clostridium perfringens* in a modified medium and selected foods. *Applied Microbiology*, *15*, 871-876.
- Kobayashi, S., Wada, A., Shibasaki, S., Annaka, M., Higuchi, H., Adachi, K., Mori, N., Ishigawa, T., Masuda, Y., Watanabe, H., Yamamoto, N., Yamaoka, S., & Inamatsu, T. (2008). Spread of a large plasmid carrying the cpe gene and the tcp locus amongst *Clostridium perfringens* isolates from nosocomial outbreaks and sporadic cases of gastroenteritis in a geriatric hospital. *Epidemiology & Infection*, *137*, 108-113.
- Kokai-Kun, J.F., & McClane, B.A. (1997). Deletion analysis of the *Clostridium perfringens* enterotoxin. *Infection and Immunity*, *65*, 1014-1022.
- Kokai-Kun, J.F., Songer, J.G., Czczulin, J.R., Chen, F., & McClane, B.A. (1994). Comparison of Western immunoblots and gene detection assays for identification of potentially enterotoxigenic isolates of *Clostridium perfringens*. *Journal of Clinical Microbiology*, *32*, 2533-2539.
- Kreske, A.C., Ryu, J.H., & Beuchat, L.R. (2006). Evaluation of chlorine, chlorine dioxide, and peroxyacetic acid-based sanitizer for effectiveness in killing *Bacillus cereus* and *Bacillus thuringiensis* spores in suspensions, on the surface of stainless steel, and on apples. *Journal of Food Protection*, *69*, 1892-1903.
- Kroos, L., Zhang, B., Ichikawa, H., & Yu, Y.-T.N. (1999). Control of σ factor activity during *Bacillus subtilis* sporulation. *Molecular Microbiology*, *31*, 1285-1294.
- Kusumaningrum, H.D., Riboldi, G., Hazeleger, W.C., & Beumer, R.R. (2003). Survival of foodborne pathogens on stainless steel surfaces and cross-

- contamination to foods. *International Journal of Food Microbiology*, 85, 227-236.
- Labbe, R. (1980). Relationship between sporulation and enterotoxin production in *Clostridium perfringens* type A. *Food Technology*, 34, 88-90.
- Labbe, R., & Huang, T.H. (1995). Generation times and modeling of enterotoxin-positive and enterotoxin-negative strains of *Clostridium perfringens* in laboratory media and ground beef. *Journal of Food Protection*, 58, 1303-1306.
- Labbe, R.G. (1989). *Clostridium perfringens*. *Food-borne bacterial pathogens*, 192-234.
- Lahti, P., Heikinheimo, A., Johansson, T., & Korkeala, H. (2008). *Clostridium perfringens* type A strains carrying a plasmid-borne enterotoxin gene (genotype IS1151-cpe or IS1470-like-cpe) as a common cause of food poisoning. *Journal of Clinical Microbiology*, 46, 371-373.
- Lawrie, R.A. (1991). Meat and human nutrition. In: Lawrie, R.A. (Ed.), *Meat science* (pp. 225-234). Oxford, England: Pergamon Press.
- LeDeaux, J.R., & Grossman, A.D. (1995). Isolation and characterization of *kinC*, a gene that encodes a sensor histidine kinase homologous to the sporulation sensor kinases KinA and KinB in *Bacillus subtilis*. *Journal of Bacteriology*, 177, 166-175.
- LeDeaux, J.R., Yu, N., & Grossman, A.D. (1995). Different roles for KinA, KinB, and KinC in the initiation of sporulation in *Bacillus subtilis*. *Journal of Bacteriology*, 177, 861-863.
- Leggett, M.J., McDonnell, G., Denyer, S.P., Setlow, P., & Maillard, J.-Y. (2012). Bacterial spore structures and their protective role in biocide resistance. *Journal of Applied Microbiology*, 113, 485-498.
- Leuschner, R.G.K., & Lillford, P.J. (1999). Effect of temperature and heat activation on germination of individual spores of *Bacillus subtilis*. *Letters in Applied Microbiology*, 29, 228-232.
- Levinson, H.S., & Hyatt, M.T. (1970a). Activation energy for glucose-induced germination of *Bacillus megaterium* spores. *Journal of Bacteriology*, 103, 270-271.
- Levinson, H.S., & Hyatt, M.T. (1970b). Effects of temperature on activation, germination, and outgrowth of *Bacillus megaterium* spores. *Journal of Bacteriology*, 101, 58-64.
- Li, J., & McClane, B.A. (2006a). 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. *Applied and Environmental Microbiology*, 72, 7620-7625.
- Li, J., & McClane, B.A. (2006b). Further comparison of temperature effects on growth and survival of *Clostridium perfringens* type A isolates carrying a chromosomal or plasmid-borne enterotoxin gene. *Applied and Environmental Microbiology*, 72, 4561-4568.

- Li, J., & McClane, B.A. (2010). Evaluating the involvement of alternative sigma factor SigF and SigG in *Clostridium perfringens* sporulation and enterotoxin synthesis. *Infection and Immunity*, 78, 4286-4293.
- Lindström, M., Heikinheimo, A., Lahti, P., & Korkeala, H. (2011). Novel insights into the epidemiology of *Clostridium perfringens* type A food poisoning. *Food Microbiology*, 28, 192-198.
- Liu, C., Duan, J., & Su, Y.C. (2006). Effects of electrolyzed oxidizing water on reducing *Listeria monocytogenes* contamination on seafood processing surfaces. *International Journal of Food Microbiology*, 106, 248-253.
- Løvdal, I.S., Hovda, M.B., Granum, P.E., & Rosnes, J.T. (2011). Promoting *Bacillus cereus* spore germination for subsequent inactivation by mild heat treatment. *Journal of Food Protection*, 74, 2079-2089.
- Luciano, F.B., Hossinian, F.S., Beta, T., & Holly, R.A. (2008). Effect of free-SH containing compounds on allyl isothiocyanate antimicrobial activity against *Escherichia coli* O157:H7. *Journal of Food Science*, 73, M214-M220S.
- Lynch, M., Painter, J., Woodruff, R., & Braden, C. (2006). Surveillance for foodborne-disease outbreaks - United States, 1998-2002. *Morbidity and Mortality Weekly Report*, 55, 1-42.
- Marriott, N.G. (1999). Sanitizers. In: Marriott, N.G. (Ed.), *Principles of food sanitation* (pp. 139-157). Gaithersburg: Aspen Publishers, Inc.
- McClane, B.A. (2001). *Clostridium perfringens*. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), *Food Microbiology: fundamentals and frontiers* (pp. 351-372). Washington, D.C.: ASM Press.
- McClane, B.A. (2007a). *Clostridium perfringens*. In: Doyle, M.P., Beuchat, L.R. (Eds.), *Food Microbiology: fundamentals and frontiers* (pp. 423-444). Washington, D.C.: ASM Press.
- McClane, B.A. (2007b). *Clostridium perfringens*. In: Doyle, M.P., Beuchat, L.R. (Eds.), *Food Microbiology Fundamentals and Frontiers* (pp. 423-444). Washington, D.C.: ASM Press.
- McClane, B.A., Lyster, D.J., Moncrief, J.S., & Wilkins, T. (2000). Enterotoxigenic Clostridia. In: Fischetti, V.A., Novick, R., Farretti, J., Potney, D., Rood, J.I. (Eds.), *Gram-positive pathogens*. Washington D.C.: ASM Press.
- McClane, B.A., Uzal, F.A., Miyakawa, M.E.F., Lyster, D., & Wilkins, T. (2006). The enterotoxigenic clostridia. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), *The Prokaryotes* (pp. 698-752). New York: Springer New York.
- McDonnell, G., & Russell, A.D. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clinical Microbiology Reviews*, 12, 147-179.
- McDonnell, J.L. (1986). Toxins of *Clostridium perfringens* type A, B, C, D, and E. In: Dorner, F., Drews, J. (Eds.), *Pharmacology of bacterial toxins* (pp. 477-517). Oxford: Pergamon Press.
- Miki, Y., Miyamoto, K., Kaneko-Hirano, I., Fujiuchi, K., & Akimoto, S. (2008). Prevalence and characterization of enterotoxin gene-carrying *Clostridium*

- perfringens* isolates from retail meat products in Japan. *Applied and Environmental Microbiology*, 74, 5366-5372.
- Miljkovic, V. (1969). Disinfectant properties of peracetic acid. *Mikrobiologija*, 6, 145-149.
- Moir, A. (2006). How do spores germinate? *Journal of Applied Microbiology*, 101, 526-530.
- Moir, A., Corfe, B.M., & Behravan, J. (2002). Spore germination. *Cellular and Molecular Life Sciences*, 59, 403-409.
- Molle, V., Fujita, M., Jensen, S.T., Eichenberger, P., Gonzalez-Pastor, J.E., Liu, J.S., & Losick, R. (2003). The Spo0A regulon of *Bacillus subtilis*. *Molecular Microbiology*, 50, 1683-1701.
- Montville, T.J. (1984). Characterization of a halo-acid-tolerant subpopulations from *Clostridium botulinum* Applied and Environmental Microbiology, 46, 311-316.
- Montville, T.J., De Siano, T., Nock, A., Padhi, S., & Wade, D. (2006). Inhibition of *Bacillus anthracis* and potential surrogate bacilli growth from spore inocula by nisin and other antimicrobial peptides. *Journal of Food Protection*, 69, 2529-2533.
- Montville, T.J., Jones, S.B., Conway, L.K., & Sapers, G.M. (1985). Germination of spores from *Clostridium botulinum* B-aphis and Ba410. *Applied and Environmental Microbiology*, 50, 795-800.
- Mpamugo, O., Donovan, T., & Brett, M.M. (1995). Enterotoxigenic *Clostridium perfringens* as a cause of sporadic diarrhoea. *Journal of Medical Microbiology*, 43, 442-445.
- Munakata, N. (1974). Ultraviolet sensitivity of *Bacillus subtilis* spores upon germination and outgrowth. *Journal of Bacteriology*, 120, 59-65.
- Myers, G.S., Rasko, D.A., Cheung, J.K., Ravel, J., Seshadri, R., DeBoy, R.T., Ren, Q., Varga, J., Awad, M.M., Brinkac, L.M., Daugherty, S.C., Haft, D.H., Dodson, R.J., Madupu, R., Nelson, W.C., Rosovitz, M.J., Sullivan, S.A., Khouri, H., Dimitrov, G.I., Watkins, K.L., Mulligan, S., Benton, J., Radune, D., Fisher, D.J., Atkins, H.S., Hiscox, T., Jost, B.H., Billington, S.J., Songer, J.G., McClane, B.A., Titball, R.W., Rood, J.I., Melville, S.B., & Paulsen, I.T. (2006). Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. *Genome Research*, 16, 1031-1040.
- Nakamura, M., Kato, A., Tanaka, D., Gyobu, Y., Higaki, S., Karasawa, T., & Yamagishi, T. (2004). PCR identification of the plasmid-borne enterotoxin gene (cpe) in *Clostridium perfringens* strains isolates from food poisoning outbreaks. *International Journal of Food Microbiology*, 294, 261-265.
- Nerandzic, M.M., & Donskey, C.J. (2010). Triggering germination represents a novel strategy to enhance killing of *Clostridium difficile* spores. *PLoS ONE*, 5, 1-8.
- Novak, J.S., & Juneja, V. (2002). *Clostridium perfringens*: hazards in new generation foods. *Innovative Food Science & Emerging Technology*, 3, 127-132.
- Novak, J.S., Peck, M.W., Juneja, V.K., & Johnson, E.A. (2005). *Clostridium botulinum* and *Clostridium perfringens*. In: Fratamico, P.M., Bhunia,

- A.K.Smith, J.L. (Eds.), *Foodborne pathogen: Microbiology and molecular biology* (pp. 383-408). Norfolk, UK: Caister Academic Press.
- Paidhungat, M., Ragkousi, K., & Setlow, P. (2001). Genetic requirements for induction of germination of spores of *Bacillus subtilis* by Ca^{2+} -dipicolinate. *Journal of Bacteriology*, *183*, 4886-4893.
- Paidhungat, M., & Setlow, P. (1999). Isolation and characterization of mutations in *Bacillus subtilis* that allow spore germination in the novel germinant D-alanine. *Journal of Bacteriology*, *181*, 3341-3350.
- Paidhungat, M., & Setlow, P. (2001). Localization of a germinant receptor protein (GerBA) to the inner membrane of *Bacillus subtilis* spores. *Journal of Bacteriology*, *183*, 3982-3990.
- Paidhungat, M., & Setlow, P. (2002). Spore germination and outgrowth. In: Hoch, J.A., Losick, R., Sonenshein, A.L. (Eds.), *Bacillus subtilis and its relatives: From Genes to cells* (pp. 537-548). Washington, DC: ASM Press.
- Paik, H.D., Kim, H.J., Nam, K.J., Kim, C.J., Lee, S.E., & Lee, D.S. (2006). Effect of nisin on the storage of *sous vide* processed Korean seasoned beef. *Food Control*, *17*, 994-1000.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009). Role of GerKB in germination and outgrowth of *Clostridium perfringens* spores. *Applied and Environmental Microbiology*, *75*, 3813-3817.
- Paredes, C.J., Alsaker, K.V., & Papoutsakis, E.T. (2005). A comparative genomic view of clostridial sporulation and physiology. *Nature Review Microbiology*, *3*, 969-978.
- Paredes-Sabja, D., Gonzalez, M., Sarker, M.R., & Torres, J.A. (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*, *72*, M202-M206.
- Paredes-Sabja, D., Raju, D., Torres, J.A., & Sarker, M.R. (2008a). Role of small, acid-soluble spore proteins in the resistance of *Clostridium perfringens* spores to chemicals. *International Journal of Food Microbiology*, *122*, 333-335.
- Paredes-Sabja, D., Setlow, B., Setlow, P., & Sarker, M.R. (2008b). Characterization of *Clostridium perfringens* spores that lack SpoVA proteins and dipicolinic acid. *Journal of Bacteriology*, *190*, 4648-4659.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009a). GerO, a putative $\text{Na}^+/\text{H}^+-\text{K}^+$ antiporter, is essential for normal germination of spores of the pathogenic bacterium *Clostridium perfringens*. *Journal of Bacteriology*, *191*, 3822-3831.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009b). The protease CspB is essential for initiation of cortex hydrolysis and dipicolinic acid (DPA) release during germination of spores of *Clostridium perfringens* type A food poisoning isolates. *Microbiology*, *155*, 3464-3472.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009c). Role of GerKB in germination and outgrowth of *Clostridium perfringens* spores. *Applied and Environmental Microbiology*, *75*, 3813-3817.

- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2009d). SleC is essential for cortex peptidoglycan hydrolysis during germination of spores of the pathogenic bacterium *Clostridium perfringens*. *Journal of Bacteriology*, *191*, 2711-2720.
- Paredes-Sabja, D., Setlow, P., & Sarker, M.R. (2011). Germination of spores of *Bacillales* and *Clostridiales* species: mechanisms and protein involved. *Trends in Microbiology*, *19*, 85-94.
- Paredes-Sabja, D., Torres, J.A., Setlow, P., & Sarker, M.R. (2008c). *Clostridium perfringens* spore germination: characterization of germinants and their receptors. *Journal of Bacteriology*, *190*, 1190-1201.
- Paredes-Sabja, D., Udombijitkul, P., & Sarker, M.R. (2009e). Inorganic phosphate and sodium ions are cogerminants for spores of *Clostridium perfringens* type A food poisoning - related isolates *Applied and Environmental Microbiology*, *75*, 6299-6305.
- Parkar, S.G., Flint, S.H., Palmer, J.S., & Brooks, J.D. (2001). Factors influencing attachment of thermophilic bacilli to stainless steel. *Journal of Applied Microbiology*, *90*, 901-908.
- Perego, M., Cole, S.P., Burbulys, D., Trach, K.A., & Hoch, J.A. (1989). Characterization of the gene for a protein kinase which phosphorylates the sporulation-regulatory proteins Spo0A and Spo0F of *Bacillus subtilis*. *Journal of Bacteriology*, *171*, 6187-6196.
- Perego, M., & Hoch, J.A. (2002). Two-component systems, phosphorelays, and regulation of their activities by phosphatase. In: Sonenshein, A.L., Hoch, J.A., Losick, R. (Eds.), *Bacillus subtilis and its closet relatives: From genes to cells* (pp. 473-482). Washington, DC: ASM Press.
- Petit, L., Gilbert, M., & Popoff, M.R. (1999). *Clostridium perfringens*: toxinotype and genotype. *Trends in Microbiology*, *7*, 104-110.
- Philippe, V.A., Mendez, M.B., Huang, I.H., Orsaria, L.M., Sarker, M.R., & Grau, R.R. (2006). Inorganic phosphate induces spore morphogenesis and enterotoxin production in the intestinal pathogen *Clostridium perfringens*. *Infection and Immunity*, *74*, 3651-3656.
- Phillips, A.D., & Strauch, M.A. (2002). *Bacillus subtilis* sporulation and stationary phase gene expression. *Cellular and Molecular Life Sciences*, *59*, 392-402.
- Piggot, P.J., & Coote, J.G. (1976). Genetic aspects of bacterial endospore formation. *Bacteriological Reviews*, *40*, 908-962.
- Piggot, P.J., & Hilbert, D.W. (2004). Sporulation of *Bacillus subtilis*. *Current Opinion in Microbiology*, *7*, 579-586.
- Piggot, P.J., & Losick, R. (2002). Sporulation genes and intercompartmental regulation. In: Sonenshein, A.L., Hoch, J.A., Losick, R. (Eds.), *Bacillus subtilis and its closet relatives* (pp. 483-517). Washington, D.C.: ASM Press.
- Plowman, J., & Peck, M.W. (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. *Journal of Applied Microbiology*, *92*, 681-694.

- Raju, D., Waters, M., Setlow, P., & Sarker, M.R. (2006). Investigating the role of small, acid-soluble spore proteins (SASPs) in the resistance of *Clostridium perfringens* spores to heat. *BMC Microbiology*, 6, 50.
- Ramirez, N., & Abel-Santos, E. (2010). Requirements for germination of *Clostridium sordellii* spores *in vitro*. *Journal of Bacteriology*, 192, 418-425.
- Ramseier, H.R. (1960). The influence of nisin on *Clostridium butyricum*. *Archives of Microbiology*, 37, 57-94.
- Rayman, M.K., Aris, B., & Hurst, A. (1981). Nisin: A possible alternative or adjunct to nitrite in the preservation of meats. *Applied and Environmental Microbiology*, 41, 375-380.
- Reddy Velugoti, P., Rajagopal, L., Juneja, V., & Thippareddi, H. (2007). Use of calcium, potassium, and sodium lactates to control germination and outgrowth of *Clostridium perfringens* spores during chilling of injected pork. *Food Microbiology*, 24, 687-694.
- Rode, L.J., & Foster, J.W. (1962). Ionic germination of spores of *Bacillus megaterium* QM B 1551. *Arch Mikrobiol*, 43, 183-200.
- Rooney, A.P., Swezey, J.L., Friedman, R., Hecht, D.W., & Maddox, C.W. (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.
- Rosenberg, E., Brown, D.R., & Demain, A.L. (1985). The influence of gramicidin S on hydrophobicity of germinating *Bacillus brevis* spores. *Archives of Microbiology*, 142, 51-54.
- Ross, C., & Abel-Santos, E. (2010). The Ger receptor family from sporulating bacteria. *Current Issues in Molecular Biology*, 12, 147-158.
- Rowley, D.B., & Feeherry, F. (1970). Conditions affecting germination of *Clostridium botulinum* 62A spores in chemically defined medium. *Journal of Bacteriology*, 104, 1151-1157.
- Ruhr, E., & Sahl, H.G. (1985). Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. *Antimicrobial Agents and Chemotherapy*, 27, 841-845.
- Russell, A.D. (1990). Bacterial spores and chemical sporicidal agents. *Clinical Microbiology Reviews*, 3, 99-119.
- Russell, A.D., Jones, B.D., & Milburn, P. (1985). Reversal of the inhibition of bacterial spore germination and outgrowth by antibacterial agents. *International Journal of Pharmaceutics*, 25, 105-112.
- Ryter, A. (1965). Etude morphologique de la sporulation de *Bacillus subtilis*. *Ann Institut Pasteur Actuali*, 108, 40-60.
- Ryu, J.H., & Beuchat, L.R. (2005). Biofilm formation and sporulation by *Bacillus cereus* on a stainless steel surface and subsequent resistance of vegetative cells. *Journal of Food Protection*, 68, 2614-2622.
- Sarker, M.R., Carman, R.J., & McClane, B.A. (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. *Molecular Microbiology*, 33, 946-958.
- Sarker, M.R., Shivers, R.P., Sparks, S.G., Juneja, V.K., & McClane, B.A. (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. *Applied and Environmental Microbiology*, 66, 3234-3240.
- Satola, S., Baldus, J.M., & Moran, C.P. (1992). Binding of Spo0A stimulates spoIIIG promoter activity in *Bacillus subtilis*. *Journal of Bacteriology*, 174, 1448-1453.
- Scallan, E., Griffin, P.M., Angulo, F.J., Tauxe, R.V., & Hoekstra, R.M. (2011a). Foodborne illness acquired in the United States - unspecified agents. *Emerging Infectious Diseases*, 17, 16-22.
- Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.-A., Roy, S.L., Jones, J.L., & Griffin, P.M. (2011b). Foodborne illness acquired in the United States - major pathogens. *Emerging Infectious Diseases*, 17, 7-15.
- Scannell, A.G.M., Ross, R.P., Hill, C., & Arendt, E.K. (2000). An effective lacticin biotreservative in fresh pork sausage. *Journal of Food Protection*, 63, 370-375.
- Schweigert, B.S., Guthneck, B.T., Kraybill, H.R., & Greenwood, D.A. (1949). The amino acid composition of pork and lamb cuts. *Journal of Biological Chemistry*, 180, 1077-1083.
- Scott, V.N., & Taylor, S.L. (1981). Effect of nisin on the outgrowth of *Clostridium botulinum* spores. *Journal of Food Science*, 46, 117-126.
- Setlow, B., Loshon, C.A., Genest, P.C., Cowan, A.E., Setlow, C., & Setlow, P. (2002). Mechanisms of killing spores of *Bacillus subtilis* by acid, alkali and ethanol. *Journal of Applied Microbiology*, 92, 362-375.
- Setlow, B., Melly, E., & Setlow, P. (2001). Properties of spores of *Bacillus subtilis* blocked at an intermediate stage in spore germination. *Journal of Bacteriology*, 183, 4894-4899.
- Setlow, B., Peng, L., Loshon, C.A., Li, Y.-Q., Christie, G., & Setlow, P. (2009). Characterization of the germination of *Bacillus megaterium* spores lacking enzymes that degrade the spore cortex. *Journal of Applied Microbiology*, 107, 318-328.
- Setlow, B., & Setlow, P. (1993). Binding of small, acid-soluble spore proteins to DNA plays a significant role in the resistance of *Bacillus subtilis* spores to hydrogen peroxide. *Applied and Environmental Microbiology*, 59, 3418-3423.
- Setlow, P. (2003). Spore germination. *Current Opinion in Microbiology*, 6, 550-556.
- Setlow, P. (2006). Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology*, 101, 514-525.
- Setlow, P., & Johnson, E.A. (2007). *Spores and their significance*. In: Doyle, M.P. Beuchat, L.R. (Eds.), *Food Microbiology: fundamentals and frontiers* (pp. 35-68). Washington, D.C.: American Society for Microbiology.
- Shah, I.M., Laaberki, M.H., Popham, D.L., & Dworkin, J. (2008). A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. *Cell*, 135, 486-496.

- Shi, X., Wegener-Feldbrügge, S., Huntley, S., Hamann, N., Hedderich, R., & Søgaard-Andersen, L. (2008). Bioinformatics and experimental analysis of proteins of two-component systems in *Myxococcus xanthus*. *Journal of Bacteriology*, *190*, 613-624.
- Simmonds, P., Mossel, B.L., Intaraphan, T., & Deeth, H.C. (2003). Heat resistance of *Bacillus* spores when adhered to stainless steel and its relationship to spore hydrophobicity. *Journal of Food Protection*, *66*, 2070-2075.
- Sonenshein, A.L. (2000). Control of sporulation initiation in *Bacillus subtilis*. *Current Opinion in Microbiology*, *3*, 561-565.
- Song, M.S., Kim, C.J., Cho, W.I., & Sunwoo, H.H. (2009). Growth inhibition of *Clostridium perfringens* vegetative cells and spores using chicken immunoglobulin Y. *Journal of Food Safety*, *29*, 511-520.
- Sonnhammer, E.L., von Heijne, G., & Krogh, A., 1998. A hidden Markov model for predicting transmembrane helices in protein sequences, p. 175-182. In: Glasgow, J., Littlejohn, T., Major, F., Lathrop, R., Sankoff, D., Sensen, C. (eds.), Proceedings of the Sixth International Conference on Intelligent Systems for Molecular Biology. AAAI Press, Menlo Park, CA.
- Sparks, S.G., Carman, R.J., Sarker, M.R., & McClane, B.A. (2001). Genotyping of enterotoxigenic *Clostridium perfringens* fecal isolates associated with antibiotic-associated diarrhea and food poisoning in North America. *Journal of Clinical Microbiology*, *39*, 883-888.
- Spiegelman, G.B., Bird, T.H., & Voon, V. (1995). Transcription regulation by the *Bacillus subtilis* response regulator Spo0A. In: Hoch, J.A., Silhavy, T.J. (Eds.), *Two-component signal transduction* (pp. 159-179). Washington, DC: ASM Press.
- Squeglia, F., Marchetti, R., Ruggiero, A., Lanzetta, R., Marasco, D., Dworkin, J., Petoukhov, M., Molinaro, A., Berisio, R., & Silipo, A. (2011). Chemical basis of peptidoglycan discrimination by PrkC, a key kinase involved in bacterial resuscitation from dormancy. *Journal of the American Chemical Society*, *133*, 20676-20679.
- Steiner, E., Dago, A.E., Young, D.I., Heap, J.T., Minton, N.P., Hoch, J.A., & Young, M. (2011). Multiple orphan histidine kinases interact directly with Spo0A to control the initiation of endospore formation in *Clostridium acetobutylicum*. *Molecular Microbiology*, *80*, 641-654.
- Stephenson, K., & Hoch, J.A. (2002). Evolution of signalling in the sporulation phosphorelay. *Molecular Microbiology*, *46*, 297-304.
- Stephenson, K., & Hoch, J.A. (2004). Developing inhibitors to selectively target two-component and phosphorelay signal transduction systems of pathogenic microorganisms. *Current Medicinal Chemistry*, *11*, 765-773.
- Stergiou, V.A., Thomas, L.V., & Adams, M.R. (2006). Interactions of nisin with glutathione in model protein system and meat. *Journal of Food Protection*, *69*, 951-956.

- Stuy, J.H. (1956). Studies on the mechanism of radiation in activation of microorganisms. III. Inactivation of germinating spores of *Bacillus cereus*. *Biochimica et Biophysica Acta*, 22, 241-246.
- Tanaka, D., Isobe, J., Hosorogi, S., Kimata, K., Shimizu, M., Katori, K., Gyobu, Y., Nagai, Y., Yamagishi, T., Karasawa, T., & Nakamura, S. (2003). An outbreak of foodborne gastroenteritis caused by *Clostridium perfringens* carrying the *cpe* gene on a plasmid. *Japanese Journal of Infectious Diseases*, 56, 137-139.
- Tang, T., & Frank, H.A. (1972). Cysteine-induced germination and emergence of putrefactive anaerobe 3679h spores. In: Halvorson, H.O., Hanson, R.Campbell, L.L. (Eds.), *Spore V* (pp. 378-383). Washington, D.C.: American Society for Microbiology.
- Tennen, R., Setlow, B., Davis, K.L., Loshon, C.A., & Setlow, P. (2000). Mechanisms of killing of spores of *Bacillus subtilis* by iodine, glutaraldehyde and nitrous acid. *Journal of Applied Microbiology*, 89, 330-338.
- Thippareddi, H., Juneja, V.K., Phebus, R.K., Marsden, J.L., & Kastner, C.L. (2003). Control of *Clostridium perfringens* germination and outgrowth by buffered sodium citrate during chilling of roast beef and injected pork. *Journal of Food Protection*, 66, 376-381.
- Thomas, L.V., & Delves-Broughton, J. (2005). Nisin. In: Davidsson, P.M., Sofos, J.N.Branen, A.L. (Eds.), *Antimicrobials in food* (pp. 237-274). Boca Raton: CRC Press.
- Thomson, J.E. (1978). Free amino acids in broiler chicken meat in relation to initial pH. *Poultry Science*, 57, 1468-1470.
- Trach, K.A., & Hoch, J.A. (1993). Multisensory activation of the phosphorelay initiating sporulation in *Bacillus subtilis*: identification and sequence of the protein kinase of the alternate pathway. *Molecular Microbiology*, 8, 69-79.
- Trujillo, P., & Laible, N. (1970). Reversible inhibition of spore germination by alcohols. *Applied Microbiology*, 20, 620-623.
- Udompijitkul, P., Paredes-Sabja, D., & Sarker, M.R. (2012). Inhibitory effects of nisin against *Clostridium perfringens* food poisoning and nonfood-borne isolates. *Journal of Food Science*, 77, M51-56.
- Underwood, S., Guan, S., Vijayasubhash, V., Baines, S.D., Graham, L., Lewis, R.J., Wilcox, M.H., & Stephenson, K. (2009). Characterization of the sporulation initiation pathway of *Clostridium difficile* and its role in toxin production. *Journal of Bacteriology*, 191, 7296-7305.
- USDA, & FSIS, 2010. Safe and Suitable Ingredients Used in the Production of meat, poultry, and egg products, FSIS Directive 7120.1 Revision 2, Washington, DC.
- Vaishnavi, C., & Kaur, S. (2008). *Clostridium perfringens* enterotoxin in antibiotic-associated diarrhea. *Indian Journal of Pathology and Microbiology*, 51, 198-199.
- van der Voort, M., Garcí, D., Moezelaar, R., & Abee, T. (2010). Germination receptor diversity and germination responses of four strains of the *Bacillus cereus* group. *International Journal of Food Microbiology*, 139, 108-115.

- Van Opstal, I., Bagamboula, C.F., Vanmuysen, S.C.M., Wuytack, E.Y., & Michiels, C.W. (2004). Inactivation of *Bacillus cereus* spores in milk by mild pressure and heat treatments. *International Journal of Food Microbiology*, *92*, 227-234.
- Vary, J.C., & Halvorson, H.O. (1965). Kinetics of Germination of *Bacillus* Spores. *Journal of Bacteriology*, *89*, 1340-1347.
- Wang, G., Zhang, P., Paredes - Sabja, D., Green, C., Setlow, P., Sarker, M.R., & Li, Y.Q. (2011a). Analysis of the germination of individual *Clostridium perfringens* spores and its heterogeneity. *Journal of Applied Microbiology*, *111*, 1212-1223.
- Wang, G., Zhang, P., Paredes-Sabja, D., Green, C., Setlow, P., Sarker, M.R., & Li, Y.-Q. (2011b). Analysis of the germination of individual *Clostridium perfringens* spores and its heterogeneity. *Journal of Applied Microbiology*, 1-12.
- Wax, R., & Freese, E. (1968). Initiation of the germination of *Bacillus subtilis* spores by a combination of compounds in place of L-alanine. *Journal of Bacteriology*, *95*, 433-438.
- Wen, Q., & McClane, B.A. (2004). Detection of enterotoxigenic *Clostridium perfringens* type A isolates in American retail foods. *Applied and Environmental Microbiology*, *70*, 2685-2691.
- Wiedemann, I., Breukink, E., Van Kraaij, C., Kuipers, O.P., Bierbaum, G., De Kruijff, B., & Sahl, H.G. (2001). Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. *Journal of Biological Chemistry*, *276*, 1772-1779.
- Wolf, J., & Thorley, C.M. (1957). The effects of various germination agents on the spores of some strains of *Bacillus subtilis*. *Journal of Applied Bacteriology*, *20*, 384-389.
- Wolgamott, G.D., & Durham, N.N. (1971). Initiation of spore germination in *Bacillus cereus*: a proposed allosteric receptor. *Canadian Journal of Microbiology*, *17*, 1043-1048.
- Wörner, K., Szurmant, H., Chiang, C., & Hoch, J.A. (2006). Phosphorylation and functional analysis of the sporulation initiation factor Spo0A from *Clostridium botulinum*. *Molecular Microbiology*, *59*, 1000-1012.
- Wrigley, D.M. (2001). *Clostridium perfringens*. In: Hui, Y.H., Pierson, M.D., Gorham, J.R. (Eds.), *Foodborne disease handbook* (pp. 139-168). New York: Marcel Dekker, Inc.
- Xiao, Y., Francke, C., Abee, T., & Wells-Bennik, M.H. (2011). Clostridial spore germination versus bacilli: Genome mining an current insights. *Food Microbiology*, *28*, 266-274.
- Xiao, Y., Wagendorp, A., Moezelaar, R., Abee, T., & Wells-Bennik, M.H. (2012). A wide variety of *Clostridium perfringens* type A food-borne isolates that carry a chromosomal cpe gene belong to one multilocus sequence typing cluster. *Applied and Environmental Microbiology*, *78*, 7060-7068.
- Yasuda-Yasaki, K., Namiki-Kanie, S., & Hachisuka, Y. (1978). Inhibition of *Bacillus subtilis* spore germination by various hydrophobic compounds: demonstration

- of hydrophobic character of the L-alanine receptor site. *Journal of Bacteriology*, 136, 484-490.
- Zhao, Y., & Melville, S.B. (1998). Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (*cpe*) of *Clostridium perfringens*. *Journal of Bacteriology*, 180, 136-142.