You and your partner are cordially invited to attend the public defense of my thesis entitled:

*Clostridium perfringens sporulation, germination and outgrowth in food: a functional genomics approach*

on June 17th 2014 at 16:00 h in the Aula of Wageningen University. Generaal Foulkesweg 1a, 6703BG Wageningen.

After the ceremony you are invited to join me at the reception at the same location and a celebration party held at Sports Pub “De Bongerd” starting from 20:00. Sportscentre, Wageningen UR, Bornsesteeg 2, 6708PE Wageningen.

Yinghua Xiao
yinghua.xiao002@gmail.com

Paranymphs:
Ellen Wemmenhove
ellenwemmenhove@yahoo.com
Arjen Wagendorp
arjen.wagendorp@nizo.com
Clostridium perfringens sporulation, germination and outgrowth in food: a functional genomics approach

Yinghua Xiao
Thesis committee

Promotor
Prof. Dr T. Abee
Personal chair at the Laboratory of Food Microbiology
Wageningen University

Co-promotor
Dr M.H.J. Wells-Bennik
Principle scientist Food Safety
NIZO food research, Ede

Other members
Prof. Dr M. Lindström, University of Helsinki, Finland
Prof. Dr H. Smidt, Wageningen University
Prof. Dr J. Kok, University of Groningen
Dr L.M. Wijnands, The National Institute for Public Health and the Environment (RIVM), Bilthoven

This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Clostridium perfringens sporulation, germination and outgrowth in food: a functional genomics approach

Yinghua Xiao

Thesis
submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 17 June 2014
at 4 p.m. in the Aula.
# Table of contents

**Abstract**

9

**Chapter 1**

General introduction and thesis outline

25

**Chapter 2**

Clostridial spore germination versus bacilli: Genome mining and current insights

47

**Chapter 3**

Genome-wide transcriptional profiling of *Clostridium perfringens* SM101 during sporulation extends the core of putative sporulation genes and genes determining spore properties and germination characteristics

71

**Chapter 4**

A wide variety of *Clostridium perfringens* type A food-borne isolates that carry a chromosomal cpe gene belong to one multilocus sequence typing cluster

91

**Chapter 5**

Differential outgrowth potential of *Clostridium perfringens* food-borne isolates with various cpe-genotypes in vacuum-packed ground beef during storage at 12°C

105

**Chapter 6**

Discussion and conclusions

125

**Samenvatting**

129

**Acknowledgements**

133

**List of publications**

135

**Overview of completed training activities**

137

**About the author**

139
Abstract

At the heart of foodborne disease caused by *Clostridium perfringens* lays its ability to form spores. The ubiquitous presence of *C. perfringens* is due to the fact that spores are resilient and are able to survive harsh environmental conditions. As a result, spores of *C. perfringens* can be found in many food ingredients and are able to survive a range of processing steps in the food industry. Subsequent spore germination in foods - followed by outgrowth - can lead to product spoilage and foodborne illness. Yet, not all *C. perfringens* strains can cause foodborne illness; only certain *C. perfringens* strains that produce enterotoxin (CPE) have this ability. Consumption of high levels (more than 10⁵ CFU/g) of such CPE producing strains can lead to diarrhea when cells sporulate, concomitant with release of the spores and CPE in the gut.

One of the aims of this thesis was to obtain better insight in genes that play a role in the process of sporulation and germination of *C. perfringens* compared with those in the better studied *Bacillus* species. This was achieved through *in silico* analysis of germination genes in the genomes of *Clostridium* and *Bacillus* species. Overall, it was found that the numbers of *ger* genes, encoding germinant receptors of the GerA family, are lower in clostridia than in bacilli. Moreover, various *Clostridium* species are predicted to produce cortex-lytic enzymes that are different from the ones encountered in bacilli.

So far, studying gene function and regulation in clostridia has been hampered by the lack of genetic tools, but novel insights in genes putatively involved in sporulation and germination were obtained through whole genome transcriptome analysis during sporulation. The majority of previously characterized *C. perfringens* germination genes showed significant upregulated expression profiles in time during sporulation. Such upregulated expression profiles during sporulation were also observed for other genes, including *C. perfringens* homologs of *Bacillus* sporulation and germination genes. A comprehensive homology search revealed that approximately half of the upregulated genes are conserved within a broad range of sporeforming Firmicutes; these genes may add to the repertoire of genes with roles in sporulation and determining spore properties including germination behavior.

Food borne disease by *C. perfringens* can only be caused by strains that carry the *cpe* gene, encoding the enterotoxin. This gene can be carried on the chromosome (C-cpe) or on a plasmid (P-cpe). To characterize *C. perfringens* strains present in foods, isolates from a nationwide survey were subjected to 16S rDNA sequence analysis, multi locus sequence typing (MLST) and toxin gene profiling. This revealed that the current standard cultivation method gives false-positive results in ~30% of cases (i.e. species other than *C. perfringens* were identified). Of the confirmed *C. perfringens* isolates, only ~10% carried the *cpe* gene. For these *cpe*-positive strains, the gene was predominantly carried on the chromosome, but P-cpe strains were also found. MLST analysis showed that the C-cpe strains are evolutionarily distant from cpe-negative and P-cpe strains (with the latter two associated with the gut). Overall, these results highlight a need for improved methods to detect *C. perfringens* with higher specificity, ideally simultaneously allowing for discrimination between C-cpe, P-cpe and cpe-negative strains.

The growth potential of cpe-negative, C-cpe or P-cpe strains was further assessed in a model food. Spores were obtained from 15 diverse strains and spore heat resistance was the highest for C-cpe strains. Spores of the individual strains were inoculated in raw minced beef prior to vacuum packaging and heating, and their germination and growth potential was assessed during storage at 12°C and 25°C. This showed lower outgrowth potential of C-cpe strains than of cpe-negative and P-cpe strains at 12°C, suggesting that the cpe-negative and P-cpe strains may have a competitive advantage over C-cpe strains when these are present in products at low storage temperatures (refrigeration abuse temperatures). Overall, the C-cpe strains produce spores that are relatively heat resistant and if such spores survive (insufficient) heat treatments, they may be able to multiply rapidly if cooling regimes are not appropriate. Once the product reaches temperatures below 12°C, growth will be limited. Therefore, rapid cooling is very important and a critical control point. The P-cpe strains, on the other hand, produce spores that are rather heat sensitive and these will normally be inactivated even by relatively low heat treatments. However, if such strains are introduced as a post-processing contamination, for instance by food handlers, and the product is held for prolonged times at abuse refrigeration conditions, they may pose a risk for food borne infections. The post-processing hygienic measures in combination with adequate cooling may therefore be the most critical with respect to control of P-cpe strains.

Overall, this thesis has provided new insights in the genes involved in germination of *C. perfringens* versus the well-known *Bacillus* species, and potential new candidate genes that play a role in sporulation and germination of spore forming Firmicutes were identified. It was apparent that cultivation methods that are currently available for *C. perfringens* rendered a large percentage of false positives. Further genetic analysis of *C. perfringens* isolates obtained from foods showed that C-cpe strains – which are usually associated with food - belong to a cluster that is distinct from P-cpe and cpe-negative strains. Strains belonging to the C-cpe and P-cpe clusters showed different heat resistance characteristics and outgrowth potential in a model food system at low temperatures. This points to different critical control points to prevent foodborne outbreaks due to *C. perfringens*; for C-cpe strains an adequate heat treatment is required to inactivate spores and rapid cooling is needed to prevent outgrowth in the event any spores survived. For P-cpe strains, spores are rather sensitive to heat treatment, but it is particularly important to prevent post-heating contamination and maintain low
Abstract

storage temperatures of products.

Chapter 1

General introduction and thesis outline
1. Foodborne clostridia and their significance

Clostridium is a genus of Gram-positive anaerobic sporeforming bacteria, classified within the phylum Firmicutes. Individual cells are rod-shaped, and their name is derived from the Greek word κλωστήρ (kloster, spindle). The genus Clostridium consists of around 100 species, including important pathogens as well as commercially valuable solventogenic C. butyricum (the type species) and C. acetobutylicum (used for production of acetone, ethanol, and butanol from sugars, starch, even cellulose; the so-called ABE fermentation). Amongst the pathogens, five main species can cause disease in humans, namely, C. botulinum (causing flaccid paralysis), C. perfringens (causing foodborne illness and gas gangrene), C. tetani (causing tetanus), C. difficile (causing antibiotic-associated diarrhea), and, possibly leading to pseudomembranous colitis) and C. sordellii (causing fatal infection in exceptionally rare cases after medical abortions).

Clostridium species are obligate anaerobes and therefore do not thrive in oxygenated environments like their aerobic counterparts, the Bacillus species. Both the anaerobic Clostridium and the aerobic Bacillus are capable of producing dormant endospores which are extremely resistant to environmental stress conditions. This extraordinary property makes that Bacillus and Clostridium species are ubiquitously present in soil, dust, the aquatic environment, the intestines of warm-blooded human and animal hosts, and as a consequence they are routinely isolated from environmental samples and food stuffs. Spores can also be quite resistant to processing and preservation treatments, and in processed foods, spores may survive. If such spores can subsequently germinate, followed by growth of their vegetative cells, this can lead to food spoilage, or – in the case of pathogens – food poisoning upon consumption of such a food. Clostridia have the ability to cause spoilage of a wide range of foods including dairy products, meat and poultry products, fresh and canned fruits and vegetables, typically producing gas and/or putrid odors (details see Table 1.1). Most species typically associated with food spoilage are not known to cause illness, and due to gas production, strong off flavors or tastes, consumers are unlikely to ingest the food product. However, clostridia can also cause foodborne illness; two species – namely C. perfringens and C. botulinum - are most commonly involved in food-borne diseases (EFSA, 2005).

These species can also cause non-foodborne human and veterinary diseases (e.g. gas gangrene and wound botulism). In addition, the C. botulinum neurotoxin is used in the cosmetic field under the commercial name Botox and locally applied to paralyze facial muscles to reduce the signs of aging.

In 2011, 165 foodborne outbreaks caused by C. perfringens, C. botulinum and other clostridia were reported by 15 European Union member states, representing 2.9% of all reported foodborne outbreaks (EFSA, 2013). In the United States, C. perfringens causes an estimated one million illnesses each year, making it the second most common bacterial cause of foodborne illness, whereas the burden of C. botulinum is estimated to be 55 cases per year (Scallan et al., 2011). In Japan, approximately 20 to 40 outbreaks of C. perfringens foodborne disease were identified from 2000 to 2005 and an estimated 4,000 people became sick each year (Miki et al., 2008). In The Netherlands, the overall disease burden caused by C. perfringens is 536 Disability Adjusted

---

1 In names of Clostridium species, the genus name is normally abbreviated as "C.", but in some cases "CL" can be used to avoid confusion with other genus names with "C" as initial letter.

---

### Table 1.1 Foodborne Clostridium species (summarized from Volume Three The Firmicutes in Bergey’s Manual of Systematic Bacteriology (Schleifer, 2009))

<table>
<thead>
<tr>
<th>Specie (types)</th>
<th>Type (reference strain)</th>
<th>Genbank accessions (16S rRNA)</th>
<th>Foodborne isolation sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. algaepurum</td>
<td>DSM 15099</td>
<td>AF127023, X77876</td>
<td>spoiled, vacuum-packed cooked pork</td>
</tr>
<tr>
<td>C. algihydrate</td>
<td>DSM12273</td>
<td>AF092549</td>
<td>vacuum-packed, temperature abused raw lamb</td>
</tr>
<tr>
<td>C. beijerinckii</td>
<td>ATCC 25752</td>
<td>X64179</td>
<td>spoiled candy; olives</td>
</tr>
<tr>
<td>C. butyricum</td>
<td>ATCC 19398</td>
<td>AB05768, M59985</td>
<td>cheese; olives; fruit juice</td>
</tr>
<tr>
<td>C. difficile</td>
<td>ATCC 638</td>
<td>AB05769, X75906</td>
<td>cheese foudne; canned tomatoes; vacuum packed smoked fish</td>
</tr>
<tr>
<td>C. botulinum (A, proteolytic B and F)</td>
<td>ATCC25768(A), L37585 (A), L37593 (F)</td>
<td></td>
<td>improperly preserved vegetables, meat and fish</td>
</tr>
<tr>
<td>C. botulinum (E, saccharolytic B and F)</td>
<td>ATCC 9964 (E), ATCC 25765 (B), ATCC 27321 (F)</td>
<td>X61473 (B)</td>
<td>food</td>
</tr>
<tr>
<td>C. carrisi</td>
<td>ATCC 25777</td>
<td>M39091</td>
<td>milk</td>
</tr>
<tr>
<td>C. esteretheticum</td>
<td>ATCC 51377</td>
<td>Y18813</td>
<td>chill-stored vacuum-packed beef</td>
</tr>
<tr>
<td>C. esteretheticum subsp. esteretheticum</td>
<td>DSM 8809</td>
<td>S46734, X68181</td>
<td>chill-stored vacuum-packed beef</td>
</tr>
<tr>
<td>C. esteretheticum subsp. laramiense</td>
<td>DSM 12271</td>
<td>AF069742</td>
<td>temperature abused vacuum-packed beef</td>
</tr>
<tr>
<td>C. gaugenes</td>
<td>DSM 12272</td>
<td>AF092548</td>
<td>vacuum-packed, chilled lamb that showed &quot;blown-pack&quot; spoilage, which causes gas production and pack distension in vacuum-packed meat</td>
</tr>
<tr>
<td>C. pasteurianum</td>
<td>ATCC 6013</td>
<td>M23930</td>
<td>fruit juice</td>
</tr>
<tr>
<td>C. perfringens</td>
<td>ATCC13124(T)</td>
<td>M59103</td>
<td>food products (usually warm meat or meat products)</td>
</tr>
<tr>
<td>C. putrificum</td>
<td>ATCC 43978</td>
<td>X71857, X73444</td>
<td>rotten potatoes, a cavity spot lesion in a carrot</td>
</tr>
<tr>
<td>C. putrificus</td>
<td>ATCC 25786</td>
<td>AF127024, Y18177</td>
<td>spoiled hams</td>
</tr>
<tr>
<td>C. saepegena</td>
<td>ATCC 25775</td>
<td>M59104</td>
<td>contaminated food</td>
</tr>
<tr>
<td>C. sporogenes</td>
<td>ATCC 3584</td>
<td>M59115</td>
<td>preserved meat and dairy products</td>
</tr>
<tr>
<td>C. sporohausnidae</td>
<td>ATCC 25781</td>
<td>M59116</td>
<td>canned food</td>
</tr>
<tr>
<td>C. typhiuticum</td>
<td>ATCC 25755</td>
<td>M59113</td>
<td>dairy products (semi-hard cheese, late-blowing)</td>
</tr>
<tr>
<td>Tissierella praeacuta (C. hastiforme)**</td>
<td>ATCC 25539 (T)</td>
<td>X80833 (X80841)</td>
<td>milk</td>
</tr>
</tbody>
</table>

** Only one strain name from common public culture collection is recorded here.

* Tissierella praeacuta and C. hastiforme have been proven synonyms, where T. praeacuta has priority (Bae et al., 2004)
Life Years (DALYs), ranking as the fourth bacterial food pathogens in 2009 (Havelaar et al., 2012). The number of cases due to *C. botulinum* is generally low, but given the high mortality and hospitalization rates in the case of *C. botulinum* intoxication, illness caused by this bacterium has enormous financial and personal impact. Moreover, *C. botulinum* contamination of foods can have detrimental effects on food businesses, leading to high costs related to recalls and reputation damage. In early August 2013, a New Zealand dairy company announced that a whey protein concentrate that it produced for baby formula contained *C. botulinum*, leading to recalls of infant formula products. Although it turned out that the contaminant was not *C. botulinum* after all and there have been no reports of any public health issues from this incident, it raised the level of concern for the safety of these products with respect to *C. botulinum* (ICMSF, 2013), and it had a dramatic influence on the sales of major infant formula suppliers in emerging markets, *e.g.* China (Danone, 2013).

Both industry and regulatory authorities have invested significantly in resources that are aimed at the control of *Clostridium* species, particularly the food pathogens in food stuffs. Yet, these organisms are generally less well studied and understood than their aerobic counterparts, the *Bacillus* species. A lot remains to be learned about clostridial pathology, epidemiology and physiology.

The range of methods for the detection of *Clostridium* species in food stuffs is broad and very complex. This is due to the broad variety of *Clostridium* species as well the number of food stuffs in which *Clostridium* species can be encountered, as recently extensively described by Fischer et al. (2012). These methods have historically relied on sulfite reduction for the differentiation between *Clostridium* species and competitive microflora. Most clostridia of interest are able to reduce sulfite, of which *C. perfringens* and *C. botulinum* are the most important species associated with foodborne disease. Therefore, sulfite-reducing clostridia have been used as indicators for the presence of particular pathogenic clostridia and other clostridia that may cause food poisoning and food spoilage. However, in addition to clostridia, other non-sporoforming microorganisms in the competitive flora (*e.g.* *Salmonella* and Proteus) are also able to reduce sulfite. When no pasteurization step is applied to samples before plating (which will detect *C. perfringens* vegetative cells as well as other flora), the term “sulfite-reducing bacteria” (SRB) will be used instead of “sulfite reducing clostridia” (SRC) when only sporeforming bacteria are recovered (see Figure 1.1).

Recently, several reviews on *C. botulinum* have been published (Peck, 2009; Johnson, 2012; Lund et al., 2013). The next sections will mainly focus on *C. perfringens*, the organism central to the work presented in this thesis.

### 2. *C. perfringens* and its specific sporulation-coupled food poisoning pathway

*C. perfringens*, originally known as “*Bacillus aerogenes* capsulatus”, “*Bacillus perfringens*”, or “*Clostridium welchi*”, is a Gram-positive, spore-forming, nonmotile, anaerobic, encapsulated rod-shaped bacterium. If present as spores, *C. perfringens* can survive cooking procedures and is able to multiply rapidly between 37 and 45°C (Heredia and Labbé, 2013). *C. perfringens* commonly inhabits the soil, freshwater sediments and is occasionally isolated from the gastrointestinal tracts of warm-blooded animals including healthy humans.

The importance of *C. perfringens* as a pathogen was recognized during the World War I because it was the primary pathogenic agent causing gas gangrene, leading to the death of injured soldiers. Spores of *C. perfringens* conceivably originating from soil can germinate and grow fast in anaerobic deep wounds, destroying the host tissue by producing exotoxins and generating gas within a few hours. Therefore, *C. perfringens* was also called “flesh-eater” by Prof. Tohru Shimizu 2, who completed and published the first complete genome sequence of *C. perfringens*, gas gangrene isolate Strain 13 (Shimizu et al., 2002). With the introduction of antibiotics, including penicillin to which *C. perfringens* is very sensitive, the incidence of gas gangrene has declined.

Various combinations of more than 18 different toxins and several extracellular hydrolytic enzymes may be produced by different *C. perfringens* strains (Amimoto et al., 2007; McClane, 2007; Keyburn et al., 2008). In general, these virulence factors...
function to establish infection and/or to facilitate the acquisition of essential nutrients from the environment, including the infected hosts (Petit et al., 1999; Levin, 2010). Four major exotoxins, α-, β-, ε- and ι-, are used for classifying all C. perfringens strains into five types (A-E). The assigned type of a given strain depends on the particular combinations of these four toxins (Table 1.2).

<table>
<thead>
<tr>
<th>Type</th>
<th>α-toxin (plc or cpa)</th>
<th>β-toxin (cph1)</th>
<th>ε-toxin (ctx)</th>
<th>ι-toxin (up, ibp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The α-toxin is a multifunctional phospholipase causing hydrolysis of membrane phospholipids, resulting in cell lysis. This toxin is considered the main lethal toxin of C. perfringens. In principal, all C. perfringens strains carry the plc (or cpa) gene that allows for the production of α-toxin, which is essential for the development of gas gangrene. Type A strains produce higher levels of α-toxin than other strains (Albini et al., 2008; Levin, 2010) which makes that type A strains are commonly associated with gas gangrene. This causes mucosal necrosis resulting in central nervous system symptoms in domestic animals. The ε-toxin is a protein toxin responsible for lethal enterotoxaemia in livestock. The ι-toxin increases vascular permeability and is dermonecrotic. An additional toxin β2 is associated with enteric diseases in piglets and horses (McClane et al., 2006; Levin, 2010).

C. perfringens enterotoxin (CPE) causes a common form of foodborne disease, resulting in diarrhea (McClane et al., 2006). The protein was characterized as a single polypeptide comprised of 319 amino acids (M, 35,317) (Czeczulin et al., 1993), and the sequence is highly conserved amongst most of the CPE-positive type A strains (Collie et al., 1998). CPE binds to eukaryotic claudin receptors, forming a small complex. Several small complexes interact to form a ~450 kDa CH-1 prepore. This prepore then inserts into membranes to form an active pore, allowing a Ca2+ influx. Morphological damage caused by alterations in membrane permeability allows for unbound CPE to access to the basolateral surface, resulting in formation a second CH-2 complex (~600 kDa). This large complex can induce tight junction rearrangement and thereby cause massive damage to the intestinal epithelium. One of the physiological responses of the eukaryotic host to toxin exposure is diarrhea.

C. perfringens type A food poisoning due to CPE toxin production is associated with sporulation as illustrated in Figure 1.2. Initially, a food becomes contaminated with a cpe-positive C. perfringens type A isolate (usually in the form of spores of this anaerobic bacterium) through ingredients, processing and handling, or packaging materials. If the food undergoes temperature abuse, the spores can germinate and the emerging vegetative cells can multiply rapidly, followed by possible exposure of the consumer to high numbers of C. perfringens upon ingestion of the contaminated food. Many of the ingested vegetative cells of C. perfringens will die when exposed to stomach acidity, but if the contamination level is more than 10⁶ C. perfringens cells per gram, a fraction of the ingested cells can survive passage through the stomach and remain viable when entering the small intestine, where they multiply and sporulate. Therefore, one of the requirements for implicating C. perfringens in a foodborne outbreak is the presence of more than 10⁵ cells per gram in the incriminated food (CDC, 2000; Heredia and Labbé, 2013). CPE is only expressed and synthesized in sporulating cells. The CPE protein constitutes a significant portion of the total bacterial cell protein and is present in the form of a crystal that is clearly visible by electron microscopy (Labbe, 2005). When sporulating cells lyse to release their endospores, the CPE is released into the intestinal lumen. Once released, CPE quickly binds to intestinal epithelial cells and exerts its action, which induces intestinal tissue damage. The symptoms include abdominal cramps, sometimes with vomiting and fever, and intestinal fluid loss, clinically manifesting as diarrhea (McClane et al., 2012). The symptoms generally develop about 8-18 hours after ingestion of contaminated food and then resolve spontaneously within 12 to 24 hours (McClane et al., 2006). The concomitantly produced spores are flushed out with the diarrheal fluid and may end up in the food chain where they may eventually be taken up by a new host.

![Figure 1.2 Sporulation-coupled food poisoning pathways of C. perfringens enterotoxigenic strains.](image)
C. perfringens possesses several properties that contribute significantly to its ability to cause foodborne illness: (1) a ubiquitous distribution throughout the natural environment, giving it ample opportunity to contaminate foods; (2) the ability to form heat-resistant spores, allowing it to survive incomplete cooking of foods or improper sterilization; (3) the ability of fast growth in foods, allowing it to reach the high levels required to cause food poisoning; and (4) the ability to produce an intestinally active enterotoxin, responsible for the characteristic gastrointestinal symptoms of C. perfringens food poisoning (Heredia and Labbé, 2013). Therefore, understanding of C. perfringens spore formation (sporulation), spore properties including resistance to heat and the ability to germinate, and growth potential in food stuffs is essential for functional strategies to control this pathogenic bacterium. Moreover, insight in the diversity amongst intensively studied reference strains and foodborne isolates is needed.

 Appropriated cooking and cooling temperatures are currently the major control measures to prevent C. perfringens type A food poisoning. Thorough cooking and rapid cooling may be challenging for certain meat products such as large roasts and whole poultry, it is rather difficult to reach the internal temperatures needed to inactivate C. perfringens spores due to the product sizes. Appropriate cooking temperatures, rapid cooling and storage at non-permissive conditions (either refrigeration temperature or temperatures above 58-60°C) are required to prevent C. perfringens foodborne outbreaks (McClane et al., 2012). Accordingly, microbiological performance standards have been put in place by the U.S. Department of Agriculture, Food Safety and Inspection Service (USDA/FSIS) for the production of ready-to-eat and partial cooked meat and poultry products. During cooling, the maximum internal temperature of the product should not remain between 54.4°C and 26.6°C for more than 1.5 hours or between 26.6°C and 4.4°C for more than 5 hours (USDA/FSIS, 1999). Specifically, the objective “no more than 1-log growth of C. perfringens and no growth of C. botulinum may occur during product stabilization” should be achieved for all RTE meat and poultry products (USDA/FSIS, 2001; Haneklaus et al., 2011). This guideline is also given by EFSA within Europe (EFSA, 2005).

3. Bacterial spore formation and spore properties

The endospore is a metabolically dormant and environmentally resistant form of living organisms, capable of surviving extremes of temperature, desiccation and ionizing radiation. Estimates of endospore longevity range from thousands to millions of years, although it is more likely on the lower end of that range. Figure 1.3 shows the taxonomy of spore-forming bacteria. Following recent developments in taxonomic methodology and the reclassification of the Bacillus genus, aerobic endospore-forming bacteria alone embrace more than 25 genera and over 200 species nowadays (Fritze, 2004). The reclassification of anaerobic spore formers has been proposed recently (Yutin and Galperin, 2013), but is not included in Figure 1.3.
Sporulation, which is the process of bacterial spore formation, is generally characterized by an asymmetric cell division, generating a mother cell and a smaller forespore that carries its own genome. The mother cell ultimately engulfs the forespore, as a cell in a cell (see Figure 1.4). Sporulation is quite well understood in the *Bacillales* in which sporulation is commonly induced by starvation for one or more nutrients (Setlow, 2013). Via a series of sensor kinases (KinA-E) and a phosphorelay system including Spo0F and Spo0A, cells commit to sporulation. In clostridia, the initiation of sporulation is less well understood; for instance, the genes encoding these kinases and the phosphorelay component Spo0F are usually missing in clostridia (Paredes et al., 2005). Overall, the initiation of sporulation in clostridia is believed to be a more ancestral mechanism and has so far been studied in most detail in *C. acetobutylicum* (Dürre, 2011). As sporulation proceeds, the forespore matures due to gene expression and protein synthesis in both the mother cell and the forespore compartment under the regulation of mother cell specific sporulation sigma factors E and K, and forespore specific sigma factors F and G (see also Figure 1.4). The current detailed understanding of *Bacillus* sporulation has been summarized systematically in a few recent reviews (Higgins and Dworkin, 2012; Setlow and Johnson, 2012).

The various layers of typical *Bacillus* spores are shown in Figure 1.5. Genomic DNA is maintained in the spore core and is saturated by so-called Small acid-soluble proteins (SASPs), which are encoded by *ssp* genes. SASPs are thought to protect spore DNA from various damages and contribute significantly to spore resistance (Setlow, 1992; Setlow, 2007). Another known factor that contributes to spore resistance is Ca$^{2+}$-dipicolinic acid (DPA) which accumulates to high levels during the late stages of sporulation (Li et al., 2012). The spore core is surrounded by the inner membrane, which contains many functional proteins that play a role in spore germination, e.g. germination receptors (Moir, 2003). The next layer is the cortex, consisting mostly of peptidoglycan with extra spore specific muramic δ-lactam residues instead of muramic acid. The cortex is required to maintain the low water activity in the spore core. Cortex degrading enzymes are embedded in different layers of the spore cortex and are required to achieve full spore germination. The cortex is subsequently surrounded by the outer spore membrane, which originates from the mother cell; the composition of this membrane is similar to the regular cell membrane of vegetative cells. Lastly, some spores contain an exosporium. A detailed description of the assembly and functions of the multilayered spore coat is given in a recent review (McKenney et al., 2013).

Spores can rapidly ‘come back to life’ and lose dormancy/resistance. This process is known as germination and can be triggered by various compounds, including specific nutrients, non-nutrient compounds, such as Ca-DPA, dodecylamine and by the application of high hydrostatic pressure. Spore germination involves two major events: upon sensing an environmental germination trigger (nutrient or non-nutrient) the spore core is rehydrated. This event is characterized by a transition of phase bright to phase dark under a phase contrast microscope. This step is concomitant with release of spore core monovalent cations and Ca-DPA. Subsequently, the spore cortex must be degraded by hydrolysis of the spore cortex peptidoglycan, allowing for expansion of the spore core. This leads to a well-hydrated spore protoplast in which metabolism and macromolecular synthesis can start. Subsequently, a vegetative cell can emerge (Setlow, 2013). The similarity and difference between *Bacillus* and *Clostridium* spore germination will be discussed in detail in Chapter 2.

4. Research approach and thesis outline

The work presented in this thesis focused on the essential steps of *C. perfringens* sporulation, spore resistance, germination and growth potential in foods, leading to more insight in potential efficient strategies to control this food pathogen in food stuffs.

Chapter 1 provides an introduction to the current knowledge on foodborne *Clostridium* species and their significance in food production and consumer’s health, followed by the current understanding of spore formation and properties.

At a molecular level the process of spore germination is better understood in *Bacillus* species than in their anaerobic relatives. In Chapter 2, the occurrence of known and putative germination-related genes was analyzed in *Clostridium* species and in a representative number of *Bacillus* species. Overall, the numbers of ger operons encoding germinant receptors were found to be lower in clostridia than in bacilli, and various *Clostridium* species were predicted to produce cortex-lytic enzymes that are different from the ones encountered in *Bacillus*.

In Chapter 3, whole genome transcriptome analysis during sporulation was per-
formed. This confirmed the specific expression of the majority of C. perfringens germination genes during sporulation. Moreover, this result revealed novel putative sporulation genes, which are likely candidates to play a role in sporulation and/or spore properties including germination.

The focus of Chapter 4 laid on the phenotypic and genetic diversity of 98 suspected food-borne C. perfringens isolates that were obtained from a nationwide survey by the Netherlands Food and Consumer Product Safety Authority (NVWA). Based on multi-locus sequence typing (MLST) analysis, we found that chromosomal cpe strains (including food strains and isolates from food poisoning cases) belong to a distinct cluster that is significantly distant from all the plasmid-cpe and cpe-negative strains. These results suggest that different groups of C. perfringens have undergone niche specialization and that a distinct group of food isolates sharing specific genetic backgrounds.

In Chapter 5, the outgrowth of spores derived from 15 different food isolates of C. perfringens, including C-cpe, P-cpe and cpe-negative strains, was evaluated in vacuum-packed ground beef. The C-cpe strains showed lower outgrowth potential in cooked ground beef stored at 12°C than the P-cpe-packed ground beef. The C-cpe strains showed lower outgrowth potential in food isolates from non-foodborne human gastrointestinal diseases. These results suggest that different groups of C. perfringens have undergone niche specialization and that a distinct group of food isolates sharing specific genetic backgrounds.

Finally, Chapter 6 contains the summarizing discussion, concluding remarks and outlook based on the research described in this thesis.

References:


Danone (2013). Danone welcomes the findings of the New Zealand authorities’ investigation of Fonterra which conclude that all ingredients used by Danone are in full compliance with the highest standards of quality. http://www.danone.com/no_cache/en/for-you/journalists/news-press-releases/pdownload/8585/


Chapter 2

Clostridial spore germination versus bacilli:
Genome mining and current insights

Y. Xiao, C. Francke, T. Abee, and M.H.J. Wells-Bennik
Published in Food microbiology (2011), vol. 28(2):266-274
Bacilli and clostridia share the characteristic of forming metabolically inactive endospores. Spores are highly resistant to adverse environmental conditions including heat, and their ubiquitous presence in nature makes them inevitable contaminants of foods and food ingredients. Spores can germinate under favourable conditions, and the following outgrowth can lead to food spoilage and foodborne illness. Germination of spores has been best studied in Bacillus species, but the process of spore germination is less well understood in anaerobic clostridia. This paper describes a genome mining approach focusing on the genes related to spore germination of clostridia. To this end, 12 representative sequenced Bacillus genomes and 24 Clostridium genomes were analyzed for the distribution of known and putative germination-related genes and their homologues. Overall, the number of ger operons encoding germinant receptors is lower in clostridia than in bacilli, and some Clostridium species are predicted to produce cortex-lytic enzymes that are different from the ones encountered in bacilli. The in silico germination model constructed for clostridia was linked to recently obtained experimental data for selected germination determinants, mainly in Clostridium perfringens. Similarities and differences between germination mechanisms of bacilli and clostridia will be discussed.

Key words: spore germination; Clostridium; Bacillus; germinant receptors

1. Introduction

Spore-forming bacteria play an important role in food spoilage and disease (Setlow and Johnson, 2007) and food industries actively employ strategies to ensure adequate inactivation of spores and control outgrowth, both for species that potentially lead to spoilage and foodborne pathogens. With regard to bacterial foodborne disease, two major bacterial spore-forming species, namely B. cereus and C. perfringens, are accountable for an estimated 1.3% and 4.0% of cases, respectively (EFSA, 2005).

Survival and persistence of Bacillus and Clostridium species largely depends on their ability to produce endospores under conditions that are unfavourable for growth, whereas their pathogenicity resides in the fact that the spores can germinate under favourable conditions. The very first stage of the germination process involves sensing specific compounds, named germinants, or can be due to physical factors. Subsequent events required for full germination include the hydrolysis of the cortex peptidoglycan, rehydration of the core, and resumption of metabolic activity and the degradation of small acid-soluble proteins (SASPs) by germination protease GPR (Sussman and Setlow, 1991; Moir, 2006; Setlow, 2008).

The process of spore germination is irreversible and eventually results in a complete and viable vegetative cell. The first detectable events of germination are the release of Zn²⁺, K⁺, Na⁺, dipicolinic acid and Ca²⁺ (Ca-DPA) and a rise in spore internal pH (Paidhungat et al., 2002). Potassium ions are subsequently reabsorbed by an energy-dependent process (Swerdlow et al., 1981). The initial events are accompanied by a loss of spore heat resistance and dormancy, and can be recognized microscopically by the transition from phase bright to phase dark. Nutritional inducers of germination include L-alanine and a combination of L-asparagine, D-glucose, D-fructose, K⁺ (AGFK) for B. subtilis (Moir and Smith, 1990), and L-alanine and inosine for B. cereus (Barlass et al., 2002; Hornstra et al., 2005). Non-nutritional germinants include chemicals such as dodecylamide and Ca-DPA. Also, peptidoglycan fragments have recently been shown to induce germination (Shah et al., 2008). Other non-nutritional physical factors that can trigger germination include high hydrostatic pressure (HHP), heat, abrasion and ageing (Raso et al., 1998; Moir et al., 2002). Spore germination in clostridia often involves a combination of nutrient germinants and generally proceeds more slowly than Bacillus species (Peck, 2009). Similar to germination in bacilli, non-proteolytic Clostridium botulinum spores can germinate in response to L-alanine and various other amino acids and nutrients such as sugars, lactate and nicotinamide (Plowman and Peck, 2002). For C. perfringens spores it has been established that the following compounds can trigger germination: L-asparagine, KCl, a mixture of L-asparagine and KCl, Ca-DPA and a mixture of Na⁺ and inorganic phosphate (NaPi) (Paredes-Sabja et al., 2008; Paredes-Sabja et al., 2009). The cholate derivatives and the amino acid glycine in bile act as cogerminants of Clostridium difficile spores (Sorg and Sonenshein, 2008). Clostridium tetani spore germination has been reported to be triggered by a mixture of methionine, lactate, nicotinamide and Na⁺ in phosphate buffer (Shoesmith and Holland, 1972).

At a molecular level the process of spore germination is better understood in Bacillus species than in their anaerobic relatives, thanks to decennia of research on model organisms such as B. subtilis and B. cereus, which are genetically accessible and for which whole genome sequences have been available, with B. subtilis being amongst one of the first bacterial genome sequences to be completed (Kunst et al., 1997). In the last decennium, the complete genomes of a number of Clostridium species have been sequenced and annotated, enabling comparisons of genes involved in spore germination of Bacillus and Clostridium using comparative genomics approaches. In the present study, the occurrence of known and putative Bacillus germination-related genes was analyzed in Clostridium species, and in a representative number of Bacillus species. The presence of genes involved in germination will be discussed with a particular focus on the clostridia.
Chapter 2 Clostridial spore germination

2. Materials and Methods

The occurrence of known and putative Bacillus germination-related genes was analyzed in Clostridium species by searching for homologues in 24 sequenced Bacillus genomes and 12 sequenced Clostridium genomes, representing a total of 47 Bacillus genomes (Table 2.1). For Bacillus, 12 genomes were selected; these selected genomes generally cover two main Bacillus groups, namely, the B. subtilis group and B. cereus group, according to a taxonomy review on aerobic spore-forming bacteria (Fritze, 2004) and an article on the ecological diversity of B. cereus group strains (Guinebretiere et al., 2008). Another selection criterion was the way in which gene annotation of sequenced genomes had been performed, with a preference for manually verified annotations (not merely performed by software).

A list of genes encoding germination-related proteins of Bacillus species was generated based on published literature (Table 2.2). The amino acid sequences of these proteins were used to identify putative homologues in the Bacillus and Clostridium genomes listed in Table 2.1. The data were extracted using the genetic analysis platform ERGO (Overbeek et al., 2003) and using the BLAST cut-off scores listed in Table 2.2. Meanwhile, domain analysis was performed via Pfam (Finn et al., 2008) and transmembrane helices were identified using TMHMM (Krogh et al., 2001). The identified sequences were aligned using Clustal X2 (Larkin et al., 2007). Bootstrapped Neighbour Joining trees were generated via Clustal X2 and the sequences were classified using LOFT (van der Heijden et al., 2007). When necessary, sequences were edited using Jalview (Waterhouse et al., 2009). Finally, the genomic context of the related gene clusters, (i.e. the ger clusters or csp clusters), was used to further classify the identified homologues.

3. Results and discussions

The distribution of germination-related genes in sequenced genomes of Bacillus and Clostridium species has been investigated in this study and the outcome has been linked with experimentally obtained data that is available for only a sub-set of the related species (as discussed in detail below). Based on intensive studies on the model species B. subtilis and B. cereus, a model of physiological germination was put forward that includes interaction between germinants and specific receptors (germination receptors), ion fluxes during germination and cortex enzymatic lysis (Moir, 2006). Spores of Bacillus and Clostridium species have comparable multi-layer structures and their germination and outgrowth shows similar morphological events. The genes and their encoded proteins can be categorized into three main function groups, involving (1) environmental sensing, (2) cortex degradation and (3) hydrolysis of core proteins. In the following sections, the function and role of each member of these distinct functional groups will be described with emphasis on similarities and differences in
their presence in Clostridium versus Bacillus.

3.1 Environmental sensing

3.1.1 Specific spore germination receptors

Germination in response to nutrients is mediated by receptors that reside in the inner spore membrane of B. subtilis spores (Hudson et al., 2001; Paidhungat and Setlow, 2001). The germination receptor is composed of 3 proteins (A, B and C), normally encoded within a tricistronic operon e.g. the gerA operon. Screening of B. subtilis mutants defective in germination identified the presence of three homologous gene clusters: the gerA operon (L-alanine response) (Moir et al., 1979), the gerB operon and the gerK operon (AGFK response) (Corfe et al., 1994). Secondary structure analyses of the gene products of these operons suggested that GerAA contains five to six transmembrane helices, that GerAB is a member of the amino acid/polyamine/organocation (APC) superfamily of transporters (subfamily of spore germination proteins) containing ten membrane-spanning regions (Jack et al., 2000), while GerAC is a lipoprotein (Zuberi et al., 1987). In Bacillus species, all three subunits are believed to be essential for receptor function.

We used a component A-C crossing approach to identify putative ger operons in sequenced genomes and took the genetic context of gene clusters into account. Thereby only operons containing component A or C were considered as germinant receptor operons. Spores of Bacillus species can contain 4 to 8 different germinant receptor operons with different organisation of genes. Our analysis of the sequenced whole genomes of Clostridium species revealed lower numbers of genes encoding germinant receptors than in Bacillus species, while in addition many incomplete operons or operons with unusual structures were found compared with the Bacillus ABC tricistronic operons (summarized in Figure 2.1).

Recent functional studies on ger mutants in C. perfringens SM101 demonstrated that not all three subunits are required for receptor functionality. This food-poisoning strain, which produces C. perfringens enterotoxin (CPE), contains a bicistronic operon, gerKA-KC, and oriented in the opposite direction, an upstream monocistronic gene gerKB. In addition, this strain contains an orphan GerA homologue, named gerA. Deletion or interruption of these genes in strain SM101 demonstrated an essential role for gerKA and gerKC in potassium-initiated spore germination (Huang et al., 2007; Paredes-Sabja et al., 2008), whereas gerKB and gerA only played an auxiliary role in spore germination under some conditions (Paredes-Sabja et al., 2008; Paredes-Sabja et al., 2009). Our in silico analysis showed that these four genes are conserved in the other two available C. perfringens genomes (gas gangrene strains Strain 13 and ATCC13124), but their exact function in these non-food-poisoning strains has not been established experimentally. The divergon-like structure of the C. perfringens gerK operon has been found in nine other clostridial genomes, but not in any of the Bacillus species included in this study. We found that the protein encoded by the orphan gerA in C. perfringens has a higher similarity to Stage V sporulation protein AF of B. subtilis than with GerAA and, consequently, all the C. perfringens gerA homologues consistently cluster with Bacillus Stage V Sporulation protein AF in a Neighbour Joining tree. However, their putative function and involvement in the sporulation process have not been confirmed experimentally.

Based on our in silico analysis of the clostridial ger operons (Figure 2.1), the available 10 sequenced C. botulinum genomes can be divided into three groups. Two complete tricistronic ger operons and a pentacistronic ger operon with a BABCB make-up are found in the genomes of all C. botulinum proteolytic strains except in the Loch Mare strain, which lacked one of B genes. The structure containing multiple B genes in one
ger operon was also found in genomes of *Clostridium acetobutylicum*, *B. cereus* E33, *Bacillus halodurans*, *Bacillus licheniformis* (this study) and a *Clostridium sporogenes* genome (Broussole et al., 2002), but its function is unclear. In the genomes of strain Okra and Langeland, a *C. perfringens* gerK-like BAC divergon is also present, while non-proteolytic strains Eklund 17B (type B) and Alaska E43 (type E) contain only one ger BAC divergon. Our results are in line with the conclusions presented in a recent review, in which very different pattern of germination receptor genes were also found between proteolytic and non-proteolytic strains of *C. botulinum* (Peck, 2009).

*Clostridium kluwyeri*, *C. acetobutylicum* and *Clostridium cellulolyticum* genomes also contain ger operons with unusual gene order. The gerA gene is consistently located upstream of the gerC gene within the same operon, whereas the location of the gerB gene varies. The ger operons of *Clostridium novyi* and *Clostridium thermocellum* have a regular structure.

Interestingly, the *C. acetobutylicum* genome contained a plasmid encoded ger cluster, whereas GerA homologues were absent in the genome of *Clostridium beijerinckii* and the human pathogen *C. difficile*, as described earlier (Paredes-Sabja et al., 2008; Paredes-Sabja et al., 2008). The fact that spores of *C. difficile* can germinate upon exposure to bile indicates that an alternative mechanism to trigger germination must exist in this species (Sorg and Sonenschein, 2008).

### 3.1.2 Novel pathway: peptidoglycan-fragment-triggered germination

An alternative and very different mechanism for initiating germination has recently been reported, namely, that spore germination of *B. subtilis* and several other *Bacillus* species can be triggered by low concentrations (<1 pg/ml) of muropeptides through a receptor that binds these peptides. Muropeptides are produced by degradation of the peptidoglycan layer that comprises the cell wall of most Gram-positive bacteria. Degradation of peptidoglycan is a normal feature of bacterial cell growth. PrkC, a member of the serine/threonine protein kinase family, was identified and associated with the peptidoglycan layer of *B. subtilis* (Peck, 2009).

The presence of PrkC homologues was also investigated in the available genomes. It turned out that prkC is highly conserved in the genomes of bacilli and clostridia, including the genome of *C. difficile*, which entirely lacks GerA homologues. The prkC gene of *C. perfringens* contains one additional PASTA repeat compared to the *B. subtilis* homologue. The diversity of PASTA repeats (in number and in sequence) found among species is suggestive of differences in specific binding to peptidoglycan fragments. So far, no experimental data are available on *Clostridium* species with regard to this novel germination mechanism.
Furthermore, the involvement of an antiporter (belonging to the family of CPA-2) in spore germination has been demonstrated for GerN in *B. cereus* (Southworth et al., 2001; Thackray et al., 2001) and for GrmA in *B. megaterium* (Tani et al., 1996). GerN encodes a ‘Na’/‘H’-‘K’ antiporter essential for the *B. cereus* germination response upon exposure to inosine (Thackray et al., 2001).

Homologues of the GerN protein were found in some clostridial genomes, including *C. botulinum*, *C. perfringens*, and *C. tetani* (Table 2.2). One of two GerN homologues identified in the *perfringens* genome, named GerO, appears to be essential for normal germination. Another GerN homologue in *C. perfringens*, named GerQ, has less profound effects on germination (Paredes-Sabja et al., 2009).

### 3.2 Cortex degradation

Germination is completed after the full degradation of the spore coat and spore cortex. The cortex surrounds the normal primordial cell wall and has a unique structure; instead of muramic acid this layer contains muramic δ-lactam residues (Popham et al., 1996; Atrih and Foster, 2001). This loosely cross-linked peptidoglycan layer is required for the maintenance of the dehydrated state of the spore core (Popham et al., 1995; Popham et al., 1996) but needs to be removed before full rehydration of the cell and spore swelling can occur. Hydrolysis of the cortex in the genus *Bacillus* is most likely performed by enzymes which reside near the cortex-coat boundary (Moriyama et al., 1999; Shimamoto et al., 2001; Bagyan and Setlow, 2002) and which act specifically on cortex peptidoglycan by recognizing the muramic δ-lactam moiety.

In *B. subtilis*, two germination-specific cortex-lytic enzymes, namely, CwlJ and SleB, are responsible for peptidoglycan degradation. CwlJ is known to be activated by Ca-DPA; activation of SleB depends on the presence of YpeB which is required for either the function, expression, localization or activation of SleB (Boland et al., 2000). Homologues of CwlJ, SleB and YpeB have been found in *C. cellulolyticum*, *C. kluyveri*, *C. novyi*, *C. wychwoodi*, and in most *C. botulinum* genomes. This suggests that these *Clostridium* species employ similar mechanisms for cortex lysis as *Bacillus*. Additionally, another group of genes, present in most *Clostridium* genomes except for *C. thermocellum*, are predicted to encode spore cortex lytic enzymes with the same function as SleB; however, the products of these genes share relatively low similarity with the *Bacillus* SleB homologues.

**Table 2.2 Homologues of previously characterized germination-related factors and their predicted functions (continue)**

<table>
<thead>
<tr>
<th>Factors</th>
<th>Genes*</th>
<th>Functions</th>
<th>P-score cut off**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ger receptors</td>
<td>ger cluster</td>
<td>Germinant receptors located on spore inner membrane, initiating germination by sensing environment</td>
<td>1×10⁻⁰⁶</td>
</tr>
<tr>
<td>PkC</td>
<td>pkC</td>
<td>Serine/threonine protein kinase (EC 2.7.11.1), responsible in mureptides-induced germination of <em>B. subtilis</em></td>
<td>1×10⁻⁰⁶</td>
</tr>
<tr>
<td>GerN</td>
<td>gerN or napA</td>
<td>Na⁺/H⁺/K⁺ antiporter</td>
<td>1×10⁻⁰⁶</td>
</tr>
<tr>
<td>SleB</td>
<td>sleB</td>
<td>Spore cortex lytic enzymes</td>
<td>1×10⁻⁰⁶</td>
</tr>
<tr>
<td>YpeB</td>
<td>ypeB</td>
<td>SleB-stabilizing protein, required for expression, localization and activation of SleB</td>
<td>1×10⁻⁰⁶</td>
</tr>
<tr>
<td>CwlJ</td>
<td>cwlJ</td>
<td>Cortex lytic enzymes</td>
<td>1×10⁻⁰⁶</td>
</tr>
<tr>
<td>Sle/F/YaaH</td>
<td>sle/YaaH</td>
<td>Spore peptideglycan hydrolase (N-acetylglucosaminidase) (EC 3.2.1.-)</td>
<td>1×10⁻⁰⁶</td>
</tr>
<tr>
<td>SleB-like</td>
<td>sleB</td>
<td>Spore cortex lytic enzymes or cell wall hydrolase</td>
<td>1×10⁻⁰⁶</td>
</tr>
<tr>
<td>SleM</td>
<td>sleM</td>
<td>N-acetylglutamyl-L-alanine amidase (EC 3.5.1.28)</td>
<td>1×10⁻⁰⁶</td>
</tr>
<tr>
<td>SleC-pro</td>
<td>sleC</td>
<td>Spore cortex lytic enzymes pre-pro-form, a bifunctional enzyme possessing lytic transglycosylase activity and N-acetylglutamyl-L-alanine amidase activity after activation by Gsp</td>
<td>1×10⁻⁰⁶</td>
</tr>
<tr>
<td>GSP</td>
<td>gspA,B,C</td>
<td>Germination specific protease, activating SleC</td>
<td>1×10⁻⁰⁶</td>
</tr>
<tr>
<td>GPR</td>
<td>gpr</td>
<td>Germination protease in spore core, degrading SASPs</td>
<td>1×10⁻⁰⁶</td>
</tr>
<tr>
<td>SASP (sleB)</td>
<td>supA,B,C,D</td>
<td>s/l-type small acid soluble proteins in spore core, protecting spore DNA</td>
<td>0.001</td>
</tr>
<tr>
<td>SASP (supEF)</td>
<td>supE, supF</td>
<td>A minor SASP in <em>B. subtilis</em> or associated to heat resistance of some <em>C. perfringens</em> strains</td>
<td>0.001</td>
</tr>
<tr>
<td>SASP (y)</td>
<td>supY</td>
<td>y-type SASP playing no role in spore resistance but providing amino acids essential for protein synthesis</td>
<td>0.001</td>
</tr>
<tr>
<td>GerP</td>
<td>gerP cluster</td>
<td>Proteins in spore coat, facilitating transfer of germinants</td>
<td>1×10⁻⁰⁶</td>
</tr>
<tr>
<td>GerD</td>
<td>gerD</td>
<td>Modulating action of germinant receptors</td>
<td>1×10⁻⁰⁶</td>
</tr>
</tbody>
</table>

* Only csp clusters at upstream of sleC genes are counted
** Contain one *C. perfringens* SspF homologue and the other is *Bacillus* SspF homologue
*** The feature names in *B. subtilis* and/or *C. perfringens* genomes are preferably used here
**** P-scores, match fasta probability score, generated from sequence blast function of ERGO platform. Lower P-score indicates higher similarity. P-score cut off is used during homologous identification as a reference

### 3.1.3 Ion fluxes in germination

It is believed that the proteins encoded by the ger operon are involved in ligand binding (Sammons et al., 1981; Paidhungat and Setlow, 1999). However, the signal-transduction pathway that subsequently mediates germination has not yet been elucidated. Possibly, the germinant receptor (or receptor complex) activates specific antiporters, thereby altering the electrochemical gradient across the membrane locally in such a way that the bulk movement of ions observed during the early stages of germination can take place. This can be achieved either by changing membrane structure (e.g. the spore membrane might exist in a compressed semicrystalline state) (Stewart et al., 1980) or is due to the activation of other transporters. Experiments performed with specific ion-channel blockers on germination of *Bacillus megaterium* spores showed that germination was inhibited by specific Ca²⁺, K⁺ and Na⁺ blockers (Mitchell et al., 1986). Furthermore, the involvement of an antiporter (belonging to the family of CPA-2 monovalent cation-proton transporters) in spore germination has been demonstrated for GerN in *B. cereus* (Southworth et al., 2001; Thackray et al., 2001) and for GrmA in *B. megaterium* (Tani et al., 1996). GerN encodes a ‘Na’/‘H’-‘K’ antiporter essential for the *B. cereus* germination response upon exposure to inosine (Thackray et al., 2001).

Recent studies suggest that cortex-hydrolytic enzymes in *C. perfringens* differ in terms of structure and mechanism of activation from those present in bacilli. In *C. perfringens* S40, which is a heat sensitive strain that does not produce enterotoxin (CPE), germination-specific cortex-lytic enzymes have been identified that act in a cooperative manner: spore cortex-lytic enzyme (SCLE), cortical fragment-lytic
enzyme (CFLE) and germination-specific protease (GSP) (Makino and Moriyama, 2002; Huang et al., 2007). CFLE (SleM) was shown to be an N-acetylmuramidase (Chen et al., 1997) and SCLE (SleC) is most likely a bifunctional enzyme with lytic transglycosylase and N-acetylmuramoyl-L-alanine amidase activities (Kumazawa et al., 2007). The C. perfringens SCLE (SleC) is produced as an inactive precursor, with a C-terminal pro-sequence and an N-terminal pre-pro sequence (Miyata et al., 1995; Urakami et al., 1999). During spore maturation the N-terminal pre-sequence and C-terminal pro-sequence are removed, generating N-terminal pro-SleC (Okamura et al., 2000), which is transported to the cortex-coat boundary (Miyata et al., 1997). Here it waits to be proteolytically activated by a germination specific protease (GSP) through removal of the N-terminal pro-sequence, yielding an active SleC.

The GSP also resides at the exterior of the cortex layer (Shimamoto et al., 2001) and is encoded by three genes of C. perfringens S40, cspA, cspB and cspC, which are located just upstream of the sleC gene. The involvement of SleC in cortex degradation has recently been demonstrated in C. difficile strain 630 (Burns et al., 2010) and foodborne C. perfringens strain SM101 (Paredes-Sabja et al., 2009). We only detected one orphan csp gene in the C. perfringens SM101 genome, whereas tricistronic cspABC operons were found in the two other C. perfringens strains (ATCC13124 and strain 13). Whether csp gene products form a complex or act as separate enzymes remains to be established. Homologues of SleM, SleC and GSPs of C. perfringens were only found in C. beijerinckii, C. botulinum Eklund 17B and partly in C. acetobutylicum, C. difficile, C. botulinum Alaska E43 genomes, but not in any Bacillus species.

Overall, the studied Clostridium species appear to contain two significantly different cortex degradation mechanisms, namely, the Bacillus-like system consisting of SleB, CwlJ, and YpeB homologues and the C. perfringens-like system containing SleM, SleC and GSPs homologues.

### 3.3 Hydrolysis of core proteins

Small acid-soluble proteins (SASPs) in the spore core region play an important role in protecting spore DNA from environmental conditions such as UV exposure and heat treatment. In dormant spores high level α/β-type SASPs (5-10% of total core protein) are present which bind to DNA thus providing protection to DNA (Setlow, 2007; Lee et al., 2008). To enable DNA transcription in later stages of spore outgrowth, the SASPs must be removed upon germination. During completion of germination, the water content in the spore increases, leading to dissociation of SASP from the spore DNA; subsequently, degradation of SASPs is initiated by pre-embedded germination protease, GPR (Sanchez-Salas et al., 1992) and the cleavage products are further degraded to amino acids that support protein synthesis and energy metabolism in early spore outgrowth (Setlow, 1994; Setlow, 1995; Sonenshein et al., 2002).

B. subtilis spores contain three major SASPs (α, β, γ), which are encoded by the sspA, sspB and sspE genes. In addition, a large number of minor SASPs are encoded by genes sspC, sspD, and sspF through to ssp P. Of the latter group, sspC and sspD encode SASPs with high homology to α/β-type SASPs and exhibit low homology to γ-type SASP, while no sequence relatedness to the rest of minor SASPs was found (Cabrera-Hernandez et al., 1999; Cabrera-Hernandez and Setlow, 2000)

A previous comparative study showed that the level of conservation of α/β-type SASP encoding genes is higher in aerobic spore formers than in their anaerobic relatives (Setlow, 2007). Several C. perfringens isolates, including heat resistant strains, contain three identical α/β-type SASP genes (ssp1, ssp2 and ssp3), which were shown to be expressed at similar levels (Raju et al., 2006; Raju and Sarker, 2007). A single amino acid replacement within SASP ssp4 has been reported to be associated with heat resistance in C. perfringens strains, where the heat resistant strains contain a protein with Asp at position 36 and the heat sensitive strains a protein with a Gly at that particular position (Li et al., 2009).

According to our analysis, the C. perfringens ssp4 has higher similarity to B. subtilis sspF, encoding a minor SASP, than to α/β-type ssp genes. The residue at position 36 is highly variable for the genomes studied, and neither Asp nor Gly occurs at equivalent positions in any of the other non-perfringens species/strains. Interestingly, no homologue of known major SASP has been found in C. tetani, which was highlighted in a recent study (Wang et al., 2008).

The GPR homologues are present and highly conserved in Bacillus and Clostridium genomes including C. tetani. Two residues Asp127 and Asp193 were shown to be essential for activity and autophosphorylation of B. subtilis GPR, which might be an aspartate protease (Carroll and Setlow, 2005). These two Asp residues are conserved for all 36 genomes.

### 3.4 Other germination related genes

Germinant permeation proteins are proteins that facilitate the translocation of nutrient germinants through outer layers of the spore, in particular through the spore coat. For the GerP germination permeation cluster, encoded at the gerP locus of B. subtilis and B. cereus, it has been demonstrated that the encoded proteins form a structural feature of the spore coat (Behravan et al., 2000). We identified homologues of the gene products of the gerP locus in most of the Bacillus genomes, but could not detect any in the available Clostridium species genomes. The exact mechanism of these GerP proteins and their relation to specific germinant compounds remains to be established. Another protein that plays a role in nutrient specific germination is GerD, a lipoprotein that is...
produced in the forespore compartment of the sporulating cell. GerD was detected in the inner membrane of spores, at a high level in *B. subtilis* spore integuments (comprising coat, cortex and germ cell wall layers), and to some extent in the soluble fraction. The mechanism by which this protein influences nutrient induced spore germination remains to be established (Pelczar and Setlow, 2008; Mongkolthanaruk *et al.*, 2009). Again, gerD homologues were only found in *Bacillus* species, but not in *Clostridium* species.

The absence of gerP and gerD homologues in clostridia prompts the question whether similar mechanisms play a role in germination of clostridia through yet unidentified proteins, or whether differences in the outer layers of the spore affect nutrient accessibility of the inner membrane.

**4. Conclusions and perspectives**

The capacity to form dormant endospores renders *Bacillus* and *Clostridium* species resistant to adverse conditions and strongly contributes to their ubiquitous presence in the environment. Even though spores of clostridia and bacilli show comparable morphological structures, their response to potential environmental stimuli that trigger germination differs significantly. Clostridial genomes contain homologues of putative germination-specific gene products that have been discovered in aerobes and facultative anaerobes. However, overall, the numbers of ger operons are lower in clostridia than in bacilli and varied amongst the species. In clostridia, two major groups of germination-specific lytic enzymes could be distinguished: one with close homology to *Bacillus* Cw1, SleB and YpeB, and one with homology to *C. perfringens* SleM, SleC and GSPs. *C. cellulolyticum*, *C. kluyveri*, *C. novyi*, *C. tetani* and most of the *C. botulinum* strains contained homologues of CwlJ, SleB and YpeB, whereas *C. beijerinckii* and *C. botulinum* Ekland 17B contained homologues of SleM, SleC and GSPs. *C. acetobutylicum*, *C. difficile* and *C. botulinum* Alaska E43 genomes contained one or more of the latter group, while these were consistently absent in the genomes of the *Bacillus* species included in the study. Lastly, all bacilli and clostridia encode germination proteases GPR, required for SASP degradation in the germinating spore. The differences observed between clostridia and the bacilli might point to different germination strategies or the involvement of so far unidentified germination proteins.

Functional studies on clostridial spore germination mechanisms have been hampered by the lack of suitable genetic tools and difficulties with transformation of clostridia, believed to be due to DNA restriction digestion systems in clostridia (Heap *et al.*, 2009). Despite these limitations, significant progress has been made in recent years, in particular in *C. perfringens* strain SM101 (Paredes-Sabja *et al.*, 2008). As a result, the gene products playing a role in *C. perfringens* spore germination and their predicted functions can be illustrated in **Figure 2.2**. Recently, a powerful clostridial gene knockout system with a motile group II intron approach has been developed and can be applied in clostridial research (Heap *et al.*, 2007; Heap *et al.*, 2010). It is to be expected that these tools will enhance mechanistic understanding of clostridial spore germination.

**Acknowledgements:**

We thank Dr Bernadet Renckens, Prof. Dr Roland Siezen and Dr Roy Moezelaar for advice and support and Dr Floris Bosveld for preliminary analyses and illustrations of *C. perfringens* germination factors.
References:


Page 42


Chapter 3

Genome-wide transcriptional profiling of *Clostridium perfringens* SM101 during sporulation extends the core of putative sporulation genes and genes determining spore properties and germination characteristics

Y. Xiao, S.A.F.T. van Hijum, T. Abee, and M.H.J. Wells-Bennik

Submitted for publication
The formation of bacterial spores is a highly regulated process and the ultimate properties of the spores are determined during sporulation and subsequent maturation. A wide variety of genes that are expressed during sporulation determine spore properties such as resistance to heat and other adverse environmental conditions, dormancy and germination responses. In this study we focused on the identification of genes that have so far not been associated with sporulation and properties of spores. To this end, the sporulation stages of \textit{C. perfringens} enterotoxic strain SM101 were characterized based on morphological characteristics and biological indicators. Subsequently, whole genome expression profiling during key stages of the sporulation process was performed using DNA microarrays, and genes were clustered based on their time-course expression profiles during sporulation. The majority of previously characterized \textit{C. perfringens} germination genes showed upregulated expression profiles in time during sporulation and belonged to two main clusters of genes. These clusters with up-regulated genes contained \textit{C. perfringens} homologues of \textit{Bacillus} genes with roles in sporulation and germination; this study suggests that the homologues are functional in \textit{C. perfringens}. A comprehensive homology search revealed that approximately half of the upregulated genes in the two clusters are conserved within a broad range of sporeforming Firmicutes. Another 30\% of upregulated genes in the two clusters were found only in clostridia species, while the remaining 20\% appeared to be specific for \textit{C. perfringens}. These newly identified genes may add to the repertoire of genes with roles in sporulation and determining spore properties including germination behavior. Their exact roles remain to be elucidated in future studies.

Key Words: Sporulation, germination, time-course expression profile, \textit{C. perfringens}

1. Introduction

\textit{Clostridium perfringens} is the causative agent of various animal and human diseases including clinical gas gangrene and foodborne diarrhoea (McClane, 2007). Its dormant endospores are highly resistant to environmental insults, such as heat, drought, sanitizing agents, and preservatives. This allows for the widespread occurrence of this anaerobe in food materials and the intestinal tract of human and animals (Hassan and Paulsen, 2011). For \textit{Bacillus} and \textit{Clostridium} species, the process of spore formation, called sporulation, starts with asymmetric cell division and is characterized by different stages in which a forespore is formed and engulfed, followed by formation of a cortex layer and completion of spore coat layer (Paredes et al., 2005). Noteworthy is that certain \textit{C. perfringens} strains produce and release diarrhoea-causing enterotoxin (CPE) during sporulation – the production of this toxin is strictly associated with spore formation and mother cell lysis in the gastrointestinal tract (McClane et al., 2006).

Morphological changes of sporulating cells and genes involved in the sporulation cascade have been intensively studied in \textit{Bacillus} species, particularly in \textit{B. subtilis} (Eichenberger, 2012). For clostridia, these phenomena have been investigated for industrially relevant \textit{C. acetobutylicum} and clinically relevant \textit{C. difficile} (Alsker and Papoutsakis, 2005; Burns and Minton, 2011). So far, detailed studies on global gene expression during sporulation has not been reported for \textit{C. perfringens}. The sporulation cascade of bacilli and clostridia share many common features, but also have distinct features, with one of the major differences being indirect versus direct Spo0A phosphorylation during the initiation stage of sporulation (Paredes et al., 2005; Galperin et al., 2012).

Bacterial spores can germinate under favourable conditions. This process is characterized by a transition from phase bright to phase dark under a phase contrast microscope and is associated with the release of the major spore core component Ca\(^{2+}\)-dipicolinic acid (DPA) (Setlow et al., 2006). Germination is irreversible and upon germination the spore loses its resistance to heat and other treatments. Full germination furthermore requires coat and cortex degradation, upon which a vegetative cell can emerge and return to vegetative growth (Setlow, 2003). For \textit{C. perfringens}, growth under optimal conditions is fastidious. In case of foodborne illness, the pathogen causes disease at relatively high numbers (>10\(^5\)) of vegetative cells, associated with sporulation in the human gut (Shimizu et al., 2002; Huang et al., 2007). To control \textit{C. perfringens} in foods, different strategies can be envisioned, including complete prevention of germination or complete germination in combination with a moderate inactivation treatment to kill the sensitive germinated spores. However, the efficacy of such approaches may be affected by diversity in germination behaviour between species and strain, and heterogeneity in germination (Paredes-Sabja et al., 2008; Akhtar et al., 2009; Xiao et al., 2012).

Various germination-related genes of \textit{C. perfringens} have previously been identified (Xiao et al., 2011), among which only one \textit{ger} divergen (\textit{gerKB}, \textit{gerKA}-\textit{gerKC}), encoding a germination receptor complex. The presence of \textit{prkC}, encoding a Ser/Thr kinase in \textit{C. perfringens}, suggests the existence of an alternative germination pathway triggered by environmental peptidoglycan fragments, as described for \textit{B. subtilis} (Shah et al., 2008; Shah and Dworkin, 2010; Xiao et al., 2011). \textit{C. perfringens} also carries the gene \textit{gpr}, encoding a germination specific protease, which also plays an essential role in the degradation of small acid-soluble proteins (SASPs), which stabilize and protect \textit{B. subtilis} spore DNA from lethal damages (Setlow, 2007). Cortex degradation of \textit{C. perfringens} has been shown to require a spore cortex-lytic enzyme, cortical fragment-lytic enzymes and a germination-specific protease, encoded by genes \textit{sleC}, \textit{sleM} and...
of the stock culture was inoculated in 10 ml pre-reduced Fluid Thioglycollate Medium (Labbe and Rey, 1979; Xiao et al., 2012). In short, 50 μl aliquots of a stock sporulating medium (Labbe and Rey, 1979; Xiao et al., 2012) was performed as previously described in 500 ml modified Duncan-Strong (mDS) medium and incubated at 37°C. Sporulation of bacilli (Paredes-Sabja and Sarker, 2009; Eichenberger, 2012), but there is evidence that their appearance in clostridia during sporulation is not strictly confined to late stage sporulation (Kirk et al., 2012; Pereira et al., 2013).

Recently, Galperin and his colleagues (2012) summarized all reported sporulation genes in a wide range of species, providing an excellent foundation to identify putative C. perfringens homologues of known sporulation genes (Galperin et al., 2012). Actual experimental data on the expression of such homologues in C. perfringens during sporulation would substantiate their role in the sporulation process of this bacterium.

In the current study, we characterized the sporulation stages of C. perfringens SM101 by assessing changes in morphology and by measuring biological indicators throughout the sporulation process, including biomass accumulation (OD₆₀₀), the total viable counts of cells plus spores, and the viable count of heat resistant spores alone, pH in the supernatant, CPE production and DPA accumulation. So far, strain SM101 is the only publicly available chromosomal-cpe strain with a completed genome sequence (Myers et al., 2006) and this strain has been subject of various studies on sporulation and spore germination (Sarker et al., 1999; Paredes-Sabja et al., 2008; Orsborn et al., 2009). Whole genome microarray analysis was performed at various stages during sporulation. This confirmed expression of many known and predicted sporulation and germination genes in C. perfringens. Moreover, it revealed novel putative sporulation genes that were specifically up-regulated during sporulation and conserved amongst bacilli and clostridia. Such genes are likely candidates to play a role in sporulation and/or spore properties.

2. Materials and Methods

2.1 Bacterial strain, growth conditions and sampling

C. perfringens SM101 (kindly provided by Dr Melville, Blacksburg, VA, US) was stored in Cooked Meat Broth (BD, Sparks, US) at 1°C and used as a working stock. Sporulation was performed as previously described in 500 ml modified Duncan-Strong (mDS) sporulating medium (Labbe and Rey, 1979; Xiao et al., 2012). In short, 50 μl aliquots of the stock culture was inoculated in 10 ml pre-reduced Fluid Thioglycollate Medium (FTM, BD, Sparks, USA), heated at 75°C for 15 min, and subsequently anaerobically incubated at 37°C overnight in an anaerobic chamber (gas mixture: 10% H₂, 5% CO₂, 85% N₂, v:v:v). An exponential-phase inoculum was prepared by mixing 1 ml of the overnight culture and 9 ml FTM followed by 4 h incubation; the fast dividing vegetative cells produced substantial amounts of gas. An inoculum (1:10,000, v:v) was added to 500 ml of pre-reduced mDS medium and incubated at 37°C. This sporulating culture was continuously mixed using a magnetic stirrer. Samples of the sporulating culture were taken every 30 min starting 2 h after inoculation. 25 μl aliquots of the culture were collected of which 1 ml was used to determine the total viable counts (vegetative cells plus spores) and the remaining 24 ml was taken out of the chamber to assess the other biological indicators (see below).

Cultures without sporulation were obtained by inoculating an exponential-phase FTM inoculum in TGY broth (3% tryptic soy broth, 2% glucose, 1% yeast extract, 0.1% L-cysteine). Samples were taken during the following 6 h incubation at 37°C. Absence of spore formation in TGY was confirmed microscopically and by plating after heating for 10 min at 75°C.

2.2 Analyses during sporulation

The following indicators of biological event were assessed to determine stages of sporulation using the 24 ml sample mentioned above (see also Figure 3.1).

Biomass accumulation was determined by measuring the optical density at 600 nm (OD₆₀₀) using an Ultraspec 3100 pro spectrophotometer (Amersham Bioscience, Freiburg, Germany).

Total viable counts of C. perfringens were determined by plating serial dilutions on BHI agar plates (BD, Sparks, US) in the anaerobic chamber. Spore counts were determined by first heating the sample to 75°C for 10 min, followed by plating on BHI plates. Colonies were counted after 24 h of anaerobic incubation.

The culture pH and CPE release were measured as follows: a 5 ml culture was subjected to centrifugation (16,000 xg for 5 min) at 4°C. The supernatant and the pellet, that contained the cells and spores, were separated. The supernatant was further filter-sterilized using 0.2 syringe filters (Whatman, Germany). The pH in the filter-sterilized supernatant was measured using a pH meter (WTW, Weilheim Germany), and the CPE concentration was measured using the PET-RPLA kit (Oxoid, Hampshire, UK). Besides measuring CPE in the supernatant, it was also assessed whether CPE had accumulated in sporulating cells. To this end, half of the pellets were disrupted using Lysing Matrix B (0.1mm silica spheres, MP Biomedicals) and re-suspended in 2.5 ml of the sterilized supernatant, followed by CPE measurement using the PET-RPLA test.

DPA accumulation was measured using the other half of the harvested pellet, which was...
Figure 3.1 Changes of measured biological indicator following sporulation of C. perfringens SM101. Results of measured biological indicators in time course of C. perfringens SM101 sporulation and phase contract microscopical images taken at representative time points of five sporulation stages (magnification: 1000×). The selected indicators include: A. total viable counts (♦) and spore counts (□); B. OD_{600} (▲) and DPA accumulation in forespores/spores (○); C. pH (-) and CPE in supernatant (×). The upper detection limit of CPE concentration measured by PET-RPLA is 128 ng/ml. The saturated range is indicated as stash line (C).

2.3 Preparation of total RNA samples

Total RNA was extracted from cells and spores throughout the sporulation process in mDS and during growth in TGY (in which sporulation did not occur). First, a 3 ml mixture of acidified phenol-ethanol (v:v=1:9) was added to 12 ml culture samples immediately upon sampling. After short mixing using a vortex, this sample was kept on ice for 30 min. Cells/spores were then harvested by centrifugation at 16,000×g for 5 min at 4°C, and resuspended in ice cold TRIzol Reagent (Ambion, US). This procedure was used to minimize the effects of exposure to e.g. oxygen, chilling and centrifugation on transcripts and to prevent RNA degradation. 0.6 ml aliquots of the mixture were transferred to Lysing Matrix B tubes prefilled with 0.1mm silica spheres. These samples were frozen in liquid N\textsubscript{2} and kept at -80°C until use.

For final RNA extraction, 0.4 ml ice-cold TRIZol Reagent was added to the frozen sample and homogenization was performed immediately - without thawing - using Savant FastPrep FP120 (QiBiogene, Carlsbad, US) for 40 sec at Speed 6, in total 3 times with interval cooling on ice. After phase separation, RNA precipitation and washing was performed as described in the instructions of the manufacturer. The total RNA extracts were further purified using a RNaseasy kit (QIAGEN, Hilgen, Germany). On-column DNA digestion was performed during purification using RNase-Free DNase.
Experimental design, labeling and hybridization. Based on analyses of the ORFs of strain SM101 were represented on the microarray by at least one probe. 4×44K probes glass slides. 96.7% (2532/2619) of protein-coding open reading frames (GPL accession number will be provided during review), which were spotted on and (v) low-complexity: 9.1%. This custom probe design contained 26,876 probes hybridization: 39.0%, (ii) delta Tm: 26.0%, (iii) folding: 13.0%, (iv) position: 13.0%, The total probe score was based on weighting of the individual scores for (i) cross-

2.4 RT-PCR

In our previous genome-mining study, a set of genes were predicted to play essential roles in germination of clostridia (Xiao et al., 2011). RT-PCR was employed prior to whole genome expression profiling to verify expression of a number of genes that are known to be involved in germination and expressed during sporulation. Therefore, purified total RNA samples of all samples that were taken every 0.5 h during sporulation were subjected to reverse transcription using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Paisley, UK) according to the instructions of the manufacturer. The generated cDNA was subsequently amplified by real-time PCR using the primer pairs listed in Table 3.1 for the corresponding targets. Serial dilutions of a genomic DNA sample of strain SM101 were prepared as described before (Xiao et al., 2012) and this was used as a control in this multiple-plate application. Transcripts of genes dnaE and sigH (Alsaker and Papoutsakis, 2005; Kirk et al., 2012) were used to normalize the resulting Ct values. The results are shown in Figure 3.2A and 3.2B.

2.5 Time-course genome expression profiling and clustering

Probe design. Microarrays were obtained from Agilent technologies (Santa Clara, CA, US). Probes were specified for ORFs that were present in the genomes of C. perfringens SM101. Probe design was performed separately for the plasmid and chromosomal ORFs. The chromosomal ORFs were aligned with BLAST (Kent, 2002) and chromosomal ORFs that are (virtually) identical were grouped based on a 95% or higher nucleotide identity over the full length of the ORF. The virtually identical ORFs were aligned by Clustal W v2.0.1 (Thompson et al., 1994), and the identical regions (that are most suited for probe design) were selected as consensus ORF fragments using an in-house developed script. Probe design was performed on the plasmid ORFs, the unique chromosomal ORFs and the consensus fragments of near-identical ORFs, resulting in about 5 probes per ORF. All probes were designed with an aim length of 60 bp with a minimum of 55 bp and a minimum probe distance of 100 bp using OligoWiz v2.1.3 (Wernersson et al., 2007) using default parameters for prokaryotic long-mers. The total probe score was based on weighting of the individual scores for (i) cross-hybridization: 39.0%, (ii) delta Tm: 26.0%, (iii) folding: 13.0%, (iv) position: 13.0%, and (v) low-complexity: 9.1%. This custom probe design contained 26,876 probes (GPL accession number will be provided during review), which were spotted on 4×44K probes glass slides. 96.7% (2532/2619) of protein-coding open reading frames (ORFs) of strain SM101 were represented on the microarray by at least one probe.

Experimental design, labeling and hybridization. Based on analyses of the C. perfringens SM101 cultures throughout growth and sporulation, five time points were selected that represented different stages of the spore-forming procedure, namely, exponential growth (T1), cell elongation (T2), early sporulation (T3), late sporulation (T4) and cell lysis (T5). These different stages were assessed in two independent experiments, performed on different days. The exact time between inoculation and the typical events in the different stages was not identical between the duplicates. For experiment A, the five sampling times – namely T1 to T5 which are typical for the five observed stages - were at 2.2, 3.5, 4.5, 5.0, and 6.0 h after inoculation. For Experiment B, the sampling times representing the five typical stages were at 2.6, 4.1, 5.1, 5.6 and 6.1 h after inoculation.

For each sample taken at the different time points, 3 µg of total RNA was subjected to cDNA synthesis using random nonamer priming and indirect labeling approaches as previously described (Stevens et al., 2010). For each sample at each time point, both Cy3- and Cy5-labeled cDNA was prepared using a Cyscribe postlabeling kit (Amersham Biosciences, UK) according to the protocol of the manufacturer. A hybridization scheme was designed that allowed for comparison of samples taken at time points of experiments A and B, which were performed independently (Figure...
Chapter 3 Transcriptional profiling during sporulation

3. Results

3.1 Five stages of sporulation defined by morphological changes and biological indicators

To select time points during sporulation that are representative for the different stages of *C. perfringens* sporulation, we performed microscopic analysis to assess morphological changes during the fast sporulation process of this bacterium. At the same time, a set of biological indicators was measured to determine key events during spore formation. These data are presented in Figure 3.1. Based on the biological events, five stages could be distinguished:

**Stage I** consisted of exponential growth (Figure 3.1A). Starting from an inoculum of 1×10⁵ CFU/ml, the culture reached a level of 1×10⁸ CFU/ml in approximately 3 h. This stage of exponential growth was characterized by an increase in total viable counts with a calculated generation time of 11 min, and biomass accumulation to an OD₆₀₀ of 0.3. During this stage, spores were not formed, DPA and CPE were not detectable and the culture pH remained high at around 7.5. Microscopical analysis showed actively dividing cells at relatively low densities. A representative time point is indicated by T₁.

During **Stage II**, the total viable counts and the OD₆₀₀ were stable and at their...
maximum level. Microscopical analysis clearly showed fore-spores that were formed in the mother cells, with a small portion of the spores appearing phase bright. At this stage, the spores were not heat resistant. DPA levels were increasing (corresponding with spore formation) and CPE was detectable with increasing levels during this stage. The pH of the culture decreased further to approximately 6.7. Time T₄ shows a representative time point of Stage III.

**Stage IV** was characterized by the occurrence of heat resistant spores; within a timeframe of about one hour, the level of heat resistant spores increased from undetectable to approximately 1×10⁷ CFU/ml, *i.e.* the maximum level. During this stage the levels of CPE and DPA increased rapidly to the maximum levels. Microscopical analysis revealed that the majority of cells consisted of mother cells carrying phase bright spores. Time T₅ is a representative point in the middle of Stage IV.

**Stage V** was characterized by a stable concentration of heat resistant spores and DPA, indicative of completion of the spore formation process, albeit that the majority of the spores were still retained in the mother cells. The total viable counts started to decrease significantly of the spore formation process, but at a lower level (around 2 log₂-folds and maximally 3 h later, respectively.

Subsequently, genome wide transcriptional gene expression profiling was performed using custom microarrays. The expression of the above mentioned germination genes from the time-course microarray experiment revealed that their expression levels were increased one or more log₂-fold during sporulation (Figure 3.2A). These genes were not expressed during exponential growth (*T*=2.5 h) but specifically expressed during *C. perfringens* sporulation (the following time points, see Figure 3.1). When *C. perfringens* was cultured in TGY, the viable counts increased from 10³ CFU/ml at 3.0 h to 10⁹ CFU/ml at 6.0 h, but sporulation did not occur (data not shown). The selected germination genes were not expressed in this medium (Figure 3.2B), confirming that their expression is specifically associated with *C. perfringens* sporulation. The expression patterns in time of the assessed genes in mDS showed similar patterns for most target genes, however, in the case of prkC and "sleB" (CPR_2348) the onset of the gene up-regulation was later than the other target genes, namely around 1.5 h and 3 h later, respectively.

3.3 Identification of genes (putatively) involved in sporulation based on expression profiles and their occurrence in other sporeforming bacteria

*k*-means clustering of gene expression profiles during the *C. perfringens* sporulation process - based on point-wise similarity with equal weight - yielded six clusters of temporal expression. These clusters are presented in Figure 3.3 for the two independent experiments A and B. Many genes were upregulated during sporulation to different extents: the expression levels of 106 genes in Cluster 1 exceeded five log₂-folds. 294 genes in Cluster 2 showed levels of up-regulation between three and five log₂-folds (Figure 3.3). Lastly, Cluster 3 also contained genes that were up-regulated during sporulation (total 451 genes), but at a lower level (around 2 log₂-folds and maximally 3
log₂-folds; Figure 3.3).

Known germination-related genes of \textit{C. perfringens} - tested earlier using RT-PCR - were mainly found in Cluster 1 (\textit{gpr, csp, sleC}) and Cluster 2 (\textit{gerKA, gerKC, gerKB, "sleB"}), while \textit{prkC} and \textit{sleM} were found in Cluster 3. This showed that members of Cluster 1 and 2 can be associated with genes that are expressed during \textit{C. perfringens} sporulation.

<table>
<thead>
<tr>
<th>Function category</th>
<th>Cluster 1</th>
<th>Cluster 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>General sporulation</td>
<td>\textit{CPR_1738 (apg)}, \textit{CPR_1782 (spoIVF)&quot;}, \textit{CPR_2019 (spoIVC)}, \textit{CPR_2018 (spoIVD)}, \textit{CPR_2017 (spoIVE)}, \textit{CPR_2491 (spoVT)}</td>
<td>\textit{CPR_2020 (apg)}, \textit{CPR_1732 (apg)}, \textit{CPR_2542 (spoIVA)}, \textit{CPR_2541 (spoVA)}, \textit{CPR_2022 (spoIVF)}, \textit{CPR_2021 (spoIVD)}, \textit{CPR_2158 (spoIVD)}, \textit{CPR_2475 (spoIVF)}, \textit{CPR_1778 (spoIVG)}, \textit{CPR_2023 (spoIVI)}, \textit{CPR_2157 (&quot;spoIV&quot;)}, \textit{CPR_1801 (spoIVC)}, \textit{CPR_1800 (spoIVD)}, \textit{CPR_1790 (spoIVG)}, \textit{CPR_2475 (spoIVF)}, \textit{CPR_1778 (spoIVG)}, \textit{CPR_2023 (spoIVI)}, \textit{CPR_2157 (&quot;spoIV&quot;)}, \textit{CPR_1801 (spoIVC)}, \textit{CPR_1800 (spoIVD)}</td>
</tr>
</tbody>
</table>

**Locus tags of \textit{C. perfringens} homologues are shown with their assigned \textit{C. perfringens} gene names, gene names of \textit{C. difficile} genes are bracketed, and locus tags of \textit{C. difficile} 630 are preceded by CD.**

In a recent review, Galperin \textit{et al.} (2012) summarized the genes involved in sporulation, based on results of experimental functionality studies on \textit{B. subtilis}, comparative genomics studies on sporeforming bacteria and proteomics studies on \textit{C. difficile} spores (de Hoon \textit{et al.}, 2010; Galperin \textit{et al.}, 2012). \textit{C. perfringens} homologues of known sporulation genes which were found to be up-regulated more than three log₂-fold during sporulation of \textit{C. perfringens} (belonging to Cluster 1 and 2) are shown in Table 3.2, listed per functional category. Additionally, supplemental Table S2 lists all \textit{C. perfringens} homologues (i.e. present in clusters 1-6) of (putative) sporulation genes summarized in Galperin \textit{et al.} (2012). Per gene, the expression during sporulation is visualized for the \textit{C. perfringens} homologues of these (putative) sporulation genes. The expression levels of the \textit{C. perfringens} homologues varied, leading to their distribution over the 6 clusters (see Table S2).

The majority of the genes with increased expression during sporulation (Cluster 1 and 2) belong to the functional categories "general sporulation"; SASPs, spore cortex, spore germination and transport. Other genes in these clusters had homologues that belong to functional categories such as housekeeping, cell division/DNA replication and cell wall metabolism (Table 3.2). The current study established the actual expression of a range of genes in \textit{C. perfringens} during sporulation, including homologues of genes with established roles in sporulation in other species. This is a strong indication that these genes in \textit{C. perfringens} have a functional role in sporulation and/or determine spore properties of this organism.

### 3.4 New candidate sporulation and germination genes

Genes with the highest level of up-regulation during sporulation, namely those belonging to Cluster 1 (more than 5 log₂-fold) and Cluster 2 (3-5 log₂-fold), were subjected to further analysis. A particular focus was on genes that showed significantly increased expression during sporulation, but showed no homologues in the list drawn up by Galperin \textit{et al.} (2012).

Firstly, an inventory was made to establish the occurrence of the homologues of the genes in the Cluster 1 genes in the genomes of publicly available sporeforming Firmicutes. Cluster 1 contains 106 genes including 25 genes that show homology with genes on the Galperin list (see Table 3.2). The analysis of the occurrence of the homologues of the Cluster 1 genes in publicly available sporeforming Firmicutes revealed that 49 of 106 genes in the Cluster 1 were conserved amongst these strains. Within these conserved genes, 32 genes were not included in the Galperin list and they potentially play roles in mechanisms involved in sporulation and may determine spore properties. In addition, 33 genes were \textit{Clostridium} specific and the remaining 24 genes were \textit{C. perfringens}-specific (Figure 3.4).
More *C. perfringens* homologues of known sporulation genes were found in Cluster 2, namely 64 on a total of 294 (see Table 3.2). All these 294 genes were also subjected to a homology search in sporeforming Firmicutes. The presence/absence of the 294 genes homologues that belong to Cluster 2 in other Firmicutes is shown in Table S2. Approximately 50% of the Cluster 2 genes, including 48 known genes, were highly conserved amongst spore-forming Firmicutes, while approximately 30% of the genes were *Clostridium* specific and the remaining 20% *C. perfringens*-specific.

Our study established that expression of a number of likely candidate genes involved in spore formation actually occurs in *C. perfringens*. This include CPR_2486, a homologue of *B. subtilis* yabP (involved in spore coat assembly) (van Ooij et al., 2004); CPR_1593, a homologue of *yybI* (located at inner coat of spores) (Eichenberger et al., 2003), CPR_2191, a homologue of *yabG* (encoding a protease involved in modification of spore coat proteins (Kuwana et al., 2006), and CPR_2044, a homologue of *ytaF* which is an essential membrane protein in sporulation (Abecasis et al., 2013). However, besides enterotoxin-coding gene *cpe*, the majority of *Clostridium*-specific and *C. perfringens* specific genes are still poorly characterized or uncharacterized.

### 4. Discussion

In this study, the succession of events during growth and the sporulation process of *C. perfringens* SM101 were characterized in mDS medium in detail. Representative time points for different stages throughout this process were selected and whole genome expression profiling was performed throughout sporulation. This confirmed expression of known sporulation genes, genes predicted in other studies, but also of genes that have so far not been associated with sporulation which may play a role in sporulation and potentially determine spore properties, including spore germination behaviour.

*C. perfringens* strain SM101 showed fast exponential growth in mDS at 37°C with generation times of 11 min. Subsequent cell elongation and formation of phase bright, heat resistant spores took place within six hours after inoculation. Similar growth rates and time to sporulation have been reported for its parental food isolate NCTC8798 (Duncan, 1973). In previous transcriptomics studies on clostridial sporulation, determination of different sporulation stages were often based on optical density measurements, representing biomass accumulation (Alsaker and Papoutsakis, 2005; Bassi et al., 2013). The combined analyses of optical densities, total viable counts, heat resistant spore counts, and microscopic imaging in this study showed a discrepancy between the increase of the OD600 and the total viable counts due to cell elongation. In addition, it was evident that only ~1% of the total number of cells produced mature, heat resistant spores. This percentage is low given the microscopic observation that a high
proportion of vegetative cells produced phase bright spores (see microscopic image at T4 in Figure 3.1). A possible explanation for this phenomenon is that immature spores were actively lysed and provided essential nutrients for a subpopulation to complete the formation of resistant spores. Similar observations have recently been reported during B. subtilis sporulation, with a role for the skf and sdp operons, encoding spore killing factor and the cannibalism toxin SDP, respectively (Westers et al., 2005; Allenby et al., 2006; Lamsa et al., 2012; Morales, 2013). Clostridium ‘cannibalism’ during sporulation has not been reported so far, and no skf or sdp homologues were found in the C. perfringens SM101 genome (data not shown).

In recent years, a substantial number of available genome sequences of spore forming bacteria have become available. This has allowed for comparative genomics studies to establish similarities and differences in the sporulation process and germination properties of relatively well-studied Bacillus species (particularly B. subtilis) and Clostridium species. The predicted presence of (putative) orthologues of known Bacillus sporulation genes in Clostridium species suggests that the sporulation cascades of these two genera overlap at least in part with a core sporulation genome that has been reported by Galperin et al. (2012). However, there are also clear differences (Jones et al., 2008; Burns and Minton, 2011; Xiao et al., 2011). One of the hallmark differences in sporulation is that the initiation of this process in bacilli is mediated by phosphorylation of Spo0A through a phosphorylation cascade (Dürre, 2011), while in clostridia, Spo0A is believed to be phosphorylated directly (Dürre, 2009). In addition, bacilli and clostridia can possess different germination mechanisms (Xiao et al., 2011).

In this study, the expression during sporulation of known C. perfringens germination genes with experimentally established roles was confirmed, namely gerKA, gerKC (Paredes-Sabja et al., 2008), gerKB (Paredes-Sabja et al., 2009), sleC (Paredes-Sabja et al., 2009), cspB (Paredes-Sabja et al., 2009) and sleM (Masayama et al., 2006). In addition, the actual expression of a large number of so far merely predicted sporulation and germination genes was confirmed in C. perfringens in this study. This included most of the genes that were listed in the publication of Galperin et al. (2012) which are deemed essential for sporulation, spore properties and germination. A substantial number of orthologues of genes that were listed by Galperin et al. (2012) showed high upregulation, including 25 genes in Cluster 1 and 64 in Cluster 2 (see Table 3.2).

Furthermore, it was found that a range of other genes were upregulated during sporulation of C. perfringens that were also highly conserved in other Firmicutes, pointing to so far uncharacterised genes that play a role in sporulation and/or determine spore properties.

For the C. perfringens genes with the highest levels of up-regulation during sporulation (i.e. Cluster 1 members) a search was performed for homologues in spore-forming Firmicutes (presented in Figure 3.4). The genes were grouped according to their conservation levels and their functional classes. Around half of the genes belonging to Cluster 1 were found to be conserved in sporeforming Firmicutes. Cluster 1 also includes genes that encode predicted proteins that are involved in amino acid/ferrous ion transport and enzymatic modifications of functional groups (transferases), which have previously not been associated with the sporulation process in particular. These genes putatively encode proteins associated with energy generation, e.g. oxidoreductase (CPR_1769), flavodoxin (CPR_0519), ferrous iron transport (operon CPR_0970-CPR_0971-CPR_0972), aminotransferases (operon CPR_0567-CPR_0568-CPR_0569) and carbonate polymerization (CPR_2159). These findings strongly suggest that these mechanisms are associated with sporulation, and the genes potentially have an influence on properties of the formed spores. The other half of the genes in Cluster 1 had homologues only in clostridia, or even more particularly, only in C. perfringens. The functions of most Clostridium-specific and C. perfringens-specific genes in Cluster 1 remain uncharacterized, except for the cpe gene, encoding the enterotoxin.

A similar analysis was performed on the genes belonging to Cluster 2, which showed less pronounced - yet significant - upregulation during sporulation. Cluster 2 contained 64 homologues of known sporulation genes out of 294 in total (see Table 3.2). In addition, 48 genes were expressed in C. perfringens during sporulation and showed orthologues in most other sporulating Firmicutes. These genes are also important candidates and may play a role in sporulation of Bacillus and Clostridium spp., such as CPR_0449 (annotated as a sensor histidine kinase, may potentially play a role in sensing environmental signal) and CPR_0558 (belongs to Mg²⁺ transport ATPase MgtC family).

Another 75 genes with orthology to genes from the Galperin list (Galperin et al., 2012) showed no significant up-regulation or levels of up-regulation that were below three log₂-folds, and therefore belonged to Cluster 3, 4, 5 or 6 (Table S1) despite having established roles in sporulation in other species. Potential explanations for such observations could include the following: the level of up-regulation of genes involved in initiation of sporulation or early stage sporulation may be underestimated as the time point of sampling is close to the reference time point (Tₜ) of exponential growth; sporulation is tightly regulated and certain genes have inhibiting effects on sporulation genes, e.g. genes encoding anti-transcriptional factors (e.g. spoIIAA); genes are expressed during sporulation but the required levels of transcripts are below three log₂-folds (e.g. genes encoding sigma-factors); pseudo genes may be present that have high sequence similarity with known sporulation genes but their expression levels were very low or completely silenced throughout the sporulation process. Nevertheless, we demonstrated that 89 genes of the Galperin list (Galperin et al., 2012) do not only have orthologues in C. perfringens, but that these genes are actually expressed during

In summary, the results of this study demonstrate the complex regulation of sporulation and germination in C. perfringens. The expression of a significant number of genes involved in various aspects of the sporulation process, such as spore killing, germination and energy generation, has been confirmed. Furthermore, the potential involvement of orthologues of known sporulation genes in other Firmicutes was investigated, indicating the presence of unique mechanisms in C. perfringens that may contribute to its sporulation and germination processes.
sporulation of this pathogen, indicative of a functional role of these orthologues.

The current study provided a genomic insight in expression patterns during sporulation of *C. perfringens* with a specific focus on sporulation and germination genes, thereby confirming expression of known and predicted genes with a role in these processes. Moreover, the study identified expression of genes during late stage sporulation of this bacterium that are highly conserved in other Firmicutes and may play so-far unidentified roles in sporulation and determine spore properties, including germination behavior.

**Acknowledgments**

The authors would like to thank Mr. Arjen Wagendorp and Mr. Erwin Berendsen for their essential help during sample preparation and Mr. Roger Bongers for his help with the microarray experiments. Dr Michiel Wels’s input during transcriptomics analysis is greatly appreciated. We thank Dr Roy Moezelaa for valuable feedback and support.

**References**


Chapter 3 Transcriptional profiling during sporulation
Chapter 3 Transcriptional profiling during sporulation


Chapter 4

A wide variety of Clostridium perfringens type A food-borne isolates that carry a chromosomal cpe gene belong to one multilocus sequence typing cluster

Y. Xiao, A. Wagendorp, R. Moezelaar, T. Abee, and M.H.J. Wells-Bennik

Published in Applied and Environmental Microbiology (2012), vol. 78(19):7060-7068
Out of 98 suspected foodborne Clostridium perfringens isolates obtained from a nationwide survey by the Food and Consumer Product Safety Authority in The Netherlands, 59 strains were identified as C. perfringens type A. Using PCR-based techniques, the cpe gene encoding enterotoxin was detected in eight isolates, showing a chromosomal-location for 7 isolates and a plasmid-location for one isolate. For further characterization of these strains, (GTG)\textsubscript{5}-rep-PCR fingerprinting analysis could distinguish C. perfringens from other sulphite reducing clostridia, but did not allow for differentiation between various types of C. perfringens strains. To characterise the C. perfringens strains further, multi locus sequence typing (MLST) analysis was performed on eight housekeeping genes of both enterotoxic and non-cpe isolates, and the data were combined with a previous global survey covering strains associated with food poisoning, gas gangrene, and isolates from food or healthy individuals. This revealed that the chromosomal cpe strains (food strains and isolates from food poisoning cases) belong to a distinct cluster that is significantly distant from all the other plasmid-cpe and cpe-negative strains. These results suggest that different groups of C. perfringens have undergone niche specialization and that a distinct group of food isolates has specific core genome sequences. Such findings have epidemiological and evolutionary significance. Better understanding of the origin and reservoir of enterotoxic C. perfringens may allow for improved control of this organism in foods.

Keywords: C. perfringens; enterotoxin; MLST; food safety

1. Introduction
The anaerobic sporeforming bacterium Clostridium perfringens can be classified into five types (A to E), based on the production of different toxins that include alpha, beta, epsilon, and iota toxins (major C. perfringens toxins). The different types are associated with different veterinary or human diseases, including gas gangrene (Rood, 1998; Petit et al., 1999; McClane et al., 2006). In addition, certain C. perfringens strains are responsible for the third most common foodborne illness in the United States and Europe (Andersson et al., 1995; 2005; EFSA, 2005; McClane, 2007); C. perfringens foodborne illness is caused by type A strains (producing alpha toxin encoded by the cpa gene, also known as phospholipase C, encoded by the plc gene) that concomitantly produce the C. perfringens enterotoxin (CPE). Production and release of CPE in the gastrointestinal tract causes diarrhea and has been associated with spore formation and lysis of the mother cell in the gut (McClane et al., 2006).

The CPE-encoding gene cpe is located either on the chromosome (C-cpe) or on a plasmid (P-cpe) (Goldner et al., 1986). Interestingly, the C. perfringens strains that carry the cpe gene on the chromosome seem to have a different specific preferred growth niche than the strains that carry the cpe gene on a plasmid. Both vegetative cells and spores of representative C-cpe strains showed overall higher resistances to heat or other environmental stresses than the P-cpe strains (Sarker et al., 2000; Li and McClane, 2006; Li and McClane, 2006; Grant et al., 2008; Lindström et al., 2011). This may result in better survival and potential outgrowth of the C-cpe strains during food processing, storage and inappropriate handling by consumers, resulting in a more frequent association with food poisoning. Strains that are commonly found to harbour the cpe gene on a plasmid are more prevalent in the human gastrointestinal tract (Li et al., 2007). These strains have been implicated in CPE-associated diarrhoea in individuals undergoing antibiotic treatment, but such P-cpe strains have also been associated with food poisoning outbreaks (Lahti et al., 2008). Recent studies suggest that food poisoning by P-cpe strains is mediated by people carrying these strains and handling foods or raw food materials (Heikinheimo et al., 2006; Tanaka et al., 2007; Lindström et al., 2011).

Standard methods to identify C. perfringens in foods are traditionally based on isolation of sulphite-reducing colonies which appear black on iron-containing agar supplemented with cycloserine. Colonies are further confirmed by nitrite reduction, lactose fermentation and motility assays (Rhodehamel and Harmon, 2001; ISO, 2004). The presence of the cpe gene or CPE production by isolates is normally only carried out in diagnostic procedures of food poisoning investigations. This could also be relevant for food isolates, considering that the cpe gene is only carried by 1-5% of C. perfringens isolates in the previously studied general populations (Smedley and McClane, 2004; Li et al., 2007). Also, the current standard methods do not distinguish between different geno-types that are typically associated with foods or with the human gastrointestinal tract. Although the reservoir for C. perfringens isolates that lead to foodborne illness and their entry into the food chain is still not completely understood, progress is being made through strain analysis and comparisons at the genomic level (Lindström et al., 2011).

The Food and Consumer Product Safety Authority in The Netherlands (further referred to as VWA) has accumulated a collection of C. perfringens isolates from a variety of foods and raw materials following regular inspections and investigations of consumer complaints and food poisoning outbreaks, by using the standard methods. In this study, the presence of the cpe gene was assessed amongst the identified C. perfringens strains, and these enterotoxic strains were further evaluated for CPE production and sporulation ability. Moreover, (GTG)\textsubscript{5}-fingerprinting patterns of strains in this collection were compared, and multilocus sequence typing (MLST) results of a selection of food-related isolates (C-cpe, P-cpe, or cpe absent) were combined in a global survey (Deguchi et al., 2009; Miyamoto et al., 2011), providing insight in niche specialization of C. perfringens enterotoxic strains.
2. Materials and methods

Bacterial strains, culturing and sporulation. 102 foodborne isolates of *C. perfringens* were obtained from VWA. These strains were isolated from various food samples during general food inspections, food poisoning outbreaks and customer’s complaint cases in the period 2000-2008. All these isolates were cultivated in Brain Heart Infusion (BHI) broth from the culture in the sulfite cycloserine azide (SCA) tubes (de Jong et al., 2002) under anaerobic conditions and incubated at 37°C in a Simplicity 888 automatic atmosphere chamber (PLAS LABS, Lansing, US) with an anaerobic gas mixture (5% CO2, 10% H2 and 85% N2). Growth indicated by culture turbidity and gas production was checked after 24 hours or extended 48 hours for slow growing strains. In total, 98 isolates were recovered. 50 μl aliquots of recovered cultures were inoculated in Cooked Meat Medium (BD, Sparks, US) followed by overnight anaerobic incubation at 37°C. These cultures were subsequently stored at 1°C as stocks. Furthermore, 13 reference strains of various toxinotypes (9 type A strains and one for each of types B-E) were obtained from public culture collections (see Figure 4.1) and enterotoxic strain SM101 was kindly provided by Dr Melville, Blacksburg, VA, USA. Strains were cultured in the recommended tryptone-glucose-yeast extract (TGy) broth (Chen et al., 2005).

Sporulation was performed by inoculating 50 μl overnight incubated Fluid Thioglycollate Medium (FTM, BD, Sparks, USA) culture in 5 ml modified Duncan-Strong (mDS) sporulating medium in which raffinose was used as carbon source instead of starch (Duncan and Strong, 1968; de Jong et al., 2002) and incubated for another 48 hours. It is known that the ability of *C. perfringens* strains to sporulate and produce CPE can depend on the sporulation medium. For this reason, we tested the sporulation capacity of 11 different *cpe*-positive strains in DS as well (24 hrs FTM culture as inoculum). Sporulation ability was determined using phase-contract microscopy and four qualitative levels were given according to observations of at least 10 microscopic views (using 10×100 magnification): no phase-bright spores visible (-), 1-3 spores per view (+), less than 50% spores per view (++) and more than 50% spores per view (+++).

DNA isolation. DNA was isolated from BHI cultures of food isolates and TGy cultures of reference strains by using a GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, US) following manufacturer’s instruction for Gram-positive bacteria. The concentration and purity of the DNA samples was determined using the Nanodrop analyzer (Thermo Fisher Scientific, Wilmington, U.S.). For a number of samples, the DNA yield was very low, and this appeared to be due to high levels of spores in BHI broth, that were not lysed by the DNA extraction method applied. DNA of these isolates was isolated from vegetative cultures following a shorter incubation period of 6 hrs; this way, the quantity and quality of the obtained DNA was comparable to DNA of the other isolates. The DNA was stored at -20°C. No DNA degradation was observed by 1% agarose electrophoresis (data not shown).

16S rRNA gene sequencing. The identity of the 98 recovered VWA isolates was verified based on the sequence of *perfringens*-specific 16S rDNA fragments, amplified by PCR using the primers P4 and pH (see Table 4.1) (Wang et al., 1994). The amplicons were purified using the GFX PCR DNA and gel band purification kit (Amersham Biosciences, Buckinghamshire, United Kingdom). Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, US), DyeEx 2.0 Spin Kit (GIAGEN, Hilden, Germany) and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, US).

PCR detection of *plc*, *cpe* and the other genes encoding *C. perfringens* major toxins. A duplex Taqman quantitative PCR reaction was performed as described by Albini et al. (Albini et al., 2008), targeting the following two genes: *plc* (also named *cpa*) encoding alpha toxin which is ubiquitously present in *C. perfringens* (Li et al., 2007; Grant et al., 2008), and *cpe* encoding CPE. Strain SM101 and ATCC13124 were included as positive and negative control (Myers et al., 2006). In addition, the genes *cph*, *ctx* and *iap*, encoding β-, ε- and ι-toxins respectively, were detected by using the multiplex PCR method described by Baums et al. (Baums et al., 2004). The strains carrying none of these three genes were assigned to type A (Songer, 1996). Strain ATCC13124 (A), LMG10468 (B), NCTC3180 (C), NCTC8346 (D) and NCTC8084 (E) were used as the reference of each type.

Further characterization of enterotoxic food isolates: *cpe* gene context, sporulation in mDS and in vitro CPE release. Eight *cpe*-positive foodborne VWA isolates plus six enterotoxic type A reference strains (SM101, NCTC8239, NCTC10613, NCTC8679, NCTC8799 and NCTC11144) were further characterized (boxed in Figure 4.1). The cpe gene context was determined using a multiple PCR method as described by Miyamoto et al. (Miyamoto et al., 2002). Additional PCR analysis was performed to evaluate proximity of cpe gene to the IS1470 element using one primer specific for the cpe gene (forward primer cpe4F) and the other specific for the IS1470-element (reverse primer IS1470R1.3). All primers are listed in Table 4.1. Furthermore, their ability to sporulate was determined by enumerating spores formed in mDS liquid medium (cultures were heated for 15 min at 70°C followed by enumeration on BHI agar plates), and in vitro release of CPE in the supernatant was determined using the PET-RPLA kit (Oxoid, Hampshire, UK), according to the manufacturer’s instruction.

(GTG)$_6$-rep-PCR-fingerprinting. 67 pure VWA *Clostridium* sp. food isolates identified by 16S rRNA gene sequencing were subjected to rep-PCR fingerprinting with the primer (GTG)$_6$ (Versalovic et al., 1995; Gevers et al., 2001), with combination
Chapter 4 Genetic diversity of C. perfringens food isolates

Chapter 4 Genetic diversity of C. perfringens food isolates

of 14 reference strains including 5 with various toxin type, 6 enterotoxic type A and additional 3 cpe-negative type A (LMG12224, NCBBC47071 and NCTC8768). The PCR products were electrophoresed in a 1.5% (wt/vol) agarose gel (15 × 20 cm) for 9 h at a constant voltage of 55 V in 1 × TAE buffer (40 mM Tris–Acetate, 1 mM EDTA, pH 8.0) at 4°C. The rep-PCR profiles were visualized under UV light after staining of the gel with ethidium bromide, and digital image capturing was performed using IMAGO compact imaging system (B&L Systems, Maarsen, The Netherlands). The reproducibility of (GTG)$_5$-PCR was verified by amplifying DNA from twelve randomly chosen strains several times, showing consistent banding patterns. The fingerprints of all strains were analyzed using the BioNumerics version 5.10 software package (Applied Maths, Sint-Martens-Latem, Belgium). The similarity among digitized profiles was calculated using the curve based Cosine coefficient and a UPGMA dendrogram was derived from the profiles.

MLST of 8 cpe positive and 8 cpe-negative C. perfringens food isolates. For all eight cpe positive strains obtained from VWA and eight randomly selected cpe-negative C. perfringens foodborne strains further typing was performed by comparing sequences of eight housekeeping genes with those present in the global MLST dataset (obtained from Genbank with accession number AB477535-AB477966 (Deguchi et al., 2009), AB604033-AB604035 (Miyamoto et al., 2011) and NC003366, CP000312, NC008261 for completely sequenced strains strain 13, SM101, and ATCC13124). The typing procedure was performed as previously described by Deguchi et al. (Deguchi et al., 2009), and was based on the sequence analysis of the following genes: colA, groEL, sod, plc, gyrB, sigK, pgk and nadA. Data analysis was performed using the BioNumerics with a specific MLST plugin (version 1.001).

The annotated sequence data obtained in this study were deposited in Genbank with accession numbers JX307715- JX307850. A publicly accessible C. perfringens MLST

Figure 4.1 Clustering of (GTG)$_5$-rep patterns of identified pure Clostridium sp. foodborne isolates in this study with reference strains (type A-E). The resulting fingerprints were analyzed using the BioNumerics version 5.10 software package (Applied Maths, Sint-Martens-Latem, Belgium). The left dendrogram shows the similarity among (GTG)$_5$-rep patterns of test strains/isolates (Curve-based Cosine coefficient). The cpe-positive food isolates and reference strains are boxed.

* Sporulation ability in mDS sporulating culture was estimated using phase-contrast microscopy (magnification 1000×) and four levels of spore production were defined: no phase-bright spores/view (+), 1-3 spores/view (++), less than 50% spores formed (+++) and more than 50% spores formed (+++); spore counts (spore/ml) of cpe-positive C. perfringens strains were noted in brackets. The details are described in the Methods section.

** Cpe-types were determined using a multiplex PCR genotyping assay. Strain VW8012 and NCTC11144 could carry IS1151-cpe gene on plasmid.

*** PET RPLA kit was used to detect in vitro enterotoxin release in mDS sporulating culture of cpe-positive strains. The results were interpreted following the instructions supplied by the manufacturer.
database including all data used in this study have been hosted at PubMLST (http://pubmlst.org/cperfringens/) (Jolley and Maiden, 2010)

3. Results

Identification of C. perfringens food-related isolates. 16S rDNA sequencing of DNA extracted from 98 recovered cultures in BHI broth rendered unambiguous sequences for 67 strains; 59 of these strains were C. perfringens. In addition, these strains were plc-positive (plc encodes the alpha toxin) and their (GTG)_5-patterns belonged to the same cluster. Of these 59 C. perfringens isolates, 8 strains were found to contain the cpe gene (see Table 4.2); these strains had the following origins and were genotyped further: meat dish (VWA009), salmon (VWA012), poultry dish (VWA085), potato dish (VWA031), soup (VWA019, VWA326) and miscellaneous meat and meat products (VWA001, VWA300) (see Figure 4.1). Within these eight strains, only VWA009 was associated with a food poisoning case; VWA012, VWA031 and VWA300 were isolated from complaint samples; and the rest were isolated from samples during regular inspections. Meanwhile, another 5 isolates were associated with food poisoning cases but they do not carry a cpe gene. In the 59 isolates, the other three C. perfringens major toxin genes (encoding beta, epsilon, and iota toxin) were not detected, while PCR reactions using template DNA from the control strains were positive.

The 8 pure non-C. perfringens isolates were identified as C. baratii (3 strains), C. bifermentans (4 strains) and C. sordellii (1 strain) by 16S rDNA sequencing. These strains were plc- and cpe-negative as shown by PCR and rendered significantly different banding patterns in the (GTG)_5-fingerprints compared with C. perfringens, clearly not clustering with C. perfringens (Figure 4.1). In addition, 31 of the 98 recovered cultures were not pure, since 16S rDNA sequencing results were ambiguous and it was not possible to recognize clear (GTG)_5-patterns (data not shown). Duplex qPCR results on DNA of these cultures revealed the presence of the plc gene in 13 of these 31 cultures, indicating that C. perfringens was part of a mixed culture. In these 13 cultures, the cpe gene could not be detected. The other 18 impure isolates were plc-negative.

In summary, out of 98 recovered isolates, 59 cultures were pure C. perfringens type A (including 8 cpe positive strains), 13 cultures contained C. perfringens (cpe-negative) mixed with other strains, 8 cultures were pure strains of C. baratii, C. bifermentans, and C. sordellii, and 18 cultures were impure without C. perfringens. The prevalence of enterotoxigenic strains (8) in the pure (59) and impure (13) C. perfringens-containing foodborne isolates in this study is 11%.

Sporulation ability and enterotoxin release from enterotoxigenic strains. The ability of the selected C. perfringens strains to sporulate when incubated in mDS broth, a medium that is known to support sporulation of C. perfringens, was overall poor, but highly variable as some strains sporulated well. The ability to sporulate did not correlate with the presence of the cpe gene or the source of isolation of a strain. The non-C. perfringens food isolates showed very high sporulation efficiencies, e.g. all four
The spore yields of cpe-positive strains VWA001, NCTC8678, NCTC8679, NCTC8799 and NCTC10613 were < 1.3 log_{10} CFU/ml in mDS and DS medium. For strains SM101, VWA009, VWA085, VWA326, NCTC8239 and NCTC11144, the spore yields were 5.8, 6.5, 5.6, 2.7, 1.6 and 6.5 log_{10} CFU/ml respectively in mDS and lower in DS, namely 4.8, 6.4, <1.3, <1.3, <1.3 and <1.3 log_{10} CFU/ml, respectively. For this reason all subsequent sporulation experiments were performed in mDS.

For the 8 cpe-positive food isolates identified in the current study plus the reference strains SM101 and five enterotoxic food poisoning strains from the NCTC collection, the sporulation behaviour and in vitro enterotoxin release was assessed in more detail. The spore counts of these strains in mDS culture revealed a group of strains with good sporulation (>10^7 spores/ml) including SM101, NCTC8239, NCTC11144, VWA009, VWA085, VWA326, NCTC8679, VWA012, VWA019 and VWA031), and a group of strains that sporulated poorly (<10 CFU/ml) consisting of NCTC8799, NCTC10613, VWA001 and VWA300. In vitro CPE toxin release in the culture supernatant correlated with the ability of strains to sporulate in the culturing medium (Figure 4.1); strains that sporulated best released the highest concentrations of enterotoxin in the medium (marked as "+++"), while in four poor sporeforming cultures enterotoxin toxin was not detected in the supernatant. The correlation between sporulation capacity and enterotoxin production is in line with previous observations (Lindström et al., 2011).

Genotyping of C. perfringens cpe-positive food isolates. The presence of the cpe gene was demonstrated in 8 out of the 59 pure C. perfringens strains by specific Taqman PCR.

C. bifermentans strains fully sporulated (Figure 4.1).

Based on (GTG)_5-rep-PCR fingerprinting patterns of all identified C. perfringens strains no significant correlation between cpe occurrence and chromosomal organization was identified, and moreover, cpe-positive strains did not form clear clusters amongst cpe-negative isolates and reference strains. Furthermore, no correlation was observed between toxinotypes and chromosomal organizations of the isolates and reference strains (Figure 4.1).

Further genotyping MLST was performed to establish whether diversity between cpe positive strains is due to site-mutagenesis following evolutionary adaptation to specific environmental niches or due to plasmid transfer or IS element translocation, which has been suggested by a recent epidemiological study on worldwide strains (Deguchi et al., 2009). To this end, eight housekeeping genes were sequenced for eight cpe-positive and eight randomly selected cpe-negative food isolates obtained in this study, with addition of P-cpe strain NCTC11144. These sequences were merged with a dataset containing sequences of 62 C. perfringens strains published (Deguchi et al., 2009; Miyamoto et al., 2011). The results show that six out of seven C. perfringens food isolates with chromosomal cpe genes cluster with all other previously analysed C. perfringens cpe-negative isolates and reference strains. Furthermore, no correlation was observed between group I and II and the occurrence and chromosomal organization of the isolates and reference strains (Figure 4.1).
range. Enterotoxic VWA009 is positioned as an intermediate between Group I and II. Six food isolates from Japan (M01-08 excluding M-04) are close to each other and clustered as Group III (in yellow).

4. Discussion
According to a recent estimation by Scallan et al (2011), 10% of foodborne illnesses in the U.S.A. (about 1.0 million cases per year) are caused by the pathogen C. perfringens. In The Netherlands, the estimated number of cases is approximately 160,000 (Haagsma et al., 2009; Wijnands et al., 2011). Only a fraction of C. perfringens foodborne isolates analysed in this study contained the enterotoxin encoding gene cpe and can potentially lead to C. perfringens food poisoning. The prevalence of cpe-positive strains in this study was slightly higher than previously reported incidences of 1-5%, which included non-enterotoxic veterinary isolates from animals (except a few type C and E strains) and clinical gas gangrene isolates (Smedley et al., 2005; Li et al., 2007; Lindström et al., 2011). A prevalence of cpe positive strains in 11% of the isolates is closer to recent results of a simultaneous study in The Netherlands, in which 15.6% of all identified foodborne isolates were cpe-positive (Wijnands et al., 2011).

Our results show that the CPE production by cpe positive strains correlated with the ability of strains to sporulate, which is in line with earlier findings on C. perfringens strains including clinical isolates (Lindström et al., 2011). Recent evidence that mechanistically links sporulation to cpe production was presented in a study by Huang et al, who showed that disruption of the gene spo0A, encoding the central sporulation transcription factor, results in the inability of cells to produce spores and enterotoxin production in strain SM101 (Huang et al., 2004). Furthermore, sporulation-specific sigma factors SigE and SigK also play an important role in controlling CPE synthesis (Harry et al., 2009). Since a significant proportion of the cpe positive strains do not sporulate well under laboratory conditions, as observed also in our study, this phenomenon may have implications for application of CPE-targeted immunological assays (e.g. PET-RPLA or ELISA) to identify enterotoxic C. perfringens isolated from food samples, even though these assays have been proven functional to detect CPE in faeces samples of food poisoning patients. This potential disagreement between clinical evidence and origin isolation may lead to conflicting diagnostic results and difficulty to build up association. Meanwhile, the ability of C. perfringens to sporulate...
and thus produce CPE toxin strongly depends on a range of factors including strain-specific properties, inoculation ratio, incubation temperature, and environmental factors (de Jong et al., 2002; Le Marc et al., 2008). Cpe-positive strains that show a negative result in the toxin assay may well be able to produce CPE in the GI tract and lead to food poisoning. Therefore, molecular detection of the cpe gene is a more reliable way to identify (potential) food poisoning strains, especially since factors supporting efficient sporulation of \textit{C. perfringens} are not fully understood, and universal \textit{in vitro} sporulation conditions remain to be defined (Lindström et al., 2011). In this study classical culturing and confirmation methods for \textit{C. perfringens} gave false positive results in approximately 30% of cases, while only approximately 11% of identified \textit{C. perfringens} isolates was able to produce enterotoxin. Clearly, there is a need for specific cultivating methods for \textit{C. perfringens} strains that can cause food poisoning.

A simple oligonucleotide repeat, \((\text{GTG})_5\), can be used to rapidly classify or accurately genotype species from various genera including nontuberculous \textit{Mycobacterium} spp. (Cilliers et al., 1997), \textit{Enterococcus} spp. (Svec et al., 2005), and \textit{Lactobacillus} isolates (Gevers et al., 2001). In this study, \textit{C. perfringens} strains could be distinguished from other closely related sulfate-reducing \textit{Clostridium} spp. using \((\text{GTG})_5\)-fingerprinting. However, relatively low discrimination was observed between different \textit{C. perfringens} strains using \((\text{GTG})_5\)-rep-typing: food isolates, reference strains carrying cpe or strains belonging to different toxin-types did not show distinct profiles, even though analysis of the available \textit{C. perfringens} genome sequences showed diverse G+C content, chromosomal sizes and organizations for different \textit{C. perfringens} strains (Myers et al., 2006; Xiao et al., 2011). This technique therefore does not seem suitable for clustering \textit{C. perfringens} strains.

Therefore, another more discriminative typing approach, using MLST, was taken to investigate strain diversity in specific niches. MLST allowed for a detailed classification of \textit{C. perfringens} strains. This technique is powerful for comparison of strains when sufficiently large databases of sequences of housekeeping genes are available. MLST profiles have been established for veterinary isolates of \textit{C. perfringens} isolates with various sets of housekeeping genes and primers; these data are publicly accessible (Jost et al., 2006; Chalmers et al., 2008; Neumann and Rehberger, 2009; Hibberd et al., 2011). More recently, Deguchi et al set up a MLST scheme to cluster \textit{C. perfringens} strains associated with food poisoning, gas gangrene, and isolates from food or healthy individuals (Deguchi et al., 2009). In the current study, we applied this method on the 8 cpe-positive and on 8 cpe-negative \textit{C. perfringens} food isolates, a P-cpe reference strains NCTC11144, and then interpreted our data based on the existing dataset that so far covers 62 strains (Deguchi et al., 2009; Miyamoto et al., 2011). The original clustering was based on similarity of the concatenated sequences, which has been improved in this study by applying BioNumerics and its MLST plugin (Pot et al., 2011). The genetic type assignment and sequence similarity comparison were performed based on an individual housekeeping gene (locus); then the entries were clustered by normalized locus types.

The enterotoxigenic strains carrying the cpe gene on the chromosome in this study belong to Group I (Figure 4.3) and correspond with Cluster I strains as described by Deguchi et al. (Deguchi et al., 2009), all sharing a distinct genetic background. These strains are typically associated with foodborne gastrointestinal infections (Deguchi et al., 2009) and their occurrence may be associated with certain phenotypic properties that favor their persistence in a food-poisoning infectious life cycle, which include different environmental niches, e.g. the human gut (sporulation and enterotoxin release), soil (dormant spores in aerobic atmosphere) and food (survival of more heat resistant spores following heat treatments).

The distinction between members of Group I and those of Group II and III is significant. Interestingly, strain VWA009 is an intermediate strain between Group I and Group II/III. Members of group II and III are more closely related and as the identified group III members were all from Japan, geographic isolation might explain this difference (Figure 4.3). The minimal spanning tree (Figure 4.3) includes the previously described clusters III-XII by Deguchi et al. (Deguchi et al., 2009), and based on this analysis of the strains belonging to cluster III-XII could effectively merged in a general Group II. The presence of the plasmid cpe gene does not correlate with a specific genetic background, which is in support of horizontal transfer of cpe genes among cpe-negative and P-cpe \textit{C. perfringens} strains.

Further studies on these isolates would render a better understanding the evolutionary separation between these three groups of \textit{C. perfringens}. Even though MLST renders a high level of resolution among various \textit{C. perfringens} strains, this analysis is not very suitable for routine analyses due to the relatively high costs associated with PCR amplification and sequence analysis of eight housekeeping genes of individual strains. Spices and herbs are considered to be an important source of \textit{C. perfringens} in the food industry (EU regulation EC 2073/2005). Spices/herbs and prepared foods are the two main food commodities in which the presence of \textit{C. perfringens} is routinely monitored, and in \textit{C. perfringens} foodborne outbreaks, such products are tentatively implicated. In a recent survey of 8,495 food samples by VWA, 92% of investigated samples were categorized into different food origins, including herbs and spices and prepared foods (Wijnands et al., 2011). Strikingly, in the current study, all 7 C-cpe strains were isolated from prepared or handled foods, and clustered in Group I. The reason that only C-cpe strains were found in this product group in our study is not entirely clear. It could be due to relatively high heat resistances of strains belonging to Group I (Huang et al., 2007), which survive during food processing, and/or due to...
outgrowth of other species that compete with C. perfringens during enrichment and subsequent cultivating steps. No cpe-positive strains were originally isolated from spices/herbs, which is in agreement with an RIVM study which showed that prevalence of cpe-positive isolates in prepared foods was much higher than in spices/herbs (23% versus 4.5%, respectively) (Wijnands et al., 2011). Following this observation, we isolated additional 22 C. perfringens strains from spices and herbs in 2010; these were also found to be cpe negative (data not shown). Our study lends support to a recent study by Lindstrom et al. (Lindström et al., 2011), who indicated that operators or processing environments might be important reservoirs for food poisoning C. perfringens strains instead of ubiquitous contamination as presumed so far.

In conclusion, this study shows that the currently used isolation and confirmation method for C. perfringens may lead to a number of false positive results (in the current study approximately 30% of cases). Meanwhile, only in about 10% of identified C. perfringens strains from spices and herbs in 2010; these were also found to be cpe negative (data not shown). Our study lends support to a recent study by Lindstrom et al. (Lindström et al., 2011), who indicated that operators or processing environments might be important reservoirs for food poisoning C. perfringens strains instead of ubiquitous contamination as presumed so far.

Acknowledgements
The authors would like to thank Dr Arieke de Jong from VWA for providing C. perfringens strains from food sources and Dr Lucas Wijnands for cross-comparison on the results obtained in this study and the survey at RIVM.

References:
Chapter 4 Genetic diversity of C. perfringens food isolates


Chapter 5

Differential outgrowth potential of *Clostridium perfringens* food-borne isolates with various *cpe*-genotypes in vacuum-packed ground beef during storage at 12°C

Y. Xiao, A. Wagendorp, T. Abee, and M.H.J. Wells-Bennik

Submitted for publication
In the current study, the outgrowth of spores derived from 15 different food isolates of *C. perfringens* including C-cpe, P-cpe and cpe-negative strains was evaluated in vacuum-packed ground beef during storage at 12°C and 25°C. The 15 strains were selected from a larger group of strains that were first evaluated for their ability to sporulate in mDS medium. Sporulation ability varied greatly between strains but was not associated with particular cpe genotypes. In line with previous studies, the tested C-cpe strains produced spores with significantly higher heat resistance than the cpe-negative and P-cpe strains (both IS1151 and IS1470-like) with the exception of strain VWA009. Following inoculation of vacuum-packed cooked ground beef with spores, the heat resistant C-cpe strains showed lower outgrowth potential in cooked ground beef stored at 12°C than the P-cpe and cpe-negative strains, while no significant differences were observed at 25°C. These results suggest that the latter strains may have a competitive advantage over C-cpe strains at reduced temperatures during storage of foods that support the growth of *C. perfringens*.

Keywords: *Clostridium perfringens*, low temperature, growth, cpe

Highlights:

- The ability of 26 *C. perfringens* food isolates and type strains to sporulate in mDS medium varies greatly between species and is independent of cpe genotypes.
- The tested C-cpe strains (except VWA009) produced spores with significantly higher heat resistance than the cpe-negative and P-cpe strains (both IS1151 and IS1470-like).
- The heat resistant C-cpe strains showed lower outgrowth potential in cooked ground beef than the P-cpe and cpe-negative strains at 12°C.

1. Introduction

*Clostridium perfringens* ubiquitously exists in soil, sewage, food and in the normal intestinal flora of humans and animals. This organism is generally considered a food pathogen since some isolates are capable of producing and releasing the enterotoxin CPE in the gastrointestinal tract, resulting in diarrhoea (McClane et al., 2006). The CPE-coding gene *cpe* is only carried by 1 to 5% of *C. perfringens* isolates in general (Li et al., 2007), and by 11 to 15.6% in foodborne isolates (Wijnands et al., 2011; Xiao et al., 2012). These *cpe* genes are frequently located on the chromosome (C-cpe), flanked by an upstream IS1469 and a downstream IS1470 element. The organization of the cpe locus on plasmids commonly contains a upstream IS1469 element, but varies in the downstream IS1470-like or IS1151 element, as reported previously (Li et al., 2013).

Until recently, *C. perfringens* food poisoning was mainly associated with strains carrying the C-cpe gene (Li et al., 2007), but plasmid-borne (P-cpe) strains are now also recognized as causative agents of food poisoning (Tanaka et al., 2003; Lahti et al., 2008). Both vegetative cells and spores of C-cpe strains display enhanced robustness compared to P-cpe strains as reflected by their higher resistance to heat, osmotic stress and nitrates (Sarker et al., 2000; Li and McClane, 2006). Deletion of the C-cpe gene or curing of plasmids in P-cpe strains does not alter resistance characteristics of these strains, suggesting that the resistance properties of C-cpe strains are linked with parameters other than CPE production (Raju and Sarker, 2005; Li and McClane, 2006). Recent molecular epidemiological studies demonstrated that the C-cpe strains belong to a distinct MLST cluster (Deguchi et al., 2009; Xiao et al., 2012). It appears that the P-cpe strains belong to a lineage that is commonly found in the gut, whereas the C-cpe strains may be more common in the food environment in which the higher robustness of the latter strains might allow for a competitive advantage of this lineage. The distinct resistance properties might be the result of horizontal gene transfer and vertical evolutionary adaption in a specific niche or environmental selection.

In the current study, we evaluated the outgrowth of different *C. perfringens* types in cooked ground beef. Individual food isolates of *C. perfringens* (C-cpe, P-cpe and cpe-negative strains) were first evaluated for their ability to sporulate and the heat resistance of their spores was assessed. Subsequently, spores of individual strains were inoculated in beef and the outgrowth of these individual strains in vacuum-packed cooked ground beef was assessed during storage at 12°C and 25°C.

2. Materials and methods

*C. perfringens* strains and spore preparation. *C. perfringens* food isolates and type strains were obtained from epidemiological surveys in The Netherlands and public...
collections. In total, 26 C. perfringens food isolates were included in the current study. The toxinotypes and cpe-genotypes of these strains were characterised as described in the previous study (Xiao et al., 2012) (see Table 5.1). All strains were routinely typable using multiplex PCR (no PCR product).

### Table 5.1 Clostridium perfringens food isolates used in the current study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolate source</th>
<th>Toxino type*</th>
<th>cpe-type**</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC13124</td>
<td>gas gangrene, type strain</td>
<td>A</td>
<td>negative</td>
<td>Myers et al. 2006</td>
</tr>
<tr>
<td>C903</td>
<td>soup</td>
<td>A</td>
<td>P-cpe, IS1470-like</td>
<td>This study</td>
</tr>
<tr>
<td>C200</td>
<td>croquette</td>
<td>A</td>
<td>P-cpe, IS1470-like</td>
<td>This study</td>
</tr>
<tr>
<td>C198(Cp10)</td>
<td>peanut sauce</td>
<td>A</td>
<td>P-cpe, IS1470-like</td>
<td>This study</td>
</tr>
<tr>
<td>C200(Cp11)</td>
<td>meat, Foe Wong Hay</td>
<td>A</td>
<td>P-cpe, IS1470-like</td>
<td>This study</td>
</tr>
<tr>
<td>C102</td>
<td>soup</td>
<td>A</td>
<td>P-cpe, IS1470-like</td>
<td>This study</td>
</tr>
<tr>
<td>NCTCL0240</td>
<td>bird, chicken</td>
<td>A</td>
<td>C-cpe</td>
<td>Melville et al. 1994</td>
</tr>
<tr>
<td>NCTCI1144</td>
<td>beef</td>
<td>A</td>
<td>P-cpe, IS1351</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>NCTCR829</td>
<td>salt beef</td>
<td>A</td>
<td>C-cpe</td>
<td>Melville et al. 1994</td>
</tr>
<tr>
<td>SM101</td>
<td>derivative of NCTC8798</td>
<td>A</td>
<td>C-cpe</td>
<td>Myers et al. 2006</td>
</tr>
<tr>
<td>VW4001</td>
<td>meat</td>
<td>A</td>
<td>C-cpe</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW4006</td>
<td>meat</td>
<td>A</td>
<td>negative</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW4009</td>
<td>meat</td>
<td>A</td>
<td>C-cpe</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW4012</td>
<td>salmon</td>
<td>A</td>
<td>P-cpe, IS1351</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW4019</td>
<td>soup</td>
<td>A</td>
<td>C-cpe</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW4031</td>
<td>meat</td>
<td>A</td>
<td>C-cpe</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW4039</td>
<td>ice cream</td>
<td>A</td>
<td>negative</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW6080</td>
<td>meat</td>
<td>A</td>
<td>negative</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW6085</td>
<td>soup</td>
<td>A</td>
<td>C-cpe</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW6114</td>
<td>powder</td>
<td>A</td>
<td>negative</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW6121</td>
<td>powder</td>
<td>A</td>
<td>negative</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW6128</td>
<td>meat</td>
<td>A</td>
<td>negative</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW6264</td>
<td>bird</td>
<td>A</td>
<td>negative</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW6300</td>
<td>meal</td>
<td>A</td>
<td>C-cpe</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW6326</td>
<td>soup</td>
<td>A</td>
<td>C-cpe</td>
<td>Xiao et al., 2012</td>
</tr>
<tr>
<td>VW6331</td>
<td>powder</td>
<td>A</td>
<td>negative</td>
<td>Xiao et al., 2012</td>
</tr>
</tbody>
</table>

* Genes encoding C. perfringens major toxins, determining toxino-types, were detected by using the multiplex PCR method described by Baumes (2004).
** Presence of cpe gene was detected by using specific duplex Taqman PCR method (Ahmiet al., 2008; Xiao et al., 2012); cpe types, chromosomal (IS1470) and plasmid borne (IS1351 and IS1470-like) were further determined using multiplex PCR genotyping assay (Miyamoto et al., 2008). For strains C033 and C211, carrying cpe gene suggested by Taqman PCR, are not typable using multiplex PCR (no PCR product).

#### Heat resistance

The heat resistance of spores of selected strains that yielded more than 3.5 log_{10} spores per ml of mDS culture was assessed at different heating regimes. Therefore, 400 μl of spore suspensions were heated, using 50-μl aliquots distributed over eight PCR tubes of a strip in a GeneAmp 9700 Thermocycler (Applied Biosystems, Foster City, CA). Spore suspensions were exposed to preheating for 10 min at 75°C to heat activate the spores, and then exposed to 95°C for 0, 5, 10, 20 or 30 minutes. After forced cooling to 4°C and storage on ice, the eight aliquots of each spore suspension were pooled and enumerated on BHI plates. Heating experiments for spores of the more heat-sensitive strains were performed at 95°C for 0, 5, 10, 20, 30 and 60 sec, respectively, following the same preheating step. The decimal reduction times at 95°C (D_{95}, in minute) of spores of the tested strains were calculated as previously described (Sarker et al., 2000).

#### Growth potential in cooked ground beef

Beef was purchased from a local butcher, and freshly ground using sanitised equipment to ensure a low background flora. A batch of ground beef (500g per bag) was stored at -20°C, and thawed at 4°C overnight before inoculation with C. perfringens spores. Portions of 25 g ± 0.5 g meat, were aliquoted in a homogenization bag with filter (12 cm×19 cm) and inoculated with 10^5 spores of individual C. perfringens strain (calculated based on pre-assessed spore concentrations) to yield a final spore population of approximately 1.6 log_{10} spores/g, to mimic a low contamination level in food. Controls without inoculation with C. perfringens were also included. For each strain, the outgrowth in time was assessed in duplicate by preparing two individual pouches with ground beef for each sampling point in time, which were opened and analysed at specific time points. All these pouches were firstly homogenised for 30 sec using a stomacher (MiniMix, Interscience, St Nom la Bretêche, FR) to reach uniform distribution of spores, then vacuum packed using a negative pressure of 1,000 MPa followed by sealing (Vacuum system 200, Henkelman, ’s-Hertogenbosch, The Netherlands) and storage on ice. To start the experiment, the sealed pouches were subjected to a heat treatment of 10 min at 75°C in a water bath (Julabo, Seelbach, Germany), followed by cooling on ice and subsequent incubation at 12°C for 0, 1, 4 and 7 days in a fan-equipped refrigerating incubator (Airtast Solution, Malden, The Netherlands) or at 25°C for 24 h.

All enumerations were performed in duplicate. The initial counts were assessed immediately after inoculation (T=0). The pouches were individually opened and homogenised with 10 ml PFZ (peptone physiological salt solution, 8.5 g/l NaCl, 1 g/l peptone) by mixing with the stomacher for 2 min. A special triple-layer pour plating technique (Heikinheimo et al., 2004) using selective egg yolk free TSC medium (Oxoid, Basingstoke, Hampshire, UK) was applied, using serial dilutions (two samples for each pouch) of the fluid from the fluid side of filter. Using this technique, all colonies of the C.
perfringens strains used resulted in black colonies. Enumeration was performed using samples directly but also after heating at 75˚C for 10 min (to inactivate vegetative cells and count spores). Typical black colonies were counted after 24 and 48 h of anaerobic incubation, resulting in enumeration of the total C. perfringens population in the meat and the spore counts only. All other stored pouches were processed in the same way.

3. Results and Discussion

Sporulation capacity of C. perfringens type strains and food isolates in mDS. The numbers of mature spores that were produced in mDS cultures were highly variable per strain and ranged from less than 10 CFU/ml (i.e. below the detection limit) to 8 log_{10} CFU/ml (Figure 5.1). No significant correlation was found between sporulation capacity and cpe-genotypes of the tested strains, and the amounts of spores yielded by eleven cpe-positive strains in the current study were not significantly different from levels obtained in mDS in a previous study (Xiao et al., 2012) (Student-T, p=0.15). In conclusion, the ability of 26 C. perfringens type strains and food isolates to sporulate in mDS medium is diverse and shows no apparent clustering of cpe-negative, C-cpe and P-cpe genotypes (see Figure 5.1).

Spores of most chromosomal cpe strains show significantly higher heat resistances than those of other cpe-genotypes, except for strain VWA009. The resistance of spores to heating at 95˚C has been used to characterize C. perfringens strains as heat sensitive (inactivation > 1 log within 1.5 min), heat resistant (inactivation > 1 log requiring more than 30 min) or intermittently heat resistant (inactivation > 1 log between 1.5 and 30 min) (Grant et al., 2008). Using this definition, heat sensitive spores were produced by all tested cpe-negative and P-cpe strains, but also by strain VWA009 (a C-cpe strain), while the rest of the C-cpe strains produced heat resistant spores. Strains with an intermediate heat resistance were not encountered (Figure 5.2). The higher resistance to heat of spores of C-cpe strains compared with P-cpe strains has been attributed to the composition of the small acid soluble protein Sp4 with an Asp on amino acid residue 36 for C-cpe strains and a glycine on residue 36 for other C. perfringens strains (Li and McClane, 2008; Li et al., 2009). If spore heat resistance is influenced by a single amino acid residue in Sp4 that directly corresponds with C-cpe or P-cpe strains, this lends support to the hypothesis that C-cpe strains have a unique genetic and epidemiological background, possibly following vertical evolution. Interestingly, strain VWA009 carries the cpe gene on the chromosome but shows a heat sensitive phenotype; based on MLST typing, this strain belongs neither...
to the genetically homogeneous cluster of C-cpe strains (Group I) that are typically associated with food poisoning, nor to P-cpe strains, gastrointestinal isolates and others (Group II/III) (Xiao et al., 2012). The occurrence of a strain like VW A009 and also of an exceptionally heat resistant P-cpe strain as reported by Grant et al (Grant et al., 2008) exemplifies that grouping C. perfringens isolates based on their spore heat resistance— which appears to be linked to Ssp4 in C-cpe or P-cpe strains— is not always corresponding with their genotypes. Besides typing techniques that are based on absence or presence of the type of virulence gene (cpe) or on a set of housekeeping genes (MLST), a whole genome microarray-based comparative genomic hybridization (aCGH) analysis recently revealed genetic variety amongst C. perfringens strains. Further phenotyping demonstrated differences in myo-inositol, ethanolamine, and cellobiose metabolism between C-cpe and P-cpe strains (Lahti et al., 2012), which may be useful markers in future typing if based on phenotypic properties.

Cpe-negative and IS1470-like P-cpe strains showed enhanced growth potential compared with C-cpe strains in meat stored at 12°C. Cooked ground beef has widely been used as a model food to assess the growth potential of C. perfringens and to evaluate the effect of cooling schemes, storage temperatures and preservatives. Spore cocktails of three cpe-positive strains were routinely applied (Huang, 2003; 2006; Juneja et al., 2006; Juneja et al., 2006). In the current study, ground beef was inoculated with low levels of spores from individual C. perfringens food isolates with various cpe- genotypes, and outgrowth was assessed after vacuum packaging and cooking the beef at 75°C for 10 min followed by storage at 12°C and 25°C. The temperature of 12°C was selected since it is usually considered as the upper limit of refrigerating temperatures and used in challenge test as the worst case scenario of refrigeration temperature abuse. After 24 h of storage, no heat resistant spores were detected in each of the tested pouches stored at 12 or 25°C, indicating that all spores had germinated.

At 12°C, levels of total C. perfringens populations showed no increase compared with the counts immediately after heating at the initial time point for most strains (see Figure 5.3). Exceptions were observed for strains VWA012 and VWA039, which showed a significant reduction in counts in the first 24 h. In this study, the inoculation levels of spores were low (less than 2 log10 higher than the detection limit) to mimic low level contamination levels occurring in foods, and heat-activated surviving spores can germinate and grow out during subsequent chilled storage (Juneja and

![Figure 5.3 Total C. perfringens concentrations in cooked ground beef stored at 12°C for a maximum of 7 days. The tested strains were grouped according to their cpe-genotypes. The error bars show standard deviations of results of each strain at specific time points (see legend in insert) from a total of four samples taken from two pouches.](image-url)

![Figure 5.4 Growth of tested C. perfringens food isolates in cooked ground beef at ambient temperature (25 °C). The total C. perfringens counts at 0 day are shown in white bars, the counts at 24 h in black bars. The vertical error bars indicate standard deviations of replicates. The control samples were not inoculated with C. perfringens and the counts would come from in situ C. perfringens background in the ground beef used in this study (at t=0 below detection limit).](image-url)
Thippareddi, 2004). With initial counts as control, we could demonstrate germination of C. perfringens in meat at 12°C, but their growth within 24 h appeared to be limited, reaching maximum numbers of 100 CFU/g.

It was reported that growth of strain NCTC8239 – a C-cpe strain - in similarly prepared ground beef pouches held at 12°C was effectively prevented within 40 h (Juneja et al., 1994). However, in our study, C-cpe strains of C. perfringens showed a 2 log\(_{10}\) increase in viable numbers at 12°C when the incubation at 12°C was extended to 7 days. This increase in viable numbers was even more pronounced for cpe-negative and P-cpe IS1470-like strains, which reached 3 log\(_{10}\) CFU/g after four days, and more than 5 log\(_{10}\) CFU/g after seven days storage at 12°C. The rest of the C-cpe strains that produce heat resistant spores, including food poisoning isolate NCTC8239 grew relatively poorly compared with the strains producing heat sensitive spores. We did detect low level of C. perfringens from the meat pouch without inoculation (control samples), 1 log CFUs/g in) after 7-day incubation at 12°C and nearly 3 log CFUs/g after 24 hours at 25°C (see Figure 5.3), which indicates the presence of C. perfringens in the ground beef used in this study. These low levels did not affect the outcome of the studies with the spiked samples.

For C-cpe food isolates VWA009, VWA085 and VWA326, growth ceased between 4 and 7 days. These results clearly show that the strains that produce more heat resistant spores actually have lower growth rates with an optimal nutrient supply under cold conditions than strains that produce heat sensitive spores. Based on our observations, it is conceivable that cpe-negative and P-cpe strains outcompete C-cpe strains and become the dominant C. perfringens population in refrigerated foods.

When the ground beef was stored at abuse temperatures of 25°C, growth performance determined after 24 h incubation showed similar counts were reached for all the C. perfringens strains tested (see Figure 5.4), i.e., 6.5-7.0 log\(_{10}\) CFU/g, approaching the reported maximal cell density of 8.03 log\(_{10}\) CFU/g under comparable conditions (Huang, 2003). Earlier work revealed that the two C-cpe strains NCTC8798 and FD-1041 grew faster than two other cpe-negative and P-cpe strains at a temperature range from 41°C to 46°C (Labbe and Huang, 1995). However, these temperatures are far above the upper limit of storage temperatures and only occur when food product is improperly cooled down (USDA/FSIS, 2001).

In conclusion, the current study demonstrated that the ability of C. perfringens strains to sporulate varies greatly amongst 26 different isolates including many C-cpe and P-cpe strains from various food products. Moreover, the heat resistances of spores of C-cpe strains was significantly higher than those of spores of cpe-negative and P-cpe (both IS1151 and IS1470-like) strains, but this was not a general rule: A notable exception was observed for C-cpe strain VWA009 that produces heat-sensitive spores and which does not cluster with C-cpe strains on a phylogenetic basis (Xiao et al., 2012). Lastly, growth of different C. perfringens strains in meat showed that C-cpe, P-cpe and cpe negative strains reached similar high levels after incubation for 24 h at 25°C. However, when the meat was stored at a lower temperature of 12°C, the levels reached after 7 days were overall greater for cpe-negative strains and P-cpe strains than for C-cpe strains. Such findings suggest that during cooled storage P-cpe and cpe-negative strains may have a competitive advantage over the C-cpe strains. The varying responses of C. perfringens spores to heat and the differences in outgrowth capacity at different temperatures are factors to be considered in challenge tests and predictive modelling of C. perfringens. Our data have added further insight in differences in behaviour of C-cpe, P-cpe and cpe negative strains of C. perfringens.

Acknowledgements

We thank Lucas Wijnands (RIVM, The Netherlands) for providing C. perfringens IS1470-like P-cpe strains.

References


Chapter 6
Discussion and conclusions
1. Introduction

At the heart of foodborne disease caused by *Clostridium perfringens* lays its ability to form spores. The ubiquitous presence of *C. perfringens* is due to the fact that spores are resilient and are able to survive harsh environmental conditions. As a result, spores of *C. perfringens* can be found in many food ingredients and are able to survive a range of processing steps in the food industry. Subsequent spore germination in foods - followed by outgrowth - can lead to foodborne illness. Yet, not all *C. perfringens* strains can cause foodborne illness; only certain *C. perfringens* strains that produce enterotoxin (CPE) have this ability. Consumption of high levels of such CPE producing strains can lead to diarrhea when cells sporulate, concomitant with release of the spores and the CPE toxin in the gut.

One of the aims of this thesis was to obtain better insight in genes that play a role in the process of sporulation and germination of *C. perfringens* compared with those in the better studied *Bacillus* species. This was achieved through *in silico* analysis of germination genes in *Clostridium* and *Bacillus* species (Chapter 2). So far, studying gene function and regulation in Clostridia is hampered by the lack of genetic tools, but novel insights were obtained through whole genome transcriptome analysis during sporulation (Chapter 3). In addition, it is apparent that only certain variants of this bacterium cause foodborne disease. Therefore, the variation between strains of this species was investigated using multi locus sequence typing and toxin gene profiling (Chapter 4). Finally, the ability of strains of distinct types to germinate and grow was investigated in a model meat product (Chapter 5). The various aspects of the work described in these Chapters are discussed in more depth in the following sections of the discussion.

2. Spore formation and germination: *Clostridium* versus *Bacillus*

The mechanism of spore germination at the molecular level is better understood in *Bacillus* species than in their anaerobic relatives. Starting with *Bacillus* and *Clostridium* genes with established functionalities in germination, an *in silico* analysis was performed on available whole genome sequences of *Clostridium* species and on a representative number of *Bacillus* species.

In addition, transcriptome analysis was performed during sporulation. During this process, genes that encode germination specific proteins are expressed in the fore-spore and in the mother cell. This analysis confirmed sporulation-specific expression of genes in *C. perfringens* that are homologues of known *Bacillus* sporulation and germination genes. In addition, a large number of *C. perfringens* genes showed expression profiles with similar levels and time-course patterns as those of established germination genes. Such genes with specific up-regulation during sporulation are potential new candidates with a role in sporulation or germination. Various homologues of these up-regulated genes (many with unknown function) were highly conserved in other spore forming Firmicutes (Chapter 3). In the following paragraphs, specific aspects related to germination of *Clostridium* spores are discussed in more detail.

2.1 Clostridial germination receptors

The results presented in Chapter 2 suggest that *Bacillus* and *Clostridium* species differ in the way in which the spores sense environmental germination triggers and in which subsequent cortex degradation takes place. For other specific aspects of germination, namely, the PrkC-mediated germination pathway and the hydrolysis of the spore-specific small acid soluble proteins (SASPs), it appears that the mechanisms are similar for *Bacillus* and *Clostridium* species.

Sensing of environmental germination triggers can be mediated by germination receptors (GRs). The specificity of GRs for nutrient germinants is generally thought to be strain and species specific, and may reflect adaptions of endospore formers to their specific environmental niches. In many cases, the nutrients that can trigger germination are those found in environments that are favored by certain strains or species (Paredes-Sabja et al., 2011). It is generally believed that nutrient specificity of GRs is due to specific interaction of the germinant with proteins that make up the GR complex (Setlow, 2013). These GR proteins are synthesized in the forespore and their expression during sporulation is under the control of the forespore-specific sigma factor, σ^p. The association between GRs and nutrient germinants have been specifically studied in *B. subtilis* (strain 168), the model species of spore forming Firmicutes (Setlow, 2003; Moir, 2006). *Bacillus* GR operons are usually tricistronic and consist of three genes encoding the component A, B, C, respectively.

*B. subtilis* contains five individual ger operons, encoding the well-known GerA, GerB and GerK receptor complexes and two operons with pseudogenes, respectively (Setlow, 2003; Moir, 2006). GerA responds to L-alanine or L-valine alone, while GerB and GerK cooperate to respond to a germinant mixture of L-asparagine (or L-alanine), D-glucose, D-fructose and K^+ ion – also known as “AGFK”. Loss of the operons with the GRs containing pseudogenes does not affect *B. subtilis* spore germination with the germinants mentioned above (Setlow, 2013). The numbers of GRs encoded in the genome can vary between different strains and species. For instance, *B. cereus* strain 14579 encodes 7 GRs (Hornstra et al., 2006). The responses of GRs of *B. cereus* to specific germinants and their interactions have been studied and were recently reviewed by Abee et al., (2011). All three components of GRs appear necessary to form a functional receptor in *Bacillus* species (Moir and Smith, 1990).

The comparative genomics analysis as described in Chapter 2 rendered insight in the
occurrence and organization of GRs in Clostridium species. C. perfringens gerK is the only complete GR operon in the genome of this organism, and is the GR that has been studied best within clostridia. The gerKA and gerKC genes are co-transcribed while gerKB is transcribed divergently. This divergon-like GR operon structure was found in nine other clostridial genomes but not in any of the Bacillus genomes that were subject of study in Chapter 2. While the C. perfringens GR is called GerK, its function differs significantly from its B. subtilis GR homologue. The C. perfringens GerK receptor responds to a wide range of germinants, including KCl, L-asparagine, a mixture of KCl and L-asparagine, or a mixture of sodium and inorganic phosphate (Paredes-Sabja et al., 2008; Paredes-Sabja et al., 2009; Paredes-Sabja et al., 2009). Furthermore, in B. subtilis the three genes in the GerK operon all seem to be required for proper functioning of the receptor, whereas in C. perfringens it was recently shown that GerKC alone plays a major role (Banawas et al., 2013). These experimental results highlight the importance of the C-component of GRs, at least in the clostridia. Such findings may be significant to understand germination of clostridia better and it remains to be established whether a GR C-component by itself suffices for germinant induced germination, for instance in the case of C. kluveri, where in silico analysis of the genome revealed a sole orphan GR C-component in absence of other GR genes. Clearly, there is a need to verify the importance of the GR C-component not only in the transformable laboratory strain SM101 (Myers et al., 2006) but also in other Clostridium strains.

As indicated above, the Bacillus GR operons usually encompass three genes (encoding the A, B and C component). In various instances, GR operons were found to be tetracistronic or pentacistronic, with multiple genes in the operon encoding the GR B-component. This was the case in genomes of C. acetobutylicum ATCC824, B. cereus E33, B. licheniformis, B. halodurans, and in all C. botulinum proteolytic strains except strain Loch Maree, as reported in Chapter 2. A similar structure was also found in C. sporogenes (Broussolle et al., 2002), B. cytotoxicus (Abee et al., 2011) and B. megaterium (Christie et al., 2008). While multiple copies of the GR B component can be found in various ger operons, multiple copies of the GR A- or C-component in the same operon have not been observed so far. Specific amino acid substitution in the GerVB of B. megaterium (a tetracistronic operon containing two GerB genes) showed the particular importance of these amino acids in defining the specificity and apparent affinity for germinants (Christie et al., 2008; Christie and Lowe, 2008). However, the exact structure and molecular functionality of the Ger receptors remains to be elucidated and so far, it is not exactly known how germinants interact with the receptor complex.

In addition to the three well known GR complexes, a putative germination gene encoding a protein called “D subunit” (named gerAD) of 75 amino acid residues was found, containing two predicted transmembrane (TM) domains. In B. megaterium and B. subtilis, loss of this gene affected the GR function, despite the fact that levels of GR A, B and C subunits were not influenced (Ramirez-Peralta et al., 2013). This gene has also been found downstream of a number of likely GR-encoding operons of C. botulinum ATCC19397, C. kluveri, B. subtilis, B. megaterium, B. anthracis, B. cellulosiyticus and B. cytotoxicus (Paredes-Sabja et al., 2011). The exact role of this “D subunit” remains to be elucidated.

As mentioned above, GRs respond to nutrient germinants upon interaction with these small molecules. Highly conserved regions in GR complexes may be associated with the response to specific germinants; however at this stage, not enough is known about the binding of germinants to the GRs and the exact molecular mechanism that triggers germination. Phylogenetic analysis on GRs was performed for B. cereus group members, and revealed that an enormous diversity in germinant receptors exists within the studied genomes. It is conceivable that in addition to common germinant triggers, each species can also respond to unique (combination of) germinants and/or germination triggers (Abee et al., 2011).

The complexity and diversity of GR genes of spore formers leads to differences in gene calling of (in)complete GRs in certain genomes. An exceptional case was discussed in Chapter 2 for a C. perfringens gene that encodes an orphan protein (CPR_1053 in strain SM101). This protein has high similarity with the amino acid sequence of B. subtilis GerA, and was therefore called gerA when first described (Paredes-Sabja et al., 2008). A more detailed phylogenetic analysis showed that this protein has higher similarity with B. subtilis sporulation protein SpoVAF, which led to the exclusion of this protein as a GR protein in Chapter 2. In the expression profiling study described in Chapter 3, this gene was found to be expressed during sporulation, which was in line with its predicted function as a stage V sporulation protein. In another homology study that used a similar approach, this gene was considered to encode a monocistronic GR A protein (Paredes-Sabja et al., 2011). This example illustrates that the differences in gene calling leads to an apparent variation in the number of GRs in a specific genome. Overall, it was found that the number of ger operons (gerA family) are lower in clostridia than in bacilli, it that it varies amongst species, and that the organization is very heterogeneous.

B. subtilis GerA-like GR genes are missing in the genomes of C. difficile, C. bartletti and C. beijerinckii, even the individual components. Despite this absence, the spores of these species can germinate upon exposure to specific germinants, such as a mixture of glycine and bile salts like sodium cholate or sodium taurocholate for C. difficile, suggesting that alternative germination mechanism(s) must exist in this human pathogen. Recent investigations on C. difficile spore germination (Heeg et al., 2012; Dembek et al., 2013; Saujet et al., 2013) and outgrowth (Nerandzic and Donskey, 2013) have focused on such alternative mechanisms.
2.2 An outlook on Clostridium-specific germination pathways

*C. difficile* lacks *B. subtilis* GerA-like GRs but its spores germinate in response to bile acids present in the environment, suggesting an alternative pathway of spore germination in this species. Recently, a germination-specific protease, CspC, was identified as the sensor of the bile acids in *C. difficile* (Francis et al., 2013). This CspC is a homologue of *C. perfringens* germination-specific proteases CspA, B and C. In *C. difficile*, only CspB contains a complete catalytic triad while CspA and CspC have lost their catalytic residues. The authors found that mutations in cspC alter the specificity of germinant recognition or abrogate the ability of *C. difficile* spore to germinate in response to bile acids. Furthermore, their results suggest a unique mechanism for *C. difficile* spore germination through direct stimulation of cortex hydrolysis by a spore germinant. The Csp-SleC cortex degradation pathway was discovered and characterized, where SleC exists as an inactive zymogen (pro-SleC) and early in germination, the pro-region is cleaved by germination specific proteases, converting pro-SleC into the active cortex lytic enzyme (CLE), leading to further hydrolysis of the spore cortex (see Chapter 2 and Paredes-Sabja et al., 2011). In *C. perfringens*, CspA, CspB and CspC all have complete catalytic triads, suggesting that any one of these proteins can activate SleC-mediated cortex hydrolysis. Further investigations of CspA, B and C in *C. perfringens* strains (perhaps not in strain SM101 since it only carries CspB, and not CspA and C) will shed light on the role of these proteins in germination and sensing of environmental signals that trigger germination (see Figure 6.1).

As mentioned in Chapter 2, the presence of the divergon GR (gerK) operon in *C. perfringens* is likely correlated with the presence of the Csp-SleC cortex degradation system which requires csp-encoding GSPs to activate SleC. Since the presence of GSP-SleC cortex degradation may provide an alternative environmental sensing pathway which is independent from *B. subtilis* GerA-like GRs, this correlation may explain why some *Clostridium* species have significantly fewer but heterogeneous GRs in their genomes. For some species whose spore life cycle is highly niche specific, such as *C. difficile*, it may not be necessary to maintain *B. subtilis* GerA-like GRs.

From an evolutionary point of view it is not entirely clear which germination mechanisms appeared first. *Bacillus* and *Clostridium* species are thought to have differentiated as a result of environmental/niche-specialized selection (during the Great oxidation, 2.3 billion year ago (Canfield et al., 2013)). It is believed that *Clostridium* maintained the ancestral mechanisms, including e.g. sporulation initiation (Dürre, 2011). It is plausible that the Csp-SleC mediated germination pathway is an ancestral mechanism which is advantageous in anaerobic environments, where chemicals are generally maintained in their reduced form. Following increased availability of oxidized forms of small nutrient molecules in the environment during the Great oxidation, GRs encoded by divergon operons responding to broad-spectrum germinants (e.g. *C. perfringens* GerK receptor) and possibly the ones encoded by tetracistronic or pentacistronic operons containing multiple B-components may have appeared. This development possibly diminished the importance of the ancestral system. This trend may have continued following adaptation of spore-forming Firmicutes to other niches, possibly requiring more efficient regulation of germination and an ability to respond to more complicated environmental signals. This may have led to the occurrence of multiple specialized GRs encoded by tricistronic operons, which individually or cooperatively respond to specific germinants and are found in abundance in *Bacillus* species. Following such developments, it is conceivable that the *Clostridium* Csp-SleC mechanism was substituted by the YpeB-SleB-CwlJ-GerQ system in *Bacillus*.

2.3 Common features of spore germination

The PrkC-mediated germination pathway. Shah et al., (2008) reported that muropeptides, which are fragments of cellular peptidoglycan, can act as germinants of dormant *B. subtilis* spores via a eukaryotic-like Ser/Thr kinase called PrkC. The PrkC protein was shown to be highly conserved in the studied *Bacillus* and *Clostridium* genomes, and further analysis on these PrkC homologues revealed that the PASTA repeats would possibly determine selective binding to peptidoglycan (also described...
in Chapter 2). This germination pathway may have a similar function in (most of) the spore forming Bacillus and Clostridium species. In subsequent studies, more details on the functionality of PrkC and the germination pathway it mediates were described (Dworkin and Shah, 2010; Lee et al., 2010); however, the relevance of this system in germination activation of spores and its role in germination of spores in foods and/or host environments remains to be established (Abee et al., 2011).

Spore core hydration. Following full hydrolysis of the spore cortex, full core hydration is accompanied by expansion of the core volume, loss of resistance properties and loss of dormancy. This stage of the spore germination can be considered as the last stage in the germination process and can also be considered as the starting stage of spore outgrowth (Setlow, 2013). Germination protease (GPR) and its substrate, the small acid soluble proteins (SASPs), play a major role to release the genome that was packed in the core during sporulation, and are needed for the initiation of the metabolic activity in future vegetative cells. GPR and SASPs are highly conserved amongst the Firmicutes (Setlow, 2007). A specific SASP, encoded by C. perfringens gene spp4, was studied in detail and it appeared that a single amino acid residue substitution increased spore heat resistance (Li and McClane, 2008; Li et al., 2009). The SAPS-encoding genes in C. tetani were initially missed and not annotated in the original annotation due to mostly short ORFs that were not translated in silico (Bruggemann et al., 2003) as pointed out by Galperin et al., (2012). Generally, the presence of SASPs is compulsorily in bacterial spores. Moreover, inactivation of gene gpr, encoding the GPR germination protein, leads to very slow SAPS degradation. The time taken for Agyr spores to return to vegetative growth was much longer than for the wild type spores (Sanchez-Salas et al., 1992). Site-directed mutagenesis and structural studies on GPR in spores of Bacillus species suggest that GRP is an atypical aspartate protease (Carroll and Setlow, 2005). Considering that all GPRs studied in Chapter 2 are highly conserved at the sequence level, including the domains encoding the enzymatic activity center, the general mechanisms during core hydration is believed to be comparable for Bacillus and Clostridium species.

2.4 Discovery of putatively novel germination/sporulation associated factors - Clostridium specific genes and common genes in spore forming Firmicutes

Comparative genomics approaches can help bridge the knowledge between clostridia and bacilli. Such approaches can be used not only to transfer knowledge on spore germination from Bacillus to the less well-known Clostridium species, but also to discover novel germination associated genes (Gupta et al., 2013) or mechanisms (Galperin et al., 2012; Eijlander et al., 2013) that are present in both Bacillus and Clostridium species or unique to Clostridium species. After establishing the presence or absence of putative (novel) germination genes in certain genomes, analysis of their specific expression during sporulation aids in the assessment whether a gene is functional during sporulation and/or germination. Moreover, genes sharing expression patterns with known sporulation or germination genes during sporulation can provide leads to narrow down the candidate list of genes that are associated with sporulation or spore properties including germination.

In Chapter 3, this approach was employed by performing genome expression profiling during C. perfringens sporulation in an attempt to discover novel putative targets. The results of a comprehensive homology search within sporeforming Firmicutes revealed expression of genes during sporulation that were partly C. perfringens specific genes, whereas others were commonly present in spore forming Firmicutes. Various studies have now been performed on Clostridium species including time-course genome expression profiling using array- or RNAseq-analyses in various experimental set-ups, including the use of mutants that lack sporulation-specific transcription regulators (sigma-factors) (Alsaker and Papoutsakis, 2005; Wang et al., 2012; Bassi et al., 2013; Dembek et al., 2013; Finlaid et al., 2013; Sauer et al., 2013). Together with the comprehensive proteomic profiling of C. difficile spores (Lawley et al., 2009; Abhyankar et al., 2013), the body of knowledge on sporulation and germination is increasing. Meanwhile, further functionality analysis has been hampered by the lack of functional Clostridium genetic tools but recent developments are thought to resolve these technical difficulties.

At the time of carrying out the practical work described in this thesis, the genetic accessibility of wild type strains and the availability of genetic tools for clostridia were limited, even though some basic tools were available for the laboratory strain C. perfringens SM101.

As mentioned at the end of Chapter 2, we obtained the 2nd generation Clostron system from Prof. N. Minton (Nottingham University) as described by Heap et al. (2007) and performed experiments to knock out three (then partly putative) germination-related genes in C. perfringens, gerKA (CPR_0615), gerKC (CPR_0614), gerO (CPR_0227) and prkC (CPR_1709). The protocol (based on the one provided by Minton's lab) was adjusted for use of the system with C. perfringens, and conjugation was replaced by our optimized electroporation step for Targetron application (Chen et al., 2007). Erythromycin-resistant integrant colonies of the first three constructs were obtained. There was extremely low integration efficiency in the case of prkC, which may have been due to the anti-sense strand orientation of this gene. Subsequently, single Group II intron directed insertions to the three target loci were confirmed using PCR screening, gene-walk sequencing and southern blotting. However, we found that the constructed KO mutants sporulated very poorly in various sporulating media whereas the wild type strain forms dormant spores efficiently. We speculate that this could be due to sensitivity of the C. perfringens sporulation process to this 1.3 kb intron insertion, which might interrupt the genomic replication clock during sporulation. At the same
time, the functional analysis of our target genes was also performed and published by Paredes-Sabja and his colleagues (Paredes-Sabja et al., 2008; Paredes-Sabja et al., 2009; Paredes-Sabja et al., 2009; Paredes-Sabja et al., 2009). Given the technical difficulties in obtaining spores with the Clostron mutants and the appearance of the published papers, an alternative approach to identify novel germination genes was taken using a transcriptomics approach as described in Chapter 3. While noting the limitation of the application of Clostron and related Group II intron based techniques to study C. perfringens spore properties including germination, we do recognize that these systems provide powerful genetic tools to achieve directed mutagenesis in Clostridium species, particularly in which allele exchange hardly occurred, such as C. difficile (Burns et al., 2010), C. acetobutylicum (Steiner et al., 2011), and C. botulinum (Selby et al., 2011; Lindström et al., 2012). Since then, progress has been made on such genetic tools for use in Clostridia; two clostridial mutagenesis systems were reported to be used to successfully obtain random insertion mutant using mariner-based transposon (Cartman and Minton, 2010; Ng et al., 2013) and double-crossover allelic exchange mutants facilitated by an inducible counter selection marker (Al-Hinai et al., 2012). Development and practical implementation of these methods will enable a functional genomics approach in studying these important and poorly understood bacteria.

As discussed so far, it can be concluded that for both Bacillus and Clostridium species, endospores are essential for survival in challenging and dynamically changing environmental conditions. The multiple layer structure of spores allows for maintenance of dormancy and protection of genomic DNA, yet still provides the possibility to return to a vegetative state by recognizing environmental signals. This provides the opportunity to germinate under the right conditions using various strategies to adapt to specific niches. This strategy of these spore forming bacteria seems to be obtained from their common ancestor.

3. Genetic and phenotypic diversity of C. perfringens foodborne isolates

3.1 C-cpe type A isolates, a distinct group of foodborne pathogenic C. perfringens

C. perfringens is generally considered to be a pathogen as it is the causative agent of gas gangrene upon wound entry, often initially in the form of dormant spores. C. perfringens alpha-toxin (PLC) is essential in the etiology of gas gangrene and production of this toxin during growth is considered a housekeeping capacity of all C. perfringens strains that have been isolated so far (Chen et al., 2007). Therefore, the gene encoding the alpha-toxin, plc, can be used as a solid marker to identify C. perfringens isolates.

The answer to the question whether C. perfringens is a general foodborne pathogen is more complicated. First of all, most of collected C. perfringens strains carry no cpe gene and are therefore not able to produce CPE that causes foodborne diarrhea. The cpe-negative C. perfringens even has been used as rising agent during making bread in North America (Brown and Bardwell, 2014). Until recently, it was the general belief that foodborne isolates of C. perfringens that actually cause diarrhea carry the cpe on their chromosome (C-cpe) while isolates from other non-food gastrointestinal (GI) diseases, such as antibiotic-associated diarrhea (AAD) and sporadic diarrhea (SD), carry cpe on a transferable plasmid (P-cpe) (Sparks et al., 2001; Lindström et al., 2011). However, these associations have been challenged since it was demonstrated that P-cpe strains were involved in foodborne GI disease following outbreak investigations in Europe and Japan (Tanaka et al., 2007; Grant et al., 2008; Lahti et al., 2008). Both C-cpe and P-cpe C. perfringens isolates are capable of producing intestinally active enterotoxin and can thereby cause food poisoning. Other attributes of C. perfringens that play a major role in its ability to cause food borne illness include its ubiquitous presence in the natural environment in the formation of spores that can contaminate foods, the ability of (heat) resistant spores to survive incomplete cooking of foods, and finally the ability of spores to germinate, followed by rapid vegetative growth during storage or holding, to levels above 10^6 cells per g in the incriminated food.

A large collection of suspected C. perfringens foodborne isolates that were isolated during a nationwide survey in The Netherlands provided a great opportunity to investigate enterotoxic strains which are isolated from food. C. perfringens strains are more commonly obtained from stools of patients during food poisoning outbreaks, which may not be representative for strains found in foods. As described in Chapter 3, the C. perfringens strains that were isolated from foods in this survey were typed and it was found that only ~10% of the strains carried the cpe gene. For these cpe-positive strains, the gene was predominantly carried on the chromosome, but P-cpe strains were also found. The finding that a mix of C-cpe, P-cpe and cpe-negative strains can be isolated from foods in surveys was in line with previous outcomes of other surveys (Lin and Labbe, 2003; Wen and McClane, 2004; Aguilera et al., 2005; Corigliano et al., 2011; Kanneko et al., 2011).

Using multi locus sequence typing (MLST) analysis, it was shown that the C-cpe strains are evolutionarily distant from cpe-negative and P-cpe strains (with the latter two associated with the gut). These findings suggest different niche specializations of the P-cpe en C-cpe enterotoxigenic strains. Phenotypes of C. perfringens strains isolated from food samples (either cpe negative, C-cpe or P-cpe) were further assessed, including their ability to sporulate. Spore production was good for 15 diverse strains (cpe-negative, C-cpe or P-cpe) and the spore heat resistance of these strains was determined. In addition, their germination and growth potential was individually assessed in a model food using different storage temperatures.
Of the tested strains, the C-\textit{cpe} strains produced spores with significantly higher heat resistance than the P-\textit{cpe} strains and the \textit{cpe}-negative strains (with the exception of strain VWA009), which is in line with previous findings (Sarker et al., 2000). \textit{C-cpe} strains are also known to display higher resistance to NaCl, nitrite, and high/low temperatures than P-\textit{cpe} and \textit{cpe}-negative strains (Li and McClane, 2006; Li and McClane, 2006; Grant et al., 2008). Such stresses are commonly encountered in food processing and preservation and possibly favor the survival and outgrowth of C-\textit{cpe} strains. A demonstrated factor that contributes to the elevated heat resistance of C-\textit{cpe} spores over P-\textit{cpe} spores is the presence of a specific SASP encoded by gene \textit{ssp4} (Li and McClane, 2008; Li et al., 2009). Although increased spore heat resistance of C-\textit{cpe} strains appears to be associated with the presence of \textit{ssp4}, it is not clear what the nature of the correlation between the chromosomal location of the \textit{cpe} gene and the presence of the \textit{ssp4} gene is. The fact that C-\textit{cpe} strain VWA009 does not produce heat resistant spores indicates that this correlation does not always apply. Further investigations on this strain using array-based comparative genomics hybridization (aCGH) or full genome sequencing would generate more insight on its gene content and could reveal the genetic basis for the presence of the C-\textit{cpe} gene.

According to the USDA/FSIS guideline, the growth potential of \textit{C. perfringens} can be tested using defined conditions to evaluate the effect of cooling schemes, storage/holding temperatures, preservatives and combinations thereof. Notably, according this guideline, the test conditions require the use of spore cocktails of \textit{C. perfringens} strains NCTC 8238, NCTC 8239 and ATCC10288 that all carry the \textit{cpe} gene (Sanchez-Plata et al., 2005; Junega, 2006; Junega et al., 2006; Junega et al., 2006; Junega et al., 2006; Junega et al., 2007; Junega and Friedman, 2007; Reddy Velugoti et al., 2007; Velugoti et al., 2007; Junega et al., 2008; Miguel-Garcia et al., 2009; Junega et al., 2010; Singh et al., 2010; Valenzuela-Martinez et al., 2010; Junega et al., 2013). When different strains in a cocktail have different abilities to survive spore heating, and have different potential for outgrowth, this cannot be dissected in the end result. Therefore, in the study in Chapter 5, individual \textit{C. perfringens} strains with \textit{C-cpe}, \textit{P-cpe} and \textit{cpe}-negative genotypes were assessed for their growth potential starting from spores. The results surprisingly showed lower outgrowth potential of \textit{C-cpe} strains in cooked beef stored at 12°C, suggesting that the \textit{cpe}-negative and \textit{P-cpe} strains may have a competitive advantage over \textit{C-cpe} strains at low temperatures during storage (refrigeration abuse temperatures).

### 3.2 A need for improved methods to detect enterotoxigenic \textit{C. perfringens} in foods?

The current isolation and confirmation methods for \textit{C. perfringens} (ISO, 2004) are laborious and time consuming. More importantly, this standard method has several limitations which would lead to inaccuracy to diagnostic results as reviewed by Lindström et al., (2011). Methods to detect \textit{C. perfringens} require improvement with respect to improved specificity and discrimination between \textit{cpe}-positive (even C-\textit{cpe} versus P-\textit{cpe}) and \textit{cpe}-negative strains:

**Improved specificity.** Chapter 4 shows that the current method gives false-positive results in ~30% of cases (\textit{i.e.} species other than \textit{C. perfringens} were found), suggesting that additional confirmation at the species level for purified isolates is required (for instance using molecular techniques or MALDI-TOF analysis). On the other hand, the current culturing techniques can also render false negatives for \textit{cpe}-positive \textit{C. perfringens} strains, as described by Lindström et al., (2011). These authors reported that within several dozens of strains isolated from various sources even \textit{cpe}-positive strains on fresh plates were often rendering transparent or yellowish colonies without developing the characteristic black colour on sulphite and iron-containing agar (Lindström et al., 2011).

**Discrimination between \textit{cpe}-positive and \textit{cpe}-negative strains.** As discussed in the previous section, only \textit{cpe}-positive \textit{C. perfringens} strains are able to cause food poisoning. However, their incidence in food samples is very low, making single-colony-PCR with the aim to detect \textit{cpe} gene highly unreliable. MLST clustering of \textit{C. perfringens} strains (Chapter 4) showed that \textit{C-cpe} strains that were isolated from all over the world share a very similar genetic background. Hence, these strains might carry other unique features (associated with the \textit{cpe} gene), for instance specific metabolic enzymes. Such features could be employed to develop culturing media that are specific for \textit{C-cpe} strains (\textit{i.e.} only supporting their growth) or, even better, that show the presence of \textit{C-cpe} strains amidst other clostridia, for instance a chromogenic substance that can be converted by \textit{C-cpe} strains only, in a non-selective \textit{Clostridium} medium. A resent aCGH results, complemented with growth studies, demonstrated different myo-inositol, ethanolamine, and cellobiose metabolism between the \textit{C-cpe} and \textit{P-cpe} strains (Lahti et al., 2012), providing leads to develop an assay for the direct identification of \textit{C-cpe} or \textit{cpe}-positive \textit{C. perfringens} isolated from foods.

### 4. Strategies to control of \textit{C. perfringens} in foods

Once spores have germinated, the phase dark spores become susceptible to killing by heat and other stresses. Therefore, induction of germination could be a potential strategy to facilitate eradication of \textit{Clostridium} spores. This strategy has been studied as a possible measure to eliminate spores from food products (\textit{i.e.,} addition of germinants to reduce heat resistance of spores) (Akhtar et al., 2009). Following such a strategy, increased sporidical activity of commonly used disinfectants was achieved for \textit{C. perfringens} spores upon triggering germination using a mixture of L-asparagine and KCl (Udompittkul et al., 2013). Enhanced killing of \textit{C. difficile} spores with ultraviolet-C radiation and heat was also observed after addition of a germination...
solution containing amino acids, minerals, and taurocholic acid (Nerandzic and Donskey, 2013). The largest challenge for such a strategy is the heterogeneity and diversity of spore populations and their germination behavior. Germination efficiency can be highly heterogeneous between species and strains/isolates and may depend on spore permeability properties and possibly threshold germinant concentrations (not the same for all spores) to prevent germination of mature spores in low nutrient environments (Abee et al., 2011). Such a strategy can only be successful if the spores germinate sufficiently in response to germinants.

Current valid strategies in the food industry to control clostridial spores in products constitute a combination of routine SRC counting (aimed at assessing the initial levels), spore inactivation, and appropriate temperature control to control outgrowth if full inactivation cannot be guaranteed. The USDA-FSIS guideline gives food manufacturers strict guidelines on the maximum allowed growth of the organism, “no more than 1-log growth of \textit{C. perfringens} and no growth of \textit{C. botulinum} may occur during product stabilization”. A critical control point in this overall scheme is rapid cooling, as \textit{C. perfringens} is a prolific grower at temperatures between 45 and 37°C.

When dissecting food processing in term of heat treatment, cooling, and storage at low temperatures, differences in \textit{C. perfringens} populations may come into play. The C-cpe strains have spores that are relatively heat resistant and if such spores survive (insufficient) heat treatments, they may be able to multiply rapidly if cooling regimes are not appropriate. Once the product reaches temperatures below 12°C, growth will be limited. Therefore, in line with the USDA-FSIS guidelines, rapid cooling is very important and a critical control point. The P-cpe strains, on the other hand produce spores that are rather heat sensitive and these will normally be inactivated even by relatively low heat treatments. The probability of encountering spores of these strains in heat treated products will hence not be high. However, it is assumed that the P-cpe strains have a gut origin, and such species may be therefore be introduced as a post-processing contamination for instance by food handlers. Based on our findings that the P-cpe strains grow quite well at 12°C and reach levels well above $10^5$ CFU/g within 7 days at 12°C, these strains pose a larger risk for food borne infections than their C-cpe counterparts under such conditions. The post-processing hygienic measures may be the most critical with respect to P-cpe strains.

Concluding remarks

Overall, this thesis has provided new insights in the genes involved in germination of \textit{C. perfringens} versus the well-known \textit{Bacillus} species, and potential new candidate genes that play a role in sporulation and germination of spore forming Firmicutes were identified. In addition, the work presented in this thesis showed that C-cpe strains belong to a cluster that is distinct from P-cpe and cpe-negative strains. Strains belonging to these C-cpe and P-cpe clusters showed different heat resistance characteristics and outgrowth potential in a model food system and this points to different critical control points to prevent foodborne outbreaks due to \textit{C. perfringens}.
References:


**Chapter 6 Discussion and conclusions**


**Samenvatting**

_Clostridium perfringens_ veroorzaakt éénvandemeestvoorkomende voedselgerelateerde infecties bij de mens. Het vermogen van dit anaerobe microorganisme om sporen te vormen speelt een belangrijke rol in de verspreiding van dit organism. Sporen van _C. perfringens_ zijn resistent en komen wijder voor, waaronder in vele levensmiddelingenrijdënten. De sporen zijn in staat een groot aantal gangbare bewerkingssappen te overleven die worden toegepast in de levensmiddelenindustrie. Wanneer sporen vervolgens kunnen ontkiemenv in een bereid levensmiddel en wanneer uitgegroei kan plaatsvinden, is het mogelijk dat consumptie van een dergelijk product leidt tot ziekte. Het vermogen van verschillende _C. perfringens_ stammen on toxinen te produceren loopt sterk uiteen. Voedselinfekties worden veroorzaakt door die stammen, die het _C. perfringens_ enterotoxine (CPE) kunnen produceren. Consumptie van een product met hoge concentraties van een CPE producerende stam (meer dan 10³ kolonievormende eenheden per gram) kan leiden tot diarree wanneer vegetatieve cellen in de darm overgaan tot CPE productie en sporulatie.

Het proces van sporenvorming en -ontkieming is vrij goed bekend in _Bacillus_ soorten, met name in _Bacillus subtilis_. Eén van de doelen van dit proefschrift was om een beter inzicht te krijgen in de genen die een rol spelen in deze processen in _C. perfringens_. Uit een _in silico_ analyse van ontkiemingsgenen in de genomen van Clostridia en Bacilli bleek dat het aantal _ger_ genen (welke coderen voor de ontkiemingsreceptoren van de GerA familie) doorgaans lager is in _Clostridium_ soorten dan in _Bacillus_ soorten. Daarnaast bleek dat cortex-lytische enzymen, welke een rol spelen in de afbraak van de sporen cortex, verschillen in Clostridia ten opzichte van Bacilli.

Er zijn slechts beperkte mogelijkheden om gen-functie en gen-regulatie te bestuderen in Clostridia doordat het gebrek aan genetische methoden voor deze bacteriën. Er konden echter nieuwe inzichten worden verkregen in genen die betrokken zijn bij sporulatie en ontkieming door karakterisatie van de gen-expressie tijdens sporulatie. De meerderheid van de tot dusver gekarakteriseerde ontkiemingsgenen in _C. perfringens_ vertoonden significant% verhoogde expressie profielen in de tijd tijdens sporulatie. Ook andere genen hadden soortgelijke expressie profielen tijdens sporulatie, waaronder _C. perfringens_ homologen van _B. subtilis_ sporulatiegenen. Ongeveer de helft van de genen met geïnduceerde expressie had homologen in vrijwel alle sporenvormende Firmicuten; deze zijn mogelijk onderdeel van het repertoire aan genen die een rol speelt tijdens sporulatie en eigenschappen van sporen, waaronder ontkiemingsbedrog en hitte resistentie.

Voedselgerelateerde ziekte door _C. perfringens_ wordt veroorzaakt door stammen die drager zijn van het _cpe_ gen, dat codeert voor enterotoxine. Dit gen kan op het chromosoom (C-cpe) of op een plasmide (P-cpe) gelegen zijn. In dit proefschrift zijn
C. perfringens isolaten uit een groot aantal verschillende levensmiddelen – afkomstig uit een nationaal onderzoek - gekarakteriseerd door middel van 16S rDNA analyse en multi locus sequence typing (MLST). Tevens werd voor de isolaten vastgesteld of het toxine gen al dan niet aanwezig was. Dit liet zien dat de huidige standaard kweekmethode in ongeveer 30% van de gevallen vals-positieve resultaten geeft, waarbij andere soorten dan C. perfringens werden geïdentificeerd. Verder bleek dat van alle bevestigde C. perfringens isolaten slechts ca. 10% het cpe gen droeg. In deze cpe positieve stammen was het cpe gen doorgaans op het chromosoom geïntegreerd, maar in het aantal gevallen was het gen aanwezig op een plasmide. MLST analyse liet zien aan dat de C-cpe stammen evolutionair ver verwijderd zijn van cpe-negatieve en P-cpe stammen, waarbij de laatste twee groepen gegroepeerd worden met de darmflora. Deze resultaten benadrukken dat de huidige detectiemethodes voor C. perfringens tekort schieten ten aanzien van hun specificiteit voor het aantonen van dit organism. Daarnaast is het momenteel niet mogelijk onderscheid te maken tussen C-cpe, P-cpe en cpe-negatieve stammen op basis van kweek.

Het vermogen van verschillende C. perfringens stammen om uit te groeien werd vervolgens getest in een voedselproduct. Daartoe werden 15 individuele cpe-negatieve, C-cpe of P-cpe stammen geselecteerd. De hitterestistenties van de sporen van deze stammen werden bepaald; hieruit bleek duidelijk dat de sporen van C-cpe stammen de hoogste hitteresistentie hadden. Sporen van de individuele stammen werden vervolgens geïnoculeerd in rauw rundergehakt en na vacuumverpakking en verhitting van dit product werd de ontkiemings- en uitgroei-gedrag van de stammen bepaald gedurende bewaring bij 12°C en bij 25°C. Het vermogen van de C-cpe stammen om uit te groeien bij 12°C bleek aanzienlijk kleiner dan dat van de cpe-negatieve en P-cpe stammen; hieruit volgt dat cpe-negatieve en P-cpe stammen wellicht een competitief voordeel hebben ten opzichte van C-cpe stammen wanneer deze aanwezig zijn in een product dat onvoldoende gekoeld wordt bewaard (bijvoorbeeld in een slecht werkende koelkast). C-cpe stammen produceren sporen die relatief hitte resistent zijn maar hun groei bij temperaturen beneden 12°C is beperkt. Wanneer deze sporen een (onvoldoende) verhittingsbehandeling overleven en het product niet snel genoeg wordt gekoeld zal snelle groei tijdens afkoeling kunnen optreden. Voor de C-cpe stammen is snelle koeling tot temperaturen beneden de 12°C een belangrijke aandachtspunt voor beheersing van uitgroei. In tegenstelling tot de C-cpe stammen produceren de P-cpe stammen sporen die relatief hittegevoelig zijn, waardoor een grote mate van afkoeling zal plaatsvinden tijdens verhitting ten opzichte van C-cpe sporen. Echter, als een product na verhitting wordt besmet met dit soort stammen, bijvoorbeeld tijdens verpakking, en het product wordt vervolgens bij de hoge koeltemperaturen bewaard, dan kunnen met name P-cpe stammen een potentiële risico vormen. Hygiëne maatregelen om besmetting na verhitting te voorkomen en goede koeling zijn daarom zeer belangrijk voor de beheersing van P-cpe stammen.
Dear readers,

I am glad that you have opened this section of my doctoral thesis, and I hope that this is not the only section you are going to read. Indeed, a significant part of my time in the past years was invested to complete this study, but still it is not fair to put my sole name on the cover and front pages. The co-authors and major collaborators have been acknowledged in the chapters separately, but for many of you, I have to express my sincere appreciation here with such limited space. While some people will be mentioned in person below, this list is by no means complete and I hereby wish to truly thank all of those who contributed to the work presented in this thesis.

First of all, I would like to express my gratitude to my promotor Prof. Tjakko Abee. Thank you for your trust and providing me the opportunity to perform this PhD study. Many times, your encouragements brought me confidence to face and solve difficulties. I really enjoy my time working with you.

Secondly I would thank my co-promotor Dr. Marjon Wells-Bennik. All words become pale to describe my appreciation to you. It has been a long and tough journey since I started working on *C. perfringens*. Obviously I was not fully aware of the following challenges given by this anaerobe. I am sure that without your encouragement, support and advice, this booklet would have never been delivered. But more importantly, maturation in scientific work is the greatest gift I received from you.

Thanks to all the reviewers of this thesis and individual chapters. Thank you for spending your valuable time on my work and providing critical reviews and comments which are essential. I still believe peer review is one of the key foundations of science, which requires purely selfless contribution from each scientist. I can only return such favours to you by reviewing scientific articles in the future.

Next, I would like to express my appreciation to all co-authors and collaborators, named and unnamed so far. I would like to thank you for your contribution. I have to acknowledge that not all the efforts you have put into my project is addressed in this booklet. I am still looking for opportunities to incorporate the remaining unpublished results to incoming article(s), and I will make sure your credits will be guarded.

My paranymphs, Ellen Wemmenhove and Arjen Wagendorp, you have been the closest witnesses during my PhD study. You are the ones with whom I shared my happiness and sadness most in the lab, office or on the way between Ede and Wageningen. I greatly appreciated your company all this time.

I also would like to thank Jan Hoolwerf, Dr. Jan Rademarker, Roger Bongers, Patrick Janssen and many other (former) NIZO colleagues. Thanks for your support, understanding and the bible. To perform practical research on pathogens at NIZO, where many facilities are equipped for food-grade use, my work was confined to the
class 2 laboratories. I really enjoyed the years I spent at NIZO.

Dear Erwin Berendsen, Dr. Oylum Kutahya, Dr. Carol Mosca, Sven Menschel, Onur Ercan, Dr. Marloes Schepens, Dr. Marleen Van Ampting, Dr. Sander Sieuwerts..., we are so called NIZO-AIOs, always being symbiotic between the university and the institute, and obtaining a unique experience. We enjoyed a happy time before and are all currently embarking on the next steps in our careers. Good luck!

Thanks to my (former) colleagues at the Laboratory of Food Microbiology (FHM, WU). Since my arrival at The Netherlands in 2005, my life has never been disconnected from the department, whether it was as an MSc student, an exchange fellow, a trainee, a PhD student and now a postdoc researcher. When I made achievements, I got congratulations from you; when I met hurdles, you gave me the hints and showed me the solutions; when I suffered with the stress and conflicts caused by handling current research work and thesis finalization simultaneously, you always offered great understanding and kind encouragement.

Special thanks to Dr. Clint van Melis, not only for being my wingman these years, always helping me to explain Bacillus spore germination before my Clostridium story, but also for your great contribution during finalization of this book: the outlook on GR specificity in Chapter 6 was directly triggered by our office discussion; the Samenvtting was translated by you during the weekend; and your InDesign template makes the layout manageable.

Also I would appreciate Dr. Greetje Castelijn, Elissavet Gkogka (Lisa), Sachin Kadam, Dr. Lidia Rebele-Lima and many other FHM PhD (former) candidates for the good time we spent together, including the PhD trips to Canada and Switzerland-Germany.

I would send greetings to TIFN and industrial partners. Thanks for providing me such opportunity (funding and interesting topic) to develop in science, project management and the skills to solve practical problems from industry using advanced techniques. Particular thanks go out to Dr. Roy Moezelaar, the project manager of the theme in which I participated, and to Dr. Jan-Willem Sanders, Francois Baron and other experts.

Thanks to the clostridia community, particularly Prof. Nigel Minton and Jacque Minton, Prof. Wolfgang Liebl and Prof. Simon Cutting. The difficulty of studying these anaerobic spore formers limits the number of scientists in this field, even though clostridia show such significance in the fields of food, clinical and applied microbiology. This difficulty also unites the remaining but excellent ones to a constructive network with a friendly research atmosphere. I was connected with this community via CLOSTNET and spore/clostridia conferences, and obtained valuable help and support.

I also appreciate other mentors over the years: Prof. Beizhong Han (China Agricultural University), Dr. Jeroen Rijk (RIKILT on the time), Prof. Gary Smith (UC Davis), and
List of publications


Xiao, Y., S.A.F.T. van Hijum, T. Abee, and M.H.J. Wells-Bennik. “Genome-wide transcriptional profiling of Clostridium perfringens SM101 during sporulation extends the core of putative sporulation genes and genes determining spore properties and germination characteristics.” Submitted for publication.

Xiao, Y., A. Wagendorp, T. Abee, and M.H.J. Wells-Bennik. “Differential outgrowth potential of Clostridium perfringens food-borne isolates with various cpe-genotypes in vacuum-packed ground beef during storage at 12°C.” Submitted for publication.
Overview of completed training activities

Discipline specific activities

Courses
Molecular epidemiology and genetics of foodborne pathogenic bacteria, Helsinki, Finland (2009)
Genetics and physiology of food-associated microorganisms (2010)
CLOSTNET - Transcriptomics workshop, Munich, Germany (2011)

Meetings
Clostridia 10, Wageningen (2008)
Conference: *Clostridium perfringens*, Torquay, UK (2008, giving oral presentation)
Spore forming bacteria in food, Quimper, France (2009, giving oral presentation)
European spores conference, London, UK (2012, giving oral presentation)
KNVM conference, Wageningen (2012, giving oral presentation)

Other activities

General courses
PhD week, VLAG (2008)
Arts of writing, CENTA (2009)
Philosophy and ethics of food science and technology, WGS-VLAG (2011)
Career Perspectives, WGS (2011)

Optionals
Preparation PhD research proposal
TIFN team meetings, WE days and other events (2008-2012)
Microbiology Lunch meetings, Microbiology, NIZO (2008-2012)
VLAG PhD trip, Laboratory of Food Microbiology, Canada (2008)
VLAG PhD trip, Laboratory of Food Microbiology, Switzerland (2010, organization)
About the author

Yinghua Xiao was born on April 7th, 1981, in Jinzhou, Liaoning province, P. R. China. He developed a passion to work with food pathogens during his first independent research project supervised by Prof. Beizhong Han, China Agricultural University (CAU), Beijing. His BSc thesis entitled “Staphylococcus aureus and Salmonellae biofilms formation on food contact surfaces and effects of ultra-sonic treatment” brought him Outstanding Bachelor Thesis Scholarship of CAU, 2004. As encouraged by Prof. Han who obtained his own PhD degree in Laboratory of Food Microbiology, Wageningen University (WU), Xiao decided to continue his adventure in food microbiology in The Netherlands, 10,000 km away from home.

In the following two years (2005-2007), Xiao passed all courses of MSc program of Food Safety the first time, performed a toxicological study using microarray techniques at RIKILT on anabolic effects of natural hormonal compound diosgenin in bovines, and in between he stayed on another continent for seven-month, investigating the interaction between lipids composition and transporter functionality of Listeria monocytogenes under chilling conditions, under the supervision by Prof. Gary Smith, UC Davis, US. In 2007, Xiao was selected as one of the high-potential food science graduates of WU to join the Food Valley Ambassador Program. Within this one-year training program, Xiao worked as a research assistant at NIZO food research (Ede), where he was involved in research projects, aimed at the control of potential microbiological risks in the dairy production chain; meanwhile, he followed several training sessions in business development and networking.

Xiao stayed at NIZO for another four years (2008-2012), where he carried out his doctoral research as part of the TI Food and Nutrition project “Spores and Biofilms”. The output is described in this thesis with the title: “Clostridium perfringens sporulation, germination and outgrowth in food: a functional genomics approach”. As of July 2012, Xiao has been employed as a junior Postdoc researcher at the Laboratory of Food Microbiology, WU, working on Bacillus cereus superdormant spores.
The research described in this thesis was financially supported by the Top Institute Food & Nutrition (TIFN), Wageningen, The Netherlands and conducted at NIZO food research BV, Ede, The Netherlands.