Clostridium perfringens sporulation, germination and outgrowth in food: a functional genomics approach Y.Xiao 2014

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

Yinghua Xiao

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.

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

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Yinghua Xiao

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Table of contents

Abstract **6**

Chapter 1 **9**
General introduction and thesis outline

Chapter 2 **25**
Clostridial spore germination versus bacilli: Genome mining and current insights

Chapter 3 **47**
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

Chapter 4 **71**
A wide variety of *Clostridium perfringens* type A food-borne isolates that carry a chromosomal *cpe* gene belong to one multilocus sequence typing cluster

Chapter 5 **91**
Differential outgrowth potential of *Clostridium perfringens* food-borne isolates with various *cpe*-genotypes in vacuum-packed ground beef during storage at 12°C

Chapter 6 **105**
Discussion and conclusions

Samenvatting **125**

Acknowledgements **129**

List of publications **133**

Overview of completed training activities **135**

About the author **137**

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 10^5 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

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 *kloster* (κλωστήρ, spindle). The genus *Clostridium* consists of around 100 species, including important pathogens as well as commercially valuable solventogenic *C. butyricum* [1] (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

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))

Specie (types)	Type (reference strain*)	Genbank accessions (16S rRNA)	Foodborne isolation sources
C. algidicarnis	DSM 15099	AF127023, X77676	spoiled, vacuum-packed cooked pork
C. algidixylanolyticum	DSM12273	AF092549	vacuum-packed, temperature abused raw lamb
C. beijerinckii	ATCC 25752	X68179	spoiled candy; olives
C. butyricum	ATCC 19398	AB075768, M59085	cheese; olives; fruit juice
C. bifermentans	ATCC 638	AB075769, X75906	cheese fondue; canned tomatoes; vacuum packed smoked fish
C. botulinum (A; proteolytic B and F)	ATCC25763(A), ATCC 7949 (B), ATCC 25764 (F)	L37585 (A), L37593 (F)	improperly preserved vegetables, meat and fish
C. botulinum (E; saccharolytic B and F)	ATCC 9564 (E), ATCC 25765 (B), ATCC 27321 (F)	X68173 (B)	food
C. carnis	ATCC 25777	M59091	milk
C. estertheticum	ATCC 51377	Y18813	chill-stored vacuum-packed beef
C. estertheticum subsp. estertheticum	DSM 8809	S46734, X68181	chill-stored vacuum-packed beef
C. estertheticum subsp. laramiense			vacuum-packed beef primals
C. frigidicarnis	DSM 12271	AF069742	temperature abused vacuum-packed beef
C. gasigenes	DSM 12272	AF092548	vacuum-packed, chilled lamb that showed "blown-pack" spoilage, which causes gas production and pack distension in vacuum-packed meat
C. pasteurianum	ATCC 6013	M23930	fruit juice
C. perfringens	ATCC13124 (T)	M59103	food products (usually warm meat or meat products)
C. puniceum	ATCC 43978	X71857, X73444	rotting potatoes, a cavity spot lesion in a carrot
C. putrefaciens	ATCC 25786	AF127024, Y18177	spoiled hams
C. scatologenes	ATCC 25775	M59104	contaminated food
C. sporogenes	ATCC 3584	M59115	preserved meat and dairy products
C. sporosphaeroides	ATCC 25781	M59116	canned food
C. tyrobutyricum	ATCC 25755	M59113	dairy products (semi-hard cheese, late-blowing)
*Tissierella praeacuta (C. hastiforme)***	ATCC 25539 (DSM 5675)	X80833 (X80841)	milk

* 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)

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

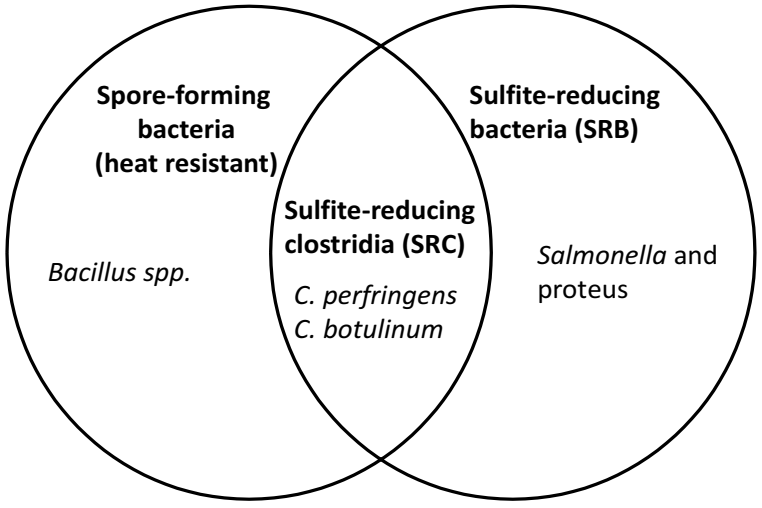

Figure 1.1: Sulfite reducing clostridia (SRC) and sulfite reducing bacteria (SRB)

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 produces for infant formula, beverages and animal feed was potentially contaminated with *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-sporeforming 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 welchii*", 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

2 Prof. Dr Shimizu sadly passed away at the moment when this thesis was in preparation. He will be memorised for his significant contribution to the *Clostridium* research field.

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 1.2 Typing of *C. perfringens* strains based on toxins produced

Type	Major toxins (encoding genes)			
	α-toxin (*plc* or *cpa*)	β-toxin (*cpb1*)	ε-toxin (*etx*)	ι-toxin (*iap, ibp*)
A	+			
B	+	+	+	
C	+	+		
D	+		+	
E	+			+

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_r 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 Ca^{2+} influx. Morphological damage caused by alterations in membrane permeability allows for unbound CPE to access to the basolatedral 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 10^5 *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 10^5 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.

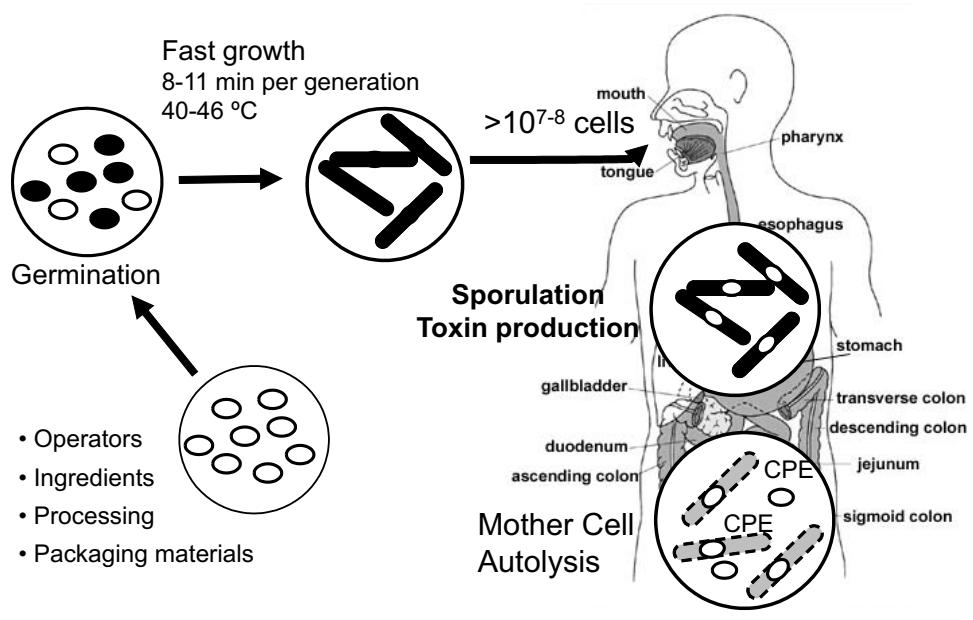

Figure 1.2 Sporulation-coupled food poisoning pathways of *C. perfringens* enterotoxic strains.

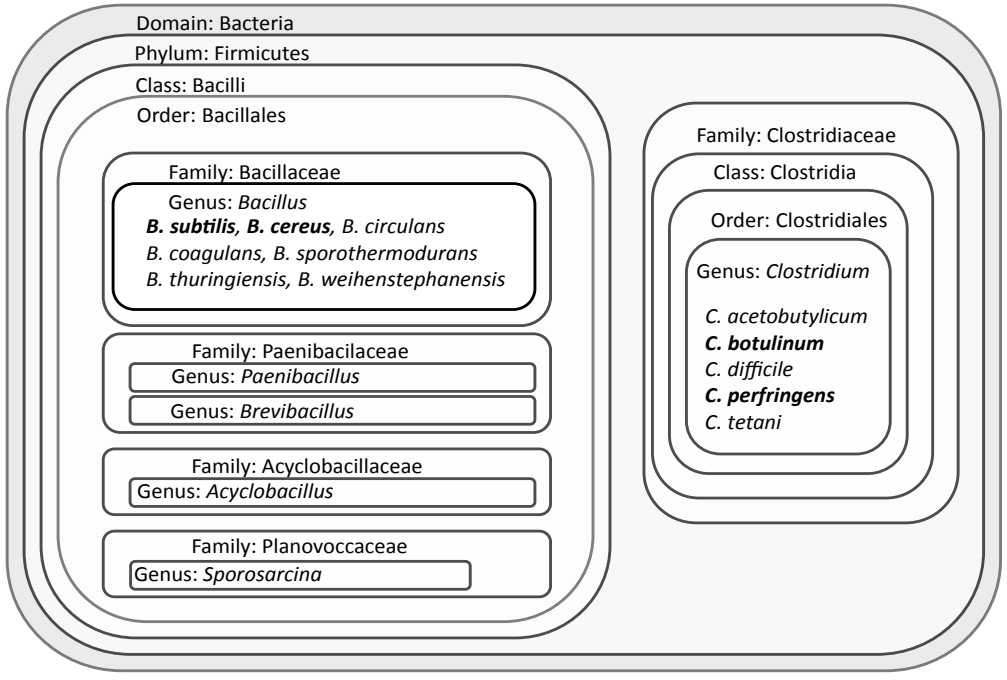

Figure 1.3 Taxonomy of spore forming bacteria

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.

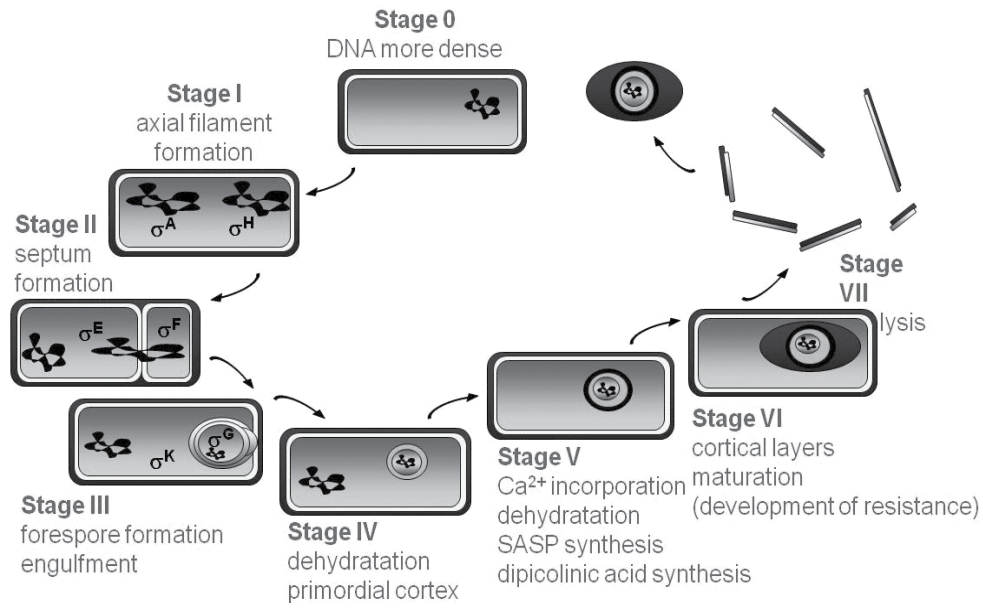

Figure 1.4 Stages of sporulation and their major aspects

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

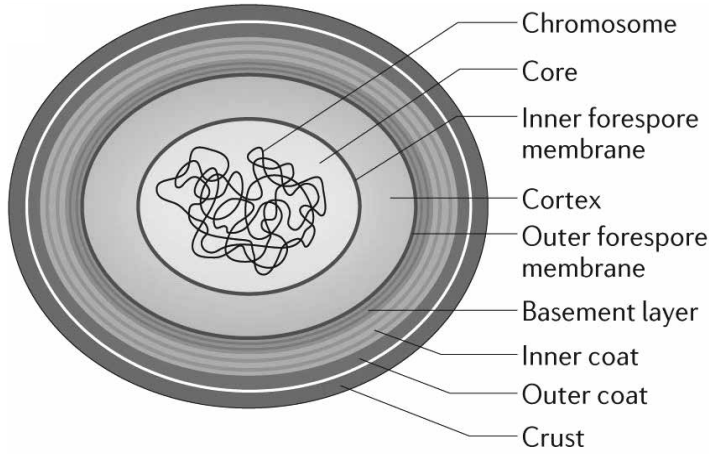

Figure 1.5 Multi-layer structures of spores (extracted from McKenney et al., (2013))

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 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 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* 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*.

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

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Chapter 2

Clostridial spore germination versus bacilli: Genome mining and current insights

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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 Zn^{2+}, K^+, Na^+, dipicolinic acid and Ca^{2+} (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.

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 *Clostridium* genomes and 12 sequenced *Bacillus* 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

Table 2.1 **Representative *Bacillus* genomes and *Clostridium* genomes included in the analysis**

ERGO code	Organism	DNA source /project	Plasmid	GenBank	Reference
BAN	*Bacillus anthracis* Ames	TIGR		AE016879	Read *et al.*, 2003
BAH	*Bacillus anthracis* str. Ames 0581 'Ames Ancestor'	J. Craig Venter Institute; TIGR	2	AE017334	Ravel *et al.*, 2009
BAR	*Bacillus anthracis* str. Sterne	Joint Genome Institute	1	AE017225	Rasko *et al.*, 2005
BCR	*Bacillus cereus* ATCC 10987	TIGR	1	AE017194	Rasko *et al.*, 2004
ZC	*Bacillus cereus* ATCC 14579 IG-20	Integrated Genomics DARPA	1	AE016877	Ivanova *et al.*, 2003
BSC	*Bacillus cereus* E33L JGI	Joint Genome Institute	5	CP000001	Han *et al.*, 2006
BCA	*Bacillus clausii* KSM-K16	Kao Corporation	1	AP006627	
HD	*Bacillus halodurans* C-125	JAMSTEC		BA000004	Takami *et al.*, 2000
BLH	*Bacillus licheniformis* DSM 13, ATCC 14580 Goettingen	Goettingen Genomics Laboratory		AE017333	Veith *et al.*, 2004
BS	*Bacillus subtilis* 168	International Consortium		AL009126	Kunst *et al.*, 1997
BTR	*Bacillus thuringiensis* serovar konkukian str. 97-27	Joint Genome Institute	1	AE017355	Han *et al.*, 2006
BWE	*Bacillus weihenstephanensis* KBAB4	DOE Joint Genome Institute	4	CP000903	Rasko *et al.*, 2005
CA	*Clostridium acetobutylicum* ATCC-824D	Genome Therapeutics,		AE001437	Nolling *et al.*, 2001
CBE	*Clostridium beijerinckii* NCIMB 8052	DOE Joint Genome Institute		CP000721	
CBJ	*Clostridium botulinum* A str. ATCC 19397	DOE Joint Genome Institute		CP000726	
CBI	*Clostridium botulinum* A str. Hall	DOE Joint Genome Institute		CP000727	
CBM	*Clostridium botulinum* A2 str. Kyoto	DOE Joint Genome Institute		CP001581	
CBG	*Clostridium botulinum* A3 str. Loch Maree	DOE Joint Genome Institute	1	CP000962	Smith *et al.*, 2007
CB	*Clostridium botulinum* ATCC-3502	Sanger Institute	1	AM412317	Sebaihia *et al.*, 2007
CBB	*Clostridium botulinum* B str. Eklund 17B	DOE Joint Genome Institute	1	CP001056	
CBD	*Clostridium botulinum* B1 str. Okra	DOE Joint Genome Institute	1	CP000939	Smith *et al.*, 2007
CBO	*Clostridium botulinum* Ba4 str. 657	DOE Joint Genome Institute	2	CP001083	Smith *et al.*, 2007
CBL	*Clostridium botulinum* E3 str. Alaska E43	DOE Joint Genome Institute		CP001078	
CBH	*Clostridium botulinum* F str. Langeland	DOE Joint Genome Institute	1	CP000728	
CCE	*Clostridium cellulolyticum* H10	Joint Genome Institute		CP001348	
DF	*Clostridium difficile* 630	Sanger Institute	1	AM180355	Sebaihia *et al.*, 2006
CBK	*Clostridium kluyveri* DSM 555	Gottingen Genomics Laboratory G2L	1	CP000673	
CLU	*Clostridium kluyveri* NBRC 12016	Research Institute of Innovative Technology for the Earth RITE, Microbiology research group	1	AP009049	
CNO	*Clostridium novyi* NT	Johns Hopkins Kimmel Comprehensive Cancer Center		CP000382	Bettegowda *et al.*, 2006
CPE	*Clostridium perfringens* 13	University of Tsukuba		BA000016	Shimizu *et al.*, 2002
CPF	*Clostridium perfringens* ATCC 13124	TIGR		CP000246	Myers *et al.*, 2006
CPR	*Clostridium perfringens* SM101	TIGR	2	CP000312	Myers *et al.*, 2006
CPY	*Clostridium phytofermentans* ISDg	DOE Joint Genome Institute		CP000885	
CLO	*Alkaliphilus oremlandii OhILAs* (*Clostridium sp.* OhILAs)	Joint Genome Institute		CP000853	
CTT	*Clostridium tetani* E88	Gottingen Genomics Laboratory	1	AE015927	Bruggemann *et al.*, 2003
CTH	*Clostridium thermocellum* ATCC-27405 JGI	Joint Genome Institute, EntrezNC_009012		CP000568	

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

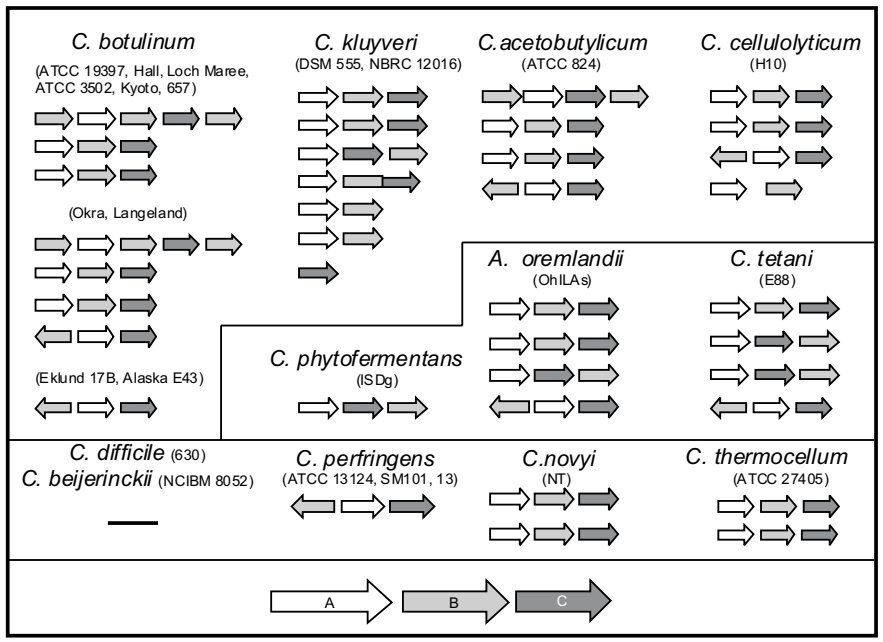

Figure 2.1Germination receptor operons of the GerA family present in *Clostridium* species. The various gene clusters encoding germinant receptor GerA homologues found in the 24 studied Clostridial genomes could be classified on basis of the organization and composition. In *B. subtilis*, each germinant receptor consists of three subunits A, B and C (with the genes are represented by arrows). The Clostridial homologues of subunit A contain five to six predicted transmembrane helices and those of subunit B contains ten predicted transmembrane helices and subunit C is a lipoprotein. *C. perfringens* and *C. kluyveri* strains share gene clusters with identical organisation. The BAC divergon present in *C. perfringens* appears also present in *C. tetani* and *C. cellulolyticum*. On basis of the *gerA* cluster organization the *C. botulinum* strains (total 10) can be divided in 3 groups: type A and Ba4 strains, type B1 and F strains and type B and E3 strains. No GerA homologues were found *C. difficile* and *C. beijerinckii* genomes. In *C. kluyveri*, an ORF containing both subunit B and C coding sequences is shown as a joint arrow.

six transmembrane helices, that GerAB is a member of the amino acid/polyamine/organo-cation (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 germination 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* genomes 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 (Broussolle *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 strain of *C. botulinum* (Peck, 2009).

Clostridium kluyveri, *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 Sonenshein, 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 to this novel mechanism. PrkC contains multiple PASTA (penicillin and serine/threonine kinase associated) repeats and these repeats have been proposed to bind species-specific peptidoglycan fragments (Setlow, 2008; Shah *et al*., 2008).

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.

Table 2.2 Homologues of previously characterized germination-related factors and their predicted functions

Organism Names	ERGO code	Ger receptor	PrkC	GerN	SleB	YpeB	CwlJ	SleL/ YaaH	SleB -like	SleM	SleC -pro	GSP*	GPR	SASP (α/β)	SASP (*ssp4/F*)	SASP (γ)	GerP	GerD
B. anthracis Ames	BAN	6	+	2	+	+	2	+	-	-	-	-	+	7	+	+	+	+
B. anthracis str. Ames 0581 'Ames Ancestor'	BAH	7	+	2	+	+	2	+	-	-	-	-	+	7	+	+	+	+
B. anthracis str. Sterne	BAR	6	+	2	+	+	2	+	-	-	-	-	+	7	+	+	+	+
B. cereus ATCC 10987	BCR	6	+	2	+	+	2	+	-	-	-	-	+	5	+	+	+	+
B. cereus ATCC 14579 (IG-20)	ZC	8	+	2	+	+	2	+	-	-	-	-	+	6	+	2	+	+
B. cereus E33L (JGI)	BSC	7	+	2	+	+	2	+	-	-	-	-	+	7	-	+	+	+
B. clausii KSM-K16	BCA	7	+	-	+	+	+	+	-	-	-	-	+	4	-	+	-	+
B. halodurans C-125	HD	6	+	-	+	+	+	+	-	-	-	-	+	4	+	+	-	+
B. licheniformis DSM 13, ATCC 14580	BLH	4	+	-	+	+	+	2	-	-	-	-	+	3	+	+	+	+
B. subtilis 168	BS	5	+	-	+	+	+	2	-	-	-	-	+	6	+	+	+	+
B. thuringiensis serovar konkukian str. 97-27	BTR	7	+	2	+	+	2	+	-	-	-	-	+	10	-	+	+	+
B. weihenstephanensis KBAB4	BWE	6	+	2	+	+	3	+	-	-	-	-	+	5	+	2	+	+
C. acetobutylicum ATCC-824D	CA	4	+	-	+	+	-	+	+	-	+	+	+	3	+	-	-	-
C. beijerinckii NCIMB 8052	CBE	-	+	-	-	-	-	-	+	+	2	+	+	11	+	-	-	-
C. botulinum A str. ATCC 19397	CBJ	3	+	+	+	+	+	-	+	-	-	-	+	4	+	-	-	-
C. botulinum A str. Hall	CBI	3	+	+	+	+	+	-	+	-	-	-	+	4	+	-	-	-
C. botulinum A2 str. Kyoto	CBM	3	+	+	+	+	+	-	+	-	-	-	+	4	+	-	-	-
C. botulinum A3 str. Loch Maree	CBG	3	+	+	+	+	+	-	+	-	-	-	+	5	+	-	-	-
C. botulinum ATCC-3502	CB	3	+	+	+	+	+	-	+	-	-	-	+	4	+	-	-	-
C. botulinum B str. Eklund 17B	CBB	+	+	+	-	-	-	-	+	+	2	+	+	5	+	-	-	-
C. botulinum B1 str. Okra	CBD	4	+	+	+	+	+	-	+	-	-	-	+	5	+	-	-	-
C. botulinum Ba4 str. 657	CBO	3	+	+	+	+	+	-	+	-	-	-	+	5	+	-	-	-
C. botulinum E3 str. Alaska E43	CBL	+	+	+	-	-	-	-	+	-	2	+	+	5	+	-	-	-
C. botulinum F str. Langeland	CBH	4	+	+	+	+	+	-	+	-	-	-	+	3	+	-	-	-
C. cellulolyticum H10	CCE	4	+	+	+	+	+	+	+	-	-	-	+	4	2**	-	-	-
C. difficile 630	DF	-	+	-	-	-	-	-	-	-	+	-	+	2	+	-	-	-
C. kluyveri DSM 555	CBK	7	+	+	+	+	+	+	+	-	-	-	+	9	+	-	-	-
C. kluyveri NBRC 12016	CLU	7	+	+	+	+	+	+	+	-	-	-	+	10	+	-	-	-
C. novyi NT	CNO	2	+	+	+	+	+	+	+	-	-	-	+	2	+	-	-	-
C. perfringens 13	CPE	+	+	+	-	-	-	-	+	+	+	2	+	4	+	-	-	-
C. perfringens ATCC 13124	CPF	+	+	+	-	-	-	-	+	+	+	2	+	4	+	-	-	-
C. perfringens SM101	CPR	+	+	+	-	-	-	-	+	+	+	2	+	4	+	-	-	-
C. phytofermentans ISDg	CPY	+	+	-	-	-	-	+	-	-	+	-	+	2	+	-	-	-
A. oremlandii OhILAs	CLO	4	+	-	+	+	-	+	-	-	-	-	+	4	-	-	-	-
C. tetani E88	CTT	4	+	+	+	+	-	+	+	-	+	+	+	-	-	-	-	-
C. thermocellum ATCC-27405 (JGI)	CTH	2	+	-	2	+	+	-	-	-	-	-	+	2	2**	-	-	-

- : homologue (cluster) does not exist or very low homology; + : one homologue (cluster) exists; number: more than one homologues or clusters exist
(continue)

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

Factors	Genes***	Functions	P-score cut off****
Ger receptors	*ger* cluster	Germinant receptors located on spore inner membrane, initiating germination by sensing environment	1×10^{-20}
PrkC	*prkC*	Serine/threonine protein kinase (EC 2.7.11.1), responsible in muro-peptides-induced germination of B. subtilis	1×10^{-20}
GerN	*gerN* or *napA*	$Na^+/H^+/K^+$ antiporter	1×10^{-20}
SleB	*sleB*	Spore cortex lytic enzymes	1×10^{-40}
YpeB	*ypeB*	SleB-stabilizing protein, required for expression, localization and activation of SleB	1×10^{-40}
CwlJ	*cwlJ*	Cortex lytic enzymes	1×10^{-40}
SleL/YaaH	*sleL/yaaH*	Spore peptidoglycan hydrolase (N-acetylglucosaminidase) (EC 3.2.1.-)	1×10^{-40}
SleB-like	*sleB*	Spore cortex lytic enzymes or cell wall hydrolase	1×10^{-40}
SleM	*sleM*	N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	1×10^{-40}
SleC-pro	*sleC*	Spore cortex-lytic enzymes pre-pro-form, a bifunctional enzyme possessing lytic transglycosylase activity and N-acetylmuramoyl-L-alanine amidase activity after activation by GSP	1×10^{-40}
GSP	*cspA,B,C*	Germination specific protease, activating SleC	1×10^{-20}
GPR	*gpr*	Germination protease in spore core, degrading SASPs	1×10^{-20}
SASP (α/β)	*sspA,B,C,D*	α/β-type small acid soluble proteins in spore core, protecting spore DNA	0.001
SASP (*ssp4/F*)	*sspF, ssp4*	A minor SASP in *B. subtilis* or associated to heat resistance of some *C. perfringens* strains	0.001
SASP (γ)	*sspE*	γ-type SASP playing no role in spore resistance but providing amino acids essential for protein synthesis	0.001
GerP	*gerP* cluster	Proteins in spore coat, facilitating transfer of germinants	1×10^{-20}
GerD	*gerD*	Modulating action of germinant receptors	1×10^{-20}

* Only *csp* clusters at upstream of *sleC* genes are counted

** Contain one *C. perfringens* Ssp4 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 homologue 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 Ca^{2+}, 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).

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 *C. 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. tetani* 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.

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 found essential for activity and autoprocessing 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

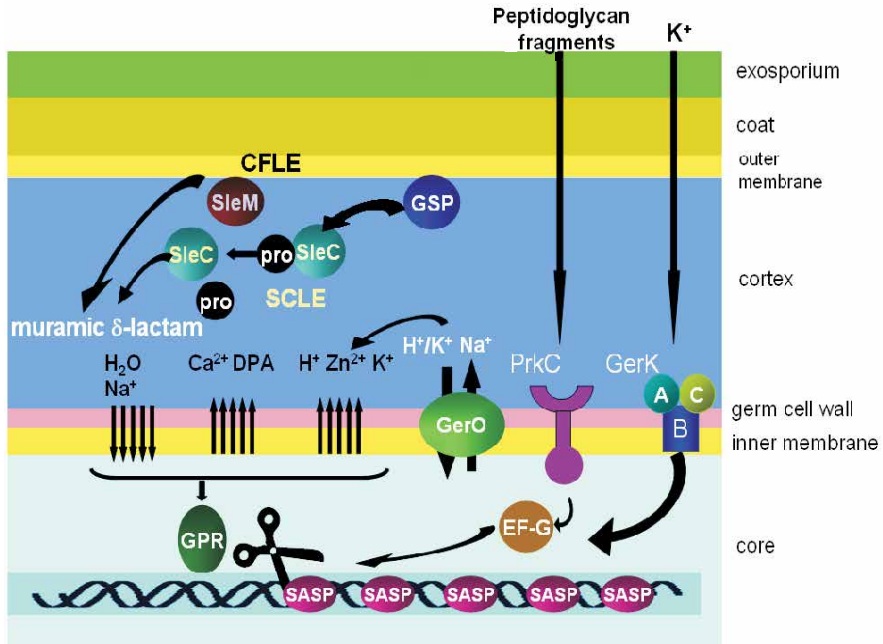

Figure 2.2 Schematic overview of the factors involved in *C. perfringens* spore germination. PrkC mediated germination of *C. perfringens* spores can be initiated by binding of peptidoglycan fragments to PASTA repeats regions of the PrkC protein. Germination mediated by the Ger receptor is initiated by interaction between K^+ and subunit A or C of the GerK receptor. This event is believed to be followed by activation of the Na^+/H^+-K^+ antiporter GerO (*B. subtilis* GerN homologue), leading to alteration of the electrochemical gradient across the membrane and bulk movement of ions. Cortex degradation is required for full germination, and is mediated through Spore Cortex Lytic Enzymes (SCLE) and Cortex Fragment Lytic Enzymes (CFLE). In the spore cortex, a bifunctional enzyme SleC possessing lytic transglycosylase activity and N-acetylmuramoyl-L-alanine amidase activity is activated by *csp*-coded germination specific protease (GSP) by cutting its pro-site, leading to changes of cortex structures, which are necessary before muramidase SleM can function. As a consequence of increasing water activity, germination protease GPR becomes active and degrades the small acid-soluble proteins (SASPs).

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* CwlJ, 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* Eklund 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.

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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

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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 *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 *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 *C. perfringens* homologues of *Bacillus* genes with roles in sporulation and germination; this study suggests that the homologues are functional in *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 *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, *C. perfringens*

1. Introduction

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, draught, 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 *Bacillus* and *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 *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 *Bacillus* species, particularly in *B. subtilis* (Eichenberger, 2012). For clostridia, these phenomena have been investigated for industrially relevant *C. acetobutylicum* and clinically relevant *C. difficile* (Alsaker and Papoutsakis, 2005; Burns and Minton, 2011). So far, detailed studies on global gene expression during sporulation has not been reported for *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 *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 *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 *C. perfringens* have previously been identified (Xiao *et al.*, 2011), among which only one *ger* divergon (*gerKB*, *gerKA-gerKC*), encoding a germination receptor complex. The presence of *prkC*, encoding a Ser/Thr kinase in *C. perfringens,* suggests the existence of an alternative germination pathway triggered by environmental peptidoglycan fragments, as described for *B. subtilis* (Shah *et al.*, 2008; Shah and Dworkin, 2010; Xiao *et al.*, 2011). *C. perfringens* also carries the gene *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 *B. subtilis* spore DNA from lethal damages (Setlow, 2007). Cortex degradation of *C. perfringens* has been shown to require a spore cortex-lytic enzyme, cortical fragment-lytic enzymes and a germination-specific protease, encoded by genes *sleC*, *sleM* and

csp, respectively (Kumazawa *et al.*, 2007; Paredes-Sabja *et al.*, 2009; Paredes-Sabja *et al.*, 2009). *In silico* analysis suggested this mechanism is present in *C. perfringens*, *C. difficile*, *C. beijerinckii* and specific strains of *C. botulinum* (Kumazawa *et al.*, 2007; Xiao *et al.*, 2011). The expression of the majority of genes that encode characterized germination proteins, including *gerKA, gerKC, sleC, sleM, csp*, is highly sporulation-specific (Masayama *et al.*, 2006; Paredes-Sabja *et al.*, 2008) and under the control of the sporulation σ factors σ^G or σ^K. These σ factors normally appear during late stage 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 (OD_{600}), 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; Orsburn *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

2.1Bacterial 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% H_2, 5% CO_2, 85% N_2, 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 ml 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 (OD_{600}) 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 ×g 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 supernatant, followed by CPE measurement using the PET-RPLA test.

DPA accumulation was measured using the other half of the harvested pellet, which was

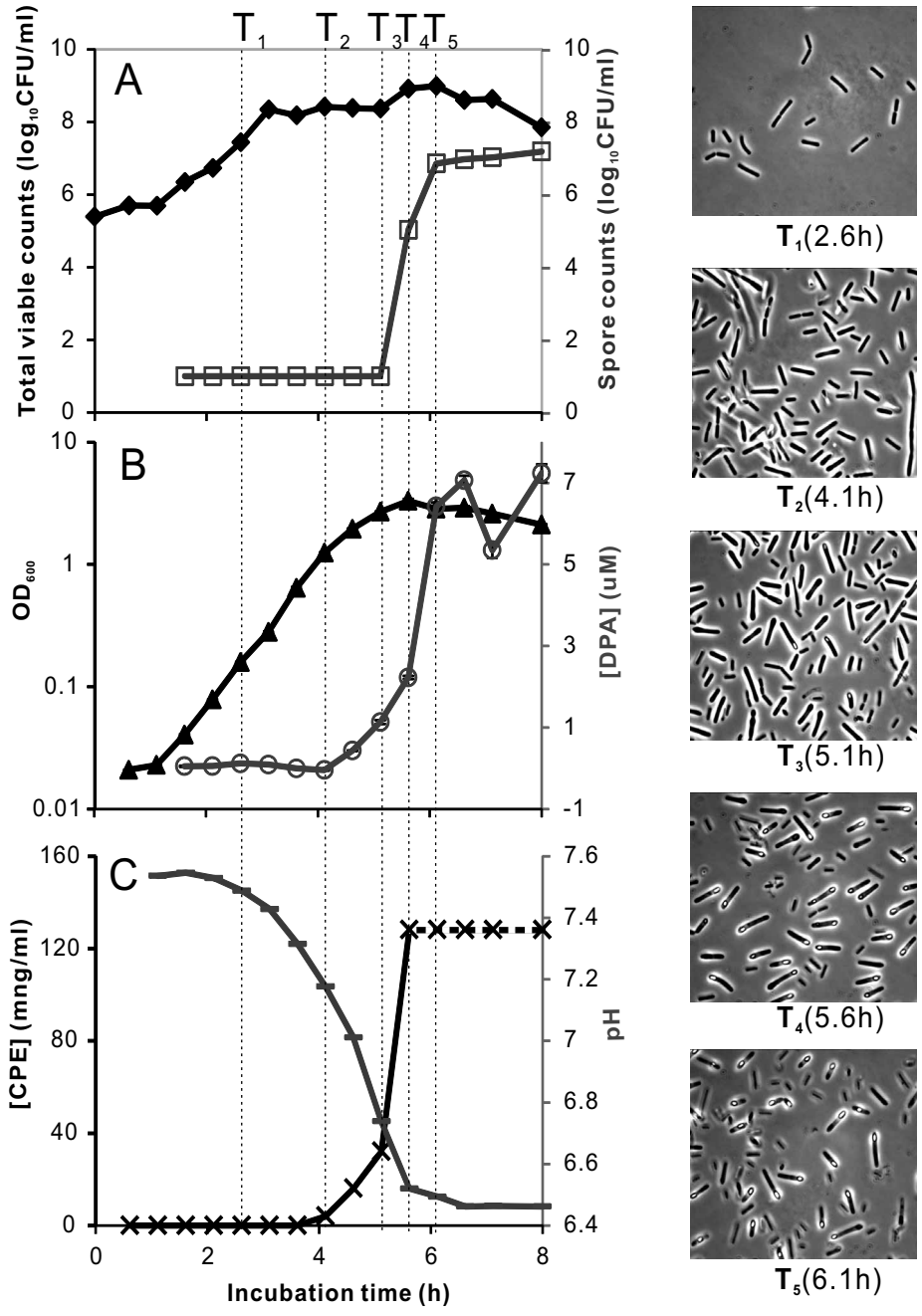

Figure 3.1 Changes of measured biological indicator following sporulation of *C. perfringens* SM101. Results of measured biological indicators in time course of *C. perfringens* SM101 sporulation and phase contract microscopical images taken at representative time points of five sporulation stages (magnification: 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).

washed in phosphate buffered saline (PBS, containing 130 mM NaCl, 10 mM sodium phosphate, pH 7.4) and autoclaved at 121°C for 15 min to release DPA from forespores and spores. DPA concentrations were determined by measuring the emission at 545 nm of the fluorescent terbium-DPA complex in a Safire II plate reader equipped with B122253 fluorescence top module (TECAN, Austria) as previously described (Kort *et al.*, 2005).

Furthermore, samples were examined microscopically. Fixation and microscopic imaging was performed as follows: harvested cells/spores originating from 5 ml of a culture were fixed upon removal from the anaerobic chamber using 3 ml cold 4% paraformaldehyde (Sigma, Steinheim, Germany) solution followed by incubation at 4°C for 16 h. The fixated samples were washed once with a mixture of PBS and 0.1 % Tergitol-type NP-40 (Sigma-Aldrich, St. Louis, MO, US) (9:1, v:v), and once with PBS only. The pellet was then re-suspended in a cold mixture (1:1, v:v) of PBS and ethanol (99.9 %). 100 µl aliquots were made and stored at -20°C for at least 1 h. Complete fixation of spores was confirmed by spreading 0.1 ml fixated *C. perfringens* spore suspension on BHI agar. No colony formation was observed on the plates after anaerobic incubation at 37°C for 3 days. The morphological stages of sporulation were examined for all samples using phase-contract microscopy (magnification 1000×) (see Figure 3.1). The microscopic images were collected using an imaging system (Zeiss Axioplan microscope with Zeiss HRc camera).

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_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 (Qbiogene, 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 RNeasy kit (QIAGEN, Hilgen, Germany). On-column DNA digestion was performed during purification using RNase-Free DNase

(QIAGEN, Hilgen, Germany). RNA concentrations were determined using a Nanodrop analyzer (Thermo Fisher Scientific, Wilmington, US) and RNA was confirmed to be intact using a Bioanalyzer (Agilent technologies, Palo Alto, CA, US).

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.*

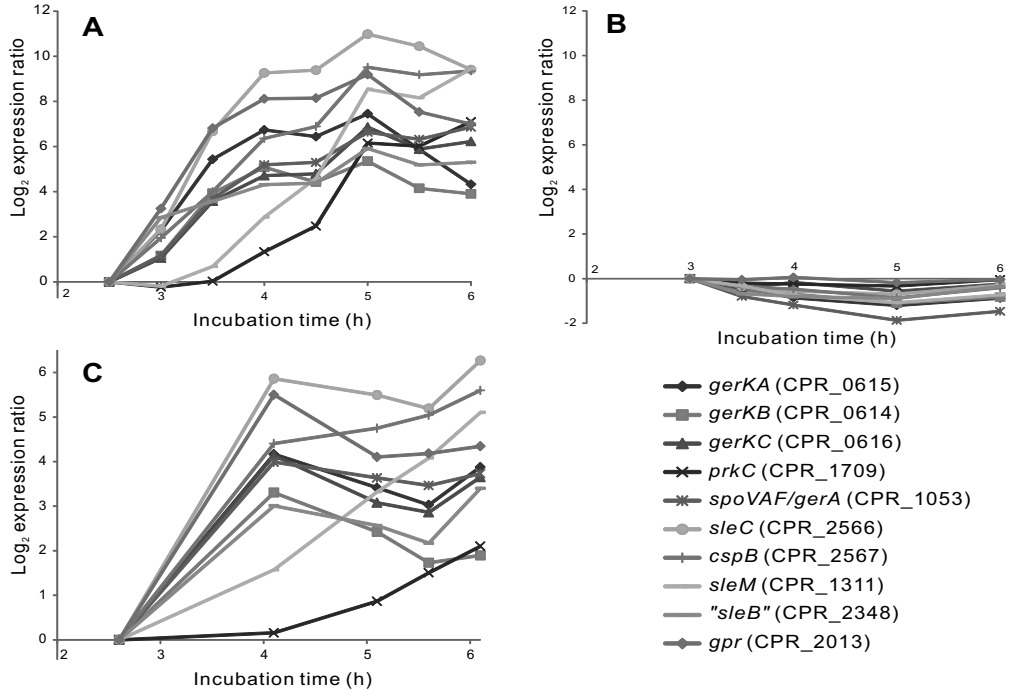

Figure 3.2 Time course expression of germination-related genes assessed by RT-PCR and microarray. Samples were taken from cultures growing in mDS (A) and TGY in which cells did not sporulate (B) every 0.5 h after inoculation. *Ct* values resulted from RT-PCR were normalized with combination of *dnaE* and *sigH*. RNA samples taken at the five representative time points were used for microarray expression profiling (C). The data extraction and presentation is described in the Materials and Methods section.

perfringens SM101 cultures throughout growth and sporulation, five time points were selected that represented different stages of the spore-forming procedure, namely, exponential growth (T_1), cell elongation (T_2), early sporulation (T_3), late sporulation (T_4) and cell lysis (T_5). 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 T_1 to T_5 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

S1). The subsequent steps in the microarray expression profiling experiments included concentration of labeled cDNA mixtures, hybridization, washing, slide scanning, spot quantification and normalization. This was performed as described previously (Leimena *et al.*, 2012), with adjustments to reaction volumes according to instructions of the manufacturer for 44K arrays. The microarray data have been deposited in NCBI's Gene Expression Omibus (GSE accession number will be provided during review).

Clustering. The expression at time points T_2 to T_5 (sporulation stages) were compared with T_1 (exponential growth phase). Genes with similar transcription profiles were clustered with a *k*-means cluster analysis using Euclidean distance,50 maximum iterations and 6 predefined clusters (Genesis, v1.7.6) (Sturn *et al.*, 2002). The results of two independently performed time-course experiments were subjected to the clustering (see Figure 3.3). Sporulation-specific upregulated genes were grouped in the Clusters 1 and 2, and genes belonging to these two clusters were the focus of further analyses as described below.

2.6 Homologue search in sporeforming Firmicutes

To evaluate the occurrence of homologues in a wider range of endospore-forming Firmicutes, genes present in 113 publicly available genome sequences which belong to the Firmicutes were investigated. Briefly, the chromosomal and protein coding sequences of these strains were obtained from the National Center for Biotechnology Information (NCBI) site. The genes were first assigned to Clusters of Orthologous Groups of proteins (COGs) (Tatusov *et al.*, 1997) using KOGNITOR (Snel *et al.*, 2002). The remaining genes were clustered using OrthoMCL with default parameters (Li *et al.*, 2003) resulting in orthologous groups of genes (OGs). COGs are more generic and the additional OGs likely signify more specific gene functions.

Predicted operons in the genome of *C. perfringens* SM101 were obtained from two independent sources using different methods (Price *et al.*, 2005; Li *et al.*, 2009) . MGcV (Overmars *et al.*, 2013) was used to visualize all genes of Cluster 1 and 2 together with the transcriptional terminators predicted using TransTerm (Ermolaeva *et al.*, 2000). The genes with potential co-expression are labeled in Figure 3.4 for Cluster 1 and Table S3 for Cluster 2.

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

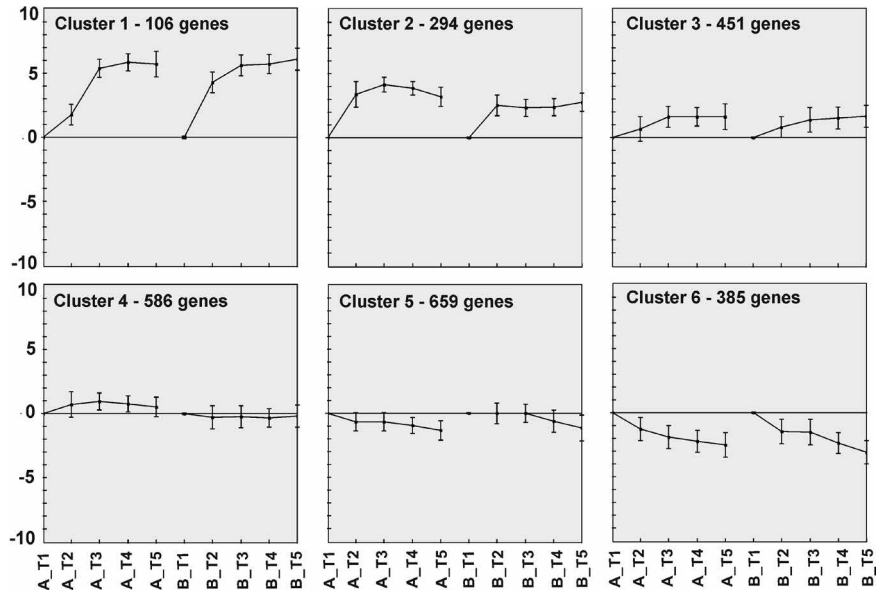

Figure 3.3 Results of *k*-means clustering of whole genome expression profiles. Six clusters were generated based on comparison of point-wise similarity of genome-wide gene expression profiles with Euclidean distance (Genesis v 1.7.6). The samples collected at representative time points (T_1-T_5) of independent experiment duplicates (A and B) are equally weighted (see Methods and materials). The data points show the average $\log_2$-transformed expression compared to exponential phase (T_1) of all cluster members with standard deviation indicated by the error bars.

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 1×10^5 CFU/ml, the culture reached a level of 1×10^8 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 OD_{600} of 0.3. During this stage, spores were not formed, DPA and CPE were not detected 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 T_1.

During **Stage II,** the total viable counts remained constant (Figure 3.1A) while the OD_{600} continued to increase (Figure 3.1B); this could be explained by elongation of part of the cells as observed using microscopical analysis. During this stage, no bright-phase spores were observed (T2 in Figure 3.1). DPA and CPE were not detectable and the pH of the culture showed a slight decrease to approximately 7.2. A representative time point of this stage is indicated by T_2.

During **Stage III**, the total viable counts and the OD_{600} 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. T_3 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 1×10^7 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 T_4 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 and approached the level of spore counts. Time point T_5 is at the very early stage of this phase, and was selected as a sampling point to minimize the effect of cellular RNase

Table 3.1 Primers for RT-PCR used in this study

Locus tag	Gene	Primer	Sequence (5'-3')	Product size (bp)	Reference
CPR_1053	*spoVAF (gerA)*	gerA-403F	AGGGGTTCTAGGGATGGCTTTGT	236	This study
		gerA-644R	ACAAGGCTTTGTTCGCTCATGGT		Paredes-Sabja, et al., 2008
CPR_0615	*gerKA*	gerKA-F1	GTATAGGGAGGTGGATACAG	192	This study
		gerKA-190sR	TCGTTTTTATCCTTTGACCAATTT		
CPR_0614	*gerKB*	gerKB-006F	TTTGGGAAAGCTAAATACAAGACA	112	This study
		gerKB-118R	TCCAAGTATCTCTTCCGCCTA		
CPR_0616	*gerKC*	gerKC-622F	TTAAGCGGAGGAGCTTTGTT	128	This study
		gerKC-752R	GGGTCTTGAGGGTTCATAACTTC		
CPR_1709	*prkC*	prkC-1182F	TTGGAACAACAACTGGAGACA	167	This study
		prkC-836-426sR	AATAATGAAAGCTATGGAAAAGGA		
CPR_2566	*sleC*	sleC-698F	CGGCTTTTGATCATGCTTTT	170	This study
		sleC-868R	CCCATTGAGTCATCCAACCT		
CPR_2348	*"sleB"*	sleB-423F	TGACGTAGTCCCTGATGGTGATAGC	127	This study
		sleB-550R	CGACGCCTTTCATCCACGAGCA		
CPR_1311	*sleM*	sleM-638F	ATTTAAACTGGGGGCCAAAT	233	This study
		sleM-871R	TTGGCTTTCCTTTTGGAAGA		
CPR_2013	*gpr*	gpr-295F	ACGGCTTTAGTAGTTGGGCTTGGA	165	This study
		gpr-460R	TGCCTAAAACTCCAGGTGCT		
CPR_2418	*sigH*	sigH-195F	TCAAGAGGGAATGATTGGATT	134	This study
		sigH-329R	TTTTGCCTTGTTGCAGTTTTT		
CPR_0339	*dnaE*	dnaE-1F	TCATCAACTCACGCTGCGGGA	128	This study
		dnaE-1R	TCCACAGCATCACGCATAACAGTT		
CPR_2567	*cspB*	cspB-1368F	TGGTAGGGGCGTTGTTAGAC	152	This study
		cspB-1520R	AGAAGAGCGCATATCCCAGA		

released by lysing mother cells.

3.2 Sporulation-specific gene expression patterns are shared by known germination-related genes

Expression of 12 known germination related genes of *C. perfringens* SM101 was assessed throughout growth and sporulation in mDS sporulation medium (Figure 3.2A), and additionally in glucose-rich TGY medium in which the organism does not form spores (Figure 3.2B). RT-PCR on germination genes that are normally expressed during sporulation was performed prior to whole genome expression profiling as a pilot experiment and to test the quality of the isolated RNA. The RT-PCR results revealed that the expression levels of the selected germination genes in mDS were significantly upregulated during sporulation (4-12 $\log_2$-fold, 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 10^3 CFU/ml at 3.0 h to 10^9 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.

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 $\log_2$-fold during sporulation (Figure 3.2C). The data obtained using RT-PCR and microarray analysis followed similar expression patterns in time for the different genes, albeit that the log expression rates were lower for the microarray based analyses than for the RT-PCR (Figure 3.2C).

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 $\log_2$-fold increase. 294 genes in Cluster 2 showed levels of up-regulation between three and five $\log_2$-fold (Figure 3.3). Lastly, Cluster 3 also contained genes that were up-regulated during sporulation (total 451genes), but at a lower level (around 2 $\log_2$-folds and maximally 3

log$_2$-folds; Figure 3.3).

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

Table 3.2 Cluster 1 and 2 *C. perfringens* homologues of known sporulation genes summarized previously in Galperin *et al*, (2012)*

Function category	Cluster 1	Cluster 2
General sporulation	CPR_1738 (*sigK*), CPR_1782 (*spoIVB*)**, CPR_2019 (*spoVAC*), CPR_2018 (*spoVAD*), CPR_2017 (*spoVAE*), CPR_2491 (*spoVT*).	CPR_2020 (*sigF*), CPR_1732 (*sigG*), CPR_2542 (*spmA*), CPR_2541 (*spmB*), CPR_2022 (*spoIIAA*), CPR_2021 (*spoIIAB*), CPR_2158 (*spoIID*), CPR_2475 (*spoIIE*), CPR_1778 (*spoIIM*), CPR_2012 (*spoIIP*), CPR_2157 ("*spoIIQ*"), CPR_1801 (*spoIIIAA*), CPR_1800 (*spoIIIAB*), CPR_1799 (*spoIIIAC*), CPR_1798 (*spoIIIAD*), CPR_1797 (*spoIIIAE*), CPR_1796 (*spoIIIAF*), CPR_1795 (*spoIIIAG*), CPR_1794 (*spoIIIAH*), CPR_2156 (*spoIIID*), CPR_1724 (*spoIVA*), CPR_2101 (*spoIVFB*), CPR_1053 (*spoVAF/gerA*), CPR_2490 (*spoVB*),CPR_1619 (*spoVB*), CPR_0528 (*spoVD*), CPR_1332 (*spoVR*)
SASPs	CPR_1411/CPR_2035 (*ssp*)	CPR_1870 (*ssp4*)
Spore cortex	CPR_2486 ("*yabP*"), CPR_2566 (*sleC*)	CPR_1699 ("*ylbJ*"), CPR_1993 ("*ygfC*"), CPR_1992 ("*yqfD*") CPR_2361 (*cwlD*), CPR_2348 (*sleB*)
Spore coat	CPR_0933 (*cotJB*), CPR_0934 (*cotJC*), CPR_2191 ("*yabG*"), CPR_1593 ("*yybI*")	CPR_2192 (*cotS*), CPR_1397 ("*yhbB*")
Spore coat maturation		CPR_1770 (*dacB*)
Spore germination	"CPR_2567 (*cspB*), CPR_2070 ("*cspA*"), CPR_2071 ("*cspC*"), CPR_2013 (*gpr*)	CPR_0615 (*gerKA*), CPR_0614 (*gerKB*), CPR_0616 (*gerKC*)
Signaling		CPR_2131 ("*ykuL*"), CPR_1334 (*prkA*)"
House cleaning		CPR_0840 (*rbr*)
Cell division, DNA replication		CPR_1383 (*lonB*)
Transport		CPR_0437 ("*ytrB*"), CPR_2026 ("*yloB*"), CPR_2256 ("*oppC*"), CPR_2255 ("*oppD*"), CPR_2254 ("*oppF*") CPR_2662 ("*ytvI*")
Cell wall metablism	CPR_1775 (*dacF*) ,CPR_1836 (*pdaA*) CPR_2453 ("*ykfA*")	CPR_1878 ("*ybaN*")
Poorly characterized (R COGs)		CPR_0756 (*hmp*)
Uncharacterized (S COGs)	CPR_1859 (*ytxC*), CPR_0147 (CD1511), CPR_1448 (CD3032), CPR_2589 (CD3613)	CPR_1348 (*ydfR/ydfS*), CPR_2517(*yerB*), CPR_1333 (*yhbH*), CPR_1731 (*ymxH*), CPR_1929 (*yunB*), CPR_1626 (*yuzA*), CPR2663 (*yyaC*), CPR2565 (*yyaD/ykvI*), CPR_0309 (CD0546), CPR_1322 (CD1594), CPR_2328 (CD2809), CPR_2525(CD3522), CPR_2297 (CD3580)

* Homologues of the known sporulation genes summarized in Galperin *et al* (2012) but that do not have specific sporulation-associated increased expression (grouped in Cluster 3-6) can be retrieved from Table S1.

** Locus tags of *C. perfringens* homologues are shown with their assigned *C. perfringens* gene names, gene names of the *Bacillus* genes are bracketed, and locus tags of *C. difficile* 630 are preceded by 'CD'.

sporulation.

In a recent review, Galperin *et al.* (2012) summarized the genes involved in sporulation, based on results of experimental functionality studies on *B. subtilis*, comparative genomics studies on sporeforming bacteria and proteomics studies on *C. difficile* spores (de Hoon *et al.*, 2010; Galperin *et al.*, 2012). *C. perfringens* homologues of known sporulation genes which were found to be up-regulated more than three log$_2$-fold during sporulation of *C. perfringens* (belonging to Cluster 1 and 2) are shown in Table 3.2, listed per functional category. Additionally, supplemental Table S2 lists all *C. perfringens* homologues (i.e. present in clusters 1-6) of (putative) sporulation genes summarized in Galperin *et al.* (2012). Per gene, the expression during sporulation is visualized for the *C. perfringens* homologues of these (putative) sporulation genes. The expression levels of the *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 *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 *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 log$_2$- fold) and Cluster 2 (3-5 log$_2$-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 *et al* (2012).

Firstly, an inventory was made to establish the occurrence of the homologues of 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 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 *Clostridium* specific and the remaining 24 genes were *C. perfringens*-specific (Figure 3.4).

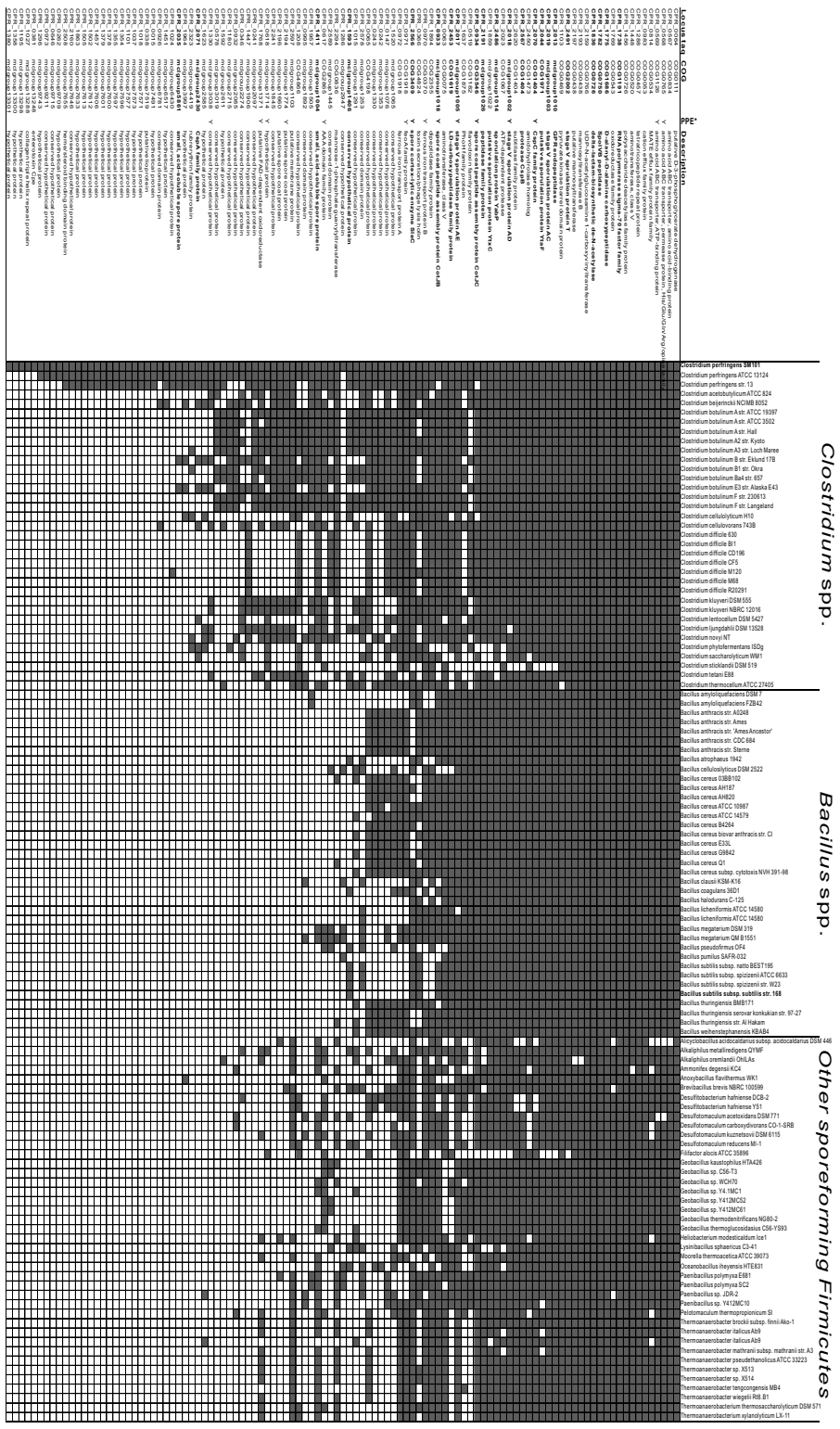

Figure 3.4 Cluster 1 genes and the occurrence of their homologues in other spore forming Firmicutes. The genes in Cluster 1 are highly up-regulated during *C. perfringens* sporulation. The genes with known sporulation-related function are indicated in bold against a grey background; the ones with putative functions (poorly characterized and uncharacterized) have a grey background only, while the others without specific highlights have not been associated with sporulation or known to determine spore properties. PPE is an abbreviation for the Potential of Polar Effect, as co-expression may occur due to high levels of expression of upstream genes in the same operon.

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 OD_{600} 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 difference 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 Mg^{2+} 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 $\log_2$-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 (T_1) 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 $\log_2$-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

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.

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Chapter 3 Transcriptional profiling during sporulation

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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

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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, $(GTG)_5$-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 sulfite-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, $(GTG)_5$-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.*, 2003) 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% CO_2, 10% H_2 and 85% N_2). 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

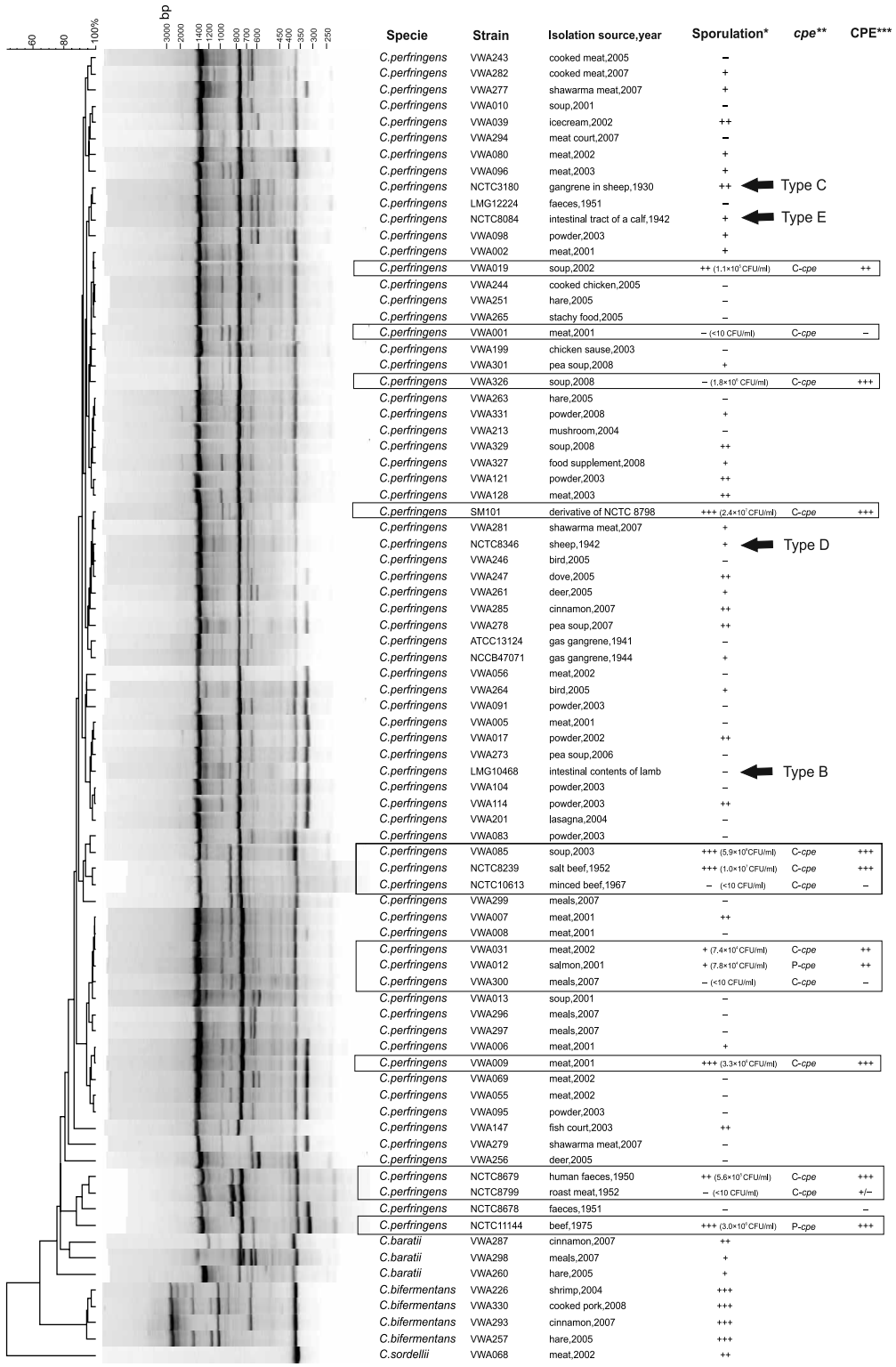
of the other isolates. The DNA was stored at -20°C. No DNA degradation was observed by 1% agarose electrophoresis (data not shown).

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 pA 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 *cpb*, *etx* 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.

$(GTG)_5$-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 $(GTG)_5$ (Versalovic *et al.*, 1995; Gevers *et al.*, 2001), with combination



Specie	Strain	Isolation source,year	Sporulation*	*cpe***	CPE***	
C.perfringens	VWA243	cooked meat,2005	–			
C.perfringens	VWA282	cooked meat,2007	+			
C.perfringens	VWA277	shawarma meat,2007	+			
C.perfringens	VWA010	soup,2001	–			
C.perfringens	VWA039	icecream,2002	++			
C.perfringens	VWA294	meat court,2007	–			
C.perfringens	VWA080	meat,2002	+			
C.perfringens	VWA096	meat,2003	+			
C.perfringens	NCTC3180	gangrene in sheep,1930	++			Type C
C.perfringens	LMG12224	faeces,1951	–			
C.perfringens	NCTC8084	intestinal tract of a calf,1942	+			Type E
C.perfringens	VWA098	powder,2003	+			
C.perfringens	VWA002	meat,2001	+			
C.perfringens	VWA019	soup,2002	++ (1.1×10^5 CFU/ml)	C-*cpe*	++	
C.perfringens	VWA244	cooked chicken,2005	–			
C.perfringens	VWA251	hare,2005	–			
C.perfringens	VWA265	stachy food,2005	–			
C.perfringens	VWA001	meat,2001	– (<10 CFU/ml)	C-*cpe*	–	
C.perfringens	VWA199	chicken sause,2003	–			
C.perfringens	VWA301	pea soup,2008	+			
C.perfringens	VWA326	soup,2008	– (1.8×10^5 CFU/ml)	C-*cpe*	+++	
C.perfringens	VWA263	hare,2005	–			
C.perfringens	VWA331	powder,2008	+			
C.perfringens	VWA213	mushroom,2004	–			
C.perfringens	VWA329	soup,2008	++			
C.perfringens	VWA327	food supplement,2008	+			
C.perfringens	VWA121	powder,2003	++			
C.perfringens	VWA128	meat,2003	++			
C.perfringens	SM101	derivative of NCTC 8798	+++ (2.4×10^7 CFU/ml)	C-*cpe*	+++	
C.perfringens	VWA281	shawarma meat,2007	+			
C.perfringens	NCTC8346	sheep,1942	+			Type D
C.perfringens	VWA246	bird,2005	–			
C.perfringens	VWA247	dove,2005	++			
C.perfringens	VWA261	deer,2005	+			
C.perfringens	VWA285	cinnamon,2007	++			
C.perfringens	VWA278	pea soup,2007	++			
C.perfringens	ATCC13124	gas gangrene,1941	–			
C.perfringens	NCCB47071	gas gangrene,1944	+			
C.perfringens	VWA056	meat,2002	–			
C.perfringens	VWA264	bird,2005	+			
C.perfringens	VWA091	powder,2003	–			
C.perfringens	VWA005	meat,2001	–			
C.perfringens	VWA017	powder,2002	++			
C.perfringens	VWA273	pea soup,2006	–			
C.perfringens	LMG10468	intestinal contents of lamb	–			Type B
C.perfringens	VWA104	powder,2003	–			
C.perfringens	VWA114	powder,2003	++			
C.perfringens	VWA201	lasagna,2004	–			
C.perfringens	VWA083	powder,2003	–			
C.perfringens	VWA085	soup,2003	+++ (5.9×10^5CFU/ml)	C-*cpe*	+++	
C.perfringens	NCTC8239	salt beef,1952	+++ (1.0×10^7 CFU/ml)	C-*cpe*	+++	
C.perfringens	NCTC10613	minced beef,1967	– (<10 CFU/ml)	C-*cpe*	–	
C.perfringens	VWA299	meals,2007	–			
C.perfringens	VWA007	meat,2001	++			
C.perfringens	VWA008	meat,2001	–			
C.perfringens	VWA031	meat,2002	+ (7.4×10^7 CFU/ml)	C-*cpe*	++	
C.perfringens	VWA012	salmon,2001	+ (7.8×10^7 CFU/ml)	P-*cpe*	++	
C.perfringens	VWA300	meals,2007	– (<10 CFU/ml)	C-*cpe*	–	
C.perfringens	VWA013	soup,2001	–			
C.perfringens	VWA296	meals,2007	–			
C.perfringens	VWA297	meals,2007	–			
C.perfringens	VWA006	meat,2001	+			
C.perfringens	VWA009	meat,2001	+++ (3.3×10^5 CFU/ml)	C-*cpe*	+++	
C.perfringens	VWA069	meat,2002	–			
C.perfringens	VWA055	meat,2002	–			
C.perfringens	VWA095	powder,2003	–			
C.perfringens	VWA147	fish court,2003	++			
C.perfringens	VWA279	shawarma meat,2007	–			
C.perfringens	VWA256	deer,2005	–			
C.perfringens	NCTC8679	human faeces,1950	++ (5.6×10^5 CFU/ml)	C-*cpe*	+++	
C.perfringens	NCTC8799	roast meat,1952	– (<10 CFU/ml)	C-*cpe*	+/–	
C.perfringens	NCTC8678	faeces,1951	–		–	
C.perfringens	NCTC11144	beef,1975	+++ (3.0×10^5 CFU/ml)	P-*cpe*	+++	
C.baratii	VWA287	cinnamon,2007	++			
C.baratii	VWA298	meals,2007	+			
C.baratii	VWA260	hare,2005	+			
C.bifermentans	VWA226	shrimp,2004	+++			
C.bifermentans	VWA330	cooked pork,2008	+++			
C.bifermentans	VWA293	cinnamon,2007	+++			
C.bifermentans	VWA257	hare,2005	+++			
C.sordellii	VWA068	meat,2002	++			

of 14 reference strains including 5 with various toxin type, 6 enterotoxic type A and additional 3 *cpe*-negative type A (LMG12224, NCCB47071 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, Maarssen, 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-contract microscopy (magnification 1000×) and four levels of spore production were defined: no phase-bright spores/view (-), 1-3 spores /view (+), less than 50% spores (++) 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 VWA012 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 enterotoxic 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 enterotoxic strains. The ability of the selected *C. perfringens* strains to sporulate when incubated in mDS broth, a

Table 4.1 Primers and probes used for PCR amplifications in this study

Primer/probe	Sequence (5'-3')*	Application	Reference
CPALPHTOX1TM-L	AAGAACTAGTAGCTTACATATCAACTAGT-GGTG	Taqman qPCR to detect *cpe*	Albini *et al.*, 2008
CPALPHTOX1TM-R	TTTCCTGGGTTGTCCATTTCC		
CPALPHTOX1TM-S	VIC-TTGGAATCAAAACAAAGGATG-GAAAAACTCAAG-TAMRA		
CPENT-L	AGCTGCTGCTACAGAAAGATTAAATTT		
CPENT-R	TGAGTTAGAAGAACGCCAATCATATAA		
CPENT-S	FAM-ACTGATGCATTAAACTCAAATC-CAGCT-TAMRA		
pA	AGAGTTTGATCCTGGCTCAG	16S rRNA gene sequencing	Wang *et al.*, 1994
pH	AAGGAGGTGATCCAGCCGCA		
$(GTG)_5$	GTGGTGGTGGTGGTG	$(GTG)_5$-fingerprinting	Gevers *et al.*, 2001
IS1470R1.3	CTTCTTGATTACAAGACTCCAGAAGAG	*cpe*-typing	Miyamoto *et al.*, 2004
cpe4F	TTAGAACAGTCCTTAGGTGATGGAG		
3F	GATAAAGGAGATGGTTGGATATTAGG		
4R	GAGTCCAAGGGTATGAGTTAGAAG		
IS1470-likeR1.6	CTTTGTGTACACAGCTTCGCCAATGTC		
IS1151R0.8	ATCAAAATATGTTCTTAAAGTACGTTC		
CPA5L	AGTCTACGCTTGGGATGGAA	Toxiotyping	Baums *et al.*, 2004
CPA5R	TTTCCTGGGTTGTCCATTTC		
CPBL	TCCTTTCTTGAGGGAGGATAAA		
CPBR	TGAACCTCCTATTTTGTATCCCA		
CPEL	GGGGAACCCTCAGTAGTTTCA		
CPER	ACCAGCTGGATTTGAGTTTAATG		
CPETXL	TGGGAACTTCGATACAAGCA		
CPETXR	TTAACTCATCTCCCATAACTGCAC		
CPIL	AAACGCATTAAAGCTCACACC		
CPIR	CTGCATAACCTGGAATGGCT		
CPB2L	CAAGCAATTGGGGGAGTTTA		
CPB2R	GCAGAATCAGGATTTTGACCA		
gyrB-F	ATTGTTGATAACAGTATTGATGAAGC	MLST	Deguchi *et al.*, 2009
gyrB-R	ATTTCCTAATTTAGTTTTAGTTTGCC		
sigK-F	CAATACTTATTAGAATTAGTTGGTAG		
sigK-R	CTAGATACATATGATCTTGATATACC		
sod-F	CAAAAAAAGTCCATTAATGTATCCAG		
sod-R	TTATCTATTGTTATAATATTCTTCAC		
groEL-F	TACAAGATTTATTACCATTACTTGAG		
groEL-R	CATTTCTTTTTCTGGAATATCTGC		
pgk-F	GACTTTAACGTTCCATTAAAAGATGG		
pgk-R	CTAATCCCATGAATCCTTCAGCGATG		
nadA-F	ATTAGCACATTATTATCAAATTCCTG		
nadA-R	TTATATGCCTTTAATCTTAAATCCTC		
colA-F	ATTAGAAAGTTTATGTACAATAGGTG		
colA-R2	AAGACATTCTATTATTTCTATCGTAAGC		
plc-F	AGGAACTCATGATTGTAACTC		
plc-R	GGATCATTACCCTCTGATACATCGTG		

* PCR tag abbreviations: TAMRA, 6-carboxytetramethylrhodamine; FAM, 6-carboxyfluorescein. The VIC reagent is a trademarked product of Applera Corporation.

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

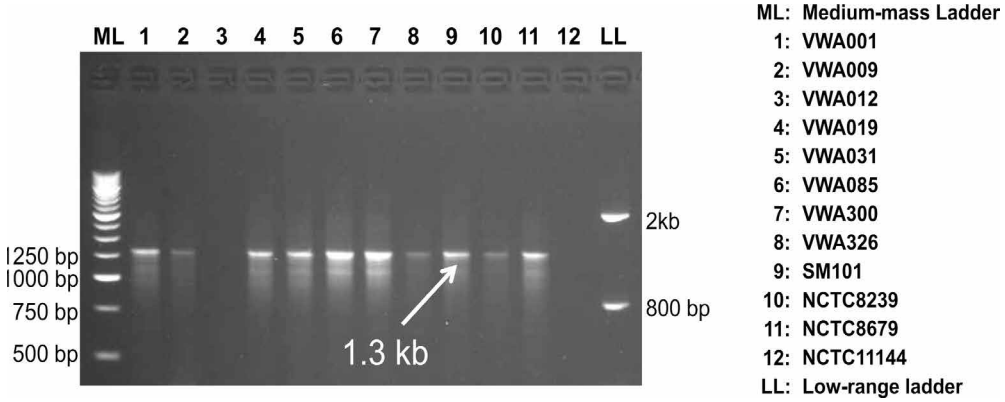


Figure 4.2 PCR results to evaluate the proximity of the cpe gene to the *IS1470* elements for the studied enterotoxic strains, using one primer specific for the *cpe* gene (forward primer cpe4F) and the other specific for the *IS1470*-element (reverse primer IS1470R1.3). For all *IS1470*-cpe-type strains, a 1.3 kb product was found and no product was amplified for two *IS1151*-type strains (VWA012 and NCTC11144), indicating that the fragment size between the *cpe* gene and the *IS1470* element is 1.3 kb.

C. bifermentans strains fully sporulated (Figure 4.1).

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 log10 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 (>10^5 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 used (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

Table 4.2 Identification of enterotoxic *C. perfringens* in 98 recovered cultures

Culture type	*plc*-positive		*plc*-negative
	cpe-positive	*cpe*-negative	
Pure	8	51	8
Impure	0	13	18
Total	8	64	26

(Albini *et al.*, 2008), while the *cpe* gene was not detected in the 13 impure strains. For 7 out of these 8 strains, the *cpe* gene was accompanied by a downstream *IS1470* element which is conserved for chromosomal *cpe* genes, *i.e.* these *cpe* genes were located on the chromosome (Miyamoto *et al.*, 2002; Miyamoto *et al.*, 2004). To verify the proximity of the *cpe* gene to the *IS1470* sequences, the PCR experiment resulted 1.3 kb fragments for these strains, as shown in Figure 4.2. In one case, namely for strain VWA012 which was isolated from sliced salmon, the *cpe* gene was located on a locus with an *IS1151* sequence, identical to that in reference strain NCTC11144. According to previous literature, the *cpe* genes of these two strains could be located on plasmids (Miyamoto *et al.*, 2002; Miyamoto *et al.*, 2004).

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 food poisoning strains (Group I in the green matrix) according to the minimal spanning tree shown in Figure 4.3. The majority of *C. perfringens* including P-*cpe* and *cpe*-negative strains was typically separated from Group I and formed a much less conserved cluster, with the core (Group II in red) including the type strain of *C. perfringens*, ATCC13124. 6 of 8 *cpe*-negative food isolates in the current study were included in Group II, while the other 2 and P-*cpe* VWA012 are in the surrounding

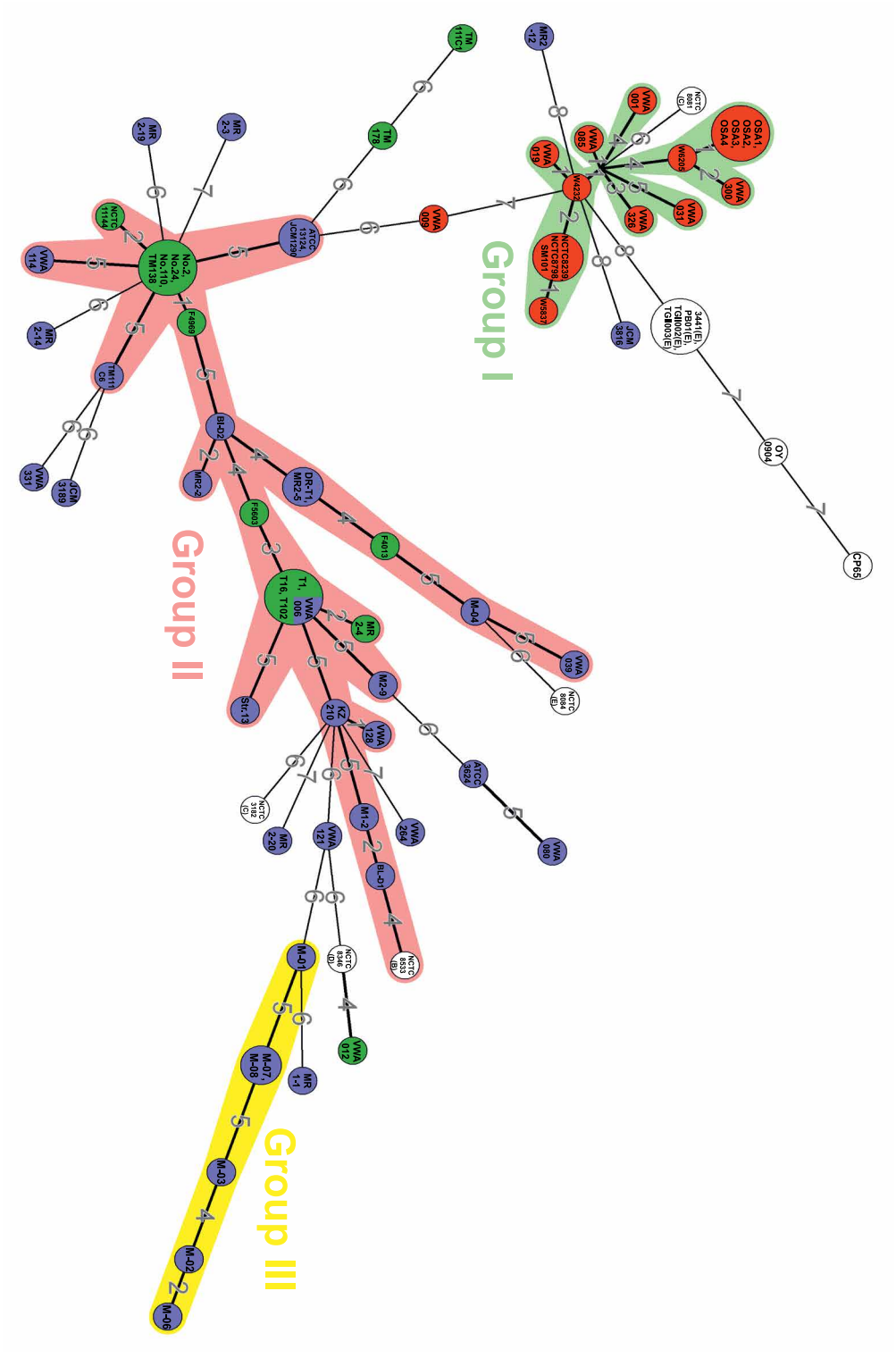


Figure 4.3 Minimal spanning tree of MLST analysis on 17 *C. perfringens* strains in this study combined with a worldwide survey, revealing three clusters correlating to environmental niches. The seven chromosomal *cpe* food isolates are clustered with all recorded food poisoning strains (Group I, in light green), when plasmid-*cpe*, gas gangrene and healthy human isolates are clustered in Group II (red) and Group III (yellow) mainly contains non-*cpe* food isolates. The tree was constructed by using BioNumerics version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium) with allowed hypothetical types, categorical coefficient. The size of the nodes indicates the number of strains sharing the same MLST type and the node colours show C-*cpe* type A in red, P-*cpe* type A in dark green, non-*cpe* type A in blue and other types in white. The clusters were created for at least three types within 5 changes of neighbour distances.

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 *C. perfringens* are not fully understood, and universal *in vitro* sporulation conditions remain to be defined (Lindström *et al.*, 2011). In this study classical culturing and confirmation methods for *C. perfringens* gave false positive results in approximately 30% of cases, while only approximately 11% of identified *C. perfringens* isolates was able to produce enterotoxin. Clearly, there is a need for specific culturing methods for *C. perfringens* strains that can cause food poisoning.

A simple oligonucleotide repeat, $(GTG)_5$, can be used to rapidly classify or accurately genotype species from various genera including nontuberculous *Mycobacterium* spp. (Cilliers *et al.*, 1997), *Enterococcus* spp. (Svec *et al.*, 2005), and *Lactobacillus* isolates (Gevers *et al.*, 2001). In this study, *C. perfringens* strains could be distinguished from other closely related sulfite-reducing *Clostridium* spp. using $(GTG)_5$-fingerprinting. However, relatively low discrimination was observed between different *C. perfringens* strains using $(GTG)_5$-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 *C. perfringens* genome sequences showed diverse G+C content, chromosomal sizes and organizations for different *C. perfringens* strains (Myers *et al.*, 2006; Xiao *et al.*, 2011). This technique therefore does not seem suitable for clustering *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 *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 *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 *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 *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 enterotoxic 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.* 2009 (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* *C. perfringens* strains.

Further studies on these isolates would render a better understanding the evolutionary separation between these three groups of *C. perfringens.* Even though MLST renders a high level of resolution among various *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 *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 *C. perfringens* is routinely monitored, and in *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 culturing 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* isolates, the *cpe* gene encoding enterotoxin causing food poisoning is present. We found that a wide variety of *C. perfringens* type A foodborne isolates that carry a chromosomal *cpe* gene belong to one particular MLST cluster. There is a need to better understand the origin and reservoir of *C. perfringens cpe* positive strains, allowing for a better control of this organism in foods.

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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

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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 nitrites (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

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

Strain	Isolate source	Toxino type*	*cpe*-type**	Reference
ATCC13124	gas gangrene, type strain	A	negative	Myers *et al.* 2006
C003	soup	A	P-*cpe*, *IS1470*-like	This study
C020	croquette	A	P-*cpe*, *IS1470*-like	This study
C198(Cp10)	peanut sauce	A	P-*cpe*, *IS1470*-like	This study
C200(Cp11)	meal, Foe Yong Hay	A	P-*cpe*, *IS1470*-like	This study
C202	soup	A	P-*cpe*, IS*1470*-like	This study
NCTC10240	bird; chicken	A	C-*cpe*	Melville *et al.*, 1994
NCTC11144	beef	A	P-*cpe*, *IS1151*	Xiao *et al.*, 2012
NCTC8239	salt beef	A	C-*cpe*	Melville *et al.*, 1994
SM101	derivative of NCTC8798	A	C-*cpe*	Myers et al. 2006
VWA001	meat	A	C-*cpe*	Xiao *et al.*, 2012
VWA006	meat	A	negative	Xiao *et al.*, 2012
VWA009	meat	A	C-*cpe*	Xiao *et al.*, 2012
VWA012	salmon	A	P-*cpe*, *IS1151*	Xiao *et al.*, 2012
VWA019	soup	A	C-*cpe*	Xiao *et al.*, 2012
VWA031	meat	A	C-*cpe*	Xiao *et al.*, 2012
VWA039	ice cream	A	negative	Xiao *et al.*, 2012
VWA080	meat	A	negative	Xiao *et al.*, 2012
VWA085	soup	A	C-*cpe*	Xiao *et al.*, 2012
VWA114	powder	A	negative	Xiao *et al.*, 2012
VWA121	powder	A	negative	Xiao *et al.*, 2012
VWA128	meat	A	negative	Xiao *et al.*, 2012
VWA264	bird	A	negative	Xiao *et al.*, 2012
VWA300	meal	A	C-*cpe*	Xiao *et al.*, 2012
VWA326	soup	A	C-*cpe*	Xiao *et al.*, 2012
VWA331	powder	A	negative	Xiao *et al.*, 2012

* Genes encoding *C. perfringens* major toxins, determining toxino-types, were detected by using the multi PCR method described by Baumes (2004).

** Presence of *cpe* gene was detected by using specific duplex Taqman PCR method (Albini *et al.*, 2008; Xiao *et al.* 2012); *cpe* types, chromosomal (*IS1470*) and plasmid-borne (*IS1151* and *IS1470*-like) were further determined using a multiplex PCR genotyping assay (Miyamoto *et al.*, 2004). For strains C033 and C211, carrying cpe gene suggested by Taqman PCR, are not typable using multiplex PCR (no PCR product).

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 maintained in cooked meat medium (BD, Sparks, MD) at 1˚C. Anaerobic cultivation was generally performed at 37˚C in a Simplicity 888 automatic atmosphere chamber (PLAS Labs, Lansing, MI). To obtain spores, Fluid thioglycollate culture medium (FTM, BD, Sparks, MD) was inoculated with the stock culture and incubated anaerobically overnight. A 1 ml aliquot of this culture was inoculated into 5 ml fresh FTM medium. After 4 h of sub-culturing, 2 ml of this exponentially growing culture was inoculated in 200 ml modified Duncan-Strong (mDS) sporulating medium, in which raffinose was used as the carbon source instead of starch (Duncan and Strong, 1968). After anaerobic incubation at 37˚C for 48 h, a 1.5 ml aliquot of each sporulation culture was heated (75 ˚C for 10 min) and the spores were enumerated using BHI agar plates (BD, Sparks, MD). *C. perfringens* spores were further harvested, washed and stored at 4˚C (Juneja *et al.*, 2010). The spore concentrations in the spore crops were assessed before

use.

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 10^3 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 (Airtest 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.*

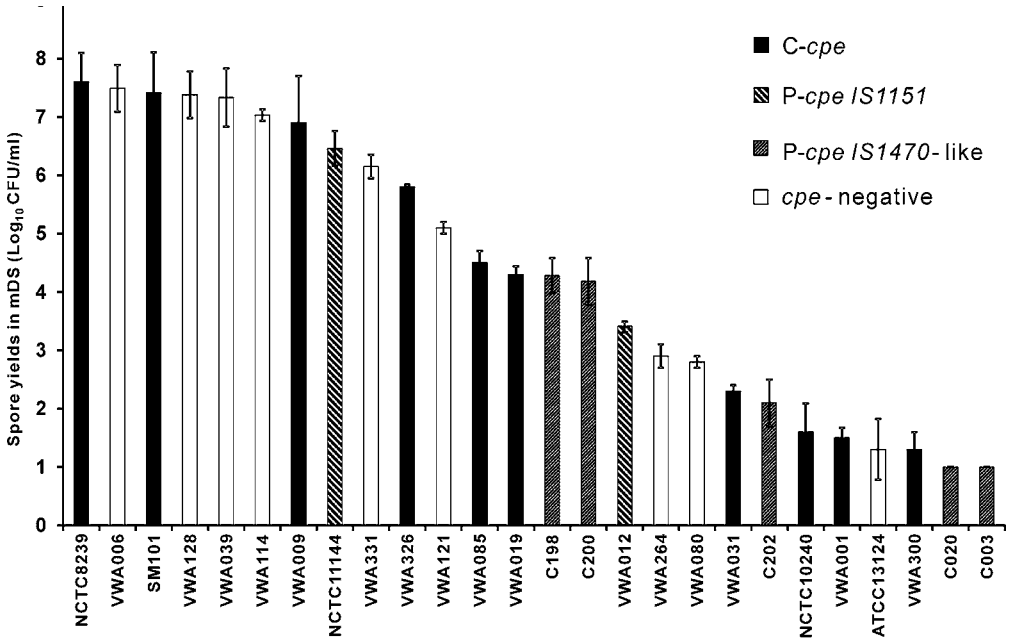

Figure 5.1 Spore yields of various *C. perfringens* foodborne isolates in modified mDS. Error bars are showing the standard deviation of plate counting performed in triplicate. The detection limit is 10 spores/ml.

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 intermediately 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 strainVWA009 (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 Ssp4 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 Ssp4 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

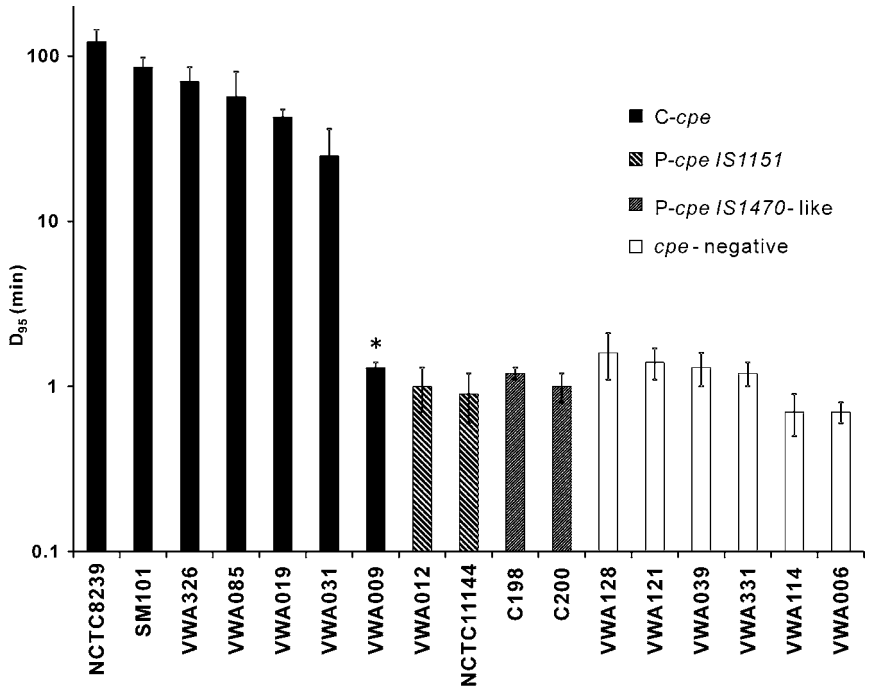

Figure 5.2 Heat resistances of spores formed by *C. perfringens* foodborne isolates with various *cpe*-types. The strains of each type were ordered based on their Decimal reduction time at 95˚C (D_{95}) in minutes. The D_{95} of C-*cpe* food isolate VWA009 significantly varied from other tested C- *cpe* strains and was comparable with the P-*cpe* and *cpe*-negative strains.

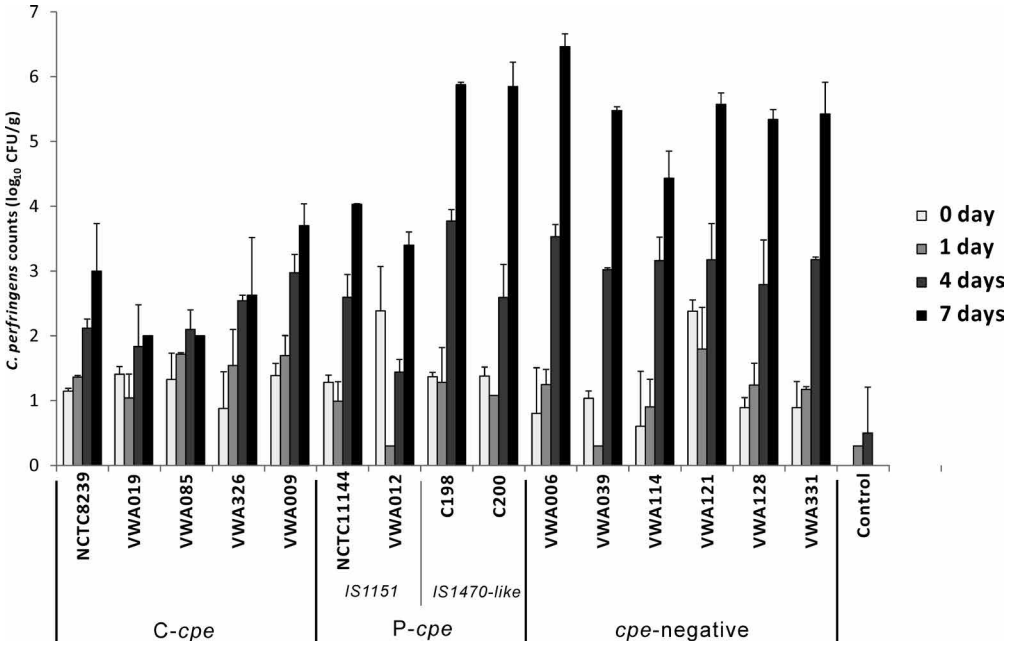

Figure 5.3 Total *C. perfringens* concentrations in cooked ground beef stored at 12˚C for a maximum of 7 days. The tested strains were grouped according to their *cpe*-genotypes. The error bars show standard deviations of results of each strain at specific time points (see legend in insert) from a total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

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 VWA009 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 $\log_{10}$ 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

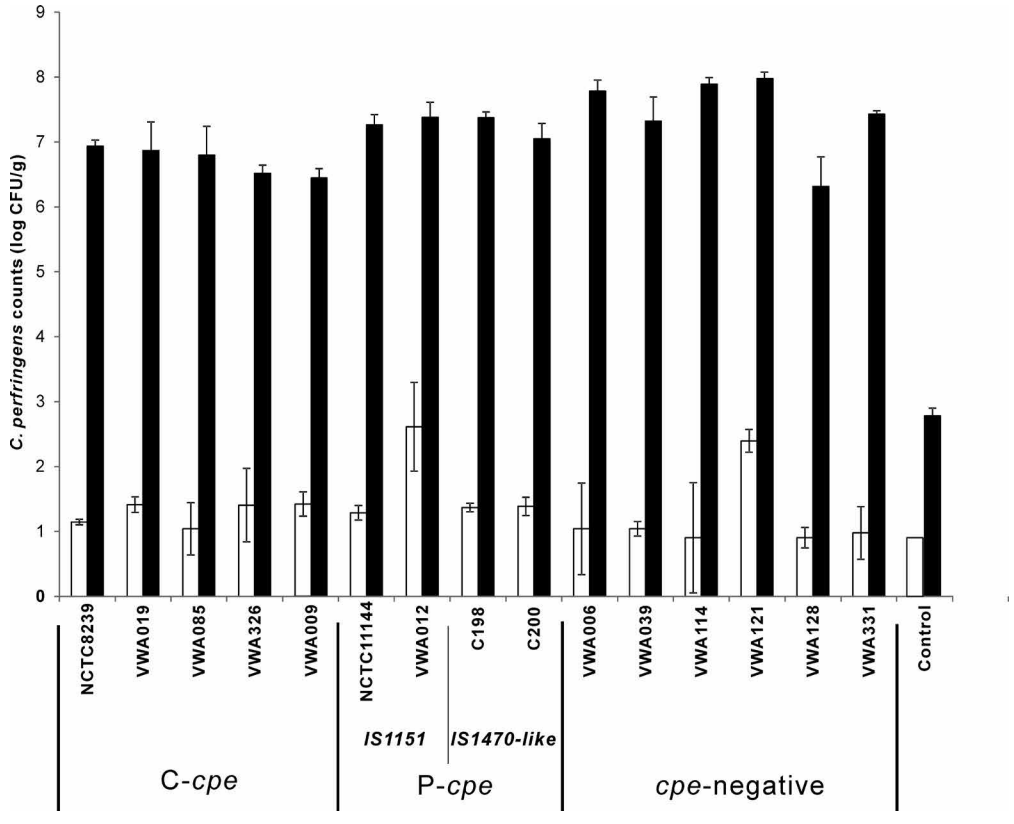

Figure 5.4 Growth of tested *C. perfringens* food isolates in cooked ground beef at ambient temperature (25 ˚C). The total *C. perfringens* counts at 0 day are shown in white bars, the counts at 24 h in black bars. The vertical error bars indicate standard deviations of replicates. The control samples 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).

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 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 are 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 higher 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*.

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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^G. 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. kluyveri,* 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. kluyveri*, *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 components 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. Phylogenic 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, 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. bartlettii 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

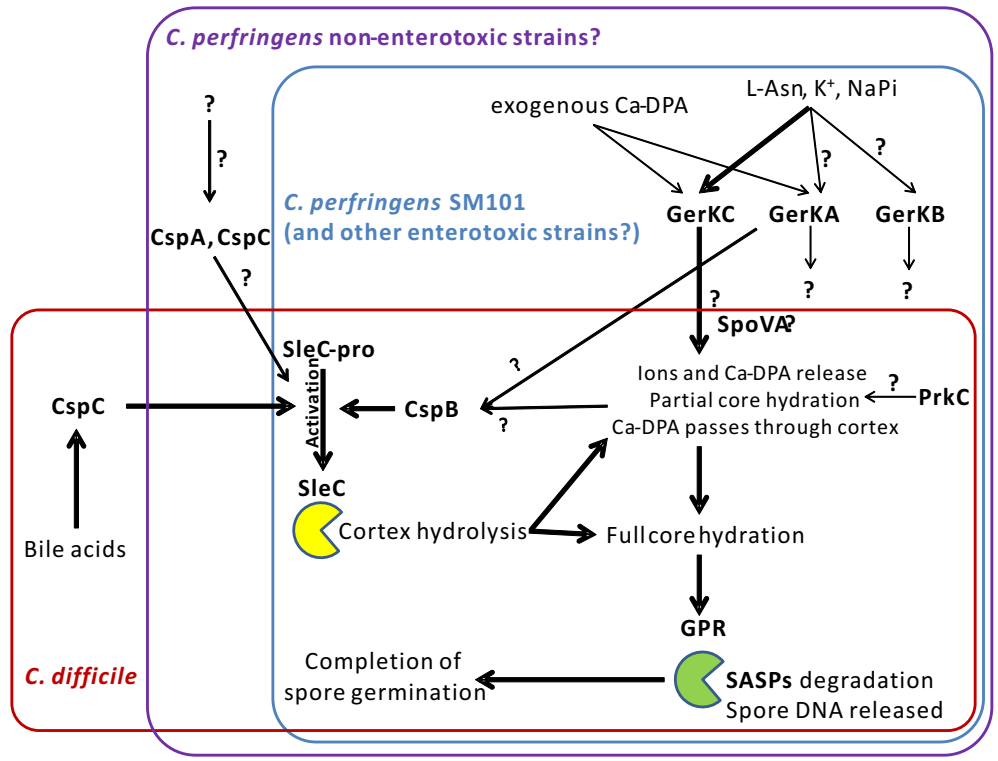

Figure 6.1 Tentative model for information flow during the germination of *C. perfringens* and *C. difficile* spores.

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 *ssp4,* 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 SASP-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 SASP degradation. The time taken for *Δgpr* 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; Fimlaid *et al.*, 2013; Saujet *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; 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 10^5 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; Kaneko *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-*cpe* strains produced spores with significantly higher heat resistance than the P-*cpe* strains and the *cpe*-negative strains (with the exception of strain VWA009), which is in line with previous findings (Sarker *et al.*, 2000). C-*cpe* strains are also known to display higher resistance to NaCl, nitrite, and high/low temperatures than P-*cpe* and *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-*cpe* strains. A demonstrated factor that contributes to the elevated heat resistance of C-*cpe* spores over P-*cpe* spores is the presence of a specific SASP encoded by gene *ssp4* (Li and McClane, 2008; Li *et al.*, 2009). Although increased spore heat resistance of C-*cpe* strains appears to be associated with the presence of *ssp4*, it is not clear what the nature of the correlation between the chromosomal location of the *cpe* gene and the presence of the *ssp4* gene is. The fact that C-*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-*cpe* gene.

According to the USDA/FSIS guideline, the growth potential of *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 *C. perfringens* strains NCTC 8238, NCTC 8239 and ATCC10288 that all carry the C-*cpe* gene (Sanchez-Plata *et al.*, 2005; Juneja, 2006; Juneja *et al.*, 2006; Juneja *et al.*, 2006; Juneja *et al.*, 2006; Juneja *et al.*, 2007; Juneja and Friedman, 2007; Reddy Velugoti *et al.*, 2007; Velugoti *et al.*, 2007; Juneja *et al.*, 2008; Miguel-Garcia *et al.*, 2009; Juneja *et al.*, 2010; Singh *et al.*, 2010; Valenzuela-Martinez *et al.*, 2010; Juneja *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 *C. perfringens* strains with C-*cpe,* P-*cpe* and *cpe*-negative genotypes were assessed for their growth potential starting from spores. The results surprisingly showed lower outgrowth potential of C-*cpe* strains in cooked beef stored at 12°C, suggesting that the *cpe*-negative and P-*cpe* strains may have a competitive advantage over C-*cpe* strains at low temperatures during storage (refrigeration abuse temperatures).

3.2 A need for improved methods to detect enterotoxigenic *C. perfringens* in foods?

The current isolation and confirmation methods for *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 *C. perfringens* require improvement with

respect to improved specificity and discrimination between *cpe*-positive (even C-*cpe* versus P-*cpe*) and *cpe*-negative strains:

Improved specificity. Chapter 4 shows that the current method gives false-positive results in ~30% of cases (*i.e.* species other than *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 *cpe*-positive *C. perfringens* strains, as described by Lindström *et al.*, (2011). These authors reported that within several dozens of strains isolated from various sources even *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 *cpe*-positive and *cpe*-negative strains. As discussed in the previous section, only *cpe*-positive *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 *cpe* gene highly unreliable. MLST clustering of *C. perfringens* strains (Chapter 4) showed that 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 C-*cpe* gene), for instance specific metabolic enzymes. Such features could be employed to develop culturing media that are specific for *C-cpe* strains (i.e. only supporting their growth) or, even better, that show the presence of *C-cpe* strains amidst other clostridia, for instance a chromogenic substance that can be converted by *C-cpe* strains only, in a non-selective *Clostridium* medium. A resent aCGH results, complemented with growth studies, demonstrated different myo-inositol, ethanolamine, and cellobiose metabolism between the C-*cpe* and P-*cpe* strains (Lahti *et al.*, 2012), providing leads to develop an assay for the direct identification of C-*cpe* or *cpe*-positive *C. perfringens* isolated from foods.

4. Strategies to control of *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 *Clostridium* spores. This strategy has been studied as a possible measure to eliminate spores from food products (i.e., addition of germinants to reduce heat resistance of spores) (Akhtar *et al.*, 2009). Following such a strategy, increased sporicidal activity of commonly used disinfectants was achieved for *C. perfringens* spores upon triggering germination using a mixture of L-asparagine and KCl (Udompijitkul *et al.*, 2013). Enhanced killing of *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 *C. perfringens* and no growth of *C. botulinum* may occur during product stabilization". A critical control point in this overall scheme is rapid cooling, as *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 *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 *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. 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 *C. perfringens*.

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Samenvatting

Clostridium perfringens veroorzaakt één van de meest voorkomende voedselgerelateerde infecties bij de mens. Het vermogen van dit anaerobe microorganisme om sporen te vormen speelt een belangrijke rol in de verspreiding van dit organisme. Sporen van *C. perfringens* zijn resistent en komen wijdverbreid voor, waaronder in vele levensmiddeleningrediënten. De sporen zijn in staat een groot aantal gangbare bewerkingsstappen te overleven die worden toegepast in de levensmiddelenindustrie. Wanneer sporen vervolgens kunnen ontkiemen in een bereid levensmiddel en wanneer uitgroei 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. Voedselinfecties worden veroorzaakt door díe stammen, die het *C. perfringens* enterotoxine (CPE) kunnen produceren. Consumptie van een product met hoge concentraties van een CPE producerende stam (meer dan 10^5 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 door 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 dat een rol speelt tijdens sporulatie en eigenschappen van sporen, waaronder ontkiemingsgedrag 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 gelocaliseerd, 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 geassocieerd 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 organisme. 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 hitte-resistentie hadden. Sporen van de individuele stammen werden vervolgens geïnocculeerd in rauw rundergehakt en na vacuumverpakking en verhitting van dit product werd het 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 belangrijk 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 afdoding 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 te hoge koeltemperaturen bewaard, dan kunnen met name P-*cpe* stammen een potentieel risico vormen. Hygiëne maatregelen om besmetting ná de hitte behandeling te voorkomen en goede koeling zijn daarom zeer belangrijk voor de beheersing van P-*cpe* stammen.

Dit proefschrift heeft nieuwe inzichten opgeleverd ten aanzien van de genen die betrokken zijn bij de ontkieming van *C. perfringens* sporen en er zijn nieuwe kandidaat-genen geïdentificeerd die mogelijk een rol spelen in sporulatie en ontkieming van sporevormende Firmicuten. Verder bleek dat de huidige kweekmethoden voor *C. perfringens* een groot aantal vals positieve resultaten gaf. Nadere genetische analyse van *C. perfringens* isolaten uit levensmiddelen liet zien dat C-*cpe* stammen – welke doorgaans voedsel-geassocieerd zijn - tot een cluster behoren dat duidelijk verschilt van de clusters van P-*cpe* en *cpe*-negatieve stammen. Pathogene C-*cpe* of P-*cpe* stammen vertoonden verder duidelijk verschillen ten aanzien van sporen hitteresistentie en hun vermogen tot uitgroei in een voedselproduct bij lage temperaturen. Dit duidt op verschillen in de 'critical control points' (CCP's) die beheerst dienen te worden voor verschillende *C. perfringens* stammen om voedselinfecties door deze ziekteverwekker te voorkomen; voor C-*cpe* stammen is een voldoende hoge hittebehandeling nodig voor inactivatie van sporen en snelle koeling na verhitting om uitgroei van eventueel overlevende sporen te voorkomen. P-*cpe* stammen produceren relatief hitte gevoelige sporen, echter nabesmetting na verhitting dient voorkomen te worden terwijl ook een lage bewaartemperatuur geborgd moet worden.

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.

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Sincerely yours,

Yinghua Xiao

List of publications

Xiao, Y., C. Francke, T. Abee and M. H. J. Wells-Bennik (2011). "Clostridial spore germination versus bacilli: genome mining and current insights." Food Microbiol 28(2): 266-274.

Xiao, Y., A. Wagendorp, R. Moezelaar, T. Abee and M. H. J. Wells-Bennik (2012). "A wide variety of *Clostridium perfringens* type A food-borne isolates that carry a chromosomal *cpe* gene belong to one multilocus sequence typing cluster." Appl. Environ. Microbiol. 78(19): 7060-7068.

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 off July 2012, Xiao has been employed as a junior Postdoc researcher at the Laboratory of Food Microbiology, WU, working on *Bacillus cereus* superdormant spores.

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