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Clostridium perfringens and foodborne infections

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1. Introduction

Clostridium perfringens causes human gas gangrene and two very different foodborne diseases: the relatively mild, classic Type A diarrhea, which is among the more common of its kind in the industrialized world and the very serious but rare Type C human necrotic enteritis (Granum, 1990). The bacteria are also the cause of many animal diseases such as enterotoxemia and necrotic enteritis in birds. Usually, the production of one or more of *C. perfringens*' many toxins is the major cause of the disease (Songer, 1996).

C. perfringens is a spore-forming bacterium and a natural inhabitant of soil and the intestinal tract of many warm-blooded animals and humans. The ubiquitous nature of this bacterium and its spores makes it a frequent problem for the food industry and establishments where large amounts of foods are prepared (Andersson et al., 1995), and most food-poisoning cases involving *C. perfringens* are reported from restaurants, hospitals and homes for elderly people. Through proper cleaning and disinfection, it should be relatively easy to control foodborne diseases caused by *C. perfringens*, but unfortunately, large outbreaks, sometimes with fatal outcome due to *C. perfringens* food poisoning, are still frequently reported (Labbé, 2000).

In this review, we will describe the foodborne diseases caused by *C. perfringens*, concentrating on the cause of the common diarrheal food poisoning caused by Type A enterotoxin-positive strains, while Type C necrotic enteritis and the involved toxins will be described briefly.

2. Characteristics of the organism/reservoir

C. perfringens is a spore-forming, Gram-positive, anaerobic, non-motile rod which form large, regular, round and slightly opaque and shiny colonies on the surface of agar plates. Colonies usually show a double-zone hemolysis on blood agar plates with a clear inner theta-toxin zone and a hazy outer zone caused by alpha-toxin production. They can grow between 15 and 50 °C with an optimum of 45 °C for most strains. The generation time (G_t) for most strains at temperatures between 33 and 49 °C is below 20 min, and G_t of 8 min has been reported. (Labbé, 2000). *C. perfringens* can produce over 13 different

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Table 1
The toxins used for typing *C. perfringens* as well as the enterotoxin and their genetic location

Type	α-toxin	β-toxin	ε-toxin	ι-toxin	Enterotoxin
A	+	–	–	–	+
B	+	+	+	–	+
C	+	+	–	–	+
D	+	–	+	–	+
E	+	–	–	+	+
Gene	*plc*	*cpb*1	*etx*	*iap*	*cpe* (not used for typing)
		*cpb*2		*ibp*	
Genetic location	Chromosome	Plasmid	Plasmid	Plasmid	Plasmid/chromosome

toxins although each bacterium only produces a subset of these (Petit et al., 1999). The production of four major lethal toxins are used to type isolates (A–E), and three of these are located on plasmids (Canard et al., 1992) (see Table 1).

C. perfringens is an ubiquitous bacterium found in virtually all environments tested including soil, water, milk, dust, sewage and the intestinal canal of humans and animals (Hatheway, 1990). The presence in soil and feces and the longevity of the spores make *C. perfringens* a suitable indicator of both distant and intermittent fecal contamination (Fujioka and Shizumura, 1985) and for the inactivation and removal of viruses and protozoan cysts in drinking water treatment (Payment and Franco, 1993). Based on these characteristics, *C. perfringens* is used as an indicator parameter in surface water sources in Europe (Council Directive 98/83/EU). Many surveys have shown that *C. perfringens* is found in raw and processed foods, most notably, raw meat products and spices (Labbé, 2000).

3. Characteristics of diseases/infective dose

3.1. Type A food poisoning

The disease is due to the production of the enterotoxin (CPE) (Skelkvåle and Uemura, 1977; Sarker et al., 1999). CPE is produced in the small intestine after ingestion of at least 10^7 *C. perfringens* cells. About 8–12 h (6–24 h) after eating contaminated food, the symptoms start with acute abdominal pain, nausea and diarrhea. The contaminated food is almost always heat-treated, which kills competing flora while the *C. perfringens* spores survive. *C. perfringens* is then frequently the dominating flora, sometimes accompanied by other spore formers such as *Bacillus cereus* (Andersson et al., 1995). The disease is mostly self-limiting and lasts for about 24 h. Deaths may occur due to dehydration, mainly seen in elderly and very young patients.

3.2. Type C

C. perfringens Type C food poisoning is rare in the industrialized world today and has not been recorded in Europe during the last decade. The incubation time is at least 5–6 h, and symptoms start with an acute sudden onset of severe abdominal pain and diarrhea (often bloody), sometimes with vomiting, followed by necrotic inflammation of the small intestine. If not treated, the disease is often fatal and has a mortality rate of 15–25% even with treatment. The disease is mainly due to the production of the β-toxin, with contribution from the δ-toxin and θ-toxin (Granum, 1990; Jolivet-Reynaud et al., 1986). These toxins are all produced during the vegetative growth of *C. perfringens* Type C. It is associated with individuals with low levels of proteolytic enzymes in their intestines, most often caused by low protein intake. As recent as the first few years after World War II, several outbreaks were recorded mostly in Europe and mainly due to underprosessed home canned foods and probably due to the scarcity of meat. The disease is now rarely seen outside the Highland of Papua New Guinea, where it occurs mainly due to the eating habits during traditional feasts. The population has a staple diet of sweet potatoes, which contains a trypsin inhibitor, and large amounts of spit-grilled (and presumably contaminated) pork are consumed during the festival, but due to the sweet potato diet, the β-toxin is

not degraded. Due to normal trypsin activity, the duodenum and small intestine will normally inactivate the β-toxin by cleaving the toxin at a two-lysine residue site in the active β-barrel part of the toxin (Granum, 1990; Hunter et al., 1993; Steinthorsdottir et al., 2000).

4. Foodborne outbreaks due to *C. Perfringens* Type A

The spore-forming ability and rapid growth rates at a range of temperatures are features which allow the bacteria to multiply and survive in food. Most cases of *C. perfringens* food-poisoning outbreaks have occurred in institutions and food service establishments which cook large amounts of food well in advance of serving. If the food is cooled down too slowly and/or not sufficiently reheated, the numbers of bacteria increase rapidly. Due to the rather mild nature of the sickness and to the relatively short duration of the symptoms, most people do not come into contact with health authorities. *C. perfringens* food poisoning is not a reportable disease, and the number of cases is probably greatly underestimated, but even so, enough outbreaks are registered that it is documented as one of the most common foodborne diseases in industrialized nations (McClane, 1997). In Norway, *C. perfringens* was registered as the most common cause of food-poisoning cases (almost one third) in the period from 1988 to 1995 (Granum, 1996). The number of recorded cases varied between 202 and 1240 in the USA, 288 and 4571 in Japan, and 562 and 1716 in England and Wales during the period from 1983 to 1994 (Labbé, 2000).

C. perfringens lacks the ability to produce 13 of the 20 essential amino acids and is therefore associated with protein-rich foods, and 75% of the foodborne outbreaks can be traced to meat and meat products (Johnson and Gerding, 1997). The types of foods that have been involved in the outbreaks include corned beef, Mexican food, pea soup, stew, salmon, lasagne, reindeer and vacuum-packed pork (Hatheway, 1990). It should be noted that modified atmosphere packaging without refrigeration does not hinder *C. perfringens* growth, and temperature-abused sous vide products present a possible public health risk (Labbé, 2000).

5. Virulence factors

5.1. Type A enterotoxin

The enterotoxin (CPE) has been shown to be the major virulence factor in the common form of food poisoning. Stark and Duncan (1971) first showed that all clinically significant properties were linked to the enterotoxin, human volunteer studies strengthened the theory (Skelkvåle and Uemura, 1977), and gene deletion studies (Sarker et al., 2000) gave the definitive proof that the effects seen are solely due to the production of enterotoxin.

5.2. Biochemistry

CPE was first isolated in the 1970s (Stark and Duncan, 1971), and the protein was sequenced (Richardson and Granum, 1985) and has been cloned and sequenced by several groups (Iwanejko, 1989; Van Damme-Jongsten et al., 1989; Czeczulin et al., 1996). The sequence of the toxin itself has been found to be highly conserved in Type A strains, while defect copies have been found to be associated with the iota toxin in Type E strains (Billington et al., 1998). CPE is a single, 319 amino acid polypeptide of 3.5 kDa with an isoelectric point of 4.3 and with no significant similarity to other known proteins, except for the limited homology with a *C. botulinum* complexing protein (Kokai-kun and McClane, 1997). The secondary structure appears to be ca. 80% $\beta-$sheet and with 20% random coil (Granum and Stewart, 1993). It is both heat- and pH-labile, but limited trypsination and chymotrypsination increase the biological activity (Granum and Richardson, 1991; Granum et al., 1981). The protein has a two-domain structure. The C-terminal end (aa 290–319) contains the binding region which binds to the protein receptor in the intestine (Fig. 1). The receptor has been shown to be 22-kDa claudin proteins, which are proteins located in tight junctions of many cell types (Katahira et al., 1997; McClane, 2000). Antibodies against this binding region neutralise CPE cytotoxicity. The first 44 N-terminal aa region and three C-terminal aa can be removed without loss of the activity (Kokai-kun and McClane, 1997) and the first 25–34 aa are probably trypsinated/chymotrypsinated in the intestine (Granum,

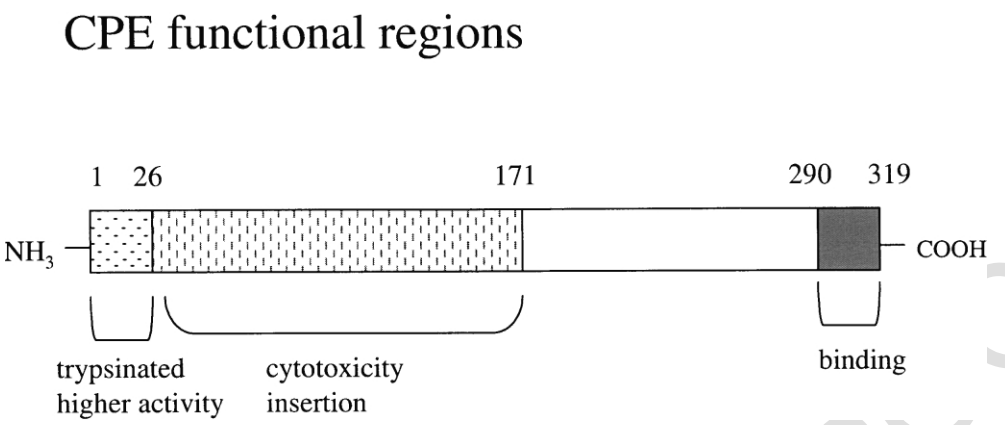

Fig. 1. A schematic diagram showing the functional regions of CPE. The enterotoxin has greater activity when aa 1–26 (34) are removed, the aa 290–319 are essential for binding, and aa 26–171 are involved in the insertion in the membrane and cytotoxicity.

1990). Amino acids 44–171 have been shown to be involved in insertion and cytotoxicity (Fig. 1).

The current model of CPE action is based on a number of studies which show that CPE is found in two different complexes. The first complex is formed when CPE binds to the claudin receptor and this binding can take place at 4 °C. After a physical change in this "small complex," it interacts with a ca. 70-kDa protein to form a very hydrophobic "large complex," which causes small molecule permeabilities to develop (Fig. 2). The large complex does not form at 4 °C (McClane, 1997). Clamp patch studies

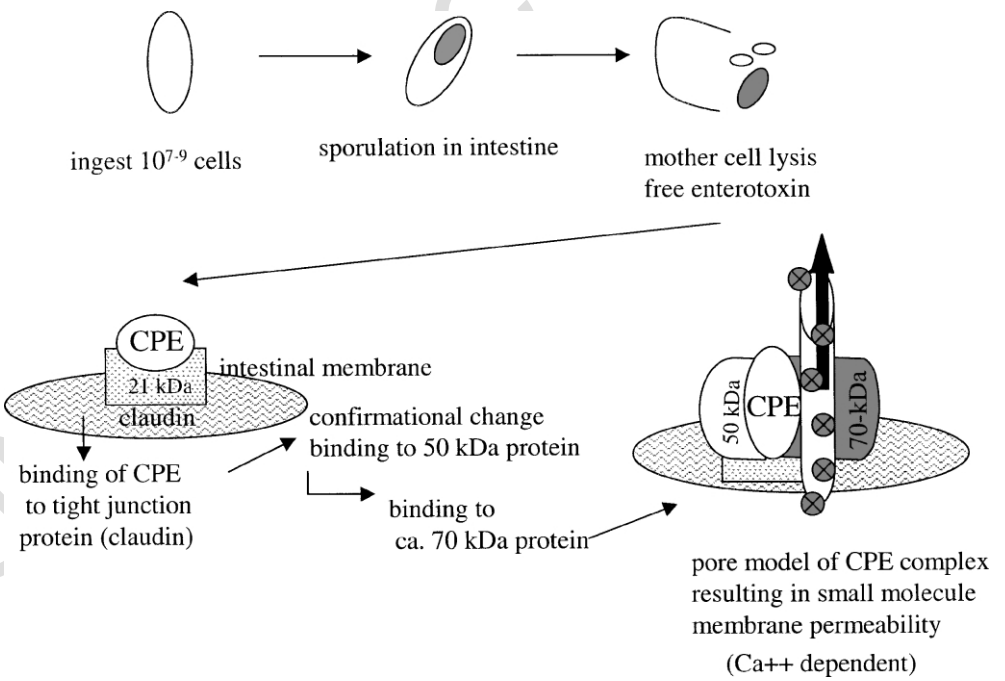

Fig. 2. A schematic diagram showing the major steps in *C. perfringens* food-poisoning mechanisms.

indicate that *C. perfringens* enterotoxin is able to form cation-permeant pores in the apical membrane of human intestinal CaCO-2 epithelial cells and that the increases in the short-circuit current can be prevented by pre-exposure to zinc ions (Hardy et al., 1999). The mechanism of small molecule permeability appears to be substantially different from other known pore-forming toxins.

5.3. Genetics and regulation of production

The enterotoxin gene is in a single copy on a hypervariable region of the chromosome (Canard et al., 1997) in an apparent transposon in most of the food-poisoning isolates and is situated in the same genetic location between two housekeeping genes in the isolates tested (Brynestad et al., 1997) (see Fig. 3). The isolates have different genetic backgrounds and are not a single clone which is dispersed (Ridell et al., 1998; Collie et al., 1998). There is evidence that the transposon can excise (Brynestad and Granum, 1999), and this could explain the loss of the enterotoxin production which is sometimes observed (Petit et al., 1999). The animal isolates and the non-foodborne diarrhea strains have *cpe* on a large plasmid (Cornillot et al., 1995; Collie and McClane, 1998), which had been shown to be conjugative and transferred at rates up to 10^{-3} transconjugates/donor (Brynestad et al., in preparation). The *cpe*-positive *C. perfringens* Type A strains tested to date have an IS*200*-like element (IS*1469*) ca. 1 kb upstream of *cpe* and the plasmid-associated *cpe* is in association with IS*1151* (Cornillot et al., 1995). The conjugative transfer of *cpe* and the association with mobile elements indicate that the low level of *cpe*-positive strains found in nature could be sufficient as the reservoir for *cpe*. The transfer of *cpe* from positive strains to negative strains in situations where *cpe* production is an advantage, presumably in kitchen environments and in the gut, could account for the appearance of new *cpe*-positive strains.

The production of enterotoxin is regulated by sporulation, and up to 15% and possibly 30% of the total protein produced during sporulation is CPE in *cpe*-positive strains. Sporulation mutants, Western blots and mRNA studies have all confirmed that enterotoxin is only produced in large amounts during sporulation although small amounts are produced by some cells during vegetative growth (McClane, 1997). CPE is not secreted but is released upon lysis of the mother cell. Three promoters have been mapped from

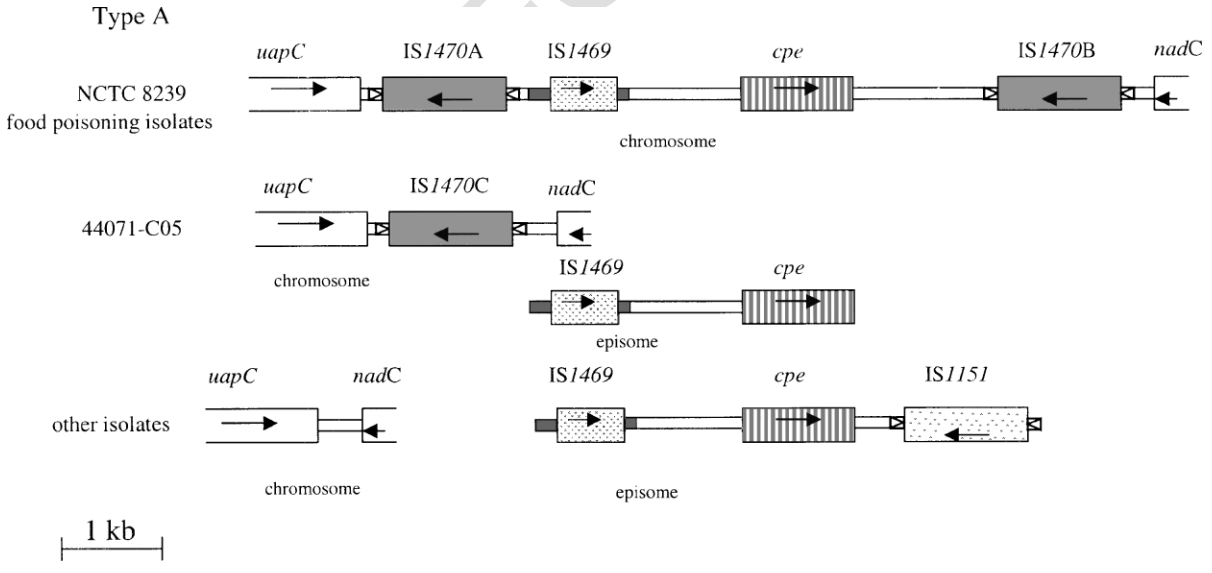

Fig. 3. The genetic placement of *cpe* in human food-poisoning strains and other isolates. Arrows indicate the direction and coding strand. *uap*C and *nad*C are housekeeping genes. *cpe*, the associated IS elements and the genomic location are indicated. The genetic configuration of strain 44071–C05 is included to illustrate apparent genetic movement of *cpe*.

58 to 143 bp upstream of the initiation codon and sequences upstream of P1, P2 and P3 shows significant similarity to the sporulation-dependent sigma factor SigK- and SigE-dependent promoters. Transcription of P2 and P3 was initiated at the entrance into the stationary phase, and deletion studies showed that these promoters were necessary for the sporulation-controlled expression of *cpe* (Zhao and Melville, 1998). The *cpe* mRNA seems to be very stable with a half-life of up to 58 min (Labbé and Duncan, 1977). The *C. perfringens* background appears to be essential and sufficient for the production of large amounts of CPE as cloned copies on multiple copy vectors in *E. coli* and *Bacillus subtilis* do not result in appreciable amounts of toxin even during sporulation of the latter (Melville et al., 1994), but *cpe* introduced into *cpe*-negative strains resulted in normal enterotoxin production (Czeczulin et al., 1996). An additional possible regulation mechanism could be related to a transition state regulator related to Hpr found in *B. subtilis* as conserved Hpr consensus binding sites are found up- and downstream of *cpe* (Brynestad et al., 1994).

6. Survival in foods/control

Although *C. perfringens* spores are the main source of concern in food products, vegetative cells may occasionally cause problems in non-heat-treated foods or by recontamination of heat-treated foods. Proper disinfection of critical surfaces in restaurants and in food production industries is the most efficient way of controlling the problem of *C. perfringens* food poisoning. The *C. perfringens* spores are only killed by the use of hypochlorite at a pH below 8.5 (Granum and Magnussen, 1987) or by the use of UVC light. When vegetative *C. perfringens* cells are present in foods, they will grow (with sufficient protein sources) at temperatures between 15 and 50 °C. Optimum temperature is about 43–46 °C, where the generation time may be as low as 7–8 min (Labbé, 2000). Although this organism is an anaerobe, it will usually grow at E_h below +350 mV (Labbé, 2000), while the final levels can reach below −400 mV. *C. perfringens* will grow at pH values ranging from 5 to 9, with an optimum between 6 and 7. It is not especially tolerant to low a_w, and different strains will stop growing somewhere between 0.95 and 0.97 (Labbé, 2000). $D_{95\ °C}$ values for the spores can be as high as 200 min (Labbé, 2000). It has recently been shown that vegetative cells of strains with a chromosomal copy of *cpe* have ca. 2-fold higher $D_{55\ °C}$ values than strains with *cpe* on a plasmid or *cpe*-negative strains, and the spores have ca. 60-fold higher $D_{100\ °C}$ values. This heat resistance could be part of the explanation of the association of *C. perfringens* with chromosomal copies of *cpe* in food poisoning outbreaks (Sarker et al., 2000).

7. Detection

The confirmation of foodborne outbreaks is often difficult, and some outbreaks involving *C. perfringens* have been especially challenging. It is not enough to demonstrate that *C. perfringens* is suspected in food and stools since many healthy people, especially the elderly, often have high numbers of *C. perfringens* spores in their feces. The ability of the isolates from suspect food and stools of affected individuals to produce enterotoxin as well as the confirmation that the strains from the food and the affected individual are the same needs to be confirmed in outbreak situations. There are commercially available kits for the detection in fecal specimens (ELISA-TECHLAB) and from sporulating culture (PET-RPLA, Oxoid). Not all *C. perfringens* strains will sporulate in the sporulation media, and there is the possibility of false positives due to a cross-reaction with a vegetatively produced protein and incompletely sporulated cultures (Brynestad and Synstad, unpublished observations), which complicate this type of CPE detection. The fact that a bacterium must have *cpe* in order to cause food poisoning and that the *C. perfringens* Type A strains tested to date, which carry a complete *cpe*, are able to produce enterotoxin when they sporulate, make PCR of the enterotoxin gene itself a good alternative for confirmation in the diagnostic work (Kokai-kun et al., 1994). Several PCR-typing studies have been successfully performed on both *cpe* and other toxins in *C. perfringens* (Meer and Songer, 1997; Yamagishi et al., 1997). Pulsed field gel electrophoresis (PFGE) can be used to identify the presence of the same strain in the food and patient although the presence of multiple *cpe*-positive clones in the same outbreak can make the

interpretation of these results somewhat difficult (Ridell et al., 1998).

8. Concluding remarks

Although *C. perfringens* food poisoning is a relatively mild form of food poisoning, it is commonly enough in industrialized nations to cause considerable economic loss. The spores are ubiquitous, long-lived and resistant to heat and many cleaning procedures, and these characteristics make the spores good indicators of the effectiveness of the disinfection routines in food, food production environments and in water. Even though the *cpe* gene is only found in ca. 5% of the environmental isolates, the increased heat resistance seen in the isolates, which have a chromosomal copy of *cpe*, the fact that the enterotoxin gene is transferable and the presence of multiple *cpe*-positive clones in single outbreaks indicate that the presence of *cpe* can confer a selective advantage, and the low level of *cpe*-positive strains can suffice as a reservoir for the enterotoxin. Only good disinfection routines and attention to proper food handling practices will remove these problem bacteria.

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