Published in final edited form as:

Anaerobe. 2008 April ; 14(2): 102–108.

***Clostridium perfringens* toxin genotypes in the feces of healthy North Americans**

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Abstract

We investigated the frequency of *C. perfringens* in the normal fecal flora of healthy North Americans. About half of 43 subjects were colonized with *C. perfringens* at levels of $\sim 10^6$ cfu/g feces. Only type A strains were recovered. Spores sometimes outnumbered vegetative cells. Several genotypes were found. Some donors carried two genotypes, some only one. We found no alpha, beta2 or enterotoxin in the stools of any donors. Though some isolates carried toxin genes (e.g. *cpe* and *cpb2*) on plasmids, we saw no indication that healthy humans are the reservoir for the chromosomally-borne *cpe* recovered from cases of *C. perfringens* food poisoning.

Keywords

Clostridium perfringens; health; genotype; enterotoxin; beta2

1. INTRODUCTION

Clostridium perfringens is a Gram positive, spore-forming, rod-shaped anaerobe. This bacterium is commonly classified into one of five types depending on production of alpha, beta, epsilon and iota toxins. Its primary habitat is the bowel of some healthy humans and probably some individuals of most other warm-blooded animal species. It is also found in soil where animals congregate and where manure has been used to boost agricultural fertility.

Ostensibly a non-pathogenic member of the healthy human gut flora, the ecology of *C. perfringens* is complicated by its carriage of various combinations of toxin genes and by the location of these genes on either the chromosome or a plasmid. For example, type A strains carrying the *cpe* gene encoding the *C. perfringens* enterotoxin (CPE) cause, under suitable conditions, food poisoning [1,2,3], sporadic out-patient diarrhea [4,5] and nosocomial antibiotic associated diarrhea [6–16]. Since the *cpe* gene in food poisoning isolates is usually chromosomal while in other diarrheas it is predominantly plasmid-borne [3,17,18], identifying reservoirs for the type A plasmid and chromosomal *cpe* isolates is important for understanding disease transmission and for rational design of intervention strategies to block transmission of

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virulent strains. It has been clearly shown that American foods can be contaminated with chromosomal *cpe* isolates at the time of retail purchase [19] but it is not yet clear how or when those foods become contaminated. However, two recent studies reported the presence of a few chromosomal *cpe* isolates in healthy Japanese and Finnish people, possibly suggesting healthy people as a potential reservoir for chromosomal *cpe* food poisoning isolates.

Though no longer as widely accepted as before, the relative levels of *C. perfringens* type A in human feces is thought to be helpful in establishing a causal role for this bacterium in cases of diarrhea, especially food poisoning. Thus, while counts below 10^5 to 10^7 /g were considered “normal”, higher counts were said to be clinically relevant, though it was sometimes unclear whether this meant only spores or all *C. perfringens*. Consequently, reports of *C. perfringens* in healthy humans’ feces ranging anywhere from none to 10^8 /g feces are confusing and contradictory. For example, with good anaerobic technique coupled with non-selective, feces-mimicking medium, the mean carriage rate among 191 healthy adults was $10^{9.6}$ cfu/g, though surprisingly only 8 subjects (~4%) were colonized (the late WEC Moore, VPI Anaerobe Lab, VA Tech, Blacksburg, VA, Personal communication). Using a variety of selective and differential media, Harmon and Kautter [20] found that even their best medium missed some positives recovered by one or more of the less effective media. Thus without paying close attention to the details, reports of counts that range anywhere from none to 10^8 /g are difficult to interpret. Nor is it clear why an individual's carriage rate, though stable for weeks at a time, may still vary considerably over 12 months [21]. Furthermore the carriage of particular strains is sporadic and individuals may carry more than one strain at a time [22]. Finally, age affects carriage rates; *C. perfringens* is more abundant in healthy neonates and the elderly than in adults. Tonooka et al., [23] found *C. perfringens* in 28% of healthy month old babies, with rates highest among those receiving formula milk, while carriage rates went up significantly in the elderly [24].

Our studies were designed to assess the relative incidence of the different toxin genotypes of *C. perfringens* in healthy North Americans in order to test the hypothesis that healthy people are reservoirs for type A chromosomal *cpe* isolates capable of causing food poisoning or for type A plasmid *cpe* isolates capable of causing non-foodborne gastrointestinal diseases. We looked at the rates of carriage in individuals, the relative abundance of spores and vegetative cells in feces, the presence or not of toxins in stool, the presence of *cpe* and *cpb2* genes, and, lastly, the type of *cpe* locus present in recovered type A *cpe*-positive isolates.

2. MATERIALS AND METHODS

2.1. Fecal samples

TechLab Inc IRB #1 approved the collection of Informed Consent, self-reported information about age, diet and health, and a fecal sample from 43 healthy subjects. We recruited only those donors reporting themselves in good general health, free of underlying intestinal illnesses, having taken no antibiotics for at least 12 weeks and being free of diarrhea over the same period. 20 donors were males, 23 were females. Donors provided their first feces of the day. Immediately on arrival at the laboratory, usually within 60 min of defecation, feces were homogenized, dispensed aseptically under anaerobic gasses (90% nitrogen, 10% carbon dioxide) into airtight glass tubes. The samples were then frozen (-70°C), usually within 120 min of defecation. At the time of use, they were thawed at room temperature while still anaerobic. All assays run on a fecal sample (as opposed to the subsequent testing of isolated bacteria) were done on the same day.

2.2. Fecal toxins

We used the *C. perfringens* Enterotoxin TEST (TechLab, Inc., Blacksburg, VA), a research-use-only assay, for the detection of fecal enterotoxin. This is a polyclonal antibody based assay that detects both intact, cytotoxic CPE and fragments of CPE that, though no longer biologically active, are still immunoreactive. Thus a positive would not automatically mean that sample would also be cytotoxic. Cytotoxic activity in feces was detected using Vero cells [25]. Some samples were sufficiently voluminous and liquid to be readily filterable. The others were centrifuged ($8000 \times g$, 10 min, 4 °C) to generate a supernatant with which to work. All were filtered (0.22 μm pore size). Serial doubling dilutions were prepared and loaded into wells to give in-the-well dilutions of 1/20 to 1/2560. The reciprocal highest dilution producing a cytopathic effect within 48 h was the titer. A second 8-well row was used to neutralize any cytotoxic activity by adding 50 μL of a 1/50 dilution of goat anti-*C. perfringens* enterotoxin (the concentrated component that is diluted for inclusion in the *C. perfringens* Enterotoxin Test, TechLab, Inc.) in PBS to each well. Neutralization of a cytotoxic sample would indicate the presence of active CPE. This assay would reveal incidentally all cytotoxicities active on Vero cells. Any *C. perfringens* alpha toxin, which is active on Vero cells, would thus be apparent in this assay.

2.3. *Clostridium perfringens* counts

Aseptically and anaerobically, 10-fold dilution series (out to 10^{-6}) were made in pre-reduced anaerobically sterilized (PRAS) diluents [26; Anaerobe Systems, Morgan Hill, CA]. The total count was obtained by spreading 100 μL from serial dilutions onto PRAS blood agar (Hardy Diagnostics, Santa Maria, CA). To select for spores by eliminating vegetative cells, we pre-incubated 1 mL aliquots of each dilution of a sample in an equal volume of non-denatured 95% ethanol for 60 minutes at room temperature [6]. Inocula (200 μL , equal to 100 μL of diluted feces) were plated on to blood agar. Both sets of plates were incubated anaerobically at 37 °C for 24 h. *C. perfringens* was presumptively identified based on colonial and cellular morphology, patterns of hemolysis and Gram stain. Counts were adjusted for dilution and the mass of feces added to the initial tube to give count/g feces. The minimum level of detection of vegetative cells or spores or both was $\sim 10^2$ cells/g feces, depending on the precise mass of stool in the first dilution tube. Up to five isolates from each positive sample were subcultured in PRAS chopped meat broths (Anaerobe Systems) to provide stock cultures for subsequent studies. Each isolate was confirmed as *C. perfringens* using PCR and species-specific 16S rRNA primers [23]. The mean bacterial counts and their standard deviations were calculated using absolute numbers that, for presentation, were transformed into $\log_{10}$ count/g.

2.4. Toxin genotyping of *Clostridium perfringens*

Template DNA for PCR genotyping analysis were prepared as described before [19]. Each PCR mixture contained 2 μL of template DNA, 17 μL of TAQ Complete 1.1 Master Mix (Gene Choice), and 0.5 μL of each primer pair (1 μM final concentration). The multiplex PCR reactions were performed in a Techne (Buckingham, Germany) thermocycler using previously described conditions and primer sets capable of detecting the presence of genes encoding alpha toxin, beta toxin, epsilon toxin, iota toxin, enterotoxin, and beta2 toxin [27]. PCR products were separated on 2% agarose gels and visualized with ethidium bromide staining.

The above prepared template DNA was also used for multiplex PCR *cpe* genotyping analysis. Primers and amplification conditions were used as described before [28] to identify the enterotoxin gene (*cpe*) locus present in each recovered type A *cpe*-positive *C. perfringens* fecal isolate. Specifically, this assay can distinguish between those isolates carrying a chromosomal *cpe* locus flanked by IS1470 sequences, a pCPF4969-like plasmid with an IS1470-like

sequence downstream of *cpe*, or a pCPF5603-like plasmid with an IS1151 sequence downstream of *cpe*.

2.5. Detection of CPE by Western immunoblotting of *Clostridium perfringens* culture fluids

CPE production by *C. perfringens* sporulating cultures of *cpe*-positive isolates (as detected by PCR assays) was assessed by inoculating a 0.1 mL aliquot of an overnight thioglycollate broth culture into 10 mL of Duncan-Strong sporulation medium [29,30]. Those cultures were then incubated for ~14 h, at which time sporulation was examined by phase-contrast microscopy. Sporulating cells in the cultures were sonicated (Sonicator 3000) to release their internal CPE. After centrifugation, supernatants from the sonicated sporulating culture lysates were used for CPE Western blotting [30]. For CPE Western blotting, a 100 μ L aliquot of supernatant from each sporulating (prepared as described above) culture was mixed with 100 μ L of SDS sample buffer and 30 μ L of that mixture was then electrophoresed on 10% acrylamide gels containing SDS (no sample boiling). After electrophoresis and transfer onto nitrocellulose, CPE Western blotting was performed using a rabbit polyclonal CPE antiserum as primary antibody, followed by a goat anti-rabbit IgG peroxidase conjugate (Sigma) as secondary antibody. Blots were developed with SuperSignal West Pico Chemiluminescent substrate (Pierce).

3. RESULTS

No donor excreted *C. difficile* or its toxins (data not shown). All samples were negative for evidence of biologically active alpha toxin, a probable indication of good health that we discuss elsewhere in this report. They were also negative for evidence of both cytotoxic and immunoreactive CPE. All were negative for fecal lactoferrin, a biomarker of inflammation, indicating the absence of intestinal inflammation (data not shown). Thus, these negative data support the donors' self-reported good health.

3.1. *C. perfringens* in stool

A combination of spores and vegetative cells of *C. perfringens* were present in 22 of 43 (or 51%) of healthy donors. Spores alone were found in 5 additional subjects, so altogether 27 (or 63%) subjects carried *C. perfringens* (Table 1). The mean incidence for total *C. perfringens* (vegetative cells and spores) in the 27 carriers was $10^{6.50}$ /g. The standard deviation, $10^{7.15}$ cfu/g, exceeded the mean, reflecting the wide range of obtained fecal counts, from as high as $10^{7.83}$ down to 10 individuals carrying fewer than 10^3 /g. There were no significant differences between rates of carriage in males and females (t-test, $p < 0.05$).

Multiplex toxin genotyping PCR analyses showed that only type A *C. perfringens* (i.e. PCR positive for the alpha toxin gene but negative for genes encoding beta, epsilon and iota toxins) isolates were recovered from the feces of healthy human subjects (Table 2). We recovered 34 type A isolates (each distinct within the sample from which it was recovered) from 23 individuals (Table 2). There were nine subjects who had neither *cpe* nor *cpb2*, 11 were *cpb2* positive, two had both *cpe* and *cpb2*, and one had *cpe* only. Multiplex *cpe* genotyping PCR analysis showed that two people carried type A isolates with a pCPF5603 *cpe* locus and a *cpb2* gene, presumably both on a pCPF5603-like plasmid. Lastly, one *cpb2*-negative type A isolate was recovered that carried a pCPF4969 *cpe* locus.

Individual spore counts tended to be nearly the same or greater than the total count (data not shown). In 14 of the 27 colonized individuals the spore count was 100% or more than the total count, while spores were on average 124% of the total count in colonized individuals (Table 1).

3.2. Genotypes in individuals

We isolated and genotyped the *C. perfringens* type A isolates from 23 healthy donors; 11 donors carried only one type A genotype, the other 13 donors carried two. None carried three (Table 2). When only one strain was present, it was always a type A without either the *cpe* or *cpb2* genes.

3.3. *In vitro* CPE production by fecal isolates

7% (3) of all 43 subjects, and 3 of 27 (11%) colonized donors, carried *cpe*-positive isolates. Of the 14 *cpe*-positive type A isolates recovered from those 3 colonized people, 12 could sporulate *in vitro*, as required to demonstrate CPE expression [29]. Only two of the 12 sporulating strains produced CPE levels detectable by Western blotting. The *cpe* open reading frame (ORF) was sequenced in two of the 12 sporulation-capable, *cpe*-positive isolates that failed to produce CPE. No changes from the consensus *cpe* ORF was detected in one of those isolates but the other carried a premature termination codon in its *cpe* ORF.

4. DISCUSSION

4.1. Fecal carriage

About half our donors were colonized with, on average, slightly over 10^6 *C. perfringens*/g (Table 1), a finding that lies within the broad range of reported rates. Some colonized donors carried no vegetative cells. Many had spore counts that exceeded their total counts, an odd result that needs an explanation. In this study spores were selected by alcohol wash, though it could equally have been by heat shock. Stressors, such as ethanol, are believed to enhance the rate of germination of spores [31]. This effect may help explain how a spore count could exceed its corresponding total count, especially if the majority of viable *C. perfringens* was already present as spores, not vegetative cells. The skew towards spores may have occurred after the samples were collected, as an artifact of the processing although we saw no evidence of this in preliminary studies of fresh and frozen aliquots of the same fecal samples. A second possibility is that the alcohol wash eliminated the inhibitory effects of the normal flora thus elevating the relative spore count (established with no background flora thanks to the wash) at the expense of the total count (established against a high and therefore inhibitory background). A third alternative is that the trend points to *C. perfringens* vegetative cells occurring high in the bowel, perhaps in the terminal ileum, while spores of this bacterium are more common further along the bowel, perhaps where conditions are less hospitable. Five extreme instances of this trend, spores alone ($10^{1.93}$ to $10^{4.93}$ /g), were observed during our study. Assuming there were neither within-donor nor within-sample variations of the distribution of spores, $10^{1.9}$ spores/g coupled with a probable daily fecal mass of over 100 g suggests donors excreted almost 10,000 spores each day. This is indicative of colonization, not the transient passage of ingested spores.

4.2. Genotypes

CPE is the primary virulence factor for enteric diseases caused by CPE-positive type A isolates [3]. In a series of studies [32–35] the *cpe* gene has been localized to either the chromosome, as in the overwhelming majority of food poisoning outbreaks, or on one of two plasmid families, represented by prototype *cpe* plasmids pCPF5603 and pCPF4969. The latter *cpe* plasmid family is known to be conjugative [36]. When chromosomal, the *cpe* gene is flanked by the insertion sequence, IS1470. In contrast, the pCPF5603 plasmid family carries an IS1151 sequence downstream of its *cpe* gene. pCPF5603-like plasmids usually also carry the *cpb2* gene. pCPF4969-like plasmids have an IS1470-like sequence downstream from *cpe* but do not themselves carry *cpb2*. However, *cpb2* can be present on an additional plasmid in strains carrying pCPF4969-like *cpe* plasmids. The beta2 toxin (CPB2) produced by expressing

pCPF5603-borne *cpb2* is about 10-fold more active on Caco-2 cells than the equivalent gene product produced by isolates carrying pCPF4969-like plasmids and a separate *cpb2* plasmid [32].

Finnish workers [37] recently studied *C. perfringens* fecal carriage in 136 healthy food workers. By amplifying *cpe* from enrichment broths they identified 25 (18% of the total study population) *cpe*⁺ fecal samples, whereas after enrichment only 11 (8%) of 136 samples were also culture positive for *cpe*⁺ isolates. Given the reputation of *C. perfringens* for ease of isolation, it is somewhat surprising that 14/25 samples were PCR positive but culture negative for *cpe*. In contrast, without enrichment we detected 7% of donors as carrying *cpe*⁺ isolates. Of the 11 *cpe*⁺/culture⁺ donors identified in the Finnish study, 10 carried both *cpe*⁺ and *cpe*⁺ isolates, while one carried only *cpe*⁺ isolates. From among those 11 donors, the Finns found one with a strain carrying a chromosomally-located *cpe* (IS1470-*cpe*), several that by implication had *cpe* on the plasmids pCPF5603 or pCPF4969 and, lastly, two with untypeable *cpe*⁺ strains. We found no IS1470-*cpe* or untypeable strains in our study, only isolates with a pCPF5603 or pCPF4969 *cpe* locus. The Finns also saw one instance of both *cpe* plasmid families in the same sample; that individual also carried an additional *cpe*⁺ strain, making three in total. Though we saw no instances of three genotypes in the same sample (Table 2), we often found two. Further analysis of their chromosomal DNA might resolve the strains into, perhaps, one with and one without a *cpe* or *cpb2* plasmid or show a single strain occurring with and without a toxin-encoding plasmid.

Japanese workers have also studied the distribution of *cpe*⁺ isolates in health and disease [38]. They recovered 16 *cpe*⁺ fecal isolates from an unspecified number of healthy donors. 11 of those isolates apparently carried a pCPF5603-like *cpe* plasmid, three had a pCPF4969-like *cpe* plasmid, and two isolates had a chromosomal *cpe* gene. Thus all three groups of workers, us, the Finns [37] and the Japanese [38], have found both *cpe* plasmid families in healthy donors, at low and comparable frequencies. In contrast, Miyamoto et al. [28] found nearly equal numbers of pCPF4969-like and pCPF5603-like plasmids among European nonfoodborne gastrointestinal disease isolates but suggested a skew in favor of pCPF4969-like plasmids among North American nonfoodborne gastrointestinal disease isolates.

With our data, the presence of chromosomal *cpe* isolates has now been surveyed in the feces of healthy Finns, Japanese and Americans. While a few fecal samples have tested positive (at apparently low levels) for these bacteria, these survey results collectively indicate that most healthy people are not routinely colonized by chromosomal *cpe* type A isolates. Whether the few carriers of these bacteria represent an important reservoir for food poisoning transmission remains to be determined.

The finding of *cpe*⁺ spores in both our and the Finnish studies is important. If, as *in vitro*, the *in vivo* regulation of sporulation and enterotoxin production are co-regulated, the presence of *cpe*⁺ spores suggests possible *in vivo* expression of CPE. Despite all 11 colonized donors having at least one readily sporulating *cpe*⁺ isolate recovered originally as a spore, we saw no cytotoxicity from CPE. In fact no sample at all elicited any type of cytopathic effect, implying the absence of any and all cytotoxic activities. This would include the alpha toxin, the cytotoxic phospholipase C, of *C. perfringens* whose production is constitutive *in vitro*, and that by implication should have been detectable in all 27 colonized healthy donors but was not. This would be explained if the *in vitro* degradation of alpha toxin by proteases [39,40] also occurred *in situ* as a result of both host and bacterial enzymes. In fact, the capacity of stool to inhibit alpha toxin activity has been long known [41]. Reports of active alpha toxin in diarrheic stool [42] coupled with its absence from healthy feces [43] suggest an innate mechanism of resistance during health, possibly mediated by host or bacterial proteases or both. Degradation by proteases might also explain the lack of CPE cytotoxic activity in any healthy donors' feces

and arguably in the majority of symptomatic patients with CPE food poisoning [44]. Perhaps in some healthy donors, *cpe* expression and sporulation both occur in the ileo-cecal region but normal proteolytic activity eliminates, in a stepwise manner, firstly the biological activity and finally, even the immunoreactivity.

We saw three donors colonized with *cpe*⁺ isolates. From all three at least one readily sporulating *cpe*⁺ strain was recovered after alcohol shock. Those stools might have contained expressed CPE but did not. One possible explanation for the lack of CPE in the three donors colonized with *cpe*⁺ isolates is the inability of all isolates but one to produce CPE *in vitro*, as detectable by Western blot with specific monoclonal antibodies [45]. One isolate had a stop codon located within its *cpe* sequence (McClane and Li, unpublished data) that could explain its deficiencies in *cpe* expression. This isolate is interesting since it represents, to our knowledge, the first identified type A isolate carrying a defective *cpe* ORF. A lack of expression by the remaining *cpe*⁺ isolates may be nothing more than a measure of our lack of success in establishing the appropriate *in vitro* conditions for expression.

Of our 43 subjects, 8 (23%) were colonized with isolates carrying the *cpb2* gene (Table 2). Conversely, Fisher et al., [32] have shown that over 75% of *C. perfringens* recovered from cases of sporadic diarrhea or nosocomial antibiotic associated diarrhea (AAD) caused by *C. perfringens* were *cpb2*⁺ and almost all the *cpb2*⁺ isolates they studied expressed CPB2 *in vitro*. However, some care should be taken with these data since the North American AAD isolates included as part of that survey came mainly from two nosocomial outbreaks of *C. perfringens* AAD that, if typical of earlier outbreaks (6), may be associated with a single strain at each site. Apparently *cpb2* may be found in health or in disease and the possible involvement of beta2 toxin in human GI disease remains intriguing but unproven. The toxin can clearly damage Caco-2 human enterocyte-like cells [32]. However, there are no published reports, successful or not, of attempts to fulfill Koch's Postulates using strains that produce CPB2, nor have we been able, thus far, to show any enterotoxic effect of partially-purified or purified CPB2 in rabbit ileal loops (M.R. Sarker and R.J. Carman, unpublished data; D.J. Fisher, F.A. Uzal and B.A. McClane, unpublished data). Despite this, a body of literature is developing that may support a clinical role for CPB2.

5. CONCLUSIONS

We saw no evidence that healthy humans are a reservoir for the chromosomal-*cpe* typical of isolates from instances of food poisoning. About half of all subjects were colonized with *C. perfringens* type A, with on average about 10⁶/g feces, though with a broad variance. Often spores outnumbered vegetative cells. A variety of type A genotypes were found, often more than one per person. There was no evidence for the accumulation of detectable alpha, CPE or CPB2 toxins during health. Though recovered from healthy individuals, some type A genotypes (*cpe*⁺, *cpb2*⁺, conjugative plasmid) were suggestive of potential virulence.

ACKNOWLEDGMENTS

This research was generously supported, in part, by National Research Initiative Competitive Grant 2005-53201-15387 from the USDA Cooperative State Research, Education and Extension Service and by T32 AI060525-01A1 (a Ruth L. Kirschstein National Service Award) from the National Institute of Allergy and Infectious Diseases.

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Table 1Mean counts of *Clostridium perfringens* and its spores in the feces of healthy human subjects

	Mean <i>C. perfringens</i> count/g feces $\pm$ SD		Mean spores as % of total count $\pm$ SD
	Total count	Spores	
All samples (n=43)	$10^{6.21 \pm 7.01}$	$10^{5.30 \pm 5.92}$	72 ± 133
Only samples with <i>C. perfringens</i> (n; % of all 43 samples)	$10^{6.50 \pm 7.15}$ (n=22; 51%)	$10^{5.50 \pm 6.01}$ (n=27; 63%)	124 ± 153

Table 2*Clostridium perfringens* Type A genotypes recovered from the feces of 23 healthy human subjects

Donor #, Isolate #	Genotype
1	<i>plc</i>
2	<i>plc</i>
3.1	<i>plc</i>
3.2	<i>plc cpb2</i>
4.1	<i>plc</i>
4.2	<i>plc cpe</i> _{F4969} - like
5.1	<i>plc</i>
5.2	<i>plc cpb2</i>
6.1	<i>plc</i>
6.2	<i>plc cpb2</i>
7.1	<i>plc</i>
7.2	<i>plc cpb2</i>
8	<i>plc cpb2</i>
11	<i>plc</i>
12.1	<i>plc</i>
12.2	<i>plc cpb2</i>
14.1	<i>plc</i>
14.2	<i>plc cpb2</i>
18	<i>plc</i>
19	<i>plc</i>
21	<i>plc</i>
25.1	<i>plc</i>
25.2	<i>plc cpb2</i>
29.1	<i>plc</i>
29.2	<i>plc cpb2</i>
30	<i>plc</i>
32	<i>plc cpb2 cpe</i> _{F5603}
33	<i>plc cpb2 cpe</i> _{F5603}
36	<i>plc</i>
39.1	<i>plc</i>
39.2	<i>plc cpb2</i>
40.1	<i>plc</i>
40.2	<i>plc cpb2</i>
43	<i>plc</i>

All isolates were type A, i.e. they carried *plc*, encoding alpha toxin with its phospholipase C activity, but not beta, epsilon or iota toxin genes. Only Isolate 33 produced immunologically detectable CPE. Isolate 4.2 did not express CPE despite sporulating. Strain 32 neither sporulated nor produced CPE. All other *cpe*⁺ isolates were efficient spore formers.