Genotyping and Phenotyping of Beta2-Toxigenic *Clostridium perfringens* Fecal Isolates Associated with Gastrointestinal Diseases in Piglets

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Although *Clostridium perfringens* is recognized as an important cause of clostridial enteric diseases, only limited knowledge exists concerning the association of particular *C. perfringens* toxinotypes (type A to E) with gastrointestinal (GI) diseases in domestic animals. Some *C. perfringens* isolates also produce the newly discovered beta2-toxin (CPB2). Recent epidemiological studies suggested that *C. perfringens* isolates carrying the gene encoding CPB2 (*cpb2*) are strongly associated with clostridial GI diseases in domestic animals, including necrotic enteritis in piglets and typhlocolitis in horses. These putative relationships, obtained by PCR genotyping, were tested in the present study by further genotyping and phenotyping of 29 *cpb2*-positive *C. perfringens* isolates from pigs with GI disease (pig GI disease isolates). PCR and restriction fragment length polymorphism analysis reconfirmed the presence of *cpb2* gene sequences in all the disease isolates included in the study. Furthermore, genotyping by pulsed-field gel electrophoresis analyses showed that the pig GI disease isolates included in this study all carry a plasmid *cpb2* gene, yet no clonal relationships were detected between the *cpb2*-positive pig GI disease isolates surveyed. Finally, CPB2-specific Western blotting demonstrated CPB2 expression by all of the *cpb2*-positive isolates surveyed. The CPB2 proteins made by five of these pig GI disease isolates were shown to have the same deduced amino acid sequences as the biologically active CPB2 protein made by the original type C isolate, CWC245. Collectively, our present results support a significant association between CPB2-positive *C. perfringens* isolates and diarrhea in piglets.

Clostridium perfringens is a gram-positive, endospore-forming, anaerobic bacterium that has long been recognized as a significant cause of both histotoxic and gastrointestinal (GI) diseases in humans and domestic animals (20). The virulence of this bacterium largely results from its ability to produce at least 15 different *C. perfringens* toxins (21). A commonly used classification scheme (21) assigns *C. perfringens* isolates to one of five types, types A to E, depending upon the isolate's ability to produce the four major lethal toxins (i.e., the alpha, beta, epsilon, and iota toxins). *C. perfringens* type A strains are defined as producing alpha toxin, and type C strains are defined as producing alpha and beta toxins. The major lethal toxins, however, are not the only biomedically important toxins; some *C. perfringens* isolates (mostly belonging to type A) produce *C. perfringens* enterotoxin (CPE), and some type C isolates produce a newly discovered *C. perfringens* toxin, the beta2 toxin (CPB2) (10).

CPB2, a 28-kDa protein, was first purified from *C. perfringens* type C strain CWC245, which was isolated from a piglet

that died of necrotizing enterocolitis (16). Purified CPB2 was reported to be cytotoxic for Chinese hamster ovary cells and to induce hemorrhagic necrosis of the intestinal mucosa in a guinea pig ligated intestinal loop (16). In 1997, the gene encoding this 28-kDa protein was cloned from *C. perfringens* type C strain CWC245, and its nucleotide sequence was determined (10). Since the deduced amino acid sequence showed no significant homology with beta toxin, the respective protein corresponded to a new *C. perfringens* toxin, referred to as CPB2.

C. perfringens type C is generally considered to be the primary cause of necrotic enteritis in piglets aged 0 to 2 weeks (29), but type A isolates have also been linked to enteric disease in suckling and feeding pigs with mild necrotic enterocolitis and villous atrophy (8, 15, 22, 24, 25). The role of alpha toxin (produced by all *C. perfringens* toxinotypes) as a virulence factor in type A enterotoxemia remains unclear (29). Type A enterotoxemia syndrome could be produced by oral inoculation of type A isolates into gnotobiotic colostrum-deprived pigs as well as conventional weaner pigs (14). However, this effect could not be explained by the contribution of alpha toxin alone because purified alpha toxin was unable to produce significant lesions and fluid loss in a pig ileal loop assay (25, 29). Although CPE has been implicated in porcine diarrheal disease (5, 29, 32), only nonenterotoxigenic *C. perfringens* type A and type C

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strains were isolated from some diarrheic piglets (9, 10, 17). Despite the probable central role of beta toxin, typical type C disease cannot be produced in the pig model by use of that toxin alone. Experimental reproduction of the disease in pigs requires viable bacteria as well as toxin (as crude culture supernatants) (23).

The availability of the *cpb2* gene sequence has provided a powerful genetic tool for the detection of CPB2-positive *C. perfringens* isolates, which has greatly improved our understanding of the association between *C. perfringens* toxinotypes and animal GI diseases. For example, by using a multiplex PCR assay incorporating *cpb2* gene-specific primers, *cpb2*-positive *C. perfringens* type A isolates could be detected from diarrheic piglets (9, 17), horses with typhlocolitis (13), diarrheic dogs (31), an African elephant with ulcerative enteritis (1), and calves with enterotoxemia (19).

In two studies performed in Switzerland and The Netherlands, all *C. perfringens* isolates obtained from diarrheic piglets were genotyped as nonenterotoxigenic types A and C (17). Interestingly, however, the *cpb2* gene was found to be highly prevalent in isolates from diarrheic piglets in both studies. These data suggest a causal relationship between *cpb2*-positive strains and digestive tract diseases in piglets (17). In a recent PCR genotyping study (9), most of the *C. perfringens* isolates from pigs with GI disease (pig GI disease isolates) were found to be genotype A. Of the 33 isolates from piglets with diarrhea examined, 27 (82%) were positive for the *cpb2* gene, whereas none of the isolates from the piglet controls were positive for the *cpb2* gene, consistent with *cpb2*-positive type A isolates being significantly associated with diarrhea in piglets. However, these putative associations between *cpb2*-positive isolates and GI diseases in piglets remain tentative because this conclusion was drawn only on the basis of the results of PCR genotyping, in which only the presence of *cpb2* sequences and not the expression of CPB2 were demonstrated.

To better appreciate the involvement of *cpb2*-positive *C. perfringens* isolates in GI diseases of piglets, the present study genotypically and phenotypically characterized 35 pig fecal *C. perfringens* isolates. Notably, this study includes the first indepth genotypic analysis of *cpb2*-positive fecal isolates obtained from piglets with CPB2-associated GI diseases. Results from the present study indicate that all *cpb2*-positive *C. perfringens* fecal isolates carry the *cpb2* gene on a plasmid and express CPB2, confirming their virulence potential. These new findings hold potential epidemiologic significance, as they are consistent with *cpb2*-positive *C. perfringens* isolates being responsible for GI diseases in piglets.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *C. perfringens* isolates used in this study are listed and described in Table 1. Each strain was from a separate disease instance in piglets, as described earlier (9, 10, 17). A starter culture (6 ml) of each *C. perfringens* isolate was prepared by overnight growth at 37°C in fluid thioglycolate broth (FTG; Difco), as described previously (7). For DNA isolation or culture supernatant protein preparation, an aliquot (0.2 ml) of each FTG culture was inoculated into 10 ml of TGY broth (3% Trypticase, 2% glucose, 1% yeast extract, 0.1% cysteine) (7), which was then incubated at 37°C overnight.

***cpb2*-specific PCR analysis.** Total *C. perfringens* DNA was isolated from the overnight TGY cultures by a previously described protocol (7). The isolated DNA was then subjected to screening by a *cpb2*-specific PCR with two sets of primers. The primer set 5′-AAACTGAATTTTTAAATGGTGC-3′ (primer 1F) and 5′-TCCACATCCAATGATCTACAA-3′ (primer 2R) was used to amplify a

cpb2 gene-specific internal 318-bp PCR product. The primer set 5′-GCTCTAG AGGATATCTTAAATTTAGCACAG-3′ (primer 3F) and 5′-CCGGAATTCT TTTTTAAGCTCAATTTTTACTGG-3′ (primer 4R) was used to amplify a 1,373-bp DNA fragment carrying the entire *cpb2* open reading frame (ORF) and 263-bp upstream and 312-bp downstream sequences. These PCRs used 100 ng of template DNA, 25 pM each primer, 200 μM deoxynucleoside triphosphates (Roche), 2.5 mM MgCl₂, and 1 U of *Taq* DNA polymerase (Fermentas) in a total volume of 50 μl. The reaction mixture was placed in a thermal cycler (Techne) for an initial period of 5 min at 94°C (denaturation) and was then subjected to 28 cycles, each consisting of 1 min at 94°C, 1 min at 58°C (annealing), and 1 min at 72°C (extension), followed by an additional period of extension for 10 min at 72°C. After PCR, the presence of an amplified product was analyzed by subjecting an aliquot of each PCR sample to electrophoresis at 100 V in 1.5% agarose gels, followed by ethidium bromide staining and visualization under UV illumination.

Preparation of DIG-labeled *cpb2* probe for Southern blotting experiments. A 318-bp digoxigenin (DIG)-labeled, double-stranded *cpb2*-specific DNA gene probe was prepared by a two-step PCR amplification method. Briefly, a 318-bp *cpb2* internal fragment (nucleotides 581 to 899 of the *cpb2* ORF) (10) was first amplified from the total DNA of *C. perfringens* type C strain CWC245 by PCR, as described above.

The 318-bp PCR product obtained from this amplification was then gel purified by using the Prep-A-Gene DNA Purification system (Bio-Rad) and was used as the template for a second PCR in order to produce a 318-bp PCR product containing DIG-labeled nucleotides. This second PCR involved the incubation of 50 ng of the 318-bp purified product obtained from the previous PCR; 2.5 mM MgCl₂; 100 μM dATP, dCTP, and dGTP; 65 μM dTTP; and 35 μM DIG-dUTP (Roche) under the same amplification conditions described above.

Restriction fragment length polymorphism (RFLP) and Southern blotting analyses. Isolated *C. perfringens* DNA samples, prepared as described above, were digested to completion with *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hpa*I, or *Xba*I (New England Biolabs); separated by electrophoresis on 1% agarose gels; transferred to positively charged nylon membranes (Roche); and fixed to the membranes with UV light, as described previously (26). The blots were hybridized with the DIG-labeled *cpb2* probe, and the hybridized *cpb2* probe was then detected with a DIG chemiluminescence detection system with CSPD [disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3,3.1.13.7]decan}-4-yl) phenyl phosphate] ready-to-use substrate (Roche), as described earlier (26).

Pulsed-field gel electrophoresis (PFGE) and Southern blotting analyses. Cells from overnight TGY cultures were collected by centrifugation and used to prepare agarose plugs containing genomic *C. perfringens* DNA, as described previously (2, 3, 6, 27, 30). The undigested total unsheared genomic DNA in 100 μl of an agarose plug was electrophoresed at 200 V with a Bio-Rad CHEF-DRII apparatus, with pulse times ramped from 50 to 90 s over 20 h (27, 30). These pulsed-field gels were then subjected to *cpb2* Southern blotting analysis by the same procedure described above for RFLP and Southern blotting analyses.

PFGE studies for clonal relationships. Total DNA in 100 μl of an agarose plug was digested overnight with *Sma*I at 30°C or with *Mlu*I at 37°C in 200 μl of the buffer solution recommended by the enzyme manufacturer. Both *Sma*I- and *Mlu*I-digested DNA samples were then analyzed by PFGE with 1% agarose gels prepared with PFGE-grade agarose (Bio-Rad), with pulse times ramped from 5 to 120 s over 46 h at 150 V (4). After PFGE, these gels were subjected to ethidium bromide staining and photographed under a UV transilluminator (Bio-Rad).

Isolation of culture supernatant proteins. An aliquot (0.2 ml) of each *C. perfringens* FTG starter culture was inoculated into two tubes containing 10 ml of TGY broth and grown overnight at 37°C. The culture supernatant (~20 ml) was separated from the bacterial pellet by centrifugation. To precipitate the supernatant proteins, ammonium sulfate [4.76 g of (NH₄)₂SO₄/10 ml of supernatant] was added, and the mixture was incubated overnight at 4°C. The precipitated proteins were then collected by centrifugation and resuspended in 25 μl of sterile water and 25 μl of sample buffer. The prepared proteins were immediately used for Western blotting or were stored at −80°C.

Isolation of total cell proteins. Total cell protein was prepared from the bacterial pellet of the 20-ml TGY culture that was used to prepare the culture supernatant proteins. After aspiration of the culture supernatant, the bacterial pellet was resuspended in 500 μl of sterile water, transferred to an Eppendorf tube, and then washed twice with 500 μl of sterile water. The resultant pellet was resuspended in 500 μl of sterile water and 500 μl of sample buffer, and the mixture was boiled for 5 min. Five microliters of this total cell protein was immediately electrophoresed and Western blotted or stored at −80°C.

CPB2 Western blotting analysis. The prepared proteins were electrophoresed on a 0.1% sodium dodecyl sulfate (SDS)–12% polyacrylamide gel and trans-

TABLE 1. Summary of genotypic and phenotypic characterization results obtained in this study for *cpb2*-positive *C. perfringens* isolates

Source and strain	Type	*cpb2* PCR result[a]	*cpb2* RFLP pattern (kb) with *Hpa*I	CPB2-specific Western blotting results[b]	*cpb2* location determined by PFGE	Reference
cpb2-positive strain CWC245	C	+	~5	+	Plasmid	10
Healthy pigs						
JGS1537	A	−	−	−	ND[c]	9
JGS1540	A	−	−	ND	ND	9
JGS1569	A	−	−	ND	ND	9
JGS1580	A	−	−	ND	ND	9
D9703881	A	−	−	ND	ND	9
106527	C	−	−	−	−	17
Diarrheic pigs						
106526	A	+	~5	+	Plasmid	17
JGS1528	A	+	~5	+	ND	9
JGS1538	A	+	~5	+	ND	9
JGS1541	A	+	~5	+	Plasmid	9
JGS1548	A	+	~6	+	ND	9
JGS1552	A	+	~5	+	Plasmid	9
JGS1561	A	+	~5	+	Plasmid	9
JGS1570	A	+	~5	+	ND	9
JGS1580	A	−	−	ND	ND	9
JGS1807	A	+	~5	+	Plasmid	9
JGS1817	A	+	~5	+	Plasmid	9
JGS1818	A	±	>20	+	Plasmid	9
120-98	A	+	~5	+	Plasmid	9
170-98	A	+	~5	+	Plasmid	9
855-97#3	A	+	~5	+	ND	9
855-97#4	A	+	~5	+	Plasmid	9
2011	A	+	~8	+	Plasmid	9
2142	A	+	~5	+	ND	9
9461-97	A	+	~5	+	Plasmid	9
9777colon	A	+	~5	+	Plasmid	9
10618 J	A	+	~5	+	Plasmid	9
10728	C	+	~5	+	ND	9
97-4029	C	+	~6	+	ND	9
JGS1071	C	+	~5	+	Plasmid	9
JGS1090	C	+	~5	+	Plasmid	9
JGS1495	C	+	~5	+	ND	9
JGS1504	C	+	~5	+	ND	9
JGS1508	C	+	~5	+	ND	9
106640	C	+	~5	+	Plasmid	17

[a] +, presence of both 1,373-bp and 318-bp products; −, absence of both 1,373-bp and 318-bp PCR products; ±, presence of 318-bp product but absence of 1,373-bp product.

[b] +, presence of ~28-kDa immunoreactive band; −, absence of ~28-kDa immunoreactive band.

[c] ND, not determined.

ferred to Sequi-Blot polyvinylidene difluoride membrane (Bio-Rad) as described before (18, 26). The membrane was then washed once in 10 ml of Tris-buffered saline (TBS) for 5 min and blocked by incubation in 10 ml of 5% bovine serum albumin (BSA) for 2 h at room temperature, followed by three washes with Tween plus TBS (TTBS; 10 ml for each wash). The membrane was then incubated overnight in a rabbit polyclonal CPB2 antibody solution (1:500 dilution in 5% BSA) (10). After four more washes in TTBS, the membrane was incubated in anti-rabbit–horseradish peroxidase antibody (Pierce) solution (1:20,000 dilution in 5% BSA) for 1 h. Finally, the membrane was washed three times in TTBS and twice in TBS, incubated for 5 min in SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the protocol of the manufacturer, and exposed to autoradiography film (Kodak).

Cloning and sequencing of the *cpb2*-containing fragments from various *cpb2*-positive isolates. To facilitate nucleotide sequencing, the 1,373-bp DNA fragment carrying the *cpb2* ORF and the ~262-bp upstream and ~312-bp downstream sequences from each of several representative pig GI disease isolates were first amplified by PCR and cloned into *Escherichia coli*. To reduce the PCR-induced mutation(s) introduced during the amplification process, the strategy described by Collie et al. (4) was adopted. Briefly, the 1,373-bp *cpb2*-con-

taining DNA fragment from each *cpb2*-positive isolate was amplified in five identical, but independent, reaction tubes, as described above. After confirmation of the presence of the expected amplification products in all reaction tubes by agarose gel electrophoresis, the five individual PCR mixtures with one isolate's DNA were combined. These mixed PCR products were then ligated into the pCR-XL-TOPO vector by using the TOPO XL cloning kit (Invitrogen), and TOP10 *E. coli* was transformed with the ligated mixture according to the instructions of the manufacturer. Plasmid-containing transformants were then selected on tryptic soy agar plates containing kanamycin (50 μg/ml). The presence and the orientation of the expected *cpb2*-containing insert in plasmids isolated from these transformants were confirmed by restriction analysis with *Eco*RI or *Hin*dIII. Five clones carrying the *cpb2* insert of one *C. perfringens* isolate, placed in the same orientation in pCR-XL-TOPO, were then grown as separate overnight cultures in 3.0 ml of tryptic soy broth with kanamycin (50 μg/ml). These overnight cultures were combined, and plasmid DNA was isolated by using a Qiagen mini plasmid prep kit, according to the instructions of the manufacturer. The insert present in the plasmid DNA was then sequenced by the fluorescence-based automated DNA sequencing facilities available in the Center for Gene Research
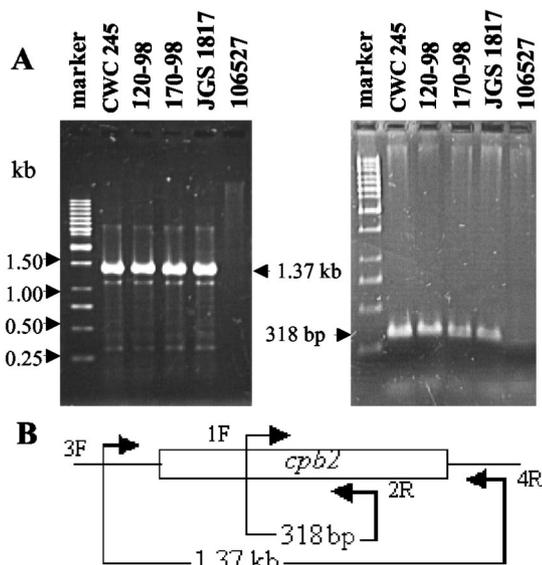
FIG. 1. PCR analysis of pig GI disease isolates. (A) Representative results of a PCR assay with primers designed to amplify 1,373- and 318-bp PCR products. The migration of the PCR products derived from each primer set is indicated between the two gels. Results are shown for control strains CWC245 (a known *cpb2*-positive *C. perfringens* type C strain) and 106527 (a *cpb2*-negative type C strain) and representative pig GI disease isolates 120-98, 170-98, and JGS1817. DNA size markers (GeneRuler 1-kb ladder; Fermentas) are shown in the left lane of each gel. (B) Schematic diagram showing the location of each primer in CWC245 DNA (10).

and Biotechnology at Oregon State University by using the M13 forward and reverse primers.

cpe-specific PCR. The *cpe*-specific PCR analysis was performed as described before (7, 26) with the primer set 5′-GGTACCTTTAGCCAATCA-3′ (primer 2F) and 5′-TCCATCACCTAAGGACTG-3′ (primer 5R).

Alpha toxin expression by *cpb2*-positive *C. perfringens* pig GI disease isolates. Alpha toxin expression by pig GI disease isolates was tested by the reverse CAMP test (12).

RESULTS

***cpb2*-specific PCR screening for presence of entire *cpb2* ORF sequences in *C. perfringens* isolates associated with GI diseases in piglets.** The isolates in our collection of pig GI disease isolates have previously been identified (9, 17) as *cpb2* positive by a multiplex PCR assay by monitoring the isolates for the presence of a 516-bp PCR product from the internal *cpb2* sequence. In our present study, we determined (Fig. 1) whether these isolates carry the entire *cpb2* ORF sequence by PCR analysis with two sets of primers designed from the CWC245 *cpb2* gene sequence published earlier (10).

To ensure the reliability of the PCR results obtained with the template DNAs isolated from our pig GI disease strains, control PCRs were run by using template DNAs isolated from CWC245 (a known *cpb2*-positive *C. perfringens* type C isolate) and 106527 (previously determined to be a *cpb2*-negative *C. perfringens* type C strain). As shown in Fig. 1, the expected 318-bp PCR product (which is internal to the *cpb2* ORF) and the expected 1,373-bp PCR product (which contains the whole *cpb2* ORF) were amplified from CWC245 DNA with primer pairs 1F-2R and 3F-4R, respectively. However, no such PCR-
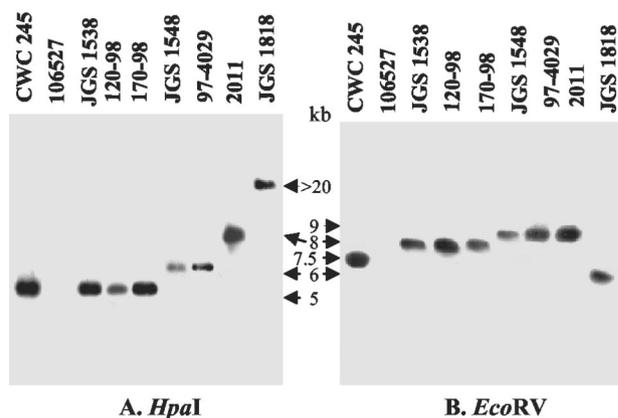

FIG. 2. RFLP and Southern blotting analyses of *Hpa*I- or *Eco*RV-digested DNA from pig GI disease isolates. Total DNA isolated from each of the specified *C. perfringens* strains was digested with *Hpa*I (A) or *Eco*RV (B) and then Southern transferred. The Southern blots were probed with a 318-bp, DIG-labeled *cpb2*-specific probe. Results are shown for control strains CWC245 (a known *cpb2*-positive type C isolate) and 106527 (a *cpb2*-negative type C strain) and representative *cpb2*-positive pig GI disease isolates JGS1538, 120-98, 170-98, JGS1548, 97-4029, 2011, and JGS1818. The migration of the hybridizing band derived from each strain is indicated between the two blots.

amplified products were obtained when DNA from *cpb2*-negative strain 106527 was used as the template. These results indicate that our PCR results are reliable and specific for the detection of isolates carrying the *cpb2* ORF sequence.

When template DNA isolated from each pig GI disease isolate was subjected to these same PCR analyses, both 318- and 1,373-bp products were obtained from the DNA of 28 of 29 isolates tested with primer pairs 1F-2R and 3F-4R, respectively (Table 1; see Fig. 1 for representative results). The 318-bp *cpb2* internal fragment but not the 1,373-bp fragment containing the whole *cpb2* ORF was amplified from the DNA of the remaining isolate, JGS1818. These results confirm that our pig GI disease isolates carry the *cpb2* ORF sequence.

Genotyping of *cpb2*-positive *C. perfringens* isolates by RFLP analysis. Next, our collection of *cpb2*-positive isolates was subjected to RFLP analysis. We first performed RFLP and Southern blotting analyses with a representative *cpb2*-positive *C. perfringens* isolate (isolate CWC245) and a representative *cpb2*-negative *C. perfringens* isolate (isolate 106527) using a series of restriction enzymes which have no cleavage site within the *cpb2* ORF. When RFLP analysis with *Xba*I, *Eco*RI, *Bam*HI, or *Bgl*II and Southern blotting assays were performed, a DIG-labeled *cpb2* probe always hybridized to a >20-kb fragment of DNA from *cpb2*-positive isolate CWC245 (data not shown). However, when RFLP analysis with *Hpa*I or *Eco*RV and Southern blotting assays were performed with that isolate (Fig. 2), the *cpb2* probe hybridized to an ~5-kb *Hpa*I DNA fragment and an ~7.5-kb *Eco*RV DNA fragment of CWC245, respectively. No hybridizing band was observed with DNA from *cpb2*-negative healthy pig isolate 106527, indicating that our *cpb2*-specific RFLP and Southern blotting analyses were reliable and specific.

When the same assay consisting of RFLP analysis with *Hpa*I and Southern blotting was applied to our collection of *cpb2*-positive pig GI disease isolates, the *cpb2*-specific probe hybrid-

ized to an ~5-kb *Hpa*I DNA fragment for 25 of 29 pig GI disease isolates (Table 1; see Fig. 2 for representative results). Four pig GI disease isolates (isolates JGS1548, JGS1818, 2011, and 97-4029) produced different hybridizing bands of >5 kb (Table 1 and Fig. 2). In order to confirm that these different results by RFLP analysis with *Hpa*I were not simply due to the loss of an *Hpa*I restriction site, further RFLP analyses were performed with *Eco*RV-digested DNA (Fig. 2). The results of RFLP analysis with *Eco*RV confirmed our results obtained by RFLP analysis with *Hpa*I, demonstrating that the patterns of these four isolates obtained by RFLP analysis with *Eco*RV are different from those of the other pig GI disease isolates surveyed (Fig. 2 and data not shown).

Finally, the specificity of the *cpb2*-specific probe used in the present RFLP studies has been confirmed by further control studies demonstrating (Table 1; see Fig. 2 for representative results) that this probe does not hybridize to *Hpa*I-digested DNA from *C. perfringens* healthy pig isolates (isolates 106527, JGS1537, JGS1540, JGS1569, JGS1580 and D9703881) that are genotyped as *cpb2*-specific PCR negative (Table 1).

PFGE evidence supporting the plasmid localization of *cpb2* in isolates from pigs with GI diseases. To determine whether the *cpb2* gene is located on the chromosome or plasmid of *cpb2*-positive pig GI disease isolates, representative *cpb2*-positive *C. perfringens* isolates were subjected to PFGE and Southern blotting analyses, which have successfully been used (2, 3, 6, 27, 30) to formally establish the chromosomal or plasmid localization of the *cpe* gene. Briefly, the principle of this method is that, without any restriction enzyme digestion, unsheared *C. perfringens* chromosomal DNA is too large to enter a pulsed-field gel. However, because of its smaller size, at least some plasmid DNA should enter a pulsed-field gel, even without any restriction enzyme digestion. Therefore, when total DNA from *cpb2*-positive isolates is subjected to this PFGE analysis and then Southern blotted for *cpb2*, either of two specific profiles should be observed: (i) if the *cpb2* gene is located on the chromosome, all *cpb2*-containing DNA should remain in the gel wells and no hybridizing band should be observed in the gel; or (ii) if the *cpb2* gene is located on the plasmid, some *cpb2*-containing plasmid DNA should enter the pulsed-field gels and should produce a hybridizing band in the blot.

When PFGE genotyping experiments were performed with the DNA of control *cpb2*-positive type C isolate CWC245, *cpb2*-containing DNA entered the pulsed-field gel in the absence of restriction enzyme digestion and produced a hybridizing band of ~50 kb (Fig. 3). These PFGE results confirmed previous observations that the *cpb2* gene is located on the plasmid of *cpb2*-positive isolate CWC245 (10). The apparent size of the CWC245 plasmid carrying *cpb2* matches the calculated size of the plasmid from type A strain 13 whose *cpb2* gene was sequenced (28).

When similar PFGE genotyping analyses were performed with the DNA of representative *cpb2*-positive isolates, their *cpb2*-containing plasmid DNA entered the pulsed-field gels and produced hybridizing bands that comigrated with the *cpb2*-containing plasmid DNA from CWC245 (Table 1; see Fig. 3 for representative results). These results indicate that both type A and C *cpb2*-positive *C. perfringens* pig GI disease isolates carry the *cpb2* gene on a plasmid.
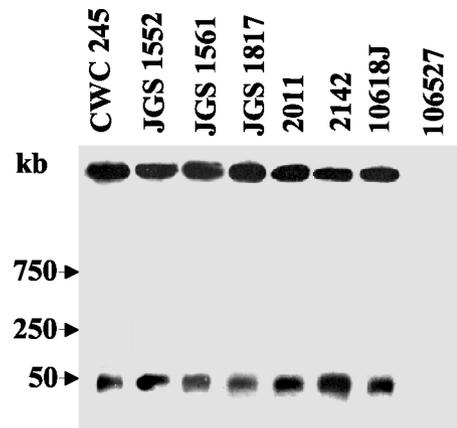

FIG. 3. PFGE evidence supporting the plasmid localization of *cpb2* in pig GI disease isolates. PFGE and Southern hybridization were used to analyze undigested DNA, prepared in agarose plugs, from each of the *C. perfringens* isolates specified. The blots were probed with a 318-bp *cpb2*-specific probe. Results are shown for control strains CWC245 (a *cpb2*-positive type C isolate carrying the *cpb2* gene on a large plasmid) and 106527 (a cpb2-negative type C isolate) and representative *cpb2* pig isolates JGS1552, JGS1561, JGS1817, 2011, 2142, and 10618J. The pulsed-field gel was calibrated with bacteriophage lambda DNA markers, whose migration is shown at the left of the blot.

PFGE analysis for clonal relationship between *cpb2*-positive isolates. The final series of experiments conducted for the genotyping of *cpb2*-positive isolates involved the use of PFGE analyses to examine the possibility that *cpb2*-positive pig isolates share a common clonal relationship with each other. PFGE analyses of *Sma*I-digested DNA identified no clonal relationship between the 17 pig GI disease isolates tested (see Fig. 4 for representative results). In order to confirm that the results of the *Sma*I digestions were not due to uneven migra-
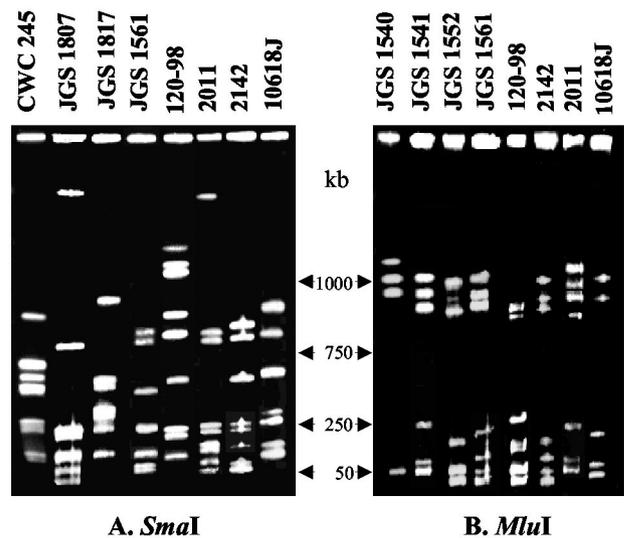

FIG. 4. Analysis of clonal relationships among *C. perfringens* pig GI disease isolates. DNA from each of the specified *C. perfringens* isolates in agarose plugs was digested with *Sma*I (A) or *Mlu*I (B) and subjected to PFGE and ethidium bromide straining. The gel was calibrated with bacteriophage lambda ladder DNA. The molecular sizes of the DNA markers are between the two gels.
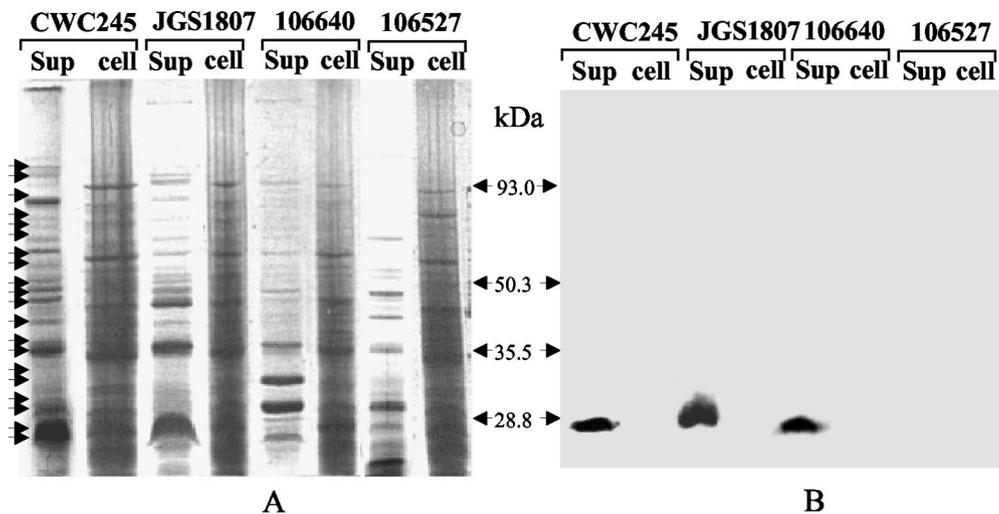
FIG. 5. Western blotting analysis of CPB2 expression by selected pig GI disease isolates. (A) Culture supernatant proteins (sup) or total cell (cell) proteins, prepared from each of the *C. perfringens* isolates specified, were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. The migrations of the supernatant proteins are shown by arrows. (B) Western blot of the gel shown in panel A. The blot was probed with CPB2 antibodies and developed by chemiluminescence detection to identify immunoreactive species. Results are shown for control strains CWC245 (a *cpb2*-positive type C strain) and 106527 (a *cpb2*-negative type C strain) and representative pig GI disease isolates JGS1807 and 106640. Molecular mass markers (in kilodaltons) are shown between the gels.

tion throughout the gel, further PFGE analyses were performed with *Mlu*I-digested gel blocks. The results of PFGE with *Mlu*I-digested DNA confirmed the lack of a clonal relationship between 17 pig GI disease isolates (see Fig. 4 for representative results).

CPB2 expression by *cpb2*-positive isolates. The phenotypic characteristic of the *cpb2*-positive *C. perfringens* pig GI disease isolates examined in this study was whether these isolates can, in fact, express CPB2. Because *cpb2* sequencing information (10) supports the fact that CPB2 is a secreted protein, we first analyzed culture supernatant proteins from *cpb2*-positive *C. perfringens* isolates. *C. perfringens* culture supernatants (Fig. 5) contained more than 15 protein bands in SDS-polyacrylamide gels stained with Coomassie blue. When CPB2-specific Western blotting was performed with supernatant proteins from *C. perfringens* CWC245 (Fig. 5), an ~28-kDa immunoreactive band was obtained, which is consistent with the results presented previously (10). However, no CPB2 expression could be detected in the culture supernatant of *cpb2* PCR-negative isolate 106527. These results indicate that our CPB2-specific Western blotting analyses are specific and reliable.

When similar CPB2-specific Western blotting analyses were performed with culture supernatant proteins prepared from our 29 unknown *cpb2*-positive pig GI disease isolates, an ~28-kDa immunoreactive band that comigrated with the 28-kDa band of CWC245 was observed (Table 1; see Fig. 5 for representative results). These results indicate that all of our pig GI disease isolates surveyed express CPB2.

To investigate whether CPB2 is truly a secreted protein and not just a lysed cell product, we compared the results of CPB2-specific Western blotting with culture supernatant proteins and CPB2-specific Western blotting with total cell proteins (Fig. 5) for the detection of CPB. As expected, an ~28-kDa immunoreactive band was observed with the culture supernatant proteins but not with the total cell proteins of CWC245 (Fig. 5).

When similar Western blotting analyses were performed with pig GI disease isolate JGS1807 or 106640, an immunoreactive band that comigrated with the ~28-kDa immunoreactive band of CWC245 was observed in the assays with the culture supernatant proteins but not in the assays with the total cell proteins (Fig. 5). These results support the fact that the CPB2 detected in the culture supernatant proteins is most likely an excreted product and not a product of cell lysis.

Comparison of *cpb2* ORFs present in different *cpb2*-positive isolates. To examine whether the *cpb2* ORF of pig GI disease isolates encodes a CPB2 protein with an amino acid sequence similar to that of the classical CPB2 protein of pig type C isolate CWC245 (10), the nucleotide sequences of both strands of a 1,373-bp PCR-amplified DNA insert (as shown in Fig. 1) carrying the *cpb2* ORFs from five different *cpb2*-positive pig GI disease isolates (three type A isolates and two type C isolates) were determined. These analyses revealed (data not shown) that the *cpb2* ORF sequences present in isolates JGS1817, 120-98, and 170-98 (three U.S. type A isolates) and isolate 97-4029 (a U.S. type C isolate) are identical to those present in isolate 106640 (a French type C isolate). Furthermore, the *cpb2* ORF sequences present in five of these isolates exactly match the *cpb2* ORF sequence from CWC245 (a type C strain isolated from a diseased pig in France) determined previously (10). Comparison of the *cpb2* upstream and downstream sequences present in all five isolates revealed that both the upstream and the downstream sequences are highly conserved in all five isolates and match the *cpb2* upstream and downstream sequences of CWC245 determined previously (10).

***cpe*-specific PCR screening for presence of *cpe* gene in *cpb2*-positive isolates.** The *cpb2*-positive *C. perfringens* pig GI disease isolates used in this study had been identified earlier as *cpe* negative by multiplex PCR analysis (9). In this study, we reevaluated whether the *cpb2*-positive *C. perfringens* isolates that we surveyed carry the *cpe* gene by a single *cpe*-specific

PCR analysis with primers 2F and 5R (7, 25) (note that primers 2F and 5R are different from the primers used in the multiplex PCR). These PCR analyses confirmed (data not shown) that none of the isolates examined in this study, regardless of their source, carry the *cpe* gene; i.e., all type A isolates examined in this survey were classified as *cpe*-negative type A *C. perfringens* isolates. The reliability of the *cpe*-specific PCR assay for the detection of the genes encoding CPE, if they are present, was demonstrated by blinded control studies with reference *C. perfringens* type A isolates that were *cpe* negative (isolate ACTC 3624) (18) and *cpe* positive (isolate NCTC 8239) (18) (data not shown).

Alpha toxin expression by *cpb2*-positive pig GI disease isolates. The *cpb2*-positive *C. perfringens* pig GI disease isolates used in this study were characterized for their alpha toxin-producing capabilities. By using the reverse CAMP test (12), all *cpb2*-positive type A or type C isolates surveyed in this study produced (data not shown) the arrow-shaped zone of synergistic hemolysis indicative of alpha toxin expression.

DISCUSSION

The present study offers several significant contributions to our understanding of the pathogenesis of recently discovered CPB2-positive *C. perfringens* isolates. First, the results of PCR and RFLP analysis presented in this study for 6 isolates from healthy pigs and 29 isolates from pigs with GI diseases previously genotyped by multiplex PCR, which reconfirmed the presence of *cpb2* only in the diseased pig isolates and not in the healthy pig isolates, significantly strengthen the hypothesis (9, 10, 17) that *cpb2*-positive isolates are highly associated with GI diseases in pigs.

Second, the present study reports the first PFGE genotypic characterization of *cpb2*-positive isolates. Our PFGE and Southern blotting results for 14 of the *cpb2*-positive type A pig GI disease isolates indicates that 14 of these type A isolates carry their *cpb2* gene on a large plasmid. When these results are coupled with similar results for type C isolates obtained in this study and a previous study (10), it is now clear that the *cpb2* gene has a plasmid location in *C. perfringens* pig GI disease isolates, regardless of their toxinotypes or geographical origins. Furthermore, the PFGE and RFLP analyses performed in this study demonstrated no clonal relationship among the pig GI disease isolates surveyed. Collectively, these PFGE results suggest that the acquisition of the putative *cpb2*-containing plasmid may be a critical step for *C. perfringens* isolates to become enteropathogenic for animals. Further studies are needed to determine (i) whether this *cpb2*-containing plasmid, like the *cpe*-containing plasmid (2), can be mobilized between *C. perfringens* isolates and (ii) whether virulence genes other than *cpb2* might be encoded on the *cpb2*-containing plasmid.

Third, the present study's most significant finding is the presentation of the first evidence that CPB2 is expressed by many or all *cpb2*-positive type A isolates originating from pigs with CPB2-associated GI diseases. Our CPB2-specific Western blotting results provide direct evidence that the CPB2 produced by *cpb2*-positive type A isolates from pigs with GI diseases is a secreted protein, as it is for type C isolate CWC245 (10). Furthermore, the results of the present study also show

that type A isolates secrete a CPB2 protein of the same size as that from type C isolates and that the sequences of the CPB2 proteins from the two types are identical (10; this study). Collectively, these findings offer important evidence supporting the virulence potential of type A isolates and suggest that the *cpb2* present in many, if not all, type A isolates is regulated similarly to that present in type C isolates.

The similarity between the CPB2 protein produced by the isolates surveyed and the CPB2 protein produced by original *cpb2*-positive strain CWC245 (10) receives further support from the nucleotide sequencing data obtained in this study, which reveal that the *cpb2* ORF sequences present in five different isolates (originating from different geographic sources) are identical to each other and to a previously determined (10) *cpb2* ORF sequence from CWC245. These sequencing results also provide the strongest evidence to date that the *cpb2* ORF is highly conserved, if not identical, in most or all *cpb2*-positive pig GI disease isolates.

The involvement of CPB2-positive *C. perfringens* type C isolates in GI diseases in piglets raises an important, but still unanswered, question about the pathogenesis of CPB2-associated GI diseases in animals; i.e., is CPB2 responsible for most or all GI symptoms of CPB2-associated GI diseases, or do these symptoms also result from the expression of another *C. perfringens* toxin(s)? All of the type A pig GI disease isolates surveyed in this study were classified as *cpe*-negative type A isolates, arguing that the expression of CPE or the beta, iota, or epsilon toxin is not required for a CPB2-producing type A isolate to cause GI diseases in piglets. Since the present study confirms that *cpb2*-positive type A porcine disease isolates can produce both CPB2 and alpha toxin, it is possible that the enteropathogenicities of these CPB2-producing type A isolates are augmented by either alpha toxin (particularly since alpha toxin has been implicated in some veterinary enteric illnesses [11]) or their ability to produce one or more of the minor *C. perfringens* toxins (21). Further gene knockout studies should address the relative contributions of CPB2 versus those of other toxins in CPB2-associated GI diseases in animals.

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