

Atypical *cpb2* Genes, Encoding Beta2-Toxin in *Clostridium perfringens* Isolates of Nonporcine Origin

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Received 25 August 2004/Accepted 3 September 2004

Beta2-toxin, encoded by *cpb2*, is implicated in the pathogenesis of *Clostridium perfringens* enteritis. However, *cpb2* genes from nonporcine *C. perfringens* isolates were not always expressed, at least in vitro. Nucleotide sequencing identified atypical *cpb2* genes with 70.2 to 70.7% DNA identity to previously identified (consensus) *cpb2*. Atypical beta2-toxin displayed 62.3% identity and 80.4% similarity to consensus beta2-toxin. No porcine type C isolates ($n = 16$) and only 3.3% of porcine type A isolates ($n = 60$) carried atypical *cpb2* genes. However, 88.5% of nonporcine isolates carried atypical *cpb2* ($n = 78$), but beta2-toxin was not expressed. Almost half of the nonporcine consensus *cpb2* genes (44.4%) carried a frameshift mutation ($n = 9$), resulting in an absence of beta2-toxin expression. These findings strengthen the role of beta2-toxin in the pathogenesis of enteritis in neonatal pigs. However, the identification of apparently nonexpressed, atypical *cpb2* genes raises the question of whether this protein plays the same role in enteritis in other animal species.

In addition to gas gangrene and food poisoning in humans, *Clostridium perfringens* is a cause of economically important disease in livestock, such as cattle and swine (reviewed in reference 11). *C. perfringens* is classified into five types (A to E) on the basis of differential production of the four toxins, alpha-toxin, beta-toxin, epsilon-toxin, and iota-toxin. While the roles of beta-, iota-, and epsilon-toxin in the pathogenesis of enteritis are well documented (11), the roles of other toxins, such as alpha-toxin and beta2-toxin, in disease pathogenesis are still equivocal.

cpb2 encodes beta2-toxin, which was toxic to cultured epithelial cells and lethal to mice when it was administered intravenously (4). *cpb2*-positive *C. perfringens* strains are associated with the occurrence of enteric disease in domestic animals, notably pigs (2, 3, 6, 13), horses (1, 5), and dogs (12). There was an especially strong correlation between the prevalence of *cpb2* in isolates from piglets with enteritis and the absence of *cpb2* in isolates from healthy piglets (2, 3). However, apart from this epidemiologic association, there is little experimental evidence to support the role of beta2-toxin in pathogenesis. Immunohistochemistry identified beta2-toxin in 35 to 48% of small and large intestines of horses with typhlocolitis caused by *C. perfringens* (1). Furthermore, Manteca et al. demonstrated that a *cpb2*-positive isolate of *C. perfringens* produced more pronounced necrotic intestinal lesions than an isolate lacking *cpb2*. However, this study did not use isogenic strains, and factors other than beta2-toxin may have contributed to the severity of the lesions (8).

Recently, we determined that beta2-toxin may be differentially expressed in *C. perfringens* strains isolated from different host species. There was a strong correlation between beta2-toxin phenotype and genotype only in type A and C isolates obtained from diseased pigs (2). For isolates of nonporcine

origin, the correlation of phenotype and genotype was only 50% (2). This study aims to determine the basis for the absence of beta2-toxin expression.

The majority of the *C. perfringens* isolates used in this study were received through the Clostridial Enteric Disease Unit (CEDU), University of Arizona, and were from clinical cases where *C. perfringens* disease was suspected. All isolates were typed by a multiplex PCR assay which amplifies *cpa*, *cpb*, *cpb2*, *cpe*, *etx*, and *ibp* genes (3).

PCR product, amplified with primers CPB2F and CPB2R (Table 1) from a bovine type E isolate, *C. perfringens* NCIB 10784, was sequenced and found to diverge significantly from *cpb2* from a porcine type C isolate (GenBank accession number L77965). Due to the sequence divergence, primers designed to the sequence surrounding the previously described *cpb2* gene (GenBank accession number L77965), which we will refer to as the consensus *cpb2* gene, did not amplify this gene region from strain NCIB 10784. Therefore, primers upstream and downstream of the atypical *cpb2* gene were designed from preliminary nucleotide sequence from a bovine type E isolate, *C. perfringens* 853, provided by The Institute for Genomic Research, Rockville, Md. The nucleotide sequence of the entire NCIB 10784 *cpb2* gene was determined from PCR products amplified with primers EEBF2 and CPB2R and primers CPB2F and EEB2R (Table 1). The *cpb2* gene from strain NCIB 10784 displayed only 70.7% DNA identity with the consensus *cpb2* gene. Furthermore, deletion of a base at position 178 in the atypical *cpb2* gene resulted in a frameshift such that a protein of only 73 amino acids could be produced, explaining the absence of beta2-toxin expression in this isolate.

A triplex PCR was developed using consensus and atypical *cpb2*-specific forward primers (CPB2CONF and CPB2ATYPF, respectively) with a common reverse primer, CPB2R (Table 1). PCR was performed by using *Taq* DNA polymerase (Promega) and the supplied reaction buffer under the following conditions: (i) a 5-min hot start at 94°C; (ii) 35 cycles, with 1 cycle consisting of 1 min at 94°C, 1 min at 50°C, and 1 min/kb at 72°C; and (iii) a final extension step of 72°C for 5 min. PCR amplification of

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TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence (5'→3') ^a	Positions on <i>cpb2</i> (bp)
CPB2ATYPF ^b	ATTATGTTTAGGAATACAGTTA	51–72
CPB2CONF ^c	CAATTGGGGGAGTTTATCCACAA	488–510
CPB2SEQF ^c	GTAAAAATTTGATATAATTGAATTG	52–28 upstream
CPB2F	AGATTTTAAATATGATCCTAACC	225–247
CPB2R	CAATACCCTTCACCAAATACTC	770–791
EEB2F ^b	AACATAATAAATCCTATAACC	256–235 upstream
EEB2R ^b	ATAAATATAATTCTCTAAAACC	166–187 downstream
HISBETA2R ^c	GTCACCTCAGAATTCCTTCTATGCAC	791–18 downstream
HISATYPB2F ^b	CCTAATACAGTGGATCCAAATGAAGTG	73–99
HISATYPB2R ^b	TATAAATAAATAGAATTCCTCTAAAACC	166–192 downstream

^a Restriction endonuclease sites introduced for cloning (underlined) are indicated.

^b This primer does not bind to the consensus *cpb2* gene region. The position on the atypical *cpb2* gene region is shown.

^c This primer does not bind to the atypical *cpb2* gene region. The position on the consensus *cpb2* gene region is shown.

consensus and atypical *cpb2* genes produced 304- and 741-bp products, respectively (data not shown). This PCR assay was used to screen 154 *cpb2*-positive isolates, including all five *C. perfringens* types, from a variety of host sources. No porcine type C isolates (*n* = 16) and only 3.3% of porcine type A isolates (*n* = 60) carried the atypical *cpb2* gene; only 2.6% of all porcine isolates carried the atypical *cpb2* gene (Table 2). However, the predominant allele carried by nonporcine isolates was the atypical *cpb2* gene, which was present in 88.5% of these isolates (*n* = 78) (Table 2). All type B, nonporcine type C, type D, and type E isolates carried the atypical *cpb2* gene, regardless of host origin (Table 2). Most nonporcine type A isolates also carried the atypical *cpb2* gene (66.6 to 100%) (Table 2). A total of 55 *cpb2*-negative isolates (determined by a multiplex genotyping PCR [3]) were also examined. As expected, amplicons were not detected in these *cpb2*-negative isolates using the triplex PCR assay (data not shown).

A total of 23 *C. perfringens* isolates carrying the atypical *cpb2* gene were selected for nucleotide sequence analysis: 10 type A isolates (2 avian isolates, 6 bovine isolates, 1 canine isolate, and 1 equine isolate), 1 type B isolate (unknown origin), 2 type C isolates (bovine), 4 type D isolates (2 caprine isolates and 2 ovine isolates), and 6 type E (bovine) isolates (including NCIB 10784). Nucleotide sequence data from these isolates were

obtained as for the *cpb2* gene from *C. perfringens* strain NCIB 10784, and the data were compared with data for a consensus *cpb2* gene (GenBank accession number L77965).

Compared individually to the consensus *cpb2* gene, the atypical *cpb2* genes displayed 70.2 to 70.7% DNA identity. When the atypical *cpb2* genes were compared as a group, all the atypical genes displayed 93.0% DNA identity. Two bovine type A isolates carried identical 13-bp deletions at positions 73 to 85, and one avian type A isolate carried a mutation at position 606 resulting in premature termination of translation. The remaining type A, B, and C atypical *cpb2* genes displayed 99.0% DNA identity with each other and could potentially produce full-length atypical beta2-toxin protein. Five of the six type E *cpb2* genes, including the one from strain NCIB 10784, were identical and carried the frameshift mutation at position 178. In the other type E isolate, 853, the sequence was identical to that of nonporcine type A and C isolates. The type D genes were identical and most similar to type E *cpb2*, although the *cpb2* genes from type D isolates did not carry the frameshift mutations.

Nucleotide sequence upstream of the consensus *cpb2* gene or the atypical *cpb2* genes was similar, with 79.4% DNA identity over the 97 bp immediately upstream of *cpb2*. However, the consensus and atypical *cpb2* sequences were significantly more divergent 5' of this point. Primer extension analysis had identified a σ^{70} -type promoter (TTTTAA-N₁₇-TATAAT), which is present 35 bp upstream of the start of the consensus *cpb2* gene in *C. perfringens* strain 13 (9), and the identical sequence is present in the consensus *cpb2* gene from *C. perfringens* strain CWC245 (GenBank accession number L777965). Similar, but not identical, sequences are present upstream of atypical *cpb2* genes from type A, type B, nonporcine type C and type D (TTTTAAA-N₁₇-TA[T/A]AAT), and type E (TTAAAA-N₁₇-TATAAT) isolates.

When compared with sequences in GenBank, atypical *cpb2* displayed similarity with only consensus *cpb2* at either the nucleotide or amino acid level (data not shown). Most of the nucleotide changes occurring between atypical *cpb2* genes from type A, B, and C isolates conferred either no change or conservative substitutions. Figure 1 shows an amino acid alignment between a consensus beta2-toxin protein, and representatives of types A, B, and C, type D and type E atypical beta2-toxin sequences. Atypical beta2-toxin proteins were 96.2 to 98.9% identical and 97.7 to 99.2% similar to each other,

TABLE 2. Prevalence of atypical and consensus *cpb2* genes in *cpb2*-positive *C. perfringens* types isolated from various animal species

Isolate source	No. of isolates containing atypical <i>cpb2</i> genes/no. of isolates tested by type:				
	A	B	C	D	E
Avian	13/14				
Bovine	7/10		2/2		12/12
Canine	5/7				
Caprine				2/2	
Cervine	2/2				
Equine	9/10				
Feline	2/2				
Human	8/8				
Ovine	4/6			2/2	
Porcine	2/60		0/16		
Unknown		1/1			
Total no.	52/119	1/1	2/18	4/4	12/12

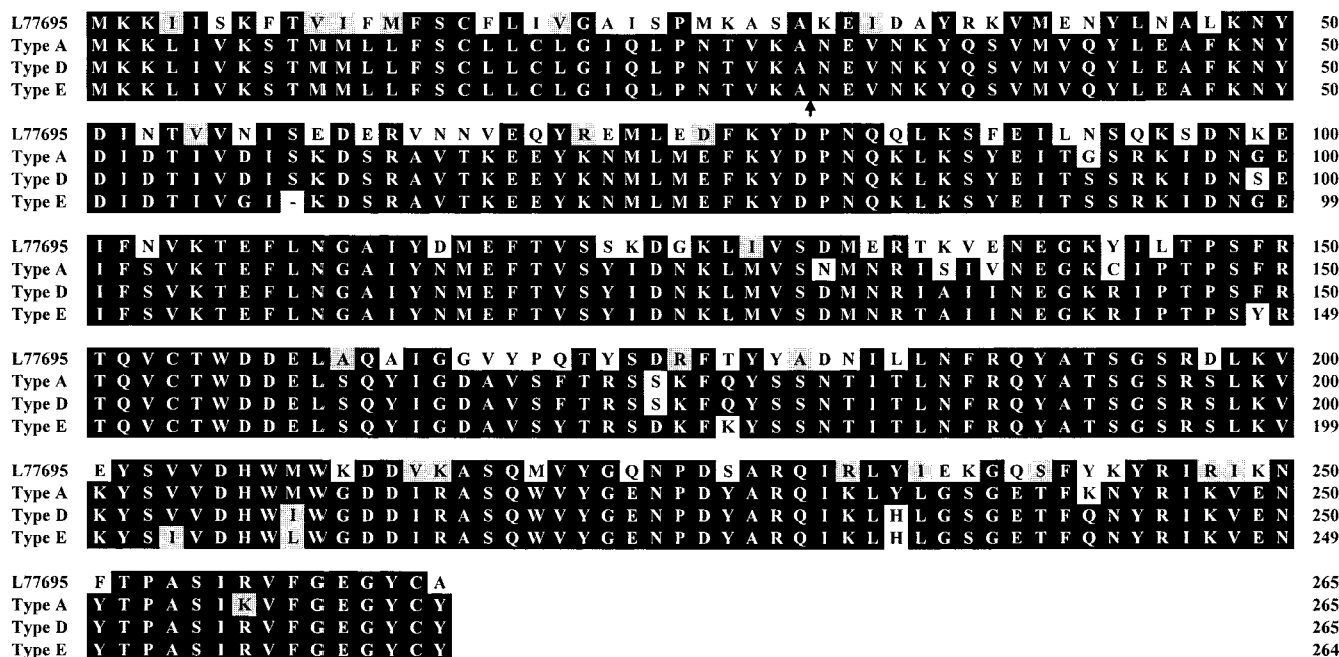


FIG. 1. Amino acid alignment of *C. perfringens* beta2-toxin proteins expressed from consensus (GenBank accession number L77695) or atypical *cpb2* genes. The type A sequence shown is the sequence of *C. perfringens* JGS4147 (bovine type A) and is representative of atypical type A, B, and C beta2-toxin sequences. The type D and E sequences are the sequences from *C. perfringens* JGS4152 (ovine type D) and NCIB 10784, respectively. The position of the frameshift mutation is indicated by the dash at position 59 in the type E sequence, and the remaining amino acid sequence shown is that which would occur in the absence of the mutation. Two or more identical amino acids in the sequences are shown on a black background, while conservative substitutions are shown on a gray or shaded background. The predicted signal sequence cleavage sites are indicated by the arrow. Amino acid numbers for each protein are indicated to the right of the sequences.

whereas atypical beta2-toxin proteins displayed only 62.3% identity and 80.4% similarity to the previously described beta2-toxin protein (4).

To ensure that antibodies raised against consensus beta2-toxin would recognize the atypical protein, a strain expressing recombinant atypical beta2-toxin was constructed. The atypical *cpb2* gene, lacking the coding region for the signal sequence, was amplified as a 917-bp product from *C. perfringens* strain JGS4142 (bovine type A) by PCR with a 5' primer containing a BamHI site (primer HISATYPB2F) and a 3' primer containing an EcoRI site (primer HISATYPB2R) (Table 1). The PCR product was digested with BamHI-EcoRI and cloned into pTrcHis B (Invitrogen), generating pJGS659. pJGS659 encoded His-atypical beta2-toxin, a 268-amino-acid protein comprising 235 amino acids of the mature atypical beta2-toxin with an N-terminal extension of 33 amino acids encoded by pTrcHis B. DNA sequencing of the insert portion of pJGS659 indicated that no mutations had been introduced during PCR.

HIS-atypical beta2-toxin was purified from *Escherichia coli* DH5α(pJGS659) to >95% homogeneity using TALON resin (2). In Western blots, monoclonal antibody (MAb) 9E10B, raised against purified HIS-beta2-toxin (2), reacted with preparations of purified HIS-atypical beta2-toxin (data not shown).

C. perfringens isolates were grown in anaerobic brain heart infusion broth (Difco) supplemented with 0.5% yeast extract and 0.05% cysteine at 37°C for 48 h. The culture supernatant fluid (CSF) was harvested by centrifugation and passage through a 0.45-μm-pore-size filter. The CSF was concentrated 40-fold by

ultrafiltration through a 10,000-molecular-weight cutoff filter (Amicon), and beta2-toxin was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with MAb 9E10B. A positive-control lane loaded with purified HIS-atypical beta2-toxin was always included, and a representative blot is shown in Fig. 2. Thirty-three isolates carrying atypical *cpb2* genes were selected from all five *C. perfringens* types and a variety of host sources and tested for beta2-toxin expression by Western blotting. As expected, beta2-toxin expression was not detected in any of the type A and E isolates that carry frameshift mutations (Table 3 and Fig. 2). However, beta2-toxin was also not detected in the 25 isolates that carried full-length atypical *cpb2* genes. Beta2-toxin expression was not detected in control isolates of *C. perfringens* that did not carry *cpb2* (*n* = 3) (Fig. 2).

Consensus *cpb2* genes were not common in nonporcine isolates, with only 11.5% of nonporcine isolates carrying the consensus *cpb2* gene (*n* = 78) (Table 2). Interestingly, only five of the nine nonporcine isolates carrying consensus *cpb2* genes expressed beta2-toxin protein (Table 3 and Fig. 2). The nucleotide sequences of these nine consensus *cpb2* genes were determined from PCR products amplified with primers CPB2SEQF and CPB2R and primers CPB2F and HISBETA2R (Table 1). Identical frameshift mutations resulting from the insertion of an additional A within a poly(A) tract from positions 4 to 10 were identified in the *cpb2* genes from type A isolates of bovine, canine, and ovine origin, including strain 13 (7). This frameshift mutation could result in translation of a truncated protein of 4.5 kDa,

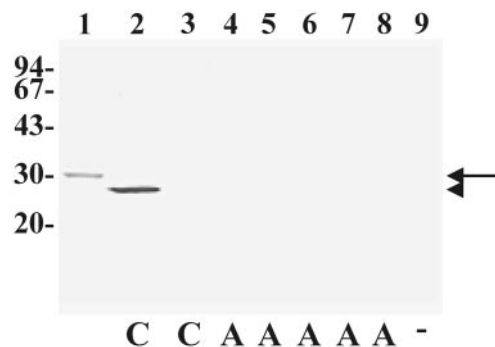


FIG. 2. Expression of beta2-toxin or atypical beta2-toxins in concentrated CSF from *C. perfringens* isolates. One microgram of HIS-atypical beta2-toxin (lane 1) or concentrated CSF from *C. perfringens* strains (lanes 2 to 9) was subjected to electrophoresis on SDS-10% polyacrylamide gels. CSF from *C. perfringens* strain 690D (porcine type A) (lane 2), strain 13 (lane 3), JGS4142 (lane 4), JGS1604 (canine type A) (lane 5), JGS1984 (unknown type origin B) (lane 6), JGS1880 (lane 7), JGS4152 (lane 8), and *cpb2*-negative, porcine type A (negative control) (lane 9) were used. The separated proteins were transferred to nitrocellulose by Western blotting and immunostained with a 1/20 dilution of MAb 9E10B. The positions of molecular mass standards (in kilodaltons) are shown to the left of the gel. The presence of consensus (C) or atypical (A) *cpb2* genes or the absence of *cpb2* (-) in that strain is indicated below the gel. The positions of HIS-atypical beta2-toxins (arrow) and beta2-toxins (arrowhead) are indicated to the right of the gel.

explaining the absence of beta2-toxin expression in these strains. Interestingly, the published sequence of *cpb2* from strain 13 does not contain this frameshift mutation (10), but this mutation was identified in the *cpb2* gene from strain 13 obtained from a different source (Bruce A. McClane, personal communication). The other five *cpb2* genes from nonporcine hosts did not contain the frameshift mutation, and beta2-toxin expression was detected in each of these isolates (Table 3).

With one exception, strain JGS1475, all strains of porcine origin carrying *cpb2* expressed beta2-toxin ($n = 4$) (Table 3 and

TABLE 3. Correlation of the presence of the *cpb2* allele with beta2-toxin expression in *C. perfringens* types isolated from various animal species

Isolate source	Type	No. of isolates positive for beta2-toxin expression/no. of isolates tested	
		Atypical <i>cpb2</i>	Consensus <i>cpb2</i>
Avian	A	0/4	1/1
Bovine	A	0/6	2/3
	C	0/2	
	E	0/12	
Canine	A	0/1	0/2
Caprine	D	0/2	
Equine	A	0/2	1/1
Ovine	A		1/2
	D	0/2	
Porcine	A	0/1	1/1
	C		2/3
Unknown	B	0/1	
Total no.		0/33	8/13

Fig. 2). In addition, we previously demonstrated that 96.9% of consensus *cpb2*-carrying porcine isolates expressed beta2-toxin protein ($n = 32$) (2). The nucleotide sequence of *cpb2* from strain JGS1475 was determined, but it was identical to the *cpb2* sequence from strain CWC245 (GenBank accession number L777965) (data not shown), so it is not known why this isolate does not express beta2-toxin. Regardless, it is clear that in general, consensus genes from porcine isolates are expressed, yet atypical genes from nonporcine *C. perfringens* isolates are not expressed. Furthermore, consensus genes from nonporcine *C. perfringens* isolates are expressed at different levels.

Given that the sequences upstream of consensus and atypical *cpb2* genes were similar, but not identical, the defect in expression of atypical genes could occur at the transcriptional level. Reverse transcription-PCR (RT-PCR) was used to identify *cpb2* transcripts in *C. perfringens* isolates carrying atypical genes. Tri Reagent (Medical Research Center) was used to extract total RNA from mid-log-phase cultures, as transcript from a consensus *cpb2* gene is abundant at this phase of the cell cycle (9). RNA was reverse transcribed into total cDNA using the Advantage RT-for-PCR kit (Clontech) and was used as a template in the multiplex PCR (3) to identify the presence of any *cpb2* transcript. *cpa* transcript was detected in all *C. perfringens* isolates tested and served as an internal positive control. Transcripts for the *cpb*, *cpe*, *etx*, and *ibp* genes, encoding other toxins, were also detected in the multiplex PCR assay, depending on the *C. perfringens* type. Reaction mixtures with no Moloney murine leukemia virus (MMLV) reverse transcriptase added were used as negative controls to determine whether contaminating bacterial DNA was present. RNA was prepared from *C. perfringens* strains JGS1880 (bovine type C), JGS1906 (bovine type A), JGS4142, NCIB 10784, and 13 and subjected to RT-PCR.

RNA from all *C. perfringens* isolates contained approximately equivalent amounts of *cpa* transcript (Fig. 3). As reported previously, strain 13 RNA contained *cpb2*-specific transcript (9). By PCR, strains JGS1880, JGS1906, and JGS4142 did not contain *cpb2*-specific product, indicating that very little or no transcription occurred in these isolates carrying atypical *cpb2* genes (Fig. 3). The absence of atypical *cpb2* gene expression in these strains probably occurs at the transcriptional level, at least in broth culture. Unexpectedly, however, RNA from strain NCIB 10784 contained *cpb2*-specific transcript (Fig. 3), but expression of beta2-toxin was not detected in this isolate due to a frameshift mutation in the *cpb2* gene. The putative -35 promoter sequence in type E atypical *cpb2* genes varies slightly from the -35 promoter sequence found in type A, B, C, and D atypical genes, and these -35 promoter sequences are slightly different from those found for consensus genes (TTAAAA, TTTAAA, and TTTTAA, respectively). The absence of transcription in the type A and C isolates examined may be a result of this divergent -35 sequence, whereas the type E *cpb2* -35 sequence may still be able to promote transcription. However, these studies were performed with bacteria grown in vitro, and there may be signals involved in up-regulation of atypical *cpb2* gene expression that are present only in the host, or possibly the type A and C isolates may lack any required activators of *cpb2* transcription. Further experiments will be necessary to confirm these hypotheses.

Consensus *cpb2* genes are found predominantly in *C. per-*

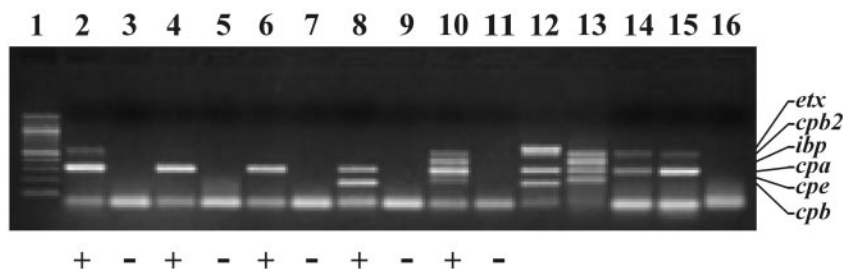


FIG. 3. Transcriptional analysis of atypical *cpb2* genes. Total cDNA, prepared by reverse transcription from RNA, was subjected to multiplex PCR analysis (3), and the amplicons were visualized after electrophoresis in a 1.5% agarose gel. Lanes: 1, 100-bp ladder; 2 and 3, strain 13 cDNA; 4 and 5, JGS1906 cDNA; 6 and 7, JGS4142 cDNA; 8 and 9, JGS1880 cDNA; 10 and 11, NCIB 10784 cDNA; 12, JGS1984 DNA; 13, strain 294 DNA (bovine type E); 14, strain 13 DNA; 15, JGS4142 DNA; 16, no-template control. MMLV reverse transcriptase was added (+) or not added (-) to the reaction mixtures in lanes 2 to 11. The positions of the 655-bp *etx*, 567-bp *cpb2*, 446-bp *ibp*, 324-bp *cpa*, 233-bp *cpe* and 196-bp *cpb* gene products are indicated to the right of the gel.

fringens strains isolated from porcine hosts. With only a few exceptions, these genes are expressed and beta2-toxin protein can be detected in culture supernatant by Western blotting. However, atypical *cpb2* genes predominate in *C. perfringens* strains isolated from nonporcine species. These genes were either not transcribed or full-length protein was not translated in bacteria grown in broth culture, although expression may still occur in vivo. Atypical genes present in types D and E are more similar to each other than those from type A and B and nonporcine type C isolates and may indicate divergent evolution of atypical *cpb2* genes in different *C. perfringens* types. As *cpb2* genes are plasmid-borne, at least in some strains (4, 10), there is the potential for mobility and the subsequent transfer of *cpb2* among strains of *C. perfringens*. However, the strong correlation of porcine isolates carrying consensus *cpb2* genes suggests that if atypical gene transfer does occur, it is not maintained in porcine isolates. Correspondingly, consensus genes transferred into nonporcine isolates are also apparently not maintained. Interestingly, almost half of the consensus genes in nonporcine isolates carry frameshift mutations (44.4%), resulting in an absence of beta2-toxin expression.

The findings presented here strengthen the possible role of beta2-toxin in the pathogenesis of enteritis in neonatal pigs. However, the presence of apparently nonexpressed, atypical *cpb2* genes in *C. perfringens* strains isolated from nonporcine hosts raises the question of whether this protein plays the same role in enteritis in other animal species. Answering these questions will require the development of appropriate animal models of *C. perfringens* enteritis.

Nucleotide sequence accession number. The atypical *cpb2* sequence data were submitted to the DDBJ/EMBL/GenBank databases under accession numbers AY609161 to AY609183.

We thank Jeremy W. Coombs for excellent technical assistance. MAb 9E10B was kindly provided by Carol W. Maddox, University of Illinois at Urbana-Champaign. We also thank Ian T. Paulsen, The Institute for Genomic Research, Rockville, Md., for the *C. perfringens* 853 nucleotide sequence.

Sequencing of the *C. perfringens* 853 nucleotide sequence was part of a genome sequencing project funded by NIAID grant 1 U01 AI49921-01.

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