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Genomic insights into *cpb2*-positive *Clostridium perfringens* and the potential biological function of *cpb2* gene

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Abstract

Clostridium perfringens, capable of causing intestinal infections in both animals and humans, represents a significant public health concern. This study aimed to assess the occurrence of the beta2 toxin-coding gene *cpb2* in *C. perfringens* from various host species and to explore the genetic contexts of this gene. The results showed an enrichment of *cpb2* in pig-derived *C. perfringens*. A comparative analysis of the detection rates of *cpb2* and pCP13-like plasmids revealed that the *cpb2* gene itself, rather than the pCP13-like plasmids, caused the enrichment. Sequence comparison of *cpb2*-positive pCP13-like plasmids showed that *cpb2* was located on the *cpb2-hp*-transcriptional regulator (PadR family) segment. Despite the diverse plasmid structures of pCP13-like plasmids, the *cpb2-hp*-transcriptional regulator (PadR family) segment was consistently observed in all *cpb2*-positive *C. perfringens* strains, suggesting the potential transmission of the *cpb2* gene on this specific genetic segment. Additionally, phylogenetic analysis of the *C. perfringens* strains harboring pCP13-like plasmids, as well as 31 pCP13-like plasmids, indicated that *cpb2* did not affect the evolutionary relationship of either pCP13-like plasmids or *C. perfringens*. Genetic markers, particularly those located on mobile genetic elements (MGEs), that can help bacteria survive in external environments are more readily enriched in the population. The high prevalence of *cpb2* in pig-derived strains indicated that it might confer a selective advantage, enhancing the survival and persistence of *C. perfringens* in the pig intestine. In conclusion, our study elucidated the genetic context, host tropism and potential biological functions of *cpb2*, which can provide references for further research.

Keywords *Clostridium perfringens*, *cpb2*, Genome, Host adaption

Introduction

Clostridium perfringens is a Gram-positive, rod-shaped, spore-forming bacterium that widely exists in natural environments and the intestinal tracts of both humans and animals [1]. *C. perfringens* strains can secrete more than 20 protein toxins or enzymes and are categorized into seven toxinotypes based on the combination of six major toxins (α-toxin, β-toxin, ε-toxin, ι-toxin, *C. perfringens* enterotoxin, and necrotic enteritis B-like toxin) [2].

Beta2 toxin (CPB2), with a molecular weight (MW) of approximately 28 kDa, was initially identified in a *C. perfringens* strain isolated from a piglet that died of necrotizing enterocolitis [3]. Subsequently, several studies have

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reported its presence in *C. perfringens* strains isolated from cases of enteritis and enterotoxaemia in both humans and animals [4–7]. Previous studies have indicated that CPB2 can be encoded by both *cpb2* genes and atypical *cpb2* (*aty.cpb2*) genes [8, 9], whereas the differences between the encoding proteins of these two genetic variants have not been elucidated. The *aty.cpb2* genes share approximately 60–80% sequence identity with *cpb2* genes but 99% identity with other *aty.cpb2* genes. However, a high number of the *aty.cpb2* genes have a frameshift mutation that prevented expression [9]. Therefore, this study focused on *cpb2*-positive *C. perfringens* strains.

The *cpb2* gene was initially identified on plasmid pCP13 (AP003515.1). In contrast to the extensively studied pCW3-like plasmids, research on pCP13-like plasmids is relatively limited. It is currently known that pCP13-like plasmids are a conjugative plasmid family with a size ranging from ~ 36 kb to ~ 58 kb. In addition, they can carry *cpb2* and the recently identified enterotoxin coding gene *becA/B* [8, 10, 11].

Epidemiological surveys and pathogenicity tests have indicated the potential involvement of CPB2 in enteric diseases and diarrhea in animals [12, 13]. Moreover, a recent study suggests that the primary effect of CPB2 is the formation of cation-selective channels [14]. High-throughput sequencing technology has been widely applied in epidemiological studies to trace and precisely control pathogenic agents. Nevertheless, there has been a lack of comprehensive research on the genetic characteristics of *cpb2*-positive *C. perfringens* strains and *cpb2* genes. This study investigated the genomic features of *cpb2*-positive *C. perfringens* strains and explored the potential biological functions of *cpb2* genes from both genomic and host-adapted perspectives, which can enhance our understanding of *cpb2* gene and provide a reference for further research.

Results

Prevalence of toxin genes and pCP13-like plasmids among the *C. perfringens* genomes

The detection of toxin genes in the 763 *C. perfringens* genomes revealed the presence of *plc* in all samples, followed by *cloSI* (755/763, 98.95%), *nanH* (754/763, 98.82%), *colA* (733/763, 96.06%), *nagH* (682/763, 89.38%), *nagJ* (678/763, 88.86%), *nanJ* (675/763, 88.47%), *nagI* (674/763, 88.34%), *nanI* (657/763, 86.11%), *pfoA* (624/763, 81.78%), *nagK* (621/763, 81.39%), *nagL* (427/763, 55.96%), *cpe* (127/763, 16.64%), *cpb2* (65/763, 8.52%), *etx* (61/763, 7.99%), *netB* (44/763, 5.77%), *tpeL* (36/763, 4.72%), *cpb* (28/763, 3.67%), *becA*/*becB* (9/763, 1.18%), and *iap*/*ibp* (8/763, 1.05%). Additionally, pCP13-like plasmids were identified in 30.3% (231/763) of the *C. perfringens* genomes.

cpb2 is associated with host species of *C. perfringens*

In order to eliminate potential bias introduced by the *cpb2*-positive strains in this study, we utilized genomic data from GenBank to compare the prevalence of *cpb2* in *C. perfringens* strains across different hosts. The 757 *C. perfringens* genomes available in GenBank were classified into seven groups: human-derived strains ($n=216$), chicken-derived strains ($n=108$), sheep/goat-derived strains ($n=75$), bovine-derived strains ($n=67$), pig-derived strains ($n=65$), strains of unknown origin ($n=46$), and others ($n=180$, including *C. perfringens* isolated from foods, environments, and other animals such as camels). The specific sources of the strains are listed in Table S1.

Among the 59 *cpb2*-positive *C. perfringens* strains available in GenBank, 25 strains were isolated from pigs, followed by humans ($n=5$), chickens ($n=3$), bovines ($n=3$), and sheep/goats ($n=0$).The remaining 23 *cpb2*-positive strains belonged to groups unknown ($n=9$) and others ($n=14$). Comparative analysis indicated that the detection rate of *cpb2* was significantly higher ($P<0.01$) in strains from pigs compared to those from humans, sheep/goats, bovines, and chickens (Table 1). This indicated that *cpb2* or the pCP13-like plasmids capable of carrying *cpb2* might affect the distribution of *C. perfringens* in different animals.

To ascertain the specific factor contributing to the high prevalence of *cpb2* in pig-derived strains, we further investigated the prevalence of *cpb2*-negative pCP13-like plasmids from different sources. The results demonstrated significantly higher detection rates of *cpb2*-negative pCP13-like plasmids in *C. perfringens* from bovines ($P=6.97\times10^{-6}$, χ^2 test) and humans ($P=0.01694$, χ^2 test) rather than pigs (Fig. 1A). These results indicated that the *cpb2* gene but not the pCP13-like plasmids attributed to the enrichment of *cpb2* in pig-derived *C. perfringens*.

Table 1 Prevalence of *cpb2* genes among the *C. perfringens* strains from pigs and other groups, including sheep/goat, chickens, bovines, and humans

Source	Total	*cpb2*-positive	Proportion
pigs	65	25	38.5%
sheep/goat	75	0	0
bovines	67	3	4.5%
chickens	108	3	2.8%
humans	216	5	2.3%

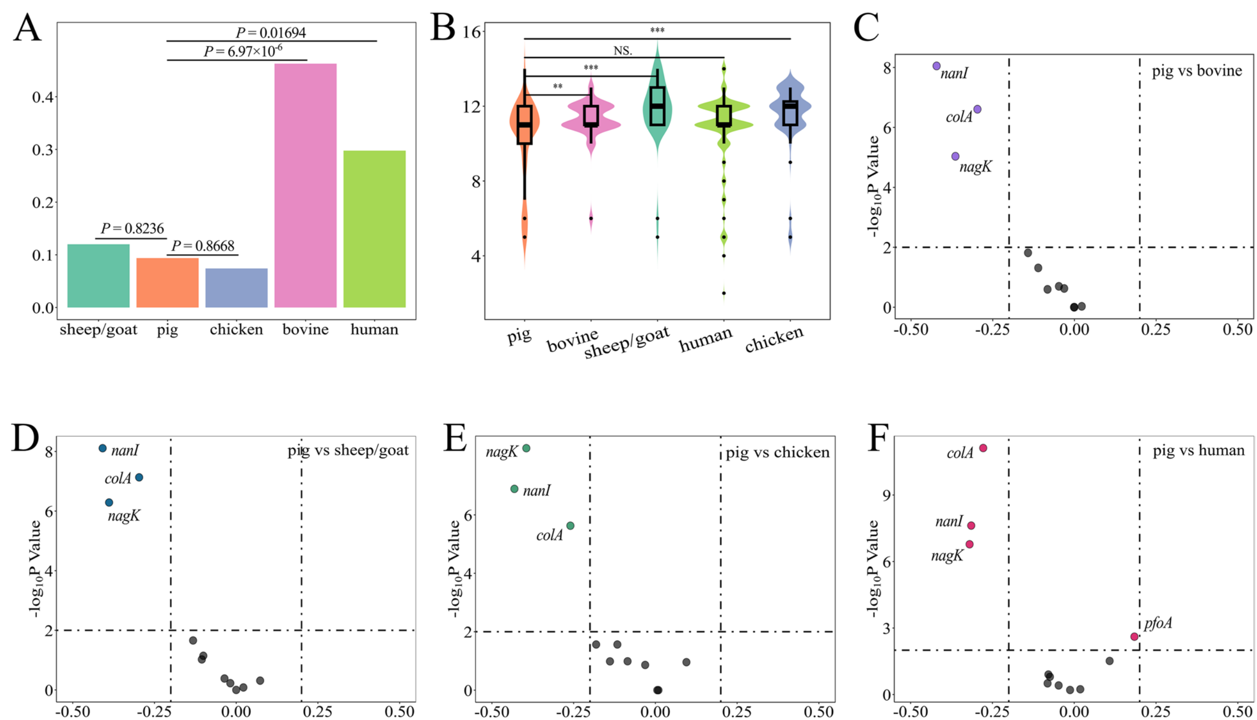

Fig. 1 Distribution of toxin genes among the *C. perfringens* strains from different sources. **A** Detection rates of *cpb2*-negative pCP13-like plasmids in different sources. **B** Box-plot of the toxin genes counts. **C** Volcano plot of the non-typing toxin genes between *C. perfringens* strains from pigs and bovines, **D** pigs and sheep/goats, **E** pigs and chickens, and **F** pigs and humans. The X-axis represents the difference in gene prevalence (in pigs-in other sources). The Y-axis represents the transformed *P* value ($-\text{Log}_{10}$ *P* value). **: $P < 0.01$, ***: $P < 0.001$, NS.: no significance

Although more *cpb2* genes were detected in the pig-derived strains, the total number of toxin genes was significantly lower in the pig-derived strains compared to those from sheep/goats, chickens, and bovines. (Fig. 1B). Therefore, we further investigated the prevalence of 11 non-typing toxin genes, including *cloSI*, *colA*, *pfoA*, *nagH*, *nagI*, *nagJ*, *nagK*, *nagL*, *nanH*, *nanI*, *nanJ*, in *C. perfringens* from different sources. The results indicated that the frequencies of *nanI*, *nagK*, and *colA* were significantly lower in pig-derived strains compared to those from sheep/goats, bovines, chickens, and humans (Fig. 1C–F). Additionally, the prevalence of *pfoA* was significantly lower in human-derived strains (Fig. 1F and Fig. S1). To eliminate any potential biases arising from researchers' preferences in strain selection and sequencing, the distribution of typing toxin genes was not examined in this study.

cpb2 doesn't affect the evolution relationship of *C. perfringens*

A total of 228 *C. perfringens* strains carrying pCP13-like plasmids, including 59 *cpb2*-positive and 169 *cpb2*-negative, passed the quality assessment of CheckM. A maximum likelihood tree was constructed based on 103,089 SNP sites across these 228 strains. The *C. perfringens* strains were divided into four clusters within the tree (Fig. 2). Approximately two-thirds of the *cpb2*-positive strains were found in Clusters I and II, while the remaining one-third were dispersed throughout the tree. Notably, the *cpb2*-positive strains in Clusters I and II typically originated from the same country or animal source, suggesting that the phylogenetic relationships among *C. perfringens* strains are more influenced by their isolation sources than the presence of the *cpb2* gene. The cluster heatmap of ANIs also revealed the higher genomic similarity of *C. perfringens* strains from the same source (Fig. S2). Interestingly, several *cpe*-positive strains from humans and animals in the UK, the Netherlands, and Germany clustered together in Cluster III, indicating the potential transmission of *cpe*-positive *C. perfringens* strains among these countries.

cpb2 locates on a conserved segment on pCP13-like plasmids

Six *cpb2*-positive pCP13-like plasmids were identified from the *C. perfringens* strains in this study: pscCP-7, pscCP-8, pscCP-25, pscCP-26, pscCP-28, and pscCP-97 (Table S2). Sequence comparisons together with plasmid pCP13 (AP003515.1) revealed that the Pcp loci were conserved across these plasmids (Fig. 3). The replication

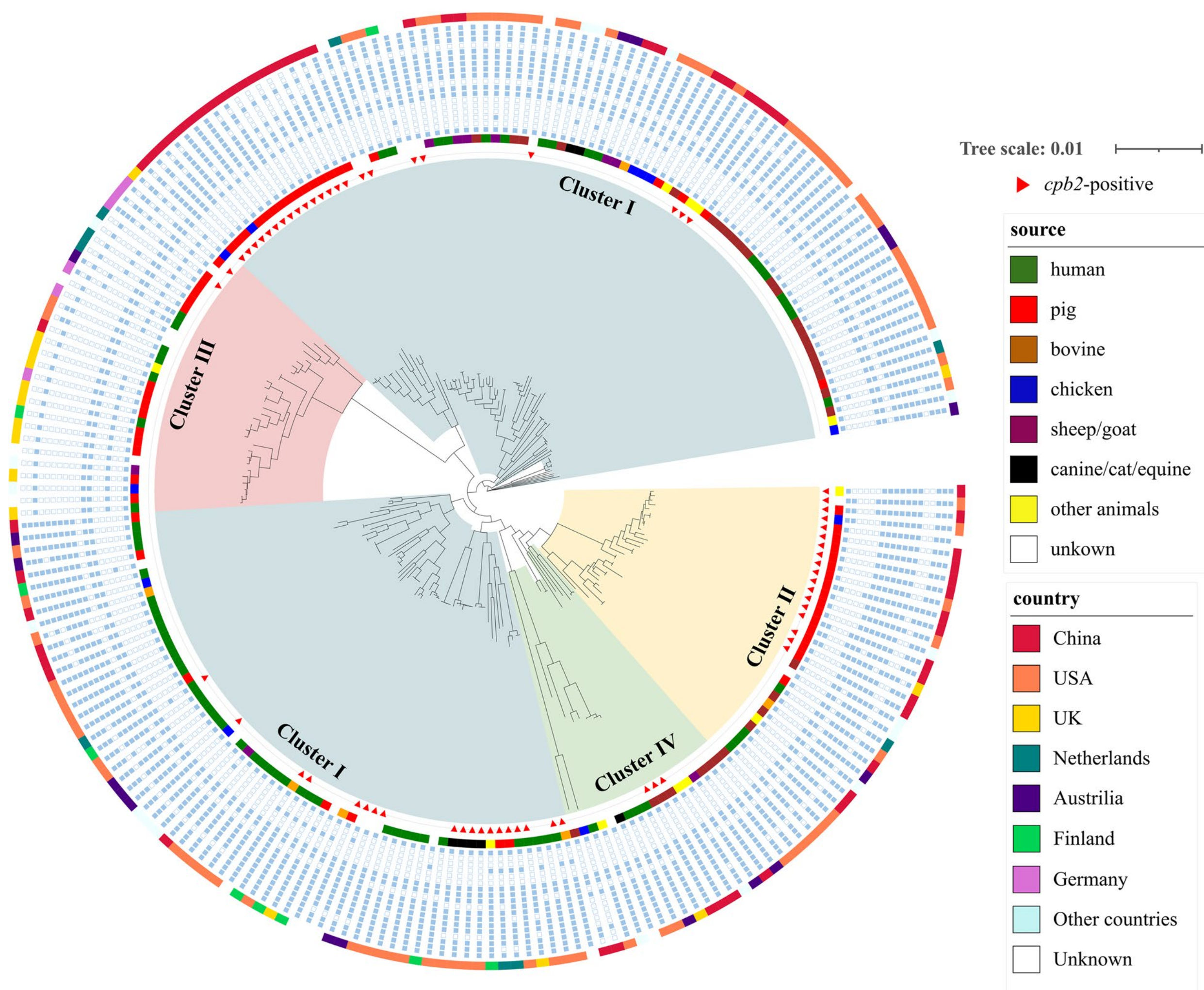

Fig. 2 Maximum likelihood tree of the 228 *C. perfringens* strains harboring pCP13-like plasmids. The toxin genes are represented by sky blue squares, *plc*, *cpb*, *etx*, *cpe*, *netB*, *iap/ibp*, *cloSI*, *colA*, *cpb2*, *pfoA*, *nagH*, *nagI*, *nagJ*, *nagK*, *nagL*, *nanH*, *nanI*, and *nanJ* in order from the inside to outside. Source and country of the *C. perfringens* strains were marked by squares with different colors

initiator gene of pCP13 was identified in pscCP-7, pscCP-8, pscCP-26, pscCP-28, and pscCP-97, but not in pscCP-25. It was observed that the *cpb2* genes on these plasmids all located on *cpb2-hp*-transcriptional regulator (PadR family) segment. In addition, the resolvase coding gene *resP* and one hypothetical protein-coding gene (designated as *tcb*) were detected upstream of the *cpb2* segments. Subsequently, we conducted gene detections of *resP*, *tcb*, and the *cpb2* segment (*cpb2*, *hp*, and transcriptional regulator (PadR family)) among the 59 *cpb2*-positive *C. perfringens* strains. The results revealed that the *cpb2* segment was detected in all *cpb2*-positive *C. perfringens* strains, whereas *resP* and *tcb* were detected from 49 and 32 of them, respectively. In addition, *resP* and *tcb* were always detected upstream of the *cpb2* segment, indicating that they coexisted with *cpb2* segment on the pCP13-like plasmids. The phylogenetic analysis of the 31 pCP13-like plasmids revealed that these plasmids with similar structural features clustered together in the evolutionary tree (Fig. S3), while the presence of *cpb2* was not strongly correlated with the evolutionary relationship of the plasmids, suggesting that *cpb2*/*cpb2-hp*-transcriptional regulator (PadR family) was more likely to be a component of inter-plasmid transfer rather than a stabilizing component that had evolved over time.

Discussion

C. perfringens is known for causing foodborne illnesses in humans and several gastrointestinal infections in animals [8, 15]. The pathogenicity of *C. perfringens* strains is primarily determined by the toxins they produce. It has been documented that *C. perfringens* can produce over 20 different protein toxins or enzymes. Among them, the biological characteristics and host pathogenicity

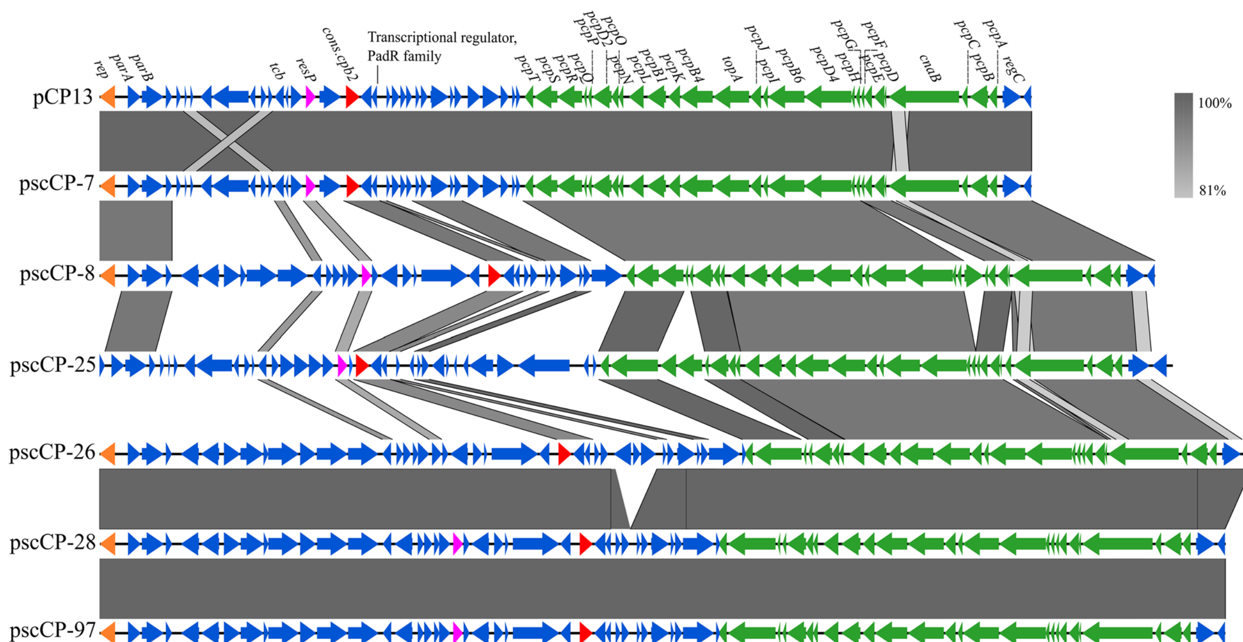

Fig. 3 Sequence comparison of the *cpb2*-positive plasmids in this study together with pCP13. Arrows indicate the directions of transcription of the genes, and shared regions are denoted by shadings. *cpb2* genes, replication initiator coding genes, the Pcp locus coding genes, and other hypothetical proteins were marked with red, orange, green, and blue boxes, respectively

of the six typing toxins have been extensively studied. Beta2 toxin is a pore-forming cytolytic toxin with less than 15% sequence homology to β-toxin [16]. Epidemiological studies have linked β2-toxin to intestinal diseases, such as necrotizing enteritis (NE) in piglets and enterocolitis in foals. Additionally, it has been suggested to be an accessory toxin in *C. perfringens*-associated non-foodborne diarrhea [1]. Although the structure of *cpb2*-positive plasmid pCP13 has been characterized to some extent [10], the genomic context of the *cpb2* gene remains poorly understood, and there are no studies focusing on its host tropism or potential biological functions.

Epidemiological studies have indicated that a considerable proportion of *C. perfringens* strains obtained from pigs are positive for *cpb2* [17, 18]. In a recent study [19], we detected toxin genes among animal-derived *C. perfringens* in China and observed that the majority of *cpb2* genes were present in pig-derived *C. perfringens* strains. Here, we screened the distribution of toxin genes among 757 *C. perfringens* genomes available in GenBank and observed that there was an enrichment of *cpb2* in pig-derived *C. perfringens*. Phylogenetic analysis of 228 *C. perfringens* strains harboring pCP13-like plasmids and 31 pCP13-like plasmids indicated that *cpb2* was more likely to be a component of inter-plasmid transfer rather than a stabilizing component that has evolved over time. Additionally, the *cpb2* genes were consistently located on the *cpb2-hp*-transcriptional regulator (PadR family) segment in all *cpb2*-positive *C. perfringens* strains, suggesting the potential horizontal transfer of *cpb2* via this segment. Comparison of the detection rates of *cpb2* and *cpb2*-negative pCP13-like plasmids across different sources indicated that *cpb2* (or *cpb2-hp*-transcriptional regulator) but not pCP13-like plasmids led to the enrichment of *cpb2* in pig-derived *C. perfringens*.

The presence or absence of genetic markers is often the result of bidirectional selection between bacterial strains and their hosts. Genetic markers that enhance bacterial resistance to external environments, without causing disease or lethality in hosts, are more likely to be enriched under selective pressure. The prevalence of *mcr-1* during periods of heavy antibiotic use, followed by its decline after the colistin ban, supports this concept [20]. A recent study indicates that infant-associated *pfoA* + strains caused significantly more cellular damage to Caco-2 cells than *pfoA* − strains [21]. The higher pathogenicity of *pfoA* + strains and the lower detection rate of *pfoA* in human-derived strains was also consistent with the above concept. Although CPB2 can form cation-selective channels in cell membrane, only a prolonged incubation with CPB2 to 22 h induced significant propidium iodide (PI) entry into cells [14]. Additionally, an in vitro experiment has indicated that CPB2 does not contribute to cell cytotoxicity caused by human and porcine *cpb2*-harboring *C. perfringens* [22]. The higher prevalence of *cpb2* among pig-derived strains suggests that the primary biological

function of CPB2 might be promoting the survival of *C. perfringens* in pig intestines. In conclusion, this study highlighted the genetic context, host tropism, and potential biological functions of *cpb2*, which could provide references for further research.

Material and methods

Genome statistics

Six *cpb2*-positive *C. perfringens* strains (designated as scCP-7, scCP-8, scCP-25, scCP-26, scCP-28, scCP-97) isolated from pigs in Sichuan province were included in this study. *C. perfringens* isolation and *cpb2* gene identification were performed in accordance with the methodology mentioned in our previous study [23]. Genomic DNA of the isolates was extracted using TIANamp Bacteria DNA Kit (Tiangen, Beijing, China) and subjected to whole-genome sequencing on the Illumina platform and Nanopore platform. Draft genomes were assembled using the hybrid mode of Unicycler 0.5.0 [24]. Genomes of 757 *C. perfringens* strains (as of November 10, 2023, genomic size > 2,700 KB, and *plc* positive) available on GenBank were analyzed together with the six *C. perfringens* strains in this study. Information on all *C. perfringens* strains is presented in Table S1.

Screening for genetic markers

Toxin genes of the *C. perfringens* strains were detected using VFDB (http://www.mgc.ac.cn/VFs/) and standalone BLASTn analyses. The conjugative locus of pCP13-like plasmids (Pcp locus) was identified by local BLASTn assays, and Pcp locus coding genes (*pcpA-J*) (AP003515.1) were used as reference sequences. Genomes positive for Pcp locus coding-genes were considered to contain pCP13-like plasmids.

Phylogenetic analysis

The genome quality of the *C. perfringens* strains carrying pCP13-like plasmids was assessed by checkM 1.0.12 [25]. Genomes with completeness > 95% and contamination < 5% were selected for further phylogenetic analysis. Average nucleotide identity (ANI) of the strains was identified by FastANI 1.34 (https://github.com/ParBLiSS/FastANI). Core genome single nucleotide polymorphisms (cgSNPs) among the *C. perfringens* strains were called using Snippy v4.6.0 (https://github.com/tseemann/snippy). A maximum likelihood tree was constructed based on the cgSNPs using FastTree 2.1.11 [26] and visualized using the iTOL online tool (https://itol.embl.de/). *C. perfringens* strains 13 (SAMD00061119) was used as the reference strain in the phylogenetic analysis. Grouping of the *C. perfringens* strains in the maximum likelihood tree was performed using rhierBAP [27].

Sequence comparison of the cpb2-positive pCP13-like plasmids

To gain insight into the genetic background of *cpb2*, we performed sequence comparison on the six *cpb2*-positive pCP13-like plasmids in this study together with plasmid pCP13. EasyFig 2.1 was used for the comparison and visualization of *cpb2*-positive pCP13-like plasmids [28]. Then, a set of 31 pCP13-like plasmids (Table S2), comprising 16 *cpb2*-positive plasmids and 15 *cpb2*-negative plasmids, available in GenBank were selected for sequence alignment and phylogenetic analysis. Sequence alignment of the plasmids was performed using MAFFT 7.520 [29], followed by phylogenetic tree construction using IQtree 2.2.5 [30]. The most appropriate substitution model was determined by the ModelFinder algorithm of IQTree.

Statistical analysis

Statistical analyses were performed using R version 4.3.2. Categorical data were analyzed using χ^2 or Fisher's exact test. Continuous data with normal and non-normal distributions were analyzed using the *t*-test and Mann–Whitney *U* test or Wilcoxon test, respectively.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44280-024-00058-8.

Supplementary Material 1.
Supplementary Material 2.

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Not applicable.

Authors' contributions
J.W. and Z.Y. conceived and supervised the study; J.W., D.Y. and K.W. designed the experiments and wrote the manuscript; K.W., Y.Y. and M.F. performed the experiments and analyzed the data; L.Z., Y.L. and X.T. helped with experiments; L.D., D.Y. and E.F. took part in the editing of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All genome assemblies of the *C. perfringens* strains were deposited in GenBank under BioProject accession number PRJNA1044929.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare no conflicts of interest regarding the publication of this paper.

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