<Research Article>

Characterization and functional analyses of fibronectin-binding protein C (FbpC) of *Clostridium perfringens*

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Summary *Clostridium perfringens* is an obligate anaerobic bacterium that can cause gas gangrene and food poisoning in humans. Our previous studies have revealed that *C. perfringens* binds to fibronectin (Fn) and has several Fn-binding proteins (Fbps) on its cell surface. FbpC, one of the Fbps identified in *C. perfringens*, does not play a major role in Fn binding, because Fn binds to *fbpC* and *fbpD* null mutants at levels comparable to those of the parental strain. In this study, we identified the *fbpC* gene promoter and performed structural and functional prediction of FbpC via *in silico* studies and evaluation of FbpC carboxypeptidase activity. FbpC was predicted to be both a cell wall-binding protein and a carboxypeptidase based on sequence similarity analysis, domain-based annotation, motif-based annotation, and tertiary structure–based functional analyses. The purified recombinant FbpC exhibited significantly higher carboxypeptidase activity than bovine serum albumin *in vitro*. FbpC thus appears to be a carboxypeptidase associated with the cell wall.

Key words: *Clostridium perfringens*, Fibronectin-binding protein C, Promoter, Carboxypeptidase, Cell wall-binding protein

1. Introduction

Clostridium perfringens, a strictly anaerobic spore-forming bacterium, is a pathogenic bacterium that can cause gas gangrene and food poisoning in humans[1]. *C. perfringens* is thought to enter the individual through wounds or surgical incisions and bind to extracellular matrix components, such as hyaluronic acid and collagen, within tissues[2,3]. Recent studies have elucidated the mechanisms by which *C. perfringens* adheres to these extracellular matrix components[2,3]. We found that *C. perfringens* binds to human fibronectin (Fn), an extracellular matrix protein[4]. This finding suggested that *C. perfringens* has some kind of fibronectin-binding protein (Fbp). So far, we have identified six Fbps [FbpA (CPE0737), FbpB (CPE0847), FbpC (CPE0625), FbpD (CPE0630), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, CPE1304), and autolysin (Acp, CPE1231)] from *C. perfringens* 13[5-8]. Although the recombinant proteins of FbpA, FbpB, FbpC, and FbpD possess Fn-binding activity, the null mutant cells did not show decreased Fn-binding

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ability, indicating that these proteins are not involved in Fn binding by *C. perfringens*[5,6,8]. GAPDH also possesses Fn-binding activity and it has been confirmed that GAPDH is present on the cell surface[7]. However, the extent to which it contributes to Fn binding to *C. perfringens* cells remains unknown. On the other hand, Acp has been revealed to contribute to *C. perfringens* binding to Fn[8]. The original roles of the other Fbps of *C. perfringens* remain largely unknown. We have previously reported that both recombinant FbpA and recombinant FbpB significantly inhibit Fn binding to dermatopontin, a 22-kDa extracellular matrix protein that accelerates normal collagen fibrillation and induces Fn fibrillation[9]. This finding suggested that FbpA and FbpB might be virulence factors for *C. perfringens*. Nothing has been reported about the functions and stereo structures of FbpC and FbpD, although both are expressed on the surface of *C. perfringens*[8].

In this study, we found that the *fbpC* gene is expressed under its own promoter. We then predicted the function and three-dimensional structure of FbpC using various databases, suggesting that FbpC is a putative carboxypeptidase. We therefore investigated whether FbpC possesses carboxypeptidase activity. Based on these findings and predictions, we attempted to elucidate the intrinsic function of FbpC.

2. Materials and methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* Top10 (Table 1) was grown in Luria-Bertani (LB) broth or on LB agar plates (Nacalai Tesque, Kyoto, Japan). *C. perfringens* strains were grown anaerobically in Gifu anaerobic medium (GAM) broth or on GAM agar plates (Nissui, Tokyo, Japan). Antibiotics were used at the following concentrations: ampicillin, 50 μg/mL; chloramphenicol, 10 μg/mL.

Table 1 Bacterial strains and plasmids used in this study

Name	Description	Origin or reference
Strain		
E. coli		
Top10	*hsdR mcrA lacZΔM15 endA1 recA1*	Invitrogen, Thermo Fisher Scientific
C. perfringens		
13	a type A wild-type strain isolated from soil	11, 12
HN13	13 Δ*galK* Δ*galT*	20
SAK3	HN13 Δ*fbpC* Δ*fbpD*	8
Plasmid		
pCR™ 2.1-TOPO™	a TA cloning vector	Invitrogen, Thermo Fisher Scientific
pJIR418	a *C. perfringens* - *E. coli* shuttle plasmid, Em^r, Cm^r	10
pfbpC	pJIR418 containing *fbpC* gene	8

Construction of SAK3/pfbpC strain

We cloned the DNA segment extending from the stop codon of the CPE0624 gene upstream of the FbpC gene to the stop codon of the *fbpC* gene into pJIR418, *a C.perfringens-E.coli* shuttle plasmid[10] (Table 1). The resultant plasmid was named pfbpC[8] (Table 1). This plasmid was transformed into a *C. perfringens* Δ*fbpC* Δ*fbpD* strain, SAK3[8] (Table 1).

Extraction of cell wall-associated proteins and western blotting analysis

Extractions of cell wall-associated proteins from *C. perfringens* cells were basically performed according to a previously described method[6]. Western blot analysis was performed as follows. *C. perfringens* cell wall-associated proteins were subjected to 12.5% SDS-PAGE. Proteins in the gel were transferred to iBlot™ gel transfer stacks PVDF, mini (Thermo Fisher Scientific, Waltham, MA, USA) using the iBlot™ dry blotting system. The polyvinylidene di-fluoride (PVDF) membrane with transferred proteins was washed three times with 20 mmol/L phosphate-buffered saline (pH 7.4) containing 0.1% Tween 20 (PBST), then blocked in 5% (w/v) ECL™ blocking agents (Cytiva, Marlborough, MA, USA) overnight at room temperature. After blocking, the membrane was washed three times with PBST and incubated at room temperature for 1 h with PBST-diluted anti-FbpC serum (1:10,000). After three washes with PBST, horseradish peroxidase-labeled anti-rabbit immunoglobulin G antibody (1:40,000) was added, then incubated at room temperature for 1 h. Following three washes with PBST, FbpC was detected by chemiluminescence using an ImageQuant™ LAS 4000 mini (GE Healthcare, Chicago, IL, USA).

Determination of the 5' end of *fbpC* mRNA and *fbpC* promoter search

Total RNA of *C. perfringens* cells grown up to OD_{600} = 0.8±0.1 (exponential growth phase) was purified using NucleoSpin® RNA kit (Takara Bio Inc., Kyoto, Japan). For determination of the 5' end of the *fbpC* transcript, the 5' RACE (rapid amplification of cDNA end) System version 2.0 (Invitrogen, Thermo Fisher Scientific) was used. One microgram of total RNA extracted from *C. perfringens* 13[11,12] (Table 1) was used as the template. A reaction was performed at 70°C for 10 min using 2.5 pmol of gene-specific primer 1 (GSP1: 5'-GTAGCTAAACTTCCATC-3'). Next, 1 μL of SuperScript™ II reverse transcriptase (Invitrogen, Thermo Fisher Scientific) was added. After a 50-min reaction at 42°C, the reaction was terminated at 70°C for 15 min to synthesize cDNA. Total RNA used as the template was degraded by adding 1 μL of RNase Mix and incubating at 37°C for 30 min. The cDNA product was purified using the Wizard® SV Gel and PCR clean-up system (Promega, Madison, Wi, USA). To add a cytosine homopolymer tail to the 3' end of the cDNA, 2.5 μL of 2 mmol/L dCTP and 1 μL of terminal deoxynucleotidyl transferase (TdT) were added to 10 μL of purified cDNA. The reaction was performed at 37°C for 10 min, followed by inactivation of TdT at 65°C for 10 min. The 5'-cytidine-tailed cDNA was amplified by PCR using 10 μmol/L GSP2 (5'-CTTTCATGATAATACCACTTATCA-3'), 10 μmol/L abridged anchor primer (5'-GGCCACGCG TCGACTAGTACGGGGGGGGGGGGGGG-3'), and Takara Ex *Taq* HS (Takara, Kyoto, Japan). The single band of cDNA derived from the RT-PCR products using total RNA from *C. perfringens* 13 was estimated to be 350 bp in length by 0.8% agarose gel electrophoresis (Fig. 1A, Lane 2). This cDNA fragment was cloned into an appropriate plasmid, pCR™ 2.1-TOPO™ vector using the TOPO™ TA cloning™ kit (Invitrogen, Thermo Fisher Scientific) (Table 1). The nucleotide sequence of the inserted fragment DNA on the resultant plasmid was determined using the dideoxy method. This nucleotide sequence identified the transcription start site of the *fbpC* gene (Fig. 1B, +1). GENETYX®-MAC version 18 (Nihon Server, Tokyo, Japan) was used for searching promoter sequences (Table 2).

Amino acid sequence of FbpC

The amino acid sequence of FbpC (CPE0625; accession No. Q8XMR3) from *C. perfringens* 13

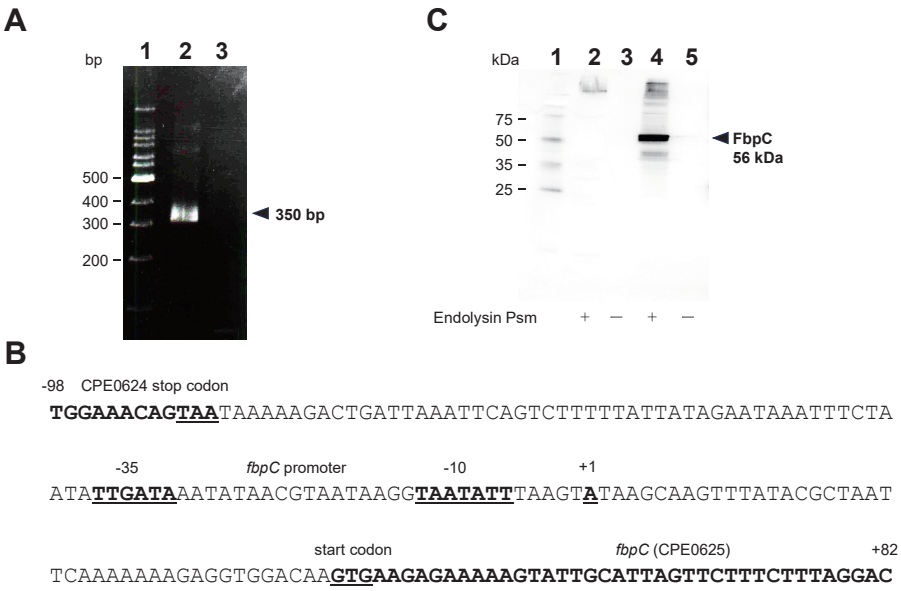

Fig. 1. Promoter region of the *fbpC* gene. A) 0.8% agarose gel electrophoresis of the 5' RACE-PCR product of *fbpC* gene. Lane 1, 100 bp DNA ladder markers; lane 2, 5' dC-tailed *fbpC* cDNA; lane 3, non dC-tailed cDNA. Note the size of the cDNA synthesized by 5' RACE RT-PCR was estimated to be about 350 bp. B) Nucleotide sequence of upstream region of the *fbpC* gene. +1 is the start point of the *fbpC* transcript as determined by the 5' RACE method. The *fbpC* promoter (-35 and -10 regions: Pribnow box) is speculated on the start site (+1) with GENETYX-MAC ver.18. C) Western blotting analysis of cell wall-associated proteins from *C. perfringens* cells with anti-FbpC serum. *C. perfringens* cells in the exponential growth phase were treated (+) or not treated (-) with endolysin Psm[18]. Lane 1, molecular weight markers; lanes 2 and 3, SAK3/pJIR418; lanes 4 and 5, SAK3/pfbpC.

was obtained from the National Center for Biotechnology Information (NCBI) database (Table 2). The FbpC amino acid sequence in FASTA format was used for further analysis.

Analysis of physicochemical properties

The physicochemical properties of FbpC were analyzed using the ExPASy server[13] (Table 2).

Function prediction by domain analysis and multiple sequences alignment

Protein functions were predicted from various database server and tools. The potential function and conserved domains of FbpC were determined using NCBI conserved domain search (CD search) (Table 2), ScanProsite[14] (Table 2), and InterPro[15] (Table 2). To identify protein homologs, a BLASTp search (Table 2) was executed on the NCBI website against the nonredundant database.

Predicting tertiary structures and functional predictions from tertiary structures

We acquired the predicted tertiary structure of FbpC and associated domain information (Domain 1, Domain 2, Domain 3, and additional regions) using the AlphaFold Protein Structure Database[16] (Table 2). The predicted tertiary structures and Protein Data Bank (PDB) files of Domain 3 and other regions (Domain 1, Domain 2, and additional regions) of FbpC were acquired using AlphaFold 2 Colab Fold[17] (Table 2). The potential functions of FbpC were predicted using the DALI server[18] (Table 2) and Foldseek server[19] (Table 2), based on the PDB files obtained as described above.

Preparation of recombinant protein and evaluation of carboxyl peptidase activity

N-terminal His_6-tagged recombinant FbpC (rFbpC) was purified as described previously[6]. Carboxypeptidase B derived from porcine pancreas were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The carboxypeptidase activities of carboxypeptidase B, rFbpC and bovine serum albumin (BSA) were evaluated using an acid

Table 2 Tools employed for computational characterization of promotor and FbpC

Function	Tools/Servers	URL
Promotor prediction	GENETYX-MAC ver.18	https://www.genetyx.co.jp/
Sequence retrieval and domain-base annotation	NCBI	https://www.ncbi.nlm.nih.gov/
Sequence similarity search	BLASTp	https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins
Physicochemical property analysis	ExPASy ProtParam	https://web.expasy.org/protparam/
Motif-base annotation	Scan Prosite	https://prosite.expasy.org/scanprosite/
	InterPro	https://www.ebi.ac.uk/interpro/
3D structure prediction and acquired PDB file	AlphaFold Protein Structure Database	https://alphafold.ebi.ac.uk/
	AlphaFold 2 Colab Fold	https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb
3D structure-based functional search	Foldseek	https://search.foldseek.com/search
	DALI	http://ekhidna2.biocenter.helsinki.fi/dali/

carboxypeptidase activity kit (Kikkoman Biochemifa Company, Tokyo, Japan). The carboxypeptidase activity of each protein was calculated as relative activity (%), with standard solution defined as 100%.

Statistical analysis

Data are presented as the mean ± SD. Statistical comparisons were performed using one-way ANOVA followed by Holm's method. A value of $p < 0.05$ was considered to indicate statistical significance.

3. Results and discussion

Promoter of *fbpC* gene

FbpC is expressed on the cell surface of *C. perfringens* HN13 (Table 1)[6,8,20]. This protein is encoded by the *fbpC* gene (CPE0625)[6]. Whether this *fbpC* gene is expressed alone or forms an operon was unclear. To clarify this, expression of FbpC in SAK3/pfbpC (Table 1) was confirmed among proteins extracted from the cell wall (Fig. 1C, Lane 4). When SAK3/pfbpC cells were not treated with endolysin Psm[21], FbpC within the cell wall could not be detected (Fig. 1C, Lane 5). These results suggested that the promoter exists immediately upstream of the *fbpC* gene on pfbpC. The *E. coli lac* promoter, however, is located upstream of multiple cloning sites in pJIR418. The possibility that *fbpC* expression is driven by the *lac* promoter remains. We therefore decided to determine the 5' end of the *fbpC* transcript using the 5' RACE method. The transcription start site of the *fbpC* gene was identified by the method (Fig. 1B, +1). Approximately 10 base pairs upstream of this transcription start site, a typical promoter sequence was speculated to be present (Promoter Sequence Score = 66.86) using GENETYX-MAC version 18 (Fig. 1B, -35 and -10 regions). These results suggested that expression of FbpC was driven by its own promoter rather than by the *lac* promoter on pfbpC. The *fbpC* gene was shown to possess its own promoter upstream of the gene and be expressed in the exponential growth phase. This suggested that FbpC might be solely responsible for the proliferation, although whether an operon is formed with surrounding genes (CPE0624 and CPE0626) remains unclear.

Physiochemical properties analysis of FbpC

Using the ExPASy ProtParam tool, various physicochemical characteristics of FbpC were assessed as described below. FbpC comprises 550 amino acids and has a molecular weight of 63,367.96 Da. The isoelectric point of FbpC was determined to

be 5.37. Calculations revealed the presence of 57 positively charged residues and 70 negatively charged residues. The instability index showed the instability of the protein *in vitro* was standard, at 40. The instability index of FbpC was 28.88, exhibiting stability *in vitro*. Grand average of hydropathicity (GRAVY) showed the hydropathicity index of protein, with a negative score reflecting a hydrophilic protein. GRAVY of FbpC was -0.768, reflecting a hydrophilic protein. Not all amino acid residues constituting FbpC are hydrophilic; rather, they include a moderate proportion of hydrophobic residues, as indicated by a GRAVY score higher than -1.0. This analyzed result thus suggested that FbpC might be more likely to represent a cell wall-binding protein rather than a secreted protein.

Sequence similarity and motif-based analysis of FbpC

BLASTp is a sequence similarity-based tool for

Table 3 BLASTp outcome indicating degree of similarity among proteins

Representative sequence	Organism	Total score	Query cover	E value	Percent identity (%)	Accession No.
N-acetylmuramoyl-L-alanine amidase family protein	*Clostridium perfringens*	650	0.57	0	100	WP_283700822.1
Cell wall-binding protein	*Clostridium perfringens*	814	0.54	$3.00e^{-145}$	100	MDU5650529.1
N-acetylmuramoyl-L-alanine amidase family protein	*Clostridium perfringens*	785	0.54	$9.00e^{-109}$	100	WP_243153931.1
Hypothetical protein	*Clostridium perfringens*	657	0.55	$7.00e^{-88}$	100	MDU6697818.1
Cell wall-binding protein, partial	*Clostridium perfringens*	816	0.74	0	99.75	WP_415336067.1
Cell wall-binding protein	*Clostridium perfringens*	1167	0.91	0	99.56	WCM69639.1
Hypothetical protein PL325_03555	*Clostridium perfringens* D	652	0.59	0	99.38	WEV16687.1
M14 family zinc carboxypeptidase	*Clostridium perfringens*	893	0.78	0	99.38	MDV5113639.1
M14 family zinc carboxypeptidase, partial	*Clostridium perfringens*	481	0.43	$3.00e^{-166}$	99.16	WP_279285559.1
M14 family zinc carboxypeptidase	*Clostridium perfringens*	1008	0.91	0	98.96	WP_243153932.1
M14 family zinc carboxypeptidase, partial	*Bacillus safensis*	325	0.29	$3.00e^{-106}$	98.74	WP_283506800.1
M14 family zinc carboxypeptidase	*Clostridium perfringens*	575	0.52	0	98.26	WP_283700825.1
Cell wall-binding protein	*Clostridium perfringens*	1080	1	0	96.55	WP_115649549.1
Glucosaminidase domain-containing protein	*Clostridium perfringens*	843	0.82	0	91.13	WP_208337915.1
Cell wall-binding protein	*Clostridium perfringens*	831	0.44	$2.00e^{-78}$	89.71	MFH5975310.1

searching for homologs using full-length amino acid sequences. BLASTp was performed on the NCBI protein database against a non-reductant protein sequence database, revealing similarities between FbpC and various proteins, particularly those containing *N*-acetylmuramoyl-L-alanine amidase family proteins, cell wall-binding proteins, and M14 family zinc carboxypeptidase (Table 3). In particular, the results identifying cell wall-binding proteins were broadly consistent with our previous findings, indicating that FbpC is localized to the cell wall[6,8].

FbpC was estimated using the NCBI-CD search, ScanProsite, and InterPro annotation tools. The zinc carboxypeptidase domain was found in FbpC from 323 aa to 509 aa by domain-based annotation using NCBI-CD search. The server generated an E-value of $7.95e^{-14}$, indicating a strong match to this domain.

We also performed motif-based annotation analysis. ScanProsite predicted that residues 57–296 aa and 288–550 aa of FbpC correspond to cell wall-binding repeats and the peptidase M14 family, respectively. The molecular function of FbpC was predicted to be GO:0004181 metallocarboxypeptidase activity using InterPro gene-ontology (GO) terms. These analytical results were consistent with our previous predictions for FbpC based on SSDB, one of the motif-based annotation analyses[6].

These sequence similarity-, domain- and motif-based analyses pointed to the possibility that FbpC has both zinc carboxypeptidase activity and cell wall-binding domains.

Prediction of FbpC tertiary structure

To investigate structural similarities between FbpC and various proteins, we performed tertiary-structure prediction for FbpC using the AlphaFold Protein Structure Database and AlphaFold2 via ColabFold (Fig. 2A–C). Based on prediction results from the AlphaFold Protein Structure Database, the confidence of the model was considered very low for amino acid positions (1–52 aa), whereas the predicted structure (53–550 aa) was of acceptable

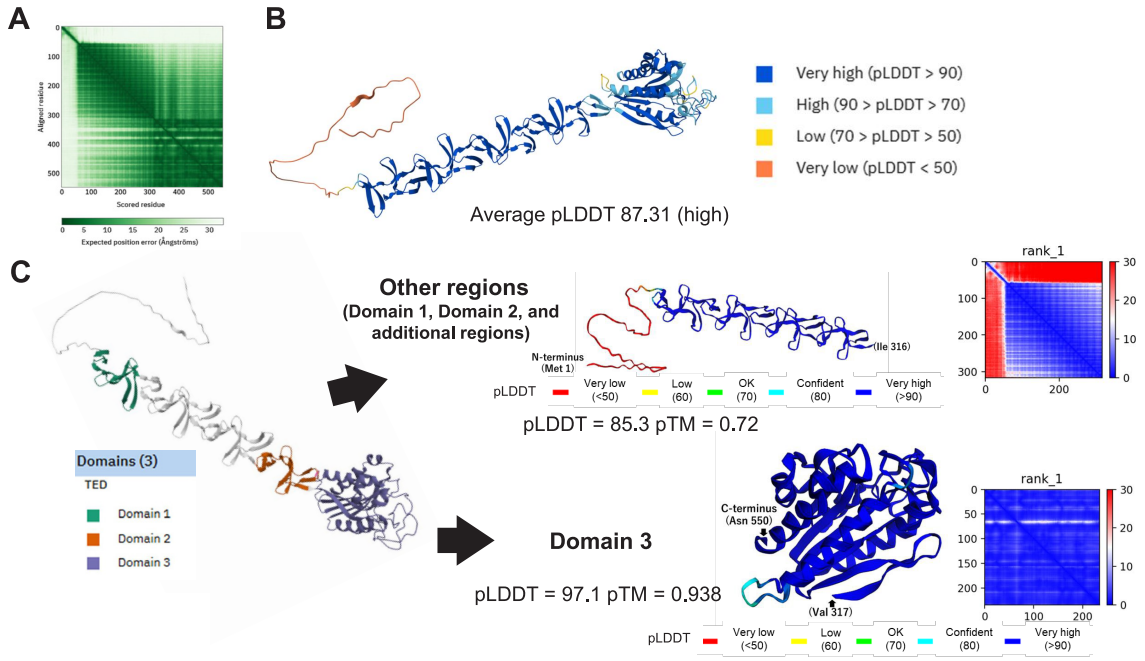

Fig. 2. Tertiary prediction of FbpC. A) The predicted alignment error (PAE) was generated using the AlphaFold Protein Structure Database. PAE provides a measure of confidence in relative positioning between pairs of amino acid residues, with darker green indicating lower predicted error. B) The predicted tertiary structure of FbpC was generated using the AlphaFold Protein Structure Database. C) The Domain 1, Domain 2, Domain 3, and additional regions of FbpC predicted by the encyclopedia of domain program. The structures of other regions (Domain 1, Domain 2, and additional regions), as well as Domain 3, were further predicted using AlphaFold2 via ColabFold.

confidence (Fig. 2A, B). The average predicted local distance difference test (pLDDT) score of FbpC was 87.31, indicating a high level of confidence (Fig. 2B). FbpC was predicted to contain three domains (Domains 1, Domain 2, and Domain 3) and additional regions according to the encyclopedia of the domain program in AlphaFold Protein Structure Database (Fig. 2C). The other regions (Domain 1, Domain 2, and additional regions; 1–316 aa) and Domain 3 (317–550 aa) exhibited a distinct structural conformation. The tertiary structure of FbpC was thus predicted by separating the other regions (1–316 aa) from Domain 3 (317–550 aa) using AlphaFold2 via ColabFold, and individual PDB files were generated for each region.

Tertiary structure–based functional analysis of FbpC

We employed the DALI server, which assesses broad similarities in overall conformation, and the Foldseek server, which assesses local structural and sequence congruence, to compare protein structures. The Z-score obtained from the DALI server indicates the statistical significance of structural similarity, and proteins with Z-scores more than 8 are considered to share the same fold structure. According to DALI server results (Z score > 11.9), the other regions (1–316 aa) were primarily structurally similar to cell wall-binding proteins, such as teichoic acid phosphorylcholine esterase, 1,4-β-*N*-acetylmuramidase, and autolysin (Table 4A), whereas Domain 3 (317–550 aa) showed structural similarity to carboxyl peptidase (Table 4B). Next, we searched for proteins with high tertiary-structure similarity using the Foldseek server and compared these results with those obtained from DALI. We were able to further refine the candidate pool by assessing results from both DALI and Foldseek servers. This approach allows for the selection of functionally more closely related homologs. The proteins commonly predicted by both DALI (Z score > 11.9) and Foldseek showed that the other regions (1–316 aa) were structurally similar to teichoic acid phosphorylcholine esterase/choline-binding protein (RCSB PDB: 2BIB), autolysin (RCSB PDB: 4X36), putative endo-β-*N*-acetylglucosaminidase (RCSB PDB: 7PL2), and toxin B (RCSB PDB: 7ML7) (Fig. 3A). Domain 3 (317–550 aa) was structurally similar to teichoic acid phosphorylcholine esterase/choline-binding protein (RCSB PDB: 1JQG), carboxypeptidase B2 (RCSB PDB: 5HGV), mureintripeptide amidase, MpaA (MPAA) (RCSB PDB: 4AXV), carboxypeptidase M (RCSB PDB: 1UWY), putative carboxypeptidase (RCSB PDB: 3K2K), Mlr6093 protein (RCSB PDB: 2QJ8), and rapid encystment phenotype protein 34 kDa (RCSB PDB: 4OKO) (Fig. 3B). The other regions (1–316 aa) were primarily matched to cell wall-binding domains by the Foldseek server (Fig. 3A). TM-score is a quantitative measure of global structural similarity between protein structures, with values greater than 0.5 generally indicating a shared fold. Root mean square deviation (RMSD) quantifies the average distance between corresponding atoms after structural superposition, with lower values indicating higher structural similarity.

However, some proteins identified for these regions, such as those corresponding to PDB entries 7PL2 and 7ML7, exhibited low TM-scores and high RMSD values, indicating that similarity was only partial (Fig. 3A). In contrast, Domain 3 was predominantly matched to hydrolases, including carboxypeptidases, and the TM-scores (0.566–0.673) and RMSD values (3.72–5.9) of the matched proteins were within acceptable ranges (Fig. 3B).

These tertiary structure–based analyses pointed to the possibility that FbpC is a carboxypeptidase associated with the cell wall.

Carboxypeptidase activity of FbpC

Collectively, analyses based on sequence similarity (BLASTp), conserved domain (NCBI-CD), conserved motifs (ScanProsite and InterPro), and tertiary structure–based functional prediction (DALI and Foldseek) consistently suggested that FbpC possesses carboxypeptidase activity.

We therefore investigated whether rFbpC actually functions as a carboxypeptidase. Compared with BSA, carboxypeptidase B at 0.50–1.50 μmol/L and rFbpC at 1.00–1.50 μmol/L exhibited a statistically significant increases in relative activity, suggesting

Table 4 3D structural similarity of FbpC using DALI (Z-score > 11.9)

(A) Other regions (Domain 1, Domain 2, and additional regions)

Chain	Z score	RMSD (Å)	%ID	Description	Organisms
2bib-A	19.7	7.5	28	Teichoic acid phosphorylcholine esterase/choline	*Streptococcus pneumoniae*
7pl2-A	18.8	9.4	21	Putative endo-β-*N*-acetylglucosaminidase	*Streptococcus pneumoniae* R6
2wwd-A	15.9	4	26	1,4-β-*N*-acetylmuramidase	*Streptococcus pneumoniae* R6
4x36-A	14.4	9.9	29	Autolysin	*Streptococcus pneumoniae* TIGR4
9mf4-A	13.9	9.3	21	Toxin B	*Clostridioides difficile*
7ml7-A	13.4 1	1.1	23	Toxin B	*Clostridioides difficile*
2j8f-A	12.2 1	8.1	31	Lysozyme	*Streptococcus phage* Cp1
9mx1-A	12.0 1	6.7	26	Toxin A	*Clostridioides difficile*

(B) Domain 3

Chain	Z score	RMSD(Å)	%ID	Description	Organisms
1jqg-A	22.8	2.2	21	Carboxypeptidase A	*Helicoverpa armigera*
5hvf-A	22.4	2.1	16	Carboxypeptidase B2	*Homo sapiens*
4axv-A	21.3	2.2	22	MPAA	*Vibrio campbellii* CAIM 519 = NBRC 15631 = ATCC 25920
3k2k-A	18.9	2.4	22	Putative carboxypeptidase	*Burkholderia mallei* ATCC 23344
1uwy-A	18.8	2.4	20	Carboxypeptidase M	*Homo sapiens*
2qvp-C	17.7	2.5	16	Uncharacterized protein	*Shewanella amazonensis* SB2B
8v3q-A	16.8	2.5	17	Cytosolic carboxypeptidase-like protein 5	*Homo sapiens*
4oko-A	16.6	2.6	16	Rapid encystment phenotype protein 34 kDa	*Francisella tularensis* subsp. novicida U112
3lwu-A	15.4	2.7	17	Succinylglutamate desuccinylase/aspartoacylase	*Shewanella frigidimarina* NCIMB 400
2g9d-A	15.4	2.9	13	Succinylglutamate desuccinylase	*Vibrio cholerae* MO10
4wck-A	14.4	2.6	14	Conserved hypothetical secreted protein	*Helicobacter pylori* G27
3cdx-A	13.8	2.6	19	Succinylglutamate desuccinylase/aspartoacylase	*Cereibacter sphaeroides* 2.4.1
2qj8-B	12.2	2.7	22	MLR6093 protein	*Mesorhizobium japonicum* MAFF 303099
7b3n-B	12	3	11	Cell wall hydrolase	*Thermus parvatiensis*

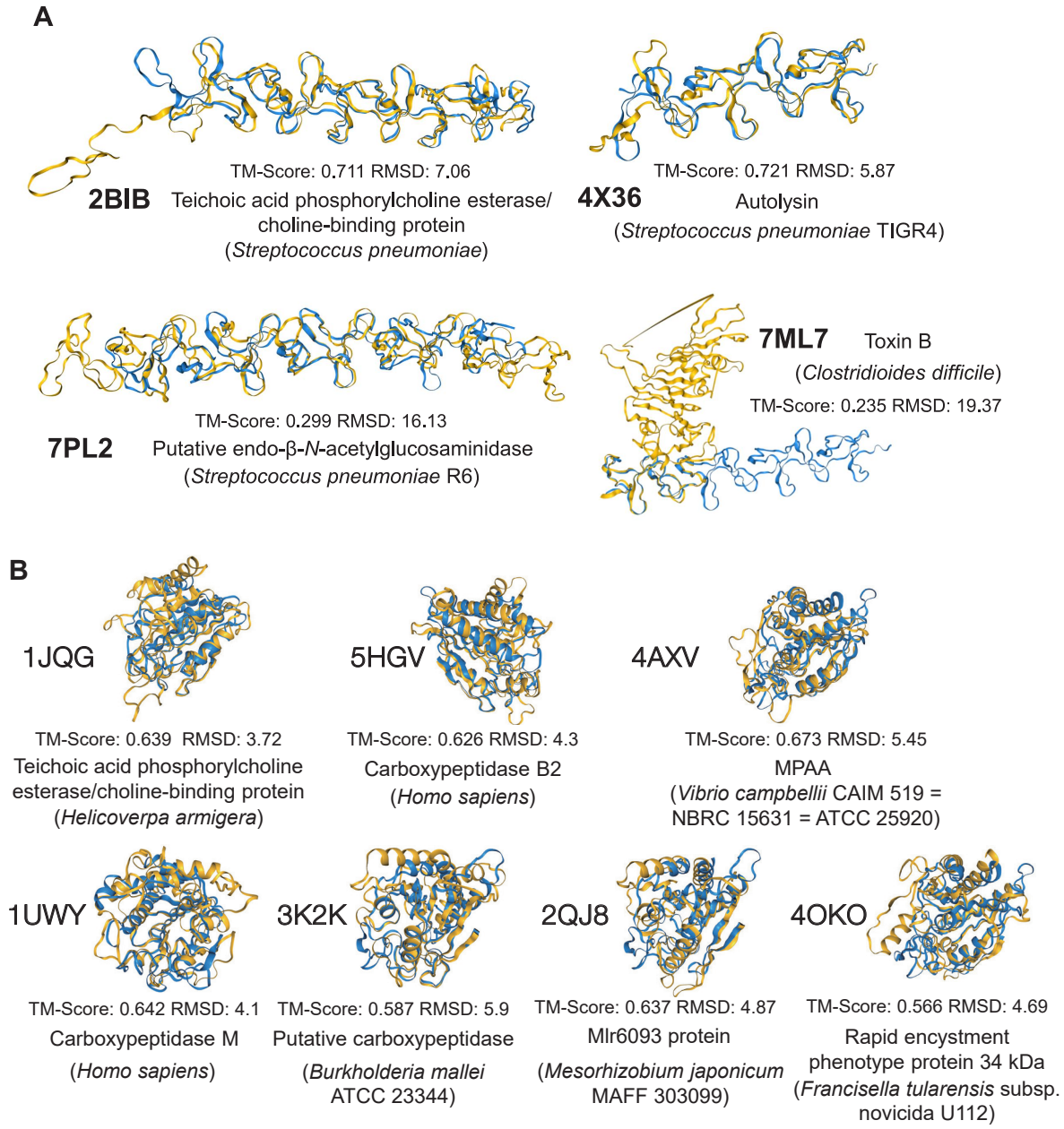

Fig. 3. Superposition of FbpC and proteins commonly predicted by both DALI (Z score > 11.9) and Foldseek. A, B) Superimposed structures of the predicted FbpC other regions (Domain 1, Domain 2, and additional regions) (A) and FbpC Domain 3 (B) with proteins matched by both DALI and Foldseek. FbpC domain and matched protein are shown as blue and beige, respectively. PDB ID, TM-score, RMSD, protein name, and organisms are shown in this figure.

that FbpC indeed possesses carboxypeptidase activity (Fig. 4).

Peptidoglycan hydrolases, including carboxypeptidase, play important role in bacterial cell division and proliferation[22,23]. Therefore, FbpC which possesses carboxypeptidase activity might be involved in *C. perfringens* cell proliferation.

Conclusion

We found that *fbpC* gene (CPE0625) possesses its own promotor. The gene was expressed in the exponential growth phase. FbpC was predicted to be both a cell wall-binding protein and a carboxypeptidase using physicochemical property analysis, similarity-based annotation, domain-based annotation, motif-based annotation, and tertiary structure–based

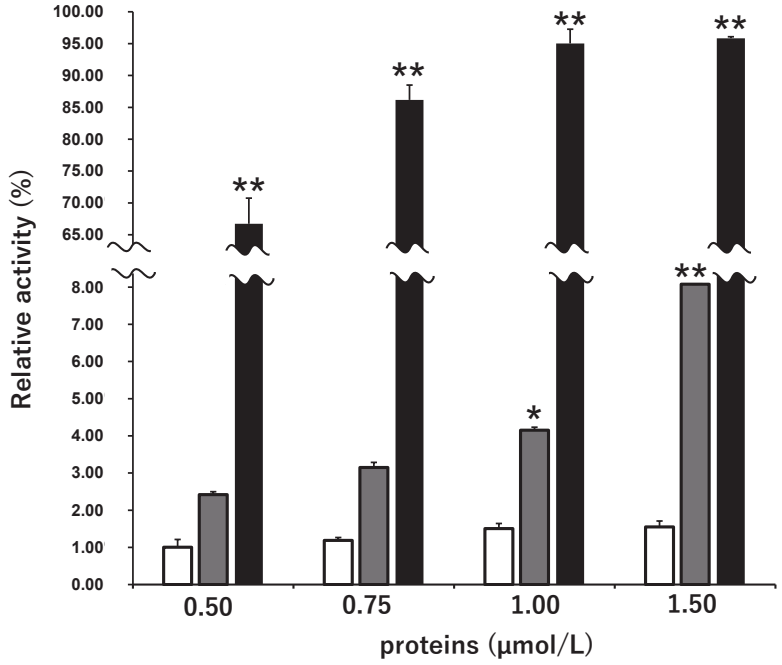

Fig. 4. Measurement of carboxy peptidase activity. The carboxypeptidase activity of BSA, rFbpC and carboxypeptidase B at 0.50–1.50 μmol/L as measured by acid carboxypeptidase activity kit. Open columns, gray columns, and closed columns indicate BSA, rFbpC, and carboxypeptidase B, respectively. Carboxypeptidase activity is determined by measuring color development of reduced nicotinamide adenine dinucleotide (NADH) in a two-step reaction, with absorbance measured at 450 nm. Relative activity (%) was calculated by setting the optical density at 450 nm of the standard solution to 100%. Data are shown as mean ± SD (n = 3). *$p < 0.05$ and **$p < 0.01$ versus BSA at each dose point (Holm's method).

functional analysis. Actually, FbpC was revealed to possess carboxypeptidase activity. These results suggest that FbpC is likely to be a carboxypeptidase associated with the cell wall, and might be involved in cell proliferation.

Conflicts of interest

The authors declare no conflict of interest.

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