〈Original Article〉

Novel cell wall–associated fibronectin-binding proteins of *Clostridium perfringens*

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Summary *Clostridium perfringens* is a Gram-positive anaerobic bacterium that causes food poisoning and gas gangrene. Human fibronectin (Fn) binds to *C. perfringens* cells. To date, only two Fn-binding proteins, FbpA and FbpB, have been identified in *C. perfringens*. However, we found that Fn also bound to null mutant cells lacking the *fbpA* and *fbpB* genes. Binding of Fn to these cells decreased following treatment with endolysin Psm, which cleaves the glucan chains in the cell wall of *C. perfringens*. This result suggested the presence of cell wall–associated Fn-binding proteins in addition to FbpA and FbpB. Ligand blotting analyses using biotinylated Fn revealed at least three Fn-binding proteins (Fbps; 56, 45, and 40 kDa) associated with the peptidoglycan layer of *C. perfringens*. We identified two genes, CPE0625 and CPE0630, which encode the 56- and 45-kDa Fbps, respectively. The 56- and 45-kDa Fbps were designated FbpC and FbpD, respectively. Biotinylated Fn bound to recombinant FbpC and FbpD as well as to recombinant FbpA. These proteins had no motifs common to other Fn-binding proteins. Therefore, we conclude that both FbpC and FbpD are novel *C. perfringens* cell wall–associated Fn-binding proteins.

Key words: *C. perfringens*, Human fibronectin, Fn-binding proteins, Cell wall

1. Introduction

Clostridium perfringens is a Gram-positive anaerobic bacterium that causes food poisoning and gas gangrene in humans and animals[1]. In order to induce gas gangrene, *C. perfringens* first invades the host through a wound or a surgical injury. Immediately following invasion, the bacterium is exposed to blood or body fluids containing many components, including fibronectin (Fn). Fn is a 450-kDa dimeric glycoprotein found in vertebrate body fluids, on cell surfaces, and in a variety of extracellular matrices[2]. Many pathogenic

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bacteria, including both Gram-positive and -negative species, express Fn-binding proteins (Fbps) on the cell surface in order to adhere to host cells[3, 4, 5].

Human Fn was shown to bind to three *C. perfringens* strains isolated from gas gangrene[6]. We previously cloned the genes (*fbpA* [CPE0737] and *fbpB* [CPE1847]) encoding FbpA and FbpB of *C. perfringens* strain 13[7]. These Fbps, however, had no signal peptides and were not detected on the cell surface[8]. Fn bound to a null mutant (MW5) lacking both the *fbpA* and *fbpB* genes[9]. These findings suggested that *C. perfringens* might express other Fbp(s) in addition to FbpA and FbpB.

In this work, we attempted to identify the novel Fbps associated with the cell wall by degradation of peptidoglycans from *C. perfringens* cells. We identified at least three Fbps on the cell surface of *C. perfringens*. The genes encoding two of these Fbps were also identified and cloned in this study. Fn bound to both recombinant Fbps, which were designated FbpC and FbpD.

2. Materials and methods

1. Bacterial strains, plasmids, and media

The strains and plasmids used in this study are listed in Table 1. *Clostridium perfringens* HN13[10] was derived from the wild-type strain 13[11], which was isolated from a case of human gangrene. Strain MW5 is a null mutant in which the *fbpA* and *fbpB* genes of HN13 were deleted[9]. The *C. perfringens* strains were grown anaerobically in Gifu anaerobic medium (GAM) (Nissui, Tokyo, Japan) or on GAM agar plates. *Eschericha coli* strains were grown in Luria-Bertani (LB) broth (Nakarai Tesque, Kyoto, Japan) or on LB agar plates. Antibiotics were used at the following concentrations: ampicillin (50 μg/ml) and chloramphenicol (10 μg/ml) for selection or (34 μg/ml) for gene overexpression.

2. Extraction of cell wall–associated proteins

The *C. perfringens* strains were grown to an optical density at 600 nm (OD_{600}) of 1.0 ± 0.1 at 37℃ in 25 ml of GAM medium. A 20-ml volume was removed from the culture and centrifuged at 8,000 rpm for 15 min at 4℃. The resulting cell pellet was washed with 20 mM phosphate-buffered saline (PBS), pH 7.4, and then resuspended in 2 ml of STMC buffer (500 mM sucrose, 50 mM Tris-HCl [pH 7.0], 25 mM $MgCl_2$, 25 mM $CaCl_2$). This suspension was divided into two aliquots (1 ml each), and 2 μl of recombinant endolysin Psm (1 mg/ml)[12] was added to one of the aliquots. All samples were then incubated at 37℃ for 10 min and then centrifuged at 16,000 × g for 10

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
***C. perfringens* strains**		
13	Type A wild-type strain isolated from human gas gangrene	11
HN13	13 Δ*galK* Δ*galT*	10
MW5	HN13 Δ*fbpA* Δ*fbpB*	9
SAK1	HN13 Δ*fbpC*	This study
SAK2	HN13 Δ*fbpD*	This study
***E. coli* strain**		
BL21-CodonPlus® (DE3) RIL	*E. coli* B F^- *ompT hsdS* (r_B^- m_B^-) *dcm*$^+$ Tet^r gal λ (DE3) *endA* Hte [*argU ileY leuW* Cm^r]	Stratagene
Plasmids		
pGALK	A vector for an in-frame deletion, *galK-Ca* (*Clostridium acetobutylicum*), Cm^r	11
pSAK3	pGALK carrying the 1-kb upstream and 1-kb downstream regions of *fbpC*	This study
pSAK4	pGALK carrying the 1-kb upstream and 1-kb downstream regions of *fbpD*	This study
pET11a	An expression vector of *E. coli*	Novagen
pNAK1	pET11a carrying His_6-tagged *fbpC*	This study
pNAK2	pET11a carrying His_6-tagged *fbpD*	This study

min. The pelleted cells were used in Fn-binding assays. Trichloroacetic acid (TCA) was added to the supernatants to a final concentration of 10%. The resulting precipitates were washed twice with acetone to remove the TCA and then dissolved in 100 μl of buffer (0.5 × SDS-PAGE sample buffer, 0.5 M Tris-HCl, pH 7.0).

3. Fn-binding assay

Binding of Fn to *C. perfringens* cells and recombinant proteins was evaluated using an enzyme-linked immunosorbent assay (ELISA) with biotinylated human Fn according to a previously described method[7].

4. SDS-PAGE and ligand blotting analysis using biotinylated Fn

Cell wall–associated proteins and recombinant proteins were electrophoresed on 10 or 12.5% SDS-PAGE gels under reducing conditions and then transferred to polyvinylidene difluoride (PVDF) membranes. Ligand blotting analyses were performed using biotinylated Fn, as described previously[7].

5. LC-MS/MS analysis

Silver-stained protein bands were excised from the gel and subjected to in-gel digestion with 10 μg/ml of trypsin (Promega, Madison, WI, USA) overnight at 37℃[13]. The resulting tryptic peptides were eluted with 0.1% formic acid and then subjected to LC-MS/MS analysis, which was performed on an LCMS-IT-TOF instrument (Shimadzu, Kyoto, Japan) interfaced with a nano reverse-phase LC system (Shimadzu). LC separation was performed using a Pico Frit Beta Basic C18 column (New Objective, Woburn, MA, USA) at a flow rate of 300 nl/min. Peptides were eluted from the column using a gradient of 5-45% acetonitrile in 0.1% formic acid and sprayed directly into the

Table 2 Oligonucleotide primers used in this study

Primer	Sequence
Overlapping PCR of the 1-kb upstream and 1-kb downstream regions of *fbpC*	
CN Fw	5'-AGCTCGGTACCCGGGGATCCTAGGAGCTACAACAATTTTAACTA-3'
	*Bam*HI
CM Rv	5'-ATAGTTTAGTTATCATTATTTTTACACTTGTCCACCTCTTTTTTTGAA-3'
	Start and stop anti-codons
CM Fw	5'-TTCAAAAAAAGAGGTGGACAAGTGTAAAAATAAGTATAACTAAACTAT-3'
	Start and stop codons
CC Rv	5'-AGTGCCAAGCTTGCATGCGATTGCTATTTTCTAACTTTGGTA-3'
	*Sph*I
Overlapping PCR of the 1-kb upstream and 1-kb downstream regions of *fbpD*	
DN Fw	5'-AGCTCGGTACCCGGGGATCCACTATGCTTGGAAAAGTGAACAAA-3'
	*Bam*HI
DM Rv	5'-AAAAATAAATTTTTATACATATTACATAATAACCTCCAATTTTTATAT-3'
	Start and stop anti-codons
DM Fw	5'-ATATAAAAATTGGAGGTTATTATGTAATATGTATAAAAATTTATTTTT-3'
	Start and stop codons
DC Rv	5'-AGTGCCAAGCTTGCATGCTCAACCAGCACATGAAGAAGAATT-3'
	*Sph*I
PCR cloning of His_6-tagged *fbpC*	
fbpC Fw	5'-TAAGAAGAAGATATACATATGCATCATCATCATCATCATGAAGGAAATAAGAACACAACTAAT-3'
	*Nde*I His_6-tag
fbpC Rv	5'-GCTTTGTTAGCAGCCGGATCCTTAGTTATTTAAAAGAATATTTAC-3'
	*Bam*HI
PCR cloning of His_6-tagged *fbpD*	
fbpD Fw	5'-TAAGAAGAAGATATACATATGCATCATCATCATCATCATCAGCCAACTAAAGAAGTATCTAAC-3'
	*Nde*I His_6-tag
fbpD Rv	5'-GCTTTGTTAGCAGCCGGATCCTTATTCTGGTAAATAGAAGAAATG-3'
	*Bam*HI

mass spectrometer. The heated capillary temperature and electrospray voltage were set at 200℃ and 2.5 kV, respectively. MS/MS data were acquired in the data-dependent mode using LCMS Solution software (Shimadzu) and converted to a single text file (containing the observed precursor peptide *m/z*, the fragment ion *m/z*, and intensity values) using Mascot Distiller (Matrix Science, Boston, MA, USA). The file was analyzed using Mascot MS/MS Ion Search (Matrix Science) to search the data and assign the obtained peptides in reference to the NCBI nonredundant database. The search parameters were set as follows: database, NCBInr; taxonomy, all; enzyme, trypsin; variable modifications, carbamidomethyl, oxidation, and propionamid; peptide tolerance, ±0.05 Da; and MS/MS tolerance, ±0.05 Da. For protein identification, the criteria were as follows: (1) Mascot scores above the statistically significant threshold ($p < 0.05$), and (2) at least one top-ranked unique peptide matching the identified protein.

6. In-frame deletion and PCR cloning of the *fbpC* and *fbpD* genes

The *fbpC* and *fbpD* genes were deleted using an in-frame (IF) deletion system, basically as reported by Nariya et al.[10]. The 1-kb upstream and 1-kb downstream regions of *fbpC* and *fbpD* were separately amplified by overlapping PCR using chromosomal DNA of *C. perfringens* strain 13 as the template, with appropriate primers (Table 2). The resulting amplicons were then cloned in tandem into the *Bam*Hl and *Sph*l sites of pGALK (Table 1). The resultant plasmids, pSAK3 and pSAK4 (Table 1), were used for deletion of either *fbpC* or *fbpD* of *C. perfringens* HN13 via recombination. Deletion of each gene was confirmed by sequencing around the respective deleted regions. The *fbpC* and *fbpD* deletion mutants were designated SAK1 and SAK2, respectively (Table 1).

The *fbpC* and *fbpD* genes were amplified by PCR using chromosomal DNA of *C. perfringens* strain 13 as the template, using primers (Table 2) with appropriate restriction sites and coding sequences for adding a His_6-tag to the N-terminus of the recombinant

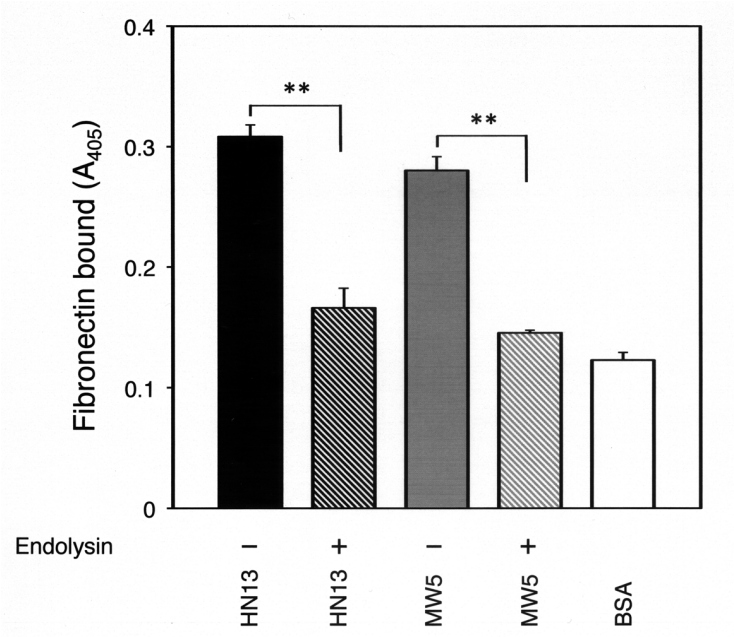

Fig. 1 Binding of Fn to *C. perfringens* cells after endolysin Psm treatment. The wells of microtiter plates were coated with 100 ml of cell suspension (OD 600 = 0.5 ± 0.1) treated (+) or not treated (−) with endolysin Psm. After blocking, 1 μg of biotinylated Fn was added to each well. After incubation at 37℃ for 30 min, binding of Fn molecules to the cells was assessed by ELISA as described in the Materials and Methods. Data are presented as the mean and standard deviation of 3 determinations. $^{**}p < 0.01$.

protein. DNA fragments of the PCR products were cloned into pET11a (Novagen, Merck KGaA, Darmstadt, Germany), an *E. coli* expression vector. The resultant plasmids were designated pNAK1 and pNAK2, respectively (Table 1). The *E. coli* strain BL21-CodonPlus® (DE3) RIL was transformed with each plasmid, and then each gene was overexpressed in the transformed *E. coli* cells. Recombinant FbpC (rFbpC) and FbpD (rFbpD) were purified using the N-terminal His_6-tags (Fig. 5A). Recombinant FbpA (rFbpA) was prepared according to a previously described method[7].

7. Protein assay

Protein concentration was measured using a Qubit® 2.0 Fluorometer and Qubit® protein assay kit (Invitrogen, Carlsbad, CA, USA), using bovine serum albumin (BSA) as the standard.

3. Results

The binding of Fn to *C. perfringens* cells was examined by ELISA using biotinylated Fn, which showed that Fn molecules bound to both *C. perfringens* HN13 (13 *ΔgalK ΔgalT*) and MW5 (HN13 *ΔfbpA ΔfbpB*) cells (Fig. 1). Cells of both strains were treated with endolysin Psm, which is an *N*-acetylmuramidase from the episomal phage phiSM101[12]. Binding of Fn to cells of both *C. perfringens* strains treated with endolysin decreased significantly ($p < 0.01$) compared with the respective controls, comparable to the level of Fn that bound to BSA (Fig. 1), suggesting that Fbp(s) other than FbpA and FbpB are associated with the peptidoglycan layer of the cell wall of *C. perfringens*.

To examine the possibility that other Fbp(s) are associated with the cell wall, *C. perfringens* cells were again treated with endolysin Psm, and proteins within the supernatants were precipitated with 10% TCA and then subjected to SDS-PAGE. The resulting gel was used for ligand blotting analysis with bitotinylated Fn. Two major bands (56 and 45 kDa) and a minor band (40 kDa) were detected in the supernatants of only HN13 and MW5 cells treated with endolysin (Fig. 2B). The major two bands (56- and 45-kDa Fbps) were designated FbpC and FbpD, respectively.

To identify the genes encoding FbpC and FbpD, the 56- and 45-kDa bands were excised from a silver-stained SDS-PAGE gel and then subjected to LC-MS/MS analysis. The results of this analysis suggested that the 56-kDa protein (FbpC) was either a putative pyruvate kinase or hypothetical protein CPE0625 and that the 45-kDa protein (FbpD) was phosphoglyc-

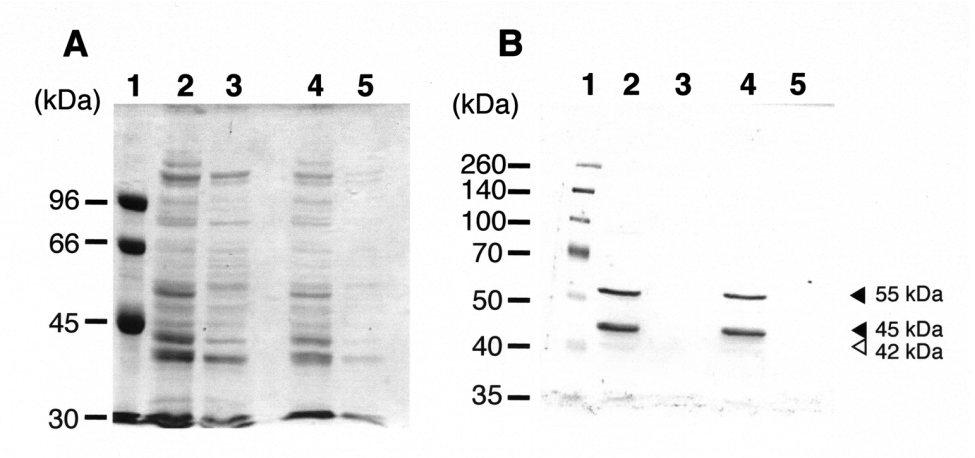

Fig. 2 Binding of Fn to *C. perfringens* cell wall–associated proteins. (A) Coomassie Blue–stained 10% SDS-PAGE gel. After treatment of *C. perfringens* cells with (+) or without (−) endolysin Psm, the supernatants were applied to the gel. (B) Ligand blot analysis with biotinylated Fn. Lane 1, molecular weight markers; lane 2, HN13 (+); lane 3, HN13 (−); lane 4, MW5 (+); lane 5, MW5 (−). Note that two Fbps bands (closed arrowheads) and a weakly staining band (open arrowhead) were detected just after treatment with endolysin in strains HN13 and MW5.

Table 3 Identification of Fbps from *C. perfringens* strain 13 by MS/MS of selected bands

Band (kDa)	Protein name	Molecular weight (Da)	Score	NCBI* ID
56	pyruvate kinase	49,440	445	gi:18309344
	hypothetical protein CPE0625	63,329	413	gi:18309697
45	phosphoglycerate kinase	42,652	522	gi:18310285
	maltose ABC transporter	43,952	508	gi:18311325
	hypothetical protein CPE0630	50,573	451	gi:18309612

*National Center for Biotechnology Information.

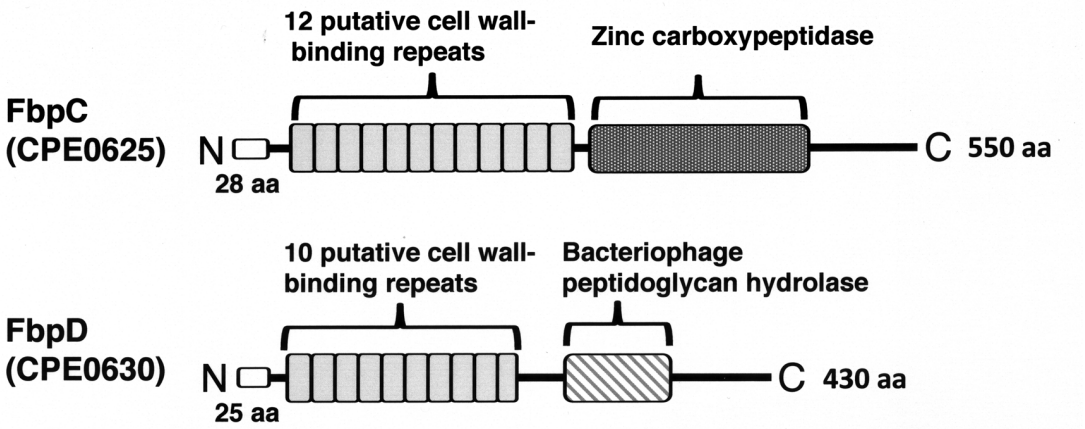

Fig. 3 Motifs of FbpC (CPE0625) and FbpD (CPE0630) as determined by SSDB motif search. Open boxes at the N-termini denote signal peptides.

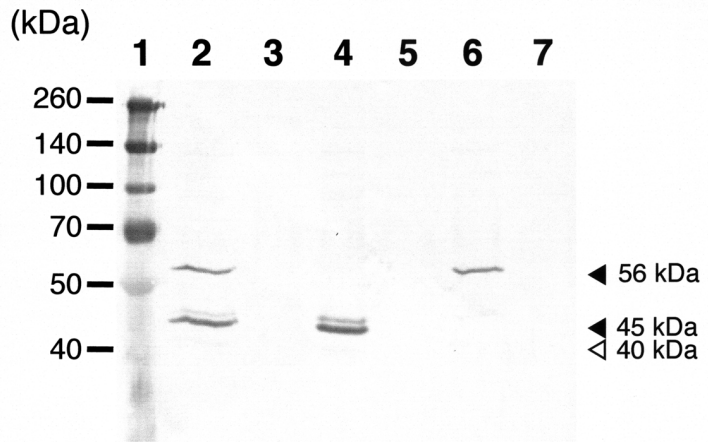

Fig. 4 Fn-ligand blot analysis of the cell wall–associated proteins from the *fbpC* and *fbpD* deletion mutants. Endolysin treatment (+); no endolysin treatment (−). Lane 1, molecular weight markers; lane 2, HN13 (+); lane 3, HN13 (−); lane 4, SAK1 (+); lane 5, SAK1 (−); lane 6, SAK2 (+); lane 7, SAK2 (−). Note that SAK1 (HN13 *ΔfbpC*) did not produce the 56-kDa FbpC and SAK2 (HN13 *ΔfbpD*) did not produce the 45-kDa FbpD.

erate kinase, maltose ABC transporter, or hypothetical protein CPE630 (Table 3). Pyruvate kinase and phosphoglycerate kinase are cytoplasmic enzymes. Maltose ABC transporter is localized in the cytoplasmic membrane. Analyses using Signal Peptide Prediction (http://bmbpcu36.leeds.ac.uk/prot_analysis/Signal.html) and SSDB motif search (www.genome.jp/kegg/) suggested that both CPE0625 and CPE0630 have signal peptides, putative cell wall–binding repeats, and a peptidase and peptido-

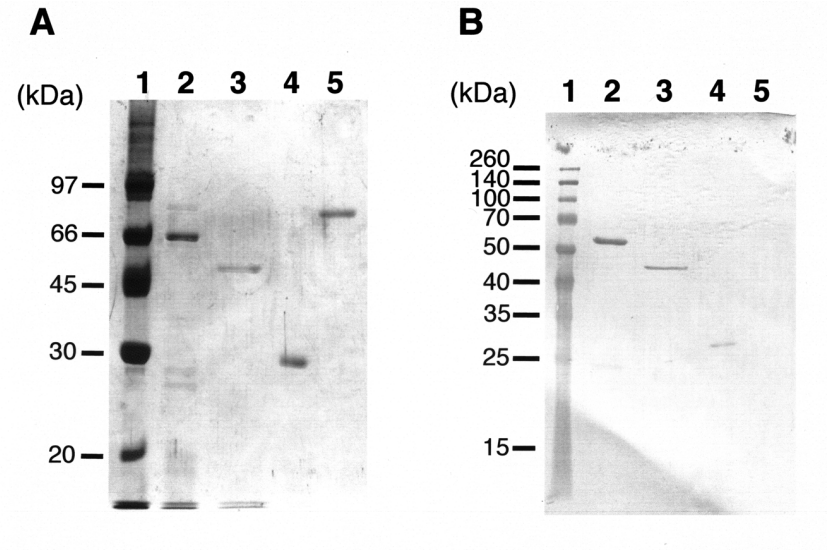

Fig. 5 Binding of Fn to recombinant proteins examined by Fn-ligand blot analysis. (A) Coomassie Blue–stained 12.5% SDS-PAGE gel. A total of 2 μg of protein was applied to each lane of the gel. (B) Fn-ligand blot analysis. Lane 1, molecular weight markers; lane 2, rFbpC; lane 3, rFbpD; lane 4, rFbpA; lane 5, BSA. Note that Fn molecules bound to rFbpC, rFbpD, and rFbpA (used as a positive control) but not to BSA, which was used as a negative control.

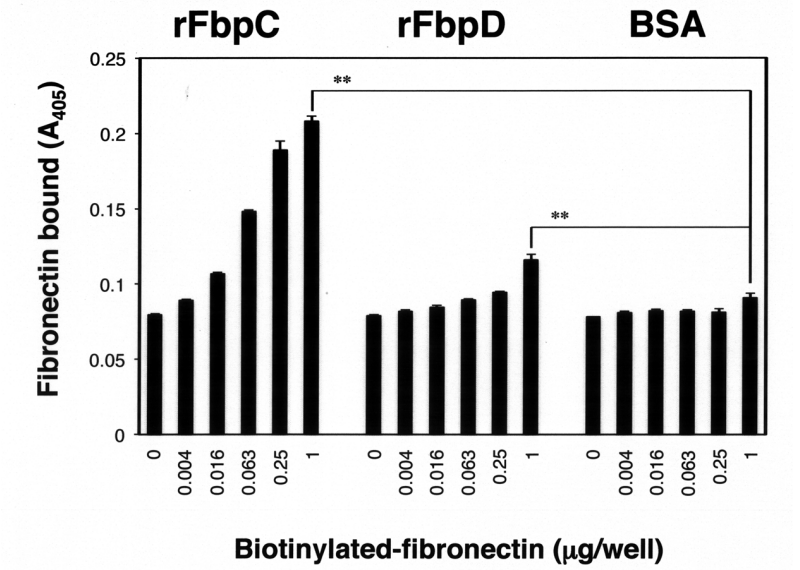

Fig. 6. Dose-dependent binding of Fn to the intact recombinant proteins, as determined by ELISA. A total of 2 μmoles of protein was allowed to adhere to each well of a microtiter plate. The binding of biotinylated Fn to the proteins was examined by ELISA as described previously[7]. Data are presented as the mean and standard deviation of 3 determinations. $^{**}p < 0.01$.

glycan hydrolase motif, respectively (Fig. 3).

To confirm whether the genes encoding CPE0625 and CPE0630 encode FbpC and FbpD, the genes were deleted in strain HN13 using an in-frame deletion system[12] as described in the Materials and Methods. Neither deletion mutant (designated SAK1 and SAK2 [Table 1]) expressed FbpD or FbpC, respectively (Fig. 4), indicating clearly that the genes encoding CPE0625 and CPE0630 encode FbpC (56 kDa) and FbpD (45 kDa), respectively.

Fn-ligand blot analyses clearly demonstrated binding of Fn not only to rFbpC and rFbpD, but

also to rFbpA, which was used as a positive control (Fig. 5B). Furthermore, Fn also bound to both rFbpC and rFbpD in ELISA in a significant dose-dependent manner (Fig. 6). In addition, rFbpC appeared to exhibit higher Fn-binding activity than rFbpD. These results clearly showed that FbpC and FbpD are Fn-binding proteins.

4. Discussion

In this work, we found that in addition to FbpA and FbpB[7], *C. perfringens* HN13 expresses at least two novel Fbps, which we designated FbpC and FbpD. These newly identified Fbps are associated with the *C. perfringens* cell wall. The genes encoding FbpC and FbpD were identified as CPE0625 and CPE0630, respectively, and were designated *fbpC* and *fbpD*. CPE0625 encodes a 63-kDa hypothetical protein, whereas CPE0630 encodes a 51-kDa hypothetical protein (Table 3). The estimated apparent molecular weights of FbpC and FbpD as determined by ligand blotting analyses were 56 and 45 kDa, respectively (Fig. 2B), which in both cases are lower than the estimated apparent molecular weights of FbpC and FbpD without their signal peptides (61 and 48 kDa). Both FbpC and FbpD appeared to migrate abnormally in the SDS-PAGE gel, although the reason for this abnormal migration was unclear. In-frame deletion analyses of *fbpC* and *fbpD*, however, indicated clearly that the genes encode 56- and 45-kDa Fbps, as determined from ligand blots (Fig. 4). A weakly staining band of 40 kDa was observed in some ligand blotting analyses using biotinylated Fn (Figs. 2B, 4B). The possibility remains that the 40-kDa minor band (Fig. 2B) is also an Fbp.

The results of the FbpC and FbpD SSDB motif search showed that neither protein contains a Fn-binding repeat (FnBR) homologous to staphylococcal or streptococcal FnBRs[4] or a LPXTG motif[14] that facilitates covalent binding to cell wall peptidoglycan. Instead, the results of this search suggested that FbpC contains 12 putative cell wall–binding repeats (17-19 amino acids per repeat)[15] at the N-terminus and a zinc carboxypeptidase domain at the C-terminus. The SSDB motif search also suggested that FbpD contains 10 putative cell wall–binding repeats (17-19 amino acids per repeat)[15] at the N-terminus and a bacteriophage peptidoglycan hydrolase (amidase) domain at the C-terminus (Fig. 3). These results imply that these Fbps are secreted via their signal peptides and that they bind to the peptidoglycan layer of the cell wall via the putative cell wall–binding repeats. Indeed, after washing *C. perfringens* cells with SDS-PAGE buffer containing 250 mM NaCl, FbpC (but not FbpD) was recovered in the washing buffer (data not shown). These data support the hypothesis that some FbpC molecules are localized on the cell surface through noncovalent bonds involving the putative cell wall-binding repeats. Although both Fbps contain either a peptidase or peptidoglycan hydrolase motif, it is not clear whether the proteins have enzymatic activity. As these Fbps exhibit Fn-binding activity and reside in the peptideoglycan layer or on the cell surface (Figs. 4, 5), it is believed that they contribute to the binding of Fn to *C. perfringens* cells.

The complete genomic sequences of *C. perfringens* strains 13[16], ATCC13124 (NCTC8237)[17], and SM101[17] have been determined. BLASTP analysis of FbpC and FbpD indicated that ATCC13124 and SM101 have homologous genes (CPF_0606 and CPF_0611 in ATCC13124; CPR_0591 and CPR_0597 in SM101, respectively). Accordingly, the genes encoding FbpC and FbpD seem to be conserved among *C. pefringens* strains. These genes were all annotated as cell wall–associated peptidases or peptidoglycan hydrolases (amidases), suggesting that C. perfringens FbpC and FbpD are multi-functional proteins that bind to the cell wall and then degrade the peptides of the peptidoglycan and also attach to Fn molecules in the environment.

In conclusion, we found that in addition to FbpA and FbpB, *C. perfringens* expresses two novel cell wall–associated Fn-binding proteins, which we designated FbpC and FbpD. As the functions of these proteins remain to be clarified, we plan to elucidate the contribution of these Fbps to Fn-binding in *C. perfringens*.

Conflicts of interest

The authors report no conflicts of interest.

Acknowledgments

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