<Brief Note>

Autolysins involved in conformation-dependent fibronectin binding to dry-fixed *Clostridium perfringens*

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Summary The pathogen *Clostridium perfringens* exploits fibronectin (Fn) to mediate adhesion to collagen. Our previous study reported that Fn under low ionic strength (l-Fn) bound significantly to dry-fixed *C. perfringens* cells compared with Fn under high ionic strength (h-Fn), and that *C. perfringens* autolysin (Acp) bound to Fn under moderate ionic strength (m-Fn). Our previous study and this study revealed that m-Fn bound to recombinant Acp C-terminal catalytic domain (rAcpCD) and to rAcpCD plus eight cell wall-binding repeats (rAcpCWB3-10+CD) but not to recombinant six cell wall-binding repeats. l-Fn binding to rAcpCWB3-10+CD exhibited saturable binding and reached a plateau at a low concentration. That of m-Fn exhibited linear, unsaturated binding across all concentration tests. That of h-Fn exhibited an attenuated concentration dependency with reduced slope. Moreover, the binding pattern of Fn to dry-fixed *C. perfringens* cells was also similar. However, all three Fn showed saturable binding to rAcpCD and reached a plateau at a low concentration. These results pointed to a possibility that the binding of Fn to dry-fixed *C. perfringens* cells involves AcpCD + cell wall-binding repeats.

Key words: Fibronectin, *Clostridium perfringens*, Fibronectin-binding protein, Autolysin

Introduction

Clostridium perfringens is an anaerobic gram-positive spore-forming bacterium that causes gas gangrene[1]. The virulence of *C. perfringens* is associated with its ability to adhere to injured tissue and produce various toxins[1]. We previously reported that *C. perfringens* cells adhered to type II collagen, type III collagen, and gelatin only when they were prebound with fibronectin (Fn), but not to collagen and gelatin alone[2]. Therefore, adhesion of *C. perfringens* cells to host connective tissue involves Fn.

Fn, found in blood and on cell surfaces, plays a key role in several cellular processes, including wound healing, tissue structure formation, and cell migration. Fn is a 230-270-kDa glycoprotein, typically existing as a dimer and covalently linked by a pair of disulfide bonds at the C-terminus. The monomer polypeptide of Fn comprises 12 Type Ⅰ, 2 Type Ⅱ, and 15-17 Type Ⅲ modules. Fn can adopt several different configurations ranging from

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compact to extended conformations in solution. Factors such as changes in pH, high ionic strength, or the presence of glycerol induce Fn to adopt an extended conformation[3-5]. In contrast, Fn adopts a compact conformation under low-ionic strength conditions[4, 5].

Fn acts as a ligand for several receptors and hence it is a common target for several bacteria[6]. Many bacteria express Fn-binding proteins (Fbps) that promote colonization and host infection via adhesion to Fn[6, 7]. In our previous report, we identified Acp (CPE1231), a peptidoglycan hydrolase required for cell division in *C. perfringens*, as an Fn binding protein[8].

Moreover, we revealed that Fn under low ionic strength conditions bound significantly more strongly to dry-fixed *C. perfringens* HN13 cells (strain 13 Δ*galK* Δ*galT*) [9] than Fn under high ionic strength conditions[10], suggesting that compared with the extended conformation (extended Fn), the compact conformation of Fn (compact Fn) preferentially binds to dry-fixed *C. perfringens* HN13 cells.

Recognition of the *C. perfringens* cell wall by Acp, which degrades peptidoglycan, is essential for cell division[11]. The Acp molecule comprises three regions, including a signal peptide, 10 tandemly repeated bacterial Src homology, domains (cell wall-binding repeats [CWBRs]) functioning as the cell wall-binding domain, and a catalytic domain (AcpCD) belonging to the glycoside hydrolase family 73 with glucosaminidase activity (Fig. 1)[11]. Our previous study using ligand blotting assay demonstrated that Fn under moderate ionic strength bound to recombinant AcpCD (rAcpCD) and to recombinant AcpCWB3-10+CD (rAcpCWB3-10+CD) (Fig. 1), which contains eight CWBRs and an AcpCD, but not to recombinant AcpCWB1-6 (rAcpCWB1-6) (Fig. 1), which contains six CWBRs[8].

To date, it remains unclear whether Acp recognizes compact Fn and/or extended Fn. In this study, we investigated the binding of various Fn conformations to rAcpCD, rAcpCWB3-10+CD, and dry-fixed *C. perfringens* HN13 cells.

Materials and methods

Bacterial strains and growth conditions

C. perfringens HN13[9] cells were derived from the wild-type strain 13[12], which was isolated from soil. The *C. perfringens* HN13 cells used for all experiments were cultured in Gifu anaerobic medium (GAM; Nissui Co., Tokyo, Japan) broth under anaerobic conditions using an AnaeroPack system (Mitsubishi Gas, Tokyo, Japan).

Preparation of recombinant proteins and Fn

His_6-tagged rAcpCWB3-10+CD, rAcpCWB1-6, and rAcpCD were prepared as described previously[13, 14]. Strain BL21-CodonPlus RIL (Stratagene, La Jolla, CA) harboring the plasmids pColdAcpHCWB3[13], pColdAcpCWB1-6H[13],

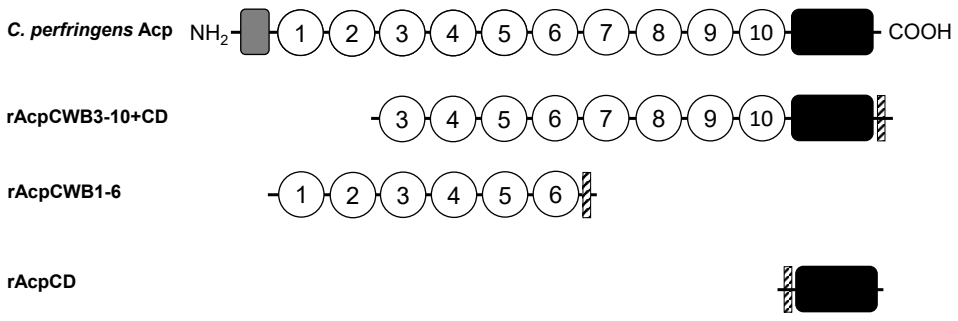

Fig. 1. Schematic of *C. perfringens* Acp and Acp fragments from this study. *C. perfringens* Acp comprises a signal peptide, 10 CWBRs, and a catalytic domain. The gray square, open circles, closed edge rectangle, and diagonal hatching represent signal peptide, CWBR, catalytic domain, and His_6-tag, respectively. rAcpCWB3-10+CD comprises eight CWBRs and a catalytic domain with a C-terminal His_6-tag, while rAcpCWB1-6 consists of six CWBRs with a C-terminal His_6-tag. rAcpCD possess six CWBRs and a catalytic domain with a N-terminal His_6-tag.

and pColdAcpCD[14] was used to express C-terminal His_6-tagged 8 CWBRs with AcpCD (AcpCWB3-10+CD), C-terminal His_6-tagged 6 CWBRs (AcpCWB1-6), and the N-terminal His_6-tagged AcpCD, respectively (Fig. 1).

Fn was purified from pooled human serum using a gelatin-Sepharose column[15]. Elution was performed with 4 mol/L urea in 5 mmol/L veronal buffer (VB) (pH 7.4) containing 50 mmol/L NaCl (50 mmol/L NaCl VB, VBS), 140 mmol/L NaCl (140 mmol/L NaCl VB), or 250 mmol/L NaCl (250 mmol/L NaCl VB). The purified Fn was then dialyzed against the corresponding buffer. Fn preparations in 50 mmol/L NaCl VB, 140 mmol/L NaCl VB, and 250 mmol/L NaCl VB were named as low ionic strength Fn (l-Fn), moderate ionic strength Fn (m-Fn), and high ionic strength Fn (h-Fn), respectively.

Biotinylation of Fn and recombinant proteins

Fn (1 mg) and recombinant proteins (1 mg each) were incubated with 50 μL of 1.63 mg/mL biotin-amidohexanoic acid 3-sulfo-N-hydroxysuccinimide ester (Dojindo Laboratories, Kumamoto, Japan) in dimethyl sulfoxide for 1 h at room temperature. Subsequently, 0.5 mol/L Tris glycine (pH 7.5) was added to a final concentration of 0.1 mol/L, followed by incubation at room temperature for over 15 min. After incubation, the buffer surrounding biotinylated Fn was replaced with 50 mmol/L NaCl VB, 140 mmol/L NaCl VB or 250 mmol/L NaCl VB, and then, unreacted biotin was removed using a desalting column (Cytiva, Tokyo, Japan).

Preparation of dry-fixed *C. perfringens* HN13 cells

Dry-fixed *C. perfringens* HN13 cells were prepared as follow. *C. perfringens* HN13 cells were cultured in GAM broth under anaerobic conditions until reaching an optical density at 600 nm (OD_{600}) of 0.5 ± 0.1. The cells were then harvested, washed three times with 140 mmol/L NaCl VB, and then resuspended in the same buffer to OD_{600} = 1.0. A 100-μL aliquot of the cell suspension was transferred to an EIA plate and then incubated for 48–72 h at 37 ℃.

Enzyme-linked avidin-biotin complex system (ELABC)

All binding assays were performed in EIA plates (Corning, Corning, NY), with wells individually coated with purified protein or dry-fixed *C. perfringens* HN13 cells. After blocking with Bovine serum albumin (BSA; FUJIFILM Wako Pure Chemical Co., Osaka, Japan), dry-fixed *C. perfringens* HN13 cells were incubated with or without biotinylated l-Fn, m-Fn, or h-Fn (0.3–5 μg/well).

rAcpCWB3-10, rAcpCWB1-6, rAcpCD, or BSA was coated onto EIA plates. For protein-coated wells, 50 μL of protein solution at a concentration of 0.02 mg/mL in 10 mmol/L borate buffer (pH 8.5) was added to indicated wells and incubated for 30 min at room temperature. After blocking with BSA, the wells were incubated with or without biotinylated l-Fn, m-Fn, or h-Fn (0.3–5 μg/well) for 1 h at room temperature.

Bound Fn was detected as follows. Wells were washed with 20 mmol/L phosphate-buffered saline (pH 7.4) containing 0.02% (v/v) Tween 20 (PBST) and incubated with 100 μL horseradish peroxidase (HRP)–streptavidin (1:1,000 dilution; Vector Laboratories, Burlingame, CA) in 20 mmol/L Tris-buffered saline (TBS, pH 7.2) for 30 min at room temperature. After washing with PBST, color was developed by adding 100 μL of 0.91 mmol/L 2,2' azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) in 0.1 mol/L citrate buffer (pH 4.1) containing 0.03% H_2O_2. The reaction was stopped using 100 μL of 0.1 mol/L citric acid containing 0.01% NaN_3. The absorbance of each well was then measured at 405 nm using a microplate reader (iMark; Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA). Data are presented as the mean ± standard deviation (SD). Statistical comparisons were performed using one-way analysis of variance followed by a Student's *t*-test or Bonferroni multiple comparison test. A p-value of <0.05 was considered statistically

significant.

Results

The binding of m-Fn to recombinant Acp fragments

Using ligand blotting analysis, we previously reported that m-Fn binds to rAcpCWB3-10+CD and rAcpCD [8]. In this study, we compared the binding strength of m-Fn to rAcpCWB3-10+CD, rAcpCD, and rAcpCWB1-6 using the ELABC assay. m-Fn bound significantly to rAcpCD and rAcpCWB3-10+CD compared to BSA, but not to rAcpCWB1-6 (Fig. 2). Furthermore, m-Fn binding to rAcpCD was increased approximately 3-fold compared with that of rAcpCWB3-10+CD (Fig. 2).

The binding of Fn under various ionic strength to dry-fixed *C. perfringens* HN13 cells and recombinant Acp fragments

Fn binding affinity to bacterial cells is affected by pH[16]. Because the environmental conditions alter Fn conformation, the expression of Fn-binding regions changes. Our previous study reported that l-Fn binds more strongly to dry-fixed *C. perfringens* HN13 cell than h-Fn[10]. Thus, we evaluated the binding affinity of l-Fn, m-Fn, and h-Fn to dry-fixed *C. perfringens* HN13 cells, rAcpCWB3-10+CD, and rAcpCD using the ELABC system. l-Fn at 0.3–5 μg/well bound 3.4–6.7 times to dry-fixed *C. perfringens* HN13 cells more strongly than BSA (Fig. 3A). The binding of m-Fn at 0.3–5 μg/well was 1.8–4.0 times higher, and that of h-Fn at 0.3–5 μg/well was 1.3–2.7 times higher (Fig. 3B and C). Particularly, l-Fn binding to dry-fixed *C. perfringens* HN13 cells reached a plateau at 1 μg/well (Fig. 3A), whereas m-Fn and h-Fn exhibited dose-dependent binding to dry-fixed *C. perfringens* HN13 cells (Fig. 3B and C). Therefore, we investigated whether l-Fn, m-Fn, and h-Fn bound to rAcpCWB3-10+CD and rAcpCD in dose-dependent manner. l-Fn at 0.3–5 μg/well bound 6.5–10.6 times to rAcpCWB3-10+CD more strongly than BSA (Fig. 3D). The binding of m-Fn at 0.3–5 μg/well was 3.0–4.9 times higher, and that of h-Fn at 0.3–5 μg/well was 2.2–3.3 times higher (Fig. 3E and F). l-Fn binding to rAcpCWB3-10+CD plateaued at 1 μg/well (Fig. 3D), while m-Fn and h-Fn showed dose-dependent binding from 0.3–5 μg/well (Fig. 3E and F). Interestingly, this binding pattern was similar to that observed for dry-fixed *C. perfringens* HN13 cells (Fig. 3 A-C). l-Fn at 0.3–5

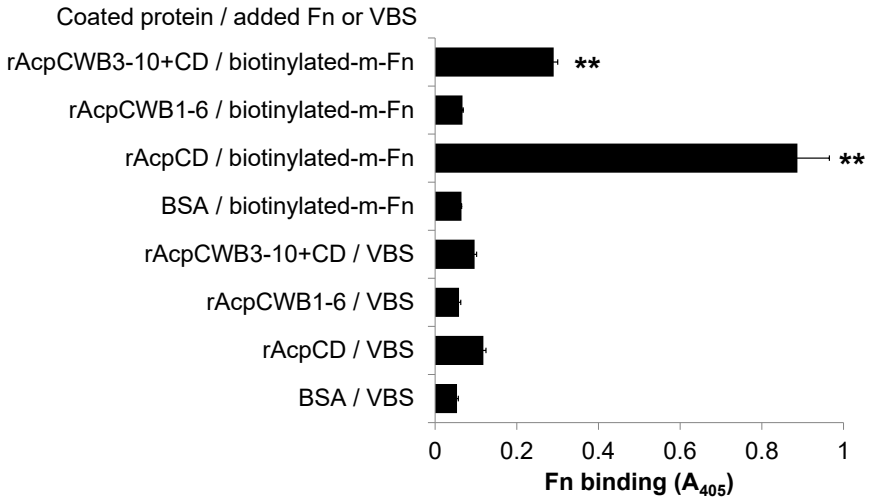

Fig. 2. m-Fn binding to various rAcp fragments. rAcpCWB3-10+CD (1 μg/well), rAcpCWB1-6 (1 μg/well), rAcpCD (1 μg/well), and BSA (1 μg/well) were coated onto microplate wells and then incubated with biotinylated m-Fn (1 μg/well). Following several washes, streptavidin-HRP was added, and bound biotinylated m-Fn was detected using a colorimetric assay. No biotinylated m-Fn was added to each control wells (veronal buffered saline: VBS). Data are shown as the mean ± SD. (n=3). **$p < 0.01$ versus BSA/biotinylated m-Fn. (Bonferroni pair-wise multiple-comparison test).

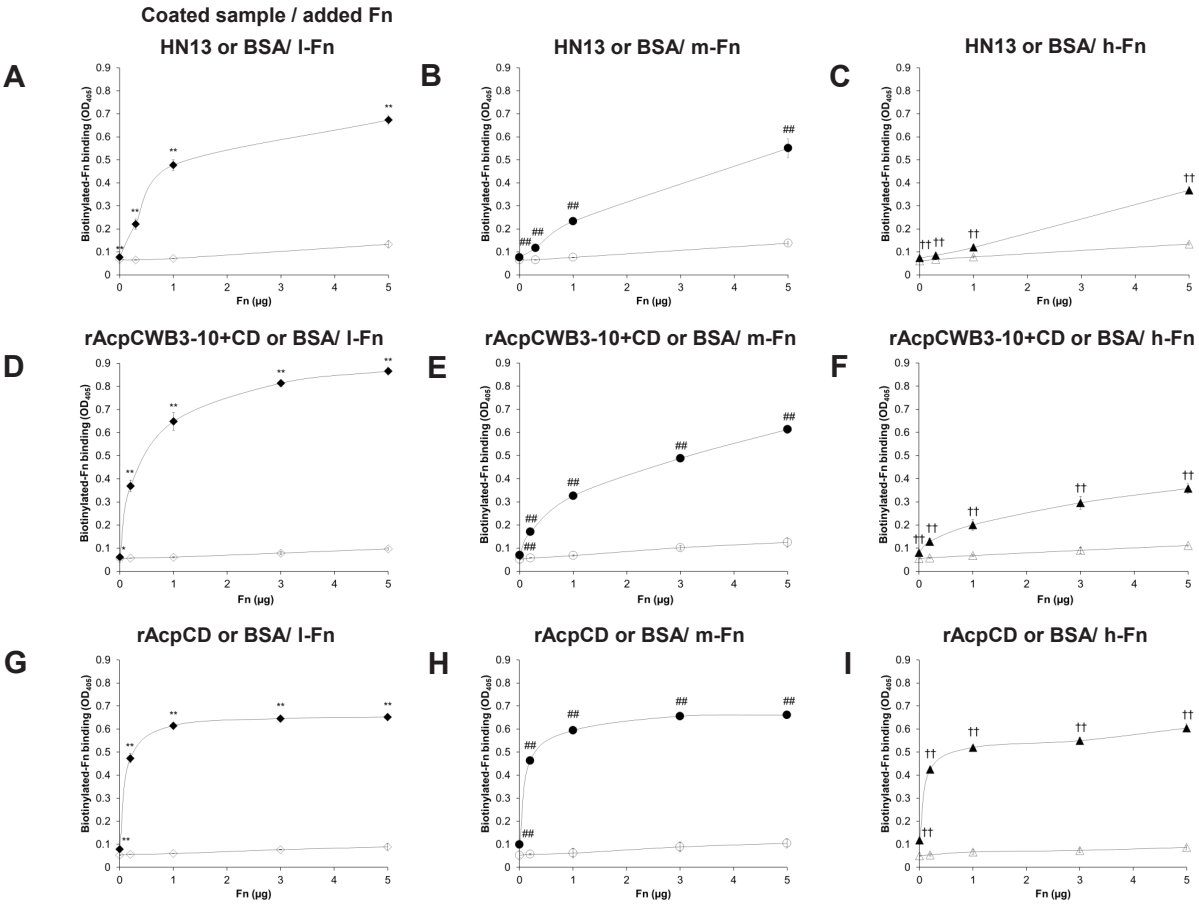

Fig. 3. Effect of ionic strength on Fn binding to dry-fixed HN13 cells or rAcp fragments. The coated dry-fixed HN13 cells, rAcpCWB3-10+CD (1 μg/well), rAcpCD (1 μg/well) and BSA (1 μg/well) were allowed to react with biotinylated l-Fn, m-Fn, or h-Fn in a dose-dependent manner. After several washes, HRP-streptavidin was added, and binding was detected using a colorimetric assay. BSA was used as a blocking reagent. Closed diamonds, closed circles, and closed triangles indicate the binding of biotinylated l-Fn (A, D, and G), m-Fn (B, E, and H), and h-Fn (C, F, and I) to coated samples (A-C: dry-fixed *C. perfringens* HN13 cells, D-F: rAcpCWB3-10+CD, G-I: rAcpCD), respectively. Open diamonds, open circles, and open triangles indicate the binding of biotinylated l-Fn (A, D, and G), m-Fn (B, E, and H), and h-Fn (C, F, and I) to coated BSA, respectively. BSA was used as a control for nonspecific binding. Data are shown as the mean ± SD (n = 3). *, $p < 0.05$ and **, $p < 0.01$ versus the binding of biotinylated l-Fn to BSA at each dose point. ##, $p < 0.01$ versus the binding of biotinylated m-Fn to BSA at each dose point. ††, $p < 0.01$ versus the binding of biotinylated h-Fn to BSA at each dose point (Student's t-test).

μg/well bound 7.3–10.3 times to rAcpCD more strongly than BSA (Fig. 3G). The binding of m-Fn at 0.3–5 μg/well was 6.3–9.6 times higher, and that of h-Fn at 0.3–5 μg/well was 7.0–7.8 times higher (Fig. 3H and I). The binding pattern of Fn to rAcpCD was different from those to dry-fixed *C. perfringens* HN13 cells and to rAcpCWB3-10+CD, and the binding of all Fn (l-Fn, m-Fn, and h-Fn) to rAcpCD plateaued at 1 μg/well (Fig. 3 G-I).

Discussion

In this study, we revealed that the binding affinity of Fn under low ionic strength conditions to dry-fixed *C. perfringens* HN13 cells involves Acp.

Fn is often targeted for adhesion by several pathogenic bacteria, including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus*

dysgalactiae, *Borrelia burgdorferi*, *Staphylococcus equisimilis*, *Clostridioides difficile*, and *Listeria monocytogenesis*[6]. Their adhesins for Fn, termed Fbps, are over 100 across bacterial species[6]. We previously identified three proteins, FbpC (CPE0625), FbpD (CPE0630), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (CPE1304), that can bind to Fn on the surface of *C. perfringens* cells[13, 17]. However, Fn bound to *fbpC* and *fbpD* knockout mutants as well as to their parent strain[8], suggesting that FbpC and FbpD are not essential for Fn binding the host wound site.

Autolysins from other pathogens, such as Aaa from *S. aureus* and AtlA from *Streptococcus mutans* have reported to be involved in Fn binding [18, 19]. Consistently, our results showed that m-Fn significantly bound to recombinant autolysin of *C. perfringens*, rAcpCWB3-10+CD and rAcpCD, compared with BSA (Fig. 2)[8]. Moreover, Acp knockout mutants exhibited significantly decreased Fn binding, while complementation with the *acp* gene recovered binding to parental levels[8]. These findings indicate that Acp plays a central role in mediating Fn binding by *C. perfringens* cells.

SFS, *Streptococcus equi* Fbps, binds stably to compact Fn compared with extended Fn, because of its two identical subsites separated by 17 residues that simultaneously target Fn $I_{8\text{-}9}$ in both subunits of the Fn dimer[20]. This observation supports our data that l-Fn, compact Fn under low ionic strength, bound more strongly to rAcpCWB3-10+CD than m-Fn and h-Fn (Fig. 3 D-F). This result is consistent with our previously observation that l-Fn showed stronger binding to dry-fixed *C. perfringens* HN13 cells compared with m-Fn and h-Fn[10]. Interestingly, all Fn isoforms (l-Fn, m-Fn, and h-Fn) at low concentration exhibited sufficient binding to rAcpCD (Fig. 3 G-I). Furthermore, the dose-dependent binding patterns of l-Fn, m-Fn, and h-Fn to dry-fixed *C. perfringens* HN13 cells (Fig. 3 A-C) were similar to that with rAcpCWB3-10+CD (Fig. 3 D-F). These results suggest that the recognition of Fn to dry-fixed *C. perfringens* HN13 cells involves both AcpCD and CWBRs.

In conclusion, this study demonstrated that Acp is one of the essential Fbp for dry-fixed *C. perfringens* HN13 cells, as was case living bacterial cells. Our previous report and this study showed that the compact Fn binds more strongly to dry-fixed *C. perfringens* cell than the extended Fn[10]. Moreover, the findings point to a possible involvement of AcpCD+CWBRs in the binding of Fn to dry-fixed *C. perfringens* cells, suggesting that dry-fixed *C. perfringens* HN13 cells may express not only AcpCD but also partial CWBRs on their surface. However, our previous report showed that non-fixed *C. perfringens* HN13 cells bound substantially to each of l-Fn-, m-Fn-, and h-Fn-prebound gelatin [10]. Furthermore, extracellular matrix-binding protein from, an *S. epidermidis* Fbps, selectively binds to extended Fn in fibrillated form while ignoring compact Fn[21], which is different from the binding pattern observed in this study. *C. perfringens* cells may express multiple Fbps, including GAPDH in addition to Acp, enabling recognition of different forms of Fn. Such versatility could suggest a sophisticated strategy for facilitating infection at different stages and in distinct host environments. Future research should address how other, unknown thus far, Fbps and GAPDH on the *C. perfringens* cell surface contribute to Fn binding and determine the specific Fn conformations they preferentially bind.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgments

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