

Short Communication

Detection of Fibronectin-Binding Proteins in *Clostridium perfringens*

Seiichi Katayama*, Nanami Nozu, Masako Yokoyama, and Yasuo Hitsumoto

Department of Life Sciences, Okayama University of Science, Okayama 700-0005, Japan

Clostridium perfringens is an anaerobic spore-forming pathogen of humans and animals. *C. perfringens* type A strains, 13, CPN50, and NCTC8237, isolated from human gas gangrene, bound specifically to human fibronectin (Fn). The trypsin-treatment of the bacterial cells significantly reduced the Fn-binding. A ligand blotting analysis of all three *C. perfringens* strains revealed that 5 protein bands of 34 kDa, 29 kDa, 26 kDa, 17 kDa, and 12 kDa specifically bound to biotinylated Fn. These results suggest that *C. perfringens* possesses certain Fn-binding proteins on the cell surface.

Key words: human fibronectin, fibronectin-binding protein, *Clostridium perfringens* (*C. perfringens*)

C *lostridium perfringens*, a gram-positive, sporulating, anaerobic pathogen of humans and animals, causes gas gangrene and food poisoning [1]. In gas gangrene, *C. perfringens* invades from wounds or surgical injuries into a host body. Immediately after the invasion, the bacterium is exposed to blood or body fluid containing many components including fibronectin (Fn).

Fn is a 450 kDa dimeric glycoprotein found in body fluids, on the cell surface, and in a variety of extracellular matrices. Fn comprises several domains, each of which specifically binds to other substances such as fibrin, collagen, gelatin, DNA, and heparin [2, 3]. Furthermore, Fn is known to have many biological activities, including the promotion of cell adhesion, activation of CD4 cells, and enhancement of phagocytosis [4, 5].

It is well known that a number of bacteria, includ-

ing *Staphylococcus* [6], *Streptococcus* [7-11], *Listeria* [12-14], and *Mycobacterium* [7], have fibronectin-binding proteins (Fbps). The function of these Fbps is considered to facilitate attachment of these pathogens to host cells [7], and to act as invasins that allow them to enter into host cells [6]. Kreutz *et al.* reported that 40 to 51% of the strains of 18 *Clostridium* species, including *C. perfringens* and *C. difficile*, could agglutinate Fn-coated latex particles [15]. The complete sequencing of the *C. perfringens* 13 genome has been performed and all the open reading frames deduced from the sequence were annotated [16]. This annotation indicated that 2 genes of CPE1847 and CPE0737 (accession No. BA000016) encode 2 putative Fbps of 67 kDa and 25 kDa respectively. The amino acid sequence of the former putative Fbp is homologous (41% identity) to the 68 kDa Fbp of *C. difficile* [17]. The latter putative Fbp is homologous (29% identity) to the 25 kDa Fbp of *L. monocytogenes* [12]. The Fbps of *C. perfringens*, however, have not yet been detected.

In this work, we investigated whether *C. perfrin-*

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*Corresponding author. Phone: +81-86-256-9691; Fax: +81-86-256-9559
E-mail: katayama@dbc.ous.ac.jp (S. Katayama)

gens type A 13, CPN50 (known as BP6K-N5), and NCTC8237, all isolated from human gas gangrene, were able to bind to human Fn. *Streptococcus pyogenes* SSI-1 isolated from a patient with a toxic shock-like syndrome, which has a kind of Fbp named FbaB [9], was used as a positive control. In addition, *S. pyogenes* TR-47, which has a disrupted *fbaB* gene (FbaB⁻) [9], was used as a negative control. *C. perfringens* and *S. pyogenes* cells were grown in Gifu anaerobic medium (GAM) broth (Nissui Co., Tokyo, Japan) and Todd-Hewitt broth (Becton, Dickinson and Company, Sparks, MD, USA) supplemented with 0.2% yeast extract (THY), respectively. In the case of *S. pyogenes* TR-47, kanamycin (300 $\mu\text{g}/\text{ml}$) was added to the THY medium for antibiotic selection. The cells that grew to an optical density at 600 nm of 0.5 were harvested and then washed with phosphate-buffered saline (PBS) 3 times. The cells were resuspended in PBS to an optical density at 600 nm of 0.5. Multiwell microtiter plates (Nunc-immunoplate Maxisorp F96, Nunc, Roskilde, Denmark) were coated with 100 μl of this bacterial suspension per well, or 100 μl of PBS per well as a blank, by being dried overnight at 37 $^{\circ}\text{C}$, and then washed with PBS 3 times. The wells were blocked with 300 μl of 2% (w/v) bovine serum albumin (BSA) in PBS by incubation for 2 h at 37 $^{\circ}\text{C}$. After washing with PBS containing 0.05% (v/v) Tween 20 (PBST), either human biotinylated Fn purified from human serum by a gelatin-affinity column or biotinylated BSA was added. After incubation for 2 h at 37 $^{\circ}\text{C}$ followed by washing with PBST, horseradish peroxidase (HRPO)-streptavidin (1:5,000; Southern Biotech, Birmingham, Ala, USA) was added and reacted for 10 min at room temperature. After washing, the wells were subjected to color development by the addition of 0.1 ml of 0.91 mM 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) in 0.1 M citrate (pH 4.1) containing 0.04% (v/v) H_2O_2 . The reaction was stopped by the addition of 0.1 ml of 0.1 M citric acid containing 0.01% (w/v) NaN_3 . Then the absorbance at 405 nm was measured by a microplate reader (SPECTRAMax 340 C, Molecular Devices Corp., Sunnyvale, CA, USA).

Biotinylated Fn, but not biotinylated BSA, bound to *S. pyogenes* SSI-1 (Fig. 1A) and all three *C. perfringens* strains (13, CPN50, and NCTC8237) (Fig. 1B). By contrast, biotinylated Fn bound less to *S.*

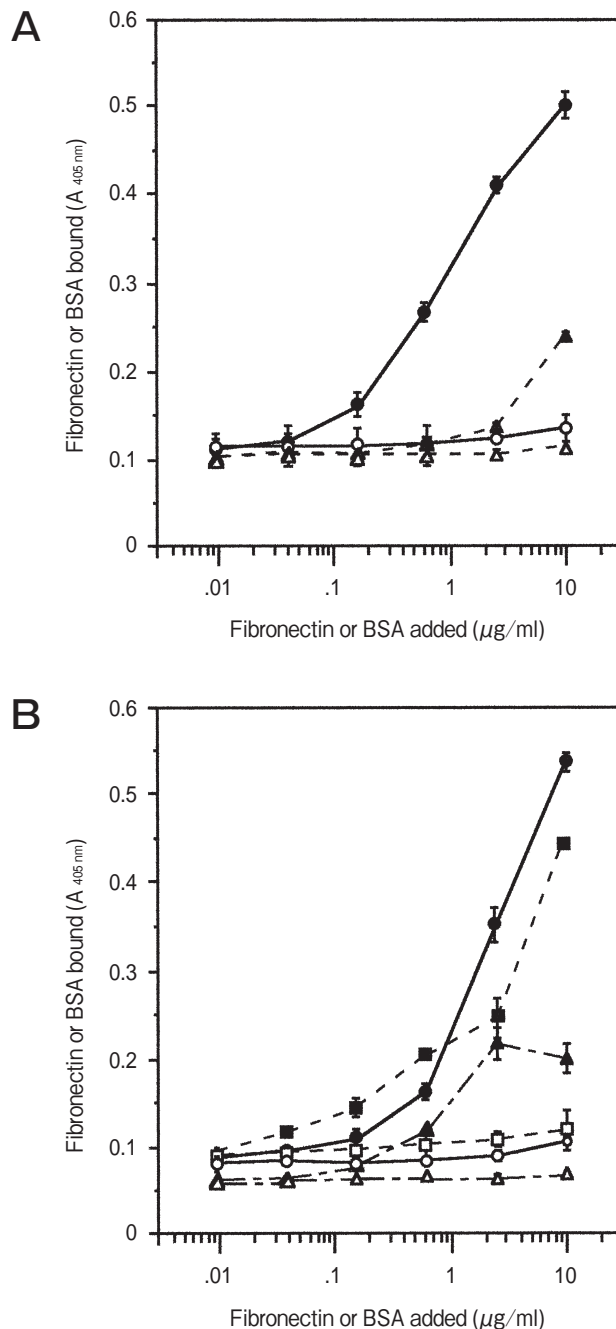


Fig. 1 Binding of Fn to *S. pyogenes* and *C. perfringens* cells. The data are presented as the means of 3 determinations \pm the standard deviations. (A) *S. pyogenes* SSI-1 (wild type): Fn (\bullet), BSA (\circ); *S. pyogenes* TR-47 (FbaB⁻): Fn (\blacktriangle), BSA (\triangle). (B) *C. perfringens* 13: (\bullet), BSA (\circ); *C. perfringens* CPN50: Fn (\blacksquare), BSA (\square); *C. perfringens* NCTC8237: Fn (\blacktriangle), BSA (\triangle).

pyogenes TR-47 (FbaB⁻) (Fig. 1A). This Fn binding to the *C. perfringens* cells was specific because the biotinylated Fn bindings were competitively inhibited in the presence of an excess amount of native Fn (data not shown). These results suggested that all three *C. perfringens* strains have certain receptor(s) that bind to Fn specifically.

To test whether the receptor(s) were protein or not, *S. pyogenes* and *C. perfringens* cells were digested with trypsin. The microtiter plate coated with *S. pyogenes* or *C. perfringens* cells were incubated for 2.5 h at 37 °C with either PBS or a solution containing trypsin (Sigma, St. Louis, MO, USA) at 10 mg/ml and 10 mM CaCl₂ in 20 mM Tris-HCl (pH 7.4) (200 μl/well), and then the Fn binding assay was performed as described above. Fig. 2 shows that the Fn bindings to the trypsin-treated *C. perfringens* cells were significantly reduced ($p < 0.01$ using Student's *t*-test) as were the Fn bindings to the trypsin-treated *S. pyogenes* SSI-1. This indicates that the Fn-binding receptor(s) are proteinaceous.

A ligand blotting analysis was performed to detect the Fbps of *C. perfringens*. *S. pyogenes* cells were

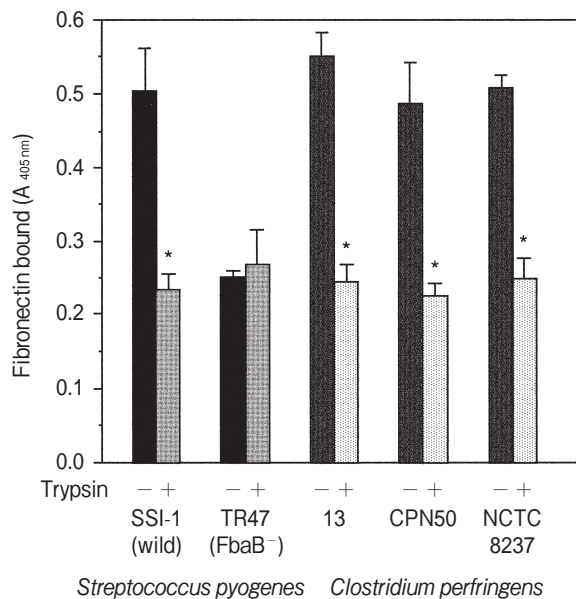


Fig. 2 Effect of trypsin-treatment of *S. pyogenes* and *C. perfringens* cells on Fn-binding. The bacterial cells were immobilized on a microtiter plate and then treated with trypsin (+) or without trypsin (-). The data are presented as the means of 3 determinations \pm the standard deviations. * $p < 0.01$.

used as controls. The bacteria grown in 100 ml appropriate media described above up to an optical density at 600 nm of 1.0 were harvested, and then washed with PBS. The cells were resuspended in 10 ml of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 % (v/v) glycerol (TEG) containing 1 mM phenylmethylsulfonyl fluoride (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The cells were then lysed by passage through a French Press (twice at 10,000 p.s.i.). After centrifugation at 1,500 \times g for 30 min, the precipitates were dissolved in sodium dodecylsulfate (SDS) sample buffer. A portion of these samples (20 μg of protein in each lane) and biotinylated low molecular weight markers (Bio-Rad, Hercules, CA, USA) were subjected to an SDS - 12 or 14% polyacrylamide gel electrophoresis (SDS-PAGE) under a non-reducing condition. The protein concentrations of the precipitates were measured using a bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL, USA) with BSA as a standard. The electrophoresed components were then transferred from the polyacrylamide gel to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) using a transblot unit (Sartorius, Goettingen, Germany). The transblotted PVDF membrane was blocked with casein blocking buffer (Sigma) for 2 h at room temperature, and then incubated with biotinylated Fn (10 μg/ml) in PBST containing 10% (v/v) casein blocking buffer for 2 h at room temperature. In a competitive analysis, the membrane was incubated with both biotinylated Fn (10 μg/ml) and native Fn (200 μg/ml) under the same condition as above. In the case of *S. pyogenes*, 1% (w/v) BSA was used instead of 10% (v/v) casein as a blocking reagent. After washing with PBST, the membrane was incubated with HRP-streptavidin (1:5,000) in PBST containing 10% (v/v) casein blocking buffer for 20 min at room temperature. After washing with PBST, the membrane was subjected to color development with 0.25 mg/ml 3,3'-diaminobenzidine (Sigma) in 50 mM Tris-HCl (pH 8.0) containing 0.01% (v/v) H₂O₂. The reaction was stopped as described above.

The competitive analysis of *S. pyogenes* revealed that the FbaB (88 kDa and 80 kDa) and M3 protein (55 kDa) were detected as ligand-specific bands, as Terao *et al.* reported [12] (Figs. 3A and 3B). As shown in Figs. 3C and 3D, the competitive analysis of *C. perfringens* revealed that at least 5 kinds of spe-

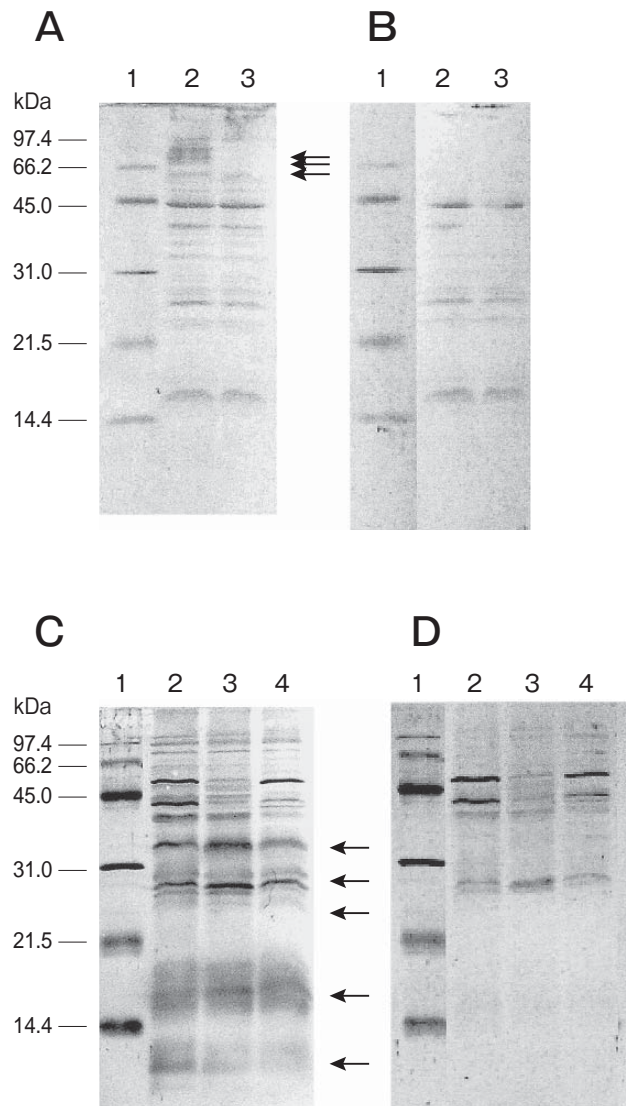


Fig. 3 Specific binding of biotinylated human Fn to some proteins of *S. pyogenes* and *C. perfringens* cells. **(A)** Ligand blotting analysis. Lanes: 1, molecular weight makers; 2, *S. pyogenes* SSI-1; 3, *S. pyogenes* TR-47. The bands of FbaB (88 kDa and 80 kDa) and the M3 protein (55 kDa) are indicated by arrows. **(B)** Competitive analysis. Lanes: 1, molecular weight makers; 2, *S. pyogenes* SSI-1; 3, *S. pyogenes* TR-47. **(C)** Ligand blotting analysis. Lanes: 1, molecular weight makers; 2, *C. perfringens* 13; 3, *C. perfringens* CPN50; 4, *C. perfringens* NCTC8237. The bands that specifically bound to Fn are indicated by arrows. **(D)** Competitive analysis. Lanes: 1, molecular weight makers; 2, *C. perfringens* 13; 3, *C. perfringens* CPN50; 4, *C. perfringens* NCTC8237.

cific Fn-binding bands (34 kDa, 29 kDa, 26 kDa, 17 kDa and 12 kDa) were detected in all three *C. perfringens* strains.

Here we indicated that all three *C. perfringens* type A strains isolated from human gas gangrene were able to bind specifically to Fn (Fig. 1). At least 5 kinds of Fn-binding protein bands (34 kDa, 29 kDa, 26 kDa, 17 kDa, and 12 kDa) were detected in each strain by a ligand blotting analysis and a competitive analysis (Figs. 3C and 3D). Among those protein bands, the 26-kDa band may be the putative 25 kDa Fbp (CPE0737). The apparent molecular weights of the other Fn-binding bands were, however, different from that of putative 67 kDa Fbp (CPE1847). It is possible that these Fbps are the products of other novel genes than both putative 67 kDa and 25 kDa Fbps. If not, then these Fbps might be the degradative products of the putative 67 kDa and 25 kDa Fbps.

The role Fbps plays in the infection of *C. perfringens* is unknown at the moment, although it has been reported that Fbps in some bacteria facilitate bacterial adherence to host tissue and bacterial colonization [6, 7]. Identification of the Fbps detected here would provide more information on their function in the infection of *C. perfringens*.

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