**Lactobacillus casei** Acquires the Binding Activity to Fibronectin by the Expression of the Fibronectin Binding Domain of *Streptococcus pyogenes* on the Cell Surface

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Abstract

Fibronectin binding domain was expressed on the cell surface of *Lactobacillus casei* strain Shirota which hardly adheres to fibronectin. DNA for the fibronectin binding domain of the *sfbI* gene, which encodes a fibronectin binding protein of *Streptococcus pyogenes* ATCC 21059, was amplified with polymerase chain reaction, cloned into a surface display vector pSAK332, and introduced into *L. casei*. The fibronectin binding domain was expressed as a fusion protein consisting of staphylokinase of *Staphylococcus aureus* and the anchor sequence of cell wall-associated 763 proteinase of *Lactococcus lactis* NCDO 763. The fibronectin binding ability of the resulting *L. casei* was confirmed with Western blot analysis, immunoelectron microscopic analysis, and adherence to fibroblast cells. These results indicate that *L. casei* has acquired a new phenotype to bind fibronectin upon the expression of the fibronectin binding domain on the cell surface. This *L. casei* also shows binding affinity to fibrinogen, indicating that fibronectin binding domain is involved in the binding to fibrinogen as well.

Introduction

Lactobacilli have been used for fermentation of dairy products. They have recently drawn much attention as probiotic bacteria for their beneficial effects on human health (Havenaar and Huis in ‘t Veld, 1992) and also been recognized as GRAS (generally recognized as safe) (Salminen et al., 1998). It has been reported that *Lactobacillus casei* strain Shirota has antitumor activity (Matsuzaki et al., 1985) and protective effect against bacterial infections (Nomoto et al., 1985) in preclinical studies. The administration of heat-killed *L. casei* strain Shirota to the model mouse of superficial bladder cancer showed high antitumor effect (T. Takahashi, unpublished).

Morales et al. (1976) first reported the use of intravesical *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) in the treatment of superficial bladder cancer, and prospective randomized trials have shown intravesical BCG to be a new treatment of superficial bladder cancer (Lamm et al., 1991). However, instillation of live organisms of BCG is occasionally associated with serious systemic side effects such as fever, serum-like syndromes, granulomatous toxicity, sepsis and even death (Lamm et al., 1992). Ratliff et al. (1988) have demonstrated that antitumor effects of BCG are due to activation of immune responses which are mediated by attachment of BCG to the bladder wall through fibronectin. *L. casei* strain Shirota, in contrast, exhibits no detectable binding to fibronectin. We could, therefore, expect higher antitumor activity of *L. casei* strain Shirota which is genetically engineered to acquire binding capacity to fibronectin.

Fibronectin binding proteins have been well characterized and several genes were cloned and sequenced for *Staphylococcus aureus* (Signäs et al., 1989, Jönsson et al., 1991), *Streptococcus dysgalactiae* (Lindgren et al., 1993), and *Streptococcus pyogenes* (Sela et al., 1993, Talay et al., 1994, Kreikemeyer et al., 1995). In the case of *S. pyogenes*, a gene coding for the fibronectin binding protein was designated as *sfbI* (Talay et al., 1992), *sfbII* (Kreikemeyer et al., 1995) or *prtF* (Hanski and Caparon, 1992). The amino acid sequences of SfbI and PrtF are 70% homologous. Fibronectin binding proteins of staphylococci and streptococci have a common feature that the repetitive sequences, which are abundant in acidic amino acid residues, play a major role for fibronectin binding.

We show here that fibronectin binding domain including four repeats of a unit of 37 amino acid residues is expressed on the cell surface of *L. casei* strain Shirota, and that the resulting *L. casei* acquires a new phenotype for binding to both fibronectin and fibrinogen. The increased adhesion of the recombinant *L. casei* to fibroblast cells is also discussed.

Results

Construction of the Plasmid pSAKStb14

A new surface display vector called pSAK332 (Figure 1) has a coding sequence for the preform (a signal sequence plus a mature form consisting of 163 amino acid residues) of staphylokinase protein (SAK) of *S. aureus* (Sako and Tsuchida, 1983) which is juxtaposed to the cell wall anchor sequence of the C-terminal 332 amino acid residues of cell wall-associated 763 proteinase of *Lactococcus lactis* NCDO 763 (Kiwaki et al., 1989). The anchor sequence contains sorting signals such as an LPXTG motif, hydrophobic domain, and a positively charged tail, which are commonly observed in gram-positive bacterial cell wall-
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associated proteins (Schneewind et al., 1992). *L. casei* harboring pSAK332 successfully expressed SAK on its cell surface and showed fibrinolysis activity (M. Kiwaki, personal communication). These results indicate that both the signal and anchor sequences function properly in *L. casei*, and that a functional domain can be expressed on the cell surface as a fusion protein with SAK and anchor sequence.

Signäs et al. (1989) reported that synthetic peptides mimicking the repetitive sequence of fibronectin binding protein of *S. aureus* interact with fibronectin. This suggests that no rigid three-dimensional structure is required for fibronectin binding. We have picked up the *sfbI* gene of *S. pyogenes* ATCC 21059 to be cloned into a surface display vector, because *S. pyogenes* ATCC 21059 is available on commercial source.

The plasmid pSAK332 has a SalI site between SAK and the anchor sequence, and this SalI site is a cloning site for this surface display vector. DNA for the fibronectin binding domain (Asn-364 through Thr-526 of SfbI protein as shown Figure 1) was amplified with polymerase chain reaction (PCR) using Sp01 and Sp02 primers which are designed to include SalI and XhoI site at the 5' region, respectively. The PCR product was digested with SalI and XhoI, and the digested product was then cloned into a SalI site of pSAK332. The resulting plasmid pSAKSfb14 was sequenced to verify that the fibronectin binding domain is inserted in the same frame as SAK and the anchor sequence (Figure 1). The plasmid pSAKSfb14 was then introduced into *L. casei* strain Shirota by electroporation.

**Detection of Fibronectin Bound on the Cell Surface of *L. casei* Strain Shirota/pSAKSfb14**

Fibronectin was incubated with *L. casei* strain Shirota (parental strain), *L. casei* strain Shirota/pSAK332 (vector plasmid not carrying the *sfbI*) and *L. casei* strain Shirota/pSAKSfb14, and then bacterial cells were washed to remove nonspecifically bound fibronectin, and were boiled to release fibronectin from the cells to the supernatant. The supernatants were separated with SDS-PAGE and fibronectin was detected by Western blot analysis. A large
amount of fibronectin was recovered from *L. casei* strain Shirota/pSAKSfb14 (Figure 2, lane 2), whereas fibronectin was not observed from either *L. casei* strain Shirota or *L. casei* strain Shirota/pSAK332 (Figure 2, lane 3, 4). The electrophoretic mobility of fibronectin released from *L. casei* strain Shirota/pSAKSfb14 was similar to that of fibronectin added to the bacteria, indicating that the fibronectin molecules are bound to *L. casei* strain Shirota/pSAKSfb14 (Figure 2, lane 2, 5). *L. casei* strain Shirota/pSAK332, which expresses SAK on its cell surface, did not trap fibronectin (Figure 2, lane 4), implying that SAK and C-terminal anchor sequence are not involved in binding to fibronectin.

The supernatant of cell culture of *L. casei* strain Shirota/pSAKSfb14 was precipitated by trichloroacetic acid and the binding capacity of precipitated proteins was analysed by Western blotting. Several proteins showed fibronectin binding ability. This is probably due to excretion of the fusion protein from the cell surface of *L. casei* strain Shirota/pSAKSfb14. However, any corresponding fragments were not detectable by Coomassie staining (data not shown). The amount of the fusion protein excreted from cell surface would be a low level.

### Table 1. Binding activity of *Lactobacillus* strains and *S. pyogenes*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Bacterial strain</th>
<th>Specific activity (units/10⁷ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibronectin</td>
<td><em>L. casei</em> strain Shirota/pSAKSfb14</td>
<td>7.80</td>
</tr>
<tr>
<td></td>
<td><em>L. casei</em> strain Shirota/pSAK332</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td><em>L. casei</em> strain Shirota</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td><em>L. acidophilus</em> YIT 0070</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td><em>L. rhamnosus</em> YIT 0227</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td><em>L. helveticus</em> YIT 0083</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td><em>S. pyogenes</em> ATCC 21059</td>
<td>12.84</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td><em>L. casei</em> strain Shirota/pSAKSfb14</td>
<td>2.77</td>
</tr>
<tr>
<td></td>
<td><em>L. casei</em> strain Shirota/pSAK332</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td><em>L. casei</em> strain Shirota</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td><em>S. pyogenes</em> ATCC 21059</td>
<td>44.72</td>
</tr>
</tbody>
</table>

\[
\text{units} = \frac{\Delta A_{650} \times v}{\varepsilon \times t \times b}
\]

\[v: 1 \text{ ml (reaction volume)}\]

\[\varepsilon: 18.5 \times 10^{-3} \text{ molar absorption coefficient}\]

\[t: 5 \text{ min (reaction time)}\]

\[b: 1 \text{ cm (path length)}\]

**Immunoelectron Microscopic Analysis**

Immunoelectron microscopic analysis was carried out to confirm that the fibronectin and fibrinogen molecules are captured on the cell surface of *L. casei* strain Shirota/pSAKSfb14. Bacterial cells incubated with fibronectin or fibrinogen were further treated with primary antibody and gold colloidal particle-labeled secondary antibody to detect fibronectin or fibrinogen molecules trapped on the cell surface. As shown in Figure 3A and 3B, gold colloidal particles were observed outside the peptidoglycan layer of *L. casei* strain Shirota/pSAKSfb14. This result indicates that *L. casei* strain Shirota/pSAKSfb14 expresses fibronectin binding domain and traps fibronectin molecules on the cell surface. On the other hand, gold colloidal particles were not detected either on the cell surface of *L. casei* strain Shirota (Figure 3C) or *L. casei* strain Shirota/pSAK332 (Figure 3D).

Fibrinogen was also detected on the cell surface of *L. casei* strain Shirota/pSAKSfb14 (Figure 3E), whereas no fibrinogen was observed on the parental strain (Figure 3F).
Figure 3. Immunoelectron microscopic detection of fibronectin or fibrinogen bound on the surface of *L. casei* cells. (A), (B) Binding capacity of *L. casei* strain Shirota/pSAKSfb14 to fibronectin. Gold colloidal particles were detected outside the cell wall peptidoglycan. (C) Binding capacity of *L. casei* strain Shirota to fibronectin. (D) Binding capacity of *L. casei* strain Shirota/pSAK332 to fibronectin. (E) Binding capacity of *L. casei* strain Shirota/pSAKSfb14 to fibrinogen. Gold colloidal particles were observed on the cell surface. (F) Binding capacity of *L. casei* strain Shirota to fibrinogen.
Adhesion to Fibroblast

Because fibronectin is secreted in large amounts in cultures of fibroblast cells (Hedman et al., 1978), we examined the adhesion of L. casei strain Shirota/pSAKSfb14, L. casei strain Shirota and S. pyogenes ATCC 21059 to murine fibroblast STO cells (Martin and Evans, 1975). Approximately 1x10^4 of bacterial strains were added to monolayers of STO cells and incubated for three hours. The wells were washed with PBS, and the STO cells were treated with 0.3% Triton X-100 in PBS to dissolve the STO cells along with the adherent bacteria. The well contents were plated to determine the number of adherent bacteria. Percent adherence was calculated by dividing the number of adherent CFU (colony forming units) by the number of inoculated CFU and multiplying by 100. As shown in Figure 4, L. casei strain Shirota/pSAKSfb14 bound to monolayers of STO cells about three times as much as its parental strain did (p<0.001). L. casei strain Shirota/pSAKSfb14 bound more than 5% of inoculum, whereas L. casei strain Shirota bound less than 2%. S. pyogenes ATCC 21059 was not recovered from monolayers of STO cells in this experiment.

We further performed inhibition experiments in the presence of anti-fibronectin antibody, i.e., monolayers of STO cells were pre-treated with anti-fibronectin antibody. The attachment of L. casei strain Shirota/pSAKSfb14 to STO cells was significantly inhibited by anti-fibronectin antibody (p<0.01), implying that L. casei strain Shirota/pSAKSfb14 adhered to fibroblast through fibronectin (Figure 4).

Discussion

For the sake of biological response modifier and safety, genetically-engineered lactic acid bacteria have been developed for antigen delivery vehicle for oral immunization (Pouwels et al., 1998, Slos et al., 1998), and intranasal immunization of mouse with Staphylococcus carnosus expressing fibronectin binding domain on the cell surface resulted in improved antibody response (Liljeqvist et al., 1999). S. carnosus is used as starter cultures in meat fermentation applications, but not used in dairy products. On the other hand, L. casei has been used in fermented milk and generally considered as safe (GRAS). Considering the simplicity of oral ingestion of fermented products, L. casei which acquires fibronectin binding capacity may have an advantage to be an effective tool for live vaccine development. There are several reports for the expression of fibronectin binding protein or fibronectin binding domain in bacteria like Escherichia coli (Flock et al., 1987, Talay et al., 1991, Lindberg et al., 1992, Sela et al., 1993), S. pyogenes.
pyogenes (Hanski et al., 1992), Enterococcus faecalis (Hanski et al., 1992) and S. carnosus (Liljeqvist et al., 1999). In contrast to these bacteria, L. casei is a GRAS and probiotic bacterium. We present here the first evidence for that fibronectin binding domain is expressed on the cell surface of probiotic bacteria.

The fibronectin binding capacity of L. casei strain Shirota/pSAK332 is lower than that of S. pyogenes ATCC 21059. (Table 1). We consider, however, that increasing of the plasmid copy number or the stability of the fusion protein could enhance the fibronectin binding capacity of L. casei strain Shirota/pSAK332. The adherence of S. pyogenes ATCC 21059 to fibroblast cells (STO cells) is not detectable in this study. Hanski and Caparon (1992) have shown that the adherence of S. pyogenes ATCC 21059 to fibroblast cells is a high level and the fibronectin binding protein plays an important role in adherence. We currently do not know the reason why S. pyogenes ATCC 21059 did not adhere to fibroblast cells. The adherence of S. pyogenes cells to fibroblast cells has not been reported yet.

L. casei strain Shirota/pSAK332 shows fibronectin binding capacity. Katerov et al. (1998) have reported that fibronectin binding protein, PrtF, of S. pyogenes type M15, binds also fibronectin, and its fibronectin binding domain is located in the N-terminal region, whereas the fibronectin binding domains are in the C-terminal region. Another fibronectin binding motif of FBP54 of S. pyogenes type M5 was revealed to be N-terminal 89 amino acid residues containing repeat sequence (Courtney et al., 1994). Those amino acid sequences involved in fibronectin binding are not homologous to the fibronectin binding domain used in this work. We present here that the repetitive sequence expressed on the cell surface showed not only fibronectin binding but also fibronectin binding activity (Table 1, Figure 3), though it is not a high level.

Bacterial attachment to host tissue is usually the first step leading to colonization and development of infectious disease. Natanson et al. (1995) have studied 109 strains of group A streptococci including clinical isolates and suggested a correlation between fibronectin binding and the pathogenic potential. To exclude the possibility of the undesirable effect by expressing fibronectin binding protein, L. casei strain Shirota/pSAK332 was injected intravenously into a rabbit model of cather-induced infective endocarditis (Durack and Beeson, 1972). L. casei strain Shirota/pSAK332 was rapidly killed and undetectable in the tissues of liver, kidney, spleen, and vegetation, and did not cause infective endocarditis (data not shown). These results imply that other factors in combination with fibronectin binding ability may be responsible for infective endocarditis. We have detected that several proteins excreted from the cell surface of L. casei strain Shirota/pSAK332 showed fibronectin binding capacity, and the possibility that those proteins are deleterious could not be denied. Excreted proteins, however, did not lead rabbit to death in the case of intravenous injection of L. casei strain Shirota/pSAK332.

Bacterial attachment to host tissue is also an important step to modulate immune system. Attachment of BCG to bladder wall was thought to be a requisite first step in the initiation of the antitumor response and fibronectin was shown to be an important mediator for attachment in vitro (Ratliff et al., 1988). The distribution of fibronectin was examined in normal human bladder mucosa (Pode et al., 1986). Fibronectin was absent from the apical surface of epithelial cells, but was observed at the basement membrane and in the submucosa. However, in areas of urothelial damage, BCG attached to the bladder wall via fibronectin and activated immune response (Ratliff et al., 1988). Recently, Zhao et al. (2000) demonstrated that fibronectin attachment protein of BCG plays an important role in the in vivo attachment of BCG to the bladder wall and in the induction of BCG-treated antitumor activity. On the other hand, a double-blinded placebo-controlled trial by oral administration of L. casei strain Shirota showed a statistically significant decrease in the recurrence of superficial bladder cancer after transurethral resection (Aso et al., 1995), and the habitual intake of L. casei strain Shirota was suggested to reduce the risk of bladder cancer (Ohashi et al., 2000). From the view of prophylaxis, long-term ingestion of lactic acid bacteria would be a preventive measure for the occurrence of bladder cancer. We expect, therefore, that probiotic bacteria with both antimtumor activity and fibronectin binding capacity have a potential for bladder cancer therapy.

We are currently studying the antitumor activity of L. casei strain Shirota/pSAK332 to the model mouse of superficial bladder cancer.

Experimental Procedures

Bacterial Strains and Growth Medium

All Lactobacillus strains were from the stock of our laboratory. S. pyogenes ATCC 21059 was obtained from American Type Culture Collection. E. coli JM109 was used as a host strain for plasmid construction. MRS medium (Difco) was used for cultivation of L. casei, Brain Heart Infusion (Difco) for S. pyogenes, and LB (Difco) for E. coli. Relevant antibiotic concentrations were 20 µg/ml of erythromycin for L. casei and 500 µg/ml for E. coli, and 100 µg/ml of ampicillin for E. coli.

Isolation of Chromosomal DNA from S. pyogenes

Chromosomal DNA was isolated from overnight culture of S. pyogenes ATCC 21059. Washed bacteria were suspended in lysis buffer (0.1% lysozyme, 0.01% N-AcetylMuramidase SG (Seikagaku Co, Japan), 10% sucrose, 50 mM Tris-HCl pH 8.0, 1 mM EDTA). Following a 90 min incubation at 37°C, SDS solution was added at the final concentration of 1%. The lysate was heated at 65°C for 90 min to inactivate nuclease, and proteinase K (Promega) was added at 200 µg/ml and incubated for further three hours at 37°C. After phenol extraction and ethanol precipitation, ribonuclease A was added to the DNA solution. Chromosomal DNA was again extracted with phenol/chloroform and recovered by ethanol precipitation.

Cloning of the Fibronectin Binding Domain of the sfbI Gene into the Surface Display Vector

The surface display plasmid pSAK332 was a generous gift from M. Kiwaki (Yakult Institute for Microbiological Research). It is a shuttle vector between E. coli and L. casei, and recombination of plasmid was completed in E.
Figure 5. Microscopic analysis of the adherence of *L. casei* cells to STO cells. (A) *L. casei* strain Shirota/pSAKSfb14. (B) *L. casei* strain Shirota. *L. casei* cells were incubated with fibroblast STO cell monolayers for three hours, washed and stained with Giemsa stain. *L. casei* cells are the small, rod shape, darkly stained objects on the surface of STO cells.

**coli.** Primers used for amplification of the *sfbI* gene were as follows:

Sp01, 5'-CGCGGTCGACAATGAAACAGGTTTTTCAG GAAATATGGTT-3' (1360-1389)

Sp02, 5'-GCGCCTCGAGGGTATCTTCAACAATGGTC ACTGTTTCAG-3' (1848-1819).

Numbers in parentheses correspond to the nucleotide number of the *sfbI* gene. Sp01 and Sp02 has *Sal* I and *Xho* I site at the 5' region, respectively. Coding sequence of *sfbI* gene is from 11th nucleotide of each primer.

The fibronectin binding domain of the *sfbI* was amplified with PCR using the primers described above. Reactions were carried out in a total volume of 50 µl in a cocktail containing 1.6 mM MgCl₂, 200 mM dNTPs, 200 ng of chromosomal DNA of *S. pyogenes*, 10 pmol of each primer, and 2.5 units of elongase (Gibco). The cycle was 94°C for 30 sec, 63°C for 30 sec, 68°C for 2 min, and repeated 35 times. The PCR product was digested with *Sal* I and *Xho* I, and the 1.2 kb fragment was extracted from agarose gel. The plasmid pSAK332 was digested with *Sal* I and dephosphorylated. The 1.2 kb fragment was cloned into the *Sal* I-digested pSAK332 to yield pSAKSfb14. The plasmid pSAKSfb14 was sequenced to verify that the fibronectin binding domain is inserted in the same reading frame as SAK and the anchor sequence.

**Electroporation**

*L. casei* strain Shirota grown exponentially were chilled on ice for 15 min. The cells were collected and washed with 10% glycerol several times to remove salt ions. Plasmid DNA was added to 40 µl of the cell suspension in a 0.2 cm cuvette (BioRad) and the mixture was pulsed using Gene Pulser (Bio-Rad) at 1.5 kV, 200 Ω, 25 µF. 0.9 ml of MRS broth was immediately added and competent cells were incubated at 37°C for 60 min. Cells were plated on MRS agar containing erythromycin at the concentration of 20 µg/ml.

**Western Blot Analysis**

Western blot analysis was performed according to van Putten *et al.* (1998) with minor modifications. Approximately 1x10⁸ bacteria in the stationary phase were collected by centrifugation at 10,000 x g at 4°C for 5 min, and washed with 500 µl of PBST buffer (0.05% Triton X-100 in PBS). For blocking, they were incubated with 250µl of 1% BSA in PBST buffer at 37°C for 60 min. Then, cells were incubated with 1 µg of fibronectin from human plasma (Boehringer Mannheim) in 100 µl of PBST buffer at 37°C for 60 min. Bacteria were collected by centrifugation, washed with 500µl of PBST buffer for three times to exclude the fibronectin non-specifically attached to bacteria. They were suspended in 100 µl of sample solution and boiled for 5 min to release the captured fibronectin to the supernatant, and then aliquots of supernatant were subjected to SDS-PAGE (7.5% gel). The proteins were electrophoretically transferred to an Immobilon-P™ membrane (Millipore) by using semidry blotter (Sartoblot II-S, Sartorius). Fibronectin was detected by incubation with the rabbit anti-human plasma fibronectin antibody (primary antibody, Becton Dickinson, dilution 1:10,000 in PBST buffer) for 60 min at room temperature, followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (secondary antibody, Promega, dilution 1:7,500 in PBST buffer) for 60 min, and then developed with nitro blue tetrazolium (NBT, Promega) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Promega) in alkaline phosphatase buffer (100 mM Tris-HCl buffer pH 9.5, 100 mM NaCl, 5 mM MgCl₂).

**Assays of Binding Activity to Fibronectin or Fibrinogen of Bacteria**

Binding activity to fibronectin or fibrinogen of bacteria was examined as follows. Approximately 1x10⁸ bacteria in the stationary phase were collected by centrifugation at 10,000 x g at 4°C for 5 min, and washed with PBST buffer. They
were incubated with 1% BSA in PBST buffer at 37°C for 60 min for blocking. Cells were then incubated with 1 µg of fibronectin or fibrinogen (human plasma, Sigma) in 100 µl of PBST buffer at 37°C for 60 min. After washing with PBST buffer, cells were suspended with anti-fibronectin antibody or anti-fibrinogen antibody (primary antibody, Beckton Dickinson, dilution 1:10,000 in PBST buffer), respectively at 37°C for 60 min. Cells were washed with PBST buffer and resuspended with the secondary antibody (dilution 1:7,500 in PBST buffer) at 37°C for 60 min. After washing with PBST buffer, 1x10^7 of cells were incubated with p-nitrophenyl phosphate (Sigma) as substrate in 1 ml of alkaline phosphatase buffer at 37°C for 5 min, and then the absorbance at 405 nm was measured. One unit of alkaline phosphatase activity is defined as the amount of alkaline phosphatase to release 1 nmol of p-nitrophenol per 1 min.

**Immunoelectron Microscopic Analysis**

Pre-embedding method was used for immunoelectron microscopic analysis. Bacteria were treated as described above in *Assays of binding activity to fibronectin or fibrinogen of bacteria* except for the use of goat anti-rabbit IgG antibody-gold conjugate (particle size 15 nm, EY Laboratories) as a secondary antibody. After incubation with the secondary antibody and washing with PBS, they were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 90 min. The cells were then embedded in 3% warm agar. The agar block was solidified, washed with 0.1 M phosphate buffer, fixed with 1% osmium tetroxide in 0.1 M phosphate buffer for two hours, and then dehydrated stepwise in ethanol (50 to 100%) at room temperature. The agar block was cut into ultrathin sections with a Reichert ultramicrotome. The sections were stained with 0.1 M phosphate buffer, fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer at 37°C for 60 min. After washing with PBST buffer, 1x10^7 of cells were incubated with p-nitrophenyl phosphate (Sigma) as substrate in 1 ml of alkaline phosphatase buffer at 37°C for 5 min, and then the absorbance at 405 nm was measured. One unit of alkaline phosphatase activity is defined as the amount of alkaline phosphatase to release 1 nmol of p-nitrophenol per 1 min.

**Adhesion Assay**

Murine fibroblast STO cells were grown to form monolayers in D-MEM medium (Difco) supplemented with 10% fetal bovine serum (Difco) at 37°C in the presence of 5% CO2 in eight-well chamber slides (Labtek II chamber, Nunc). Bacteria in the stationary phase were collected, washed three times with PBS and suspended with D-MEM medium without fetal bovine serum. The medium of STO cells was also replaced with D-MEM medium without serum. Approximately 1x10^4 of bacterial strains were added to the STO cells and incubated at 37°C in 5% CO2 for three hours. The wells were gently washed four times with PBS not to disrupt the monolayers. After rinsing, the STO cells were treated with 0.3% Triton X-100 in PBS to dissolve the STO cells along with the adherent bacteria. The well contents were agitated and the dilutions were plated on MRS agar to determine the number of adherent bacteria per well. The actual number of bacteria added to each well was determined by colony counting. Percent adherence was calculated by dividing the number of adherent CFU (colony forming units) by the number of inoculated CFU and multiplying by 100. For inhibition studies, the anti-fibronectin antibody was added to the monolayers (dilution 1:100) before adding bacteria. For the light microscopic analysis, the STO cells with adherent bacteria were stained with Giemsa stain and examined.

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**References**


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