

The Surface Layer of *Tannerella forsythia* Contributes to Serum Resistance and Oral Bacterial Coaggregation

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Tannerella forsythia is an anaerobic, Gram-negative bacterium involved in the so-called “red complex,” which is associated with severe and chronic periodontitis. The surface layer (S-layer) of *T. forsythia* is composed of cell surface glycoproteins, such as TfsA and TfsB, and is known to play a role in adhesion/invasion and suppression of proinflammatory cytokine expression. Here we investigated the association of this S-layer with serum resistance and coaggregation with other oral bacteria. The growth of the S-layer-deficient mutant in a bacterial medium containing more than 20% non-heat-inactivated calf serum (CS) or more than 40% non-heat-inactivated human serum was significantly suppressed relative to that of the wild type (WT). Next, we used confocal microscopy to perform quantitative analysis on the effect of serum. The survival ratio of the mutant exposed to 100% non-heat-inactivated CS (76% survival) was significantly lower than that of the WT (97% survival). Furthermore, significant C3b deposition was observed in the mutant but not in the WT. In a coaggregation assay, the mutant showed reduced coaggregation with *Streptococcus sanguinis*, *Streptococcus salivarius*, and *Porphyromonas gingivalis* but strong coaggregation with *Fusobacterium nucleatum*. These results indicated that the S-layer of *T. forsythia* plays multiple roles in virulence and may be associated with periodontitis.

Periodontitis is an infectious disease caused by periodontopathogenic bacteria that colonize the tooth surface and gingival sulcus (1, 2). This disease is initiated by the formation of a biofilm, known as dental plaque, which consists of many oral bacteria. Currently, many periodontal pathogens, such as *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Prevotella intermedia*, have been identified (3, 4). These pathogens produce several virulence factors and immune evasion factors, causing inflammation and destruction of periodontal tissues (5–8). *T. forsythia* is an anaerobic, Gram-negative bacterium isolated from the gingival sulci and periodontal pockets of patients with periodontitis (9, 10). Three pathogens (*T. forsythia*, *P. gingivalis*, and *T. denticola*), known collectively as the “red complex,” are strongly associated with the pathogenesis and progression of destructive forms of periodontitis (3, 9). Several *T. forsythia* virulence factors have been identified (10–12), including BspA, the S-layer, glycosidases, lipoproteins, and metalloproteinase.

The S-layer is the outermost cell envelope component of many bacteria, including archaea, Gram-positive bacteria, and Gram-negative bacteria (13, 14). Among Gram-negative bacteria, S-layers of *Campylobacter rectus* (15), *Campylobacter fetus* (16), and *T. forsythia* have been reported (17–19). The S-layer functions as a protective barrier and molecular sieve, and it plays a role in cell adhesion and surface recognition (11, 13, 14, 20–22). The S-layer of *T. forsythia* consists of two proteins, TfsA and TfsB, which are encoded by *tfsA* (TF2661 and TF2662) and *tfsB* (TF2663), respectively; these genes are located tandemly in an operon (17). Wild-type (WT) *T. forsythia* has a regularly arrayed structure at the outermost cell surface, while a mutant lacking both *tfsA* and *tfsB* completely lost its structure, and a mutant lacking either *tfsA* or *tfsB* retained smaller amounts of its structure (23). S-layer proteins are glycoproteins with apparent molecular sizes of 230 kDa

(TfsA) and 270 kDa (TfsB). Recently, the characterization and structure of oligosaccharides in S-layer proteins have been reported (24). Oligosaccharides were O-glycosidically linked to three amino acid motifs, D(S/T)(A/I/L/M/T/V), on either of the two S-layer proteins (TfsA or TfsB) (24). These two proteins are specifically recognized in the sera of patients with periodontitis (25). Also, TfsA and TfsB may mediate adhesion to, and/or invasion of, human gingival epithelial cells and epidermal carcinoma cells of the mouth and are associated with hemagglutination (11, 23). Sekot et al. have demonstrated that *T. forsythia* lacking the S-layer induces significantly higher levels of proinflammatory cytokines than WT *T. forsythia* (26), which suggests that the S-layer attenuates the host immune response by evading innate immune recognition.

In this study, we investigated the associations of the S-layer of *T. forsythia* with serum resistance and coaggregation with *Streptococcus sanguinis*, *Streptococcus salivarius*, *Streptococcus mutans*, *Streptococcus mitis*, *Aggregatibacter actinomycetemcomitans*, *F. nucleatum*, and *P. gingivalis*. Serum contains a complement, including an innate immune factor present in gingival crevicular fluid

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TABLE 1 Strains used in this study

Strain	Description ^a	Reference or source ^b
<i>Tannerella forsythia</i>		
ATCC 43037	Wild type	ATCC
Δ tfsAB mutant	TfsA and TfsB deletion mutant; Cam ^r	23
<i>Aggregatibacter actinomycetemcomitans</i>		
HK1651	Wild type	ATCC
Y4	Wild type	ATCC
<i>Fusobacterium nucleatum</i> ATCC 25586		
Wild type		
<i>Porphyromonas gingivalis</i> W83		
Wild type		
<i>Streptococcus mutans</i> UA159		
Wild type		
<i>Streptococcus mitis</i> GTC 495		
Wild type		
<i>Streptococcus salivarius</i> GTC 215		
Wild type		
<i>Streptococcus sanguinis</i> GTC 217		
Wild type		
<i>Escherichia coli</i>		
XL-II	<i>endA1 supE44 thi-1 hsdR17 recA1 gyrA96 relA1 lac</i> [F' <i>proAB lacI^rZΔM15 Tn10 (Tet^r) Amy Cam^r</i>]	Stratagene
RA31	HB101 carrying empty vector (pGEM-T Easy); Amp ^r	36
RA11	HB101 Omp100-expressing strain; Amp ^r	36

^a Cam^r, chloramphenicol resistance; Tet^r, tetracycline resistance; Amp^r, ampicillin resistance.

^b ATCC, American Type Culture Collection; GTC, Gifu type culture.

(GCF) at concentrations as high as 70%, that can kill bacteria upon activation (27). Human complement can function through three activation pathways, including the classical pathway, an alternative pathway, and the lectin pathway (28, 29). Bacterial components such as lipopolysaccharides (LPS), peptidoglycan, and lipoproteins can induce complement activation. However, many bacteria, including periodontal pathogens, are resistant to human serum (16, 30–33). Coaggregation between different bacteria plays a key role in the colonization of the gingival crevice and the organization of biofilm formation by periodontal pathogens. Among the oral bacteria, *F. nucleatum* plays an important role in bacterial coaggregation. Previously, *T. forsythia* was reported to coaggregate with *F. nucleatum* and *T. denticola* (34, 35). Overall, we concluded that the S-layer is associated with serum resistance and coaggregation with other oral bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. A *T. forsythia* mutant lacking the S-layer (Δ tfsAB) was constructed as described previously (23). *T. forsythia* was grown in brain heart infusion (BHI) medium containing heat-inactivated calf serum (CS) (5%, vol/vol), yeast extract (5 g/liter), L-cysteine (1 g/liter), N-acetylmuramic acid (10 mg/liter), hemin (5 mg/liter), and menadione (0.5 mg/liter) (TF medium) for 3 to 7 days at 37°C under anaerobic conditions using the GasPak system (Mitsubishi Gas Chemical Company Inc., Tokyo, Japan). Chloramphenicol (10 μg/ml) was added when necessary. *A. actinomycetemcomitans* was grown in Todd-Hewitt medium containing yeast extract (10 g/liter) (THY) overnight at 37°C under 5% CO₂. *F. nucleatum* was grown in BHI medium overnight at 37°C under anaerobic conditions. *P. gingivalis* was grown in BHI medium containing hemin (5 mg/liter) and menadione (0.5 mg/liter) for 2 days at 37°C under anaerobic conditions. *Streptococcus mutans*, *S. sanguinis*, *S. salivarius*, and *S. mitis* were grown in tryptic soy broth overnight at 37°C under 5% CO₂. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth overnight at 37°C.

Sera, factor H, and antibodies. Human serum was obtained from healthy volunteers, with the approval of the ethics committee of Ka-

goshima University. CS was purchased from Gibco Life Technologies Japan (Tokyo, Japan). Serum was inactivated by heat (56°C for 30 min) when necessary. Human factor H was purchased from Complement Technology (Tyler, TX). Mouse monoclonal IgG against human C3 and a mouse monoclonal antibody against human factor H were obtained from BioPorto Diagnostics A/S (Gentofte, Denmark). A mouse polyclonal antibody against human C4b-binding protein (C4BP) was acquired from Abnova (Taipei, Taiwan). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG(H+L) (Promega, Madison, WI) and Alexa Fluor 594-conjugated goat anti-mouse IgG(H+L) (Molecular Probes Inc., Eugene, OR) were used for detection.

Serum survival assay. Two methods were used to evaluate susceptibility to serum. For the growth kinetics method, 10⁵ cells of WT *T. forsythia* or the S-layer-deficient mutant grown for 5 days were added to 100 μl of modified TF medium (BHI medium containing yeast extract [5 g/liter], L-cysteine [1 g/liter], N-acetylmuramic acid [10 mg/liter], hemin [5 mg/liter], and menadione [0.5 mg/liter]) containing various concentrations of non-heat-inactivated and heat-inactivated serum (human serum at 80%, 60%, 40%, and 20%; CS at 60%, 40%, 20%, and 10%) on a 96-well plate. Then the plate was incubated for 10 days at 37°C under anaerobic conditions. The cell density (at 600 nm) was measured at 24-h intervals by a SpectraMax 340PC384 absorbance microplate reader (Molecular Devices Japan, Tokyo, Japan). For the quantitative method used to evaluate serum killing, 10⁸ WT or S-layer-deficient mutant *T. forsythia* cells grown for 5 days in TF medium, and *E. coli* strain XL-II grown in LB medium overnight, were incubated with 10 ml of 100% CS for 2 h at 37°C under anaerobic conditions. Bacterial cells were pelleted by centrifugation and were washed with 5 ml of phosphate-buffered saline (PBS). After washing with PBS, the cells were stained using the BD Cell Viability kit (BD Biosciences, San Jose, CA), which enables visualization of dead and/or live cells. Confocal laser scanning microscopy (CLSM) was performed with a Carl Zeiss LSM700 microscope (Carl Zeiss Micro Imaging Co., Ltd., Tokyo, Japan). The microscope was equipped with detectors to monitor red (excitation wavelength, 555 nm; dichroic mirror wavelength, 585 nm) and green (excitation wavelength, 488 nm; dichroic mirror wavelength, 590 nm) fluorescence. Confocal images were obtained with a 40× oil lens (numerical aperture, 1.3) for an optical section thickness of approximately 0.7 μm. The experiment was performed three times indepen-

dently. In each experiment, more than 10 microscopic fields were observed, and 3 images were randomly extracted. After three images of dead and live cells were obtained in each experiment, the areas occupied by dead and/or live cells in the CLSM images were analyzed by ImageJ 1.44i (National Institutes of Health, Bethesda, MD). Then the ratio of live bacteria to total bacteria was calculated.

Electron microscopic analysis. For transmission electron microscopy (TEM), *T. forsythia* strains grown in TF medium were washed twice with PBS. TEM was used for ultrastructural characterization of the cell, as described previously (37–39). A total of 10^{10} bacterial cells were reacted with 40 ml of CS or PBS for 2 h at 37°C under anaerobic conditions. After centrifugation, the bacterial cells were washed twice with PBS. Next, the cells were fixed with 2.5% glutaraldehyde for 12 h. The samples were dehydrated in a series of ethanol concentrations and were then embedded in Spurr's resin for 2 days at 60°C (39). Thin sections were cut using an ultramicrotome (Ultracut R; Leica, Tokyo, Japan) with a diamond knife and were examined using a JEM-1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan) at 80 kV.

Preparation of the outer membrane protein and SDS-PAGE. Sarkosyl-insoluble outer membrane protein fractions were prepared as described previously (40). Briefly, WT and S-layer-deficient mutant *T. forsythia* cells grown in TF medium for 7 days were pelleted and suspended in PBS. The cells were disrupted by sonication, and the remaining undisturbed bacterial cells were removed by centrifugation. The outer and inner membrane protein fractions were collected as a pellet by centrifugation at $100,000 \times g$ for 60 min at 4°C, and the pellet was redissolved in sodium lauryl sarcosinate (1%, wt/vol). The outer membrane protein fractions were collected as a pellet by centrifugation at $100,000 \times g$ for 60 min at 4°C, and the pellet was redissolved in sodium dodecyl sulfate (SDS) (1%, wt/vol). The outer membrane protein fractions of the WT and mutant *T. forsythia* strains were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE), and the gels were stained with Coomassie brilliant blue R-250.

Detection of deposition of complement factor C3b on the bacterial surface. WT *T. forsythia* and the S-layer-deficient mutant were grown for 5 days in TF medium. Then 2×10^8 cells were incubated in 0.5 ml of 30% human serum in PBS, or in PBS alone, for 30 min at 37°C under anaerobic conditions. The bacteria were pelleted by centrifugation; then the pellets were washed three times with 0.5 ml of PBS and were fixed with 0.5 ml of 2.5% paraformaldehyde for 10 min at room temperature. The bacteria were incubated with 0.5 ml of blocking solution (PBS containing 0.05% Tween 20 and 1% bovine serum albumin) for 30 min at 37°C. After blocking, the bacteria were treated with 1 ml of PBS-T (PBS containing 0.05% Tween 20) containing a mouse monoclonal antibody against human C3 (dilution, 1:10,000) and were incubated for 30 min at room temperature. The bacteria were washed three times with 0.5 ml of PBS, treated with 1 ml of PBS-T containing Alexa Fluor 594-conjugated goat anti-mouse IgG (1:250), and incubated for 30 min at room temperature. The bacteria were then washed three times with 0.5 ml of PBS, resuspended with 1 ml of PBS containing 4',6-diamidino-2-phenylindole (DAPI) (5 µg/ml) (Molecular Probes Inc.), and incubated for 10 min at room temperature. After washing with PBS, the bacterial cells were suspended in 10 µl of PBS. CLSM was performed with a Carl Zeiss LSM700 microscope. The areas in CLSM images occupied by cells on which C3b was deposited or by whole cells were analyzed by ImageJ 1.44i (NIH).

Factor H and C4BP binding assays. Previously, we demonstrated that Omp100, one of the outer membrane proteins in *A. actinomycetemcomitans*, had binding affinity for factor H (36). We found that *E. coli* HB101 carrying the empty vector pGEM-T Easy (RA31) was highly susceptible to serum, while *E. coli* HB101 expressing Omp100 (RA11) and *A. actinomycetemcomitans* Y4 were resistant to serum. Therefore, we used these strains as controls in the factor H binding assay. WT *T. forsythia* and the S-layer-deficient mutant were grown for 5 days in TF medium. *E. coli* strains were grown in LB broth overnight, and *A. actinomycetemcomitans* was grown in THY overnight. Then 2×10^9 bacterial cells were incubated with 0.5 ml of

50% human serum in GVB++ (Veronal-buffered saline containing 0.1% gelatin, 0.15 mM CaCl₂, and 0.5 mM MgCl₂) or with GVB++ alone at 37°C for 30 min. The bacteria were pelleted by centrifugation, and the pellets were washed three times with 0.5 ml GVB++. The pellets were then treated with 75 µl of 0.1 M glycine-HCl (pH 2.0). After centrifugation, the supernatant was mixed with 5 µl of 1.5 M Tris-HCl (pH 8.8), and the eluted fraction was collected. Next, the eluted samples and human factor H (8 ng) were separated by 7.5% SDS-PAGE and were transferred to a nitrocellulose membrane. Immunoblotting was performed as described previously (41). The membrane was blocked with 2% skim milk in Tris-buffered saline (TBS) (20 mM Tris and 137 mM NaCl [pH 8.0]) containing 0.05% Tween 20 (TBS-T) at 4°C overnight and was then incubated with a mouse monoclonal antibody against human factor H (diluted 1:10,000 in 1% skim milk in TBS-T) at room temperature for 1 h. After washing with TBS-T, the membrane was incubated with HRP-conjugated goat anti-mouse IgG (1:2,500) at room temperature for 1 h. The membrane was washed with TBS-T, and factor H was detected using a chemiluminescence detection system (Perkin-Elmer Japan Co., Ltd., Kanagawa, Japan). The intensities of factor H binding were measured with Image Lab (Bio-Rad Laboratories, Hercules, CA) and were quantified relative to the intensity of binding to WT *T. forsythia*. Data represent means \pm standard deviations (SD) of measurements performed in triplicate. We also investigated the binding of factor H or C4BP to *T. forsythia* cells by CLSM. We used the same method as that used to detect the deposition of complement factor C3b, except that an anti-factor H or anti-C4BP antibody was used.

Zymographic analysis of proteolytic activity. Since karilysin is associated with serum resistance in *T. forsythia* (42), we compared the proteolytic activities of the WT and the S-layer-deficient mutant. WT *T. forsythia* and the S-layer-deficient mutant were grown for 7 days in 10 ml of serum-free TF medium. The bacterial supernatants were collected by centrifugation and filtration. The supernatants were then concentrated 10-fold using a Centrplus YM-30 centrifugal filter unit (Millipore, Billerica, MA). The concentrated supernatants (10 µl) were mixed with nonreducing sample buffer and were separated on a 7.5% polyacrylamide gel containing gelatin (1 mg/ml). After electrophoresis, the gel was washed twice with 2.5% Triton X-100 for 30 min at room temperature. After washing, the gel was incubated with developing buffer (50 mM Tris-HCl, 10 mM CaCl₂ [pH 7.5]) at 37°C for 12 h. The gel was then stained with Coomassie brilliant blue R-250 and was destined for visualization of the proteolytic bands.

Coaggregation assay. WT *T. forsythia* and the S-layer-deficient mutant were grown for 3 or 7 days in TF medium. After the bacterial cells were harvested by centrifugation, WT *T. forsythia* and the S-layer-deficient mutant were suspended in coaggregation buffer (CAB) (1 mM CaCl₂, 1 mM MgCl₂, 150 mM NaCl, and Tris-HCl [pH 8.0]), and the suspensions were adjusted to an optical density at 550 nm (OD₅₅₀) of 1.0. Overnight cultures of *A. actinomycetemcomitans* HK1651, *F. nucleatum* ATCC 25586, *S. mutans* UA159, *S. mitis* GTC 495, *S. salivarius* GTC 215, and *S. sanguinis* GTC 217 and a 2-day culture of *P. gingivalis* W83 (stationary phase), used as coaggregation partner strains, were suspended with CAB and adjusted to an OD₅₅₀ of 1.0. Suspensions of *S. sanguinis* and *S. mutans* during exponential phase (OD₅₅₀, 0.4) were also prepared. As a preliminary, we investigated the optimal condition for the coaggregation assay. Three ratios (*T. forsythia*/partner strain ratios of 1:1, 1:2, and 2:1) using *S. sanguinis* and *S. salivarius* were investigated, and we found that a *T. forsythia*/partner strain ratio of 2:1 was most significant. Therefore, we used the *T. forsythia*/partner strain ratio of 2:1 for the coaggregation assay. WT *T. forsythia* or the S-layer-deficient mutant was mixed with the partner strain (2:1) in a disposable cuvette (12.5 by 12.5 by 45 mm), and the initial OD of the mixture was measured. Suspensions were incubated at 37°C and were measured at 15-min intervals using an Ultrospec 2000 UV/visible spectrophotometer (Pharmacia Biotech Ltd., Cambridge, United Kingdom). Decreases in absorbance were indicative of cell aggregates precipitating to the bottom of the cuvette. As controls, single bacte-

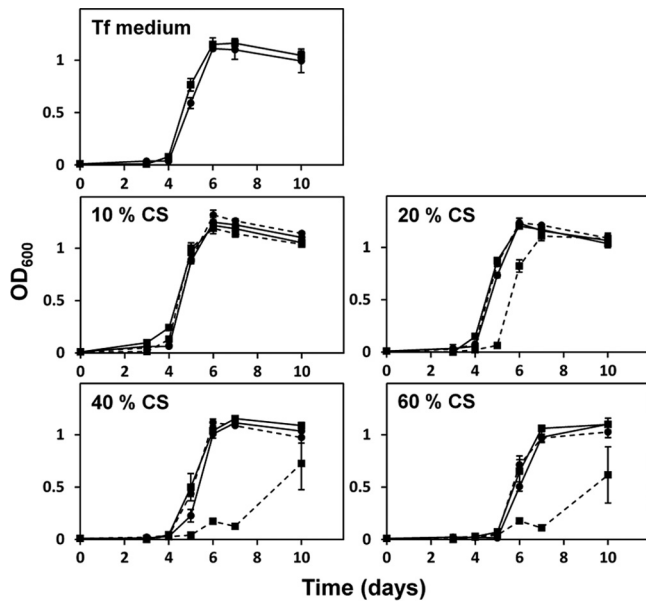


FIG 1 Growth kinetics of WT *T. forsythia* and the S-layer-deficient mutant in a medium containing serum. A total of 10^5 WT or mutant cells were inoculated into 100 μ l of TF medium containing non-heat-inactivated serum on 96-well plates and were incubated for 10 days at 37°C under anaerobic conditions. The cell density (at 600 nm) was measured at 24-h intervals. Circles and squares indicate the growth of the WT and the mutant, respectively. Solid and dashed lines indicate the results with heat-inactivated CS and non-heat-inactivated CS, respectively. Data represent the means \pm SD of measurements performed in triplicate.

rial cells were measured (the WT and S-layer-deficient mutant *T. forsythia* strains were adjusted to an OD_{550} of 0.66, while partner strain cells were adjusted to an OD_{550} of 0.33).

To observe the effects of sugars on coaggregation activity, 10 mM each sugar was added to the reaction mixture. We used *N*-acetyl-D-mannosamine, D-glucuronic acid sodium salt, L-(–)-fucose, D-(+)-xylose, and D-(+)-galactose, which were reported to be components of sugar moieties in the *T. forsythia* S-layer (24).

RESULTS

The S-layer contributes to survival in serum. To evaluate the role of the S-layer in serum resistance, we investigated the growth of WT *T. forsythia* and the S-layer-deficient mutant in TF medium containing various concentrations of calf serum (Fig. 1). For the WT, growth was gradually delayed as the serum concentration was increased. In the presence of 20% CS, the growth of the mutant was delayed for 1 day relative to that of the WT. More than 40% CS significantly suppressed the growth of the mutant, while the growth rates of the two strains were similar in heat-inactivated CS (Fig. 1). Similar results were obtained when human serum was used (data not shown). The growth of the mutant was suppressed in the presence of >40% human serum, but the mutant could grow well in the presence of heat-inactivated serum. Furthermore, we investigated the killing activity of human serum incubated for 1 to 7 days at 37°C under anaerobic conditions, and we found that the killing activity of the serum against *E. coli* XL-II was retained after 7 days of incubation (data not shown).

Hence, we quantified the effect of serum on bacterial killing. Numerous dead cells were observed when the mutant was incubated with CS, but not when the WT cells were incubated with CS

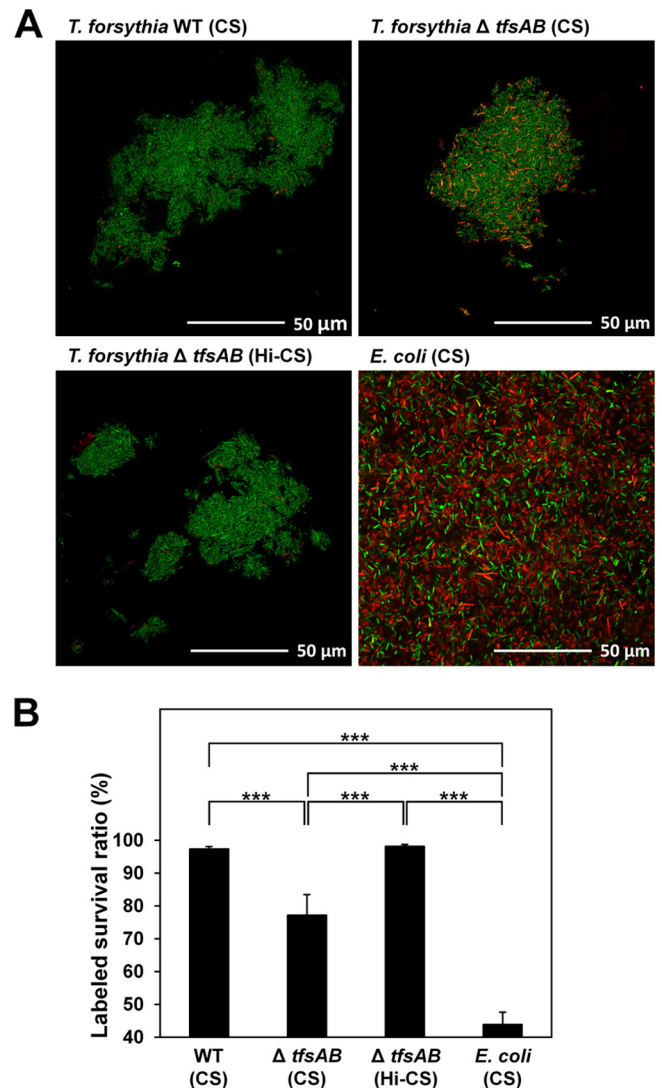


FIG 2 Effect of serum on the viability of *T. forsythia*. (A) WT *T. forsythia*, S-layer-deficient mutant, and *E. coli* XL-II cells were exposed to CS or heat-inactivated CS (Hi-CS). Details are given in Materials and Methods. In CLSM analysis, green and orange cells represent viable and dead cells, respectively. (B) The areas occupied by dead and/or live cells in the CLSM images were analyzed, and the ratio of live bacteria to total bacteria was calculated. Data represent means \pm SD of measurements performed in triplicate. Asterisks indicate significant differences (***, $P < 0.001$) as determined by Tukey's honestly significant difference test. The y axis begins with 40%.

or when the mutant was incubated with heat-inactivated CS (Fig. 2A). The survival ratio of the mutant that was reacted with non-heat-inactivated CS (76% survival) was significantly lower than that of the WT (97% survival), and *E. coli* showed a lower survival ratio than the mutant (44% survival) (Fig. 2B). Also, the mutant treated with heat-inactivated serum contained only a few dead cells.

For TEM analysis, we found that the ratio of disrupted cells of the mutant treated with CS was significantly higher than that of the WT in the low-power field of electron microscopic observation (see Fig. S1 in the supplemental material). Disruption of the cytoplasmic membrane or the outer membrane was observed in both strains. Figure 3B shows the typical features of the *T. forsythia*

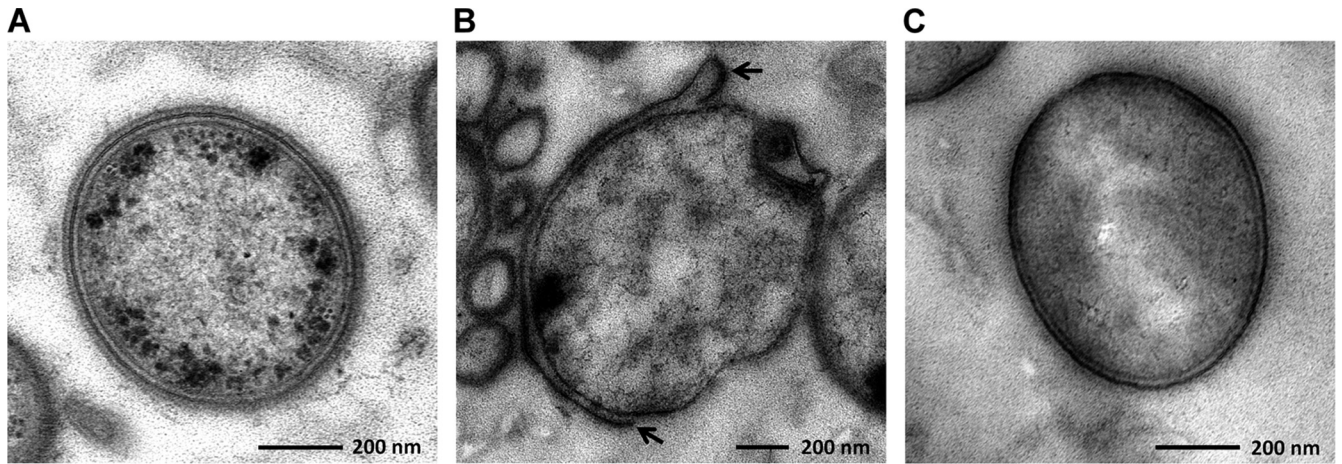


FIG 3 Electron micrographs of *T. forsythia* exposed to serum. (A and B) WT *T. forsythia* (A) and the S-layer-deficient mutant (B) were exposed to 100% CS at 37°C for 2 h. Then samples were prepared for observation by TEM. Arrows indicate the disrupted outer membrane. (C) As a control, the mutant treated with PBS was prepared.

mutant treated with CS. The outer membrane was disrupted (indicated by arrows), and cytoplasmic swelling was observed as a result. Also, in the WT, the S-layer was observed outside the outer membrane (Fig. 3A), while the mutant completely lost the S-layer at the cell surface, reducing the thickness of the cell surface relative to that of the WT (Fig. 3C). In addition, we confirmed the expression of TfsA and TfsB in the WT and the S-layer-deficient mutant. The WT expressed S-layer proteins, showing apparent molecular sizes of 230 kDa (TfsA) and 270 kDa (TfsB), while the mutant did not express either protein (Fig. 4).

Deposition of complement factor C3b is inhibited by the S-layer. Because the S-layer-deficient mutant showed decreased serum resistance, we postulated that the S-layer affects the activation of complement in serum. Initially, we investigated the deposition of complement factor C3b, which accumulates on the bacterial cell surface by activating the complement pathway, resulting in assembly of the membrane attack complex (MAC) for bacterial membrane perforation. The level of C3b deposition was significantly higher for the mutant than for the WT (Fig. 5A). The area occupied by cells on which C3b was deposited versus the area occupied by whole cells was calculated using image analysis. The areas occupied by cells on which C3b was deposited were 2.9% and

34% of the total area of the image for the WT and the mutant, respectively (Fig. 5B). When the WT and the mutant were not treated with serum, C3b deposition was not observed on cells of either strain.

Factor H and C4BP binding. Inhibition of C3b deposition by recruiting the complement-controlling protein factor H or C4BP contributes to serum resistance (30, 32, 33). Factor H inhibits the alternative pathway in complement activation, while C4BP inhibits the classical and lectin pathways. Therefore, we investigated whether the S-layer has binding affinity with factor H or C4BP. *E. coli* strain RA11, expressing Omp100, showed factor H binding affinity, while *E. coli* RA31 showed almost no affinity. The binding affinity of RA11 with factor H was 11-fold higher than that of RA31. Also, *A. actinomycetemcomitans* Y4 possessed binding affinity. Both WT *T. forsythia* and the S-layer-deficient mutant had binding affinity for factor H, and the level of binding of the mutant was slightly higher (1.7-fold) than that of the WT (Fig. 6). By CLSM analysis, the level of binding of factor H with the mutant was slightly higher than that with the WT, although the binding affinity was weak in both strains (see Fig. S2 in the supplemental material). Also, C4BP binding affinity was weak in both strains, and no difference was observed between the two strains (see Fig. S3 in the supplemental material).

Proteolytic activities of WT *T. forsythia* and the S-layer-deficient mutant. Proteolytic activity was analyzed using zymographic analysis. Two major bands, corresponding to molecular masses of 52 kDa and 48 kDa, were observed both in WT *T. forsythia* and in the mutant (Fig. 7). A previous report demonstrated that full-length karilysin undergoes autocatalytic processing to generate mature karilysin, showing a molecular mass of 48 kDa (43), suggesting that the proteolytic band of 48 kDa found in zymographic analysis is karilysin. The proteolytic activity of the mutant was similar to that of the WT (Fig. 7).

Association of the S-layer with coaggregation of *T. forsythia* with oral bacteria. Since *T. forsythia* is known to coaggregate with oral bacteria (34, 35), we investigated a possible association between the S-layer and coaggregation. Figure 8 shows the coaggregation of *T. forsythia* strains grown for 7 days with stationary-phase cells of *S. sanguinis* over time. WT *T. forsythia* aggregated

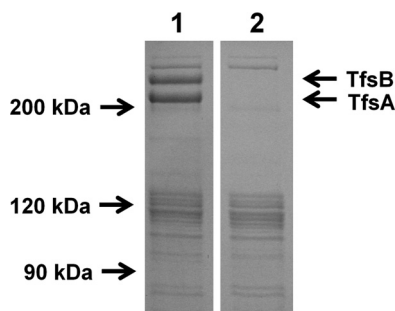


FIG 4 SDS-PAGE analysis of the outer membrane proteins in the WT and the S-layer-deficient mutant. The outer membrane protein fraction of WT *T. forsythia* and the S-layer-deficient mutant were prepared and separated on a 12.5% SDS-PAGE gel. Details are given in Materials and Methods. Lane 1, WT; lane 2, S-layer-deficient mutant.

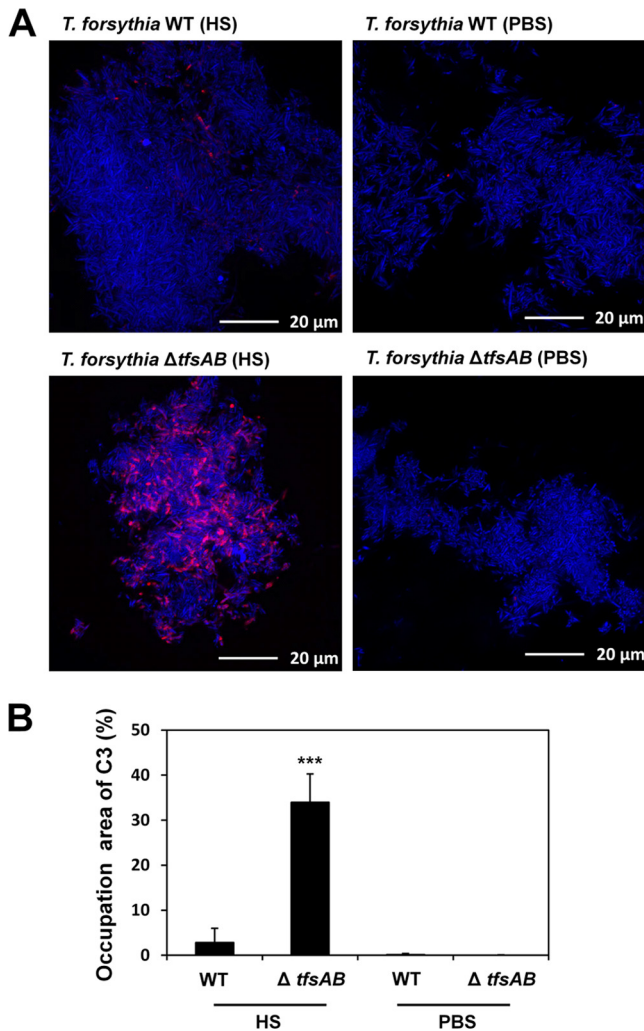


FIG 5 C3b deposition on the surfaces of WT *T. forsythia* and the S-layer-deficient mutant. (A) WT *T. forsythia* and the S-layer-deficient mutant were exposed either to 30% human serum (HS) in PBS or to PBS alone at 37°C for 30 min. After washing, the cells were reacted first with an anti-C3 antibody and then with Alexa Fluor 594-conjugated anti-mouse IgG. DAPI staining was performed as well. Blue and red cells represent whole cells and cells with C3b bound to the surface, respectively. (B) The efficiency of C3b deposition on the bacterial cell surface, as shown in the CLSM images, was analyzed. Details are given in Materials and Methods. Data represent means \pm SD of measurements performed in triplicate. Significant changes were determined using Bonferroni's *t* test (***, $P < 0.001$).

with *S. sanguinis*, while the S-layer-deficient mutant did not. The OD of the mixture of WT *T. forsythia* and *S. sanguinis* gradually decreased until 60 min, and finally a 35% reduction in the OD was observed. We further investigated the association of the S-layer with coaggregation by using stationary-phase cells of other bacteria. WT *T. forsythia* grown for 7 days coaggregated with *S. salivarius*, *F. nucleatum*, and *P. gingivalis* but not with *S. mutans*, *S. mitis*, or *A. actinomycetemcomitans* (Fig. 9A). For the mutant, no coaggregation with *S. sanguinis* or *S. salivarius* was observed, while coaggregation with *F. nucleatum* was increased over that of the WT. We investigated the coaggregation of *T. forsythia* grown for 3 days with stationary-phase cells of oral bacteria and found results similar (except for *P. gingivalis*) to those for *T. forsythia* grown for

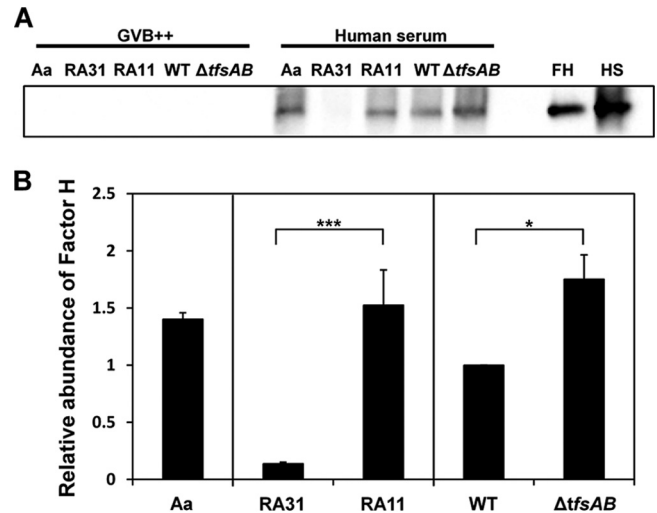


FIG 6 Binding of factor H to WT *T. forsythia* and the S-layer-deficient mutant. (A) WT *T. forsythia*, the S-layer-deficient mutant, an *E. coli* Omp100-expressing strain (RA11), *E. coli* RA31, and *A. actinomycetemcomitans* (Aa) were used. In total, 2×10^9 bacterial cells were used for the factor H binding assay, carried out as described in Materials and Methods. The samples, 8 ng of authentic human factor H (FH), and 0.1 μ l of human serum (HS) were first separated by 7.5% SDS-PAGE and then transferred to a nitrocellulose membrane. Immunoblotting was performed as described in Materials and Methods. (B) The intensities of factor H binding were measured with Image Lab and were quantified relative to that for WT *T. forsythia*. Data represent means \pm SD of measurements performed in triplicate. Significant differences between RA31 and RA11, and between WT *T. forsythia* and the S-layer-deficient mutant, were determined using Student's *t* test (***, $P < 0.001$; *, $P < 0.05$).

7 days (Fig. 9B). Furthermore, we compared the coaggregation of *T. forsythia* (after 3 or 7 days of incubation) with *S. sanguinis* and *S. mutans* cells in the exponential phase (OD_{550} 0.4) and obtained results similar to those with stationary-phase cells (data not shown). Then we investigated the effects of sugars on coaggregation activity and found that *N*-acetylmannosamine suppressed the coaggregation of WT *T. forsythia* with *S. sanguinis* and *S. salivarius*, while other sugars did not. However, the coaggregation of

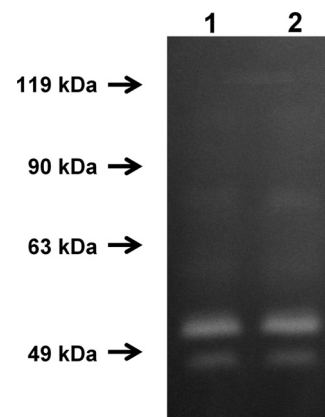


FIG 7 Zymographic analysis of WT *T. forsythia* and the S-layer-deficient mutant. The protease activities in the supernatants of WT and mutant *T. forsythia* cells were analyzed using a 7.5% polyacrylamide gel containing gelatin (1 mg/ml). Lane 1, WT; lane 2, S-layer-deficient mutant.

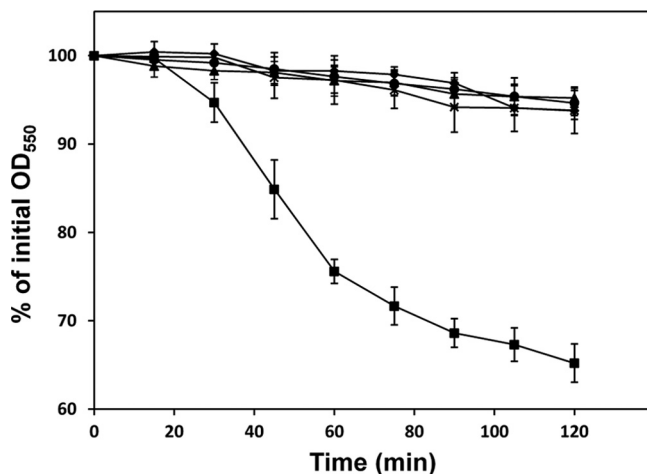


FIG 8 Coaggregation of *T. forsythia* with *S. sanguinis*. Coaggregation of WT *T. forsythia* and the S-layer-deficient mutant with *S. sanguinis* was investigated as described in Materials and Methods. A 7-day culture of WT *T. forsythia* or the mutant was mixed with an overnight culture of *S. sanguinis* (2:1) in a cuvette, and the mixture was incubated at 37°C. The OD₅₅₀ of the upper phase in the cuvette was measured using a spectrophotometer at 15-min intervals. A decrease in absorbance was considered to indicate coaggregation. Squares indicate the coaggregation of WT *T. forsythia* with *S. sanguinis*. Diamonds indicate the coaggregation of the *T. forsythia* mutant with *S. sanguinis*. As controls, the densities of single cells of WT *T. forsythia* (circles), mutant *T. forsythia* (triangles), and *S. sanguinis* (crosses) were measured. Data represent means \pm SD of measurements performed in triplicate.

WT or mutant *T. forsythia* with *F. nucleatum* was not inhibited by sugars (Fig. 10).

DISCUSSION

The mechanism of complement resistance in bacteria functions through three main pathways: protease digestion of complement components; recruitment of factors such as factor H and C4BP, which inhibit complement activation, to the bacterial cell surface; and polysaccharide-mediated suppression of complement activation (16, 30–33). In periodontal bacteria, *P. gingivalis* possesses all three resistance systems. Gingipains, surface polysaccharides, and C4BP-binding proteins in *P. gingivalis* have been characterized in previous studies (32, 33). In *T. denticola*, FhbB has been reported to have factor H-binding activity (44). In the present study, we demonstrated that the S-layer was involved in serum resistance. First, we hypothesized that the S-layer has binding affinity for factor H or C4BP, both of which are involved in the conversion of C3b to an inactivated molecule. However, we failed to observe significant differences in factor H and C4BP binding between the WT and the S-layer-deficient mutant. Finally, we observed inhibition of C3b deposition in WT *T. forsythia*, but not in the mutant, suggesting that the S-layer in *T. forsythia* inhibits the binding of C3b to the cell surface. The S-layers of *C. rectus* and *C. fetus* have been reported to mediate serum resistance by inhibiting the binding of C3b to the cell surface through the S-layer (16). Recently, a metalloproteinase, karilysin, was reported to inhibit all pathways of the complement system, contributing to serum resistance in *T. forsythia* (42). In the present study, we observed no differences in protease activity between WT *T. forsythia* and the mutant (Fig. 7). Based on these results, we concluded that the S-layer mediates serum resistance by inhibiting the deposition of C3b onto the

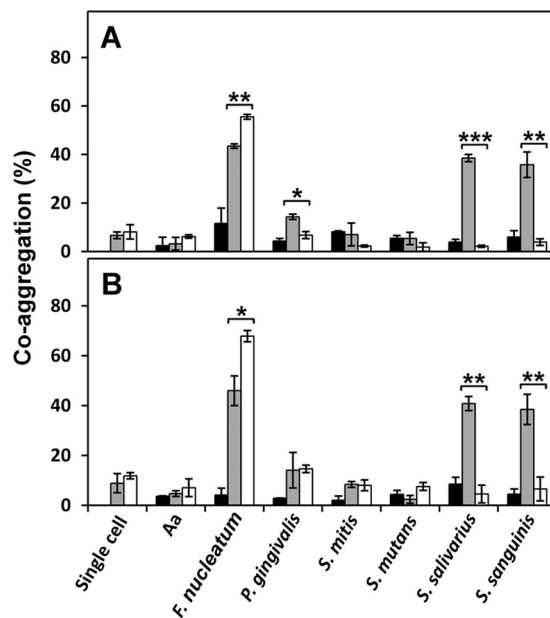


FIG 9 Coaggregation of *T. forsythia* with oral bacteria. Coaggregation of WT *T. forsythia* and the S-layer-deficient mutant with seven species of oral bacteria was investigated as described in Materials and Methods. WT *T. forsythia* (shaded bars) or the mutant (open bars) was mixed with oral bacteria (2:1) in a cuvette, and the mixture was incubated at 37°C for 60 min. The OD₅₅₀ of the upper phase in the cuvette was measured using a spectrophotometer. The percentage of coaggregation was calculated as [(initial OD – postincubation OD)/initial OD] \times 100. Filled bars indicate the percentage of autoaggregation of partner strains. Aa, *A. actinomycetemcomitans*. (A) Percentage of coaggregation of stationary-phase cells of *T. forsythia* (7-day culture) and stationary-phase cells of oral bacteria. (B) Percentage of coaggregation of exponential-phase cells of *T. forsythia* (3-day culture) and stationary-phase cells of oral bacteria. Data represent means \pm SD of measurements performed in triplicate. Significant differences in coaggregation between the WT and mutant *T. forsythia* strains were determined using Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

bacterial cell surface. In the growth experiment, the S-layer-deficient mutant grew more slowly than the WT in calf or human serum. Since complement activity in serum without bacteria did not decrease after 7 days of incubation at 37°C under anaerobic conditions (data not shown), the visible growth of both strains after 4 days was considered to result from the inactivation of complement by proteases. However, the expression of proteinases was similar for the WT and the mutant. Therefore, the slow growth of the mutant in serum was considered to be due to a significant reduction in the number of viable mutant cells by complement activity relative to the number of viable WT cells when the strains were initially exposed to serum.

Furthermore, although the S-layer-deficient *T. forsythia* mutant was more susceptible to serum than the WT, the mutant still showed a lower level of susceptibility than *E. coli* XL-II. As shown in Fig. 2, the mutant had a survival ratio almost 20% lower than that of the WT when exposed to serum, while the *E. coli* strain had a survival ratio lower than that of the mutant, suggesting that factors other than the S-layer are involved in serum resistance in *T. forsythia*. Based on the factor H binding assay, the mutant had factor H binding affinity, suggesting that a factor H binding protein(s) is localized to the cell surface. These results indicate that, as in *P. gingivalis*, several factors, including the S-layer, karilysin, and

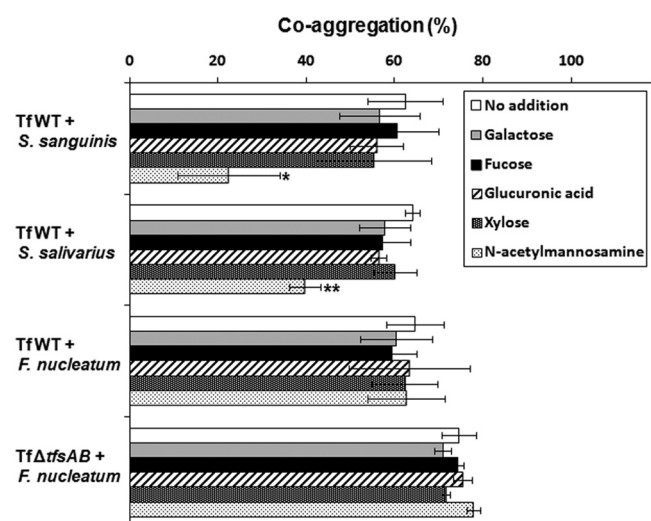


FIG 10 Effect of sugar on the coaggregation of *T. forsythia* with oral bacteria. To observe the effects of sugars on coaggregation activity, 10 mM each sugar was added to the reaction mixture. We used *N*-acetyl-D-mannosamine, D-glucuronic acid sodium salt, L-(–)-fucose, D-(+)-xylose, and D-(+)-galactose. Coaggregation activity (120 min) was investigated as described in Materials and Methods. The percentage of coaggregation was calculated as [(initial OD – postincubation OD)/initial OD] × 100. Data represent means ± SD of measurements performed in triplicate. Significant differences in coaggregation between bacteria with added sugar and bacteria with no sugar added were determined using Dunnett’s test (*, $P < 0.05$; **, $P < 0.01$).

factor H-binding protein, play a role in the serum resistance of *T. forsythia*.

Bacterial coaggregation is important for the formation of a biofilm, which is involved in resistance to various immune factors derived from the host, antibacterial agents, and disinfectants. *T. forsythia* is commonly found in dental plaques (biofilms on the tooth surface) (3, 4). Based on our results, the S-layer is associated with binding to *S. sanguinis*, *S. salivarius*, *P. gingivalis*, and *F. nucleatum*. Moreover, *F. nucleatum* coaggregates strongly with the S-layer mutant, suggesting that other surface molecules (besides the S-layer) in *T. forsythia* play a role in binding to *F. nucleatum*, but not to the oral bacteria tested in this study. *T. forsythia* BspA may play a major role in coaggregation with *F. nucleatum* (35, 45). Because BspA is a cell-associated secreted protein and belongs to the leucine-rich repeat family, deletion of the S-layer may promote coaggregation with *F. nucleatum* through BspA binding. Recently, the characterization of an S-layer protein consisting of two glycoproteins has been reported (24). Oligosaccharides containing *N*-acetylmannosaminuronic acid, 5-acetimidol-7-*N*-glycolypseudaminic acid, digitoxose, fucose, xylose, glucuronic acid, and galactose are *O*-glycosidically linked to three amino acid motifs, D(S/T)(A/I/L/M/T/V), at either TfsA or TfsB. We investigated the effects of monosaccharides, such as *N*-acetylmannosamine, fucose, xylose, glucuronic acid sodium salt, and galactose, on coaggregation activity and found that *N*-acetylmannosamine suppressed the coaggregation of WT *T. forsythia* with *S. sanguinis* and *S. salivarius* (Fig. 10). However, the coaggregation of the WT and mutant *T. forsythia* strains with *F. nucleatum* was not inhibited by sugars. Therefore, the binding site of TfsAB may differ for different bacterial species.

In conclusion, we demonstrated that the S-layer in *T. for-*

sythia contributes to serum resistance and coaggregation with other oral bacteria, suggesting that this layer has several virulence effects. Therefore, the S-layer in *T. forsythia* is important for colonization in the gingival sulcus and is associated with periodontal disease.

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