Molecular interaction of the SspB analogous peptide with periodontopathic and cariogenic bacteria

SspB ペプチドによる歯周病原細菌および齲蝕原生細菌との分子間相互作用

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Abstract

The initial stages of dental biofilm formation involved the adherence of early colonizing organisms such as *Streptococcus gordonii* on the saliva-coated tooth surface. The surface protein SspB of *S. gordonii* is known to play a role in adherence to salivary protein and mediates coaggregation with other bacteria. We previously demonstrated that the analogous SspB peptide, SspB (390-T400K-402), effectively bound to salivary agglutinin peptide SRCRP2 (scavenger receptor cysteine-rich domain peptide 2). To investigate molecular interaction of supra- and sub-gingival dental plaque biofilm formation among streptococci, periodontopathic bacteria, and salivary agglutinin as a unit, we examined the binding activity of the SspB (390-T400K-402) peptide to periodontopathic bacteria. On the other hand, SspB has extensive homology with PAc, a surface adhesin of *Streptococcus mutans*. Hence, SspB of *S. gordonii* competes with PAc of *S. mutans* for the same niche environment in the salivary pellicles. The aim of this study was to develop anti-adherence agents that enabled us to control cariogenic biofilms by using the streptococcal SspB peptide analog SspB (A4K-A11K). The binding activity of SspB (390-T400K-402) was detected by ELISA. The inhibitory effects of SspB (A4K-A11K) were then evaluated by examining *S. mutans* adhesion to saliva-coated hydroxyapatite disks (s-HA). To determine peptide interference with

biofilm formation, *S. mutans* biofilms were quantified by counting CFUs on MS agar plates and by measuring the absorbance at 492 nm of safranin-stained biofilms on s-HA. The SspB (390-T400K-402) peptide bound to *P. gingivalis* ATCC 33277, *F. nucleatum* ATCC 23726, and *F. nucleatum* #20. These bindings were reduced by the presence of whole saliva; however, the presence of SRCRP2 enhanced the binding of the SspB peptide to these bacteria. These findings suggest that the binding ability of *S. gordonii* to these periodontopathic bacteria may provide a support system for the colonization and the biofilm formation of periodontopathic bacteria. Furthermore, biofilms of *S. mutans* on s-HA were successfully reduced by pretreatment with SspB (A4K-A11K). These results suggest that SspB (A4K-A11K) peptide competitively blocked *S. mutans* adhesion to experimental pellicles through SspB-gp340 interaction, thereby inhibiting biofilm formation. These findings will contribute to the control cariogenic biofilms.

Keywords: dental plaque, biofilm, *Porphyromonas gingivalis*, *Streptococcus gordonii*, *Streptococcus mutans*, SspB (390-T400K-402), SspB (A4K-A11K)

Introduction

Dental plaque is a complex biofilm that accumulates through the colonization of large number of different species of bacteria (1, 2). Early biofilm is formed by attachment and colonization of a variety of streptococci and is dependent on both the species involved and the surface composition (3-6). Later biofilm inhabitants such as *Porphyromonas gingivalis* are capable of binding to the antecedent organisms, and these attachment mechanisms are thought to driven the temporal and spatial development of pathogenic plaque (1, 7-10). *In vitro*, *P. gingivalis* adheres avidly to sessile *Streptococcus gordonii* and, once attached, rapidly forms a biofilm comprising towering microcolonies separated by fluid-filled channels (11).

Adherence of *P. gingivalis* to *S. gordonii* is multimodal and involves a number of distinct adhesin and receptor pair on the surfaces of both organisms. These molecules include the major fimbriae and a 35-kDa protein of *P. gingivalis* and the Ssp proteins of *S. gordonii* (12, 13). The Ssp proteins (SspA and SspB, 1,500 amino acid residues), encoded by tandemly arranged genes (*sspA* and *sspB*), are members of the antigen I/II family of major streptococci surface proteins and are multifunctional adhesins (1, 8). The binding domain of the SspB to *P. gingivalis*, designated BAR (SspB Adherence Region), has been mapped to the C terminus within amino acid residues 1167 to 1250

(14). *P. gingivalis* pre-incubated with synthetic BAR peptide (1167-1193) has been shown to inhibit adherence of *P. gingivalis* to *S. gordonii*, and thereby inhibiting biofilm formation of *P. gingivalis* on the established *S. gordonii* biofilm using a dual-species open-flow biofilm culture model (15). For *in vivo* study, *S. gordonii*-colonized mice that were subsequently infected with *P. gingivalis* in the presence of BAR peptide showed levels of alveolar bone resorption similar to those of sham-infected mice; hence, the BAR peptide inhibited colonization of *P. gingivalis* on the murine oral cavity by preventing its interaction to the *S. gordonii* (16). Further, in the N-terminal region of Ssp (amino acid residues 1 to 429), the cell surface adhesins SspB and SspA showed extensive homology (63 and 60% identity, respectively, with PAc, which is surface protein antigen produced by *Streptococcus mutans*) (17). This region consists of three long and two incomplete repeating sequences likely such as the A region of PAc (17). Each repeating sequence of the N-terminal region of Ssp contains the homologous to the amino acid sequence ^{365}TYEAALKQYEADL^{377} of PAc (365-377), which is an important region for the initial attachment of *S. mutans* to the tooth surface (18, 19). We previously demonstrated the molecular mechanisms of the surface proteins of *S. gordonii* for binding to salivary components, peptides homologous to PAc (365-377) in the N-terminal region of SspB (20). The specific residues within amino acid sequences

from 283 to 295 and from 201 to 213 of SspB contribute to bind to salivary components (20). In addition, the analogous peptide SspB (390-T400K-402) peptide was the highest binding activity to the salivary components (20). This peptide had two surface positive charges at residues 390 (D) and 400 (K) in connection with the positively charged residues. The positively charged amino acid residue, i.e. lysine, is essential for binding activity on the peptide surface, the positively charged α-helical structure, to the negatively charged salivary components (21). More recently, Okuda *et al*. (22) showed the SspB (390-T400K-402) peptides significantly bound with salivary components and inhibited the binding of *S. mutans* and *S. gordonii* to saliva coated hydroxyapatite without bactericidal activity; but did not inhibit binding of *Streptococcus mitis*, a beneficial commensal.

S. mutans is the predominant etiological agent of human dental caries (23, 24). Many reports have shown correlations between elevated numbers of this organism in dental plaque biofilms and the presence of caries (reviewed by (25)). The initial adhesion of *S. mutans* to salivary acquired enamel pellicles contributes to dental biofilm maturation (26). *S. mutans* interacts with salivary proteins, such as innate immunity scavenger receptor glycoprotein-340 (gp340) (27), by means of the cell surface protein antigen PAc (28), variously designated as antigen AgI/II (29), B (30), P1 (31), and

MSL-1 (17).

Streptococci compete for adhesion binding sites on the saliva-coated tooth surface (32). SspB, which also interacts with salivary gp340, has extensive homology with PAc in *S. mutans* (17, 33); hence, *S. gordonii* competes with *S. mutans* for the same niche environment in the salivary pellicles (32, 34, 35). Recently, an SspB-derived analogous peptide, SspB (390-T400K-402), which has high-affinity binding to salivary gp340 peptide SRCRP2 (20), has been shown to competitively block *S. mutans* adhesion to experimental pellicles, thereby inhibiting biofilm formation (22).

Salivary gp340 has a high bacteria-binding capacity, and recognizes different bacterial receptors based on whether gp340 is in the fluid phase or is bound to the hydroxyapatite surface (36). Absorption of this protein onto the surfaces of teeth promotes bacterial adherence, including that of *S. mutans*. Koba et al. (21) have demonstrated that SspB (A4K-A11K) has the highest binding activity to the salivary components and to SRCRP2 in comparison to several analogous SspB peptides. Therefore, we hypothesized that SspB (A4K-A11K) would be more effective to inhibit *S. mutans* biofilm formation without antimicrobial activity.

In the present study, we evaluated the binding activity of the analogous peptide SspB (390-T400K-402) peptide to periodontopathic bacteria and the influence of

salivary components to the binding activity in order to investigate molecular interaction of supra- and sub-gingival dental plaque biofilm formation among streptococci, periodontopathic bacteria, and salivary agglutinin as a unit. An understanding of these adhesion molecules and the periodontopathic bacteria is important for elucidating the mechanisms of supra- and sub-gingival dental plaque biofilm formation. Furthermore, to develop anti-adherence agents that enable us to control cariogenic biofilms, we tested the inhibitory effects of SspB (A4K-A11K). The development of new antimicrobial compounds is essential for oral health research and for oral disease prevention strategies.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains used in this study were *Fusobacterium nucleatum strains* #20 (clinical isolate and laboratory strain in National Institute of Infectious Diseases) and ATCC 23726, *P. gingivalis* strains W83, W50 and ATCC 33277, and *Aggregatibacter actinomycetemcomitans* strains ATCC 29522, ATCC 29523 and Y4, and *S. mutans* MT 8148. *P. gingivalis* strains were grown Trypticase soy broth (Becton, Dickinson and Company; BD, Franklin Lakes, NJ, USA) supplemented with 5 μg/ml of

hemin (Sigma Chemical Co., St. Louis, Mo.) and 0.5 μg/ml of menadione (Wako Pure Chemical Industries, Osaka, Japan) under anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2) at 37˚C for 48 h. *F. nucleatum* and *A. actinomycetemcomitans* strains were grown anaerobically in Brain Heart Infusion (BHI, BD) at 37˚C for 24 h. *S. mutans* MT 8148 was maintained in BHI (BD) or on Mitis-Salivarius (MS) agar plates (Formula/Liter: 15 g of Enzymatic Digest of Casein, 5 g of Enzymatic Digest of Animal Tissue, 50 g of Sucrose, 1 g of Dextrose, 4 g of Dipotassium Phosphate, 0.075 g of Trypan Blue, 0.0008 g of Crystal Violet, 15 g of Agar, Final pH: 7.0 ± 0.2 at 25˚C) under anaerobic conditions.

Peptide synthesis

The peptide analogous to the SspB peptide, which change of T at position 400 to K in SspB (390-402), resulting in the SspB (390-T400K-402) peptide (DYQAKLAAYQKEL) and SRCRP2 (QGRVEVLYRGSWGTVC) on salivary agglutinin/gp340/DMBT1, an innate immunity scavenger receptor glycoprotein, were synthesized by Asahi Techno Glass Co. Inc. (Tokyo, Japan). SspB (A4K-A11K) peptide, DYQKKLAAYQKEL, was constructed by substitution of K (lysine) for A (alanine) at position 4 and position 11 in the consensus sequence of Ssp peptides (21). SspB

(A4K-A11K) peptide was synthesized at 95% purity by Scrum, Inc. (Tokyo, Japan), and suspended in sterile distilled water (DW) at the desired concentration immediately before use.

Human saliva collection

This study was approved by the Nihon University Institutional Review Board (approved number: EC16-031). The saliva samples from two healthy human subjects (28 and 34 years old, males) were also collected after stimulation by biting paraffin gum and placed into ice-chilled sterile bottles over a period of 5 min. The samples were then clarified by centrifugation at 10,000 × g for 10 min, filter sterilized, and used immediately for the peptide binding assay.

Peptide binding assay with SspB (390-T400K-402)

The binding activity of the SspB (390-T400K-402) peptide to periodontopathic bacteria were detected by an enzyme-linked immunosorbent assay (ELISA) technique. Briefly, 50 µl of whole periodontopathic bacterial cell (*F. nucleatum* strains #20 and ATCC 23726, *P. gingivalis* strains W83, W50 and ATCC 33277, and *A. actinomycetemcomitans* strains ATCC 29522, ATCC 29523 and Y4) suspension in

phosphate buffered saline (PBS) (1 x 10^8 cells) were used to coat each well of a 96-well plate at 37°C for 1 h. After washing three times with PBS, wells were blocked with 200 μl of 3% bovine serum albumin (BSA) in PBS at 4°C overnight. After washing, biotin-labeled SspB (390-T400K-402) peptide (50 μg/ml) in 50 μl of PBS was applied to the wells and incubated at 37°C for 1 h. The wells were then washed three times with PBS and further incubated at 37°C for 1 h with 1/1000 diluted streptavidin-conjugated alkaline phosphate (Invitrogen, Corp., Carlsbad, Calif, USA). Subsequently, SspB (390-T400K-402) adhered to periodontopathic bacteria was detected by chromogenic development using para-nitrophenyl phosphate as the alkaline phosphatase substrate. After development, absorbance at 405 nm was measured.

To prepare sonic extracts of these bacteria, washed cells were suspended in PBS (30 mg/ml, wet weight) and disrupted using sonication (Sonifier 250D, Branson Ultrasonics. Co., Danbury, CT, USA) for 10 min at 100 W on ice. The binding activity of the peptide to the sonic extracts of periodontopathic bacteria was carried out as described above with slightly modified. Briefly, 25 μg/ml or serial two-fold dilutions of sonic extracts per well in 50 μl of 50 mM carbonate buffer (pH 9.6) were used to coat each well at 4°C overnight. After washing with PBS containing 0.1% Tween 20 (PBST) and blocking, the wells were applied with 50 μl of whole saliva, 200 μg/ml of SRCRP2

peptide in 50 μl of PBS or PBS (Control), and incubated at 4°C (filter-sterilized, clarified human whole saliva) or 37°C (SRCRP2) for 1 h. After washing, biotin-labeled SspB (390-T400K-402) peptide (50 μg/ml) in 50 μl of PBS was applied to the wells and incubated at 37°C for 1 h. Following application of the peptide apply was described above.

Competitive ELISA with biotin-labeled and non-labeled SspB (390-T400K-402) peptide was carried out as described above with some of application. 25 μg/ml or serial two-fold dilutions of sonic extracts were coated each well. After blocking, 100 μg/ml of non-labeled SspB (390-T400K-402) peptide per well in 50 μl of PBS or PBS were treated with each well and incubated at 37°C for 1 h. After three times of washing with PBST, 10 μg/ml of biotin-labeled SspB (390-T400K-402) peptide in 50 μl of PBS was apply to each well and further incubated at 37°C for 1 h. Following steps were described above. All of these experiments were performed individually at least three times.

Detection of Saliva-Bound S. mutans

To detect saliva-bacterium interactions, *S. mutans* was biotin-labeled (37) for ELISA. Cultures of *S. mutans* were washed with PBS. The bacterial cells were then

biotin-labeled by incubation with NHS-LC-Biotin (Pierce) at 100 μg/ml at room temperature for 1 h. After washing with PBS, the bacterial concentration was adjusted to an optical density at 600 nm of 0.4. Ninety-six well microtiter H-plates were then coated with 100 μl of sterile whole saliva or salivary agglutinin peptide SRCRP2 (200 μg/ml) at 4˚C for 1 h. After two washes with PBST, 100 μl of biotin-labeled *S. mutans* cells were added and subsequently incubated at 37˚C for 1 h. Following steps were described above.

Peptide Binding Assay with SspB (A4K-A11K)

Binding activity of SspB (A4K-A11K) peptide to saliva was detected by sandwich assay as described by Nakai et al. (38) with some modifications. We sandwiched whole saliva between biotin-labeled and non-labeled SspB (A4K-A11K) peptides. Briefly, 96-well microtiter H-plates were coated with SspB (A4K-A11K) (650 μM) at 4˚C overnight. After 2 h blocking at 4˚C with 1% BSA in PBS containing 1 mM $CaCl_2$ (Ca-PBS), human whole saliva was added (100 μl per well) and incubated at 4˚C for 1 h. After washing, biotin-labeled SspB (A4K-A11K) peptide (650 μM) in 100 μl of sterile DW was then applied to the wells; i.e., whole saliva was placed between SspB (A4K-A11K) and biotin-labeled SspB (A4K-A11K). Reactions were detected using the

same ELISA protocol as mentioned above.

Biofilm Formation Assay Using Hydroxyapatite Disks

Biofilm formation assay using hydroxyapatite (HA) disks (10.0 mm diameter and 2.0 mm thickness; HOYA Technosurgical, Tokyo, Japan) as described by Ahn et al. (39) was performed with some modifications. Autoclaved HA disks were placed into 24-well microtiter plates and were coated with sterile whole saliva (s-HA) at 4˚C overnight. After removing the saliva, 300 μl of the SspB (A4K-A11K) peptide solution (650 μM in PBS) was added and incubated at 37˚C for 1 h. After washing with sterile PBS, 50 μl of *S. mutans* cell suspension (6.3×10^6 CFU) in PBS was added with 450 μl of tryptic soy broth without dextrose supplemented with 0.25% sucrose (TSBS) and the culture was incubated anaerobically at 37˚C for 8, 11, or 14 h.

Biofilm Evaluation

The culture medium including planktonic cells and loosely bound cells was removed, and the disks were rinsed with sterile PBS. Each disk was transferred to a conical tube containing 3 ml PBS. The adherent bacteria were detached by sonication using four 30-s pulses at 25 W with three 30-s intermittent cooling stages in an

icechilled box. The cell suspensions were serially diluted and plated on MS agar, followed by a 2 day-incubation at 37˚C. The numbers of bacterial colonies were counted and expressed as colony forming units (CFUs). To provide further confirmation, we evaluated biofilms by measuring the absorbance of safranin-stained biofilms on s-HA. After anaerobic incubation of *S. mutans* in TSBS at 37˚C for 8, 11, and 14 h on the disks, formed biofilms were rinsed with sterile PBS and air-dried, then were stained with safranin for 15 min, followed by washing with DW to remove excess dye. The biofilm mass was quantified by measuring absorbance at 492 nm.

Statistical Analyses

Data were indicated as means ± standard deviations (SD). Graph Pad Prism version 5.0 d for Mac OS X (GraphPad Software, San Diego, CA) was used to assess significance. The statistical significance of differences between two groups was determined by unpaired *t*-test. For comparisons between multiple groups, one-way analysis of variance (ANOVA) and Tukey's test were used. *P*-values less than 0.01 or 0.05 were considered to be statistically significant using two-tailed comparisons. All experiments were repeated and analyzed independently.

Results

Binding of SspB(390-T400K-402) peptide to periodontopathic bacterial cell

In order to examine the binding activity of the biotin-labeled SspB (390-T400K-402) peptide to periodontopathic bacterial cell, *P. gingivalis* strains W83, W50, and ATCC 33277, *F. nucleatum* stains #20, and ATCC 23726, and *A. actinomycetemcomitans* strains ATCC 29522, ATCC 29523, and Y4 were tested (Fig. 1). *P. gingivalis* strain ATCC 33277 showed high binding activity to this peptide. Two of *F. nucleatum* strains also showed moderate binding activity. On the other hand, *P. gingivalis* strains W50 and W83, and all of *A. actinomycetemcomitans* strains exhibited poor binding activity of the peptide. We found that SspB (390-T400K-402) possess binding ability to *P. gingivalis* strain ATCC 33277, and *F. nucleatum* strains #20 and ATCC 23726; therefore, these bacteria was used for further studies. To clarify the binding activity of the SspB (390-T400K-402) peptide to these periodontopathic bacteria, we tested the binding activity of this peptide with sonic extracts of these bacteria (Fig. 2). The SspB (390-T400K-402) peptide significantly response to sonic extract of *P. gingivalis* strain ATCC 33277, and *F. nucleatum* strains #20 and ATCC 23726. Further, we examined the binding activity of the peptide on various concentration of sonic extract of these periodontopathic bacteria (Fig. 3). The results

indicated that the binding of this peptide to these bacteria increased in a concentration-dependent manner.

Salivary components affect the binding of SspB (390-T400K-402) peptide to periodontopathic bacteria

We previously demonstrated that the SspB (390-T400K-402) peptide had the highest binding activity to salivary components as compared with the other peptides derived from *S. gordonii* SspB and *Streptococcus sobrinus* PAg (20). Thus, we hypothesized that when the extract of the periodontopathic bacteria was treated with whole saliva, the binding activity of the SspB (390-T400K-402) peptide may be increased. In order to assess this hypothesis, the sonic extracts of these bacteria treated with saliva were subjected (Fig. 4). The results showed that the binding activity of the peptide was significantly low in all of bacterial extracts treated with whole saliva compared to non-treated extracts. The SspB (390-T400K-402) peptide was also able to induce the highest level of binding to the SRCRP2 peptide, which is a member of SRCR superfamily and known for a bacterial binding feature with a multivalent character (27). Thus we investigated the binding activity of the SspB (390-T400K-402) peptide to SRCRP2 treated extraction of periodontopathic bacteria (Fig. 5). When the sonic

extracts of these bacteria were reacted with SRCRP2 peptide, the binding activity of the SspB (390-T400K-402) peptide was significantly higher than that of non-treated extraction.

Adherence of S. mutans to Salivary Components

To confirm the reproducibility of the *S. mutans*-saliva interaction, we performed ELISA where salivary components (whole saliva or salivary agglutinin peptide) were absorbed (Fig. 6). When the wells were coated with the salivary gp340 peptide SRCRP2, the highest adherence of *S. mutans* was observed of the tested conditions. Adherence of *S. mutans* to saliva-coated polystyrene plates was significantly higher than non-treated plates (control); however, it was lower than in the SRCRP2-coated condition. This suggests that salivary components, particularly gp340, promote adherence of *S. mutans* to polystyrene surfaces.

Binding Abilities of SspB (A4K-A11K) to Whole Saliva

Koba et al. (21) have demonstrated that SspB (A4K-A11K) peptide has the highest binding activity to salivary gp340 peptide SRCRP2 among several analogous SspB peptides. Hence, we hypothesized that SspB (A4K-A11K) could inhibit *S. mutans*

biofilms by competing for the same niche environment in the salivary pellicle. To assess this hypothesis, we examined the binding properties of the SspB peptide to saliva (Fig. 7). First, a sandwich assay (38) with biotin-labeled and non-labeled SspB (A4K-A11K) peptide was performed to examine the interaction between the SspB peptide and salivary components (Fig. 7). At peptide concentrations of 650 μM (Fig. 7 (a)) and 1,300 μM (Fig. 7 (b)), SspB (A4K-A11K) that had bound to saliva exhibited a positive reaction in the sandwich assay. On the other hand, low reaction levels were observed with BSA, thus suggesting that SspB (A4K-A11K) peptide has saliva-binding ability. We found sufficient saliva-binding ability of SspB (A4K-A11K) at 650 μM (Fig. 7 (a)); therefore, SspB at 650 μM was used for further studies.

Inhibitory Effects of SspB (A4K-A11K) on S. mutans Biofilm Formation on Saliva-Coated HA Disks

We next examined whether SspB (A4K-A11K) is a potential inhibitor of *S. mutans* adherence to saliva and of its biofilm formation; we therefore performed biofilm formation assays using saliva-coated HA disks (s-HA) (Fig. 8). We evaluated biofilms by measuring the absorbance of safranin-stained biofilms on s-HA (Fig. 8 (a)). Pre-treatment with the SspB analogue peptide significantly diminished biofilm mass as

compared with that of non-treatment groups at all incubation times (Fig. 8 (a)). To provide further confirmation, biofilms were evaluated by counting CFUs on MS agar plates (Fig. 8 (b)). Pre-treatment with 650 μM analogue peptide markedly reduced CFU counts compared to non-treatment at all incubation times (Fig. 8 (b)). Furthermore, growth of *S. mutans* in BHI was significantly inhibited with chlorhexidine (0.04%) ($P < 0.01$), whereas the peptide (650 μM) did not affect the bacterial growth (data not shown), suggesting that the SspB peptide has no bactericidal effect.

Discussion

Dental plaque is complex and dynamic, and the initial colonizers comprise predominantly gram-positive commensal bacteria (2, 40, 41). *S. gordonii* is an early colonizer of the salivary pellicle and its ability to bind to salivary proteins and glycoproteins are important in oral biofilm (dental plaque) development (42, 43). In addition, *S. gordonii* plays an important role in *P. gingivalis* colonization of later biofilm (44-46).

In this study, we utilized biotin-labeled SspB (390-T400K-402) peptide in our experiments to investigate molecular interaction of supra- and sub-gingival dental plaque biofilm formation among streptococci, periodontopathic bacteria, and salivary

agglutinin as a unit. We synthesized the peptide with a single N-terminal biotin label and assessed preliminary the effect of labeling of biotin in N-terminal of SspB (390-T400K-402) to binding activity of the periodontopathic bacteria. As the result, the labeling of biotin in N-terminal of SspB (390-T400K-402) was no effect to the peptide binding activity (data not shown).

In this study, we found that the analogous peptide SspB (390-T400K-402) have abilities to bind to periodontopathic bacteria, *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemcomitans* (Figs.1 and 2). In these bacteria, *P. gingivalis* strain ATCC 33277 showed the highest binding activity to SspB (390-T400K-402). *P. gingivalis* strain ATCC 33277 expressed two distinct fimbriae molecules, major fimbriae encoded by *fimA* (47) and minor fimbriae encoded by *mfa1* (48, 49). Previous reports indicated that Mfa1 of *P. gingivalis* showed to mediate adherence to surface protein SspB of *S. gordonii* (45, 46), and reported that the Mfa1-SspB mediated interaction is necessary for biofilm development. The SspB (390-T400K-402) peptide was not detected to bind to other *P. gingivalis* strains W50 and W83. These two strains express type IV fimbriae (47), while the minor fimbriae have not been expressed. These and our present results suggested that the analogous peptide SspB (390-T400K-402) bind to Mfa1, and that critical residues for binding the peptide to Mfa1 are involved within the synthetic

peptide SspB (390-T400K-402) adhesion epitope. At the same time, we found that the analogous peptide SspB (390-T400K-402) also binds to other periodontopathic bacterium, *F. nucleatum* strains #20 and ATCC 23726 (Figs. 1 and 2). These results suggested that *S. gordonii* interact with *F. nucleatum* via SspB mediated adhesion. *F. nucleatum* is considered an important bacterium in the development of complex dental plaque biofilms: a “bridge bacterium”, it mediates multiple coaggregation interactions with other oral bacteria (50). A general view concerning the formation of dental plaque suggests that *F. nucleatum* initially colonizes the tooth and gingival surfaces to form the substrate onto which later colonizers including periodontopathic bacteria adhere via coaggregation or receptor-interaction based mechanisms. In addition, our findings suggest that the ability of *S. gordonii* to bind to these periodontopathic bacteria may provide a support system for the colonization and the biofilm formation of periodontopathic bacteria.

S. gordonii is an early colonizer and has binding ability to salivary pellicle through Ssp protein (1). We previously reported that the analogous peptide SspB (390-T400K-402) strongly bind to the high-molecular-weight protein complex of salivary components and the agglutinin peptide SRCRP2 (20). While, major fimbriae of *P. gingivalis* (Type I fimbriae) can bind to salivary acidic proline-rich protein1 and

statherin through protein-protein interactions (51). *F. nucleatum* also bind to salivary proline-rich glycoprotein (1). These bacteria have the ability to bind salivary proteins, however the attachment domains of salivary protein were different between *S. gordonii* and the periodontopathic bacteria. Furthermore, recent reports and our results have shown that SspB mediates adhesion of periodontopathic bacteria. Therefore, we hypothesized that interaction between SspB and salivary components may important role for development of supra- and sub-gingival biofilm formation. We investigated the binding ability of analogous peptide SspB (390-T400K-402) and the periodontopathic bacteria in the presence of salivary protein. However, the binding activity was decreased in the presence of salivary proteins components (Fig. 4). Stinson *et al*. reported that human whole saliva and human submandibular-sublingual saliva (HSMSL) inhibit adherence of *P. gingivalis* to *S. gordonii*; and inhibition activity by HSMSL was associated with a 43-kDa protein (52). Histatins and lysozyme can also inhibit coaggregation between *P. gingivalis* and oral streptococci (40). As for *F. nucleatum*, it has strong binding ability for fibronectin, which is a glycoprotein found in saliva (50). These findings, taking the above results (Fig. 4), suggest that binding epitopes of SspB (390-T400K-402) to periodontopathic bacteria were competitively masked by these salivary components. On the other hand, when the SRCRP2 peptide was present, the

binding activity was increased (Fig. 5). Recent report indicated that *Actinomyces* species, which predominate in early dental plaque biofilm, can be interacted to salivary gp340, and also suggested that salivary gp340 has the high bacterial cell binding capacity and salivary gp340 recognizes different bacterial receptors according to whether gp340 is present in the fluid phase or surface bound (36). These findings suggest that the periodontopathic bacteria may bind to gp340 involving SRCRP2 domain. However, since the relationship between periodontopathic bacteria and gp340 is unclear at present, more research is required to address this phenomenon.

Controlling dental plaque bacteria is important in the prevention and treatment of oral diseases. In the present study, we examined the inhibitory effects of SspB (A4K-A11K) on adherence and biofilm formation of *S. mutans* in order to develop anti-adherence agents that enable us to control cariogenic biofilms.

We demonstrated that the streptococcal peptide analog SspB (A4K-A11K) derived from *S. gordonii* significantly inhibits cariogenic biofilm development formed by *S. mutans* (Fig. 8). In addition, SspB (A4K-A11K) did not show bactericidal effects (data not shown) in our preliminary study, suggesting that the diminished biofilms are irrelevant to bactericidal activity. The use of this peptide may enable us to control cariogenic biofilm formation without the risk of disruption of oral microbial

communities.

SspB of *S. gordonii* and PAc of *S. mutans* interact with salivary components including lysozyme (53, 54), amylase (53), proline-rich proteins, and an agglutinin (55). Pre-incubations of recombinant PAc (rPAc) with various concentrations of salivary agglutinin peptide SRCRP2 inhibit rPAc binding to salivary agglutinin in a dose-dependent manner, suggesting that the binding sites of PAc for SRCRP2 and agglutinin are identical or at least located in close proximity (56). Furthermore, SspB (A4K-A11K) peptide has the highest response for binding to salivary components and to salivary gp340 peptide SRCRP2 when compared with other SspBs and streptococcal adhesin-derived peptides (20, 21). These reports, together with our observations that SspB (A4K-A11K) has binding activity with saliva (Fig. 7), suggest that SspB (A4K-A11K) binds to salivary gp340.

Specific salivary proteins adsorbed onto enamel surfaces, e.g. acidic proline-rich proteins (26) and agglutinin (57), promote the adhesion of *S. mutans* by providing binding sites for bacterial adhesins. In addition, salivary sIgA, the predominant immunoglobulin found in all mucosal secretions, may promote colonization of certain strains of bacteria (58). We previously demonstrated that salivary sIgA promotes the initial attachment of *S. mutans* on the mouse tooth surface (59). In fact, saliva appears to

have a significant impact on *S. mutans* adhesion to tooth surfaces. The increase in *S. mutans* binding to saliva-coated wells (Fig. 6) suggests that the *S. mutans*-saliva interaction is reproduced in this assay. Moreover, the increase in the bacterial binding to SRCRP2-coated wells (Fig. 6) indicates that *S. mutans* interacts with salivary gp340. Indeed, rPAc has been demonstrated to bind to SRCRP2 (56). These findings provide evidence that the *S. mutans*-saliva interaction observed in this study was not induced by salivary antibodies, but rather by salivary gp340. However, some limitations are worth noting. We did not confirm whether the saliva contains sIgA specific to *S. mutans* in the present study. This assay needs to be tested further by using sIgA purified from human saliva in order to compare the binding reactions with whole saliva and SRCRP2.

Okuda et al. have reported that SspB (390-T400K-402) inhibits adhesion of *S. mutans* to s-HA beads, while *Streptococcus mitis*, a major commensal microorganism of the oral cavity of healthy humans, was not affected (22). This may be explained by the PAc-gp340 interaction being blocked by the SspB peptide. These findings, taking the above results (Fig. 6, Fig. 7) together, suggest that SspB (A4K-A11K) may be a significant and specific inhibitor for the binding of *S. mutans* to s-HA by blocking the PAc-gp340 interaction. Provided that the SspB peptide is introduced to the oral cavity, *S. mutans* may be excluded followed by immediate recolonization of *S. mitis* on the tooth

surface to keep a healthy oral flora, excluding the cariogenic bacteria.

In conclusion, the abilities of the analogous peptide SspB (390-T400K-402) derived from *S. gordonii* to bind to the periodontopathic bacteria were demonstrated. Since our findings were generated by the analogous peptide from this organism, further investigation is necessary to understand the *S. gordonii* binding mechanisms to the periodontopathic bacteria in detail. However our finding suggested that *S. gordonii* may be able to bind to the periodontopathic bacteria, subsequently the organism may play an important role of the development of late colonized bacterial biofilm as well as early colonized biofilm. The specificity of *S. gordonii* adherence with periodontopathic bacteria may represent a mechanism which contributes to colonization of the oral biofilm and may also be relevant in the infection and colonization of the periodontopathic bacteria. Furtheremore, these results suggest that the inhibitory effects on *S. mutans* biofilms by using SspB (A4K-A11K) presented herein are due to competitive inhibition of adherence of the bacteria to salivary gp340. Therefore, the SspB (A4K-A11K) peptide binding assay developed here will provide important insights into the development and employment of anti-adherence peptides in future therapies that enable us to control cariogenic biofilms.

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Figure legends

Fig. 1. Binding response of biotin-labeled SspB (390-T400K-402) peptide to periodontopathic bacteria. Whole cells of periodontopathic bacteria were coated to 96-well ELISA plate, and reacted with biotin-labeled SspB (390-T400K-402) peptide. Data were indicated as means ± SD. Fn, *F. nucleatum*; Pg, *P. gingivalis*; Aa, *A. actinomycetemcomitans* (**$P < 0.01$, *$P < 0.05$).

Fig. 2. Binding response of biotin-labeled SspB (390-T400K-402) peptide to sonic extracts of the periodontopathic bacteria. 25 μg/ml of the sonic extracts of these bacteria were coated to 96-well ELISA plate, and reacted with biotin-labeled SspB (390-T400K-402) peptide. Data were indicated as means ± SD. Fn, *F. nucleatum*; Pg, *P. gingivalis*; Aa, *A. actinomycetemcomitans* (**$P < 0.01$, *$P < 0.05$).

Fig. 3. Binding response of biotin-labeled SspB (390-T400K-402) peptide to serial dilution of sonic extracts of the periodontopathic bacteria. Serial diluted sonic extracts ( :3.2 μg/ml,  :6.3 μg/ml,  :12.5 μg/ml and  :25 μg/ml) of these bacteria were coated to 96-well ELISA plate, and reacted with biotin-labeled SspB (390-T400K-402) peptide. Data were indicated as means ± SD. Fn, *F. nucleatum*; Pg, *P. gingivalis* (vs. 3.2

μg/ml, **$P < 0.01$; *$P < 0.05$).

Fig. 4. Analysis of the effect of whole salivary components to SspB (390-T400K-402) peptide binding. Serial diluted sonic extracts of these bacteria were coated to 96-well ELISA plate. The wells were treated with whole saliva (■) or PBS (▨). Data were indicated as means ± SD. (**$P < 0.01$; *$P < 0.05$).

Fig. 5. Analysis of the effect of SRCRP2 peptide to SspB (390-T400K-402) peptide binding. 25 μg/ml of the sonic extracts of these bacteria were coated to 96-well ELISA plate. The well were treated with 200 μg/ml of SRCRP2 peptide (■) or PBS (▨). Data were indicated as means ± SD. Fn, *F. nucleatum*; Pg, *P. gingivalis* (**$P < 0.01$; *$P < 0.05$).

Fig. 6. Adherence of *S. mutans* to saliva and to salivary agglutinin peptide SRCRP2. Binding response of biotin-labeled *S. mutans* to saliva and to salivary agglutinin peptide SRCRP2 (200 μg/ml). Data were indicated as means ± SD. (**$P < 0.01$, *$P < 0.05$).

Fig. 7. Sandwich assay with biotin-labeled and non-labeled SspB (A4K-A11K).

(a) Microtiter plates were coated with non-labeled SspB (A4K-A11K) at 650 μM and (b) at 1,300 μM. Sterile saliva was added to the wells of coated plates, and then 650 μM biotinylated SspB (A4K-A11K) was added. Data were indicated as means ± SD. (**$P < 0.01$, *$P < 0.05$).

Fig. 8. Inhibition using SspB (A4K-A11K) of *S. mutans* biofilm formation on s-HA. (a) Formed biofilms were stained with safranin and were measured the absorbance at 492 nm. (b) The amounts of biofilms were expressed as a colony forming unit (CFU). Data were indicated as means ± SD. (**$P < 0.01$, *$P < 0.05$).

Figure

Fig. 1

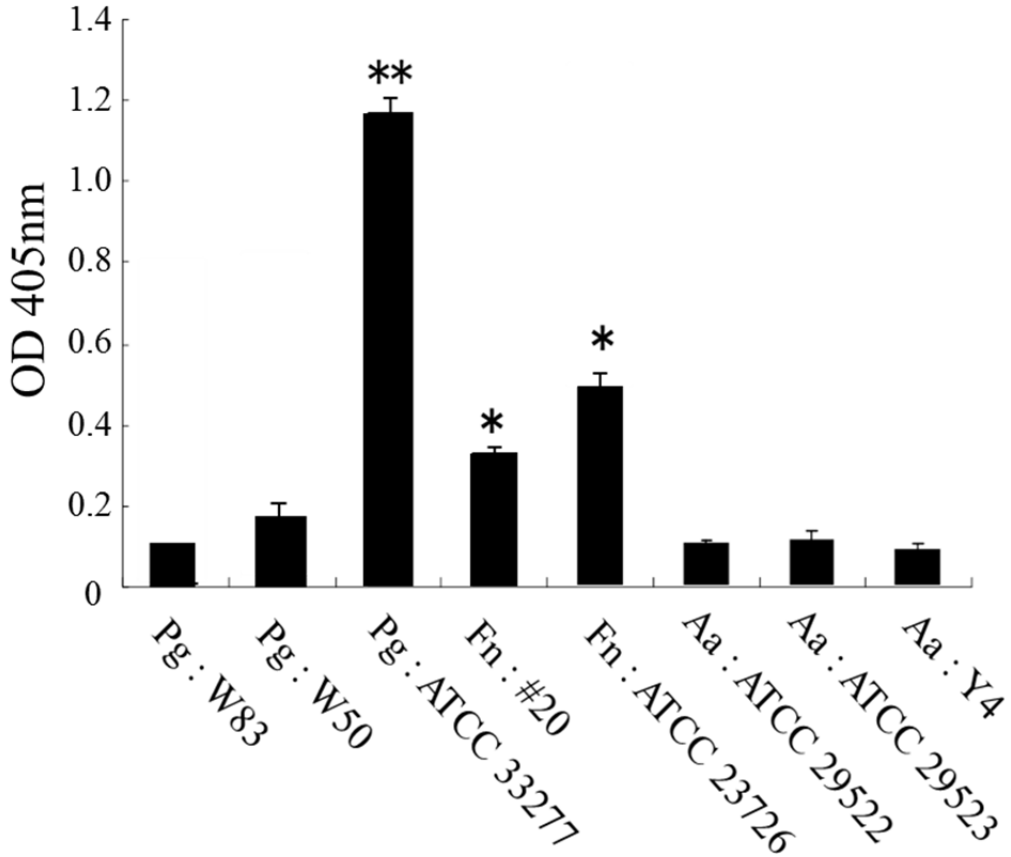

OD 405nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
*
*
Pg : W83
Pg : W50
Pg : ATCC 33277
Fn : #20
Fn : ATCC 23726
Aa : ATCC 29522
Aa : ATCC 29523
Aa : Y4

Fig. 2

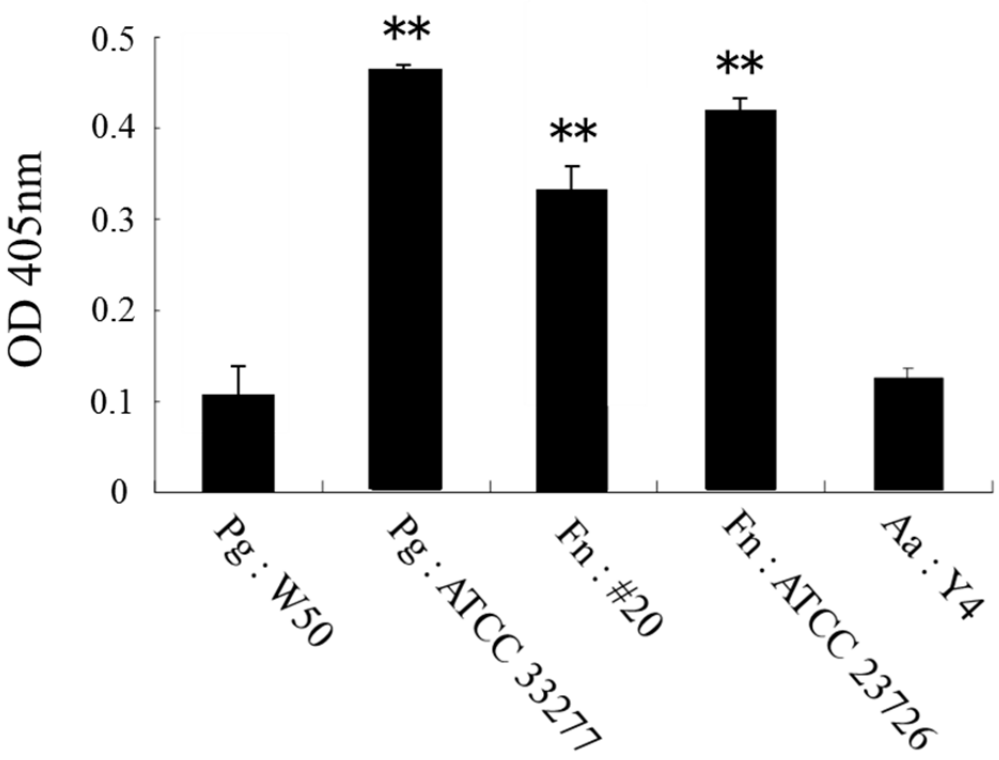

OD 405nm
0.5
0.4
0.3
0.2
0.1
0
**
**
**
Pg : W50
Pg : ATCC 33277
Fn : #20
Fn : ATCC 23726
Aa : Y4

Fig. 3

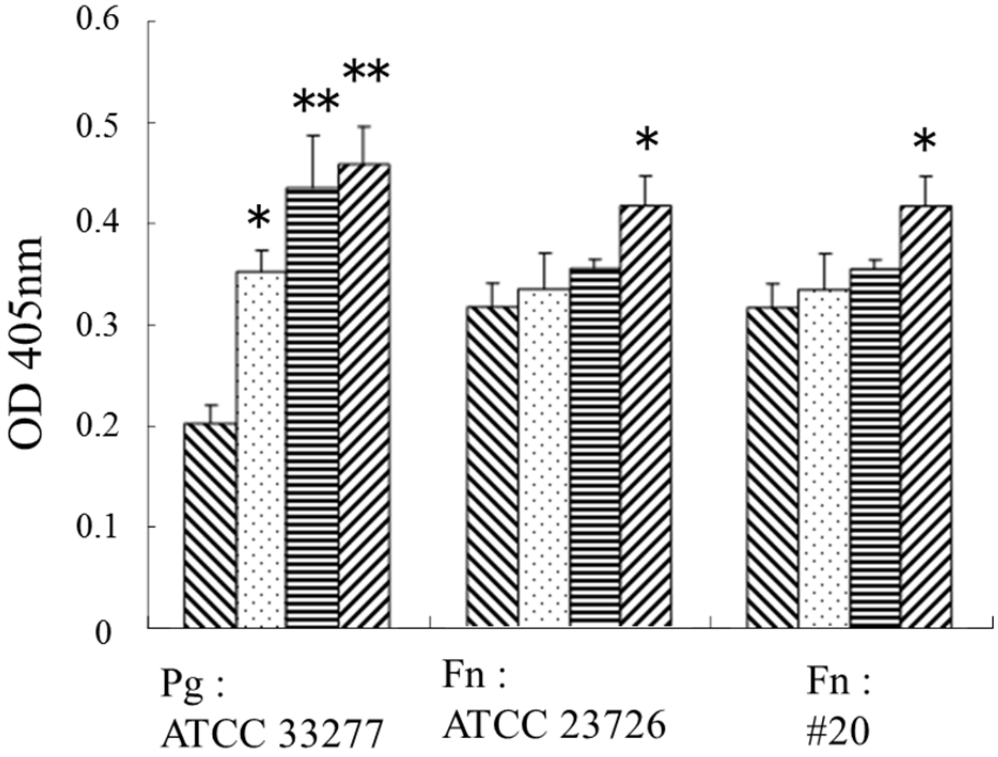


Fig. 4

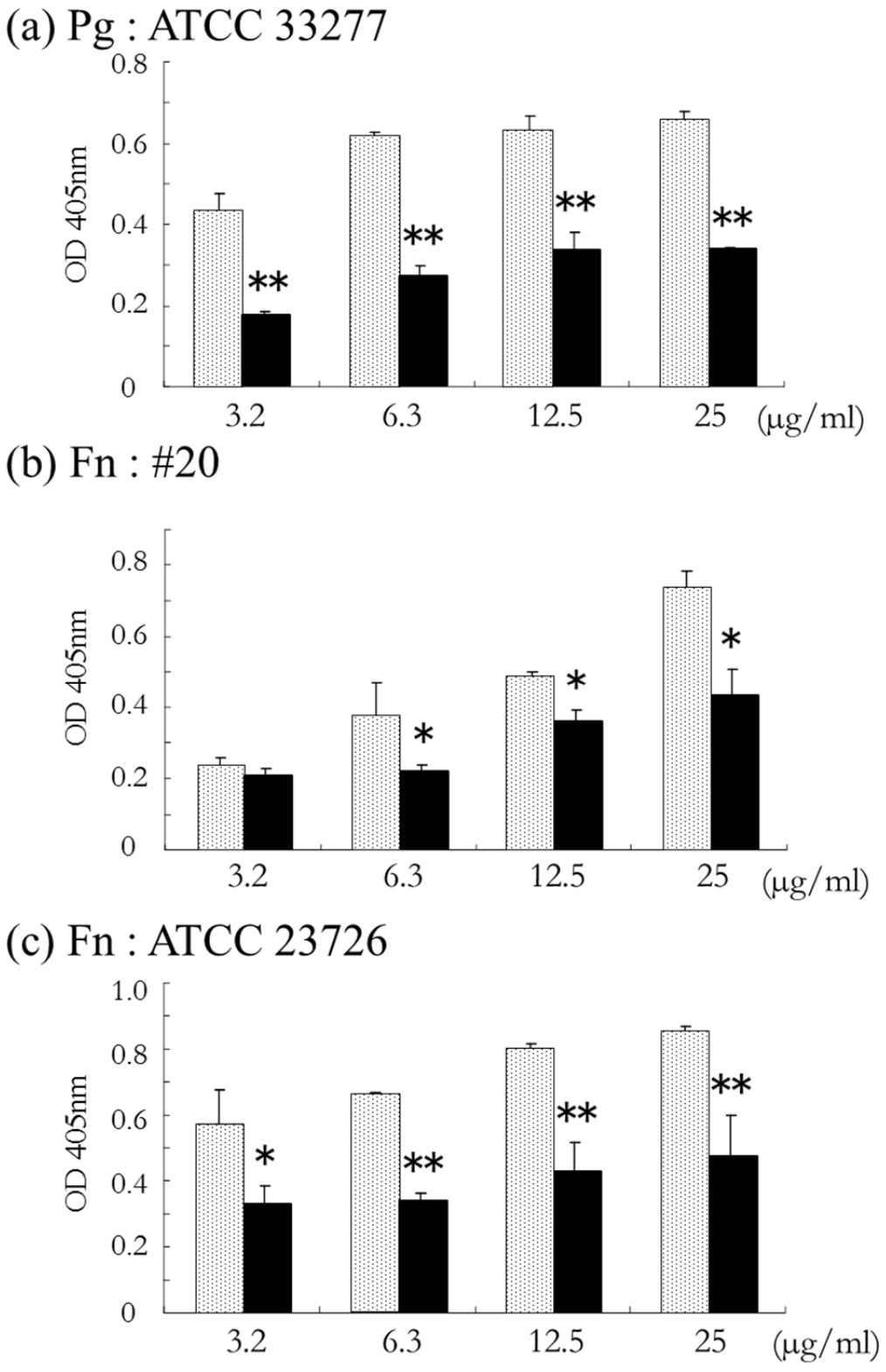
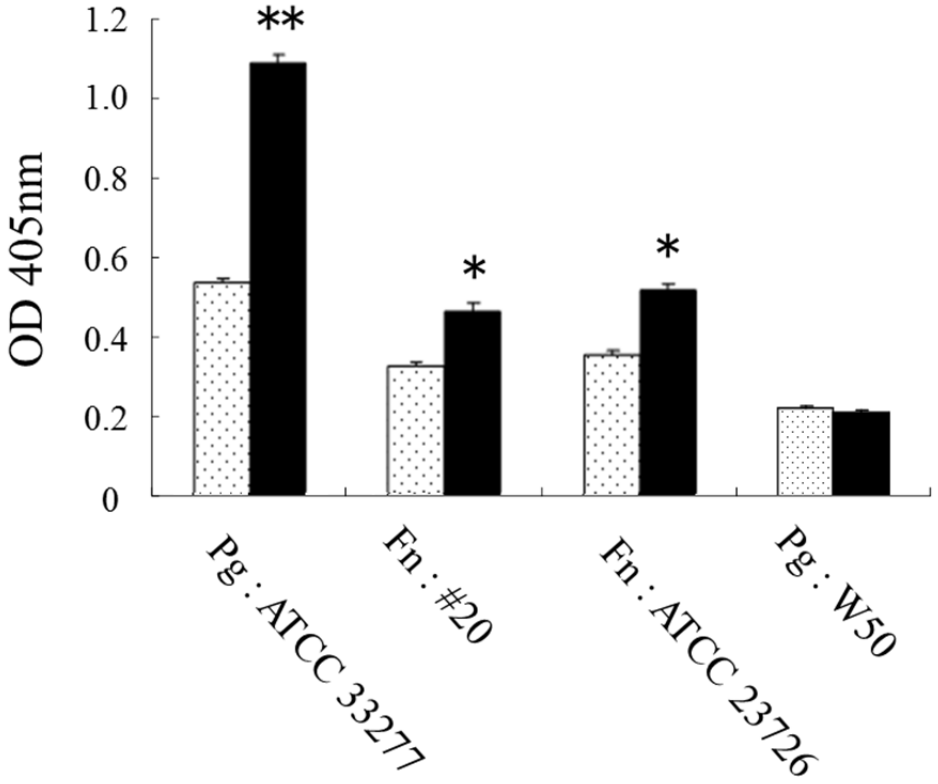

(a) Pg : ATCC 33277
OD 405nm
0.8
0.6
0.4
0.2
0
**
**
**
**
3.2
6.3
12.5
25
(μg/ml)
(b) Fn : #20
OD 405nm
0.8
0.6
0.4
0.2
0
*
*
*
3.2
6.3
12.5
25
(μg/ml)
(c) Fn : ATCC 23726
OD 405nm
1.0
0.8
0.6
0.4
0.2
0
*
**
**
**
3.2
6.3
12.5
25
(μg/ml)

Fig. 5


OD 405nm
1.2
1.0
0.8
0.6
0.4
0.2
0
**
*
*
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Fn : #20
Fn : ATCC 23726
Pg : W50

Fig. 6

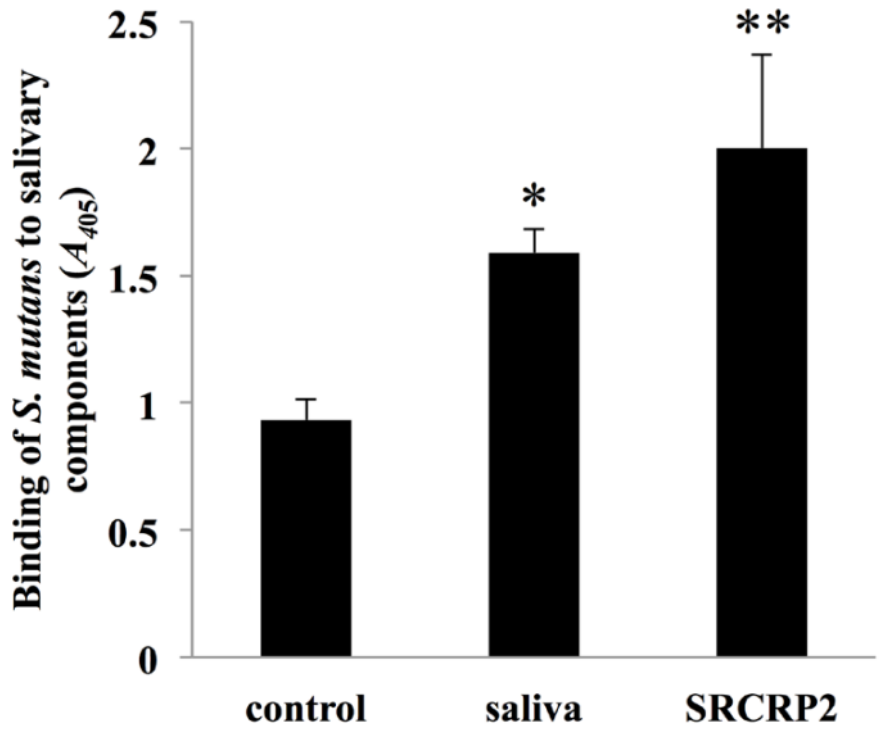

Binding of S. mutans to salivary components (A_{405})
2.5
2
1.5
1
0.5
0
*
**
control
saliva
SRCRP2

Fig. 7

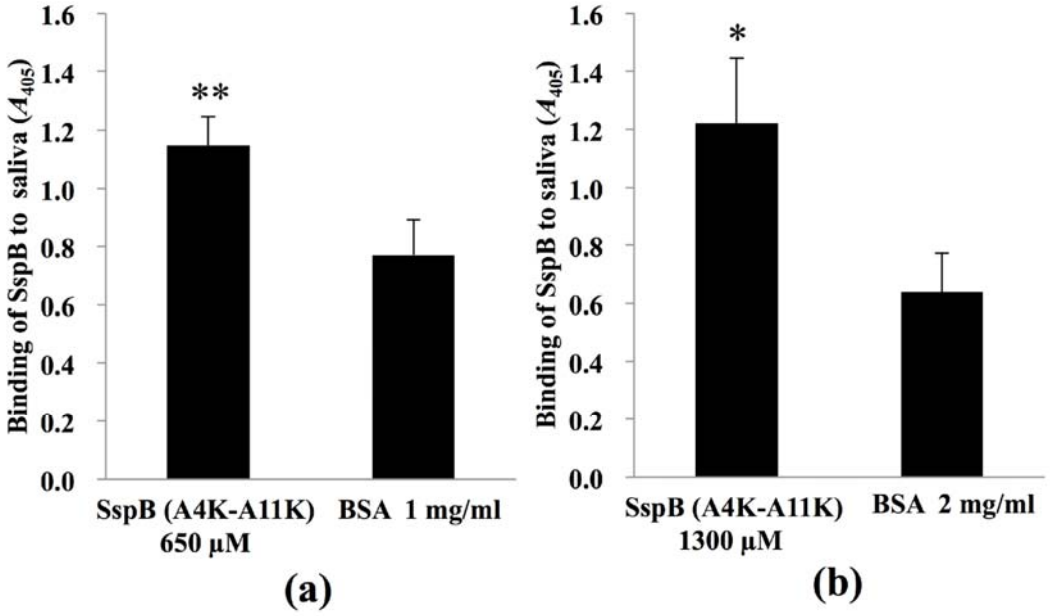
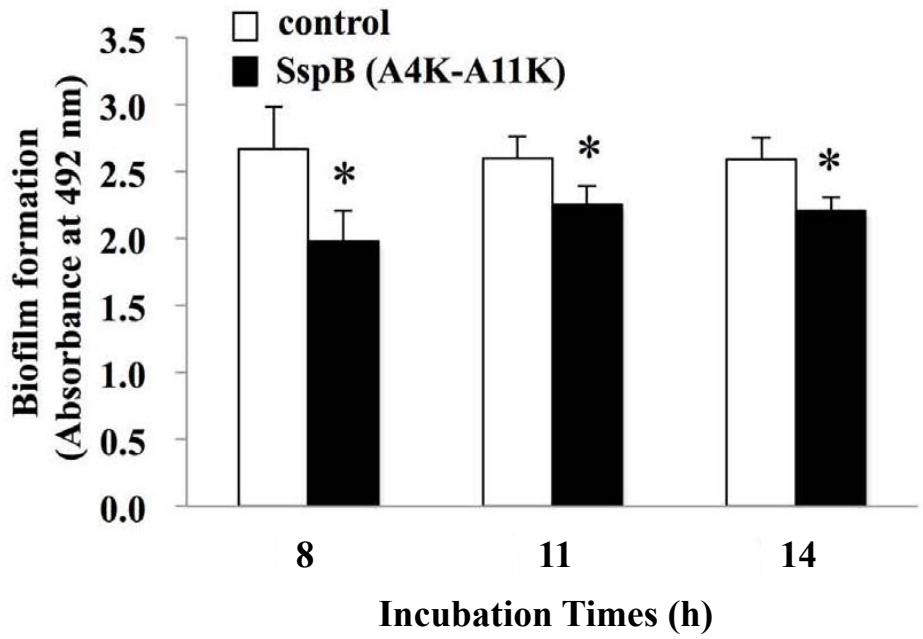
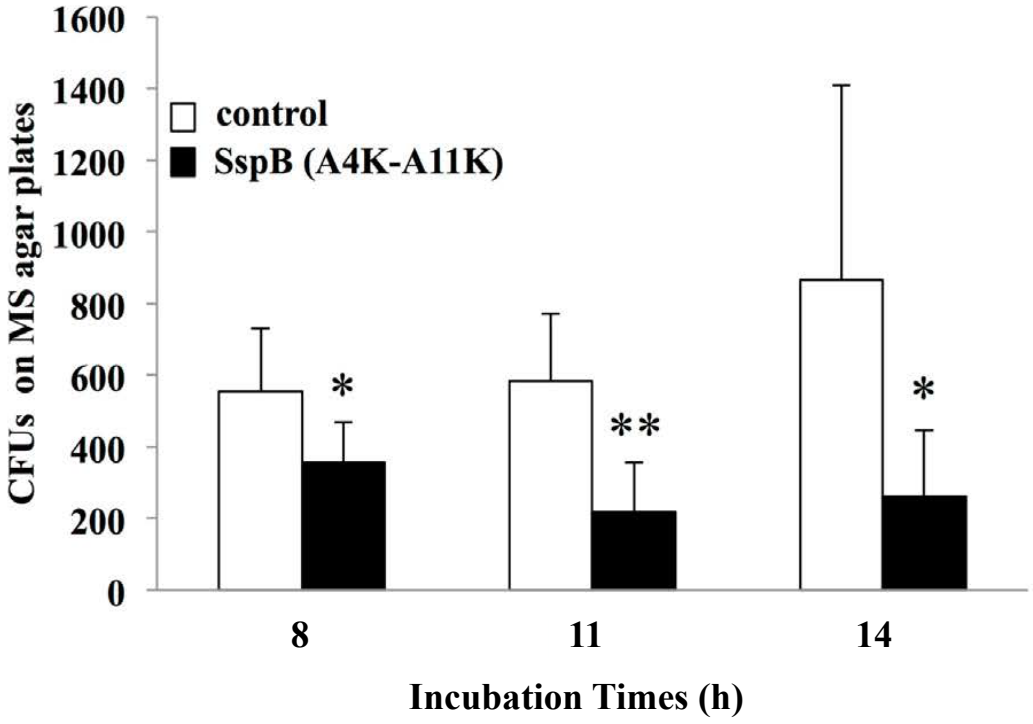

Binding of SspB to saliva (A_{405})
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
SspB (A4K-A11K) 650 µM
BSA 1 mg/ml
(a)
*
SspB (A4K-A11K) 1300 µM
BSA 2 mg/ml
(b)

Fig. 8


control
SspB (A4K-A11K)
Biofilm formation
(Absorbance at 492 nm)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
8
11
14
Incubation Times (h)

(a)


control
SspB (A4K-A11K)
CFUs on MS agar plates
1600
1400
1200
1000
800
600
400
200
0
*
**
*
8
11
14
Incubation Times (h)

(b)