INFECTION AND IMMUNITY, Nov. 2009, p. 5130–5138 Vol. 77, No. 11
0019-9567/09/$12.00 doi:10.1128/IAI.00439-09

Identification of the Binding Domain of *Streptococcus oralis* Glyceraldehyde-3-Phosphate Dehydrogenase for *Porphyromonas gingivalis* Major Fimbriae▽

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Received 20 April 2009/Returned for modification 18 June 2009/Accepted 1 September 2009

Porphyromonas gingivalis **forms communities with antecedent oral biofilm constituent streptococci. *P. gingivalis* major fimbriae bind to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) present on the streptococcal surface, and this interaction plays an important role in *P. gingivalis* colonization. This study identified the binding domain of *Streptococcus oralis* GAPDH for *P. gingivalis* fimbriae. *S. oralis* recombinant GAPDH (rGAPDH) was digested with lysyl endopeptidase. Cleaved fragments of rGAPDH were applied to a reverse-phase high-pressure liquid chromatograph equipped with a C_{18} column. Each peak was collected; the binding activity toward *P. gingivalis* recombinant fimbrillin (rFimA) was analyzed with a biomolecular interaction analysis system. The fragment displaying the strongest binding activity was further digested with various proteinases, after which the binding activity of each fragment was measured. The amino acid sequence of each fragment was determined by direct sequencing, mass spectrometric analysis, and amino acid analysis. Amino acid residues 166 to 183 of *S. oralis* GAPDH exhibited the strongest binding activity toward rFimA; confocal laser scanning microscopy revealed that the synthetic peptide corresponding to amino acid residues 166 to 183 of *S. oralis* GAPDH (pep166-183, DNFGVVEGLMTTIHAYTG) inhibits *S. oralis-P. gingivalis* biofilm formation in a dose-dependent manner. Moreover, pep166-183 inhibited interbacterial biofilm formation by several oral streptococci and *P. gingivalis* strains with different types of FimA. These results indicate that the binding domain of *S. oralis* GAPDH for *P. gingivalis* fimbriae exists within the region encompassing amino acid residues 166 to 183 of GAPDH and that pep166-183 may be a potent inhibitor of *P. gingivalis* colonization in the oral cavity.**

Coadhesion and coaggregation of *Porphyromonas gingivalis*, which is a predominant periodontopathic bacterium, and other oral bacteria are considered to be important with respect to colonization in the oral cavity (21, 33). *P. gingivalis* possesses several components as adhesins on the cell surface, such as FimA fimbriae and Mfa1 fimbriae (2, 6, 12, 14, 17, 23, 25), vesicles (10, 15, 19, 31), hemagglutinin (24), and Arg- and Lys-specific cysteine proteinases (1). Interactions between the following *P. gingivalis* cell surface components and oral gram-positive bacteria have been documented: (i) FimA fimbriae and *Actinomyces viscosus* (12), (ii) FimA and Mfa1 fimbriae and *Streptococcus gordonii* (6, 23), (iii) Arg- and Lys-specific cysteine proteinases and *A. viscosus* (1), and (iv) vesicles and *Actinomyces naeslundii* (10), *A. viscosus* (15, 31), and *Streptococcus mutans* (19). Additionally, *P. gingivalis* FimA fimbriae have been shown to interact with epithelial cells (17), cultured human fibroblasts (14), and salivary proteins (2), which indicates that *P. gingivalis* FimA fimbriae play an important role as a main adhesive component in bacterial colonization. Previously, we reported that *P. gingivalis* FimA fimbriae bind to *Streptococcus oralis*, an early colonizer in dental plaque (3). Moreover, we demonstrated that oral streptococcal cell surface glyceraldehyde-3-phosphate dehydrogenase (GAPDH) binds to *P. gingivalis* FimA fimbriae; furthermore, this interaction exhibits high affinity and high specificity (25, 26).

GAPDH is a classical glycolytic protein which is responsible for the phosphorylation of glyceraldehyde-3-phosphate, leading to the generation of 1,3-bisphosphoglycerate; however, recently, a number of diverse functions including roles in membrane fusion, microtubule binding, phosphotransferase activity, nuclear RNA export, DNA replication and DNA repair, apoptosis, and viral pathogenesis have been reported (34). An adherent function in several microorganisms has been demonstrated. For example, *Mycoplasma suis* GAPDH is involved in adhesion to erythrocytes (16); surface GAPDH proteins from group A streptococci bind a number of human proteins, including plasmin(ogen) (8, 35) and lysozyme, myosin, actin, and fibronectin (32), and adhere to human pharyngeal cells (18); *Staphylococcus aureus* and *Staphylococcus epidermidis* surface-localized GAPDHs bind transferrin (28); secreted GAPDH from *Escherichia coli* binds human plasminogen and fibrinogen (9); *Candida albicans* GAPDH binds fibronectin and laminin (13); and *Lactobacillus plantarum* GAPDH adheres to human colonic mucin (20). Moreover, we have shown previously that the cell surface GAPDHs of several oral streptococci including *S. oralis*, *S. gordonii*, *Streptococcus sanguinis*, and *Streptococcus parasanguinis* bind *P. gingivalis*

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▽ Published ahead of print on 8 September 2009.

recombinant fimbrillin (rFimA), a monomeric structural subunit of fimbriae (27).

GAPDH encoded by the *gapdh* gene, which is one of the most common housekeeping genes, is well conserved in eubacteria and eukaryotes (11). Our previous study demonstrated that *S. oralis* ATCC 9811 GAPDH possesses a high degree of homology to several other bacterial GAPDHs; its deduced amino acid sequence shares approximately 90% identity with those of *Streptococcus pneumoniae* TIGR4, *S. gordonii* FSS2, *Streptococcus pyogenes* M1, and *Streptococcus equisimilis* H46A GAPDHs and approximately 70% identity with those of *S. epidermidis* ATCC 12228, *S. aureus* N315, and *Fusobacterium nucleatum* ATCC 25586 GAPDHs (25). Therefore, we hypothesized that a common site of oral streptococcal GAPDHs may contribute to *P. gingivalis* colonization through the interaction with FimA fimbriae.

The present investigation identified the *P. gingivalis* FimA fimbria binding domain of *S. oralis* ATCC 9811 GAPDH. In addition, we examined the inhibitory effect of the synthetic peptide corresponding to the *P. gingivalis* FimA fimbria binding site of *S. oralis* GAPDH on interbacterial biofilm formation by various streptococci and *P. gingivalis* strains with different types of FimA by confocal laser scanning microscopy (CLSM).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. gingivalis* strains ATCC 33277, OMZ 314, and W 50, *S. oralis* ATCC 9811 and ATCC 10557, *S. gordonii* G9B, *S. sanguinis* ATCC 10556, and *S. parasanguinis* ATCC 15909 were maintained as frozen stocks in our laboratory. *P. gingivalis* 6/26 and HNA 99 were provided by Atsuo Amano (Osaka University). *P. gingivalis* strains were cultured in prereduced Trypticase soy broth (Becton, Dickinson and Company [BD], Sparks, MD) containing 1 mg of yeast extract (BD)/ml, 5 μg of hemin (Sigma-Aldrich Japan K. K., Tokyo, Japan)/ml, and 1 μg of menadione (Sigma-Aldrich)/ml for 24 h in anaerobic system 1025 (Forma, Marietta, OH) in an atmosphere of 80% N_2–10% CO_2–10% H_2 at 35°C. The bacterial cells were harvested by centrifugation at 5,000 × *g* for 6 min at 4°C in a high-speed refrigerated centrifuge (SRX-201; Tomy Seiko Co., Ltd., Tokyo, Japan), washed, and suspended in sterile 10 mM phosphate buffer containing 0.15 M NaCl (phosphate-buffered saline [PBS], pH 7.4). Oral streptococci were cultured at 37°C for 16 h in Todd-Hewitt broth (BD), harvested by centrifugation at 6,000 × *g* for 15 min at 4°C, washed, and suspended in chemically defined medium modified with sucrose (0.8%) as the carbon source (mCDM) (22). *E. coli* M15(pREP4) (Qiagen GmbH, Hilden, Germany) was cultured in Luria-Bertani broth (BD), and when necessary, 50 μg of ampicillin (Wako Pure Chemical Industries, Ltd., Osaka, Japan)/ml was included.

Preparation of *S. oralis* rGAPDH. *S. oralis* ATCC 9811 genomic DNA was purified with an AquaPure genomic DNA isolation kit according to the instructions of the manufacturer (Bio-Rad Laboratories, Hercules, CA) as a template for amplifying the GAPDH gene by PCR. The primer sequences (with restriction sequences underlined) were as follows: forward primer, 5′-CGCCGCGGATCC AAAGTAGTTAAAGTTGGTATTAACGGT-3′, and reverse primer, 5′-GGC GCCGAATTCGTCGACATTATTTAGCGATTTTTGCG-3′. The forward primer incorporated the BamHI and lysine (AAA) sites, whereas the reverse primer incorporated the SalI site and a translational stop site. PCR using an iCycler thermal cycler (Bio-Rad) was performed with reaction mixtures containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 1.0 μM primer, 10 ng of template DNA, and 0.025 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) in a final volume of 100 μl. The following cycling program was run: preheating at 94°C for 9 min and 33 cycles at 94°C for 45 s, 50°C for 1 min, and 72°C for 40 s. The PCR fragment was checked by DNA sequencing and cloned into plasmid pQE-30, and *E. coli* M15(pREP4) was transformed with the resulting plasmid according to the instructions of the pQE-30 manufacturer (Qiagen). His-tagged recombinant GAPDH (rGAPDH) from *S. oralis* ATCC 9811 was purified with a HisTrap HP kit (GE Healthcare UK Ltd., Buckinghamshire, United Kingdom). Purified *S. oralis* rGAPDH was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 5 to 15% Ready Gel J gel (Bio-Rad). The gel was stained with 0.1% Coomassie brilliant blue (CBB) in 40% methanol–10% acetic acid and destained by treatment with 40% methanol–1% acetic acid. A low-molecular-mass calibration kit (consisting of phosphorylase *b*, 97 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.4 kDa [Amersham Pharmacia Biotech, Buckinghamshire, England]) was used to estimate molecular masses. For the Western blot assay, rGAPDH subjected to SDS-PAGE was transferred onto a nitrocellulose membrane, after which the membrane was blocked with Block Ace (casein solution prepared from homogenized milk; Snow Brand Co., Ltd., Sapporo, Japan) for 1 h at room temperature. After three washes with PBS containing 0.05% Tween 20, the membrane was incubated with 1:1,000 penta-His horseradish peroxidase (HRP) conjugate antibodies (Qiagen) for 2 h at room temperature. His-tagged rGAPDH was visualized with an HRP conjugate substrate kit (Bio-Rad). Prestained SDS-PAGE low-range standards (phosphorylase *b*, 112 kDa; bovine serum albumin, 81 kDa; ovalbumin, 49.9 kDa; carbonic anhydrase, 36.3 kDa; soybean trypsin inhibitor, 29.9 kDa; and lysozyme, 21.3 kDa [Bio-Rad]) were used for molecular mass calibration for the Western blot assay.

Preparation of *S. oralis* rGAPDH fragments. Purified *S. oralis* ATCC 9811 rGAPDH (100 μg) was dissolved in 100 μl of 100 mM Tris-HCl (pH 8.5) containing 6 M urea (Wako) and 0.5% (wt/vol) EDTA (Dojindo Laboratories, Kumamoto, Japan), and reduction was carried out using 1 μmol of dithiothreitol at 37°C for 3 h in a nitrogen atmosphere. Alkylation was conducted by the addition of 2 μmol of iodoacetic acid (Wako) in 1 μl of 0.25 M NaOH at 25°C for 30 min in the dark. Following adjustment to pH 8.5 with Tris base (Sigma-Aldrich), rGAPDH was diluted into a 4 M urea solution; subsequently, a 400-μl solution of lysyl endopeptidase (5 μg; Wako) dissolved in 0.05% polyoxyethylene(10) octylphenyl ether (Triton X-100; Wako) was added. The mixture was incubated at 37°C for 12 h in the dark, after which 200 μl of 10% trifluoroacetic acid (TFA) was introduced to terminate the digestion. The separation of rGAPDH fragments was effected by employing reverse-phase high-pressure liquid chromatography (HPLC) involving an L-6200/6000 liquid chromatograph (Hitachi Ltd., Tokyo, Japan) equipped with a Symmetry300 C_{18} column (4.6 by 250 mm with a 5-μm particle size; Waters, Milford, MA); the column was equilibrated with 0.1% TFA in HPLC-grade water. The elution of rGAPDH fragments was performed at room temperature with a linear gradient of 0 to 60% acetonitrile in 0.1% TFA (1%/min) at a flow rate of 1 ml/min. The fractionated fragments were collected manually by monitoring the absorbance at 210 nm with an L-4000 detector (Hitachi). The peak exhibiting the strongest binding activity toward *P. gingivalis* rFimA was resubjected to chromatography on the same HPLC system utilizing an Xterra phenyl column (4.6 by 250 mm with a 5-μm particle size; Waters) equilibrated with 0.1% TFA and 10% acetonitrile in HPLC-grade water. The elution of fragments was conducted with a linear gradient of 10 to 60% acetonitrile in 0.1% TFA (1%/min) at a flow rate of 1 ml/min at room temperature. The fractionated peak displaying the strongest binding activity toward rFimA was lyophilized and digested with three proteinases.

A portion of the fragment (500 pmol) was dissolved in 100 μl of 10 mM Tris-HCl (pH 7.5) and then digested with 0.2 μg of endoproteinase Asp-N (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C for 4 h. The fragment (1 nmol) was digested with trypsin (Promega, Madison, WI) in a 100-μl volume containing 50 mM ammonium bicarbonate (pH 7.8) and 0.4 μg of trypsin at 37°C for 4 h. The resulting tryptic fragment corresponding to amino acid residues 163 to 193 of *S. oralis* GAPDH (600 pmol) was further digested with 0.2 μg of endoproteinase Glu-C (Roche Diagnostics) in 100 μl of 50 mM ammonium bicarbonate (pH 7.8) at 25°C for 4 h. Three products of digestion with the aforementioned proteinases were separated by HPLC with a Symmetry300 C_{18} column as described above, and the binding activity of each fragment was measured.

Binding of *S. oralis* rGAPDH fragments to *P. gingivalis* rFimA. *P. gingivalis* rFimA (type I fimbriae) was prepared in accordance with the methods reported previously (29). Interactions between *P. gingivalis* rFimA and the cleaved fragments of *S. oralis* rGAPDH were analyzed with a BIAcore 2000 apparatus (GE Healthcare). The carboxymethylated dextran matrix on the CM5 sensor chip (GE Healthcare) was activated with *N*-hydroxysuccinimide and *N*-ethyl-*N*-[(3-dimethylamino)propyl]carbodiimide hydrochloride (1:1) at a flow rate of 5 μl/min at 37°C. *P. gingivalis* rFimA (20 μg/ml) in 10 mM sodium acetate buffer (pH 4.5) was immobilized at 2,500 resonance units (RU) on the matrix according to the instructions in the manual from the sensor chip manufacturer (GE Healthcare). Excess active sites on the matrix were blocked with 1 M ethanolamine-HCl, and the matrix was washed with 10 mM NaOH. All materials were dissolved in 10 mM HEPES buffer containing 3 mM EDTA and 0.005% surfactant P20 (GE Healthcare), which also served as a running buffer in the experiments. Each cleaved fragment of *S. oralis* rGAPDH was injected across the active CM5

TABLE 1. Binding activities of *S. oxalis* ATCC 9811 rGAPDH fragments with *P. gingivalis* rFimA[a]

Proteinase(s) used for digestion or type of fragment	*S. oralis* ATCC 9811 GAPDH fragment		K_a (M^{-1})
	Amino acid residues	Amino acid sequence	
Lysyl endopeptidase	163–216	ALQDNFGVVEGLMTTIHAYTGDQMILDGPHRGGDLRRARAGAANIVPNSTGAAK	3.54×10^7
Endoproteinase Asp-N	166–183	---DNFGVVEGLMTTIHAYTG----------	4.51×10^7
	184–188	----------DQMIL----------	0
	189–195	----------DGPHRGG----------	0
	196–216	----------DLRRARAGAANIVPNSTGAAK	0
Trypsin	163–193	ALQDNFGVVEGLMTTIHAYTGDQMILDGPHR----------	3.67×10^7
	202–216	----------AGAANIVPNSTGAAK	0
Trypsin and endoproteinase Glu-C	163–172	ALQDNFGVVE----------	0
	173–193	----------GLMTTIHAYTGDQMILDGPHR----------	0
Synthetic peptide	166–183	---DNFGVVEGLMTTIHAYTG----------	3.84×10^8
	163–179	ALQDNFGVVEGLMTTIH----------	3.54×10^6

[a] The binding activities (K_a values) of *S. oxalis* ATCC 9811 rGAPDH and native GAPDH were determined to be 1.19×10^7 and 4.34×10^7 M^{-1}, respectively. Dashes in amino acid sequences represent missing amino acids.

surface (which had rFimA) and an empty control CM5 surface at a flow rate of 20 μl/min at 37°C. The binding of each fragment was monitored, and results are expressed as RU in a sensorgram. A result of 1,000 RU corresponded to a change in the surface concentration of 1 ng/mm^2 on the sensory chip. At the end of each run, the surface was regenerated by successive injections with 10 mM NaOH. Specific profiles of GAPDH fragment binding to the immobilized rFimA were obtained following subtraction of the control surface signal from the response signal. Analysis of these kinetic parameters was conducted with BIAevaluation 3.1, a software package (GE Healthcare), according to the operator's manual.

Amino acid analysis of *S. oralis* rGAPDH fragments. Amino acid sequencing, amino acid analysis, and mass spectrometry analysis of these fragments were performed with a Procise-cLC sequencer (Applied Biosystems), an L-8500A amino acid analyzer (Hitachi), and a Proteomics 4700 mass spectrometer (Applied Biosystems). The instruments were operated according to the manufacturers' protocols. All fragments were unambiguously identified as the peptides listed in Table 1.

Synthesis of peptides. Peptides corresponding to amino acid residues 166 to 183 and 163 to 179 of *S. oralis* ATCC 9811 GAPDH (pep166-183 [DNFGVVE GLMTTIHAYTG] and pep163-179 [ALQDNFGVVEGLMTTIH], respectively) and a control peptide containing Ala substitutions for hydrophobic amino acids of pep166-183 (DNAGAAEGAATTAHAYTG) were obtained from Invitrogen Corp. (Carlsbad, CA). The purity of each synthetic peptide was more than 96% as determined by reverse-phase HPLC.

Biofilm formation by *P. gingivalis* with streptococci. Analysis of biofilm formation by *P. gingivalis* with streptococci was conducted as described previously (22). Briefly, samples of 1.5×10^9 CFU/ml of streptococci were stained with 15 μg of hexidium iodide (HI; Molecular Probes, Carlsbad, CA) for 15 min and washed with mCDM three times. Streptococci (5×10^7 CFU) were inoculated into mCDM in individual chambers coated with human whole saliva and cultured anaerobically in a CultureWell chambered coverglass system (Grace Bio-Labs, Bend, OR) at 37°C for 16 h. *P. gingivalis* (1.5×10^9 CFU/ml) was stained with 16 μg of fluorescein isothiocyanate (FITC; Molecular Probes) for 30 min and washed with PBS three times; subsequently, samples of 5×10^6 CFU of *P. gingivalis* were added to the wells exhibiting streptococcal biofilm formation. The mixtures were incubated anaerobically at 37°C for 24 h in the dark on a rotator (Mini-Shaker 3D; BIOSAN, Riga, Latvia). In experiments investigating the inhibition of biofilm formation, *P. gingivalis* was preincubated with pep166-183 or the control peptide at room temperature for 30 min.

Biofilm analysis by CLSM. Analysis of biofilm formation was accomplished with a CLSM (LSM510 version 3.2; Carl Zeiss Co., Ltd., Oberkochen, Germany). After the wells were washed with PBS, monospecies or two-species biofilms formed on the bottoms of the wells were observed at a magnification of less than ×40 by using an argon laser (488-nm wavelength) to visualize HI-stained streptococci and an HeNe laser (543-nm wavelength) for visualization of FITC-stained *P. gingivalis.* Six fields from each CLSM image were selected randomly; biovolumes of streptococci and *P. gingivalis* were calculated using Imaris software version 5.0.1 (Bitplane AG, Zurich, Switzerland). The level of inhibition of biofilm formation by peptides was calculated as follows: percent inhibition = $(1 - A/B) \times 100$ (A, volume ratio of *P. gingivalis* to streptococci when the test peptide was added; B, volume ratio of *P. gingivalis* to streptococci when the control peptide was added). The statistical difference was analyzed by one-way analysis of variance followed by Dunnett's test; moreover, dose dependency was analyzed by utilizing the Jonckheere-Terpstra test with 2006 Excel statistics software (SSRI, Tokyo, Japan).

RESULTS

Identification of *P. gingivalis* fimbria binding domain in *S. oralis* GAPDH. *S. oralis* rGAPDH was prepared based on the amino acid sequence of *S. oralis* ATCC 9811 GAPDH. A lysine site was incorporated in the forward primer in order to facilitate the removal of the His tag with lysyl endopeptidase. Purified rGAPDH was subjected to SDS-PAGE; a band displaying a molecular mass of approximately 40 kDa was detected (Fig. 1A, left, lane 2). The purity of rGAPDH was greater than 97% according to SDS-PAGE gel staining with CBB. A Western blot assay demonstrated reaction of the band with anti-penta-His HRP conjugate antibodies, which indicated that the band was His-tagged rGAPDH (Fig. 1A, right, lane 2). Moreover, the identity of the band as *S. oralis* GAPDH was confirmed by N-terminal amino acid sequencing and mass spectrometry. The binding of *S. oralis* rGAPDH and native GAPDH to *P. gingivalis* rFimA was confirmed by the BIAcore instrument; the equilibrium association constants (K_a values) were 1.19×10^7 and 4.34×10^7 M^{-1}, respectively.

To identify the *P. gingivalis* fimbria binding domain in *S. oralis* GAPDH, rGAPDH was digested with lysyl endopeptidase and subjected to reverse-phase HPLC employing a Symmetry300 C_{18} column. The binding activity of each peak toward *P. gingivalis* rFimA was measured by the BIAcore apparatus. As shown in Fig. 1B, the strongest binding activity toward *P. gingivalis* rFimA was observed with the absorbance peak eluted at 46 min. The peak was resubjected to HPLC utilizing an Xterra phenyl column and was separated into 10 fractions (data not shown). The binding activity of each fraction was also measured by the BIAcore instrument. The fraction exhibiting the strongest binding activity toward rFimA was subjected to amino acid sequencing, amino acid analysis, and mass spectrometric analysis. The amino acid sequence of this fragment was ALQDNFGVVEGLMTTIHAYTGDQMILDGPHRGGDLR RARAGAANIVPNSTGAAK, which corresponds to amino acid residues 163 to 216 of *S. oralis* ATCC 9811 GAPDH. The

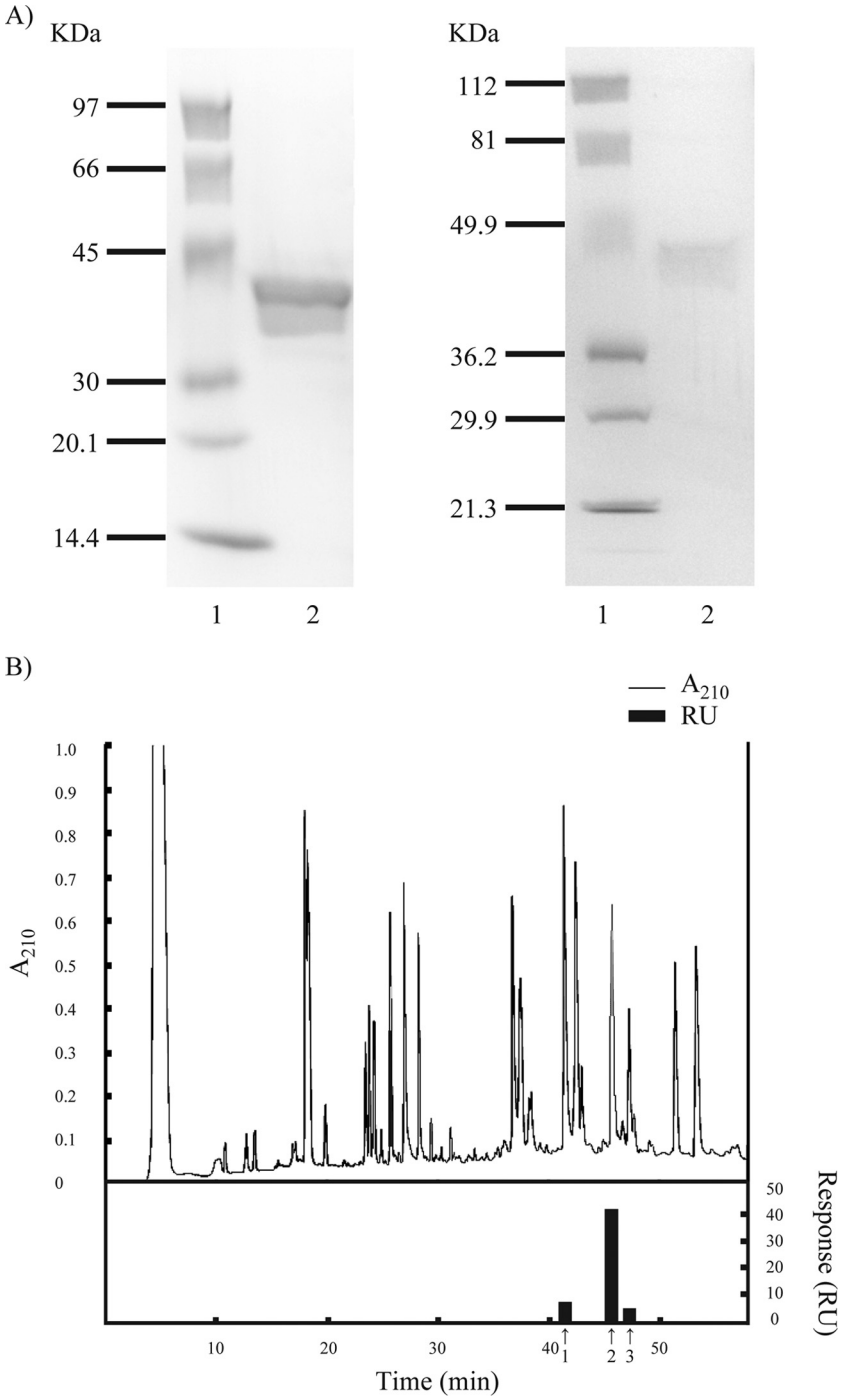
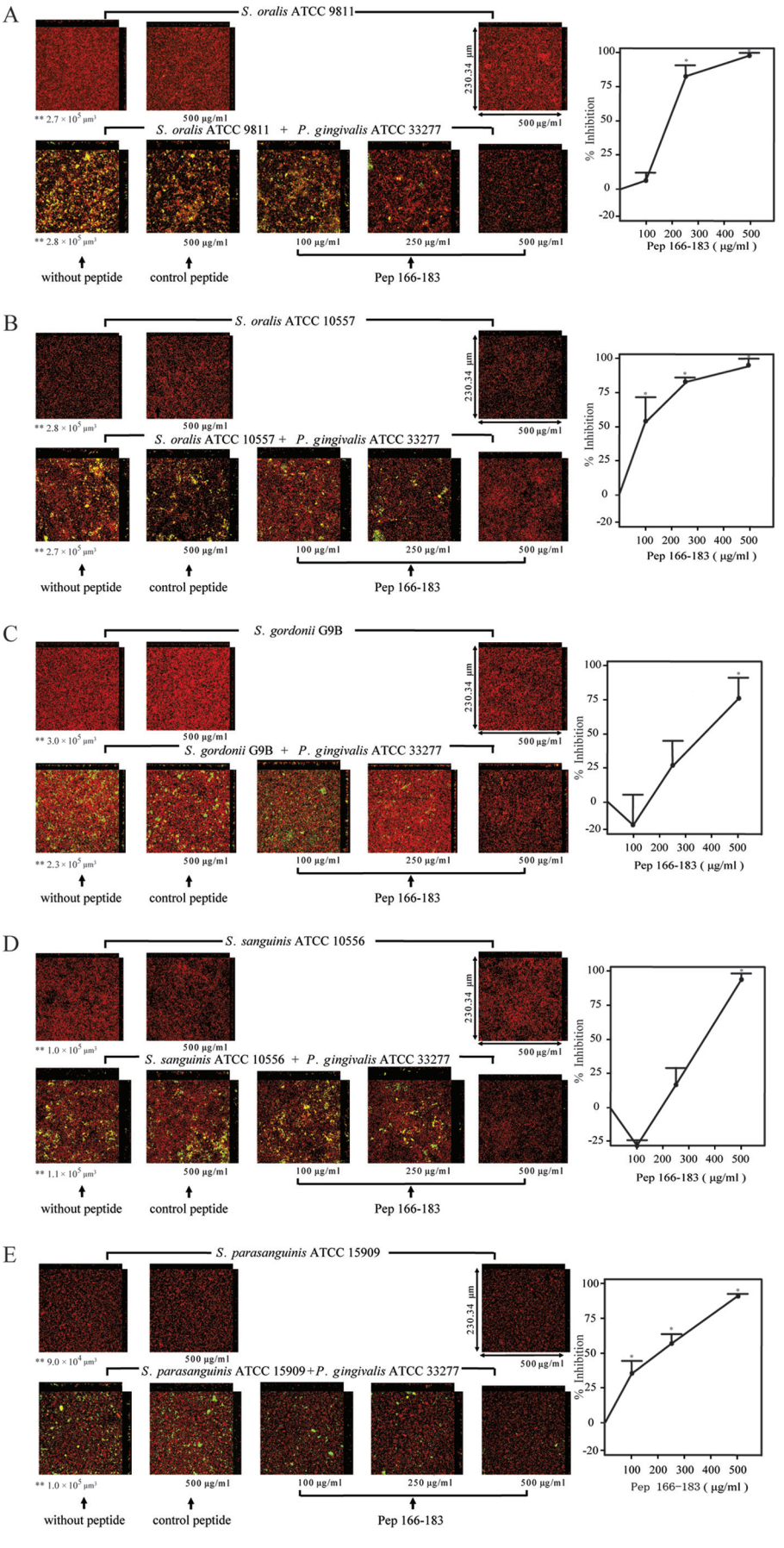


FIG. 1. (A) Results of SDS-PAGE analysis and Western blot assay of purified *S. oralis* ATCC 9811 rGAPDH. The sample was subjected to SDS-PAGE (5 to 15% gel) and electrotransferred onto a nitrocellulose membrane. After being blocked with Block Ace, the membrane was incubated with penta-His conjugate. Bound antibodies were visualized by employing an HRP conjugate substrate kit. (Left) SDS-PAGE gel with CBB staining; (right) Western blot. Lanes: 1, molecular mass standard proteins; 2, *S. oralis* ATCC 9811 rGAPDH. (B) HPLC profile and binding activity of lysyl endopeptidase-digested *S. oralis* rGAPDH with *P. gingivalis* rFimA. *S. oralis* rGAPDH was digested with lysyl endopeptidase and subjected to HPLC involving a Symmetry300 C_{18} column. The elution of rGAPDH fragments was effected with a linear gradient of 0 to 60% acetonitrile at a flow rate of 1 ml/min. Fractionated fragments were collected manually by monitoring the absorbance at 210 nm. The binding activity of each peak toward *P. gingivalis* rFimA was measured by a BIAcore apparatus. 1, absorbance peak eluted at 41.5 min; 2, absorbance peak eluted at 46 min; 3, absorbance peak eluted at 47.5 min.

K_a of the fragment according to BIAcore analysis was 3.54×10^7 M^{-1} (Table 1). The fragment was further digested with endopeptidase Asp-N; four fragments were isolated by HPLC. A fragment corresponding to amino acid residues 166 to 183 (DNFGVVEGLMTTIHAYTG) exhibited strong binding to rFimA ($K_a = 4.51 \times 10^7$ M^{-1}), whereas three other fragments (DQMIL, DGPHRGG, and DLRRARAGAANIVPNSTG AAK) demonstrated no binding activity toward rFimA as de-


A
S. oralis ATCC 9811
230.34 µm
500 µg/ml
** 2.7 × 10^5 µm^3
S. oralis ATCC 9811 + P. gingivalis ATCC 33277
** 2.8 × 10^5 µm^3
500 µg/ml
100 µg/ml
250 µg/ml
500 µg/ml
without peptide
control peptide
Pep 166-183
% Inhibition
Pep 166-183 (µg/ml)
B
S. oralis ATCC 10557
S. oralis ATCC 10557 + P. gingivalis ATCC 33277
** 2.7 × 10^5 µm^3
C
S. gordonii G9B
S. gordonii G9B + P. gingivalis ATCC 33277
** 3.0 × 10^5 µm^3
** 2.3 × 10^5 µm^3
D
S. sanguinis ATCC 10556
S. sanguinis ATCC 10556 + P. gingivalis ATCC 33277
** 1.0 × 10^5 µm^3
** 1.1 × 10^5 µm^3
E
S. parasanguinis ATCC 15909
S. parasanguinis ATCC 15909+P. gingivalis ATCC 33277
** 9.0 × 10^4 µm^3
** 1.0 × 10^5 µm^3

tected by the BIAcore system (Table 1). The fragment corresponding to amino acid residues 163 to 216 of *S. oralis* ATCC 9811 GAPDH was also digested with trypsin; subsequently, two fragments were isolated by HPLC. The fragment corresponding to amino acid residues 163 to 193 (ALQDNFGVVEGLM TTIHAYTGDQMILDGPHR) exhibited strong binding activity toward rFimA ($K_a = 3.67 \times 10^7\ M^{-1}$); in contrast, the fragment corresponding to amino acid residues 202 to 216 (AGAANIVPNSTGAAK) displayed no binding activity toward rFimA. The fragment corresponding to amino acid residues 163 to 193 (ALQDNFGVVEGLMTTIHAYTGDQMIL DGPHR) was further digested with endopeptidase Glu-C. Two fragments (ALQDNFGVVE and GLMTTIHAYTGDQ MILDGPHR) were isolated by HPLC; however, neither peptide demonstrated binding activity toward rFimA (Table 1). These results suggest that a binding domain for *P. gingivalis* FimA fimbriae may exist within amino acid residues 166 to 183 of *S. oralis* ATCC 9811 GAPDH.

The peptide corresponding to amino acid residues 166 to 183 of *S. oralis* ATCC 9811 GAPDH (pep166-183; DNFGVV EGLMTTIHAYTG) was synthesized; subsequently, the binding specificity of the peptide for *P. gingivalis* rFimA was characterized. BIAcore characterization revealed that the resonance response reflecting the *P. gingivalis* rFimA-pep166-183 interaction occurred in an analyte concentration-dependent manner. The K_a value for the interaction was $3.84 \times 10^8\ M^{-1}$, demonstrating high-affinity binding (Table 1).

Inhibitory effect of pep166-183 on biofilm formation by *P. gingivalis* ATCC 33277 with *S. oralis* ATCC 9811. The inhibitory effect of pep166-183 on biofilm formation by *P. gingivalis* ATCC 33277 and *S. oralis* ATCC 9811 was examined. As shown in Fig. 2A, pep166-183 inhibited biofilm formation by *P. gingivalis* ATCC 33277 and *S. oralis* ATCC 9811 in a dose-dependent manner (P value for trend, <0.001). *P. gingivalis* ATCC 33277-*S. oralis* ATCC 9811 biofilm formation was significantly inhibited by pep166-183 at 250 and 500 μg/ml ($P < 0.001$); the percentages of inhibition were approximately 83 and 98%, respectively. A control peptide in which hydrophobic amino acid residues were replaced with Ala (DNAGAAEGA ATTAHAYTG) was synthesized. The control peptide at 500 μg/ml exerted no effect on either *P. gingivalis* ATCC 33277-*S. oralis* ATCC 9811 biofilm formation or *S. oralis* ATCC 9811 monospecies biofilm formation (Fig. 2A). Pep166-183 at 500 μg/ml exerted no effect on *S. oralis* ATCC 9811 monospecies biofilm formation.

Inhibitory effect of pep166-183 on biofilm formation by *P. gingivalis* ATCC 33277 with various streptococci. Next, the inhibitory effect of pep166-183 on biofilm formation by *P. gingivalis* ATCC 33277 with various streptococci was examined by the same method applied in the inhibitory experiment involving *P. gingivalis* ATCC 33277 and *S. oralis* ATCC 9811. Pep166-183 inhibited biofilm formation by *P. gingivalis* ATCC 33277 with *S. oralis* ATCC 10557, *S. gordonii* G9B, *S. sanguinis* ATCC 10556, or *S. parasanguinis* ATCC 15909 in a dose-dependent manner (P value for trend, <0.001) (Fig. 2B to E). Pep166-183 significantly inhibited *P. gingivalis* ATCC 33277-*S. oralis* ATCC 10557 biofilm formation at 100, 250, and 500 μg/ml ($P < 0.001$); percentages of inhibition were approximately 69, 83, and 94%, respectively (Fig. 2B). Pep166-183 also inhibited *P. gingivalis* ATCC 33277-*S. gordonii* G9B biofilm formation; however, significant inhibition was observed solely at 500 μg/ml ($P < 0.001$), and the percent inhibition was approximately 75%. Biofilm formation by *P. gingivalis* ATCC 33277-*S. sanguinis* ATCC 10556 (Fig. 2D) and *P. gingivalis* ATCC 33277-*S. parasanguinis* ATCC 15909 (Fig. 2E) was significantly inhibited by pep166-183 at 500 μg/ml ($P < 0.001$); the percent inhibition was greater than 90% in both cases. Pep166-183 significantly inhibited *P. gingivalis* ATCC 33277-*S. parasanguinis* ATCC 15909 biofilm formation, even at a concentration of 100 μg/ml ($P < 0.001$) (Fig. 2E). Neither the tested streptococcus-*P gingivalis* ATCC 33277 biofilm formation nor monospecies streptococcal biofilm formation was affected by the control peptide at 500 μg/ml (Fig. 2B to E). Pep166-183 at 500 μg/ml exerted no inhibitory effect on monospecies biofilm formation by these streptococci (Fig. 2B to E).

Inhibitory effect of pep166-183 on biofilm formation by *S. oralis* ATCC 9811 and *P. gingivalis* strains with different types of fimbriae. The inhibitory effect of pep166-183 on biofilm formation by *S. oralis* ATCC 9811 and *P. gingivalis* in the presence of different types of fimbriae was examined. *P. gingivalis* ATCC 33277 (type I fimbriae), OMZ 314 (type II), 6/26 (type III), W 50 (type IV), and HNA 99 (type V) were selected as representative strains possessing different types of fimbriae. Pep166-183 inhibited biofilm formation by *P. gingivalis* OMZ 314 and *S. oralis* ATCC 9811 in a dose-dependent manner (P value for trend, <0.001) (Fig. 3A). The volume of biofilm formation by *P. gingivalis* 6/26 with *S. oralis* ATCC 9811 was less than that by *P. gingivalis* ATCC 33277 with *S. oralis* ATCC 9811; however, dose-dependent inhibition by pep166-183 was observed (P value for trend, <0.001) (Fig. 3B). Although *P. gingivalis* W 50 and HNA 99 exhibited biofilm formation with *S. oralis* ATCC 9811, the volume of biofilm formation was much less than that by *P. gingivalis* ATCC 33277 with *S. oralis* ATCC 9811; therefore, the inhibitory effects of pep166-183 on *P. gingivalis* W 50-*S. oralis* ATCC 9811 and *P. gingivalis* HNA

FIG. 2. Inhibitory effects of pep166-183 on biofilm formation by *P. gingivalis* ATCC 33277 with various streptococci as determined by CLSM. HI-stained streptococci (5×10^7 CFU/well; red) were inoculated into individual chambers coated with human whole saliva and cultured anaerobically at 37°C for 16 h. FITC-stained *P. gingivalis* ATCC 33277 (5×10^6 CFU/well; green) was added to the wells in which streptococcal biofilm was observed. The mixtures were incubated anaerobically at 37°C for 24 h and analyzed by CLSM. In experiments evaluating the inhibition of biofilm formation, *P. gingivalis* ATCC 33277 was preincubated aerobically with pep166-183 or the control peptide at room temperature for 30 min. Magnification, ×40. The following streptococci were tested: *S. oralis* ATCC 9811 (A), *S. oralis* ATCC 10557 (B), *S. gordonii* G9B (C), *S. sanguinis* ATCC 10556 (D), and *S. parasanguinis* ATCC 15909 (E). Representative photographs are shown. In each panel, the upper section displays the monospecies streptococcal biofilm, and the lower section displays the biofilm formed by various streptococci and *P. gingivalis* ATCC 33277. Data are the means and standard errors of results for six fields in CLSM images. *, $P < 0.001$ for comparison to results obtained for the control peptide; **, volume of streptococci.

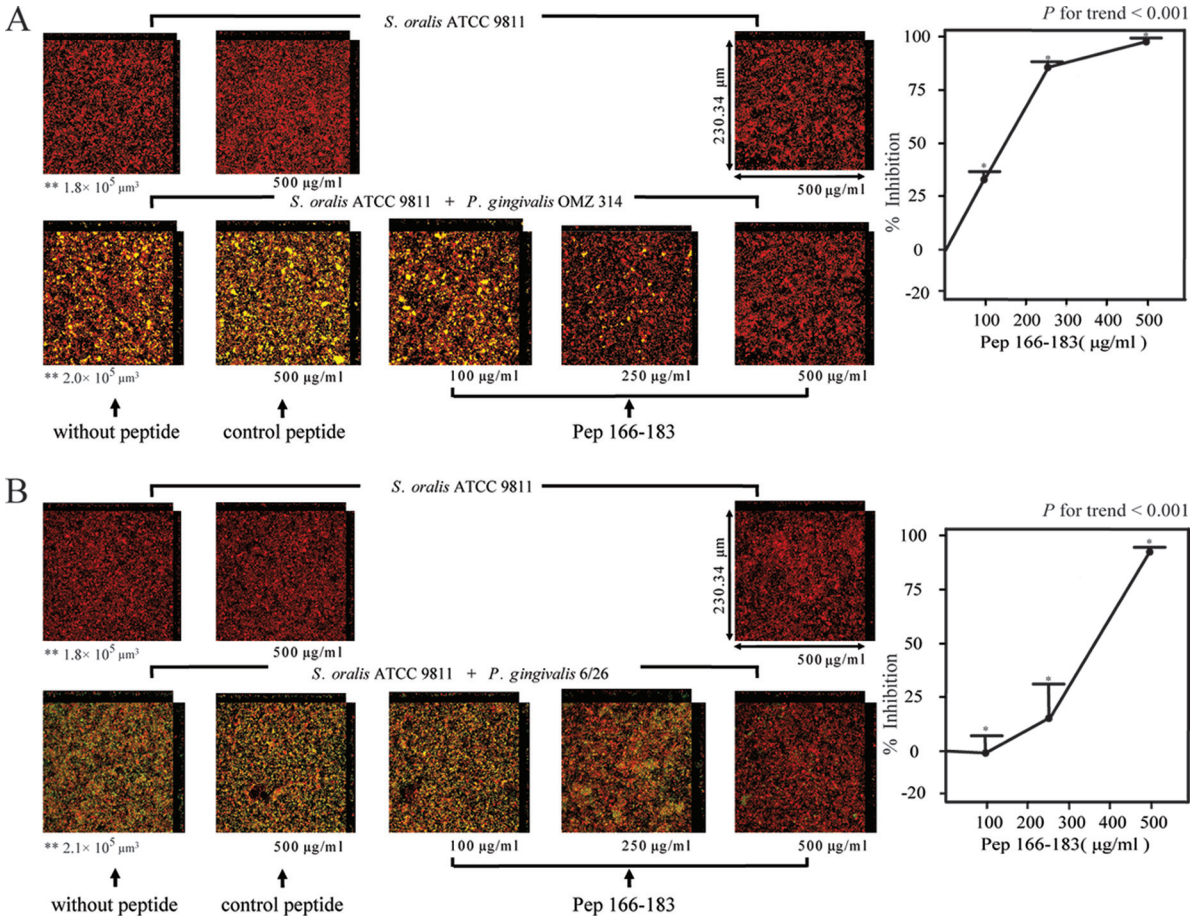


FIG. 3. Inhibitory effects of pep166-183 on biofilm formation by *S. oralis* ATCC 9811 and *P. gingivalis* strains with different types of fimbriae as determined by CLSM. HI-stained *S. oralis* ATCC 9811 (5×10^7 CFU/well; red) was inoculated into individual chambers coated with human whole saliva and cultured anaerobically at 37°C for 16 h. FITC-stained *P. gingivalis* OMZ 314 cells (A) and 6/26 cells (B) (5×10^6 CFU/well; green) were added to the wells with *S. oralis* ATCC 9811 biofilm. The mixtures were incubated anaerobically at 37°C for 24 h and analyzed by CLSM. In experiments evaluating the inhibition of biofilm formation, *P. gingivalis* was preincubated with pep166-183 or control peptide aerobically at room temperature for 30 min. In each panel, the upper section displays the *S. oralis* ATCC 9811 monospecies biofilm and the lower section exhibits the biofilm formed by *S. oralis* ATCC 9811 and *P. gingivalis*. Magnification, ×40. Representative photographs are shown. Data are the means and standard errors of results for six fields in CLSM images. *, $P < 0.001$ for comparison to results obtained for the control peptide; **, volume of streptococci.

99-*S. oralis* ATCC 9811 biofilm formation were not determined (data not shown).

DISCUSSION

This study firstly identified the *P. gingivalis* FimA fimbria binding domain in *S. oralis* ATCC 9811 GAPDH. The domain corresponding to amino acid residues 166 to 183 (DNFGVV EGLMTTIHAYTG) exhibited the strongest binding activity toward rFimA ($K_a = 4.51 \times 10^7\ M^{-1}$), indicating that the *P. gingivalis* FimA fimbria binding domain exists in this region. Consequently, pep166-183 was synthesized, and the binding of the peptide to rFimA was confirmed by BIAcore analysis ($K_a = 3.84 \times 10^8\ M^{-1}$). A peptide corresponding to amino acid residues 163 to 179 (ALQDNFGVVEGLMTTIH) of *S. oralis* ATCC 9811 GAPDH was also synthesized; subsequently, binding activity toward rFimA was assessed. This peptide also bound to rFimA; however, the K_a was lower ($3.54 \times 10^6\ M^{-1}$) than that of pep166-183 ($3.84 \times 10^8\ M^{-1}$); this result indicated that the C-terminal amino acid residues of pep166-183 (AYTG) might be important for binding to rFimA. Nagy et al. (30) showed that the 43 amino acid residues of the N terminus of human GAPDH constitute the RNA binding domain; furthermore, Jin et al. (18) reported that group A streptococcal surface dehydrogenase possesses two binding sites for human pharyngeal cells, a C-terminal strong binding site and an N-terminal weak binding site. However, no report regarding GAPDH binding site involvement in the interbacterial interaction appears in the literature. The identification of the domain of *S. oralis* GAPDH which interacts with *P. gingivalis* FimA fimbriae and the finding that the synthetic peptide corresponding to the binding domain inhibits biofilm formation by various streptococci and *P. gingivalis* strains with different types of fimbriae are significant points of the present investigation.

Employing combinatorial libraries involving substitutions for several active-site amino acid residues in a region of the *S. gordonii* SspB polypeptide (residues 1167 to 1193) designated

BAR, Daep et al. (7) reported adhesion to *P. gingivalis* minor fimbrial antigen Mfa1 and possible contributions of electrostatic and hydrophobic interactions to Mfa1-BAR binding. In the present study, the control peptide in which the hydrophobic amino acid residues were replaced with Ala exerted little inhibitory effect on *P. gingivalis* biofilm formation with streptococci; this finding indicates that the hydrophobic amino acid residues of pep166-183 are important with respect to biofilm formation. On the basis of data from Predict Protein (http://www.predictprotein.org/), pep166-183 is characterized by a β-sheet, whereas the control peptide is characterized by an α-sheet. Changes in the electric charge and conformation of the peptide as consequences of the replacement of hydrophobic amino acid residues with Ala may be the reason why the control peptide exerted no inhibitory effect on biofilm formation.

CLSM observation revealed pep166-183 inhibition of biofilm formation by *P. gingivalis* ATCC 33277 and *S. oralis* ATCC 9811 in a dose-dependent manner. Previously, Maeda et al. (27) reported a correlation between the streptococcal cell surface GAPDH activity and coaggregation activity with *P. gingivalis*; additionally, *S. oralis* ATCC 10557, *S. gordonii* G9B, *S. sanguinis* ATCC 10556, and *S. parasanguinis* ATCC 15909 exhibited high levels of cell surface GAPDH activity and coaggregation activity with *P. gingivalis*. The GAPDH amino acid sequences from these streptococci displayed identities of greater than 97% to that from *S. oralis* ATCC 9811; moreover, the amino acid sequences corresponding to residues 166 to 183 of *S. oralis* ATCC 9811 GAPDH were completely identical in these streptococci. Therefore, we hypothesized that pep166-183 might inhibit the interaction of these streptococci with *P. gingivalis* and repress interbacterial biofilm formation. As expected, pep166-183 inhibited biofilm formation by *P. gingivalis* ATCC 33277 with other tested streptococci (Fig. 2B to E). Given that the degree of homology among GAPDHs from oral streptococci was very high, the possibility that GAPDHs from other oral streptococci such as *S. mutans* and *Streptococcus criceti* might interact with *P. gingivalis* FimA fimbriae existed; however, cell surface GAPDH activities and coaggregation activities of *S. mutans* and *S. criceti* with *P. gingivalis* were very low (27). The amount of GAPDH expressed on the surfaces of streptococcal cells may be related to the degree of attachment to *P. gingivalis.*

P. gingivalis FimA fimbriae are classified into six types (I to V and Ib) based on the diversity of *fimA* genes encoding FimA (5). Amano et al. (4) reported that a majority of periodontitis patients carry type II *fimA* organisms. Based on the result that pep166-183 inhibited biofilm formation by *S. oralis* ATCC 9811 and *P. gingivalis* OMZ 314 (type II), as well as *P. gingivalis* ATCC 33277, pep166-183 may be beneficial as a potent inhibitor of *P. gingivalis* colonization with respect to the prevention of periodontitis.

In conclusion, this study demonstrated that the *P. gingivalis* FimA fimbria binding domain in *S. oralis* ATCC 9811 GAPDH exists within amino acid residues 166 to 183; furthermore, the present findings indicated that the hydrophobic amino acid residues play an important role in the binding interaction. The peptide corresponding to the binding domain for *P. gingivalis* FimA fimbriae inhibited biofilm formation by various streptococci and *P. gingivalis* strains with different types of FimA fimbriae in a dose-dependent manner. These results indicated that pep166-183 may play an important role in *P. gingivalis* biofilm formation; moreover, this peptide may be applicable as an inhibitor of *P. gingivalis* colonization.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid for scientific research (B; 17390564 and 20390534) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

We thank Mitsuko Nakatani and Yoko Takada for the expert technical assistance and Kenji Kangawa of National Cardiovascular Center Research Institute for helpful advice.

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Editor: V. J. DiRita