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Structural Characterization of Peptide-Mediated Inhibition of *Porphyromonas gingivalis* Biofilm Formation

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Porphyromonas gingivalis **is a periodontal pathogen whose primary niche is the anaerobic environment of subgingival dental plaque, but initial colonization of the oral cavity is likely to occur on supragingival surfaces that already support robust biofilm communities. Our studies have shown that *P. gingivalis* adheres to *Streptococcus gordonii* through interaction of the minor fimbrial antigen Mfa1 with a specific region of the streptococcal SspB polypeptide (residues 1167 to 1193) designated BAR. We show that a synthetic peptide comprising the BAR sequence potently inhibits *P. gingivalis* adherence to *S. gordonii* (50% inhibitory concentration = 1.3 μM) and prevents the development of *P. gingivalis* biofilms. However, a retroinverso peptide that possessed the same side chain topology as that of BAR was inactive, suggesting that interactions of Mfa1 with the peptide backbone of BAR are important for binding. A conformationally constrained analog of BAR inhibited *P. gingivalis* adherence and biofilm formation but at a lower specific activity than that of BAR. Therefore, to further define the structural features of the Mfa1-BAR interaction, we functionally screened combinatorial libraries of BAR in which active site residues (Asn^{1182}, Thr^{1184}, and Val^{1185}) were replaced with each of the 19 common amino acids. Peptides containing positively charged amino acids at position 1182 or hydrophobic residues at position 1185 bound *P. gingivalis* more efficiently than did control peptides containing Asn and Val at these positions, suggesting that electrostatic and hydrophobic interactions may contribute to Mfa1-SspB binding. In contrast, replacement of Pro or Gly at these positions was detrimental to adherence, suggesting that perturbation of the BAR secondary structure influences activity. The net effect of substitutions for Thr^{1184} was less pronounced either positively or negatively than that at the other sites. These results define physicochemical characteristics of the interacting interface of Mfa1 and SspB and suggest that peptides or peptidomimetics with greater specific inhibitory activity than that of BAR can be developed. These compounds may represent potential therapeutics that target some of the first molecular interactions that allow *P. gingivalis* to colonize the oral cavity.**

Dental plaque is a complex and dynamic biofilm that accumulates through the sequential and ordered colonization of over 700 species of bacteria (13, 24, 25, 28). An oral biofilm comprised predominantly of gram-positive commensals such as the oral streptococci and *Actinomyces* spp. can exist in the oral cavity in the absence of overt disease (21, 25). However, populational shifts in the biofilm that lead to overrepresentation of acidophiles or of gram-negative obligate anaerobes may contribute to the onset and progression of the most common oral diseases, caries and periodontal disease (5). Indeed, adult periodontitis is associated with elevated levels of several gram-negative anaerobes in subgingival plaque, including the asaccharolytic, obligate anaerobe *Porphyromonas gingivalis* (26).

P. gingivalis is a secondary colonizer of the oral biofilm, and its primary niche is the anaerobic environment of subgingival plaque. In this environment, *P. gingivalis* interacts with a variety of other gram-negative obligate and facultative anaerobes, e.g., *Fusobacterium nucleatum* (11, 12), *Treponema denticola* (8, 9), and *Tannerella forsythensis* (29) via specific receptor-ligand interactions. However, the initial colonization of the oral cavity by *P. gingivalis* is likely to occur on supragingival surfaces that already support robust biofilm communities, and the successful colonization of this niche by *P. gingivalis* is contingent upon a variety of factors such as reduced oxygen tension and sufficient nutritional sources (1, 18). Consistent with this, *P. gingivalis* has been shown to adhere to primary colonizing organisms of supragingival surfaces such as *Streptococcus gordonii* (2, 6, 16).

The adhesion of *P. gingivalis* and *S. gordonii* is multimodal and involves at least two distinct receptor-ligand pairs. The long and short fimbriae of *P. gingivalis* have both been shown to be involved in this interaction (3). The structural subunit of the long fimbriae, FimA, has been shown to interact with cell surface glyceraldehyde-3-phosphate dehydrogenase of *S. gordonii* (19, 27), whereas the minor fimbrial protein Mfa1 interacts with streptococcal SspB (23), a cell surface protein in the antigen I/II family that is expressed by virtually all of the oral streptococci (10). Interestingly, neither intact *P. gingivalis* cells nor purified Mfa1 interacts with the antigen I/II protein of *Streptococcus mutans*, even though this protein is highly similar to SspB (2). This suggests that *P. gingivalis* may selectively colonize *S. gordonii* and the related oralis group streptococci over the mutans streptococci. In addition, Demuth et al. (6) and Cook et al. (4) showed that the Mfa1-SspB interaction is essential for the development of *P. gingivalis* biofilms on a streptococcal substrate and that biofilm growth exhibits the same selectivity for streptococcal species.

These initial colonization mechanisms utilized by *P. gingivalis*

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are potentially important targets for the development of therapeutic agents, since interfering with *P. gingivalis* adherence to *S. gordonii* may block the initial colonization of the supragingival biofilm by the organism and prevent it from reaching and multiplying in its primary niche in subgingival plaque. Our previous studies have focused on the mechanism of the Mfa1-SspB interaction, and we have identified a discrete region of the SspB protein that is essential for the interaction of purified Mfa1 and intact *P. gingivalis* cells with *S. gordonii* (6, 23). In this study, we show that a synthetic peptide encompassing the active site region of SspB (designated BAR) is a potent inhibitor (50% inhibitory concentration of approximately 1.3 μM) of *P. gingivalis* adherence to *S. gordonii* cells and blocks the formation of *P. gingivalis* biofilms. In addition, a combinatorial screening approach using BAR peptide libraries with substitutions at several active site amino acid residues identified specific characteristics of BAR that are required for *P. gingivalis* adherence.

MATERIALS AND METHODS

Growth of bacterial strains. *P. gingivalis* ATCC 33277 was grown in reduced Trypticase soy broth (Difco) supplemented with 0.5% yeast extract, 1 μg/ml (final concentration) menadione, and 5 μg/ml (final concentration) hemin. Twenty-five milliliters of medium was reduced for 24 h under anaerobic conditions by equilibration in an atmosphere consisting of 10% CO_2, 10% H_2, and 80% N_2. Following equilibration, *P. gingivalis* was inoculated in the medium and grown for 48 h at 37°C under anaerobic conditions. *S. gordonii* DL-1 (22) was cultured aerobically without shaking in brain heart infusion broth supplemented with 1% yeast extract for 16 h at 37°C.

***P. gingivalis* biofilm formation with *S. gordonii*.** The formation of *P. gingivalis* and *S. gordonii* biofilms was carried out essentially as previously described by Lamont et al. (15). Reactions were carried out using a Manostat Carter 4/8 cassette peristaltic pump (Fisher Scientific, Suwanee, GA) using 0.89-mm platinum-cured silicone tubing (Fisher Scientific, Suwanee, GA) and BST FC 71 flow cells (Biosurface Technologies Corp., Bozeman, MT). A single surface of a 15- × 40-mm cover glass (Fisher Scientific, Suwanee, GA) was coated with 0.22-μm filter-sterilized saliva and incubated at 37°C for 30 min. The saliva-coated cover glass was then washed with sterile 1× phosphate-buffered saline (PBS) at a flow rate of 6 ml per hour for 30 min.

S. gordonii DL-1 cells were harvested by centrifugation at 7,000 × *g* at 4°C and suspended in 10 ml of sterile 1× PBS. *S. gordonii* cells were labeled with 20 μl of hexidium iodide (1.6 mg/ml; Molecular Probes, Eugene, OR) at 25°C for 30 min in the dark and washed with PBS. To allow streptococci to attach to the saliva-coated cover glass, *S. gordonii* cells were delivered to the flow cell at the rate of 6 ml per hour for approximately 2 h. Following inoculation with *S. gordonii*, the flow cell was washed with sterile 1× PBS for 30 min at 6 ml per hour to remove nonadherent bacteria from the cover glass.

P. gingivalis cells were harvested by centrifugation, suspended in 10 ml of sterile 1× PBS, and introduced into the flow cell at a flow rate of 6 ml per hour for 2 h to allow *P. gingivalis* to adhere and accumulate on the streptococcal substrate. Flow cells were subsequently washed with sterile 1× PBS to remove nonadherent *P. gingivalis* cells. To visualize *P. gingivalis*, rabbit anti-*P. gingivalis* polyclonal antibody at 1:5,000 in 5 ml of sterile 1× PBS was made to flow into the cell at a rate of 6 ml/hour for approximately 1 h. The flow cell was washed with sterile 1× PBS for 1 h, reacted with anti-rabbit immunoglobulin G-fluorescein isothiocyanate (FITC) conjugate (Sigma, St. Louis, MO) in sterile 1× PBS (1:5,000) for 1 h at 6 ml/hour, and received a final wash with sterile 1× PBS as described above. Under these conditions, the streptococci did not form a confluent layer on the saliva-coated cover glass but were present in small clusters of cells. *P. gingivalis* subsequently formed distinct microcolonies on the immobilized streptococci which were quantified by confocal microscopy as described below.

P. gingivalis-S. gordonii biofilms were visualized using an Olympus Fluoview confocal laser scanning microscope (Olympus, Pittsburgh, PA) under 60× magnification using an argon laser for visualization of FITC labeling and the HeNe-G laser to visualize hexidium iodide-labeled streptococci. The number and height of FITC-labeled *P. gingivalis* microcolonies were determined from 30 to 60 randomly chosen frames using the FluoView software package provided by Olympus. Microcolony depth was determined by performing *z*-plane scans from 0 μm to 30 μm above the cover glass surface. *P. gingivalis* microcolonies that formed on *S. gordonii* in the absence of inhibitor ranged from 7 to 16 μm in depth under the experimental conditions used.

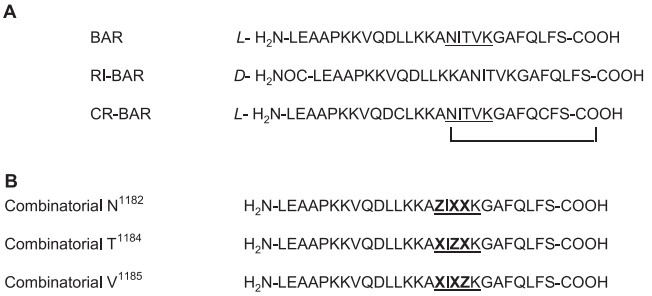

FIG. 1. (A) BAR peptide analogs used in this study. BAR peptide is comprised of amino acid residues 1167 to 1193 of the *S. gordonii* SspB protein (6). The underlined sequence, NITVK, represents active site residues involved in the adherence of *P. gingivalis* to SspB and BAR (2). RI-BAR is a retroinverso analog of BAR that was synthesized with D-amino acids and by reversing the direction of the peptide backbone of BAR. CR-BAR is a cyclic disulfide which limits the conformational flexibility of the NITVK region of BAR. (B) Biased combinatorial peptide libraries of BAR. Combinatorial libraries were synthesized with a defined amino acid occupying position "z" (cysteine excluded) and equimolar mixtures of all common amino acids (cysteine excluded) occupying positions "x." Each library contains 19 separate peptide mixtures defined by the specific "z" amino acid. Each mixture contains 361 different peptide sequences arising from the random "x" residues. Thus, each combinatorial library is comprised of 6,859 different peptide sequences.

For biofilm inhibition experiments, BAR peptide was preincubated with *P. gingivalis* cells at concentrations of 0 to 10 μg per ml for 25°C for 30 min. The *P. gingivalis*-BAR suspension was introduced into the flow cell for 2 h at a flow rate of 6 ml per hour as described above. For some experiments using structural analogs of BAR, *P. gingivalis* cells were incubated with peptide concentrations of up to 50 μg per ml. *P. gingivalis* microcolonies that formed in the presence of inhibitor were analyzed by confocal microscopy as described above. Data were analyzed using GraphPad InStat3 (GraphPad Software Co.). A nonparametric analysis of variance using Dunn's multiple comparisons test was utilized to analyze the data acquired and determine pairwise statistical differences in colony number and depth between experimental samples and the control reaction mixture that did not contain inhibitor.

Synthesis of peptides and combinatorial peptide libraries. The peptides that were used in this study are shown in Fig. 1A. The BAR peptide, a retroinverso form of BAR, and a conformationally constrained analog of BAR were synthesized by BioSynthesis, Inc. (Lewisville, TX). All peptides were obtained at greater than 90% purity. The BAR peptide comprises amino acids 1167 to 1193 of the *S. gordonii* SspB polypeptide (2). The retroinverso analog of BAR (RI-BAR) is comprised of D-amino acids and was synthesized by reversing the chirality of the peptide bond backbone of BAR. The cyclic conformationally constrained analog of BAR (CR-BAR) was synthesized by substituting cysteine for leucines 1177 and 1191 of the SspB sequence, which flank the active site NITVK region of BAR that was previously identified (6).

Biased combinatorial libraries containing substitutions at each of the three potential active site residues of BAR are shown in Fig. 1B. Typically, a biased combinatorial is built around a lead compound (i.e., the BAR peptide) that has been characterized by previous structure-activity studies, and a limited number of positions within the lead compound sequence are randomized to generate a defined collection of structurally related peptides. Thus, a biased library facilitates the directed and rational analysis of a relatively large number of structurally related compounds and facilitates the rapid correlation of functional activity with structural properties of the lead compound. Within BAR, three residues were randomized, Asn^{1182}, Thr^{1184}, and Val^{1185}. Asn^{1182} and Val^{1185} were shown by mutational analysis to be functionally relevant for *P. gingivalis* adherence and biofilm formation (6). In addition, secondary structure predictions indicated that these amino acids contribute to the local structure of BAR. Thr^{1184} was also randomized since it is present in the SspB sequence but not conserved in the functionally inactive homolog of SspB that is expressed by *Streptococcus mutans* (6).

***P. gingivalis* binding assay.** The adherence of *P. gingivalis* to the BAR peptide analogs or to the combinatorial libraries was assayed as described by Chung et al. (3). Briefly, *P. gingivalis* cultures were centrifuged at 7,000 × *g* for 40 min, washed with reduced 1× PBS, and suspended in 5 ml of 0.1 M $NaHCO_3$, pH 8.1. The bacterial cells were labeled with biotin (5 mg *N*-hydroxysuccinimidobiotin in 400 μl of dimethyl sulfoxide) and incubated at room temperature for 3 h. The biotinylated bacteria were harvested by centrifugation, washed twice with PBST (PBS supplemented with 0.05% Tween 20), and suspended in PBST to a density of 4×10^7 CFU/ml.

The BAR peptide analogs or samples of the combinatorial libraries were immobilized onto a nitrocellulose membrane using a dot blot apparatus, and the filter was blocked with 1.5% bovine serum albumin (in 1× PBS) for 2 h. For screening of the combinatorial libraries, 360 μg of each library mixture was immobilized onto the nitrocellulose filter. Thus, each spot contained approximately 1 μg of each peptide sequence that is present in the mixture. We previously showed that 1 μg of purified BAR yielded a readily detectable level of *P. gingivalis* adherence (6). A negative control consisted of PBST without peptide. The positive control was the peptide mixture from the combinatorial library that possessed the "z" amino acid that is present in BAR (e.g., N at residue 1182, T at residue 1184, or V at residue 1185), since only that mixture in the combinatorial library would contain the BAR peptide sequence. All adherence reactions were carried out in triplicate.

Membranes containing the immobilized combinatorial libraries were probed using biotinylated *P. gingivalis* for approximately 18 h at 37°C under anaerobic conditions and gently rinsed three times with PBST. Bound *P. gingivalis* was detected using streptavidin alkaline phosphatase conjugate (Bio-Rad) resuspended in PBST at a dilution of 1:5,000, followed by reaction with Sigma Fast BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium)-buffered substrate (Sigma, St. Louis, MO). Digital images were generated from the resulting membranes using an Epson Stylus CX3810 flatbed scanner, and spot intensity was determined from the digital images using ImageJ software (NIH). The mean relative intensity of each experimental sample on the membrane was then normalized to the mean intensity of the positive-control spot (*P. gingivalis* adherence to the peptide mixture from the combinatorial library that possessed the "z" amino acid that is present in BAR) after subtraction of the mean background signal (density of the negative-control spot).

RESULTS

BAR inhibits formation of *P. gingivalis* biofilms. Our previous in vitro studies showed that *P. gingivalis* bound to a synthetic peptide (designated BAR; Fig. 1) comprising the adherence-promoting region of the SspB protein of *S. gordonii* (6, 15) and that this interaction is mediated by the short fimbrial protein Mfa1 of *P. gingivalis* (23). To determine if the BAR peptide could competitively inhibit *P. gingivalis* adherence to streptococcal cells and prevent biofilm development in an open flow system, *P. gingivalis* biofilms were formed on immobilized streptococci in the presence of increasing concentrations of BAR in flow cells. *P. gingivalis* microcolonies were then visualized by confocal scanning microscopy, and the number of microcolonies that formed per microscope field and the depth of each microcolony were recorded for each peptide concentration. As summarized in Table 1, an average of approximately six *P. gingivalis* microcolonies per frame were observed in the absence of BAR (control reaction). In contrast, preincubation of *P. gingivalis* with 10 μg per ml of BAR peptide almost completely eliminated formation of *P. gingivalis* microcolonies, suggesting that the peptide competitively inhibited the adherence of *P. gingivalis* cells to the immobilized streptococci. Furthermore, the depth of the *P. gingivalis* microcolonies that formed in the presence of the peptide inhibitor was significantly reduced ($P < 0.001$, Table 1) in the presence of 5 μg per ml and 10 μg per ml BAR. Thus, a soluble synthetic 27-mer peptide representing the adherence-promoting region of SspB effectively competes with the intact 170-kDa streptococcal cell surface protein for the *P. gingivalis* short fimbrial protein Mfa1.

TABLE 1. Effect of incubation with BAR on number and depth of colonies

BAR (μg/ml)	Total no. of:		No. of colonies/frame[a] (mean ± SD)	Colony depth[b] (mean ± SD)
	Colonies	Frames		
0	166	30	5.6 ± 1.8	11.3 ± 3.7
1.0	310	50	6.2 ± 2.5	10.0 ± 4.2
2.5	268	58	4.6 ± 2.7	9.1 ± 3.8
5.0	38	30	1.3[c] ± 1.7	5.4[c] ± 4.6
10.0	13	50	0.3[c] ± 0.5	2.2[c] ± 3.9

[a] Colony number was determined from 30 to 58 frames.
[b] Colony depth was determined using confocal microscopy by analyzing *Z* stack data and is expressed in μm.
[c] $P < 0.001$.

Characterization of structural determinants required for *P. gingivalis* adherence to BAR. The specific inhibitory activity of BAR is likely influenced by a number of factors which, if controlled, might result in increased activity. For example, BAR contains five lysine residues and therefore may be highly susceptible to degradation by the Lys-gingipain expressed by *P. gingivalis*. We therefore synthesized a retroinverso analog of BAR, designated RI-BAR (Fig. 1), in which L-amino acids were replaced by D-amino acids and the direction of the peptide backbone of BAR was reversed. Thus, this peptide should possess the same side chain topology in solution as BAR does (7) but would likely be resistant to degradation by the *P. gingivalis* Lys-gingipain and potentially exhibit greater specific inhibitory activity than BAR does. However, our initial experiments using 1 to 10 μg of RI-BAR per ml as inhibitor had no effect on *P. gingivalis* biofilm formation (not shown). To determine if RI-BAR was active but at a lower specific activity than that of BAR, we repeated the experiments using higher concentrations of RI-BAR. As shown in Fig. 2, incubation of *P. gingivalis* cells with up to 50 μg per ml RI-BAR did not affect the development of microcolonies on the streptococcal substrate, indicating that RI-BAR is ineffective at inhibiting the adherence of *P. gingivalis* to immobilized streptococci, even at relatively high concentrations. These results suggest that native BAR, but not its retroinverso analog, can assume the functional conformation of SspB that is recognized by the *P. gingivalis* short fimbrial protein Mfa1.

Small peptides are structurally flexible in solution and are free to transiently assume many different conformations. To investigate more thoroughly the structural features that contribute to BAR inhibitory activity, a conformationally constrained analog of BAR and synthesized combinatorial BAR libraries was tested for biofilm-inhibitory activity. The conformationally constrained peptide (CR-BAR) maintained the chirality of BAR, but leucine residues at positions 1177 and 1191 were replaced with cysteine (Fig. 1). The resulting cyclic disulfide limits bond rotations within the peptide backbone of the intervening active site NITVK sequence that was identified by site-specific mutagenesis in our previous studies (6). As shown in Fig. 2, preincubation of *P. gingivalis* with CR-BAR resulted in a significant reduction in the number of *P. gingivalis* microcolonies that formed on the streptococcal substrate at a concentration of 50 μg per ml ($P < 0.001$). However, signifi-

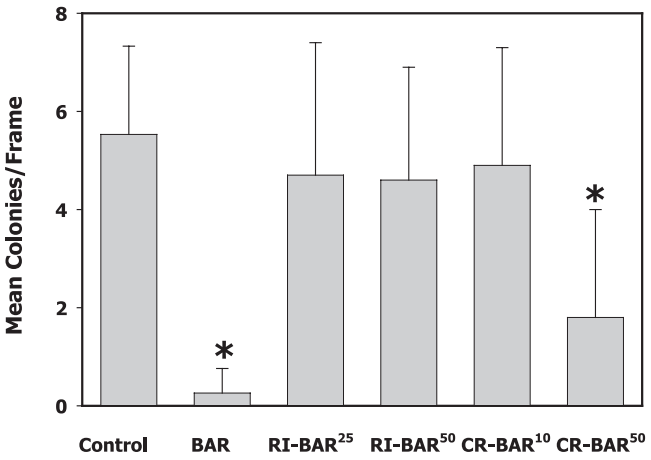

FIG. 2. Formation of *P. gingivalis* microcolonies in the presence of BAR peptide analogs. *P. gingivalis* cells were preincubated with 10 μg per ml BAR, 25 μg per ml and 50 μg per ml of a retroinverso analog of BAR (RI-BAR), or 10 μg per ml and 50 μg per ml of a conformationally constrained peptide analog (CR-BAR) and introduced into flow cells as described in Materials and Methods. *P. gingivalis* microcolonies were visualized by confocal microscopy. Thirty frames were analyzed for each peptide concentration used. A nonparametric analysis of variance using Dunn's multiple comparisons test was utilized to analyze the data acquired and determine statistical differences in colony number. *, the number of colonies was statistically significant ($P < 0.001$) compared with the control.

cant inhibition was not observed at 10 μg of CR-BAR per ml. Thus, the conformationally constrained peptide was active but exhibited lower specific inhibitory activity than the parent BAR peptide did.

Therefore, to better define the structural constraints of the NITVK region that are important for interaction with *P. gingivalis*, a combinatorial approach was undertaken to analyze BAR structure and function. To accomplish this, three combinatorial peptide libraries were synthesized in which one of the functionally active residues of NITVK (N^{1182}, T^{1184}, or V^{1185}) was replaced by each of the 19 common amino acids (cysteine was excluded to prevent incorporation of disulfide bonds in the peptide mixtures; see Materials and Methods; Fig. 1). The remaining two amino acid positions were randomized by using equimolar mixtures of the 19 common amino acids (cysteine excluded) during synthesis. Thus, each of the combinatorial libraries comprised 19 distinct peptide mixtures that differed only in the amino acid residue that occupied the "z" position (Fig. 1). Each of the 19 peptide mixtures in a given library contained 361 different peptide sequences, arising from randomization of the two amino acids occupying the "x" positions. Thus, each library is comprised of 6,859 different peptide sequences.

The adherence of *P. gingivalis* cells to immobilized samples of each of the combinatorial peptide libraries was determined. As shown in Fig. 3A, several peptide mixtures in the combinatorial library designated N^{1182} bound to labeled *P. gingivalis* better than the positive-control mixture that possessed Asn at position 1182 (i.e., the residue present in BAR at this position). Interestingly, three of the amino acid substitutions at position 1182 that exhibited greater adherence activity were positively charged amino acid residues (Arg, Lys, and His), suggesting that a basic residue is optimal at this position in BAR. Substitution of Ser for Asn at 1182 also resulted in increased adherence of *P. gingivalis*. Screening of combinatorial library V^{1185} showed that three amino acid substitutions for Val resulted in increased adherence of *P. gingivalis* (Fig. 3C). Each of these residues was hydrophobic: isoleucine, phenylalanine, and tryptophan. This suggests that hydrophobic interactions may also in part drive the adherence of *P. gingivalis* to BAR. Indeed, the requirement for a hydrophobic amino acid in position 1185 appears to be quite pronounced, since replacement of most other amino acids at this position reduced *P. gingivalis* adherence (Fig. 3C). Finally, the overall effect of amino acid substitutions for Thr at position 1184 was less pronounced, either positively or negatively, than that observed at the other sites (compare *y*-axis scales of Fig. 3B with those of Fig. 3A and 3C). The three substitutions for Thr that resulted in greatest adherence were Arg, Lys, and Ser, similar to the results obtained with combinatorial library N^{1182}.

For each of the combinatorial libraries, some amino acid substitutions resulted in decreased adherence activity and appear to be incompatible with the interaction of BAR with *P. gingivalis*. For example, substitution with Asp or Pro at any of the three positions (N^{1182}, T^{1184}, or V^{1185}) was detrimental to *P. gingivalis* adherence. In addition, the presence of Gly at position 1182 or 1185 also reduced *P. gingivalis* adherence. These results are of interest since Gly and Pro are the amino acid residues that occupy positions 1182 and 1185 in the nonfunctional homolog of SspB that is expressed by *S. mutans* (6). Furthermore, substitution of Gly for Asn or Pro for Val by site-specific mutagenesis of the full-length SspB polypeptide resulted in a significant reduction of *P. gingivalis* adherence. Therefore, results from the combinatorial library screenings are consistent with previous structure-function data of the BAR-*P. gingivalis* interaction. Taken together, the outcome of the combinatorial screen of BAR (summarized in Fig. 4) suggests that both electrostatic and hydrophobic interactions may be required for efficient adherence of *P. gingivalis* to *S. gordonii* and consequently for formation of *P. gingivalis* biofilms. Our results also suggest that replacement of active residues with helix-breaking or β-turn-inducing amino acids is detrimental for *P. gingivalis* adherence.

DISCUSSION

The human oral cavity presents a hostile environment to microorganisms as a result of the constant flow of saliva that contains numerous immune and nonimmune antimicrobial activities (20, 25). The early colonizing bacteria of the oral cavity that comprise supragingival plaque insulate themselves from these conditions by adhering tightly to oral tissues and/or to other oral organisms to form a multispecies biofilm (13, 28). The periodontal pathogen *P. gingivalis* faces an even greater challenge in colonizing the oral cavity since it is a fastidious obligate anaerobe that is asaccharolytic and acid sensitive and requires hemin for growth (17). Its primary niche is in the subgingival pocket, yet it must first colonize and survive the relatively aerobic environment of supragingival plaque. To accomplish this, our hypothesis is that *P. gingivalis* initially colonizes a specific niche in supragingival plaque by adhering tightly to organisms such as *S. gordonii* (16) and *Fusobacterium*

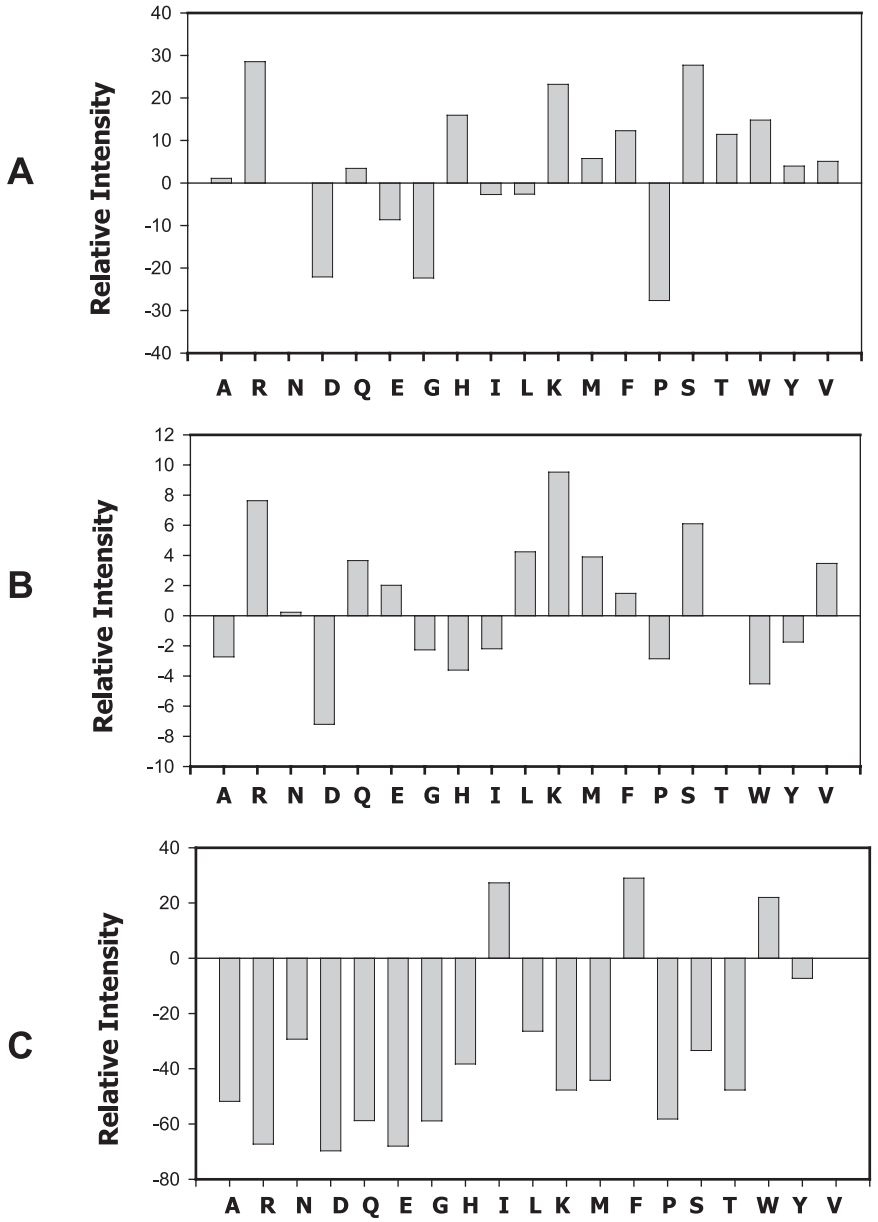

FIG. 3. Adherence of *P. gingivalis* to combinatorial peptide libraries N^{1182} (A), T^{1184} (B), and V^{1185} (C). Adherence data were normalized to the level of *P. gingivalis* adherence that was observed to the positive-control reaction mixture. Thus, the level of *P. gingivalis* adherence to the peptide mixture that possessed the "z" amino acid which is present in BAR (N at 1182, T at 1184, and V at 1185) is indicated by the horizontal reference line for each of the combinatorial libraries. Peptide mixtures that promote greater *P. gingivalis* adherence extend above the reference line, whereas mixtures that interact with *P. gingivalis* more poorly than the control mixture are shown below the reference line. The identity of the amino acid occupying the "z" position for each of the peptide mixtures is shown on the *x* axis.

nucleatum (1, 11) that may provide a reduced local environment that is not highly acidic. Since these specific interbacterial interactions may represent the first steps in *P. gingivalis* colonization, they are ideal targets for the development of therapeutic agents against adult periodontal disease.

Our previous studies have focused on the mechanism of *P. gingivalis* adherence to streptococci which leads to the subsequent development of biofilms on the streptococcal substrate. These studies showed that *P. gingivalis* adheres to *S. gordonii* cells by interaction of the short fimbrial subunit protein Mfa1 with a discrete region of the streptococcal SspB polypeptide (6, 23). Here we show that a synthetic peptide encompassing this region of SspB functions as a potent inhibitor of *P. gingivalis* adherence to *S. gordonii* and its subsequent formation of biofilms. From our results, we calculated that the number of *P. gingivalis* microcolonies that formed on the immobilized streptococcal substrate would be reduced by 50% in the presence of approximately 4 μg of peptide inhibitor per ml (50% inhibitory concentration, ~1.3 μM). This value is consistent with the kinetics of adherence of purified Mfa1 protein to the BAR peptide as determined by enzyme-linked immunosorbent assay, where half-maximal binding occurred at approximately 0.8

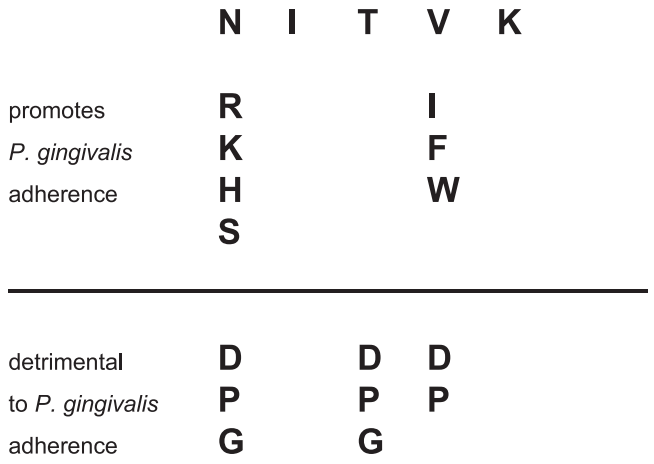

FIG. 4. Summary of the combinatorial peptide screen. Three amino acids within the active site NITVK region of BAR were randomized in the combinatorial libraries. Substitutions in two positions, N^{1182} and V^{1185}, produced peptide mixtures that exhibited greater activity than the lead compound, BAR. Positively charged residues appear to be preferred at residue 1182, whereas hydrophobic amino acids are preferred at position 1185. Aspartate and proline were detrimental to *P. gingivalis* adherence at all three positions, and replacement of glycine at residues 1182 and 1184 reduced *P. gingivalis* adherence.

μM BAR (23). This observation suggests that the interaction of Mfa1 with the BAR region of SspB is the primary driving force in *P. gingivalis* adherence to streptococci, which initiates the formation of biofilms.

Since *P. gingivalis* is a highly proteolytic organism, the specific inhibitory activity of BAR might be limited by proteolytic degradation. Indeed, based on its lysine content (5 of 27 residues), it is possible that the BAR peptide would be highly susceptible to degradation by the Lys-gingipain expressed by *P. gingivalis*. Since most proteases have not evolved to act on D-amino acid polypeptides (7), a retroinverso peptide that maintains the side chain topology of BAR might be expected to exhibit greater specific inhibitory activity. However, our results showed that a retroinverso derivative of BAR was inactive in preventing *P. gingivalis* adherence and formation of biofilms. A possible explanation for this result is that the association of Mfa1 with BAR requires crucial hydrogen bonding with the peptide backbone of BAR and reversing the chirality of the peptide bonds in BAR prevents these interactions from occurring. This has been previously suggested to represent a limitation of retroinverso peptidomimetics (7). We are currently exploring the possibility of selectively incorporating D-amino acids to limit proteolytic susceptibility of BAR while maintaining chirality in the remainder of the peptide backbone in order to facilitate potential interactions with Mfa1.

P. gingivalis adheres avidly to the SspB polypeptide of *S. gordonii* but does not interact with the highly related SpaP protein of *S. mutans* (2). Our previous studies showed that Asn^{1182}, Thr^{1184}, and Val^{1185} in BAR are not conserved in the SpaP sequence and that substitution of the corresponding *S. mutans* residues (Gly and Pro) for Asn^{1182} and Val^{1185}, respectively, of SspB resulted in a nonfunctional *S. gordonii* protein (6). These results highlighted the functional role of the NITVK region of BAR in the interaction of *P. gingivalis* with SspB but were not informative about the physicochemical nature of the interaction of SspB and Mfa1. The biased combinatorial peptide libraries analyzed in the current study allowed us to examine the functional properties of BAR peptides containing all combinations of the common amino acids occupying the nonconserved residues (Asn^{1182}, Thr^{1184}, and Val^{1185}) in the NITVK sequence. Interestingly, *P. gingivalis* efficiently bound to BAR-like peptides containing amino acid substitutions at both residues 1182 and 1185. The most active peptides were those containing a positively charged amino acid (Arg, Lys, or His) at position 1182 and a hydrophobic residue (Ile, Phe, or Trp) at 1185. This suggests that the SspB-Mfa1 interaction does not strictly depend on the specific amino acid occupying these positions in SspB but rather on the physical properties and characteristics of the amino acid residue.

Since the most active BAR peptide analogs contained a hydrophobic amino acid (Ile, Phe, Trp, or Val) at position 1185, we can speculate that the interacting interface of the *P. gingivalis* fimbrial protein Mfa1 likely contains a large hydrophobic pocket that can accommodate the bulky side chains of these residues. The fact that no other amino acids were functionally tolerated at this site in our combinatorial screen suggests that hydrophobic interactions may be essential for the association of Mfa1 with SspB. In addition, the presence of positively charged amino acids at position 1182 (Arg, Lys, or His) facilitated *P. gingivalis* adherence, whereas the replacement of N^{1182} with acidic residues (Asp and, to a lesser extent, Glu) reduced adherence. The preference for basic residues could be explained by the presence of negatively charged residues in the SspB binding pocket of Mfa1 that might contribute to binding through the formation of electrostatic interactions with SspB. However, the Mfa1-SspB interaction is clearly more complex than suggested above since the polar, but uncharged, residues Asn and Ser at position 1182 also promoted *P. gingivalis* adherence. While the exact structure of the interacting interface cannot be described in full without additional structural studies, our results suggest that both electrostatic and hydrophobic bonding may drive the protein-protein interaction of Mfa1 with SspB.

It is also of interest that several amino acids (e.g., Pro, Gly, and Asp) appeared to be generally detrimental to *P. gingivalis* adherence regardless of position in the NITVK sequence. The presence of Pro at any of the randomized positions in BAR, and Gly at residue 1182 and to a lesser extent at residue 1184, resulted in reduced adherence. In addition, substitution of Gly and Pro for N^{1182} and V^{1185} reduced adherence. This is in agreement with our previous site-specific mutagenesis data (6). Gly and Pro are both β-turn-inducing or helix-breaking residues, and it is likely that their presence in BAR alters the structure of this region. Secondary structural predictions of the BAR peptide analogs are consistent with this conclusion. For example, residues 1167 to 1180 of BAR are predicted to be α-helical, with the remainder of the peptide predicted to be β-sheet. In contrast, structural predictions of a BAR analog containing Gly and Pro at positions 1182 and 1185, respectively, differ from BAR in that residues 1167 to 1180 are predicted to be α-helical as described above, but this helix is followed by a β-turn (comprising residues 1181 to 1187) and then a second α-helix. The predicted turn in this peptide analog arises from the presence of three helix-interrupting amino

acids Gly^{1182}, Pro^{1185}, and Gly^{1187} in the NITVK region. Thus, at least some of the amino acid substitutions that reduce *P. gingivalis* adherence may alter secondary structural elements of BAR, suggesting that the overall structure of the BAR region may also be important for recognition of SspB by Mfa1.

In summary, we have shown that the formation of *P. gingivalis* biofilms can be blocked by peptide-mediated inhibition of the interaction of Mfa1 with the streptococcal SspB protein. Analysis of peptide analogs and combinatorial peptide libraries based on the sequence of BAR suggests that both conformational determinants and the characteristics of specific amino acid residues in BAR are essential for this interaction to occur. These studies highlight molecular details of the interacting regions of the streptococcal SspB protein and the *P. gingivalis* minor fimbrial subunit Mfa1 and define physicochemical characteristics of the binding domains. These results suggest that peptides or peptidomimetics with greater specific inhibitory activity than that of BAR can be synthesized.

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