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Selective substitution of amino acids limits proteolytic cleavage and improves the bioactivity of an anti-biofilm peptide that targets the periodontal pathogen, *Porphyromonas gingivalis*

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Abstract

The interaction of the periodontal pathogen, *Porphyromonas gingivalis* with oral streptococci such as *Streptococcus gordonii* precedes colonization of the subgingival pocket and represents a target for limiting *P. gingivalis* colonization of the oral cavity. Previous studies showed that a synthetic peptide (designated BAR) derived from the antigen I/II protein of *S. gordonii* was a potent competitive inhibitor of *P. gingivalis* adherence to *S. gordonii* and subsequent biofilm formation. Here we show that despite its inhibitory activity, BAR is rapidly degraded by intact *P. gingivalis* cells in vitro. However, in the presence of soluble Mfa protein, the *P. gingivalis* receptor for BAR, the peptide is protected from proteolytic degradation suggesting that the affinity of BAR for Mfa is higher than for *P. gingivalis* proteases. The rate of BAR degradation was reduced when the *P. gingivalis* lysine-specific gingipain was inhibited using the specific protease inhibitor, z-FKcK, or when the gene encoding the Lys-gingipain was inactivated. In addition, substituting D-Lys for L-Lys residues in BAR prevented degradation of the peptide when incubated with the Lys-gingipain and increased its specific adherence inhibitory activity in a *S. gordonii*-*P. gingivalis* dual species biofilm model. These results suggest that Lys-gingipain accounts in large part for *P. gingivalis*-mediated degradation of BAR and that more effective peptide inhibitors of *P. gingivalis* adherence to streptococci can be produced by introducing modifications that limit the susceptibility of BAR to the Lys-gingipain and other *P. gingivalis* associated proteases.

Keywords

Porphyromonas gingivalis; *Streptococcus gordonii*; biofilm; gingipain

1. Introduction

The sub-gingival colonization of the obligate anaerobe *Porphyromonas gingivalis* has been associated with the onset of severe periodontal disease [15,25]. However, *P. gingivalis*

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colonization of the sub-gingival pocket likely occurs only after the organism initially colonizes the supragingival microbial biofilm by adhering to bacteria such as the oralis group of streptococci [5,23,24] and/or *Fusobacterium nucleatum* [2,18,19]. These inter-species associations are mediated by specific receptor-ligand interactions that occur between the adherent organisms [3,22–24,27,35] and the interaction of *P. gingivalis* with these co-adherent species may provide physiologic support that facilitates the survival of *P. gingivalis* in the supragingival environment.

The adherence of *P. gingivalis* to *Streptococcus gordonii* is a multimodal protein-protein interaction [4], but the interaction of the *P. gingivalis* minor fimbrial protein Mfa [35] with the streptococcal antigen I/II polypeptide (e.g., SspB, [3,23] has been shown to be the driving force that mediates this interbacterial co-aggregation. Interestingly, antigen I/II paralogs are ubiquitously expressed among all oral streptococci [26] but *P. gingivalis* adheres only to select species (e.g., *S. gordonii* but not to the mutans group of streptococci, e.g., *Streptococcus mutans*) [17,24]. Functional dissection of the *S. gordonii* SspB protein identified a discrete structural domain designated BAR (SspB Adherence Region) that mediates *P. gingivalis* adherence to streptococci and the species-selective development of *P. gingivalis* biofilms on streptococcal substrates [5,10]. Studies by Demuth et al [10] and Daep et al [8] showed that the BAR region of SspB resembles a nuclear receptor (NR) box protein-protein interaction domain and that *P. gingivalis* adherence requires the amino acid motifs, EXXP, VQDLL and NITVK in BAR [7,8]. These studies also showed that amino acid substitutions in the NITVK motif accounted for the selectivity of *P. gingivalis* adherence to antigen I/II proteins and that a synthetic peptide encompassing the BAR region of SspB functioned as a potent competitive inhibitor of *P. gingivalis* adherence to streptococci [7].

P. gingivalis survival in the oral cavity is also contingent upon the successful acquisition of nutrients from the local environment. *P. gingivalis* is asaccharolytic and utilizes proteins and peptides as a carbon source [28,38–40,42]. Consistent with this, the *P. gingivalis* genome encodes numerous putative proteolytic enzymes [6,13,16,20,28,30] including several papain-like proteases that specifically cleave at lysine (the Lys-gingipain encoded by *kgp*) or arginine (the Arg-gingipains encoded by *rgpA* or *rgpB*) [20,32,36]. While previous studies showed that the BAR peptide is a potent inhibitor of *P. gingivalis*/*S. gordonii* adherence [7,8,14], this peptide contains five lysine residues, suggesting that it may be highly susceptible to cleavage by the Lys-gingipain. In this report, we show that BAR is indeed rapidly degraded by intact *P. gingivalis* cells in vitro but the peptide is significantly protected from degradation in the presence of its soluble receptor, the Mfa protein. The rate of BAR degradation was reduced by Lys-gingipain specific inhibitors, by inactivation of the gene encoding the Lys-gingipain, or when D-Lys was substituted for L-Lys residues in BAR. Finally, the cleavage-resistant D-Lys substituted peptide exhibited increased specific inhibitory activity against *P. gingivalis*-*S. gordonii* adherence. These results suggest that the Lys-gingipain is primarily responsible for proteolytic cleavage of BAR and that more potent peptide inhibitors can be produced by limiting *P. gingivalis*-mediated proteolysis.

2. Materials and Methods

2.1 Growth of bacterial strains

The *P. gingivalis* strains that were used in this study are listed in Table 1. Strains were 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. *P. gingivalis* was inoculated in pre-reduced media and grown for 48 hours at 37°C in an atmosphere consisting of 10% CO₂, 10% H₂, and 80% N₂. For the gingipain mutant strains, antibiotics were added to the medium as indicated in Table 1. *S. gordonii* DL-1 was cultured aerobically without

shaking in brain heart infusion (BHI) broth (Difco) supplemented with 1% yeast extract for 16 hours at 37°C.

2.2 Expression and purification of *P. gingivalis* Mfa

The Mfa protein was expressed from pET 30a vector system as described by Park et al [35]. Following induction in *E. coli*, the recombinant protein was extracted using CellLytic B cell lysis reagent (Sigma, St. Louis, MO). Mfa was purified by chromatography via His-tag selection using HiTrap Chelating HP column (GE Healthcare, Piscataway, NJ) and eluted with 500 mM imidazole. Purity was assessed via PAGE and Coomassie Blue staining.

2.3 Peptide synthesis

The BAR and BAR-19 peptide sequences are shown in Figure 1A. Each peptide was synthesized by Biosynthesis Inc., Lewisville, TX at $\geq 85\%$ purity and was suspended in nuclease/protease-free water (Fisher Scientific) at an initial concentration of 1000 $\mu\text{g/ml}$. Samples were diluted to the desired concentration immediately before use.

2.4 Determination of *P. gingivalis* protease activity

Approximately 1 ml of an overnight *P. gingivalis* culture was centrifuged at 8000 rpm for 5 minutes at 4°C and the bacterial pellet was washed 3 times with sterile $1\times$ PBS before suspending to a final volume of 1 ml in PBS. Approximately 5 μg of BAR (or BAR-19) was incubated with 5 μl of bacterial suspension ($\sim 1 \times 10^7$ cfu) or 5 μl of the original culture supernatant at 37°C for 0, 5, 10, 30, and 60 minutes. The presence of intact peptide was determined via polyacrylamide gel electrophoresis (PAGE) using Novex® 10–20% tricine gel (Invitrogen, Carlsbad, CA) and Coomassie Blue staining. Digital images of the stained gels were obtained using Epson Stylus CX3810 flatbed scanner (Epson America, Inc., Long Beach, California). In assays comparing the activity of purified Lys-gingipain (kindly provided by Dr. Jan Potempa) with whole *P. gingivalis* cells, the activity of the purified enzyme used was normalized against 1×10^7 cfu *P. gingivalis*, determined by the hydrolysis of a chromogenic Lys-gingipain substrate, Ac-Lys-p-nitroanilide (Bachem, King of Prussia, PA).

2.5 Stability of BAR in human saliva

Approximately 1 ml of whole saliva was collected from 5 individuals. The saliva was clarified by centrifugation at $10,000 \times g$ for 10 minutes at 4°C. The supernatant was collected and stored on ice. Approximately 5 μg of BAR was incubated with 5 μl of clarified saliva and incubated for 120 minutes. The samples were analyzed for intact peptide via PAGE using 10–20% Tricine gels as described above. Fresh saliva was collected for each subsequent experiment.

2.6 Inhibition of *P. gingivalis* protease activity

The protease activity of *P. gingivalis* was inhibited using a bacterial protease inhibitor cocktail (Sigma, St. Louis, MO) or with the Lys-gingipain specific inhibitor z-Phe-Lys-2,4,6-trimethyl benzoyloxy-methyl ketone (z-FKcK) (Bachem, King of Prussia, PA). The inhibitor cocktail consisted of 18 mM 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 0.22 mM N-(trans-Epoxy succinyl)-L-leucine 4-guanidinobutylamide (E-64), 86 mM ethylenediamine-tetraacetic acid (EDTA), 1.7 mM bestatin, and 0.29 mM pepstatin A. Approximately 1×10^7 *P. gingivalis* intact cells were incubated in serially 2-fold diluted protease inhibitor cocktail or with z-FKcK for 30 minutes at 25°C prior to the addition of 5 μg of BAR peptide. The resulting mixture was subsequently incubated at 37°C for 2 hours. The presence of intact BAR was visualized by PAGE and Coomassie Blue staining.

2.7 Formation of dual species *P. gingivalis* -*S. gordonii* biofilms

The formation of dual species *P. gingivalis*/*S. gordonii* biofilms was carried out essentially as previously described Daep et al [7,8]. Bacterial biofilms developed under flow conditions using a Manostat Carter 4/8 cassette peristaltic pump (Fisher Scientific, Suwanee, GA) with 0.89 millimeter platinum-cured silicone tubing (Fisher Scientific, Suwanee, GA) and BST FC 71 flow cells (Biosurface Technologies, Corp, Bozeman, MT). A single surface of a 24 × 60 mm cover glass (VWR International, West Chester, PA) was coated with 0.22 µm filter sterilized saliva and incubated at 37°C for 30 minutes. The saliva coated coverglass was then washed with sterile 1× PBS at a flow rate of 6 ml per hour for 30 minutes.

S. gordonii DL-1 cells were harvested by centrifugation at 4000 rpm at 4°C and suspended in 10 ml of sterile 1× PBS. *S. gordonii* cells were labeled with 20 µl of hexidium iodide (5 mg/ml in ethanol, Molecular Probes, Eugene, OR) at 25°C for 30 minutes in the dark and washed with PBS. To allow the streptococci to attach to the saliva coated cover glass, *S. gordonii* was inoculated into the flow cell at the rate of 6 ml per hour for approximately 2 hours. Following inoculation with *S. gordonii*, the flow cell was washed with sterile PBS for 30 minutes at 6 ml per hour to remove non-adherent bacteria from the cover glass and form a non-confluent layer on the saliva coated coverglass.

P. gingivalis cells were harvested by centrifugation at 4000 rpm at 4°C for 45 minutes, suspended in 20 ml of sterile 1× PBS, and introduced into the flow cell at a flow rate of 6 ml per hour for 2 hours to allow the bacteria to adhere and accumulate on the streptococcal substrate. Flow cells were subsequently washed with sterile 1× PBS to remove the non-adherent *P. gingivalis* cells. To visualize adherent *P. gingivalis*, rabbit anti-*P. gingivalis* 33277 polyclonal antibody labeled with Alexa-fluor 488 at 1:5000 dilution in 5 ml of sterile 1× PBS was flowed into the cell at a rate of 6 ml per hour for approximately 1 hour and washed with sterile 1× PBS for 30 minutes. *P. gingivalis* subsequently formed distinct microcolonies on the immobilized streptococci and were quantified by confocal microscopy as described below.

2.8 Confocal analysis of *P. gingivalis*-*S. gordonii* biofilms

P. gingivalis-*S. gordonii* biofilms were visualized using an Olympus Fluoview confocal laser scanning microscope (Olympus, Pittsburgh, PA) under 600× magnification using an Argon laser for visualization of FITC labeling and the HeNe-Green laser to visualize the hexidium iodide labeled streptococci. The number and height of FITC-labeled *P. gingivalis* microcolonies was 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 to 30 µm above the coverglass surface as *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 by Daep et al [7].

Data were analyzed using GraphPad InStat3 software (GraphPad Software Co.) using a non-parametric analysis of variance (ANOVA). Wilcoxon matched pair test was utilized to analyze the data acquired and determine the pair-wise statistical differences in colony number and between experimental samples and the control reaction which did not contain a peptide inhibitor.

3. Results

3.1 *P. gingivalis* mediated proteolysis of BAR

P. gingivalis expresses a variety of proteases that may potentially degrade BAR including the Lys-gingipain (Kgp) which may target the Lys residues that are present in the peptide sequence (Figure 1A). Indeed, incubation of BAR with intact *P. gingivalis* cells in vitro resulted in the

rapid degradation of the peptide (Figure 1B). In contrast, the cleavage of BAR was significantly slower when the peptide was incubated with cell-free culture supernatant, indicating that the majority of the *P. gingivalis* proteolytic activity that targets BAR is cell associated. Interestingly, incubation of BAR with 5 µg of purified soluble recombinant Mfa protein (the *P. gingivalis* receptor for BAR) prior to the addition of intact *P. gingivalis* cells (either the wild type or the Mfa-deficient strain SMF1) protected BAR from degradation. As shown in Figure 1C, BAR was completely degraded after incubation with intact bacteria for 5 minutes in the absence of Mfa whereas approximately 70% of the peptide remained intact after incubation for 5 minutes in the presence of Mfa. Indeed, intact BAR was still present after 30 minutes in the presence of soluble Mfa. Thus, soluble Mfa protein protects BAR from proteolytic degradation, suggesting that the peptide binds to the Mfa protein at higher affinity than it binds to *P. gingivalis* proteases. This may explain at least in part why BAR potently inhibits *P. gingivalis* adherence to streptococci even though *P. gingivalis* expresses cell surface proteases. In control reactions, BAR remained intact after incubation for up to 2 hours in buffer alone (Figure 1D) or in clarified human saliva (Figure 1E) suggesting that the peptide is stable under physiologic conditions and not susceptible to salivary proteases. Saliva itself does not contain a peptide that co-migrates with BAR in gel electrophoresis (not shown).

3.2 Gingipain-mediated cleavage of BAR peptide

BAR peptide contains five lysine residues and no arginines (Fig. 1A) suggesting that it may be highly susceptible to the Lys-gingipain but resistant to the Arg-gingipains. To determine if the Lys-gingipain contributes to BAR degradation, the peptide was incubated either with wild type *P. gingivalis* cells or with various mutant strains that lack the Lys-gingipain, the Arg-gingipains or both the Lys- and Arg-gingipains. As shown in Figure 2A, incubating 5 µg of BAR with intact wild type *P. gingivalis* 33277 resulted in the rapid degradation of the peptide (<5 minutes). In contrast, degradation of BAR occurred at a significantly slower rate when the peptide was incubated with *P. gingivalis* KDP128 (Kgp/RgpA/RgpB-deficient; Figure 2B) or with *P. gingivalis* KDP129 (Kgp-deficient; Figure 2C). The rate of BAR degradation after treating with *P. gingivalis* E8 (RgpA/B-deficient) was similar to the wild type reaction (Figure 2D). This suggests that the rapid cleavage of BAR peptide by *P. gingivalis* cells is predominantly mediated by the Lys-gingipain. However, prolonged exposure of BAR with either of the Kgp-deficient strains (i.e., 30 minutes or more) still resulted in loss of the peptide, suggesting that other *P. gingivalis* proteases are also active against BAR, albeit less efficiently than the Lys-gingipain. Although the RgpA/B-deficient *P. gingivalis* strain was constructed in a different background than strains KDP128 and KDP129, genetic analysis performed by Mikolajczyk-Pawlinska et al. [29] and Curtis et al. [6] showed that all strains of *P. gingivalis* possess highly related Lys-specific and Arg-specific gingipains and BLAST-alignment of the gingipain protein sequences of *P. gingivalis* 33277 and *P. gingivalis* W83 showed that these enzymes were identical (data not shown). In addition, our results show that the activity of *P. gingivalis* E8 against BAR was indistinguishable from strain 33277.

Two approaches were subsequently undertaken to limit the susceptibility of BAR to the Lys-gingipain. First, Lys-gingipain-mediated degradation of BAR was assessed in the presence of z-FKcK, a specific inhibitor of this protease [37]. As shown in Figure 3, incubation of BAR with intact *P. gingivalis* cells resulted in the rapid degradation of the peptide, consistent with the results shown above. However, in the presence of z-FKcK, BAR was protected from cleavage by intact bacterial cells or by purified Lys-gingipain (kindly provided by Dr. Jan Potempa), confirming that the Lys-gingipain is predominantly responsible for the rapid degradation of the peptide. In addition, a modified peptide designated BAR-19, was synthesized in which the L-Lys residues of BAR were selectively substituted with D-Lys. As shown in Figure 3, BAR-19 was resistant to *P. gingivalis*-mediated or Lys-gingipain mediated cleavage relative to the parent peptide. Together these results suggest that BAR is most

susceptible to the Lys-gingipain and that degradation of BAR can be reduced by inhibiting this enzyme or protecting BAR from cleavage at the Lys residues in the peptide.

3.3 BAR-19 is a more potent inhibitor of *P. gingivalis* adherence to streptococci

BAR was previously shown to be a potent inhibitor of *P. gingivalis* adherence with specific species of oral streptococci and the subsequent formation of *P. gingivalis* biofilms [7,8]. To determine if reducing Lys-gingipain mediated degradation of BAR improves its adherence inhibitory activity, the formation of *P. gingivalis* – *S. gordonii* dual species biofilms was examined in the presence and absence of BAR and BAR-19. As shown in Table 2, treating *P. gingivalis* with either BAR or BAR-19 inhibited adherence to *S. gordonii* and reduced the development of *P. gingivalis* microcolonies. However, BAR-19 was a more potent inhibitor and exhibited a significant increase in inhibitory activity relative to BAR at peptide concentrations of 0.85 μ M (approximately 55% inhibition versus 18% for BAR, $P \leq 0.02$) and 0.3 μ M (approximately 27% inhibition versus 16% for BAR, $P \leq 0.05$). Both peptides inhibited *P. gingivalis* adherence by greater than 80% at higher peptide concentrations (e.g., 1.7 μ M). These results show that limiting the degradation of BAR by the Lys-gingipain results in increased adherence inhibitory activity and suggest that further modifications to reduce proteolytic cleavage of BAR may result in even more potent peptide inhibitors.

4. Discussion

Periodontal disease is a microbe-induced inflammatory disorder that when untreated results in the resorption of alveolar bone, leading to tooth loss. In addition, periodontal pathogens such as *P. gingivalis* have been suggested to contribute to various systemic diseases such as atherosclerosis, nephritis, pneumonia, and stroke [9,11,31,33,34,43]. The initial colonization of the oral cavity by *P. gingivalis* involves its interaction with specific species of oral streptococci [5,10,12,18,21,24,41,44] and this adherence is mediated by a protein-protein interaction between the *P. gingivalis* Mfa protein and streptococcal antigen I/II. Daep et al. previously showed that a peptide derived from antigen I/II, designated BAR, potently inhibits the adherence of *P. gingivalis* to *S. gordonii* both in vitro [7] and in vivo (D. Demuth, manuscript submitted). Thus, BAR represents a potential therapeutic agent that may limit colonization of the oral cavity by *P. gingivalis* and reduce periodontal disease and the systemic diseases associated with *P. gingivalis*.

However, a potential confounding problem with the use of peptides as therapeutic agents is their susceptibility to proteolytic degradation and *P. gingivalis* is known to be a highly proteolytic organism. Indeed, our results show that BAR peptide is rapidly degraded in the presence of intact *P. gingivalis* cells bacteria. Interestingly, the peptide was significantly protected from degradation by soluble Mfa protein, suggesting that the affinity of BAR-Mfa interaction is greater than the affinity of BAR for the Lys-gingipain and other *P. gingivalis* proteases. This may explain in part why BAR functions as a potent inhibitor of *P. gingivalis* adherence to streptococci in the presence of cell surface associated *P. gingivalis* proteolytic enzymes.

Although the *P. gingivalis* genome encodes many putative proteases, the Lys-gingipain appears to be the primary enzyme that targets BAR since the rate of peptide degradation was significantly reduced when treated with a mutant strain lacking Lys-gingipain. In addition, the degradation of BAR was inhibited by the Lys-gingipain specific inhibitor z-FKcK and when D-Lys was substituted for L-Lys residues in BAR. However, inactivation of the *kgp* gene did not prevent the degradation of BAR upon prolonged incubation with intact *P. gingivalis* cells, indicating that other *P. gingivalis* proteases are active against the peptide, albeit with slower kinetics than the Lys-gingipain. Consistent with this, BAR was stable for up to 2 hours when incubated with bacterial cells in the presence of a commercially available protease inhibitor

cocktail that inhibits multiple classes of proteolytic enzymes (data not shown). We are currently examining the activity of the individual inhibitors that comprise the cocktail to determine if a specific class of proteases accounts for the non-Lys-gingipain mediated activity against BAR.

Our results also suggest that the adherence inhibitory activity of BAR might be increased by limiting the susceptibility of the peptide to *P. gingivalis* proteases. The long term stability of BAR in physiologic buffers (in excess of 6 weeks, data not shown) and its stability in saliva suggest that microbial proteases represent the primary obstacles that must be overcome to increase the stability and activity of BAR and to further develop it as an effective anti-biofilm peptide. Our initial attempt to limit proteolytic degradation of BAR involved synthesizing an analog in which all the amino acids in the peptide were replaced with their D-enantiomers. This peptide was completely resistant to *P. gingivalis* mediated degradation (data not shown). However, Daep et al [7] showed that the inversed-peptide was inactive and did not block *P. gingivalis* adherence to streptococci, suggesting that the stereochemistry of the peptide backbone may be important for its interaction with Mfa. In contrast, the selective replacement of D-Lys for L-Lys residues in BAR-19 prevented cleavage of BAR by the Lys-gingipain without significantly affecting its interaction with Mfa. Indeed, BAR-19 exhibited higher specific adherence inhibitory activity and was a more potent inhibitor of *P. gingivalis* biofilm formation on a *S. gordonii* substrate. Thus, this result provides the proof of principle that decreasing the susceptibility of BAR to proteolytic cleavage increases the peptide's specific adherence inhibitory activity.

In conclusion, we have shown that although BAR peptide inhibits *P. gingivalis* adherence to *S. gordonii*, it can be degraded by *P. gingivalis* proteases. However, limiting its susceptibility to proteolytic degradation increased the specific inhibitory activity of the peptide and will facilitate the development of additional peptides or peptidomimetics that are recalcitrant to proteolytic cleavage while maintaining the anti-biofilm activity of the parent BAR peptide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Glossary and Abbreviations

SspB	the <i>Streptococcus gordonii</i> surface protein in the antigen I/II family that mediates adherence to <i>Porphyromonas gingivalis</i>
Mfa	the minor fimbrial antigen of <i>P. gingivalis</i> that interacts with SspB
BAR	a peptide comprising the domain of SspB that interacts with Mfa
z-FKcK	z-Phe-Lys-2,4,6-trimethyl benzoyloxy-methyl ketone

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A) **BAR** NH₂-LEAAPKKVQDLLKKANITVKGAFQLFS-OH
BAR-19 NH₂-LEAAP(dKdK)VQDLL(dKdK)ANITV(dK)GAFQLFS-OH

D)

E)

Figure 1.

A) The amino acid sequences of BAR and BAR-19. Lysine residues that were substituted with the D-enantiomers in BAR-19 are indicated. **B)** *P. gingivalis* mediated cleavage of BAR peptide. To determine if BAR is susceptible to proteolytic cleavage, 5 μ g of the peptide was incubated with either intact *P. gingivalis* 33277 cells or with cell free culture supernatant for 0, 5, 10, 30, and 60 minutes. **C)** Mfa protein protects BAR from *P. gingivalis* mediated proteolysis. BAR peptide was incubated with intact *P. gingivalis* SMF-1 which does not express Mfa for 5 and 30 minutes in the presence or absence of soluble Mfa protein. **D)** Stability of BAR in physiologic buffer. **E)** Stability of BAR in human saliva. Saliva was collected from 5 volunteers and incubated with BAR peptide for 2 hours at 37°C.

Figure 2.

Degradation of BAR peptide by *P. gingivalis* gingipains. BAR peptide (5 μ g) was incubated with *P. gingivalis* 33277 or with strains KDP128 (deficient in Lys- and Arg-gingipains), KDP129 (deficient in Lys-gingipain) and E8 (deficient in Arg-gingipain) for 0 to 120 minutes at 37°C and analyzed by PAGE as described in the Methods.

Figure 3.

Susceptibility of BAR and BAR-19 to Lys-gingipain mediated cleavage. BAR and BAR-19 peptides were incubated with *P. gingivalis* 33277 or with purified Lys-gingipain (Kgp) that was pre-exposed for 30 minutes to buffer alone or with buffer containing the Lys-gingipain inhibitor z-FKcK. Intact peptides were visualized by PAGE.

Table 1*Porphyromonas gingivalis* strains used in this study

Strain	Background Strain	Genotype	Antibiotic Resistance	Reference
33277	33277	WT	none	ATCC strain
E8	W50	<i>rgpA⁻/rgpB⁻</i>	Clindamycin/Tetracycline	(1)
KDP129	33277	<i>kgp⁻</i>	Tetracycline/Erythromycin	(14)
KDP128	33277	<i>rgpA⁻/rgpB⁻/kgp⁻</i>	Tetracycline/Erythromycin	(14)
SMF1	33277	<i>mfa⁻</i>	Erythromycin	(22)

Table 2

Comparison of the specific inhibitory activity of BAR vs. BAR-XIX.

Peptide	Concentration (μM)	Colonies per frame ^{**} (Mean ± SEM)	% Inhibition relative to 0 μM [*]
No peptide		6.86 ± 1.04	0
BAR	0.3	5.73 ± 0.59	16.5
	0.8	5.6 ± 0.66	18.4
	1.7	1.3 ± 0.36 ^a	80.1
BAR-19	0.3	5.0 ± 0.56	27.2
	0.8	3.1 ± 0.47 ^{a,b}	54.9
	1.7	1.05 ± 0.43 ^a	84.7

^{*} Percent inhibition was determined using the following equation: % inhibition = (normalized avg. microcolonies per frame/0 μM microcolonies per frame) X 100.

^{**} A total of at least 30 frames per peptide concentration were analyzed.

^a $P \leq 0.0001$ compared to the no peptide control, a P value of 0.006 was calculated for 0.8 μM BAR-19 compared to the no peptide control.

^b $P 0.002$ compared to equal concentration of BAR.