INFECTION AND IMMUNITY, July 1999, p. 3248–3256 Vol. 67, No. 7
0019-9567/99/$04.00+0

Purification, Characterization, and Sequence Analysis of a Potential Virulence Factor from *Porphyromonas gingivalis*, Peptidylarginine Deiminase

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Received 29 January 1999/Returned for modification 23 March 1999/Accepted 7 April 1999

The initiation and progression of adult-onset periodontitis has been associated with infection of the gingival sulcus by *Porphyromonas gingivalis*. This organism utilizes a multitude of virulence factors to evade host defenses as it establishes itself as one of the predominant pathogens in periodontal pockets. A feature common to many other oral pathogens is the production of ammonia due to its protective effect during acidic cleansing cycles in the mouth. Additionally, ammonia production by *P. gingivalis* has been proposed as a virulence factor due to its negative effects on neutrophil function. In this study, we describe the first purification of a peptidylarginine deiminase (PAD) from a prokaryote. PAD exhibits biochemical characteristics and properties that suggest that it may be a virulence agent. PAD deiminates the guanidino group of carboxyl-terminal arginine residues on a variety of peptides, including the vasoregulatory peptide-hormone bradykinin, to yield ammonia and a citrulline residue. The soluble protein has an apparent mass of 46 kDa, while the DNA sequence predicts a full-length protein of 61.7 kDa. PAD is optimally active at 55°C, stable at low pH, and shows the greatest activity above pH 9.0. Interestingly, in the presence of stabilizing factors, PAD is resistant to limited proteolysis and retains significant activity after short-term boiling. We propose that PAD, acting in concert with arginine-specific proteinases from *P. gingivalis*, promotes the growth of the pathogen in the periodontal pocket, initially by enhancing its survivability and then by assisting the organism in its circumvention of host humoral defenses.

The presence of *Porphyromonas gingivalis*, a gram-negative, nonmotile, facultative anaerobe, is correlated with the prevalence of adult-onset periodontitis (5). In model systems, the implantation of this organism in the oral cavity has been shown to be sufficient for the development of the disease (15). A multitude of factors isolated from *P. gingivalis* reportedly mediate host responses at the site of infection. Many of these virulence factors are present on membrane blebs or vesicles produced by the organism, and the presence of these vesicles is thought to amplify the zone of effectiveness for the bacterium (11). The proteinases and adhesins from the organism have been of particular interest to our group due to their abundance and potency (7, 31, 32, 40).

Degradation of periodontal tissue by host (36, 37) or bacterial (11, 12, 23, 25, 40, 41) proteinases, as well as the degradation of plasma constituents (5, 14, 20), provides a nutritive milieu of peptides that sustains the growth of asaccharolytic *P. gingivalis* (40). The proteolytic enzymes from *P. gingivalis* are apparently important in the circumvention of the host's control of available metabolites, such as iron or peptide-protein energy sources (12). Additionally, these proteinases affect other host systems in such a manner as to enrich the environment for the organism (16, 17). Recent results from this laboratory have demonstrated that R-gingipains (RGPs), the major arginine-specific cysteine proteinases from *P. gingivalis* (17), activate prekallikrein to initiate the production of bradykinin (BK) (16). In vivo assays for BK production have shown that singular doses of purified RGP produce an immense physiologic response, but it was interesting to note that vesicles with equivalent RGP activity levels produced a significantly diminished response. The presence of BK in host tissue results in increased vascular permeability (18), as evidenced by the development of edema and, in the case of periodontitis, an increase in crevicular fluid flow (40). This latter effect correlates with the presence of *P. gingivalis* (12, 23), as do the increased levels of proteinases from this organism in crevicular fluid (8, 10, 26, 34). We hypothesize that the ability of *P. gingivalis* to initiate a flow of plasma ensures that the organism is sustained in the crevicular pocket. The unknown element of this pathologic mechanism is to what extent, if at all, *P. gingivalis* regulates the rate of this flow so that the organism is not washed from the pocket due to excessive exudation of plasma. Our observations suggest that *P. gingivalis* possesses nonproteolytic activities which influence the documented vascular effects induced by the proteinases from the organism in in vitro experiments. This activity may modulate the ultimate physiologic response in the vasculature.

The discovery of an activity that might affect vascular mediators, many of which are arginyl carboxyl-terminal peptides, initially occurred during a time course digestion of various peptides with an early preparation of RGP. High-performance liquid chromatography (HPLC)–reverse-phase analysis of the RGP digestion reactions showed that a contaminating activity was altering the retention times, but not the apparent composition, of arginine carboxyl-terminal products from the RGP incubation (7). The same RGP preparations used for the specificity studies mentioned above were also used to demonstrate the production of complement component C5a from C5 by RGP. The recovery of C5a activity was only 20 to 25% percent of what was expected for the amount of C5 utilized in the experiment (44), suggesting that some alteration to the func-

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tionally important carboxyl-terminal arginine of C5a was occurring. We found an answer to our unexplained observations in a report detailing the importance of arginine residues in peptides that inhibit hemagglutination. Hayashi et al. (13) showed that their hemagglutinin preparation contained a "trypsin-like" proteinase and an activity that produces a carboxyl-terminal citrulline residue from the arginyl residue. The peptide was cleaved at the internal arginine by the trypsin-like activity of the hemagglutinin preparation, and the fragment with the arginine at its carboxyl terminal retained an inhibitory potential, albeit with a lower efficacy. Interestingly, in longer incubations the antihemagglutination activity of this fragment was completely inactivated due to the conversion of the arginine at the carboxyl terminus to citrulline (deimination) by a nonproteolytic activity within the hemagglutinin preparation. This series of seemingly unrelated observations led us to propose that the deimination of arginine-dependent bioactive molecules could affect the functionality of these peptides. In keeping with this hypothesis, we propose that the cause of the diminished vascular permeability enhancement response to vesicles, compared to the RGP response (16, 17), resulted from the action of this recently identified deiminase.

The first enzyme of the arginine deiminase pathway modifies the guanidino group on arginine residues to produce citrulline and ammonia. The pathway has predominantly been recognized for its ability to provide energy during anaerobic growth, but a series of reports have shown that a number organisms associated with the oral microflora rely on the ammonia generated by this system for their ability to tolerate and neutralize acidic environments (6, 19, 22). The ability of these organisms to persist in the rapidly changing milieu of the oral cavity is believed to be a critical feature in their virulence.

The possession of an arginine deiminase activity by *P. gingivalis* seemed to be an attractive addition to the list of putative virulence factors utilized by this organism to circumvent host defenses, primarily because the production of ammonia has already been associated with its pathogenicity (29). Additionally, inactivation of biologically relevant peptides, acid tolerance, and the production of ATP by the remaining enzymes of the arginine deiminase pathway could be critical for maintaining the viability of *P. gingivalis* and promoting its growth in the periodontal pockets. Thus, a study was initiated to isolate and characterize the deiminase from this organism and to determine if this activity might have a pathophysiological function during periodontitis.

MATERIALS AND METHODS

Reagents, bacterial strains, and culture condition. Benzoyl (Bz)-L-Arg-ethyl-ester (BAEE), Bz-L-Arg-*p*-nitroanalide, Bz-L-Arg-amide (BAA), hippurl-L-Arg, Trizma base, BK, L-citrulline, flavin adenine mononucleotide (FMN), flavin adenine dinucleotide (FAD), NADPH, *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), thiourea, a random arginine-threonine-histidine polypeptide, and L-arginine were from Sigma (Saint Louis, Mo.). L-Thiocitrulline was a gift of Owen W. Griffith (Medical College of Wisconsin, Milwaukee). Ceramic hydroxyapatite, HS, 40 μm (American International Chemical, Natick, Mass.) was a gift of Mike Adams (University of Georgia, Athens). *P. gingivalis* HG66 was a gift of Roland Arnold (University of North Carolina, Chapel Hill), and the ATCC strains 33277 and 53978 (W50) were purchased from the American Type Culture Collection (Rockville, Md.).

Cultivation of Bacteria. The strains of *P. gingivalis* were grown as previously described (7) in reducing broth (10 g of yeast extract, 30 g of Trypticase soy broth, 1 g of cysteine, 100 mg of dithiothreitol (DTT), 5 mg of hemin and 2.5 mg of menadione in a 1-liter volume). The cells were grown with constant low-speed shaking (150 rpm) at 37°C for 24 h to an optical density at 660 nm of 1.5.

Enzyme purification. Culture fluid (5,600 ml) was obtained by centrifugation (6,000 × *g*; 30 min; 4°C). Chilled acetone was added to the fluid over a period of 15 min to a final concentration of 60%, with the temperature of the solution maintained below 0°C, followed by centrifugation (6,000 × *g*; 30 min; −20°C). The pellet was redissolved in a solution of 20 mM bis-Tris-HCl, 150 mM NaCl, and 1 μM FMN (pH 6.8) (buffer A) containing 1.5 mM 4,4′-dithiodipyridine to reversibly block the active-site cysteine residues of *P. gingivalis* cysteine proteinases and was dialyzed against buffer A overnight with three changes. The first change was buffer A with 4,4′-dithiodipyridine, and subsequent changes were buffer A alone or with 1 mM $CaCl_2$. The dialyzed fraction was centrifuged (27,000 × *g*; 30 min; 4°C) and concentrated to 28 ml by ultrafiltration. This solution was clarified by centrifugation (30,000 × *g*; 1 h) and applied to a Sephadex G-150 column (5 by 120 cm; 30 ml/h) previously equilibrated with buffer A. Individual fractions were assayed for citrulline formed from BAEE, and active fractions were pooled, concentrated by ultrafiltration, and dialyzed overnight against two changes of 20 mM bis-Tris-HCl–1 μM FMN–1 mM $CaCl_2$, pH 6.8 (buffer B). This sample was then applied to a DE-52 (Whatman) column (1.5 by 21 cm; 20 ml/h) previously equilibrated with buffer B and washed until the baseline was reestablished. A linear gradient of 0 to 500 mM NaCl in buffer B was applied over 3 column volumes. The active enzyme, found primarily in the first peak (about 150 mM NaCl), was pooled and concentrated to 10 ml. Buffer exchange during ultrafiltration was repeated four times. The first exchange was in buffer B without $CaCl_2$, and the remaining three were in 10 mM NaH_2PO_4–1 μM FMN, pH 6.8 (buffer C). The pooled sample was then applied to a ceramic hydroxyapatite column (2.5 by 21 cm; room temperature; 2 ml/min). A linear multistep gradient (0 to 12, 12 to 15, 15 to 37, and 37 to 100%) was initiated, using 2 column volumes for each step, with 500 mM $NaHPO_4$ in buffer C as the elution buffer. All active fractions were pooled, concentrated, and used for further analyses.

Vesicle Preparation. Culture fluid (1,500 ml) from 36 h of cultivation of *P. gingivalis* ATCC 53978 (W50) and ATCC 33277 was obtained by centrifugation of cells (6,000 × *g*; 30 min; 4°C). Ultracentrifugation of the culture fluid (100,000 × *g*; 120 min; 4°C) produced a pellet of vesicles that was washed twice and resuspended in phosphate-buffered saline containing 1 μM FMN and used as the vesicle preparation after titration for RGP activity (33). Peptidylarginine deiminase (PAD) activity was rapidly lost in aged vesicle preparations (>50% in 2 h on ice) or following lyophylization; therefore, it was necessary to use freshly prepared vesicles for each experiment.

Electrophoresis. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method devised by Shagger and von Jagow (35), using a Tris-HCl–Tricine buffer system, was used throughout this study. Proteolytic degradation was reduced by pretreatment of the samples in 10 mM TLCK or 0.05 mM Phe-Phe-Arg-chloromethyloketone followed by boiling in nonreducing SDS-sample buffer and then in reducing sample buffer when required.

Isoelectric focusing. Determination of isoelectric points was accomplished by using the Phast electrophoresis system from Pharmacia (Uppsala, Sweden) with precast Phast isoelectric focusing gels and following the program suggested by the manufacturer. Silver staining was used to visualize the bands and assess purity. Samples of PAD from each of the peaks of activity off the hydroxyapatite column, equilibrated with 25 mM bis-Tris, pH 6.8, and 5 μM FMN by dialysis, were also applied to a mono-P column (fast protein liquid chromatography system; Pharmacia). The column, conditioned in the same buffer, was then developed with 50 ml of 10×-diluted Polybuffer 74 (Pharmacia) adjusted to a pH of 4.0.

Enzyme assays. Citrulline was measured according to the chemical colorimetric method of Boyde and Rahmatullah (4), adapted for use in microtiter plates. Samples were incubated for various periods at 37°C with 5 mM BAEE in buffer containing 0.2 M Tris-HCl (pH 8.0), 1 mM EDTA, 1 μM FMN, and 10 mM cysteine. The carbidino detection reagent was assembled daily from its two components with one part A (0.5% diacetyl monoximine and 0.01% thiosemicarbazide) added to two parts B (0.25 mg of $FeCl_3$/ml in 24.5% sulfuric acid and 17% phosphoric acid). The sample and incubation buffer (50 μl) were added to 200 μl of detection reagent, and the reaction was developed in the plates by heating it at 105°C for 25 min. The wells were then optically measured at 540 nm in a Molecular Devices (Menlo Park, Calif.) microtiter plate reader. The same buffer system was used for kinetic assays but included 25 μM FMN and FAD, together with 100 μM NADPH, in plates coated overnight with 1% bovine serum albumin. Standard curves were generated with free L-citrulline. Ammonia production was detected with an ammonia electrode (model MI-470; Microelectrodes, Londonderry, N.H.) which had previously been standardized and equilibrated according to the manufacturer's instructions.

Protein and DNA sequence analysis. The protein was prepared for sequencing by SDS-PAGE separation and blotting to a polyvinylidene difluoride membrane, as described by Matsudaira (24). Amino acid sequence analysis was performed with an Applied Biosystems 4760A gas phase sequenator with the program designed by the manufacturer. Sense and antisense primers were synthesized (D_NTER, 5′-CCGGAATTC GAT AGC GTA CCA AAA CGG CTG C, and D_NREV, 5′-CGCGGATCC CTA TCC GCA TGG TTT TGC CGA CG [Microsynth 9436; BALGACH, Basel, Switzerland]) based on the protein sequence. Genomic *P. gingivalis* DNA (HG66) was subjected to PCR with the primers, and the appropriately sized band was excised from an agarose gel (1.5%). The PCR product was subcloned (*Bam*HI-*Eco*RI) into a sequencing vector (pBluescript SK; Stratagene, La Jolla, Calif.), and the DNA was sequenced by the dideoxy termination reaction with the Sequenase kit (United States Biochemical, Cleveland, Ohio). An *Eco*RI digest library constructed from *P. gingivalis* genomic DNA (W50) was screened with the PCR product from the N-terminal sequence (>10^6 colonies), and several clones were isolated. One clone, when sequenced, was found to contain a nucleotide sequence encoding the N-terminal part of

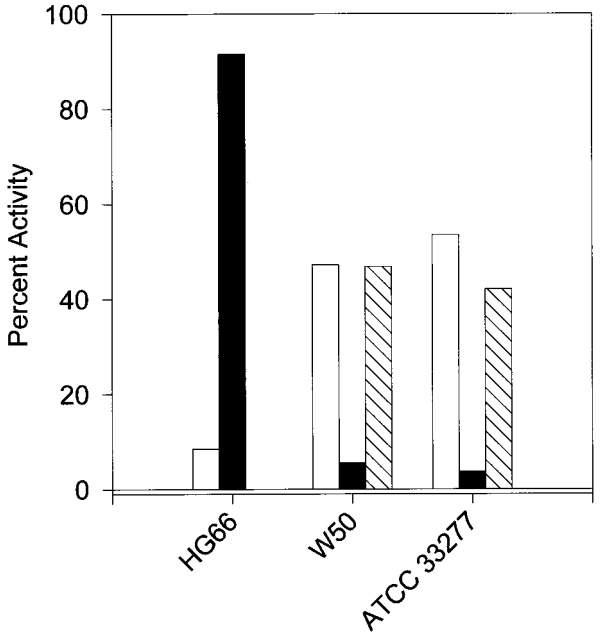

FIG. 1. Distribution of PAD in various strains of *P. gingivalis*. The production of citrulline from BAEE was determined in cellular (open bar), supernatant (solid bar), and vesicle (hatched bar) fractions from HG66, ATCC 53978 (W50), and ATCC 33277 strains of *P. gingivalis*.

PAD. The protein and DNA sequences were later compared to the incomplete database from the *P. gingivalis* genome sequencing project (31a).

Peptide analysis. Peptides and their degradation products were resolved as discrete peaks on a C_{18}/octyldecyl silane reverse-phase column (4.6 mm by 25 cm) (Beckman Instruments, Fullerton, Calif.) previously equilibrated with 0.1% trifluoroacetic acid and were eluted with a linear gradient (0 to 100% over 60 min) of 0.08% trifluoroacetic acid in 80% acetonitrile. The collected peaks were subjected to amino acid analysis with an Applied Biosystems 420A derivatizer with automated analysis. Under the separation conditions established by the manufacturer, citrulline could not be independently detected, but samples of free L-citrulline did elute at a position recognized by the data analysis program as cysteine, and this peak was taken to be citrulline in peptide analysis when cysteine residues were known not to be present. Mass analysis of both modified and native BK was performed by Jan Engheld (Duke University, Chapel Hill, N.C.).

RESULTS

Localization. The increase in total PAD activity paralleled the growth curves for three strains of *P. gingivalis*, but the distribution of this activity among cells, supernatant, and vesicles changed significantly with the growth phases. In late logarithmic growth phase, approximately 90% of the deiminase activity was cell or membrane vesicle associated for strains W50 and ATCC 33277. Strain HG66, which makes very few or no vesicles under the cultivation conditions we utilized, showed an opposite distribution, with the vast majority of activity being found in the secreted form (Fig. 1). The supernatant from late logarithmic growth of the HG66 strain ($A_{660} \approx 1.2$ to 1.5) was thus chosen as a starting point for the purification.

Enzyme purification. Precipitation of the culture fluid with cold acetone resulted in the highest recovery of stable PAD activity while leaving the majority of the pigmentous hemin in solution. The elimination of the remaining low-molecular-mass medium elements by gel filtration chromatography reliably yielded a fraction of activity in the 50-kDa range, which was pooled and used as a starting point for further purification. PAD activity eluted with the low-molecular-mass form of RGP (7), and the separation of these two activities became the primary focus of this fractionation. Anion exchange removed the remaining pigmentous materials and a portion of the RGP activity (Fig. 2), with adsorption of PAD on a ceramic form of hydroxyapatite being the final step (Fig. 3). This final procedure reliably separated the deiminase from low-molecular-mass RGP and other contaminants. Multiple peaks of deiminase activity were seen during the elution from the hydroxyapatite column, but these forms of PAD showed no differences in electrophoretic mobility and only slight differences in specific activity. Thus, all active fractions were pooled and used for further characterization. The purified PAD (Table 1) produced a single band on SDS-PAGE gels (Fig. 4), and amino

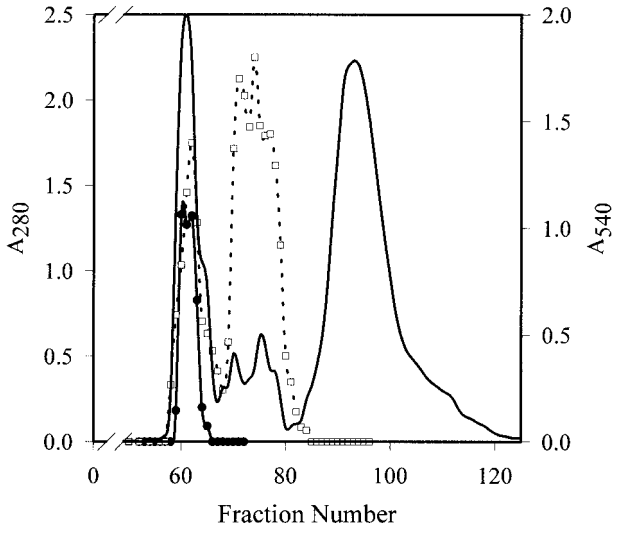

FIG. 2. Anion-exchange chromatography of 50-kDa peak from *P. gingivalis*. The active fractions from the 50-kDa range were pooled, dialyzed, and concentrated prior to being loaded onto a DE-52 anion-exchange column (1.5 by 21 cm; 20 ml/h) previously equilibrated with 20 mM Bis-Tris-HCl–1 μM FMN–1 mM $CaCl_2$, pH 6.8. A linear gradient of 0 to 500 mM NaCl in buffer B was applied over 3 column volumes. The active enzyme is found primarily in the first peak (about 150 mM NaCl). ——, A_{280}; ●, deiminase activity; □, amidolytic activity.

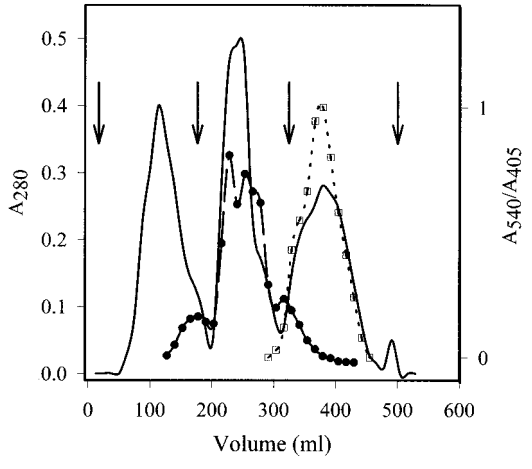

FIG. 3. Hydroxyapatite adsorption chromatography of *P. gingivalis* deiminase activity. The citrulline-producing fractions from anion-exchange chromatography were pooled and applied to a ceramic hydroxyapatite column in sodium phosphate buffer (10 mM, pH 6.5). A linear multistep gradient (arrows [left to right], 0 to 12, 12 to 15, 15 to 37, 37 to 100%) with 2 column volumes of eluent (0.5 M NaH_2PO_4, pH 6.5) for each step was applied to the column, and fractions were assayed. ——, A_{280}; ●, deiminase activity; □, amidolytic activity.

TABLE 1. Purification of PAD

Fraction	Vol (ml)	Protein (mg)	Total activity (U[a])	Sp act (U/mg)	Purification (fold)	Yield (%)
Culture medium	5,600	71,058	306,966	4.3	1	100
Acetone precipitation	280	1,187	18,068	15.2	3.5	5.9
Sephadex G-150	210	442	12,122	27.4	6.4	3.9
DE52	45	38	16,427	432.3	100	5.3
Hydroxyapatite	35	4.7	14,120	3,004	697	4.6

[a] U, nanomoles per hour at 37°C.

acid sequence analysis of the amino terminus yielded only one sequence. The combination of procedures allowed for the isolation of approximately 1 mg per liter of culture supernatant and resulted in a 700-fold purification. One might note in the purification table that the recovery increases slightly after the sizing column. This is due to the removal of a significant portion of the low-molecular-mass form of the arginine-specific proteinase, which apparently interferes with the citrulline assay, perhaps by sequestration of substrate or other means.

Isoelectric point analysis. Analysis of PAD on an isoelectric focusing gel after silver staining showed that there are three different isoforms. Two of these had pI values of 6.0 and 6.1, while the third had a pI of 4.7. Separation of PAD isoforms on a mono-P column, followed by amino acid sequence analysis, showed that all three forms of PAD contained identical amino-terminal sequences. Reapplication of the more acidic forms of PAD to the mono-P column showed that there was a progressive shift to the 6.1 pI form of PAD. Such isoform shifting was previously observed with a *Mycoplasma* deiminase when it was incubated overnight at pH 10.0 or in 50% ammonium sulfate (42). The authors noted that along with the isoform shifting, there was a distinct change in the spectral absorption pattern of this enzyme, which suggested a "loss of a nucleotide cofactor or a change in the gross conformation of the protein," but there was no loss of activity with this *Mycoplasma* deiminase (42). PAD from *P. gingivalis*, when subjected to similar treatments, or when passed over the mono-P column, lost any residual activity as the pI shift occurred.

pH optima and pH stability. Assays of PAD activity were carried out over a pH range from 3.0 to 12.0 with the universal phosphate buffer system. A pH optimum was found at 9.3, with 37% of the activity remaining at pH 3.0 (Fig. 5). The enzyme showed no appreciable loss of activity when kept frozen (−20 or −80°C) in the presence of stabilizing factors (i.e., FMN or FAD) at pHs 5.5, 7.5, and 9.0 either overnight or for several days, but, surprisingly, when it was kept at 4°C, there was appreciable loss of activity.

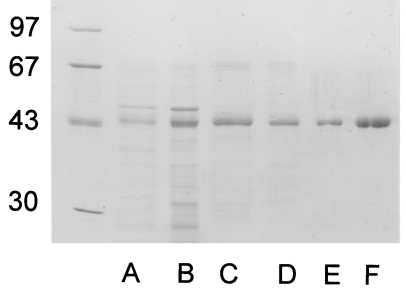

FIG. 4. SDS-PAGE of *P. gingivalis* PAD at various stages of purification. Samples were loaded for equal activity. Lane A, culture supernatant; lane B, acetone precipitate; lane C, Sephadex G-150 pooled activity; lane D, DE52 pooled activity; lane E, hydroxyapatite pooled activity (1 μg); lane F, purified PAD 5 times overloaded (5 μg). Molecular mass markers (phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; and α-lactalbumin, 14 kDa) are on the left.

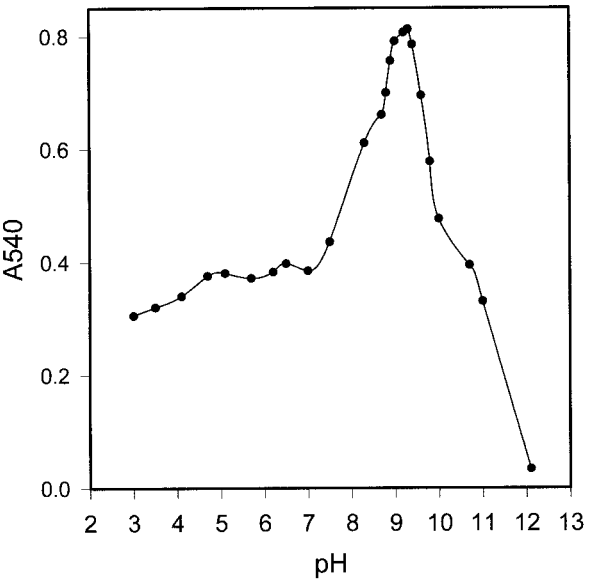

FIG. 5. Measurement of PAD activity at various pH values. PAD activity was measured at various pH values by the universal phosphate buffer system. PAD was preincubated with the 50 mM phosphate-citrate buffer with 1 mM DTT and 1 μM FMN prior to the addition of substrate.

Molecular mass analysis. PAD migrated as a single band on SDS-PAGE (Fig. 4). Computer-aided densitometry of the SDS-PAGE gel gave a molecular mass of 46.6 kDa. Elution of the PAD protein and activity from a calibrated analytical gel filtration column (TSK 3000SW) indicated a mass of 47 kDa (not shown). Attempts to purify the enzyme from the bacterial membrane were not successful. We expect that the membrane-associated form of PAD may have a somewhat larger mass than its soluble form. The predicted sequence of the PAD gene product suggests a much larger protein (see below), and as with several cases in this laboratory to date, proteins purified from the medium of the *P. gingivalis* HG66 were derived from larger precursors.

Citrulline analysis. Acid hydrolysis of PAD, followed by colorimetric determination of citrulline content for various preparations, showed that a range from 4.0 to 6.5 mol of citrulline per mol of PAD was present depending on the age of the sample. The presence of citrulline suggests that PAD undergoes automodification.

Sequence analysis. Amino-terminal sequencing of PAD through 30 cycles gave the following unique sequence: AFQET NPPAGPVRAIAEYYRRAAVLVRYPF. A search of the Swiss-Protein (release 31) and PIR (release 45) databases gave no complete matches. However, an alignment with an internal segment of carbamate kinase from *P. aeruginosa* revealed 36% identity, and this was deemed significant. It is not only known that this enzyme catalyzes the third step in the arginine deiminase pathway, but it is also reported that the enzymes of this pathway are localized to an operon and have a high level of internal homology with others in the pathway. A BLAST search (1, 2, 45) of both the protein and the DNA sequences of PAD matched to a high degree with one sequence from the *P. gingivalis* genome project (clone-P.gingivalis_112). The PAD gene encodes a 556-amino-acid protein in 1,671 bp (Fig. 6).

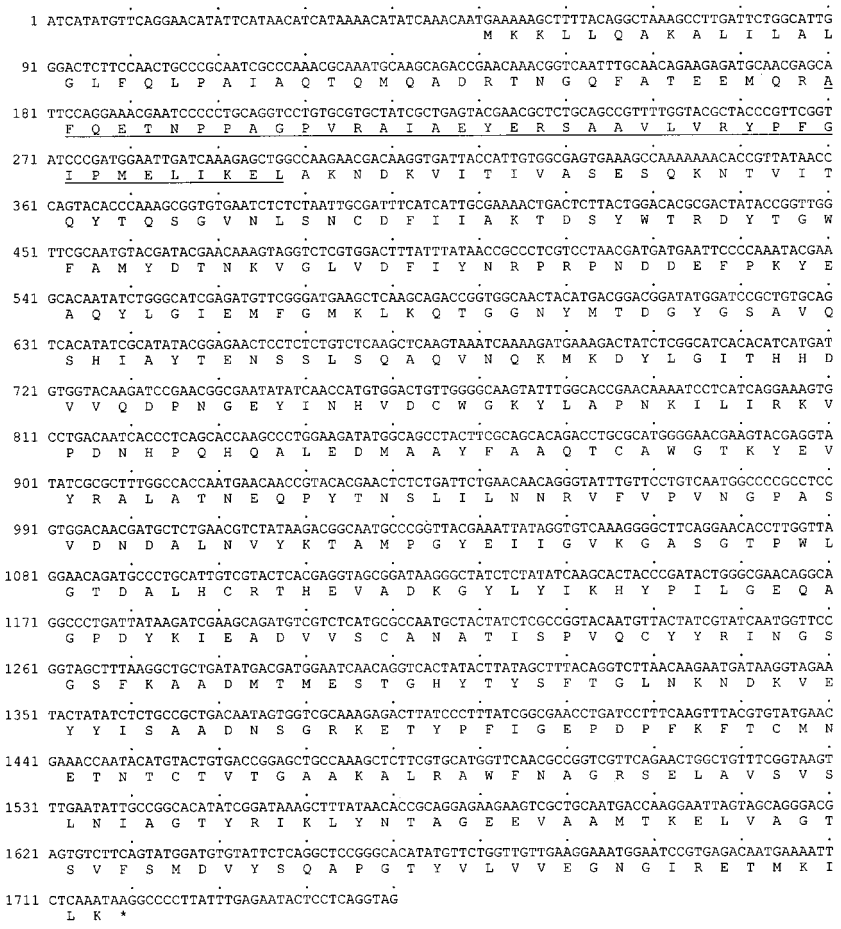

FIG. 6. PAD sequence. The underlined amino acid sequence was determined by sequencing the amino terminus of the isolated protein.

The theoretical mass and pI of the protein were 61,729.83 Da and 5.88 (3), respectively.

Cofactors. Extensive dialysis resulted in the loss of all deiminase activity, presumably by structural destabilization and/or the removal of soluble cofactors. Activity could be partially restored to a briefly dialyzed sample by the addition of FMN (25 μM), but there was no recovery of activity after extensive dialysis regardless of the concentration of FMN, FAD, or other cofactors tested. The inclusion of FMN before dialysis and in pooled fractions stabilized the deiminase activity, and it was thus integrated into all buffers. PAD purified under these precautionary measures showed slight enhancement of activity when a combination of FMN, FAD, and NADPH was included in the assay buffer (Fig. 7). This effect could be mimicked by the inclusion of other suitable electron donors or acceptors in the assay.

Stability. PAD, purified in the presence of exogenous flavin nucleotides, retained the majority of its activity after 48 h at 37°C but was completely inactivated within 5 min upon boiling. The inclusion of additional FMN, FAD, and NADPH (>5 μM) to the buffer prior to boiling increased the time of inactivation and precipitation of the protein to greater than 1 h (Fig. 8). Additionally, when incubated with RGP, the cofactor-stabilized PAD retained full activity and showed little or no fragmentation by SDS-PAGE analysis. In contrast, the inactive form was completely degraded (data not shown).

Substrate specificity and kinetic analysis. The deimination of L-arginine, small arginine-containing substrates, or BK by PAD occurred without oxidizing or reducing exogenous flavin factors, but the kinetic parameters for this reaction were nonlinear unless these cofactors were present. The K_m and V_{max} values given in Table 2 demonstrate a preference of this enzyme for peptidylarginine substrates, followed by BAA and free L-arginine. Various arginyl-*p*-nitroanilides, as well as *p*-tosyl-L-Arg methyl ester, were relatively poor substrates.

Inhibition profile. Inhibition of the PAD with natural and synthetic inhibitors containing either arginine or modified arginine residues paralleled substrate specificity, with the peptide-arginyl-aldehyde inhibitor leupeptin proving to be 100% effective at low millimolar levels. Both thiourea and thio-L-citrulline were inhibitory at high concentration, as were cysteine and TLCK. *N*α-L-arginine methyl ester (L-NAME), a potent inhibitor of nitric oxide synthase, and the fungal peptide inhibitor antipain were only 50% effective at the highest concentrations utilized (Table 3).

Reaction products. The modification of BK by PAD was confirmed by mass spectra, HPLC–reverse-phase retention, and amino acid and chemical colorimetric analysis. The altered peptide showing a single discrete product peak with a longer retention time on the reverse-phase HPLC column (BKx), indicating an increased hydrophobicity in the modified BK peptide. BKx was isolated, and its mass was found to be dif-

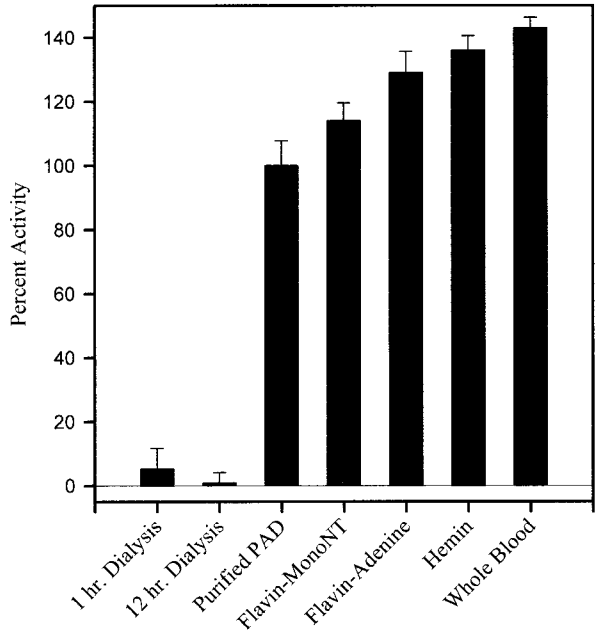

FIG. 7. Effect of electron donors and acceptors on PAD activity. The enzyme was dialyzed overnight with low levels of cofactor (50 mM Tris-HCl, 1 μM FMN, pH 8.0) and without any cofactor present (50 mM Tris-HCl, pH 8.0) as noted. Pretreatment of the dialyzed enzyme with higher levels of cofactors FMN (25 μM), FAD (25 μM), hemin (500 μM), or dilute whole blood (1:5,000; 50 mM Tris-HCl, 118 mM NaCl, 1 μM FMN, pH 8.0) was performed prior to the enzymatic assay (37°C; 10 min). Samples were then assayed for citrulline production in triplicate as described in Materials and Methods. The error bars indicate standard deviations.

ferent from BK by 0.5 Da (BK, 1062.4 Da, and BKx, 1061.9 Da). These values are clearly different from the calculated value (1062.1 Da), but the lower value for BKx is consistent with the expected mass difference (1 Da) for the citrullinated

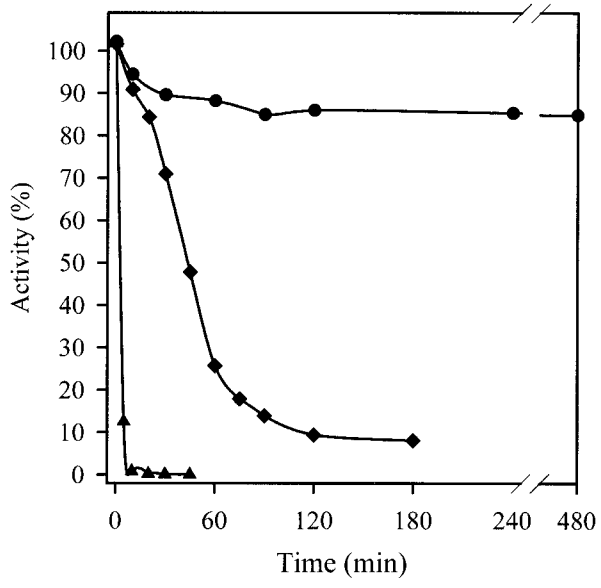

FIG. 8. Enhanced thermal stability of PAD with and without flavin nucleotides. Purified PAD was boiled with (◆) and without (▲) flavin nucleotides (FMN-FAD, 25 μM) or incubated at 37°C (●) in substrateless assay buffer (50 mM Tris-HCl, 25 μM FMN, pH 8.0). Aliquots were removed at various time points and assayed for the production of citrulline as described in Materials and Methods.

TABLE 2. Kinetic constants for the modification of synthetic substrates by PAD

Substrate	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m (10^{-3})
Benzoyl-L-arginine	2.9	104	35.9
Benzoylglycyl-L-arginine	28	186	6.6
BAA	152	77	0.51
BAEE	874	90	0.10
L-Arginine	141	26	0.18
BK	47	117	2.5

form. Sequencing of the modified BK showed that the amino-terminal arginine and internal residues were unchanged, but the carboxyl-terminal arginine was lost. Quantitative amino acid analysis of this BK analogue showed the loss of one arginine and the gain of a residue eluting under cysteine, which is assumed to be citrulline. Furthermore, the amount of this amino acid measured by the carbamino reaction (4) showed a stoichiometry of one citrulline residue per modified BK molecule. These results, taken together, verify the conversion of the carboxyl-terminal arginine to citrulline in BK after PAD treatment.

Measurement of product formation. The unusual requirement for adenine nucleotides for maximal activity and stability of PAD, as well as for the production of citrulline, parallels the requirement for the production of this compound from arginine by nitric oxide synthase (39) and prompted the measurement of nitric oxide production. Under various assay conditions and with a number of substrates it was not possible to demonstrate the production of nitric oxide with the Greisser reaction (27). Production of ammonia was demonstrated, however, by direct measurement with an ammonia electrode. The levels of ammonia measured in this manner corresponded to the amount of citrulline produced under the same reaction conditions (not shown).

DISCUSSION

Previous studies of hemagglutinin preparations, including vesicles, and partially purified low-molecular-mass RGP sug-

TABLE 3. Effects of inhibitors on deiminase activity

Inhibitor	Conc. (mM)	Activity (%)
L-NAME	0.25	100
	3.12	75
	12.5	52
Thiocitrulline	0.25	100
	6.25	30
	12.5	0
Thiourea	1.25	100
	6.25	60
	50	0
TLCK	1.25	100
	6.25	7
	12.5	2
Leupeptin	0.05	100
	2.5	55
	5	0
Antipain	0.05	100
	2	80
	8	59
Cysteine	1.25	100
	12.5	88
	25	0

gested the presence of a PAD-like activity in *P. gingivalis* (7, 13, 30). The production of ammonia from peptidylarginine substrates was of interest because of a potential relationship to the unusual function of RGP from *P. gingivalis*. Arginine deiminase enzymes modify the guanidino group of arginine residues to produce a citrulline residue and free ammonia (22). The production of ammonia by oral pathogens, long associated with the formation of caries on tooth surfaces (37), has also been linked to acid tolerance and virulence in other, non-caries-related strains (6). Interestingly, ammonia levels in the crevicular fluid from periodontal lesions are quite high, but its presence has often been attributed to other organisms (29). Our discovery that *P. gingivalis* possesses a deiminase activity suggests that this organism might also utilize this pathway to produce ammonia and provide environmental advantages over competing organisms. Several reports have suggested that deiminase activities may have in vivo functional roles, including the inactivation of peptides which inhibit the hemagglutination ability of *P. gingivalis* (13), as well as energy production (6, 22). Since the deiminase activity was only observed in the presence of other enzymes, we thought that it was important to isolate and characterize this activity fully so that its importance to the virulence of *P. gingivalis* might be assessed.

In this report we demonstrate the successful isolation of a PAD from the 50-kDa fraction of the culture medium from *P. gingivalis* HG66. The purification of a unique activity, which is distinct from the gingipain proteinases, demonstrates that the formation of citrulline-containing peptides by hemagglutination preparations and our own early preparations of RGP was the result of PAD contamination. The isolation of PAD also gives credence to the possibility that ammonia production by *P. gingivalis* may promote its viability, as it does for other oral pathogens (6, 22). In addition, and since it has been shown that the growth of *P. gingivalis* and the activity of the potent cysteine proteinase from this organism are optimal at alkaline pH (7, 25, 31, 38), we propose that PAD may be interconnected to the virulence of *P. gingivalis*, especially in relation to the function of the RGPs.

The deiminase system in *P. gingivalis* may have gone unnoticed due to its instability during common laboratory procedures, including dialysis. Initial purification attempts resulted in a rapid and irreversible loss of activity until the addition of flavin nucleotides was found to stabilize the enzyme during dialysis (Fig. 7). The use of flavins as cofactors was tested because of the similarities between the chemistry of the arginine deiminase and the nitric oxide synthase pathways. Since purification of nitric oxide synthase was only possible after flavin addition (39), it seemed possible that a comparable effect might occur with PAD. The loss of PAD activity was particularly severe regardless of flavin addition in the case of the membrane-associated forms of PAD, with more than 95% of the activity being lost within a few hours after cell or vesicle isolation. When FMN or FAD levels were maintained during the purification of the soluble protein, however, multiple peaks of PAD activity could be eluted during the final chromatography step. These exhibited the same electrophoretic mobilities and amino-terminal sequences and showed only slightly different chromatographic properties and isoelectric points, presumably due to automodification of internal arginine residues to citrulline residues.

Similar unusual results involving flavin nucleotides and multiple isoforms of an L-arginine deiminase from *Mycoplasma arthritidis* were noted by Weickmann et al. (42, 43). These authors noted that this deiminase could be transformed into an alternate form by incubation with either 50% ammonium sulfate or a high-pH buffer. The converted form had a slightly higher specific activity and an increased A_{280}/A_{260} ratio, indicating either the loss of a nucleotide factor or a conformational change in the protein (42). Attempts at reproducing the isoform conversion with the deiminase from *P. gingivalis* resulted in the production of an apoenzyme which was completely devoid of activity and which could not be reactivated by the addition of flavins. Spectral studies indicate that the flavin nucleotides do not participate in the PAD reaction and that they are not covalently bound. Rather, our data would indicate that the flavin nucleotides function by enhancing the stability of the enzyme.

Kinetic studies on low-molecular-mass arginine-containing substrates with purified PAD showed that this enzyme exhibited a strong preference for peptidylarginine substrates. However, free L-arginine was modified as well, albeit at much lower rates (Table 2). This observation separates PAD from the proteinyl arginine deiminase, which selectively modifies arginine residues within the linear backbone of a protein, and the arginine deiminase, which produces L-citrulline from free L-arginine. Clearly, PAD can perform both of these reactions, but under the conditions utilized, the deiminase from *P. gingivalis* appears to have primarily peptidylarginine specificity. Protein substrates as well as peptides containing amino-terminal arginine residues were modified at a very low rate and in some cases displayed burst-like kinetics. Furthermore, an inverse relationship between substrate concentrations, product formation, and reaction velocities was also observed for protein substrates and amino-terminal arginine substrates. Namely, as the concentration of these substrates increased, the amount of measurable citrulline formed was less and it was produced at a lower rate. It is possible that these complex kinetics are due to arginine-containing peptides interacting with an adhesion-hemagglutination domain on the deiminase protein which exhibits different binding constants than the active site. The presence of such an adhesion site is supported by the fact that PAD activity was found in *P. gingivalis* hemagglutin preparations which bound to erythrocyte ghosts by a mechanism which was sensitive to free arginine or arginyl peptides (13, 30). Additionally, a search of the databases with the PAD DNA sequence showed that there is localized homology (~28%) with the extracellular portion of the receptor for the fibroblast growth factor. The importance of this homology became apparent when Hanneken et al. showed that the soluble form of the fibroblast growth factor receptor binds tightly to the extracellular matrix (12a). We are investigating the possibility that this region of PAD may be a novel adhesion domain.

Inhibition studies indicated that the nitric oxide synthase inhibitors L-NAME and L-thiocitrulline were effective against PAD only at millimolar levels and that they most likely functioned by two different methods. L-NAME alone proved to be a substrate at the levels required for inhibition, indicating its effect could be due to competitive substrate inhibition (Table 2). The inhibition by thiocitrulline was reversible at high levels of the preferred substrate, Bz-L-Arg, but only under mildly reducing conditions (data not shown), suggesting that the thioureido group of this inhibitor was interacting with a cysteine in or near the active site, as it does with nitric oxide synthase (28). Since millimolar levels of thiourea and TLCK also inhibited the deiminase, as did the sulfhydryl-blocking reagent 4,4′-dithiodipyridine, we are convinced of the presence of a cysteine residue in or proximal to the active site of PAD.

The similarities between the chemistries of PAD and nitric oxide synthase, as well as the stabilization by flavin nucleotides, prompted the examination of the reaction mechanism of this enzyme for the direct production of nitric oxide under anaerobic and aerobic conditions. Nitric oxide, previously known as

the endothelium-dependent relaxation factor, is synthesized by an enzyme which produces citrulline as an intermediate and has a requirement for flavin nucleotides for maximal activity (28). Nevertheless, the production of nitric oxide could not be demonstrated under any conditions examined. Furthermore, the utilization of an ammonia electrode unequivocally demonstrated that citrulline and ammonia are produced in equimolar amounts by PAD (not shown), as seen with all previously described deiminase reactions (9, 22, 43). Thus, we believe that *P. gingivalis* can produce ammonia in the oral cavity or periodontal pocket from peptidylarginine substrates via the PAD mechanism.

The strategies employed by *P. gingivalis* to overcome host barriers are surprisingly elementary in their concept. By simply turning host defense mechanisms to its advantage *P. gingivalis* can obtain a foothold in the gingival sulcus or periodontal pocket at a time when there are few competitors for available nutrients. Neutralization of the highly acidic environment surrounding the oral microbes by the ammonia produced by the arginine deiminase pathway (6, 22) may allow *P. gingivalis* to survive the humoral defense of the low-pH cleansing of the mouth by saliva during food intake, which kills many nonpathogenic competitors (19). In this case, RGP may also cleave the antiadhesive humoral defense peptides, which have internal arginyl residues (9, 13, 19, 21). These cleaved peptides and other arginine carboxyl-terminal defense peptides may then be finally inactivated by PAD, not only blocking their adhesion-inhibitory roles but also providing ammonia to keep the pocket alkaline and ATP for the energy requirements of this asaccharolytic organism.

The interplay between RGPs and PAD proposed here reinforces the idea that the examination of isolated systems from *P. gingivalis* followed by confirmation of the effect by using vesicles or whole bacteria is a powerful means of elucidating the multifaceted virulence mechanisms of this organism. Now that an additional element which supports the virulence of *P. gingivalis* has been isolated, experiments examining the individual and concerted effects of the proteinase, the deiminase, and their adhesin domains should lead to a greater understanding of the function(s) of this periodontal pathogen during periodontitis.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Bjellqvist, B., G. J. Hughes, C. Pasquali, N. Paquet, F. Ravier, J. C. Sanchez, S. Frutiger, and D. Hochstrasser.** 1993. The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. Electrophoresis **14:**1023–1031.
4. **Boyde, T. R., and M. Rahmatullah.** 1980. Optimization of conditions for the colorimetric determination of citrulline, using diacetyl monoxime. Anal. Biochem. **107:**424–431.
5. **Carlsson, J., J. F. Hofling, and G. K. Sundqvist.** 1984. Degradation of albumin, haemopexin, haptoglobin and transferrin, by black-pigmented Bacteroides species. J. Med. Microbiol. **18:**39–46.
6. **Casiano-Colon, A., and R. E. Marquis.** 1988. Role of the arginine deiminase system in protecting oral bacteria and an enzymatic basis for acid tolerance. Appl. Environ. Microbiol. **54:**1318–1324.
7. **Chen, Z., J. Potempa, A. Polanowski, M. Wikstrom, and J. Travis.** 1992. Purification and characterization of a 50-kDa cysteine proteinase (gingipain) from Porphyromonas gingivalis. J. Biol. Chem. **267:**18896–18901.
8. **Eley, B. M., and S. W. Cox.** 1996. Correlation between gingivain/gingipain and bacterial dipeptidyl peptidase activity in gingival crevicular fluid and periodontal attachment loss in chronic periodontitis patients. A 2-year longitudinal study. J. Periodontol. **67:**703–716.
9. **Enwonwu, C. O., F. Ilupeju, and R. C. Warren.** 1994. Arginine metabolism in the salivary glands of protein-deficient rats and its potential association with the oral microflora. Caries Res. **28:**99–105.
10. **Gazi, M. I., S. W. Cox, D. T. Clark, and B. M. Eley.** 1996. A comparison of cysteine and serine proteinases in human gingival crevicular fluid with tissue, saliva and bacterial enzymes by analytical isoelectric focusing. Arch. Oral. Biol. **41:**393–400.
11. **Grenier, D., and D. Mayrand.** 1987. Functional characterization of extracellular vesicles produced by *Bacteroides gingivalis*. Infect. Immun. **55:**111–117.
12. **Grenier, D., and D. Mayrand.** 1987. Selected characteristics of pathogenic and nonpathogenic strains of *Bacteroides gingivalis*. J. Clin. Microbiol. **25:** 738–740.

12a. **Hanneken, A., P. A. Maher, and A. Baird.** 1995. High affinity immunoreactive FGF receptors in the extracellular matrix of vascular endothelial cells—implications for the modulation of FGF-2. J. Cell Biol. **128:**1221–1228.

13. **Hayashi, H., M. Morioka, S. Ichimiya, K. Yamato, D. Hinode, A. Nagata, and R. Nakamura.** 1993. Participation of an arginyl residue of insulin chain B in the inhibition of hemagglutination by Porphyromonas gingivalis. Oral Microbiol. Immunol. **8:**386–389.
14. **Henskens, Y. M., U. van der Velden, E. C. Veerman, and A. V. Nieuw Amerongen.** 1993. Protein, albumin and cystatin concentrations in saliva of healthy subjects and of patients with gingivitis or periodontitis. J. Periodontal Res. **28:**43–48.
15. **Holt, S. C., J. Ebersole, J. Felton, M. Brunsvold, and K. S. Kornman.** 1988. Implantation of Bacteroides gingivalis in nonhuman primates initiates progression of periodontitis. Science **239:**55–57.
16. **Imamura, T., R. N. Pike, J. Potempa, and J. Travis.** 1994. Pathogenesis of periodontitis: a major arginine-specific cysteine proteinase from Porphyromonas gingivalis induces vascular permeability enhancement through activation of the kallikrein/kinin pathway. J. Clin. Investig. **94:**361–367.
17. **Imamura, T., J. Potempa, R. N. Pike, and J. Travis.** 1995. Dependence of vascular permeability enhancement on cysteine proteinases in vesicles of *Porphyromonas gingivalis*. Infect. Immun. **63:**1999–2003.
18. **Imamura, T., T. Yamamoto, and T. Kambara.** 1984. Guinea pig plasma kallikrein as a vascular permeability enhancement factor. Its dependence on kinin generation and regulation mechanisms in vivo. Am. J. Pathol. **115:**92–101.
19. **Kanapka, J. A., and I. Kleinberg.** 1983. Catabolism of arginine by the mixed bacteria in human salivary sediment under conditions of low and high glucose concentration. Arch. Oral Biol. **28:**1007–1015.
20. **Kilian, M.** 1981. Degradation of immunoglobulins A1, A2, and G by suspected principal periodontal pathogens. Infect. Immun. **34:**757–765.
21. **Lehrer, R. I., A. K. Lichtenstein, and T. Ganz.** 1993. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. Annu. Rev. Immunol. **11:**105–128.
22. **Marquis, R. E., G. R. Bender, D. R. Murray, and A. Wong.** 1987. Arginine deiminase system and bacterial adaptation to acid environments. Appl. Environ. Microbiol. **53:**198–200.
23. **Marsh, P. D., A. S. McKee, A. S. McDermid, and A. B. Dowsett.** 1989. Ultrastructure and enzyme activities of a virulent and an avirulent variant of Bacteroides gingivalis W50. FEMS Microbiol. Lett. **50:**181–185.
24. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:** 10035–10038.
25. **Mayrand, D., and S. C. Holt.** 1988. Biology of asaccharolytic black-pigmented Bacteroides species. Microbiol. Rev. **52:**134–152.
26. **Miller, J. W., D. W. Turner, and E. D. Pedersen.** 1991. Trypsin, T. denticola and P. gingivalis levels as diagnostic markers for periodontitis. Northwest Dent. Res. **3:**12–14.
27. **Miller, K., and M. E. Neville.** 1976. Evaluation of alternate coupling reagents to replace alpha-naphthyl amine for the detection of nitrate reduction. Microbios **17:**207–212.
28. **Narayanan, K., L. Spack, K. McMillan, R. G. Kilbourn, M. A. Hayward, B. S. Masters, and O. W. Griffith.** 1995. S-alkyl-L-thiocitrullines. Potent stereoselective inhibitors of nitric oxide synthase with strong pressor activity in vivo. J. Biol. Chem. **270:**11103–11110.
29. **Niederman, R., B. Brunkhorst, S. Smith, R. N. Weinreb, and M. I. Ryder.** 1990. Ammonia as a potential mediator of adult human periodontal infection: inhibition of neutrophil function. Arch. Oral Biol. **35**(Suppl.)**:**205S–209S.
30. **Nishikata, M., and F. Yoshimura.** 1991. Characterization of Porphyromonas (Bacteroides) gingivalis hemagglutinin as a protease. Biochem. Biophys. Res. Commun. **178:**336–342.
31. **Pike, R., W. McGraw, J. Potempa, and J. Travis.** 1994. Lysine- and arginine-specific proteinases from Porphyromonas gingivalis. Isolation, characterization, and evidence for the existence of complexes with hemagglutinins. J. Biol. Chem. **269:**406–411.

31a. ***Porphyromonas gingivalis* Genome Sequencing Project.** 10 November 1997, posting date. [Online.] http://www.forsyth.org/pggp. [20 January 1999, last date accessed.]

32. **Potempa, J., R. Pike, and J. Travis.** 1995. The multiple forms of trypsin-like activity present in various strains of Porphyromonas gingivalis are due to the presence of either Arg-gingipain or Lys-gingipain. Infect. Immun. **63:**1176–1182.
33. **Potempa, J., R. Pike, and J. Travis.** 1997. Titration and mapping of the active site of cysteine proteinases from Porphyromonas gingivalis (gingipains) using

peptidyl chloromethanes. Biol. Chem. **378:**223–230.
34. **Potempa, J., and J. Travis.** 1996. Porphyromonas gingivalis proteinases in periodontitis, a review. Acta Biochim. Pol. **43:**455–465.
35. **Schagger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.
36. **Schenkein, H. A., and C. R. Berry.** 1988. Production of chemotactic factors for neutrophils following the interaction of Bacteroides gingivalis with purified C5. J. Periodontal Res. **23:**308–312.
37. **Slots, J.** 1977. Microflora in the healthy gingival sulcus in man. Scand. J. Dent. Res. **85:**247–254.
38. **Slots, J.** 1977. The predominant cultivable microflora of advanced periodontitis. Scand. J. Dent. Res. **85:**114–121.
39. **Stuehr, D. J., and M. A. Marletta.** 1985. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to Escherichia coli lipopolysaccharide. Proc. Natl. Acad. Sci. USA **82:**7738–7742.
40. **Travis, J., R. Pike, T. Imamura, and J. Potempa.** 1997. Porphyromonas gingivalis proteinases as virulence factors in the development of periodontitis. J. Periodontal Res. **32:**120–125.
41. **Uitto, V. J., H. Larjava, J. Heino, and T. Sorsa.** 1989. A protease of *Bacteroides gingivalis* degrades cell surface and matrix glycoproteins of cultured gingival fibroblasts and induces secretion of collagenase and plasminogen activator. Infect. Immun. **57:**213–218.
42. **Weickmann, J. L., M. E. Himmel, D. W. Smith, and D. E. Fahrney.** 1978. Arginine deiminase: demonstration of two active sites and possible half-of-the-sites reactivity. Biochem. Biophys. Res. Commun. **83:**107–113.
43. **Weickmann, J. L., M. E. Himmel, P. G. Squire, and D. E. Fahrney.** 1978. Arginine deiminase from Mycoplasma arthritidis. Properties of the enzyme from log phase cultures. J. Biol. Chem. **253:**6010–6015.
44. **Wingrove, J. A., R. G. DiScipio, Z. Chen, J. Potempa, J. Travis, and T. E. Hugli.** 1992. Activation of complement components C3 and C5 by a cysteine proteinase (gingipain-1) from Porphyromonas (Bacteroides) gingivalis. J. Biol. Chem. **267:**18902–18907.
45. **Zhang, J., and T. L. Madden.** 1997. PowerBLAST: a new network BLAST application for interactive or automated sequence analysis and annotation. Genome Res. **7:**649–656.

Editor: D. L. Burns