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Contribution of proteases and plasmin-acquired activity in migration of *Peptostreptococcus micros* through a reconstituted basement membrane

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Background/aims: *Peptostreptococcus micros* is a gram-positive bacterium that has been associated with chronic periodontitis and endodontic infections. The aims of this study were to investigate the production of proteases and the acquisition of plasmin activity by rough and smooth morphotypes of *P. micros*. The contribution of these properties in the migration of bacteria through a reconstituted basement membrane was also evaluated.
Methods: Protease activities were determined using chromogenic and fluorogenic substrates as well as by zymography. Plasminogen binding activity was studied using an enzyme-linked immunosorbent assay. The role of proteases and plasmin-acquired activity in tissue penetration was investigated using Matrigel.
Results: The rough morphotype strains of *P. micros*, but not the smooth morphotype strains, were found to possess chymotrypsin-like and gelatinase activities, both of which were inhibited by a serine protease inhibitor. By zymography, three gelatinase bands (165, 129, and 115 kDa) were identified. Both morphotypes of *P. micros* can bind human plasminogen on their cell surface. Once bound to *P. micros*, plasminogen activators of bacterial (streptokinase) and human (urokinase) origins were found to activate plasminogen into plasmin. Our results also showed that plasmin activity can be acquired by *P. micros* following co-incubation with human brain microvascular endothelial cells in culture. When non-coated cells were used, the rough morphotype strain (HG1262), which possesses chymotrypsin-like and gelatinase activities, showed a better capacity to penetrate a reconstituted basement membrane (Matrigel) than the smooth morphotype strain (HG1251). Penetration of the Matrigel by *P. micros* HG1262 was inhibited by the presence of a serine protease inhibitor. In addition, cells of *P. micros* with plasmin activity showed a significantly greater tissue penetration capacity.
Conclusion: Our study suggests that endogenous proteolytic activities of *P. micros* as well as plasmin-acquired activity, may facilitate dissemination of bacterial cells to surrounding periodontal tissues and blood vessels.

Key words: basement membrane; plasmin; *Peptostreptococcus micros*; periodontitis; protease

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Peptostreptococcus micros is one of the few gram-positive bacterial species for which there is evidence supporting a role in periodontitis (1, 15, 16, 18). It has also been associated with infected dental root canals (9) and with a number of extra-oral infections, including genital tract, intra-abdominal, pulmonary and brain infections (12). Van Dalen et al. (4) reported the existence of two morphotypes (rough and smooth) of *P. micros*, which differ in the presence of cell-associated fibril-like

appendages, the composition of cell wall proteins, the surface hydrophobicity, and the ability to lyse erythrocytes. Both morphotypes may be recovered from subgingival plaque and are likely acting as opportunistic pathogens, in association with gram-negative bacteria, to contribute to periodontitis (5). In a mouse model, the rough morphotype was found to be slightly more virulent than the smooth morphotype (6).

Proteases, which are enzymes that cleave peptide bonds in proteins and peptides, have been proposed to be critical virulence factors for a number of oral pathogens including *Porphyromonas gingivalis* and *Treponema denticola* (14). These enzymes have received much attention because they may play numerous roles in pathogenicity, for example, in multiplication, in adherence to host surfaces, in countering host defense mechanisms, and in the invasion of host tissues (10). In addition to its production by bacterial pathogens, protease activity may be acquired from the host through binding of inactive zymogens followed by their activation. For instance, the periodontopathogenic bacterium *Fusobacterium nucleatum* subsp. *nucleatum* has been reported to bind plasminogen and pro-matrix metalloproteinase 9 on its cell surface (7, 8). Furthermore, the binding of plasminogen by *Borrelia burgdorferi* has been shown to play a role in the *in vitro* migration of the bacterium through human umbilical vein endothelial cell monolayers and in promoting infections in a mouse model (2, 3). The aims of this study were to investigate the production of proteases by rough and smooth morphotypes of *P. micros* and to demonstrate the capacity of this bacterial species to acquire host proteolytic activity through the binding of human plasminogen and its subsequent activation into plasmin. The contribution of these activities to migration through a reconstituted basement membrane (Matrigel) was also evaluated.

Materials and methods

Bacterial strains and growth conditions

Six strains of *P. micros*, kindly provided by Dr T.J.M. van Steenbergen (Academic Center for Dentistry, Amsterdam, the Netherlands), were used in this study. The *P. micros* HG1252, HG1259 and HG1262 strains are rough morphotype isolates whereas strains HG1251, HG1253 and HG1254 are smooth morphotype isolates. Since *P. micros* grew rather poorly in a liquid medium, it was cultivated on solid plates. Lawns of bacteria were grown on Todd–Hewitt agar plates (BBL Microbiology Systems, Cockeysville, MO) for 3 days in an anaerobic chamber ($N_2 : H_2 : CO_2$, 80 : 10 : 10) at 37°C. Cells were harvested from the agar surface using a cotton swab and suspended in 50 mmol phosphate-buffered saline (PBS) pH 7.2 to an optical density at 660 nm (OD_{660}) of 0.45. This corresponds to a concentration of 2×10^9 bacteria/ml as determined in a Petroff–Hausser counting chamber. To prepare the *P. micros*-secreted material, 10 agar plates from which bacterial cells had been removed were placed at −20°C for 24 h. After thawing, the liquid phase (10 ml) was harvested and subjected to centrifugation (10,000 ***g*** for 10 min) to remove any residual bacterial cells. The resulting supernatant was considered as the secreted material.

Determination of proteolytic activities

Cell-associated and extracellular proteolytic activities of *P. micros* were determined using synthetic peptides labeled with *p*-nitroaniline (*p*Na) and self-quenched proteins labeled with fluorescein. The chromogenic synthetic peptides, obtained from the Sigma Chemical Co. (St Louis, MO), were: *N*-α-benzoyl-DL-arginine-*p*Na (for trypsin-like activity), *N*-valyl-leucine-lysine-*p*Na (for plasmin-like activity), *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*Na (for chymotrypsin-like activity), and *N*-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*Na (for elastase-like activity). Bacterial suspension or secreted material (100 µl) was incubated with 20 µl of each of the synthetic chromogenic peptides prepared at 2 mg/ml in PBS containing 10% dimethyl sulfoxide. The reaction mixtures were incubated at 37°C for 4 h (bacterial suspension) or 24 h (secreted material). Thereafter, the absorbance at 405 nm (A_{405}) was recorded with a microplate reader after removing bacteria by centrifugation when appropriate. The fluorescein-labeled substrates, purchased from Molecular Probes (Eugene, OR), were: DQ green bovine serum albumin, DQ gelatin, BODIPY® FL casein, DQ collagen type I, and DQ collagen type IV. The reaction mixtures contained 100 µl of the bacterial suspension or the secreted material and 20 µl of each of the substrates at 1 mg/ml in distilled water. The incubation was carried out at 37°C for 4 h (bacterial suspension) or 24 h (secreted material) under darkness. After removing bacteria by centrifugation (10,000 ***g*** for 10 min) when appropriate, protein degradation was monitored with a fluorometer at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Bacterial fractions treated at 100°C for 20 min were used as negative controls.

Analysis of proteases by zymography

Gelatin was incorporated at a final concentration of 0.03% into a 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS). Equal volumes of the cell suspension and sample buffer (0.06 M Tris–HCl, pH 6.8, 6% SDS, 20% glycerol, 0.01% bromophenol blue) were mixed and electrophoresis was carried out under cooling conditions. The gels were then washed for 15 min with 0.05 M Tris–HCl (pH 7.5), incubated in the equilibration buffer (2 × 15 min; 0.05 M Tris–HCl, 0.1 M NaCl, 2.5% Triton X-100, pH 7.5), and in reaction buffer (0.05 M Tris–HCl, 0.1 M NaCl, 0.01 M $CaCl_2$, pH 7.5) for 16 h at 37°C with gentle shaking. Zones of proteolysis were visualized following Coomassie blue staining and destaining (in methanol : acetic acid : water, 2 : 3 : 15).

Effect of inhibitors on protease activity

Inhibitors that were known to be specific to the different classes of proteases were used to determine the nature of the proteolytic activities produced by *P. micros* HG1262. The inhibitors tested, all obtained from Sigma Chemical Co., were: 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), ethylenediamine tetraacetic acid, 1,10-*o*-phenanthroline, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), iodoacetamide, and pepstatin A. Inhibitors were either added to the mixtures for the assays using *p*Na-labeled synthetic peptides and fluorescein-labeled proteins or incorporated into the reaction buffer when performing the zymography analysis. In this latter case, inhibition of proteolysis was estimated qualitatively after Coomassie blue staining, by comparison with a control gel developed in the absence of inhibitors.

Determination of plasminogen-binding activity

The plasminogen-binding activity of *P. micros* (strains HG1251, HG1262) was quantified by an enzyme-linked immunosorbent assay (ELISA). Bacterial suspensions (100 µl) were applied to a 96-well MaxiSorp Nunc Immuno Plate (Nalge Nunc International, Rochester, NY), which

was then covered and incubated overnight at 37°C. Unattached bacteria were then removed by liquid aspiration with a syringe (23G1 needle). Bacterial cells adhering to the bottoms of the wells were fixed by a 1-h treatment with 0.05% glutaraldehyde and the wells were washed three times with PBS containing 0.05% Tween-20 (PBST). The unreacted sites were then blocked for 1 h with PBS containing 3% gelatin. The solution was discarded and the wells were washed three more times with PBST before adding 100 µl human plasminogen (0.0294 U/ml; Sigma Chemical Co.). After a 1-h incubation at room temperature, wells were washed three times for 5 min with PBST. One hundred microliters of a goat anti-human plasminogen antibody (1/5000 dilution; Sigma Chemical Co.) in PBST–0.1% gelatin was added to each well for 1 h. After this, the wells were subjected to three–5 min washes with PBST before adding alkaline phosphatase-conjugated rabbit anti-goat immunoglobulin G antibody (1/30,000 dilution; Sigma Chemical Co.) in PBST–0.1% gelatin for 1 h. After washing the wells three times (5 min each) with PBST, the enzymatic reaction was performed by adding 100 µl *p*-nitrophenyl phosphate (1 mg/ml; Sigma Chemical Co.) in a sodium carbonate buffer (50 mM, pH 9.5). The plate was incubated for 30 min at 37°C, protected from the light, and the A_{405} was measured using a microplate reader. To determine the involvement of the plasminogen kringle domains in the interaction of the molecule with *P. micros*, the binding assay was carried out in the presence of the lysine analog ε-aminocaproic acid (10 mM).

Activation of bound plasminogen and determination of plasmin activity

The cell suspension (OD_{660} 0.45) of *P. micros* was incubated for 2 h with human plasminogen (0.15 U/ml) at 37°C with gentle shaking. Afterwards, bacteria were washed once in PBS and suspended in the same buffer before the addition of either streptokinase (25 U/ml; Sigma Chemical Co.) or urokinase (0.15 U/ml; Sigma Chemical Co.) as plasminogen activator. After a 1-h incubation at 37°C, cells were harvested by centrifugation (10,000 ***g*** for 15 min), washed twice with PBS to remove any residual plasminogen activator, and suspended in the initial volume of PBS. Bacteria (100 µl) were incubated for 4 h at 37°C with the chromogenic substrate for plasmin Val-Leu-Lys-*p*Na (20 µl of a solution at 2 mg/ml in PBS). Bacteria were then pelleted and the A_{405} of the supernatant was measured.

The capacity of *P. micros* strain HG1262 to acquire plasmin activity following co-incubation with human brain microvascular endothelial cells (BMEC) in culture was also investigated. Human BMEC, originating from a brain biopsy of an adult female with epilepsy, were kindly provided by Dr K. Kim (Johns Hopkins University School of Medicine, Baltimore, MD). These cells had been immortalized by transfection with simian virus 40 large T antigen (17) and were shown to maintain their morphological and functional characteristics. Cells were grown in RPMI-1640 medium (Hyclone, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co.), 10% Nu-serum supplement (BD Biosciences, Bedford, MA), penicillin (50 µg/ml), and streptomycin (50 µg/ml). Cultures were incubated at 37°C in a 5% CO_2 atmosphere. Cells were used before passage 35 for all experiments. After a 48 h culture in flasks, BMEC were trypsinized (0.5% for 3 min at 37°C), diluted in culture medium at 1.5×10^5 cells/ml, and 1 ml of this suspension was distributed into each well of a 24-well plate and incubated for 24 h to reach confluence. Medium was then removed and *P. micros* cells suspended in supplemented RPMI-1640 medium were added (1 ml) to give a ratio of 1000 bacteria per BMEC. Bacterial counts were determined in a Petroff–Hausser counting chamber. After 24 h of incubation, wells containing BMEC and *P. micros* were scraped and the suspension was centrifuged for 5 min at 200 ***g*** to remove BMEC. The supernatant was centrifuged for a further 20 min at 12,000 ***g*** to recover *P. micros*, which was then resuspended in the original volume of PBS. Plasmin activity associated with bacterial cells was determined as described above using the specific chromogenic peptide Val-Leu-Lys-*p*Na. A standard curve was constructed by incubating known concentrations of commercial plasmin with its specific substrate under the assay conditions for *P. micros* to estimate the quantity of plasmin activity attached to *P. micros*. The A_{415} values obtained with *P. micros* were compared to this curve to establish the number of bound plasmin units.

Penetration of a reconstituted basement membrane (Matrigel)

The *P. micros* HG1251 and HG1262 strains were radiolabeled by incubating bacterial cells suspended in Todd–Hewitt broth ($OD_{660} = 0.5$) with a mixture of ^{14}C-labeled amino acids (50 µCi/ml; Amersham Pharmacia Biotech, Baie d'Urfé, QC) at a final concentration of 10 µCi/ml at 37°C for 24 h in the anaerobic chamber. Cells were harvested by centrifugation at 10,000 ***g*** for 10 min, washed three times in PBS and resuspended in the original volume of PBS made oxygen-free by an overnight incubation in the anaerobic chamber.

Matrigel (Sigma Chemical Co.) is a solubilized basement membrane preparation containing several proteins, including laminin, type IV collagen, and proteoglycans. The Matrigel was diluted one-third in ice-cold PBS and 100 µl was placed on 8-µm filters in Transwell cell culture chamber inserts (Costar, Cambridge, MA). The Matrigel was allowed to settle at 4°C for 30 min and was then gelled at 37°C for 24 h in an anaerobic chamber. The Matrigel was rehydrated in 100 µl sterile oxygen-free PBS for 1 h at 37°C in the anaerobic chamber before proceeding with the penetration assay, while 200 µl PBS was placed in the lower chamber. Approximately 10^6 radiolabeled cells in 100 µl PBS were placed on top of the Matrigel in the double-chamber system, which was incubated in the anaerobic chamber at 37°C for 24 and 48 h. The penetration of *P. micros* cells through the Matrigel was evaluated by counting the radioactivity in the buffer recovered from the lower chamber. The effect of the serine protease inhibitor AEBSF (10 mM) on the migration of *P. micros* through the Matrigel was investigated by adding the inhibitor to the bacterial cell suspension 30 min before performing the penetration assay. The effect of treating cells at 60°C for 30 min to inactivate protease activity was also evaluated. To investigate the effect of plasmin-acquired activity on the ability of *P. micros* to migrate through Matrigel, cells incubated with human plasminogen and treated with streptokinase, as above, were used.

Statistical analysis

Data are presented as the means ± standard deviations (SD) of triplicate experiments. The statistical significance was analyzed using Student's *t*-test. Results were considered significant when the *P*-value was <0.01.

Results

Proteolytic activities

Among the four chromogenic synthetic peptides tested, only the peptide that was specific for chymotrypsin-like proteases

Table 1. Chymotrypsin-like activity of smooth and rough morphotypes of *Peptostreptococcus micros*

Strain	Hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*Na (A_{405})[1]	
	Cell suspension[2]	Secreted material[3]
Smooth morphotype		
HG1251	0.05 ± 0.02	0.04 ± 0.02
HG1253	0.12 ± 0.04	0.13 ± 0.03
HG1254	0.07 ± 0.04	0.07 ± 0.02
Rough morphotype		
HG1252	0.32 ± 0.04	0.14 ± 0.03
HG1259	0.21 ± 0.01	0.18 ± 0.03
HG1262	0.61 ± 0.04	0.33 ± 0.05

[1]Hydrolysis was determined by measuring the absorbance at 405 nm. Control values obtained in the absence of bacteria or secreted material were subtracted.
[2]Cell suspension was incubated with the substrate for 4 h.
[3]Secreted material was incubated with the substrate for 24 h.

was degraded. Overall, strains of the rough morphotype (HG1252, HG1259, HG1262) were found to be more active than those of the smooth morphotype (HG1251, HG1253, HG1254) (Table 1). Chymotrypsin-like activity was detected in both bacterial cell suspension and secreted material. The strain HG1262 was the most active whereas HG1251 showed the weakest activity. Degradation of fluorescein-labeled proteins was investigated using *P. micros* HG1262 and HG1251. As reported in Table 2, casein was strongly degraded by cell suspensions of each bacterial strain, although the rough morphotype strain was found to be more active. The cell suspension of strain HG1262, but not that of strain HG1251, showed a capacity to degrade type I collagen. Gelatin, type IV collagen, serum albumin, and elastin were not degraded, or were only weakly degraded, by *P. micros* cells. The secreted material of both strains was also capable of degrading casein. However, only the secreted material from the rough morphotype HG1262 could strongly degrade gelatin. The other substrates were poorly degraded or moderately degraded by the *P. micros*-secreted material.

The effect of inhibitors specific to each class of protease on the degradation of succinyl-Ala-Ala-Pro-Phe-*p*Na (chymotrypsin-like substrate), type I collagen, and casein by cells and secreted material of *P. micros* HG1262 is reported in Table 3. The serine protease inhibitor AEBSF inhibited all activities whereas inhibitors of metalloproteases and of aspartyl proteases had no effect. Inhibitors of cysteine proteases (TLCK and PCMP) reduced the cell-associated chymotrypsin-like and caseinase activities to various extents.

Proteolytic enzymes present in the cells of *P. micros* were analyzed by zymography using gelatin as the substrate. As shown in Fig. 1, three distinct proteolytic bands were detected in all three rough morphotype strains (HG1252, HG1259, HG1262), whereas no visible bands were detected in cell lysates from the smooth morphotype strains (HG1251, HG1253, HG1254). The molecular masses of the proteolytic bands were estimated as 165, 129, and 115 kDa. The addition of protease inhibitors during incubation of the zymogram revealed that TLCK and AEBSF completely inhibited the three gelatinase bands (data not shown). The other inhibitors had no effect on the intensity of the gelatinase bands.

Table 2. Degradation of fluorescein-labeled proteins by *Peptostreptococcus micros*

Protein	Proteolytic activity (RFU)[1]			
	Cell suspension[2]		Secreted material[3]	
	HG1251	HG1262	HG1251	HG1262
Bovine serum albumin	172 ± 30	321 ± 63	518 ± 19	494 ± 52
Casein	3911 ± 486	6031 ± 169	5315 ± 48	6701 ± 145
Gelatin	8 ± 8	19 ± 6	233 ± 27	4313 ± 58
Elastin	20 ± 12	20 ± 6	0	0
Type I collagen	12 ± 7	2585 ± 24	707 ± 145	630 ± 175
Type IV collagen	10 ± 2	326 ± 19	67 ± 12	114 ± 15

[1]Hydrolysis of the fluorescein-labeled substrates was determined by measuring the fluorescence (Relative Fluorescence Units; RFU) emitted at 520 nm following excitation at 490 nm. Control values obtained in the absence of bacteria or secreted material were subtracted.
[2]Cell suspensions were incubated with the substrate for 4 h.
[3]Secreted material was incubated with the substrate for 24 h.

Acquisition of plasmin activity

The binding of human plasminogen on the cell surface of *P. micros* smooth (HG1251, HG1253, HG1254) and rough (HG1252, HG1259) morphotypes was evaluated by ELISA. As shown in Fig. 2, all the strains bound plasminogen on their surface at a comparable level. Incorporation of the lysine analog ε-aminocaproic acid during incubation of bacterial cells with plasminogen caused complete inhibition of plasminogen attachment to *P. micros*, whereas heat treatment of the bacteria did not affect the plasminogen binding (data not shown).

The *P. micros* cells (HG1251 and HG1262) coated with plasminogen were subjected to various treatments to investigate the activation of plasminogen into plasmin. First, the secreted material of *P. micros* was tested and did not show any capacity to activate plasminogen (data not

Table 3. Effect of protease inhibitors on degradation of chymotrypsin-like substrate, type I collagen and casein by *Peptostreptococcus micros* HG1262

Class specificity	Inhibitor	Concentration	% Residual activity[1]					
			Ala-Ala-Pro-Phe-*p*Na		DQ-collagen type I		BODIPY FL-casein	
			Cell suspension	Secreted material	Cell suspension	Secreted material	Cell suspension	Secreted material
	None		100	100	100	100	100	100
Serine	AEBSF	4 mM	25.8 ± 2	25.8 ± 2	6.35 ± 1	36.35 ± 1	0.43 ± 1	34.1 ± 1
Metallo	EDTA	10 mM	100 ± 2	98.1 ± 2	98.9 ± 1	98.9 ± 1	99.8 ± 1	99.8 ± 1
	1,10-phenanthroline	10 mM	99.3 ± 1	99.3 ± 1	99.1 ± 1	99.1 ± 1	99.7 ± 2	99.7 ± 2
Cysteine	TLCK	100 μM	65.6 ± 4	95.6 ± 4	73.6 ± 1	93.6 ± 1	4.2 ± 1	94.2 ± 1
	PCMP	10 mM	62.2 ± 2	92.2 ± 2	98.6 ± 1	98.6 ± 1	4.87 ± 1	94.8 ± 1
Aspartyl	Pepstatin A	1 μM	98.4 ± 1	98.4 ± 1	99.3 ± 1	99.3 ± 1	99.7 ± 1	99.7 ± 1

[1]A value of 100% was given for the assay performed in the absence of inhibitor. Cell suspension and secreted material were incubated with the substrate for 4 and 24 h, respectively.

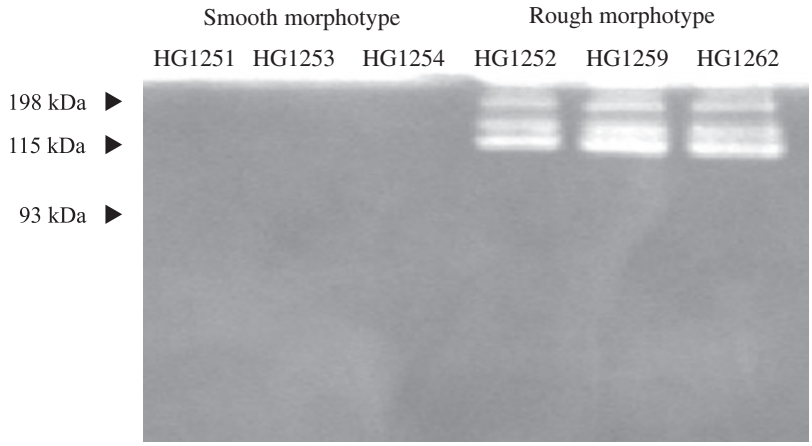

Fig. 1. Analysis of cell lysates of smooth and rough morphotypes of *Peptostreptococcus micros* by zymography on a gelatin-containing polyacrylamide gel.

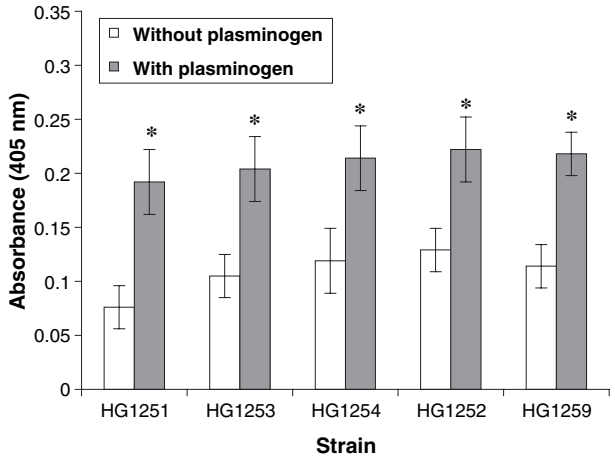

Fig. 2. Plasminogen-binding activity of smooth and rough morphotypes of *Peptostreptococcus micros* as determined by ELISA. Asterisk indicates a significant difference ($P < 0.01$) between plasminogen-treated cells and control untreated cells.

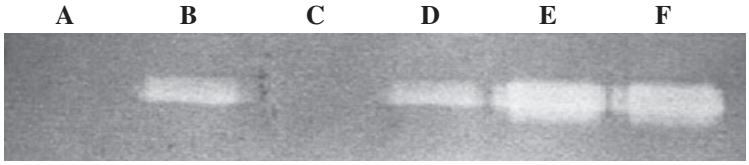

Fig. 3. Acquisition of plasmin activity by *Peptostreptococcus micros* HG1251 and HG1262 incubated with human plasminogen and treated with streptokinase as determined by zymography on a gelatin-containing polyacrylamide gel. Lane A, untreated HG1251; lane B, HG1251 incubated with plasminogen and treated with streptokinase; lane C, untreated HG1262; lane D, HG1262 incubated with plasminogen and treated with streptokinase; lane E, plasminogen treated with streptokinase; lane F, commercial plasmin.

shown). Thereafter, activation of human plasminogen bound to *P. micros* by streptokinase and urokinase was tested. Following these treatments, analysis by zymography using gelatin as a substrate revealed the appearance of a proteolytic band with a molecular mass of approximately 81 kDa corresponding to plasmin (Fig. 3). This gelatinase band was not detected when *P. micros* was covered with plasminogen but not treated with streptokinase or urokinase. Activation of the plasminogen bound to *P. micros* was confirmed by demonstrating hydrolysis of the specific chromogenic substrate for plasmin (data not shown).

Penetration of a reconstituted basement membrane

The invasive potential of *P. micros* coated, or not, with plasminogen activated into plasmin with streptokinase was evaluated by studying their migration through a reconstituted basement membrane (Matrigel). To monitor migration through the Matrigel, the cells were first radiolabeled by incubation in the presence of a mixture of ^{14}C-labeled amino acids. Cells were then deposited on the surface of the Matrigel and the radioactivity recovered in the lower well of the transwell chamber after 24 and 48 h of incubation reflected the migration of cells through the reconstituted basement membrane. Results presented in Fig. 4 show that the rough morphotype strain (HG1262) had a significantly greater capacity to penetrate the Matrigel than the smooth morphotype strain (HG1251) after both incubation periods. Observations of the content of the lower chamber by phase-contrast microscopy confirmed the presence of whole bacteria, more particularly for strain HG1262 (data not shown). Penetration of the reconstituted basement membrane by *P. micros* HG1262 was inhibited by incorporating AEBSF, an effective inhibitor of the proteases of *P. micros* as demonstrated above (Fig. 5). Treating cells at 60°C for 30 min was also associated with an inhibition of Matrigel penetration. Lastly, approximately twice as many ^{14}C-labeled *P. micros* HG1262 cells coated with plasmin activity as control cells penetrated the Matrigel after 24 and 48 h of incubation.

Discussion

Although it is well known that *P. micros* produces a large variety of aminopeptidases (4, 13), few studies have investigated the endopeptidase activities produced by this bacterium. Ng et al. (13) reported that 15 of 19 *P. micros* strains possess cell-associated gelatinase activity. More recently, Mikamo et al. (11) showed that nine of 18 strains of *P. micros* isolated from amniotic fluid linked to preterm premature rupture of membranes have elastase activity. However, no details on the above-mentioned proteolytic activities were reported. In this study, we report for the first time the production by *P. micros* of a chymotrypsin-like enzyme, which is both cell-bound and secreted. As for the chymotrypsin-like enzyme of *T. denticola*

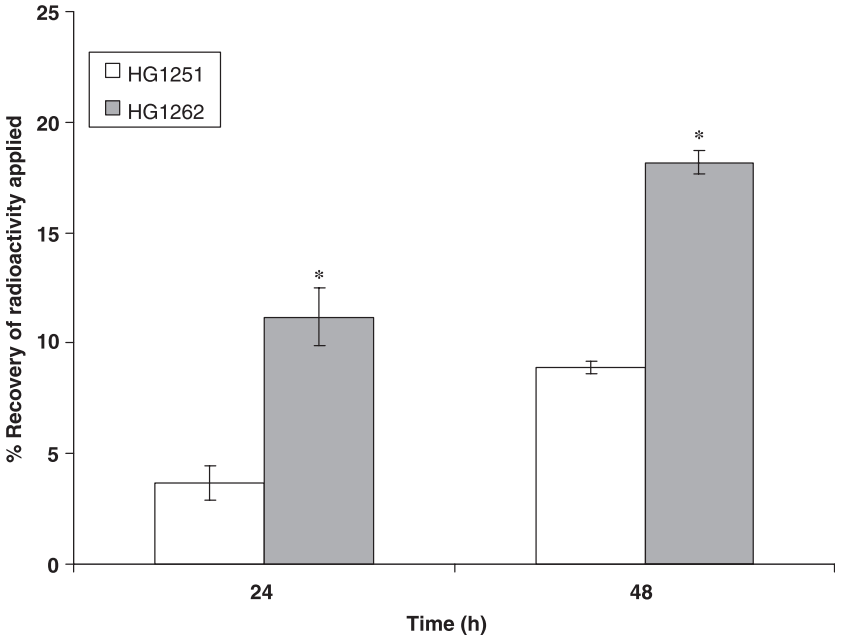

Fig. 4. Penetration of the Matrigel by *Peptostreptococcus micros* HG1251 and HG1262. Radioactivity in the lower chamber was measured with a scintillation counter after 24 and 48 h of incubation. The values represent the percentage recovery of initial radioactivity added to the upper chamber. Asterisk indicates a significant difference ($P < 0.01$) between strain HG1262 and HG1251.

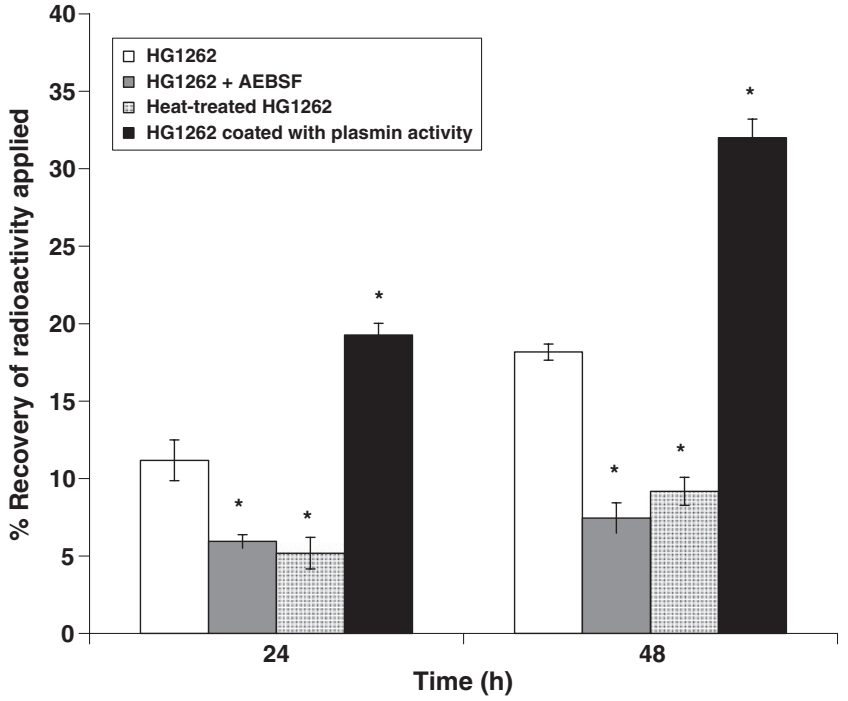

Fig. 5. Effect of various treatments of *Peptostreptococcus micros* HG1262 on its capacity to penetrate the Matrigel. Radioactivity in the lower chamber was measured with scintillation counter after 24 and 48 h of incubation. The values represent the percentage recovery of initial radioactivity added to the upper chamber. Asterisk indicates a significant difference ($P < 0.01$) compared to control untreated HG1262 cells.

(19), it belongs to the serine protease class. By zymography analysis, we confirmed the gelatinase activity previously reported in *P. micros* (13) and showed that this activity was restricted to the rough morphotypes. In addition, we showed that three enzyme species (165, 129, and 115 kDa) have the capacity to degrade gelatin. These three gelatinase bands were highly sensitive to the serine protease inhibitor AEBSF. Since we did not detect significant gelatinase activity using fluorescein-labeled gelatin as substrate, it suggests that zymography is a much more sensitive method by which to demonstrate gelatinase activity.

Few data are available concerning the differences in pathogenicity between the rough and smooth morphotypes of *P. micros*. When injected into a mouse model, the rough morphotype was reported to be more virulent, inducing the formation of more severe abscesses than the smooth morphotype (6). In this study, we showed that the rough morphotype strain, which expresses the three gelatinase bands and chymotrypsin-like activity, had a better capacity to penetrate an *in vitro* reconstituted basement membrane model (Matrigel). Using inhibitors of *P. micros* proteases, we obtained evidence for a direct contribution of these activities to the bacterial penetration through Matrigel. Further studies are required to investigate whether the higher proteolytic activity demonstrated by the rough morphotype strains may be associated with bacterial virulence in an animal model.

We also showed that both morphotypes of *P. micros* could bind human plasminogen on their cell surface. The inhibition caused by the lysine analog ε-aminocaproic acid suggests that the kringle domains found in plasminogen mediate the interactions of this molecule with the lysine residues present in cell-surface receptors. Once bound to *P. micros*, plasminogen activators of both bacterial (streptokinase) and human (urokinase) origin can activate plasminogen into plasmin. *F. nucleatum* subsp. *nucleatum* is another periodontopathogen that has been reported to acquire cell-associated plasmin activity (7). Our results also indicate that *P. micros*, when in close contact with BMEC, could acquire plasmin activity. In our assay, the plasminogen originated from fetal bovine serum added to the BMEC culture medium, whereas its activation into plasmin was mediated by plasminogen activators secreted by BMEC. Indeed, a zymography analysis revealed that the major plasminogen activator produced by the BMEC has a molecular mass of approximately 53 kDa, which is consistent with that reported for urokinase (data not shown).

In vivo, plasminogen bound to *P. micros* and subsequently activated into plasmin may interact with extracellular matrix

proteins. Using a basement membrane model (Matrigel), we present clear evidence that cells of *P. micros* coated with plasmin activity have a significantly greater tissue penetration capacity. These observations suggest that plasmin-acquired activity, in addition to endogenous proteolytic activity, may facilitate the dissemination of *P. micros* to surrounding periodontal tissues and blood vessels. This phenomenon may contribute to the capacity of this bacterial species to cause focal infections (12).

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