Oral Microbiology Immunology 2004: 19: 155–159
Printed in Denmark. All rights reserved

Oral Microbiology and Immunology

Identification of a *Fusobacterium nucleatum* 65 kDa serine protease

G. Bachrach, G. Rosen, M. Bellalou, R. Naor, M. N. Sela
Institute of Dental Sciences, The Hebrew University-Hadassah School of Dental Medicine, Jerusalem, Israel

Bachrach G, Rosen G, Bellalou M, Naor R, Sela MN. Identification of a Fusobacterium nucleatum *65 kDa serine protease.*
Oral Microbiol Immunol 2004: 19: 155–159. © Blackwell Munksgaard, 2004.

A 65 kDa protease was partially purified from extracellular vesicles of *Fusobacterium nucleatum* cultures by preparative SDS-PAGE followed by electroelution. The pH optimum of the protease is 7.5–8.0 and its activity could be inhibited by serine protease inhibitors. The protease was found to degrade the extracellular matrix proteins fibrinogen and fibronectin as well as collagen I and collagen IV which were degraded at 37°C but not at 28°C, indicating the presence of a gelatinase activity in these bacteria. The 65 kDa protease was also able to digest the α-chains of immunoglobulin A but not immunoglobulin G. The 65 kDa *F. nucleatum* protease, capable of degrading native proteins, may play an important role in both the nutrition and pathogenicity of these periodontal microorganisms. The degradation of extracellular matrix proteins by bacterial enzymes may contribute to the damage of periodontal tissues, and degradation of IgA may help the evasion of the immune system of the host by the bacteria.

Key words: extracellular matrix proteins; extracellular vesicles; *Fusobacteriuim nucleatum* proteases

Michael N. Sela, Hebrew University-Hadassah School of Dental Medicine, P.O. Box 12272, Jerusalem 911200, Israel
Tel.: +972 2 6758582; fax: +972 2 6438335;
e-mail: msela@cc.huji.ac.il
Accepted for publication November 14, 2003

Fusobacterium nucleatum is the most numerous gram-negative bacterium isolated from both healthy and diseased periodontal sites, and the most common periodontal pathogen involved in systemic diseases (12). By attaching to early colonizers of the dental plaque, fusobacteria enable late colonizers to coadhere and attach to the growing plaque. *In vitro*, fusobacteria were found to be capable of coaggregation with all the bacterial species which make up the dental plaque (10). Because of their extensive coaggregation ability, fusobacteria have been proposed to act as a bridge between early and late colonizing bacteria, assisting in the formation and accumulation of the dental plaque (9). A lack of systems for their genetic manipulation is among the reasons that little is known about the molecular mechanisms involved in the extensive coadhesion and in the virulence of these bacteria. Proteases are considered virulence factors employed by several periodontal pathogens including *Porphyromonas gingivalis* and *Treponema denticola*. While supplying the nutritional requirements of these oral microorganisms, proteases were found to degrade elements of the periodontal connective tissue and the host defense systems such as immunoglobulins and complement (1, 7). Proteases can also inactivate key components of the plasma proteinase cascade (14) and blood clotting systems (6), and degrade serum proteinase inhibitors (13). These enzymes, found both in the bacterial cell and their extracellular vesicles may contribute to bacterial survival in the antagonistic host environment, while inflicting damage to the host. With the exception of one metallo amino peptidase (17), no proteases have been described for *F. nucleatum*. The aim of this report was the identification and characterization of an *F. nucleatum* protease capable of degrading native proteins.

Materials and methods

Bacteria and growth conditions

F. nucleatum ATCC 10953, FDC 364 and PK 1594 were a gift from Dr Kollenbrander (NIH, Bethesda, MD). Strain ATCC 23726 was the kind gift of Dr. S. K. Haake (UCLA, Los Angeles, CA). The bacteria were grown in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) in Brain Heart Infusion, containing 0.25% glutamate in an atmosphere of 85% N-10% H-5% CO_2 at 37°C. Bacterial purity was determined by phase contrast microscopy and Gram staining.

Isolation of the *F. nucleatum* protease

The 65 kDa proteolytic activity was followed daily for 1 week in extracellular culture supernatants. Maximal activity was reached after 4 days. Four-day-old *F. nucleatum* FDC 364, PK 1594 and

ATCC 10953 cultures were harvested by centrifugation at 9000 ***g*** for 20 min. The cells were washed 3× with phosphate-buffered saline and further extracted with the detergent Triton X-114 as previously described (18). Briefly, cells were extracted overnight at 4°C with 2% Triton X-114 in Tris-buffered saline (100 mM Tris, 150 mM NaCl, pH 7.4). The insoluble material was removed by centrifugation at 30,000 ***g*** for 45 min at the same temperature. The supernatant was phase separated by warming for 10 min at 37°C and then centrifuged at 13,000 ***g*** for 10 min. The separated detergent and aqueous phases were washed four times as described previously (18). Before determination of enzymatic activities the Triton X-114 phase fraction was adjusted to a final detergent concentration of 1% and submitted for 2 h to Bio Beads SM-2 hydrophobic (Bio Rad, Richmond, CA) as previously described (5) to remove the detergent.

Isolation of the 65 kDa *F. nucleatum* FDC 364 protease from the extracellular culture supernatant

Four-day-old *F. nucleatum* FDC 364 cultures were harvested by centrifugation at 10,000 ***g*** for 20 min. The bacterial pellet was discarded, and the supernatant was filtered through a 0.2 μm filter. Supernatant (500 ml) was concentrated using an Amicon 100 kDa cutoff membrane to a final volume of 25 ml. To the concentrated supernatant was added 100 ml of 20 mM Tris-HCl pH 7.5, followed by reconcentration to a final volume of 25 ml. The supernatant was further concentrated with a Centriplus device (cut off 100 kDa) to a final volume of 14 ml. The concentrated supernatant was centrifuged at 100,000 ***g*** for 2 h. The supernatant was discarded, and the precipitate containing the extracellular vesicles was washed twice with 50 mM Tris-HCl (pH 7.8) by centrifugation as described above. The vesicles were examined by electron microscopy or subjected to electroelution after separation by SDS-PAGE for the purification of the protease.

Electrophoresis

Sodium dodecylsulfate-polyacryamide gel electrophoresis (SDS-PAGE) was carried out with a Mini Protean I (0.075-cm-thick; Bio-Rad Laboratory). Gels contained 240 μg human fibrinogen, gelatin or casein (Sigma, St Louis, MO) per ml. After electrophoresis, the gels were incubated for 30 min at room temperature in Tris-buffered saline (0.05 M Tris-HCl [pH 7.8], 0.1 M NaCl), containing 2% Triton X-100 and then washed three times with Tris-buffered saline. Gels were incubated overnight at 37°C. Bands of activity were revealed after staining with Coomassie brilliant blue R-250. Proteolytic activity was visualized as a clear band against a blue background.

Gels without the protein substrate were stained with Coomassie blue. Samples were either dissolved at room temperature or heated at 100°C for 5 min in sample buffer (192 mM Tris-HCl [pH 6.8] , 30% glycerol, 9% SDS). Where stated, 15% β-mercaptoethanol was added to the sample buffer. Molecular masses of protein bands were calculated by linear regression analysis of molecular mass standards.

Purification of the 65 kDa protease

The extracellular vesicles were dissolved in sample buffer (without β-mercaptoethanol, see above), centrifuged for 2 min at 10,000 ***g*** and subjected to electroelution after separation by SDS-PAGE (7.5% acrylamide). The protease was electroeluted from the gel using a Bio Trap 1000 electroeluter (Schleicher and Schuell, Germany) with Tris-glycine buffer (25–192 mM) without SDS for 2 h at 200 volts followed by 10 h at 100 volts as described before (19). The enzyme was stored at –20°C and remained active for at least 6 months.

Enzymatic activity

The enzymatic activity of the detergent phase proteins containing the 65 kDa protease was determined with a variety of synthetic chromogenic substrates: *N*-α-benzoyl-L-Arg-*p*-nitroanilide (pNA), *N*-succinyl-Ala-Ala-Pro-Phe-pNA, Ala-pNA, Leu- pNA, *N*-*p*-tosyl-Gly-Pro-Lys-pNA, Pro-pNA, *N*-acetyl-Tyr-Val-Ala-Asp-pNA, L-Ile-β-naphthylamide, L-Trp-β-naphthylamide, L-Ser-β-naphthylamide, L-His-β-naphthylamide and L-Tyr-β-naphthylamide. Hydrolysis of the pNA and naphthylamide derivatives was performed with 1.5 mM substrates and 1-5 μg/ml of the *F. nucleatum* detergent phase proteins in Tris-buffered saline (pH 8.0) at 30°C. The appearance of pNA was followed spectrophotometrically by recording the change in optical density at 405 nm. Hydrolysis of the naphthylamide derivatives was followed as previously described (11). *T. denticola* cells, *P. gingivalis* cells or human sera were used as positive controls.

The proteolytic activity of the 65 kDa protease was followed by densitometric measurement of zymograms with a Bio-imaging system 202D (Pharmacia Biotech, New Jersey) Liscap capture aplication program (Tina, invertedTiff).

Degradation of extracellular matrix proteins collagen IV (human placenta), collagen I (rat tail), fibronectin (bovine plasma), human fibrinogen, IgA and IgG was monitored by analyzing the degradation products by SDS-PAGE. The reaction mixtures contained 100 μg of protein substrate and 0.25 μg of the purified enzyme in 30 μl of Tris-buffered saline (pH 8.0). The hydrolysis was carried out for 14 h at either 28 °C or 37 °C. Samples were heated at 100 °C for 3 min in sample buffer containing 15% β-mercaptoethanol.

Effect of inhibitors, pH and temperature

The effect of a number of protease inhibitors, optimal pH and temperature on the activity of the 65 kDa protease was investigated. Digestion of fibrinogen under the different experimental conditions was followed by densitometric measurement of zymograms as described above.

Electron microscopy

Transmission electron microscopy was performed using carbon-collodium coated grids and negative staining with 1% phosphotungstic acid.

Results

The proteolytic pattern of *F. nucleatum* whole cells on zymograms containing fibrinogen as the protein substrate is shown in Fig. 1 (lane b). One proteolytic band with a molecular mass of 65 kDa was present both in the cells or secreted to the extracellular media in all *F. nucleatum* strains tested: FDC 364, PK 1594, ATCC 10953 and ATCC 23726. The same proteolytic pattern was observed when gelatin or casein replaced fibrinogen as the protein substrate (not shown). In order to investigate whether the protease is free or vesicle-bound in the extracellular supernatant, extracellular vesicles were prepared and submitted to electron microscopy. Negatively stained electron micrographs (Fig. 2A) showed the presence of extracellular vesicles protruding from the outer membrane of *F. nucleatum* cells. The sedimented material obtained by ultracentrifugation of the concentrated supernatants (see Materials and methods) revealed vesicles 50–100 nm in diameter (Fig. 2B). Zymograms of these vesicles showed the presence of the 65 kDa protease (not shown). The isolation of the 65 kDa

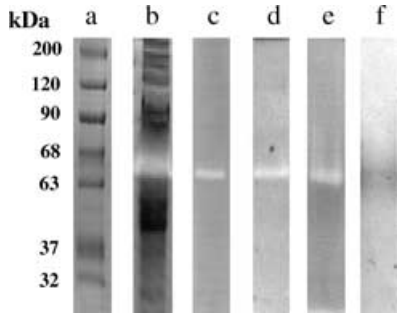

Fig. 1. F. nucleatum FDC 364 protein and protease profiles. Protease profiles of *F. nucleatum* on fibrinogen containing gels: a, molecular weight standard. b, whole cells. c, detergent phase. d, purified protease. e, detergent phase on gelatin containing gel. f, protein profile of the purified protease, unheated sample.

Table 1. Effect of inhibitors on the 65 kDa protease activity

Type of inhibitor	Agent	Concentration (mM)	Remaining activity (%)
Serine protease	PMSF	2	2
	Phenylboronic acid	5	40
Metalloprotease	EDTA	5	100
	$MgCl_2$	1	100
	$CaCl_2$	1	100
	$ZnCl_2$	1	2
Carboxyl protease	Pepstatin	50 μg/ml	100
Thiol protease	β-mercaptoethanol	5 mM	100
	Cysteine	5 mM	100
	N-ethylmaleimide	1.25	80
	Iodoacetamide	2	100

protease was first pursued by extraction of the cells with the non ionic detergent Triton X-114. The 65 kDa protease partitions into the detergent phase, as shown by zymograms of the detergent phase using fibrinogen or gelatin as substrate (Fig. 1, lanes c and e). Zymographic profiles of *F. nucleatum* extracellular supernatants as well as the detergent phase obtained from different preparations, and from the purified 65 kDa protease, occasionally revealed an additional proteolytic band of 130 kDa probably originating from dimerization of the 65 kDa protease (not shown). The specific activity associated with the 65 kDa protease in the extracellular vesicles prepared from 4-day-old cultures, was twice as high as that obtained from the detergent phase extracted from the cells by Triton X-114 and phase separation, as determined by densitometric measurement of zymograms. The protease was thus partially purified from the extracellular vesicles by electroelution after separation by preparative SDS-PAGE. By this procedure, the enzyme was purified about 15-fold over the concentrated supernatant. The recovery of activity being 12%. Zymogram and SDS-PAGE of the partially purified enzyme are shown in Fig. 1 (lanes d and f). The molecular size of the native enzyme is 65 kDa. The same protein pattern was obtained after heating the protease in the presence or absence of a reducing agent (not shown).

The optimal pH for proteolysis was 7.5–8.0 and the protease was inactivated at pH 4.5.

In order to determine the substrate specificity of the 65 kDa protease, the proteolytic activity of the detergent phase fraction enriched with the protease was tested with a number of synthetic substrates (see Materials and methods). The detergent phase fraction could not hydrolyze any of the synthetic substrates tested.

The effect of various proteinase inhibitors on the protease activity was examined (Table 1). The serine protease inhibitors PMSF and phenyl boronic acid strongly inhibited the 65 kDa protease, while the protease was not activated by sulfhydryl group reagents cysteine and β-mercaptoethanol and was not inhibited by the thiol reagents N-ethylmaleimide and iodoacetamide. The enzyme activity was also unaffected by EDTA, Ca^{+2} and Mg^{+2}, while it was strongly inhibited by Zn. Pepstatin, a carboxyl protease inhibitor, did not affect the activity of the protease.

The ability of the 65 kDa protease to degrade extracellular matrix proteins was evaluated. The protease cleaved fibrinogen (Fig. 1) and fibronectin into small degradation products that were visualized on the gel (Fig. 3A). Partial degradation of the α- and β-chains of collagen I was observed at 37°C (Fig. 3B) but not at 28°C (not shown). Partial degradation of the α-chains of collagen IV at 37°C was also observed (Fig. 3C). These data together with zymograms using bovine gelatin as substrate (Fig. 1e) demonstrate the gelatinase activity of the 65 kDa protease. The 65 kDa protease was also found to degrade both α-chains of the IgA molecule (Fig. 3D), whereas degradation of IgG by the purified enzyme could not be detected (not shown).

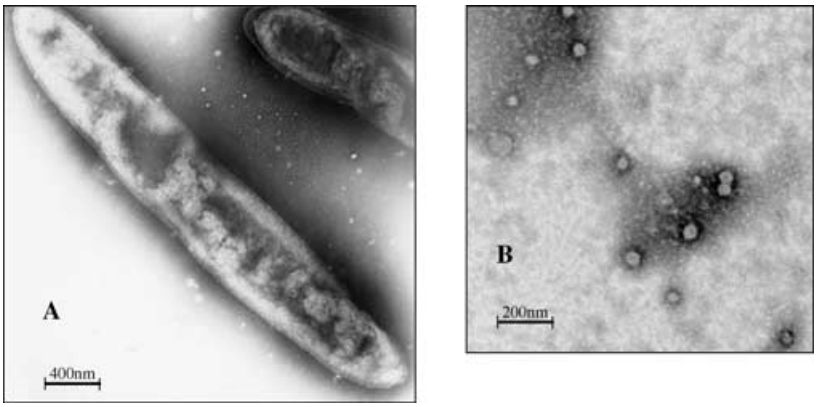

Fig. 2. Electron micrographs of *F. nucleatum* FDC 364: A, Negative staining of *F. nucleatum* (note the vesicles bound to the surface). B, Negative staining of an isolated vesicle preparation.

Discussion

This report describes the identification and characterization of an *F. nucleatum* 65 kDa serine protease capable of degrading extracellular matrix proteins and immunoglobulin A. These results disagree with earlier studies that suggested a lack of endopeptidase activity in these oral bacteria (17). Nevertheless, in comparison with the proteolytic activity of other periodontal bacteria, the specific activity of the 65 kDa protease is very low. The purified *F. nucleatum* 65 kDa protease required overnight incubation with the zymogram substrate to reach the same proteolytic zymographic intensity (per 0.2 μg enzyme, as measured by densitometric analysis) obtained by the *Treponema denticola* phenylalanine protease (19) after 2 h. The fact that *F. nucleatum* coaggregates, and is most often found

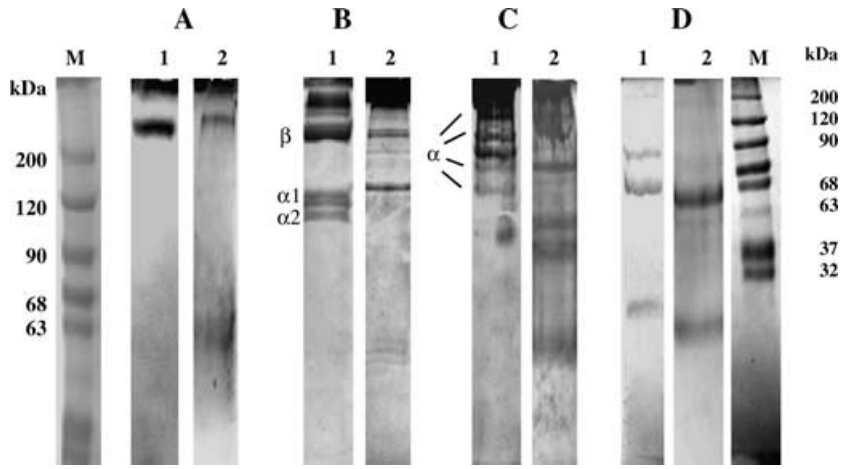

Fig. 3. Degradation of extracellular matrix proteins and immunoglobulin A by the purified 65 kDa protease. Lane A, fibronectin. Lane B, collagen I. Lane C, collagen IV. Lane D, immunoglobulin A. M: molecular weight standard. 1: proteins incubated without the enzyme at 37°C. 2: protein incubated with enzyme at 37°C. Numbers at the sides are molecular masses in kilodaltons.

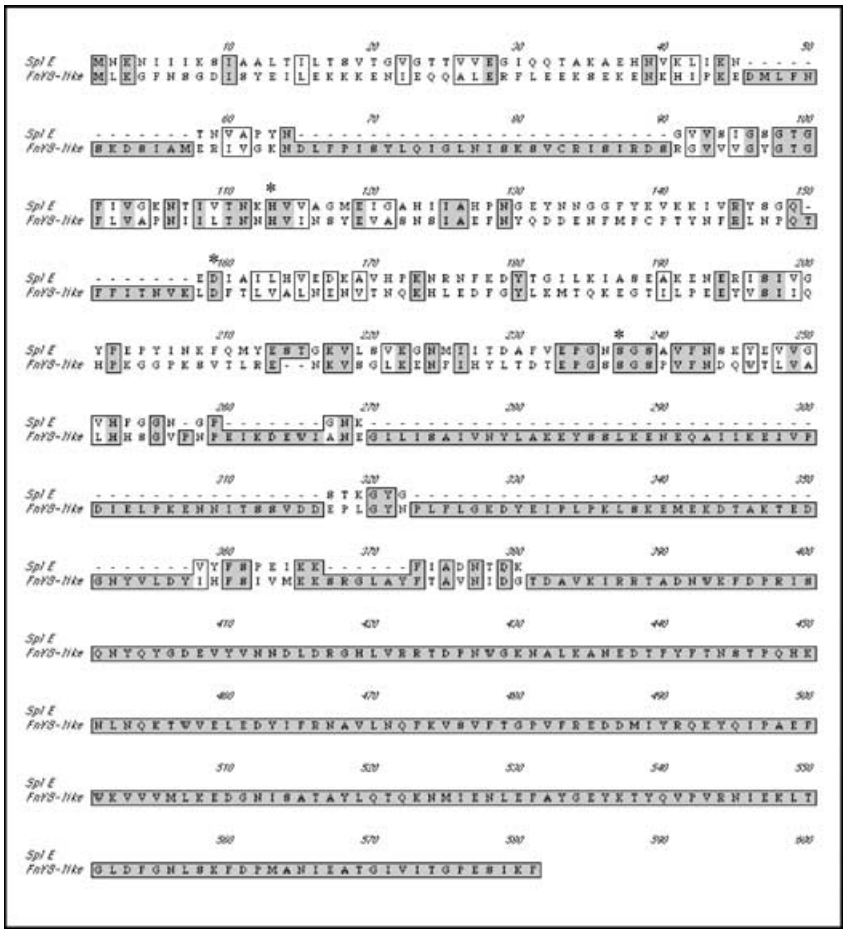

Fig. 4. Sequence alignment of SplE and the *F. nucleatum* putative V8 serine protease like protein. Asterisk indicates the SplE catalytic triad.

together with other highly proteolytic microorganisms, might reduce the necessity for *F. nucleatum* to possess a strong proteolytic activity.

The strong inhibition produced with PMSF and phenylboronic acid together with the lack of activation by thiol group reagents or inhibition by the cysteine protease inhibitors *N*-ethylmaleimide and iodoacetic acid, suggest that the 65 kDa is a serine protease. Nevertheless, the fact that zinc is a strong inhibitor of the protease, indicates a critical role for free-SH groups in the active enzyme.

F. nucleatum extracellular vesicles were reported to coaggregate with *P. gingivalis* cells (8). In this study we found proteolytic activity associated with *F. nucleatum* vesicles, similar to the proteolytic vesicles reported in two other periodontopatic gram-negative bacteria – *T. denticola* (19) and *P. gingivalis* (4). These extracellular vesicles may therefore provide the microorganisms with an effective pathogenic mechanism for the damage of periodontal tissues.

F. nucleatum was found to grow well on peptide-based medium (16, 20) or chemically defined medium provided with the four key energy-yield amino acids, Glu, His, Ser and Lys (3). These nutritional requirements point to the importance of *F. nucleatum* proteolytic activities such as the surface *F. nucleatum* aminopeptidase (17) or the 65 kDa protease described in the present work, in meeting the metabolic requirements of these bacteria. *F. nucleatum* was also found to have the capacity to bind plasminogen on its surface, which may be activated to plasmin (2), a plasma serine protease.

The Protein Extraction and Description Analysis Tool (Munich Information Center for Protein Sequences) was used to search the *F. nucleatum* ATCC 25586 genomic sequence for an open reading frame that might code for the 65 kDa serine protease. A gene coding for a putative 66 kDa serine protease belonging to the V8 family was found (GeneBank accession number NP_604177). Aligning the *F. nucleatum* 66 kDa putative protein sequence with that of the 26 kDa *Staphylococcus aureus* Serine Protease Like E protease (SplE, Fig. 4) (15) identified the conserved protease V8 catalytic triad (His-113, Asp-160 and Ser-240, putative *F. nucleatum* putative serine protease numbering). These bioinformatics results indicate that *F. nucleatum* is capable of expressing serine proteases. Closer observation of the *F. nucleatum* 66 kDa putative protein sequence revealed that amino acids 318–579 (C-terminus domain) are quite homologous to several DNA/RNA nucleases. Whether this gene generates a single 66 kDa product with serine protease activity, or codes for two products – a 27 kDa serine protease of the V8 family and a nuclease of about 30 kDa – remains to be determined.

Acknowledgment

This work was supported by the United States-Israel Binational Science Foundation, grant number 1991b.

References

1. Abe N, Kadowaki T, Okamoto K, Nakayama K, Ohishi M, Yamamoto K. Biochemical and functional properties of lysine-specific cysteine proteinase (Lys-gingipain) as a virulence factor of *Porphyromonas gingivalis* in periodontal disease. J Biochem 1998: **123**: 305–312.
2. Darenfed H, Grenier D, Mayrand D. Acquisition of plasmin activity by *Fusobacterium nucleatum* subsp. nucleatum and potential contribution to tissue destruction during periodontitis. Infect Immun 1999: **67**: 6439–6444.
3. Dzink JL, Socransky SS. Amino acid utilization by *Fusobacterium nucleatum* grown in a chemically defined medium. Oral Microbiol Immunol 1990: **5**: 172–174.
4. Grenier D, Maryand D. Functional characterization of extracellular vesicles produced by *Bacteroides gingivalis*. Infect Immun 1987: **55**: 111–117.
5. Holloway PW. A simple procedure for removal of Triton X-100 from protein samples. Anal Biochem 1973: **53**: 304–308.
6. Imamura T, Potempa J, Pike RN, Moore JN, Barton MH, Travis J. Effect of free and vesicle-bound cysteine proteinases of *Porphyromonas gingivalis* on plasma clot formation-implications for bleeding tendency at periodontitis sites. Infect Immun 1995: **63**: 4877–4882.
7. Kadowaki T, Yoneda M, Okamoto K, Maeda K, Yamamoto K. Purification and characterization of a novel arginine-specific cysteine proteinase (argingipain) involved in the pathogenesis of periodontal disease from culture supernatant of *Porphyromonas gingivalis*. J Biol Chem 1994: **269**: 21371–21378.
8. Kinder SA, Holt SC. Localization of the *Fusobacterium nucleatum* T18 adhesin activity mediating coaggregation with *Porphyromonas gingivalis* T22. J Bacteriol 1993: **175**: 840–850.
9. Kolenbrander PE, London J. Adhere today, here tomorrow: oral bacterial adherence. J Bacteriol 1993: **175**: 3247–3252.
10. Kolenbrander PE, Andersen RN, Moore VH. Coaggregation of *Fusobacterium nucleatum, Selenomonas flueggei, Selenomonas infelix, Selenomonas noxia,* and *Selenomonas sputigena* with strains from 11 genera of oral bacteria. Infect Immun 1989: **57**: 3194–3203.
11. Makinen KK, Makinen PL. Purification and characterization of two human erythrocyte arylamidases preferentially hydrolysing *N*-terminal arginine or lysine residues. Biochem J 1978: **175**: 1051–1057.
12. Moore WE, Moore LV. The bacteria of periodontal diseases. Periodontol 2000 1994: **5**: 66–77.
13. Nelson D, Potempa J, Kodula T, Travis J. Purification and characterization of a novel cysteine proteinase (periodontain) from *Porphyromonas gingivalis.* Evidence for a role in the inactivation of human α-proteinase inhibitor. J Biol Chem 1999: **274**: 12245–12251.
14. Nilsson T, Carlsson J, Sundqvist G. Inactivation of key factors of the plasma proteinase cascade systems by *Bacteroides gingivalis*. Infect Immun 1985: **50**: 467–471.
15. Reed SB, Wesson CA, Liou LE, Trumble WR, Schlievert PM, Bohach GA, Bayles KW. Molecular characterization of a novel *Staphylococcus aureus* serine protease operon. Infect Immun 2001: **69**: 1521–1527.
16. Rogers AH, Gully NJ, Pfennig AL, Zilm PS. The breakdown and utilization of peptides by strains of *Fusobacterium nucleatum*. Oral Microbiol Immunol 1992: **7**: 299–303.
17. Rogers AH, Gunadi A, Gully NJ, Zilm PS. An aminopeptidase nutritionally important to *Fusobacterium nucleatum*. Microbiology 1998: **144**: 1807–1813.
18. Rosen G, Naor R, Kutner S, Sela MN. Characterization of fibrinolytic activities of *Treponema denticola*. Infect Immun 1994: **62**: 1749–1754.
19. Rosen G, Naor R, Rahamim E, Yishai R, Sela MN. Proteases of *Treponema denticola* outer sheath and extracellular vesicles. Infect Immun 1995: **63**: 3973–3979.
20. Shah HN, Gharbia SE, Zhang MIN. Measurement of electrical bioimpedance for studying utilization of amino acids and peptides by *Porphyromonas gingivalis, Fusobacterium nucleatum,* and *Treponema denticola*. Clin Infect Dis 1993: **16**: 404–407.