Biochemical comparison of proteolytic enzymes present in rough- and smooth-surfaced capnocytophagas isolated from the subgingival plaque of periodontitis patients

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Four rough-surfaced (R) and three smooth-surfaced (S) clinical isolates of *Capnocytophaga* obtained from the subgingival plaque of periodontitis patients were studied for their peptidase and protease profiles. The results were compared with those obtained with *C. gingivalis* (which has a smooth morphology). All cell extracts obtained by ultrasonic treatment displayed high peptidase activity toward *N*-aminoacyl-2-naphthylamines, the best substrates being the arginyl, aspartyl, and leucyl derivatives. The R and S isolates did not differ in these enzyme activities. Also the protease profiles studies with 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine (PZ-PLPGA) and casein were similar. All extracts also hydrolyzed furylacryloyl-L-leucylglycyl-L-prolyl-L-alanine (FALGPA), reconstituted type I [3H]-collagen, and gelatin. *Na*-Benzoyl-DL-arginyl-2-naphthylamine was hydrolyzed faster by the R than the S strains. Comparison between cell suspensions and cell extracts of *C. gingivalis* showed the suspensions to be enzymatically more active than the extracts. In general, peptidase substrates and PZ-PLGPA were hydrolyzed at a higher rate by suspensions than by extracts, while protease substrates (such as casein) were hydrolyzed faster by the extracts. Gelatin and FALGPA were hydrolyzed by cell extracts only. Fast protein liquid chromatography of peptidases on a gel column was found to be a suitable method to differentiate between R and S isolates in diagnostics, while the chromatographic profiles of proteases were not suitable for this purpose.

Key words: periodontitis – proteolytic enzymes – capnocytophaga

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Introduction

Specific Gram-negative bacteria appear to play an important role in the etiology and pathogenesis of human periodontal disease (6, 18, 20). One of the bacterial species with evidence of periodontopathic potential is *Capnocytophaga*. Various capnocytophagas have been implicated in advanced periodontitis; for example, in juvenile diabetics (11). The capnocytophagas appear to be able to invade periodontal tissues (16) and these organisms possess several mechanisms for the destruction of tissues. Capnocytophagas degrade IgA and IgG (8), they produce aminopeptidases (9, 15, 19, 22), acid and alkaline phosphatases (9, 19, 22) and trypsin-like enzymes (9, 19). In a recent study, cell extracts of *C. ochracea* showed weak to moderate enzyme activity towards type IV collagen or fironectin (23). Capnocytophagas have also been reported to inhibit fibroblasts, alter PMN function (24) and activate polyclonal B-cells (2).

In a comparative study, *C. gingivalis, C. ochracea, C. sputigena* and a group of rough-surfaced capnocytophagas showed distinctive aminopeptidase-like activities but differed in their glycosidatic capabilities (9). The rough-surfaced capnocytopha-

gas differed also from the above type strains of *Capnocytophaga* in showing trypsin-like activity (9). It has to be stated, however, that the number and type of peptidolytic enzymes present in smooth- and rough-surfaced capnocytophagas are scantly known. The aim of the present study was to apply biochemical methods to further investigate the peptidase and protease "arsenal" of rough- and smooth-surfaced clinical isolates of *Capnocytophaga*, using *C. gingivalis* (which forms smooth-surfaced colonies) as a reference organism.

Material and methods

Source and treatment of *Capnocytophaga* strains

Seven clinical isolates of *Capnocytophaga*, four growing as spreading, rough-surfaced adherent colonies (R1, R2, R4, R5) and three growing as smooth-surfaced colonies (S2, S3, S4) on Trypticase soy agar-blood agar plates (9), were used in this study. These strains were isolated from the subgingival plaque of periodontitis patients. All R and S isolates were obtained from different patients. The reference strain, *C. gingivalis* S27 (ATCC 33624), was kindly provided by Dr. A. Tanner of the Forsyth Dental Center, Boston, MA. The cells were grown anaerobically for 4 days at 37°C in Schaedler broth (18) to the stationary phase. The cells were harvested by centrifugation (12 100 *g*, 10 min, 4°C) and washed three times with 0.1 M Tris-buffer, pH 7, containing 0.1 mM $CaCl_2$.

For experiments with whole cell suspensions the washed cell pellets were suspended in 0.1 M Tris-buffer, pH 7, containing 1 mM $CaCl_2$, to form cell suspensions with a turbidity of $A_{600} = 1.0$. The suspensions were immediately subjected to enzyme assays. For the preparation of cell extracts, the washed cell pellets were suspended in 0.1 M Tris-buffer, pH 7, containing 0.1 $CaCl_2$(the cell concentration was 30-fold compared to the original growth medium) and treated for 6 min with a Sonifier Cell Disrupter (Model W 1850D; Branson Sonic Power Company, Plainview, N.Y., USA) at setting 7 (at 4°C). The mixtures were finally centrifuged (12 100 *g*, 10 min, 4°C). The degree of cell lysis was 80–90% as determined by phase contrast microscopy.

Enzyme chromatography

The chromatographic separations were performed with a complete Pharmacia fast protein liquid chromatograph (FPLC; Pharmacia, Uppsala, Sweden) which was operated at 22°C using a Superose 12 HR10/30 gel column and a Mono-Q HR5/5 anion exchange column. The samples (50–300 µl) injected into the system were first treated with Millipore filters (Millex HA 0.45 µm or HA 0.22 µm). The FPLC programs have been described elsewhere (13, 14). The protein was monitored at either 280 mm or 214 mm.

Enzyme and protein assays

Amiopeptidase activity with 2-naphthylamine (2NA) derivatives of amino acids and trypsin-like activity with *Na*-benzoyl-DL-arginine-2NA (BANA) as substrates were determined using an azacoupling method (12). Endopeptidase ("collagenolytic") activity was studied using 4-phenylazobenzyloxycarbonyl-L-prolyl-L-leycylglycyl-L-prolyl-D-arginine (PZ-PLGPA; final concentration 1 mM) and furylacryloyl-L-leucylglycyl-L-prolyl-L-alanine (FALGPA; 1 mM) as substrates (25, 26). Proteolytic activity was studied using as substrates soluble collagen (type I from human placenta), gelatin (Baker & Adamson, Morristown, NJ, USA), hemoglobin (Pierce, Rotterdam, The Netherlands), casein, bovine serum albumin, and Polypep collagen polypeptides [high viscosity (HV) and low viscosity (LV); final concentration 1 mg/ml]. The assays were performed in 20 mM Tris-HCl buffer (pH 8.0, 1 mM $CaCl_2$), for 4–6 h at 37°C. The hydrolysis products were assayed with the Cd-ninhydrin method of Doi *et al.* (5). The collagenase assay involving reconstituted type I [3H]-collagen from rat tail tendon was performed according to the manufacturer's (New England Nuclear, Boston, MA, USA) instructions at 21°C using an incubation time of 18 h. Ten-µl aliquots of the clear incubation mixtures were subjected to liquid scintillation counting to determine the degradation of collagen. The rest of the mixtures were freeze-dried and subjected to SDS-PAGE and autoradiography as described below. Unless otherwise indicated, all reagents were from Sigma Chemical Co. (St. Louis, MO, USA). The protein concentrations were assayed with the Bio-Rad Protein Assay system (4).

Electrophoresis and autoradiography

The protein composition of the filtered cell extracts was studied using SDS-PAGE (PhastSystem®, Pharmacia; PhastGel® gradient 10–15%) and silver staining according to the manufacturer's instructions. The SDS-PAGE molecular weight standards were from Sigma. The electrophoretograms of [3H]-collagen were subjected to autoradiography. The PhastGels were treated with EN^{3}HANCE™ autoradiography enhancer (New England Nuclear, Dreieich, West Germany), air-dried overnight and

exposed to preflashed (7) Kodak XAR-5 film for 4–5 days at −76°C.

Results

Yield of enzymes

The amount of cell-bound enzyme activity not solubilized by the sonication procedure was studied using cell suspension ($A_{660}=1.0$) of the rough-surfaced *Capnocytophaga* isolate R5 and *C. gingivalis*, and employing PZ-PLGPA, casein, collagen, gelatin, and HV-Polypep as substrates. The 12 000 *g* supernatants of the cell extracts contained 70–90% of the original enzyme activities toward the above substrates. Ultracentrifugation (100 000 *g*, 1 h, + 4°C) of these supernatants reduced the enzyme activities of the supernatants additionally by 10–30%. None of the above enzyme activities was, however, affected by the centrifugation treatments. The yield of the enzymes was increased considerably by addition of NaCl (final concentration 0.5 M) to the cell extracts before centrifugation.

Substrate specificity

The filtered cell extracts from all capnocytophagas displayed broad peptidase activity with arginyl-2NA, aspartyl-2NA, and leucyl-2NA being the best arylamidase substrates (Table 1). The extracts displayed weak or no activity towards prolyl-2NA, cystinyl-2-NA, valyl-2NA, threonyl-2NA, and isoleucyl-2NA. On the whole, the peptidase profiles of the cell extracts from rough and smooth isolates were relatively similar, except in the hydrolysis of aspartyl-2NA which was hydrolyzed much faster by extracts from the rough isolates.

When tested with various endopeptidase and protease substrates, the substrate specificities and specific enzyme activities of the extracts from most rough and smooth capnocytophagas were similar. The synthetic bacterial collagenase substrate PZ-PLGPA was rapidly hydrolyzed by all strains (Table 2). The rough capnocytophagas R1 and R5 and the smooth isolate S2 as well as *C. gingivalis* hydrolyzed casein effectively. The smooth strains S3 and S4 generally showed lower specific enzyme activities towards these substrates than other strains (Table 2). All *Capnocytophaga* extracts hydrolyzed FALGPA (another synthetic collagenase substrate) slowly. Soluble type I collagen (Table 2), reconstituted type I [3H]-collagen, and gelatin (not shown) were also hydrolyzed at a slow rate.

These enzyme activities were too low to reveal any alternations in the electrophoretograms of [3H]-collagen. The LV-Polypep was hydrolyzed at a moderate rate by all organisms, suggesting that the capnocytophagas may degrade hydrolysis products of collagen. BANA was also moderately hydrolyzed by the rough-surfaced isolates, indicating the presence of trypsin-like enzyme activity (not shown). The smooth-surfaced capnocytophagas did not show measurable BANA-hydrolyzing activity. Albumin was not hydrolyzed by any of the cell extracts.

The substrate specificities of the cell extracts and the cell suspensions were compared between the rough-surfaced strain R5 and the reference organism *C. gingivalis* (which forms smooth-surfaced colonies). The cell suspensions from both organisms showed a substrate specificity towards peptidase substrates that was broader than that of the corresponding cell extracts. The cell suspensions of R5 and *C. gingivalis* hydrolyzed cystinyl-, valyl-, threonyl-, prolyl-, isoleucyl-, seryl-, and glycylgly-

Table 1. Relative specific activities in the filtered cell extracts from rough- and smooth-surfaced isolates of capnocytophagas. The enzyme assays were performed in 20 mM Tris-HCl buffer (pH 8.0; 1 mM $CaCl_2$) at 37°C. The substrate concentration was 0.167 mM. In each column, the value of 100 was given to the substrate that was most rapidly hydrolyzed by the organism indicated, and the other rates of hydrolysis were compared with 100

Substrate (amino acid-2NA)	Rough isolates				Smooth isolates			*C. gingivalis*
	R1	R2	R4	R5	S2	S3	S4	S27
Ala-2NA	6	7	5	3	6	18	14	15
Arg-2NA	100	100	100	100	100	100	100	87
Asp-2NA	48	53	55	43	10	7	11	8
γ-Glu-2NA	2	2	2	3	1	3	8	2
α-Glu-2NA	8	8	7	9	7	4	10	8
Gly-2NA	3	4	2	1	1	0	0	1
His-2NA	4	6	4	3	1	5	6	2
Hypro-2NA	0	0	0	0	0	0	0	0
Leu-2NA	96	93	88	91	18	43	91	100
Lys-2NA	7	4	6	2	11	9	13	6
Met-2NA	26	25	28	20	12	29	23	21
Try-2NA	3	2	2	1	0	0	0	0
Tyr-2NA	0	0	0	0	0	3	4	1
Leu-Gly-2NA	8	3	8	2	0	0	0	1
Gly-Pro-Leu-2NA	10	11	13	17	12	0	14	13

cyl-2NA at a considerable rate, whereas the extracts hydrolyzed these substrates weakly or not at all. It should thus be emphasized that the filtered extracts of these organisms were enzymatically much weaker then the cell suspensions. The suspensions of both organisms hydrolyzed soluble type I collagen, but at a rate 40–50% lower than did the cell extracts. The hydrolysis of the Polypep substrates was similar for suspension and extracts of both organisms. The cell suspensions of these organisms hydrolyzed neither gelatin nor FALGPA even though the corresponding extracts hydrolyzed these substrates. Casein and PZ-PLGPA were hydrolyzed by the cells and by the extracts of both organisms; the extracts hydrolyzed casein about two times faster than did the suspensions. PZ-PLGPA, however, was hydrolyzed faster by the cells. BANA was hydrolyzed at a moderate rate by the cells and the cell extracts of R5.

FPLC profiles of enzymes

The FPLC enzyme profiles were determined using substrates that were hydrolyzed at a high rate by each extract. Using leucyl-2NA as substrate, the Superose 12 enzyme profiles of the rough-surfaced capnocytophagas revealed one major peptidase peak with an approximate molecular weight of 42 000 (Fig. 1). Quantitatively similar enzyme profiles were found using aspartyl-, alanyl-, arginyl-, and methionyl-2NA. The chromatograms of the extracts from smooth capnocytophagas showed one major peptidase peak with a low molecular weight (ca. 12 000), while *C. gingivalis* displayed several enzyme peaks (Fig. 1). The FPLC enzyme profiles differed depending on the substrate used. One main peak was, however, always located in the low-molecular weight region.

Gel chromatography of rough- and smooth-surfaced *Capnocytophaga* extracts showed two peaks with activity toward PZ-PLGPA: one was fractionated in the void volume of the column, while another showed a molecular weight of ca. 54 000 (strain R5; Fig. 2). The void volume enzyme could be transformed into the low-molecular weight form with KCl treatment (0.5 M KCl for 1 h). This enzyme was probably bound to small membrane fragments (<0.2 μm). Thus, it appears that both rough- and smooth-surfaced *Capnocytophaga* isolates contained one main PZ-PLGPA-hydrolyzing enzyme with a similar molecular weight. The main enzyme peaks of the chromatogram showing hydrolysis of the Polypep substrates coincided with the peaks which exhibited activity towards PZ-PLGPA. These enzymes also hydrolyzed soluble type I collagen (not shown). The chromatograms also showed several peaks with moderate caseinolytic activity. Anion exchange FPLC of rough and smooth isolates on a Mono-Q column gave the same number of peaks for major peptidases and collagen-degrading enzymes (not shown).

FPLC and electrophoretic profiles of proteins

The Superose 12 protein profiles (A_{214} and A_{280}) were relatively similar for the cell extracts of both rough and smooth *Capnocytophaga* isolates (Figs. 1 and 2). The electrophoretic patterns of the extracts from all rough isolates and the smooth isolate S2 and *C. gingivalis* (S27) were similar. However, the smooth isolates S3 and S4 showed different protein profiles (Fig. 3). The electrophoretic patterns of the whole cell suspensions of the rough R5 strain and the smooth S27 (*C. gingivalis*) were different from each other and they were also different from the corresponding protein patterns of the extracts.

Discussion

Hydrolytic enzymes are considered to play an important role in the virulence of anaerobic bacteria associated with periodontal disease (21). Bacterial substances can diffuse into the connective tissue of

Table 2. Protease activities in the filtered extracts of rough- and smooth-surfaced capnocytophagas. The enzyme assays were performed in 20 mM Tris-HCl-buffer (pH 8.0; 1 mM $CaCl_2$) at 37°C. The substrate concentration was 1 mM for PZ-FALGPA and FALGPA and 1 mg/ml for the other substrates. For other details, see Material and Methods

	Specific enzyme activity ($\mu mol/min^{-1} \times ml^{-1} \times 10^{-3}$)							
	Rough capnocytophagas				Smooth capnocytophagas			*C. gingivalis* (smooth)
Substrate	R1	R2	R4	R5	S2	S3	S4	S27
FALGPA	0.55	1.06	1.02	0.64	0.95	0.65	0.68	0.66
PZ-PLGPA	20.91	16.44	19.92	21.10	18.23	0.78	8.64	18.71
Collagen (type I)	1.06	0.71	0.76	1.36	0.75	0.93	0.56	0.95
Hemoglobin	4.08	3.78	4.20	2.72	1.69	0.26	1.32	2.11
Casein	21.83	2.58	4.51	19.19	30.51	2.29	1.72	33.65
Polypep (HV)*	1.04	1.27	2.94	2.95	2.91	0.79	1.16	2.95
Polypep (LV)*	5.47	5.48	5.76	5.61	4.18	1.14	2.24	5.10

* High-viscosity and low-vsicosity (Sigma).

the periodontium (1, 17). Such substances very likely include various hydrolases. Bacterial proteolytic enzymes which at neutral pH attack tissue components like collagen, elastin, and fibronectin could be involved in initial stages of tissue degradation, while other peptidases may participate in the breakdown of the tissue macromolecules at later stages.

Capnocytophagas have been shown to possess periodontopathic potential even though these organisms may not be present in numbers comparable to, for example, treponemes in the subgingival floras of patients with periodontal disease (20). The present study demonstrated broad peptidase and protease activity operating at neutral pH values, in both smooth- and rough-surfaced clinical isolates of *Capnocytophaga* and *C. gingivalis*. This broad peptidase activity is in accordance with earlier studies of capnocytophagas (9, 15, 19, 22). PZ-PLGPA, a synthetic substrate used in studies of bacterial collagenases (3), was effectively split by all but one of the organisms. However, all organisms hydrolyzed collagens slowly. Earlier studies have shown no or low collagenlolytic activity in *Capnocytophaga* (20, 23). A recent study on *C. ochracea* may offer an explanation for this discrepancy: filtered extracts of this organism hydrolyzed type IV but not type I collagen (23). The degradation of the Polypep substrates (collagen-derived polypeptides) indicates that *Capnocytophaga* may participate in the hydrolysis of soluble breakdown products of collagen, possibly using the latter as growth substrates. The broad peptidase activity of these organisms should bring about the hydrolysis of a large number of different peptide bonds present in a substrate. It must be emphasized that considerable protease and peptidase activities were demonstrated in whole cells in addition to cell ex-

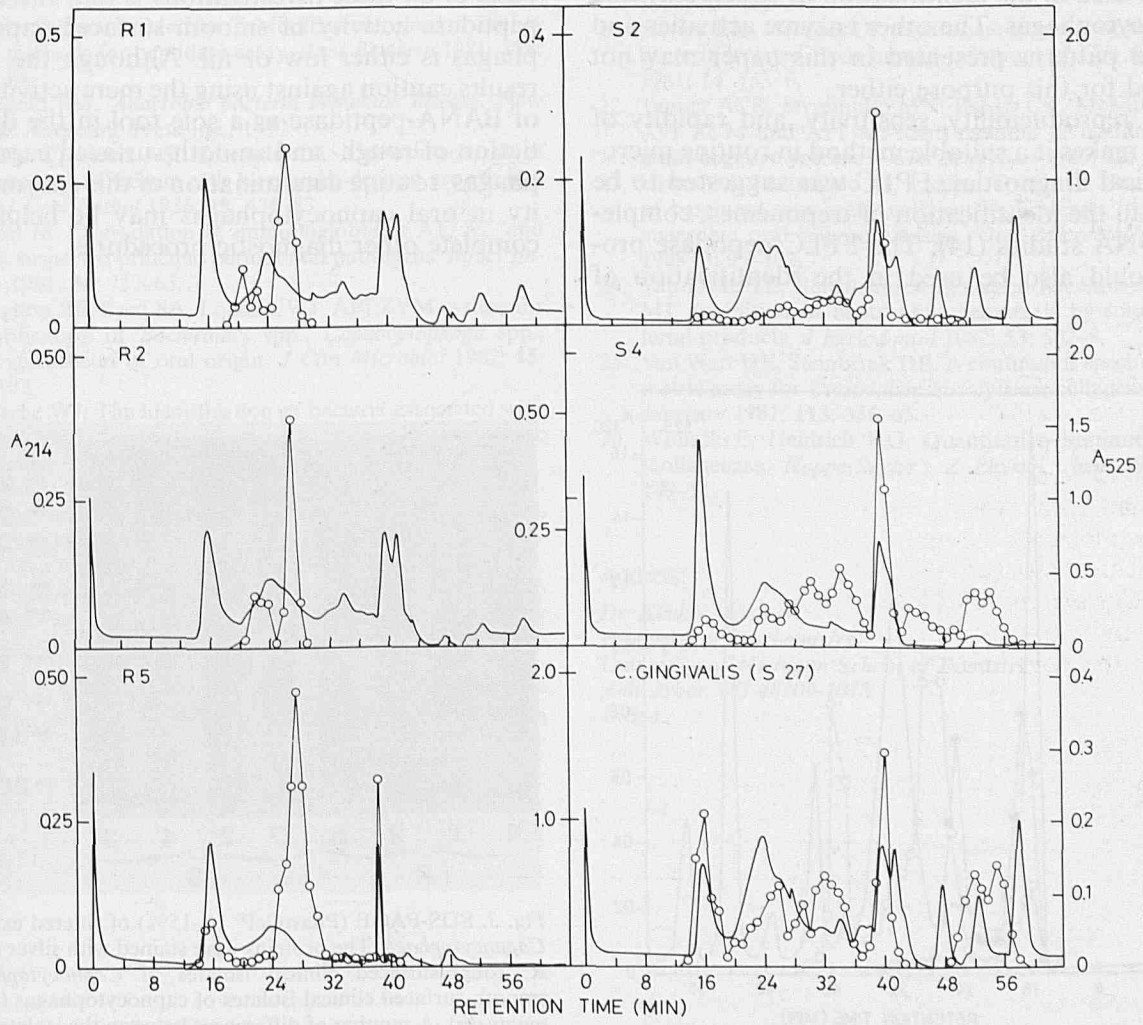

Fig. 1. FPLC on the gel column (Superose 12) of *Capnocytophaga* extracts hydrolyzing Leu-2NA. The elution buffer was 0.1 M Tris-HCl, pH 7, containing 1 mM $CaCl_2$ and 50 mM NaCl. The flow rate was 0.5 ml/min, and fractions of 1 ml were collected. ○—○, Leu 2NA, A_{525}; —— protein monitored at A_{214}. Molecular weight standards for Figs. 1 and 2: Cytochrome c (12 400), 34.5 min; carbonic anhydrase (29 000), 29.5 min; bovine serum albumin (66 000), 25.0 min. For details, see Material and Methods.

tracts. Consequently, lysis of cells was not needed for the expression of hydrolytic activities.

Trypsin-like activity (demonstrated with BANA) of anaerobic bacteria has been suggested to be a key indicator of their virulence (10). Some capnocytophagas appear to be BANA-positive (9, 20). *C. gingivalis, C. sputigena*, and *C. ochracea* have been demonstrated to possess no or weak trypsin-like activity while the majority of rough-surfaced clinical isolates of *Capnocytophaga* (different from above capnocytophagas) showed strong trypsin-like activity (9). Thus, it is possible that the virulence of the rough-surfaced capnocytophagas is stronger than that of the smooth-surfaced cells. The trypsin-like activity of the rough-surfaced organisms used in the present study was only moderate, however, whereas the present smooth isolates and *C. gingivalis* showed no activity towards BANA. It thus appears that the mere presence of trypsin-like activity or the level of this activity may not be used in the identification of rough-surfaced capnocytophagas. The other enzyme activities and protein patterns presented in this paper may not be used for this purpose either.

The reproducibility, sensitivity, and rapidity of FPLC makes it a suitable method in routine microbiological diagnostics. FPLC was suggested to be useful in the identification of treponemes, completing DNA studies (14). The FPLC peptidase profiles could also be used in the identification of smooth- and rough-surfaced capnocytophagas, because the former showed the major peptidase peak in the low-molecular weight region (ca. 12 000), while the rough-surfaced organisms showed a major peptidase peak near the 42-kD region. The fractionation patterns of proteases were unsuitable for this purpose. For the identification of treponemes, ion exchange FPLC proved to be better than gel FPLC (14). However, with the capnocytophagas ion exchange FPLC gave no additional information about the peptidase pattern. Consequently, it appears the FPLC on gel columns constitutes a suitable procedure for the differentiation between rough- and smooth-surfaced capnocytophagas in microbial diagnostics.

This paper completes other, simultaneous studies on human oral capnocytophagas, carried out at this laboratory. Different clinical isolates and different species of *Capnocytophaga* have been included in these studies. A consensus reached on the basis of all these investigations is that the BANA-peptidase activity of smooth-surfaced capnocytophagas is either low or nil. Although the present results caution against using the mere activity levels of BANA-peptidase as a sole tool in the differentiation of rough- and smooth-surfaced capnocytophagas, routine determination of this enzyme activity in oral capnocytophagas may be helpful and complete other diagnostic procedures.

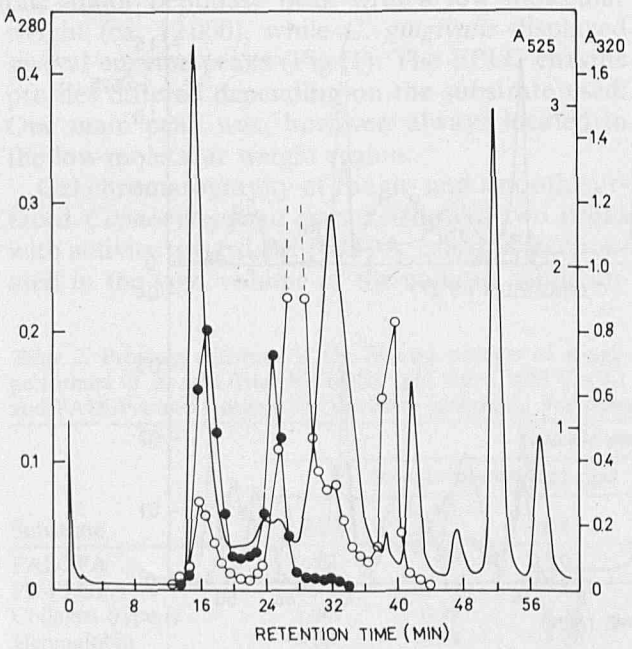

Fig. 2. FPLC on the Superose 12 column of the cell extract of a rough-surfaced isolate (R5) of *Capnocytophaga*. ○—○, *Na*-L-aspartyl-2NA, A_{525}; ●—●, PZ-PLGPA, A_{320}; —— protein monitored at A_{280}. For other details, see Fig. 2.

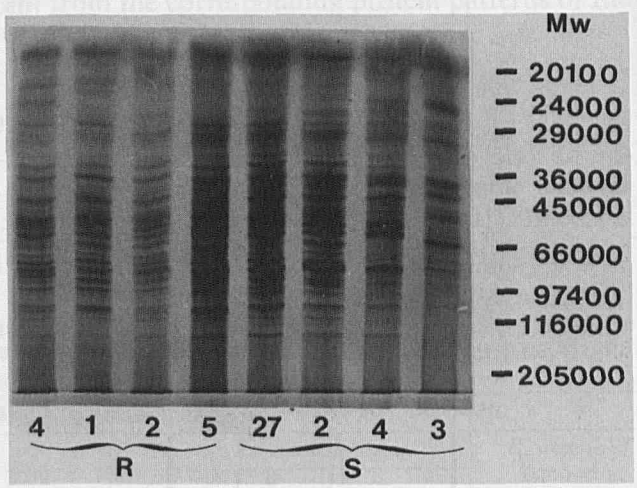

Fig. 3. SDS-PAGE (PhastGel® 10–15%) of filtered extracts of *Capnocytophaga*. The proteins were stained with silver staining. R: rough-surfaced clinical isolates of *Capnocytophaga*; S: smooth-surfaced clinical isolates of capnocytophagas (S27 = *C. gingivalis*). A number of differences between the isolates can be observed as follows: S3, missing bands near the 116–97.4-kDa region, that are present in other isolates, and a stronger band present near the 24-kDa region, not present in other isolates; R1, intense bands present near 100-, 36-, and 29-kDa regions.

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