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Characterization of immunoglobulin G-degrading proteases of *Prevotella intermedia* and *Prevotella nigrescens*

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Degradation of immunoglobulins is thought to be an important factor in the causation of periodontal diseases by hindering local host defenses and by providing nutrients to the periodontal microflora. In this study, we characterized the proteolytic activity against human immunoglobulin G (IgG) of 20 strains of *Prevotella intermedia* and *Prevotella nigrescens* isolated from periodontal pockets and oral abscesses. IgG degradation was studied by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. All strains degraded IgG within 48 h after growth in trypticase-yeast extract medium (TY) supplemented with 0.3% IgG. Incorporating IgG in TY broth enhanced bacterial growth. Protease profiles (zymography), which revealed the presence of 1–4 IgG-degrading proteolytic bands in bacterial cell extracts, became more complex after growth in the presence of IgG. A 38-kDa protease capable of degrading IgG nonspecifically was present in almost all strains. The proteolytic activity was mainly located on the surface of the cell envelope. Two strains of *P. intermedia* and *P. nigrescens* ATCC 33563 were selected for further studies. Bacterial cell suspensions in phosphate-buffered saline completely degraded human IgG, IgA and IgM within 24 h. This activity depended on reducing conditions and was inhibited at temperatures above 50°C. The pH optimum of immunoglobulin degradation was at pH 7. Strains cultured at 42°C showed a markedly reduced capacity to degrade IgG. Inhibition studies revealed that breakdown of IgG was caused by a cysteine protease(s). The capacity of *P. intermedia* and *P. nigrescens* to degrade immunoglobulins may explain their association with polymicrobial oral diseases.

Key words: proteolytic activity; immunoglobulin degradation; protease; *Prevotella intermedia*; *Prevotella nigrescens*

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In the history of oral microbiology, many organisms have been implicated in periodontal diseases (22, 23). One of these is the anaerobic black-pigmented bacterium *Prevotella intermedia*, which can be found throughout the oral cavity. This microorganism has been isolated frequently from odontogenic abscesses (31), saliva (15), the tongue and both healthy and diseased subgingival sites (2). Although its precise role in the infectious process remains unknown, it is becoming increasingly clear that *P. intermedia* is one of a group of microorganisms that may cause oral disease (28, 32, 33).

The biochemical and genetic heterogeneity of *P. intermedia* has long been known (19). A decade ago, a new species, *Prevotella corporis* (12) and recently another species, *Prevotella nigrescens*, were proposed (18). Further studies revealed that *P. intermedia* and *P. nigrescens* have different surface proteins and may have a different prevalence of isolation site (5).

Although *P. intermedia* may be less virulent than *Porphyromonas gingivalis* (30), it produces an array of virulence factors (21), of which the capacity to degrade immunoglobulins is thought to be important. Bacteria with this ability can provide themselves (8, 11) and other bacteria (27) with nutrients while simultaneously impeding host defense (3, 6). Several laboratories have isolated and characterized *P. gingivalis* proteases (4, 7, 20, 29), but less attention has been paid to those of *P. intermedia*.

The aim of our study was to determine number and type of proteases and to characterize the immunoglobulin-degrading activities of *P. intermedia* and *P. nigrescens*. The ability of these organisms to utilize human IgG as a nutrient for growth was also studied.

Material and methods

Bacterial strains and cultivation

The bacterial strains used in this study are listed in Table 1. They represent type strains, other clinical isolates and isolates from serum degrading consortia from batch and continuous culture enrichments of subgingival plaque on human serum (25, 26).

All microbiological procedures were carried out in an anaerobic chamber at 37°C in an atmosphere of 80% N_2, 10% CO_2 and 10% H_2. Bacteria were precultured in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, MD), supplemented with yeast extract (1.5 g/l), hemin (10 mg/l) and vitamin K (1 mg/l) or in a Trypticase (17 g/l; Difco Laboratories, Detroit, MI), yeast extract (1.5 g/l) medium (TY) containing NaCl (5 g/l), K_2HPO_4 (2.5 g/l), KNO_3 (1 g/l), hemin (10 mg/l) and vitamin K (1 mg/l). Unless stated otherwise, 18-h cultures were used for the determination of protease activities.

Identification of *P. nigrescens*

Colonies of *P. intermedia* were tentatively identified using the Rapid ID 32A system (Bio-Mérieux SA, 69280 Marcy l'Etoile, France). *P. intermedia* and *P. nigrescens* strains have been distinguished by van Steenbergen and coworkers (Amsterdam) using multilocus enzyme electrophoresis (18), since the Rapid ID 32A system cannot discriminate between them. Briefly, 3-day cultures of *P. intermedia* and *P. nigrescens* on blood agar plates were harvested with cotton wool swabs and resuspended in 0.1 M Tris-HCL (pH 7.5). Cell suspensions were sonificated for 30 s with an amplitude of 20 μm (on ice; Soniprep 150, MSE, Loughborough, UK), and cellular debris was removed by centrifugation. After electrophoresis of supernatants using native PhastGel 8–25% polyacrylamide gels (PhastSystem; Pharmacia, Uppsala, Sweden), glutamate dehydrogenase and malate dehydrogenase were visualized using a staining solution containing 0.1 M Tris (pH 7.0), 20 mg/ml glutamate, 10 mg/ml L-malic acid, 5 mg/ml nicotamide adenine dinucleotide, 0.04 mg/ml phenazine methosulfate and 0.02 mg/ml thiazolyl blue tetrazolium. Species differentiation was done by comparing the electrophoretic mobilities of glutamate dehydrogenase and malate dehydrogenase with those of type strains.

Preparation of cell fractions

We studied the proteolytic activities of various fractions of *P. intermedia* and *P. nigrescens* cultures. Cells (I) and

Table 1. Origin of bacterial strains used

Organism	Strain	Source*	Isolation site	Isolated from	Clinical isolate**
Prevotella intermedia	ATCC 25611	Holdeman	empyema	human	yes
	NY 363	ter Steeg	periodontal pocket	human	no
	NY 365	ter Steeg	periodontal pocket	human	no
	NY 460	Mikx	gingival sulcus	dog	yes
	NY 647	Mikx	gingival sulcus	human	yes
	NY 648	Mikx	gingival sulcus	human	yes
	NY 652	Jansen	periapical abscess	human	yes
	NY 653	Jansen	periapical abscess	human	yes
	NY 654	Jansen	periodontal abscess	human	yes
	NY 655	Jansen	periodontal abscess	human	yes
	BMH	McBride	unknown	human	yes
Prevotella nigrescens	ATCC 33563	Holdeman	Vincent's gingivitis	human	yes
	NY 646	Jansen	extraction abscess	human	no
	NY 649	Jansen	extraction abscess	human	no
	NY 650	Jansen	extraction abscess	human	no
	NY 651	Mikx	gingival sulcus	human	yes
	BH 18/23	Bowden	gingival sulcus	human	yes
	SPRO–2	Grenier	periodontal pocket	human	yes
	S19g	Sixou	gingival sulcus	human	yes
	T9	Grenier	gingival sulcus	human	yes
Other bacteria					
Porphyromonas asaccharolytica	NY 432	Mikx	gingival sulcus	dog	yes
Porphyromonas endodontalis	ATCC 35406	van Steenbergen	infected root canal	human	yes
Porphyromonas gingivalis	ATCC 33277	Coykendall	gingival sulcus	human	yes
Fusobacterium nucleatum	NY 373	ter Steeg	periodontal pocket	human	no
Peptostreptococcus micros	NY 370	ter Steeg	periodontal pocket	human	no
	NY 656	Jansen	periapical abscess	human	no
Prevotella oralis	NY 367	ter Steeg	periodontal pocket	human	no

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** Strains are either clinical isolates, including type strains, or are derived from batch and continuous culture enrichments of subgingival plaque on human serum.

supernatants (II) were obtained by centrifugation for 30 min at 10,000×*g*. Cells were resuspended in prereduced phosphate-buffered saline (PBS, 100 mM, pH 7.2). Extracts (III) were prepared by resuspending the cells in a 1/5 volume of PBS and broken by ultrasonic treatment (2×30 s, on ice, 30% duty cycle, output 5; Sonifier cell disrupter, Branson Sonic Power Co, Danbury, CT). Unbroken cells and cellular debris were then removed by centrifugation at 10,000×*g* for 20 min. Outer membranes (IV) were obtained as described by Boyd & McBride (1). Cells from a 18-h 200-ml THB culture were harvested by centrifugation at 10,000×*g* at 4°C. After washing twice, bacterial cells were resuspended in 20 ml 50 mM Tris buffer pH 8.6 containing 5 mM EDTA and sheared using a 26-gauge needle. Cell suspensions were shaken (80 rpm) at 4°C for 48 h with glass beads (150–212 μm diameter), and debris was removed by centrifugation at 10,000×*g*. Supernatants were ultracentrifuged at 100,000×*g* for 2 h at 4°C, and the outer membrane fraction in the pellet was resuspended in PBS.

SDS-PAGE

Analytical sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Mini-PROTEAN II system (thickness 0.075 cm; BioRad Laboratories, Richmond, CA) using 12.5% polyacrylamide resolving gels and Laemmli's buffer system (16). Samples contained 5 μg of protein and were boiled for 5 min in the presence of 2% SDS, 0.5% 2-mercaptoethanol, 20% glycerol, and 50 μg/ml bromophenolblue. After electrophoresis, the protein bands were stained with Coomassie Brilliant Blue R–250. Reference proteins used were myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa) (BRL, Gaithersburg, MD).

Enhancement of growth

Growth stimulation was studied by supplementing trypticase-yeast extract broth with 0.3% human immunoglobulin G (IgG) (TY/IgG). Bacteria were inoculated up to 2% in TY or TY/IgG and growth enhancement was recorded longitudinally by measuring the increase in optical density at 660 nm. The breakdown of IgG was determined by SDS-PAGE. Growth enhancement of *P. intermedia* ATCC 25611 was also estimated in TY and TY/IgG with a reduced trypticase content of 0.7%.

Determination of proteolytic activity

Growth assay. To study proteolytic activity against IgG, the strains listed in Table 1 were cultured in TY/IgG. After 48 h, supernatants (10,000×*g*, 10 min) were analyzed by SDS-PAGE.

Zymography. The profiles of proteolytic activities (zymograms) of 19 *P. intermedia* and *P. nigrescens* strains were determined by electrophoretic analysis of cell extract from 18-h THB cultures using 10% SDS-polyacrylamide gels containing covalently linked IgG or bovine serum albumin. Proteins were covalently bound to linear polyacrylamide (BDH Chemicals, Poole, UK) according to the protocol of Kelleher & Juliano (13). After electrophoresis, gels were gently washed for 30 min in 50 mM PBS (pH 7.2) containing 1% Triton X-100, washed twice and finally incubated for 24 h at 37°C in PBS with 20 mM dithiothreitol (DTT). Gels were stained for protein with Coomassie Brilliant Blue. After destaining, the proteolytic activities became visible as clear bands against a blue background.

Tube assay. The breakdown of IgG, IgA or IgM was investigated by incubating immunoglobulins (0.5 mg/ml), under anaerobiosis at 37°C, in 100 mM PBS (pH 7.2) and bacterial cells (final bacterial absorbance at 660 nm (A_{660}) was 0.3) or culture supernatant. Degradation of the immunoglobulins was determined by SDS-PAGE.

Thermal stability

The heatstability of proteolytic activity was determined by incubating cell suspensions in PBS for 30 min at 20°, 37°, 40°, 50°, 60° and 70°C, prior to adding IgG.

pH optimum

The effect of pH was studied by incubating cells and IgG in a 100 mM sodium citrate-phosphate buffer (pH 3, 4, 5, 6 and 7) or 100 mM PBS (pH 7, 7.2, 7.5, 8 and 8.5).

Effect of protease inhibitors

Various protease inhibitors have been incorporated in the IgG-degradation assay to determine their effect on the proteolytic activity. Inhibitors used and their final concentrations are as follows; iodoacetamide (10 mM), trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64) (25 μg/ml), benzamidine (10 mM), phenylmethylsulfonylfluoride (PMSF) (2 mM), *N*-tosyl-L-phenyl-alanine-chloromethylketone (TPCK) (2 mM), *Nα-p*-tosyl-L-lysine-chloromethylketone (TLCK) (10 mM), antipain (25 μg/ml), leupeptin (25 μg/ml), chymostatin (125 μg/ml), soybean trypsin inhibitor (125 μg/ml), soybean trypsin-chymotrypsin-inhibitor (125 μg/ml), pepstatin A (125 μg/ml), bestatin (125 μg/ml), ethylenediaminetetraacetate (EDTA) (10 mM) and SDS (10 mM). All protease inhibitors were obtained from Sigma Chemical Co. (St.Louis, MO).

Aminopeptidase activities

Aminopeptidase activities were characterized using nitroanilide-linked peptides (Sigma Chemical Co.). Cells at a final A_{660} in the assay of 0.2 were anaerobically incubated in PBS for 8 h at 37°C with 220 μg/ml of the following peptides: *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA; trypsinlike activity), glycyl-L-proline-*p*-nitroanilide (GPPNA; glycyl-prolyl peptidase activity), *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (SAAPPPNA; chymotrypsin-like activity) and *N*-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide (SAAAPNA; elastase-like activity). The peptidase activities were recorded semiquantitatively and by an increase in A_{405}. In the absence of peptidase activity, the assay mixture remained colorless ("−"; $A_{405} \leq 0.1$), whereas weak or strong activities, resulted respectively in a faint yellow ("±"; $0.1 < A_{405} < 0.4$) or yellow ("+"; $A_{405} \geq 0.4$) color.

Results

Our 20 test strains could be unambiguously separated into 11 strains of *P. intermedia* and 9 strains of *P. nigrescens* (Table 1). No prevalence of either bacteria was found with regard to isolation site. All strains of *P. nigrescens* and *P. intermedia* degraded IgG in the growth medium within 48 h. However, strains of the latter species were generally more

Table 2. Percentage of growth stimulation and protein degradation* observed after growth in TY broth supplemented with 0.3% IgG. Mean values (n=2) are presented

Organism	24 h (%)	48 h (%)	72 h (%)	96 h (%)
A *P. intermedia*				
ATCC 25611	52	130	126	100
NY 655	26	88	83	NT
IgG degradation	++	++	++	++
P. nigrescens				
ATCC 33563	8	39	32	NT
SPRO–2	50	136	83	NT
IgG degradation	+	++	++	NT
B *P. intermedia*				
ATCC 25611	25	50	68	77
IgG degradation	±	±	+	++

A: TY with 1.7% trypticase. B: TY with 0.7% trypticase. NT: not tested. * Breakdown of IgG as detected by SDS-PAGE; ±: little breakdown; + : partial breakdown; ++ : complete breakdown.

active (Table 2A). *P. gingivalis* and *Porphyromonas endodontalis*, which were used as positive controls, also degraded IgG. No degradation was observed after growth of *Prevotella oralis*, *Prevotella asaccharolytica*, *Fusobacterium nucleatum* and *Peptostreptococcus micros* strains. Strains of the latter 2 species acted as negative controls.

The growth of *P. intermedia* and *P. nigrescens* was enhanced by incorporating IgG into TY broth (Table 2). The degree of stimulation varied from strain to strain. The cell yield of *P. intermedia* in TY with a reduced trypticase content of 0.7%, but supplemented with IgG, exceeded that in normal TY broth (1.7% trypticase) with no IgG (Fig. 1, lines B and C). The growth rate was, however, lower. Presumably, growth rate was decreased due to a lower concentration of amino acids and peptides, which are nutrients that can be readily taken up by the bacteria. In media with 0.7% or 1.7% trypticase (Fig. 1, lines B and D), IgG was completely degraded (Table 2). These results indicate that the degradation products of IgG were used for growth.

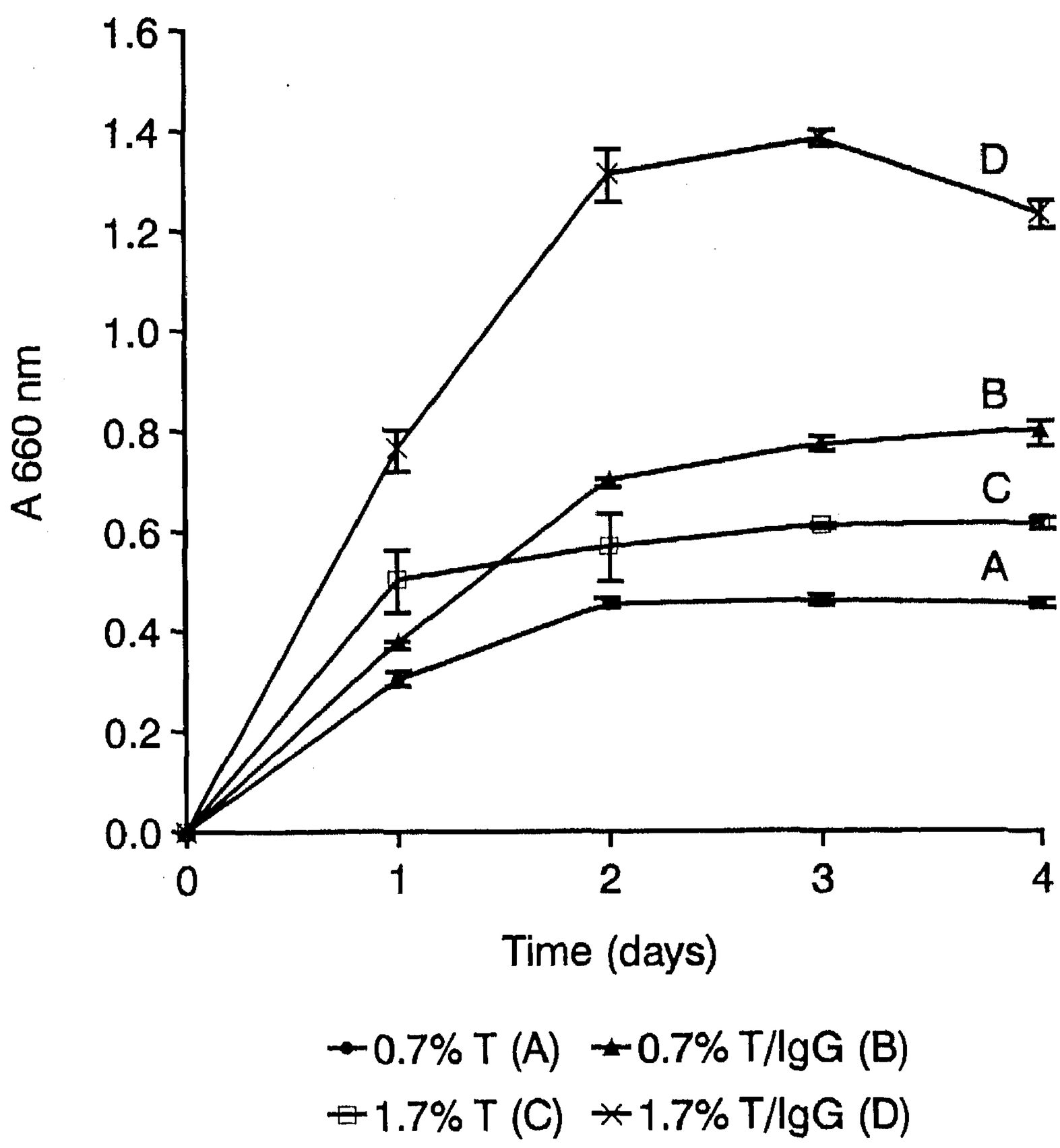

Fig. 1. Growth of *P. intermedia* ATCC 25611 (A660 nm) in trypticase-yeast extract broth with and without 0.3% human IgG. Bacteria were cultured in TY medium containing 1.7% or 0.7% trypticase. Error bars (duplicate experiment) have been included.

The protease profiles (zymograms; Fig. 2A) revealed that 16 strains elaborated at least one or two (in 13 strains), but sometimes up to 4 different bands with proteolytic activity against IgG. The enzymatic activities varied strongly between strains. Both nonspecific and what are tentatively called "specific" IgG-degrading activities were detected (Table 3). The proteolytic activity of the crude cell extract was found in the membrane fraction, which indicated that the activity was not due to soluble proteases. Similar proteolytic profiles were obtained if the preparations of outer-membranes were analyzed, suggesting that proteases were located on the surface of the cell envelope. The molecular weight of the proteases ranged from 38 kDa up to 200 kDa. A nonspecific 38-kDa protease was found with almost all strains (15 strains of 16). The proteolytic activities were present despite the growth of the organisms in the absence of IgG.

The growth of strains in TY broth with IgG to induce the production of IgG-degrading proteases or to increase their activity changed the enzymatic profile, which suggested that there had been induction of IgG-degrading proteases (Fig. 2B). From 1 to 5 proteolytic activities against IgG were detected with all 17 strains tested, including 3 strain found negative when grown in THB. In addition, the number of strains exhibiting more than one IgG-degrading protease increased from 13 of 19 (68%) to 15 of 17 strains (88%). Nearly all (96%) of these proteolytic activities were found to be nonspecific and showed a stronger activity against IgG than against bovine serum albumin. Most strains of *P. intermedia* exhibited

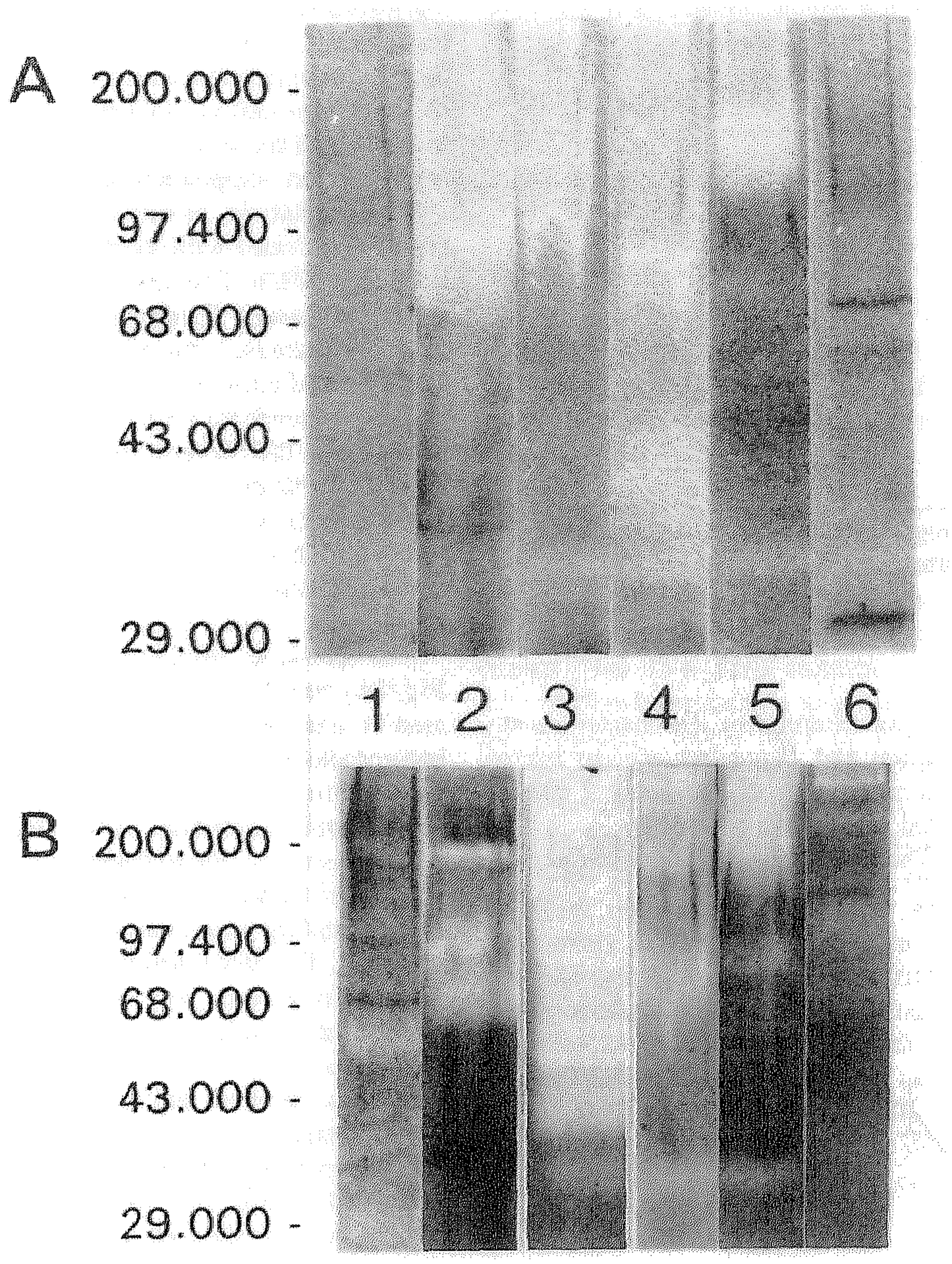

Fig. 2. Proteolytic profiles of *P. intermedia* and *P. nigrescens* on IgG-conjugated polyacrylamide gels. Cell extracts (25 μl) have been prepared of strains cultured for 18 h in either THB without IgG (gel A) or in TY medium supplemented with 0.3% aspecific human IgG (gel B). Gels have been developed for 24 h in PBS containing 20 mM dithiothreitol. Lane 1; *P. intermedia* NY 460. Lane 2; *P. intermedia* BMH. Lane 3; *P. intermedia* NY 652. Lane 4; *P. intermedia* NY 648. Lane 5; *P. nigrescens* ATCC 33653. Lane 6; *P. nigrescens* NY 652.

stronger enzymatic activities against IgG and bovine serum albumin than strains of *P. nigrescens*.

The proteolytic activities of *P. intermedia* strains NY 365, NY 653 and *P. nigrescens* ATCC 33563 against immunoglobulins have been investigated in more detail. All 3 strains completely degraded IgG, IgA and IgM within 20 h (Table 4). The proteolytic activity was found to be mainly cell-bound, which was not affected by aging of the cells up to 4 days. In a time-follow study, breakdown of IgG was analyzed after 0.5, 1, 2, 3, 4, 6, 8 and 24 h. The amount of protein gradually decreased, but accumulation of protein fragments was not observed.

The proteolytic activity was inhibited by oxygen or at temperatures above 50°C. Oxygen-inhibition was reversible and could be circumvented by incorporation of 10 mM dithiothreitol in the assay mixture. Breakdown of IgG was most efficient at pH 7–7.2, but no activity could be detected at pH-values of 4 and below or above 8.

Zymograms showed that IgG- and bovine serum albumin-degrading activities were lower in strains grown at 42°C than at 37°C, but no differences were observed in proteolytic activity or enzymatic profile between strains grown at 30°C and 37°C.

The protease inhibitors iodoacetamide and E64, which inhibit cysteine proteases, effectively prevented IgG breakdown (Table 5). No effect on proteolytic activity was observed with agents inhibiting serine, metallo and aspartic proteases.

Aminopeptidase activities (Table 3) were recorded using nitroanilide-linked peptides with 8 strains of *P. intermedia* and 4 of *P. nigrescens*. All 12 strains tested exhibited glycyl-prolyl hydrolytic activity, although this activity varied between strains. Three strains of *P. nigrescens* had weak chymotrypsin-like activity, but none of the strains showed trypsin-like or elastase-like activities.

Discussion

Microorganisms in the gingival sulcus are immersed in a flow of serum-derived tissue exudate known as gingival crevicular fluid. The protein composition of this exudate is much like that of normal human serum, and it contains immunoglobulins IgG, IgA and IgM at mean concentrations of 14.6 g/l, 1.6 g/l and 1.2 g/l respectively (9). These proteins are available in adequate amounts and are potential substrates for bacterial growth, in particular for an organism such as *P. intermedia* that generates energy and obtain cellular carbon from peptides and amino acids.

In a previous study, we demonstrated the ability of *Porphyromonas* and *Prevotella* species to use IgG as the sole source of amino acids while growing in a chemically defined medium (11). Here, using a relatively rich medium, we detected growth enhancement of *P. intermedia* and *P. nigrescens* by up to 130%. Although previous studies at our lab (10, 11, 27) and elsewhere (8, 14, 24), have reported the growth enhancement and breakdown of immunoglobulins by *P. intermedia*, characterization of the proteolytic activity has not been described before.

The hydrolysis of immunoglobulins was extensive and occurred without the accumulation of degradation products. This suggested that the immunoglobulins were rapidly degraded into fragments too small, or too low in concentration, to be detected by SDS-PAGE. Presumably due to differences in method, we were not able to confirm the cleavage of IgG into Fc and Fab as mentioned by ter Steeg et al. (27). Most strains of *P. intermedia* degraded IgG more quickly than *P. nigrescens*, which was in agreement with higher enzymatic activities observed in the zymograms.

The proteolytic activities of *P. inter-*

Table 3. Molecular mass and specifity* of IgG-degrading proteolytic bands, observed in polyacrylamide gels with covalently bound IgG, from *P. intermedia* and *P. nigrescens*, grown in either THB or TY medium with 0.3% IgG. Peptidase activities** against chromogenic substrates GPPNA and SAAPPPNA have also been included

		Proteolytic profile from strains grown in				Peptidase activity	
		THB		TY/IgG			
Species	Strain	MW (kDa)	specificity	MW (kDa)	specificity	GPPNA	SAAPPPNA
		75	IgG	180	n	+	−
P. intermedia	NY 363	38	n	38	n		
		150	n	200	n	+	−
	NY 365	38	n	38	n		
	NY 460	ND		60	IgG	+	−
		100	IgG	120	n	+	−
	NY 647	38	n	38	n		
				160	n	+	-
		90	IgG	80	n		
	NY 648	38	n	38	n		
				200	n	+	−
				80	n		
		200	n	45	n		
		100	n	40	n		
	NY 652	38	n	38	n		
				200	n	+	-
		150	n	80	n		
		90	n	45	n		
		70	IgG	40	n		
	NY 653	38	n	38	n		
				200	n	+	-
				180	n		
		130	IgG	90	n		
	NY 654	38	n	38	n		
				150	n		
		130	IgG	80	IgG		
	NY 655	38	n	38	n		
				180	n		
		100	n	90	n		
	BMH	80	n	70	n		
				180	n	+	−
		160	n	80	n		
P. nigrescens	ATCC 33563	38	n	38	n		
	NY 646	38	n	NT			
				200	n	+	±
	NY 649	38	n	140	n		
				200	n	+	±
				140	n		
	NY 650	38	n	38	n		
		200	IgG			±	±
		120	IgG	200	n		
	NY 651	38	IgG	38	n		
	BH 18/23	ND		38	n		
				200	n		
	SPRO–2	ND		140	n		
		200	n	200	n		
	S19g	38	n	140	n		
		200	n				
	T9	38	n	NT			

ND: not detected; NT: not tested; * IgG="specific", degradation of IgG, but not bovine serum albumin; n=nonspecific, degradation of IgG and bovine serum albumin. **Peptidase activities as determined by an increase in optical density (A_{405}); −=no activity ($A_{405} \leq 0.1$); ±=weak activity ($0.1 < A_{405} < 0.4$); +=strong activity ($A_{405} \geq 0.4$).

Table 4. Degradation* of IgG, IgA and IgM after incubation with *P. intermedia* or *P. nigrescens*

	IgG				IgA				IgM			
	6 h		20 h		6 h		20 h		6 h		20 h	
Strain	Heavy chain	Light chain	Heavy chain	Light chain	Heavy chain	Light chain	Heavy chain	Light chain	Heavy chain	Light chain	Heavy chain	Light chain
P. intermedia												
NY 365	C	P	C	C	P	P	C	C	P	P	C	C
NY 653	P	N	C	C	P	P	C	C	P	P	C	C
P. nigrescens												
ATCC 33563	C	P	C	C	C	C	C	C	P	P	C	C

* Degradation of immunoglobulins determined by SDS-PAGE; N: no degradation; P: partial degradation; C: complete degradation.

media and *P. nigrescens* were mainly found to be cell-associated and are located on the surface of the cell envelope. Minor hydrolysis of IgG was observed with culture supernatants, but accumulation of a 33-kDa fragment as reported by Grenier et al. (8) using a ten-fold concentrated culture supernatant was not detected. The location of proteases on the outer membrane seems reasonable given that the growth of *P. intermedia* and *P. nigrescens* depends on the uptake of peptides and amino acids derived from the breakdown of proteins.

The molecular weights tentatively assigned to the various enzymatic activities were similar in each preparation. However, they do not necessarily reflect the molecular weights of the monomeric forms of the proteases, as samples were not boiled in SDS prior to electrophoresis. In addition, one or more of the proteases might represent a modified form, for instance due to partial digestion, and not a different protease.

Proteolytic profiles obtained from strains cultured in medium without IgG revealed that nonspecific and what are tentatively called specific IgG-degrading proteases were present with *P. intermedia* and *P. nigrescens*. By growing the strains in medium with IgG, the number of IgG-degrading bands was increased and nearly all proteolytic bands exhibited nonspecific protease activities. It is likely that many of the specific IgG-degrading proteases, detected after growing strains in medium without IgG, were actually nonspecific, having an activity against bovine serum albumin too low to be detected in our zymograms. Culturing strains in the presence of protein (IgG) thus increased the level and/or activity of the proteases. To truly identify specific IgG-degrading proteases, the activity should be tested against a large number of proteins.

Inhibition studies with the sulfhydryl-blocking reagents iodoacetamide and E64 and the oxygen-protective effect of the reducing agent dithiothreitol showed that breakdown of IgG by cells of *P. intermedia* and *P. nigrescens* was due to cysteine proteases. Inhibition observed using TLCK, TPCK, antipain, leupeptin and chymostatin might indicate the presence of serine proteases, but specific serine protease inhibitors could not prevent complete breakdown of IgG. Hence, serine, metallo and aspartic proteases appear not to be important in IgG degradation, but final proof for their absence should come from inhibition studies on the individual proteolytic activities of the organisms.

The proteases of *P. gingivalis* have been well characterized, and 8 distinct proteolytic activities have been found (7). The characteristics of the proteases from *P. intermedia* and *P. nigrescens* described in this study were very similar to those of *P. gingivalis*. Most enzymatic activities of *P. gingivalis* were cell envelope-associated cysteine proteases with molecular weights ranging from 29–110 kDa. Their pH optima and thermal stability were also similar to those of *P. intermedia*. In addition, both species are able to degrade a variety of tissue (21) and serum proteins (10). Six of 8 *P. gingivalis* proteases exhibited a strong trypsin-like activity, which was not detected with either *P. intermedia* or *P. nigrescens*. The molecular weights of the other two proteases were below 38 kDa, which is the weight of the smallest protease observed with *P. intermedia* and *P. nigrescens*.

Culturing *P. intermedia* and *P. nigrescens* at temperatures above their optimal growth temperature of 37°C reduced growth yield and proteolytic activities. A similar result was observed by Mateos et al. (17), who studied the effect of growth temperature on the virulence of *Aeromonas hydrophila*.

We found glycyl-prolyl peptidase activity with *P. intermedia* and, in contrast to a study of Shah & Gharbia (18), also with *P. nigrescens*. Hence, this criterion is of little value in discriminating between these two species.

We have demonstrated that *P. intermedia* and *P. nigrescens* possess a variety of proteases that nonspecifically degrade IgG. These proteases are likely to be involved in breakdown by the organisms of an array of tissue and serum proteins (10, 21, 24), including im-

Table 5. Effect of protease inhibitors on degradation of IgG by *P. intermedia* NY 365 and NY 653 and *P. nigrescens* ATCC 33563

Protease inhibitors (final concentration)	Effect on degradation
Cysteine	
Iodoacetamide (10 mM)	+
E64 (25 μg/ml)	±
Cysteine/serine	
TLCK (10 mM)	+
TPCK (2 mM)	+
Chymostatin (125 μg/ml)	+
Antipain (25 μg/ml)	±
Leupeptin (25 μg/ml)	±
Serine	
PMSF (2 mM)	−
Benzamidine (10 mM)	−
Trypsin inhibitor (125 μg/ml)	−
Chymotrypsin-trypsin inhibitor (125 μg/ml)	−
Aspartic	
Pepstatin A (125 μg/ml)	−
Metallo	
Bestatin (125 μg/ml)	−
EDTA (10 mM)	−
SDS (10 mM)	−

−: no inhibition; ±: partial inhibition; +: complete inhibition.

munoglobulins, complement, iron-handling proteins and protease inhibitors. The breakdown of these proteins, thus undermining host defense, stimulating tissue degradation as well as bacterial growth, may explain the association of *P. intermedia* and *P. nigrescens* with periodontal infections.

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