Hydrolytic enzymes and lectin-binding activity of black-pigmented anaerobic rods

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Recent taxonomic studies on black-pigmented anaerobic rods, a group of bacteria found on mucosal surfaces of humans and animals, led to the subdivision of existing species and to the creation of new species. The aim of this study was to characterize all 11 currently recognized species of black-pigmented bacteria (55 strains) for their ability to hydrolyse a variety of natural and synthetic substrates and for their lectin reactivity. Although most of the strains demonstrated some activity against proteinaceous substrates, *Porphyromonas gingivalis* was the only species able to hydrolyse type I collagen. Most strains possessed glycylprolyl protease activity, elastase-like activity and phospholipase C activity, whereas trypsin-like activity was restricted to *P. gingivalis*, *Porphyromonas salivosa* and *Bacteroides macacae*. β -Lactamase activity was demonstrated in five strains belonging to the saccharolytic group. The lectin reactivity of the bacteria was determined by a dot-blot procedure using horseradish-peroxidase-conjugated lectins. Three lectins, LOTUS A, RCA-I and ConA, failed to react with any of the bacteria tested. WGA reacted strongly with the cell surface of human biotypes of asaccharolytic black-pigmented bacteria (*P. gingivalis*, *Porphyromonas asaccharolytica* and *Porphyromonas endodontalis*) and *Prevotella intermedia*. The animal biotype strains of *P. gingivalis* showed a higher affinity for SBA and PNA than for WGA.

Keywords: *Porphyromonas* spp., *Prevotella* spp., *Bacteroides* spp., lectin-binding activity, hydrolytic activity

INTRODUCTION

Black-pigmented anaerobic rods include a number of Gram-negative bacterial species isolated from mucosal surfaces of humans and animals. Although some species have yet to be reclassified, recent taxonomic studies recognize two genera: *Porphyromonas* for the asaccharolytic group and *Prevotella* for the saccharolytic group (Shah & Collins, 1988, 1990). Several species of black-pigmented anaerobic bacteria are recovered from the oral cavity (van Winkelhoff *et al.*, 1988). More specifically, *Porphyromonas gingivalis* and *Prevotella intermedia* are found in high numbers in periodontal pockets from patients with periodontal disease (Slots & Listgarten, 1988; van Winkelhoff *et al.*, 1988). These two species, which possess numerous virulence determinants (Holt & Bramanti, 1991; Mayrand *et al.*, 1991), are among a group of about

ten bacterial species for which there are data that associate them with periodontal disease (Tanner, 1991).

Black-pigmented bacteria produce a variety of hydrolytic enzymes which are active against natural substrates and synthetic chromogenic compounds (Seddon & Shah, 1989; Suido *et al.*, 1986, 1988). These extracellular and cell-associated hydrolytic activities may play a role in bacterial pathogenicity and may also represent an important criterion for bacterial identification. Indeed, *P. gingivalis* is the only black-pigmented bacterial species, of human origin, able to hydrolyse the synthetic chromogenic peptide *N*- α -benzoyl-DL-arginine-*p*-nitroanilide, which is the specific target for trypsin-like activity (Laughon *et al.*, 1982). Most studies on the hydrolytic activities of black-pigmented bacteria have been carried out on *Porphyromonas* spp. and *Pr. intermedia*.

Lectins are non-immune proteins or glycoproteins with selective carbohydrate reactivity. These molecules represent potential tools for the characterization of bacterial cell surfaces as well as for the identification of clinical

Abbreviation: HRP-lectin, horseradish-peroxidase-conjugated lectin (abbreviations for chromogenic substrates and lectins are defined in Methods).

Table 1. Bacterial strains and their isolation site

Species	Strain	Isolation site
<i>Porphyromonas gingivalis</i> Human isolates	ATCC 33277	Gingival sulcus (human)
	ATCC 49417	Subgingival plaque (human periodontitis)
	W83	Clinical specimen (human)
	W50	Clinical specimen (human)
	W50/BR1	Brown colonial variant from W50
	W50/BE1	Beige colonial variant from W50
	BH18/10	Subgingival plaque (human periodontitis)
	381	Subgingival plaque (human periodontitis)
	LB13D-2	Subgingival plaque (human periodontitis)
	HW24D-5	Subgingival plaque (human periodontitis)
	OMG 406	Subgingival plaque (human periodontitis)
	HG1020	Subgingival plaque (human periodontitis)
	A7A1-28	Subgingival plaque (human periodontitis)
	23A4	Subgingival plaque (human periodontitis)
	17A3	Subgingival plaque (human periodontitis)
	JKG 1	Oral cavity (human)
	16-1	Subgingival plaque (human juvenile periodontitis)
	3492	Gingival margin (cat)
	Chat 1.1	Gingival margin (cat)
	B 243	Subgingival plaque (squirrel monkey)
	T 22	Subgingival plaque (cynomolgus monkey)
	Chien 4.2	Gingival margin (dog)
	D2-9	Gingival margin (dog)
	Loup 1.1	Gingival margin (wolf)
	Coyote 1.2	Gingival margin (coyote)
	Ours 3-1	Gingival margin (bear)
<i>Porphyromonas asaccharolytica</i>	ATCC 27067	Leg wound (human)
	ATCC 25260	Empyema (human)
	BM4	Dental plaque (human)
<i>Porphyromonas endodontalis</i>	ATCC 35406	Infected root canal (human)
	H 11 a-e	Infected root canal (human)
	BN 11 a-f	Infected root canal (human)
<i>Porphyromonas salivosa</i> *	3313	Gingival margin (cat)
	3420	Gingival margin (cat)
	NCTC 11632	Gingival margin (cat)
<i>Prevotella intermedia</i>	ATCC 25611	Empyema (human)
	MS/5B/367	Subgingival plaque (human)
	BH 20/30	Subgingival plaque (human)
	NCTC 9336	Vincent's angina (human)
	Cg1265	Subgingival plaque (human)
	G8-9K-3	Subgingival plaque (human)
	5W2	Subgingival plaque (human)
<i>Prevotella denticola</i>	ATCC 33185	Maxillary antrium (human)
	LD6	Subgingival plaque (human)
<i>Prevotella melaninogenica</i>	ATCC 25845	Sputum (human)
	Cg 1198	Oral cavity (human)
	VP1 4196	Oral cavity (human)
<i>Prevotella loeschii</i>	ATCC 15930	Gingival sulcus (human)
	SA 19	Oral cavity (human)
<i>Prevotella corporis</i>	ATCC 33547	Cervical swab (human)
	HG119	Unknown
<i>Bacteroides macacae</i>	ATCC 33141	Subgingival plaque (monkey)
	MD1-21	Subgingival plaque (monkey)
<i>Bacteroides levii</i>	ATCC 29147	Rumen (bovine)
	VPI 12466	Mastitis (bovine)

* Formerly *Bacteroides salivus* (Love *et al.*, 1992).

isolates (Slifkin & Doyle, 1990). For example, lectin reactivity is a rapid and effective means for the differentiation of species of *Bacillus* (Cole *et al.*, 1984) as well as of members of the family *Neisseriaceae* (Doyle *et al.*, 1984). To our knowledge, there are no published data concerning the lectin-binding activity of black-pigmented bacteria.

In the present study, we included all the currently known species of black-pigmented anaerobic rods and characterized them for their ability to hydrolyse a variety of natural and synthetic substrates as well as for their reactivity with lectins possessing different specificities.

METHODS

Bacteria and growth conditions. A total of 55 bacterial strains, representing all 11 currently recognized species of black-pigmented anaerobic rods, were used in the study. The bacteria and their isolation site are listed in Table 1. Bacterial cells were cultivated in brain heart infusion broth (BBL Microbiology Systems) supplemented with haemin ($10 \mu\text{g ml}^{-1}$) and vitamin K ($1 \mu\text{g ml}^{-1}$). Cultures were incubated for 3 d in an anaerobic chamber ($\text{N}_2/\text{H}_2/\text{CO}_2$, 80:10:10, by vol.) at 37°C .

Determination of hydrolytic activity on natural substrates. A variety of hydrolytic activities were assayed, in a plate culture assay, according to previously described procedures: caseinase (Sokol *et al.*, 1979), gelatinase (Syed, 1976) and hyaluronidase (Grenier & Michaud, 1993). The recent method for detection of hyaluronidase activity is based on the formation of a water-insoluble salt between hyaluronic acid and the polyanion cetylpyridinium chloride. Collagen degradation was determined by assaying for the production of lower-molecular-mass fragments by SDS-PAGE, using the Laemmli buffer system (Laemmli, 1970). Briefly, bacterial cells ($50 \mu\text{l}$; OD_{660} 2.0 in Tris/HCl) were incubated in the presence of 8 mM dithiothreitol–100 mM Tris/HCl ($50 \mu\text{l}$) and acid-soluble type I collagen ($25 \mu\text{l}$, 1 mg ml^{-1} in 0.01% acetic acid). After 2 h at 37°C , the assay mixtures were boiled for 10 min in the presence of an equal volume of solubilization buffer [125 mM Tris/HCl (pH 6.8), 2%, w/v, SDS, 2%, v/v, 2-mercaptoethanol] and run on SDS-PAGE (8%, w/v, acrylamide). The proteins were stained with Coomassie Brilliant Blue. Assays were run in triplicate to ensure reproducibility.

Determination of hydrolytic activity on chromogenic substrates. (i) Peptidase. Whole bacterial cells were tested for peptidase activity according to a slight modification of the procedure described by Pederson & Lamberts (1990). The following synthetic chromogenic peptides were used: *N*- α -benzoyl-DL-arginine-*p*-nitroanilide (BAPNA; trypsin-like activity), glycyl-L-proline-*p*-nitroanilide (GPPNA; glycylprolyl protease activity), *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (SAAPPNA; chymotrypsin-like activity) and *N*-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide (SAAAPNA; elastase-like activity). Briefly, a 1% (w/v) Noble agar solution in 100 mM Tris/HCl buffer (pH 7.8) was boiled and then the chromogenic peptide was dissolved to a final concentration of 0.1%. The solution was cooled to 55°C and wells of a flat-bottomed microtitre plate were filled with $125 \mu\text{l}$ of the mixture. After solidification of the gel, the plates were sealed with a plastic wrap and kept at 4°C until used (for up to one month). Aliquots ($50 \mu\text{l}$) of the bacterial suspensions (OD_{660} 2.0 in Tris/HCl supplemented with 5 mM dithiothreitol) were placed in wells containing the chromogenic peptides immobilized in the agar gel, and the plate was incubated at 37°C

for 4 h. A positive reaction was indicated by the appearance of a yellow colour. Assays were run in triplicate to ensure reproducibility.

(ii) Phospholipase C. Phospholipase C activity was assayed using the synthetic chromogenic substrate *p*-nitrophenyl-phosphorylcholine (NPCC), based on the method described by Kurioka & Matsuda (1976). The assay was carried out as for the peptidase activity except that NPCC was used at 0.25% and 60% (w/v) sorbitol was included in the agar gel. After an incubation at 37°C for 3 h, a positive reaction was indicated by the appearance of a yellow colour. Assays were run in triplicate to ensure reproducibility.

(iii) β -Lactamase. β -Lactamase activity was tested using a chromogenic cephalosporin, nitrocefin (kindly provided by Dr J.-M. Lacroix, University of Florida, Gainesville, FL, USA), and based on the procedure of Bourgault & Rosenblatt (1979). Briefly, $50 \mu\text{l}$ nitrocefin (0.05% in Tris/HCl) was mixed with $50 \mu\text{l}$ bacterial cell extract (cell suspension at OD_{660} 2.0 in Tris/HCl and sonicated for 45 s), and incubated at 37°C for 10 min. A positive reaction was indicated by the appearance of a red colour. Assays were run in triplicate to ensure reproducibility.

Determination of lectin-binding activity. Six horseradish-peroxidase-coupled lectins (HRP-lectins; Sigma) were used: LOTUS A (*Tetragonolobus purpureus*; specific for fucose residues), Con A (*Canavalia ensiformis*; specific for mannose residues), RCA-I (*Ricinus communis*; specific for galactose residues), PNA (*Arachis hypogaea*; specific for galactose residues), SBA (*Glycine max*; specific for *N*-acetyl-D-galactosamine and galactose residues) and WGA (*Triticum vulgaris*; specific for *N*-acetyl-D-glucosamine residues). Powdered lectins were dissolved, to a concentration of 1 mg ml^{-1} , in phosphate-buffered saline pH 6.8 and stored at -20°C until used.

Aliquots ($5 \mu\text{l}$) of bacterial suspensions (OD_{660} 0.2, in Tris/HCl) were applied to a nitrocellulose membrane and allowed to dry at 37°C for 1 h. The membrane was incubated in 20 mM Tris/HCl (pH 7.5)–0.5 M NaCl (TBS) containing 3% (w/v) bovine serum albumin (BSA) for 1 h at room temperature with shaking. The nitrocellulose membrane was then transferred to TBS containing 1.5% BSA and the HRP-lectin (final concentration $1 \mu\text{g ml}^{-1}$) under investigation. The membrane was incubated at room temperature for 2 h with shaking and then washed in TBS containing 0.05% Tween-20 ($6 \times 15 \text{ min}$). The nitrocellulose membrane was stained with the Bio-Rad HRP colour development kit according to the manufacturer's instructions. Lectin labelling of bacteria was indicated by the appearance of a purple spot. Assays were run in triplicate to ensure reproducibility.

RESULTS

The ability of the black-pigmented anaerobic bacteria to degrade natural substrates is reported in Table 2. Except for one strain of *Prevotella melaninogenica* (ATCC 25845), all the other bacteria under investigation were capable of hydrolysing at least one of the two general proteinaceous substrates, casein or gelatin. Animal and human biotype strains of *P. gingivalis* were the only bacteria able to cleave acid-soluble type I collagen. Strain W50/BE1, a colonial variant of *P. gingivalis* W50, had no hydrolytic activity against collagen. Using a new specific procedure for the detection of hyaluronidase activity, none of the bacterial strains under investigation were positive.

Table 2. Hydrolytic activity of black-pigmented anaerobic rods on natural substrates

Species	No. of strains tested	Positive for hydrolysis of*:		
		Casein	Gelatin	Collagen
<i>P. gingivalis</i> (human biotype)	17	17	17	16†
<i>P. gingivalis</i> (animal biotype)	9	9	9	9
<i>P. asaccharolytica</i>	3	3	3	0
<i>P. endodontalis</i>	3	3	3	0
<i>P. salivosa</i>	3	3	3	0
<i>Pr. intermedia</i>	7	7	7	0
<i>Pr. denticola</i>	2	2	0	0
<i>Pr. melaninogenica</i>	3	0	2	0
<i>Pr. loescheii</i>	2	2	1	0
<i>Pr. corporis</i>	2	1	2	0
<i>B. macacae</i>	2	2	2	0
<i>B. levii</i>	2	2	2	0

* All the strains were negative for hydrolysis of hyaluronic acid.

† The negative result was obtained with the mutant strain W50/BE1.

Hydrolysis of a variety of chromogenic substrates by the black-pigmented anaerobic bacteria is reported in Table 3. *P. gingivalis* (human and animal biotypes), *Porphyromonas salivosa* and *Bacteroides macacae* strains possessed trypsin-like activity. By contrast, the other black-pigmented bacteria as well as the *P. gingivalis* mutant W50/BE1 had no activity against the chromogenic peptide BAPNA. All the bacteria under investigation possessed glycylprolyl protease and phospholipase C activities. The phospho-

Table 4. Lectin-binding activity of black-pigmented anaerobic rods

Species	No. of strains tested	Positive reaction with*:		
		PNA	SBA	WGA
<i>P. gingivalis</i> (human biotype)	17	0	0	16†
<i>P. gingivalis</i> (animal biotype)	9	5	5	1
<i>P. asaccharolytica</i>	3	0	0	2
<i>P. endodontalis</i>	3	0	0	3
<i>P. salivosa</i>	3	0	0	0
<i>Pr. intermedia</i>	7	0	0	6
<i>Pr. denticola</i>	2	0	2	0
<i>Pr. melaninogenica</i>	3	0	0	0
<i>Pr. loescheii</i>	2	0	0	0
<i>Pr. corporis</i>	2	0	0	0
<i>B. macacae</i>	2	0	0	0
<i>B. levii</i>	2	0	0	0

* All the strains gave a negative reaction with LOTUS A, ConA and RCA-I.

† The negative result was obtained with the mutant strain W50/BE1.

lipase C activity of *P. gingivalis* was found to be highly sensitive to oxygen (data not shown). Chymotrypsin-like activity was detected in some species of black-pigmented bacteria, more particularly in the three strains of *P. salivosa*, but not in *P. gingivalis*. Except for *P. gingivalis* W50/BE1, *P. salivosa* 3420, *Pr. melaninogenica* Cg 1198, *Prevotella corporis* HG119 and both strains of *Bacteroides*

Table 3. Hydrolytic activity of black-pigmented anaerobic rods on chromogenic substrates

Species	No. of strains tested	Positive for hydrolysis of:					
		BAPNA	GPPNA	SAAPPPNA	SAAAPNA	NPCC	Nitrocefin
<i>P. gingivalis</i> (human biotype)	17	16*	17	0	16*	17	0
<i>P. gingivalis</i> (animal biotype)	9	9	9	0	9	9	0
<i>P. asaccharolytica</i>	3	0	3	2	3	3	0
<i>P. endodontalis</i>	3	0	3	1	3	3	0
<i>P. salivosa</i>	3	3	3	3	2	3	0
<i>Pr. intermedia</i>	7	0	7	1	7	7	1
<i>Pr. denticola</i>	2	0	2	0	2	2	1
<i>Pr. melaninogenica</i>	3	0	3	1	2	3	1
<i>Pr. loescheii</i>	2	0	2	0	2	2	0
<i>Pr. corporis</i>	2	0	2	0	1	2	2
<i>B. macacae</i>	2	2	2	1	2	2	0
<i>B. levii</i>	2	0	2	1	0	2	0

* The negative result was obtained with the mutant strain W50/BE1.

levii, the other bacteria included in the study possessed elastase-like activity. β -Lactamase activity was only detected in *Pr. intermedia* MS/5B/367, *Prevotella denticola* ATCC 33185, *Pr. melaninogenica* ATCC 25845 and *Pr. corporis* ATCC 33547 and HG119.

The lectin reactivity of the black-pigmented anaerobic bacteria was determined by a dot-blot procedure using HRP-lectins. This method was simple and gave excellent reproducibility. LOTUS A, RCA-I and ConA failed to react with any of the 55 bacterial strains. These lectins are specific for fucose, galactose and mannose residues, respectively. The results presented in Table 4 indicate that most strains belonging to the species *P. gingivalis*, *P. asaccharolytica*, *P. endodontalis*, *Pr. intermedia* and *Pr. denticola* were capable of binding one or more lectins. The lectin from *Triticum vulgaris* (WGA) showed the highest reactivity with the black-pigmented anaerobic rods. This lectin, which is specific for terminal N-acetyl-D-glucosamine, reacted with the cell surface of human biotype strains of *P. gingivalis* (16/17), *P. asaccharolytica* (2/3), *P. endodontalis* (3/3) and *Pr. intermedia* (6/7). The non-reactive strain of human biotype *P. gingivalis* was the beige mutant (W50/BE1). Surprisingly, only one out of nine animal biotype strains of *P. gingivalis* reacted with WGA. On the other hand, five animal biotype strains showed a strong affinity for both SBA and PNA lectins. In all cases, incorporation of the competing carbohydrates in the lectin-binding assay reduced but did not completely prevent the reaction with the lectins.

DISCUSSION

Recent taxonomic work on black-pigmented anaerobic bacteria led to the subdivision of existing species and to the creation of new species (Shah & Collins, 1988, 1990). In addition to contributing to bacterial virulence, hydrolytic activity and lectin reactivity may represent interesting properties for bacterial differentiation. Although there are several reports (Laughon *et al.*, 1982; Pederson & Lamberts, 1990; Seddon & Shah, 1989; Suido *et al.*, 1988, 1986) on the hydrolytic activities of black-pigmented bacteria, there are no recent studies which have included all the currently known species. Furthermore, to our knowledge, no studies have evaluated the potential of lectins for the differentiation of black-pigmented anaerobic bacteria and for the characterization of their cell surfaces. We thus tested all the species of black-pigmented anaerobic bacteria for their ability to hydrolyse a variety of natural and synthetic substrates as well as for their lectin reactivity.

The present investigation showed that *P. gingivalis* (human and animal biotypes) possesses a specific collagenase activity. Other asaccharolytic species such as *P. endodontalis*, *P. asaccharolytica* and *P. salivosa*, which are closely related to *P. gingivalis*, did not hydrolyse type I collagen. This is in agreement with previous reports suggesting that *P. gingivalis* is the only black-pigmented human oral bacterium able to degrade collagen (Mayrand & Grenier, 1985; Sundqvist *et al.*, 1987). Hyaluronidase activity has been previously reported for some black-pigmented

bacteria including *P. gingivalis* (Seddon & Shah, 1989). The specific assay used in the present study indicated that none of the black-pigmented bacterial isolates under investigation were capable of hydrolysing hyaluronic acid. As we recently suggested (Grenier & Michaud, 1993), the high proteolytic activity of these bacteria coupled with the use of non-specific methods was probably responsible for the false positive reactions obtained in previous studies.

Using chromogenic substrates, it was found that trypsin-like activity was restricted to *P. gingivalis* (human and animal biotypes), *B. macacae* and *P. salivosa*. These results are in agreement with previous studies on the trypsin-like activity of black-pigmented bacteria (Laughon *et al.*, 1982; Seddon & Shah, 1989). The presence of elastase-like activity and phospholipase C activity in black-pigmented bacteria, including *P. gingivalis* and *Pr. intermedia*, was demonstrated here for the first time. These hydrolytic activities may represent additional virulence determinants for these suspected periodontopathogens.

Lectins with different specificities were chosen for characterization of the cell surface of black-pigmented bacteria. The results revealed a certain degree of homogeneity in the lectin-binding capacity of these species. The high reactivity of WGA with *P. gingivalis* human biotype, *P. asaccharolytica*, *P. endodontalis* and *Pr. intermedia* suggests the presence of terminal N-acetyl-D-glucosamine residues on the surfaces of these bacteria. As already demonstrated by Frasc (1980), in the case of *Neisseria meningitidis* and *Neisseria gonorrhoeae*, lectin reactivity may be related to the N-acetyl-D-glucosamine residues of lipopolysaccharides. Some differences in lectin reactivity between the animal and human biotype strains of *P. gingivalis* were observed, which suggest that these are variations in the cell envelope composition. Parent *et al.* (1986), using a serological approach, have already demonstrated the presence of unique antigens for both the animal and human biotypes. Several factors may influence the interactions between lectins and bacteria. These include lectin molecular mass, lectin accessibility and the involvement of hydrophobic group stabilization (Slifkin & Doyle, 1990). This is well demonstrated by the fact that PNA, which is specific for galactose residues, reacted with several strains of animal biotype *P. gingivalis*, whereas a second lectin (RCA-I) with a similar specificity did not react.

P. gingivalis W50/BE1, a non-pigmented beige variant isolated from a chemostat-grown culture of strain W50, was found to be quite different from the wild-type strain. The mutant appeared to have lost collagenolytic, trypsin-like and elastase-like activities. In addition, the cell surface of strain W50/BE1 could not bind the WGA lectin. These results are in agreement with other studies indicating that there are major differences between the beige mutant and the wild-type strain (Marsh *et al.*, 1989; McKee *et al.*, 1988).

In conclusion, the present study has confirmed previous observations and provided new data on the hydrolytic activities of black-pigmented anaerobic bacterial species.

The reactivity of selected lectins with some species of black-pigmented species was also demonstrated for the first time. However, the lectin-binding activity could not discriminate between the various species.

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