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Prevotella intermedia and *Prevotella nigrescens* serotypes, ribotypes and binding characteristics

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Abstract

Type strains and 62 clinical isolates of *Prevotella intermedia* and *Prevotella nigrescens* were typed with the use of genomic DNA fingerprints and rRNA gene probes. The strains were further serotyped with monoclonal antibodies and characterized with SDS-PAGE, enzymatic activities, hemolysis and hemagglutination and coaggregation with *Streptococcus* and *Actinomyces* spp. *P. intermedia* and *P. nigrescens* were found to have distinct ribotype patterns which correspond to previously defined serotypes I and II/III, respectively. No clear phenotypic difference related to hemolysis, hemagglutination and coaggregation with *Streptococcus* and *Actinomyces* species, or expression of aminopeptides and lipase was found between *P. intermedia* and *P. nigrescens*.

Keywords: *Prevotella intermedia*; *Prevotella nigrescens*; Monoclonal antibody; Ribosomal RNA gene restriction analysis; Coaggregation; Hemagglutination

1. Introduction

Prevotella intermedia, a black-pigmented saccharolytic anaerobic rod, is found widespread in the oral cavity. It is frequently isolated in various age groups, from individuals with healthy gingiva, and it is a common component in odontogenic abscesses [1,2]. It can therefore be argued that either this species is an opportunistic pathogen or it represents a rather heterogeneous species with respect to virulence. At present, it is not clear whether strains isolated from different oral conditions and locations belong to different genotypes or potentially represent phenotypical variants.

Two DNA homology groups were early identified among *P. intermedia* strains [3], one of which has been proposed as a new species, *Prevotella nigrescens* [4]. It (type strain ATCC 33563) was phenotypically separated recently from *P. intermedia* (ATCC 25611) by low peptidase activity, failing to cleave lipid substrates, and revealing different electrophoretic enzyme profiles. The DNA homology groups of *P. intermedia* have been correlated with 3 distinct serogroups [5]. Serogroup I, represented by type strain ATCC 25611, was predominantly found

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in deep periodontal lesions while an even distribution of all 3 serogroups (I–III) was found among isolates from adults with no periodontal disease [6].

Recent development in DNA technology has provided new approaches to bacterial taxonomy. The use of DNA fingerprinting to evaluate relatedness among isolates is somewhat hampered by numerous bands obtained in agarose gels. To overcome this problem, specific probes for identification of certain fingerprint bands containing specific DNA sequences, e.g., the highly conserved rRNA gene, have been developed [8]. The purpose of this study was (i) to investigate the genetic relatedness of 62 *P. intermedia / nigrescens* isolates, using a probe complementary to *Escherichia coli* 16S and 23S rRNA and (ii) to compare the resulting patterns with those observed by serotyping with monoclonal antibodies (MAbs) and those received by phenotypic classification as proposed [4].

2. Material and methods

2.1. Bacterial strains

Sixty-two (OMGS, Oral Microbiology, Göteborg, Sweden) strains grouped as *P. intermedia* or *P. nigrescens* based on fluorescence in long-wave UV light, glucose fermentation and indole production were investigated. They were obtained from Swedish volunteers with healthy gingival conditions (17 strains), or deep periodontal pockets (38 strains), and were characterized biochemically and serologically [6]. Additionally, 7 isolates from periodontally diseased Kenyan adults were included [7]. As reference strains for serotypes I, II and III, strain ATCC 25611 (type strain of *P. intermedia*), ATCC 25261 and ATCC 33563 (type strains of *P. nigrescens*), all provided through CCUG (Culture Collection, University of Göteborg, Sweden), were included. Strains were stored lyophilized and for each experiment fresh cell preparations were made by plating the bacteria on Brucella agar (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD) enriched with 5% horse blood, 0.5% hemolysed blood and 5 mg/l menadione. All strains were tested for aminopeptidase and lipase activities according to the methods described by Shah and Gharbia [4].

2.2. Genotyping and ribotyping

Methods for DNA extraction, restriction enzyme digestion, agarose gel electrophoresis, synthesis of probes and analyses were essentially the same as described in detail by Bowden et al. [8]. Strains were grown anaerobically using the Gas-Pak system (Gas Generation Kit, Oxoid Ltd, Basingstoke, Hampshire, England) for 2–3 days. DNA yield and purity were determined by measurement of the absorption at 260 and 280 nm. Enzyme digestion was performed with DNA from all strains using *Pst*I, and *Bam*HI (Boehringer Mannheim Scandinavia AB, Bromma, Sweden). Electrophoresis was run in a horizontal agarose gel (Bio-Rad Subcell, Bio-Rad, Solna, Sweden) at constant voltage (40 V) for 19 h at room temperature in 89 mM Tris-borate buffer pH 8.0. Digoxigenin labeled lambda DNA digested with *Hin*dIII (Marker III, Boehringer Mannheim), was used as molecular markers in the gels. The fragments were visualized after ethidium bromide staining under shortwave UV light and photographed. DNA fragments were transferred to nylon membranes (Zeta-Probe GT, Bio-Rad) for Southern blotting using a Vacuum Blotter (Bio-Rad). The preparation of the DNA probe was made with the Genius Kit (1093-657, Boehringer Mannheim), using random primers (Promega C1181, Promega Research Labs, Madison, WI) and reverse transcriptase (Gibco BRL Life Technologies, Inc., Gaithersburg, MD). Molecular masses of major bands observed by ribotyping were compared with DNA molecular markers as reference. Ribotype patterns, which were confirmed on independent gels, were estimated.

2.3. SDS-PAGE outer membrane profiles

SDS-PAGE was performed in a Mini-Protean II (Bio-Rad) Unit at 200 V run for 45 min using a vertical 0.75 mm thick slab gel containing 7.5% (w/w) polyacrylamide. Bacterial samples were prepared by sonication of whole cell suspensions at 50 W for 1 min. The preparations were heated with a SDS sample buffer at 100°C for 5 min. After electrophoresis, the gel was stained with Coomassie brilliant blue R.

2.4. Hemolysis and hemagglutination

Hemolysis was recorded after 3 days of incubation from growth on Brucella agar plates.

Hemagglutination with erythrocytes of human, sheep and rabbit origin was recorded in microtiter-plate wells using 2-fold dilution series at a bacterial density of 10^9 (measured with optical density). Hemagglutination was read after 1 h of incubation at room temperature and overnight in a refrigerator.

Hemagglutination was also tested with sheep erythrocytes and 5 *P. intermedia* or *P. nigrescens* strains following culture on various media. In addition to the Brucella agar plate, strains were cultured anaerobically for 2–3 days in BHICJ (brain heart infusion with cysteine hydrochloride and yeast autolysate) broth and semiliquid Huntoon (HCMG-SuIa) broth [9].

2.5. Coaggregation with Streptococcus and Actinomyces species

A panel of 6 streptococcal strains (*Streptococcus mutans*, 2 strains, *Streptococcus sanguis*, 2 strains, *Streptococcus oralis*, 2 strains) and 3 *Actinomyces* strains grouped as *Actinomyces naeslundii*, representing 3 different binding specificities (ATCC 12104, LY 7 and 2238) were used. Strain LY 7 was provided by R.J. Gibbons, Forsyth Dental Center, Boston, MA, USA and strain 2238 was isolated from supragingival plaque of a healthy Swedish adult. Equal volumes of bacterial suspensions (1×10^9 cells/ml in 0.001 M Tris, to pH 8.0) of two cell types were mixed and examined immediately for coaggregation. The coaggregation score was given according to the scale of Kolenbrander and London [10].

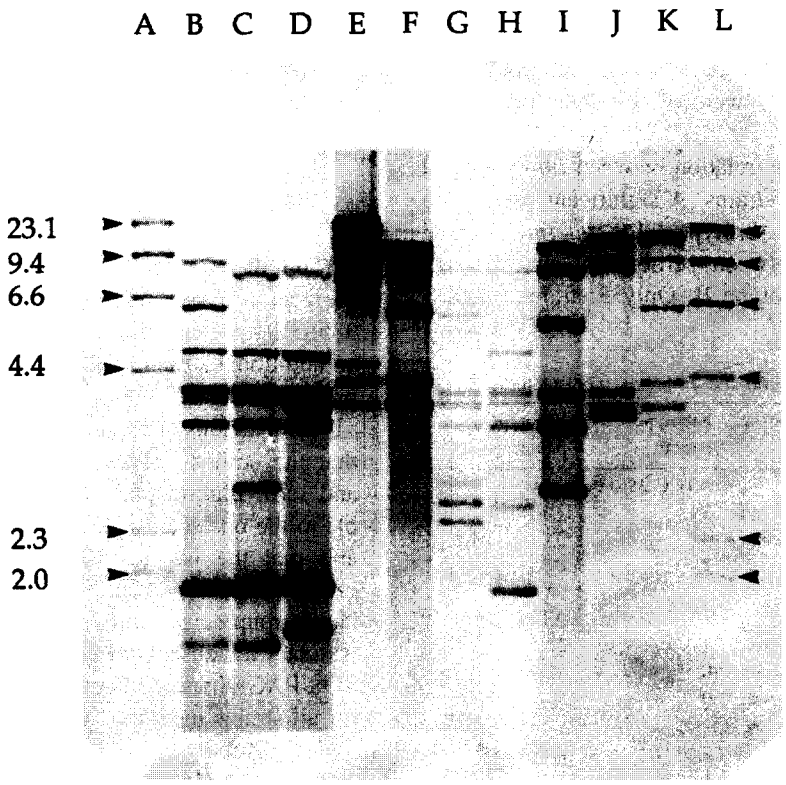

Fig. 1. Ribotype patterns generated from *P. intermedia* and *P. nigrescens* strains after treatment of chromosomal DNA with *Pst*I. Lanes B, C, D, H and I, *P. nigrescens* serotype II strains OMGS 456, OMGS 1449, OMGS 410, OMGS 457, and ATCC 33563. Lane G, *P. nigrescens* serotype III strain OMGS 447 and lanes E, F, J and K, *P. intermedia* serotype I strains OMGS 725, ATCC 25611 (obtained from CCUG, Göteborg University), OMGS 754 and ATCC 25611 (obtained from Dental Research Institute, University of Minnesota). Lanes A and L, *Hin*dIII fragments of λDNA labeled with digoxigenin.

3. Results

3.1. Enzymatic activities

None of the 62 *P. intermedia* or *P. nigrescens* reacted positively for aminopeptidase or lipase activities.

3.2. Ribotyping

The ribotypes fell into two main patterns (Fig. 1). Ribotype pattern A which included ATCC 25611 did not show fragments smaller than 3.9 kb, while ribotype pattern B including both ATCC 25261 and ATCC 33563 consistently produced small, strong bands of 2.8 kb and/or 1.8 kb.

3.3. Serotyping

All tested strains did react with the MAbs used and fell into serotype I (32 strains), II (10 strains) and III (20 strains). Of the 7 Kenyan strains 2 were of serotype I while 3 were of serotype II and 2 of serotype III.

Table 1 shows the relation between ribotypes and serotypes of the 62 strains. A significant correlation was found between serotype I (*P. intermedia*) and ribotype A and between serotype II and III (*P. nigrescens*) and ribotype B. Only 3 strains (OMGS 964, 868 and 873) did not fit into this pattern. It was not possible to find distinct different ribotype patterns between serotype II and III strains. Strains from diseased patients were overrepresented among serotype I isolates, while strains of serotypes II and III belonging to *P. nigrescens* were about equally recruited from periodontally diseased and healthy individuals.

Table 1
Ribotyping of *P. intermedia* and *P. nigrescens* isolates

Serotype	Strain category	Ribotype pattern A	Ribotype pattern B
I	Type strain	ATCC 25611	–
	Swedish diseased	24	1
	Swedish healthy	4	0
	Kenyan diseased	2	0
II	Type strain	–	ATCC 25261
	Swedish diseased	1	2
	Swedish healthy	0	4
	Kenyan diseased	0	3
III	Type strain	–	ATCC 33563
	Swedish diseased	1	8
	Swedish healthy	0	9
	Kenyan diseased	0	2
Total		32	29

Table 2
Hemolysis recorded of *P. intermedia* and *P. nigrescens* isolates

Serotype	Strain	Hemolysis
I	725	+
	754	+
	ATCC 25611	+
II	410	–
	457	–
	726	+
	ATCC 25261	+
III	447	–
	454	–
	ATCC 33563	–

3.4. SDS-PAGE

The SDS-PAGE patterns of *P. intermedia* and *P. nigrescens* type strains ATCC 25611 and ATCC 33563 both showed a strong band of 29 kDa while an additional strong band was displayed by a 25 kDa protein of ATCC 33563.

Comparisons of all isolates by SDS-PAGE showed that the strains grouped as *P. intermedia* by ribo- and serotyping displayed banding patterns comparable to the one seen with *P. intermedia* ATCC 25611 and that the isolates similarly grouped as *P. nigrescens* corresponded to ATCC 33563. Thus, the latter group consistently showed the band of 25 kDa, which was absent in all *P. intermedia* isolates. SDS-PAGE pattern confirmed the ribotype pattern for all strains including those 3, which showed serological diversity.

3.5. Hemolysis and hemagglutination

Hemolysis was registered for all *P. intermedia* strains tested (Tables 2–4), while the pattern was

Table 3
Hemagglutination pattern of *P. intermedia* and *P. nigrescens* isolates against sheep, human and rabbit erythrocytes

Strain	Serotype	Cultured on Brucella plate		
		Sheep	Human	Rabbit
1269	I	–	2+*	5+
1286	I	–	4+	6+
1281	I	–	3+	6+
861	II	2+	2+	8+
865	II	2+	3+	7+
868	II	3+	2+	6+
1271	III	2+	4+	5+
1300	III	2+	3+	6+

* + denotes the number of 2-fold dilutions of the bacterial suspension with a visible agglutination.

Table 4
Hemagglutination with sheep erythrocytes by *P. intermedia* and *P. nigrescens* after culture on various media

Strain	Serotype	Brucella	BHICJ	Huntoon
1269	I	–	–	+*
1281	I	–	–	+
1286	I	–	–	3+
861	II	2+	+	2+
865	II	2+	+	3+
868	II	3+	+	4+
1271	III	+	+	3+
1300	III	+	–	2+

* + denotes the number of 2-fold dilutions of the bacterial suspension with a visible agglutination. < /T3 >

inconsistent for the *P. nigrescens* strains. Notably, all the serotype III strains were negative.

All *P. intermedia* and *P. nigrescens* strains agglutinated with rabbit erythrocytes. Agglutination with human red blood cells was obtained for all strains, however the reaction was weaker. Five strains showed weak hemagglutination with sheep erythrocytes (Table 3) and all belonged to serotype II and III (*P. nigrescens*), whereas all hemagglutination-negative strains were serotype I (*P. intermedia*) strains. Table 4 shows that the hemagglutination was influenced by the medium in which the strain was cultured. Cells cultured in the semifluid Huntoon medium showed best over all hemagglutination including the strains of *P. intermedia*.

3.6. Coaggregation

Strains of both species showed no or very weak coaggregation with *Streptococcus* spp. The two streptococcal strains *S. oralis* 24892 and *S. mitis* N showed a high degree of autoagglutination which was potentiated particularly with *P. intermedia* strains. Presumably, *P. intermedia* and *P. nigrescens* isolates did not express receptor specificity for streptococcal species (data not shown). Both

Table 5
Coaggregation between *P. intermedia* and *P. nigrescens* and *A. naeslundii* strains Ly 7, ATCC 12104 and 2238

Bacterial species	Strain	Patient category from which the strain was isolated	Serotype	*A. naeslundii* strain		
				Ly 7	ATCC 12104	2238
P. intermedia	864	periodontitis	I	++	+++	++++
	870	periodontitis	I	+	–	+
	874	periodontitis	I	+++	++++	+++
	1277	gingivitis	I	++	++	–
	1281	gingivitis	I	+	–	+
	1300	gingivitis	I	+	–	–
	ATCC 25611	type strain	I	+	+	++
P. nigrescens	726	periodontitis (Kenya)	II	+	–	–
	865	periodontitis	II	++	+++	++++
	868	periodontitis	II	++	++	+
	904	periodontitis	II	–	–	–
	ATCC 33563	type strain	II	+	+	+
	866	periodontitis	III	–	–	–
	1302	gingivitis	III	–	+	+
	ATCC 25261	type strain	III	+	+++	+

species showed varying coaggregation ability with *A. naeslundii* strains (Table 5). Three strains, 2 *P. intermedia* and 1 *P. nigrescens* strains, showed expressed coaggregation with all three *A. naeslundii* strains. One *P. nigrescens* (OMGS 866) strain did not coaggregate with any of the *A. naeslundii* strains. The difference in coaggregation between the *A. naeslundii* strains was small.

4. Discussion

Identification of *Prevotella* species in the oral cavity has been carried out mainly on the basis of colony morphology and biochemical reaction profile. Thus black-pigmented saccharolytic *Prevotella* species separated into two main groups, one proteolytic and one non-proteolytic. It has been long been known that the proteolytic group comprises two DNA homology groups, now specified as *P. intermedia* (homology group I) and *P. nigrescens* (homology group II).

In spite of a distinct genotypic difference these two species show a remarkable similarity in phenotypic characteristics, which makes it difficult to rapidly distinguish between isolates of *P. intermedia* and *P. nigrescens*. Shah and Gharbia [4] recently proposed to separate these two species after having found appropriate phenotypic variation. However, in our hands, those methods failed to show a distinct difference between *P. intermedia* and *P. nigrescens* isolates.

Most of the isolates studied in the present investigation have previously been subjected to biochemical classification and serotyping using rat MAbs, which showed that all strains of the two species expressed one of three serological reactivity patterns [5]. Type I coincided with homology group I (*P. intermedia*) and type II and III strains with homology group II (*P. nigrescens*).

This is confirmed by the present finding and that *P. nigrescens* serotypes showed 16S and 25S ribotyping patterns distinctly different from that of *P. intermedia* isolates.

Serological species distinction is based on the binding of MAb 40BI3.2.2 to the surface of *P. intermedia* but not to *P. nigrescens*. Two strains belonging to *P. nigrescens* according to the ribotype pattern with the use of *Pst*I and *Bam*HI endonucleases, expressed the antigen which binds to MAb 40BI3.2.2. *P. nigrescens* species may therefore contain subtypes which share the *P. intermedia* surface antigen. The antigen detected by this MAb has not been characterized. On the basis of a few preliminary experiments, Gmür and Wyss [11] have speculated on the possibility of a cell surface protein of approximately 150 kDa.

In the present study, the outer membrane profile detected by SDS-PAGE showed some distinct differences between the two species. One band of 25 kDa was consistently present in *P. nigrescens* but lacking in *P. intermedia* isolates. The distinct *P. nigrescens* band of 31 kDa protein shown by Gharbia et al. [12] may correspond to the band of 29 kDa in the present study. However, this band was consistently present in all strains of both species. By SDS-PAGE Frandsen et al. [13] found a band of 20 kDa while the band of 29 kDa was found in strains of both species which was used to separate the strains into *P. intermedia* and *P. nigrescens*. This apparent heterogeneity in outer membrane protein profiles may in part be explained by different electrophoretic conditions. More likely it reflects the variations in surface protein expression due to culture conditions which also was found in this study to significantly alter the hemagglutination.

P. nigrescens strains seemed to show slightly more hemagglutination after 3 days of culturing on Brucella agar plates, which may distinguish *P. nigrescens* from *P. intermedia*. This difference was not obtained after culturing in the two different broth media. Variation in adherence to oral epithelial cells and hemagglutination was suggested to be related to types of surface appendages expressed by the two species [14–16]. Okuda et al. [14] showed a similar hemagglutinating activity for *P. intermedia* ATCC 25611 and *P. nigrescens* ATCC 33563 against intact or neuraminidase treated cells. Nesbitt et al. [15] showed coaggregation with various *Actinomyces* species by testing 2 strains of *P. intermedia* belonging to the ATCC 25611 DNA hybridization group and differing distinctly in their coaggregation pattern. *P. nigrescens* may also coaggregate with *Actinomyces* [16], however, the coaggregation was not related to the fibril type of *P. intermedia* and *P. nigrescens* or to *Actinomyces viscosus* strains lack-

ing type 1 or type 2 or both types of fimbriae. Similarly, the differences obtained in outer membrane binding proteins in the present study did not give a species related variation in the hemagglutinating activity or coaggregation.

Rapid differentiation between the two species is of interest due to a suggested closer relation of *P. intermedia* to pathological conditions in the oral cavity such as periodontal disease and endodontic infections [6,12]. However, even if this association is statistically proven, it may simply be due to environmental factors in the periodontal pocket favoring the establishment of *P. intermedia*. In an experimental study in wound chambers in rabbits a similar abscess formation capability and recovery rate from the abscess was obtained for *P. intermedia* and *P. nigrescens* and the pathological difference between the two species was questioned [17].

It is therefore concluded that despite genetic and serological differences there is still a lack of clear phenotypic differences disclosed for the rapid identification of *P. intermedia* and *P. nigrescens*.

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