Biochemical and genetic characterization of a *Prevotella intermedia/nigrescens*-like organism

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Thirty-three previously non-typable faintly pigmented Gram-negative anaerobic bacterial isolates, biochemically most closely related to *Prevotella intermedia* and *Prevotella nigrescens*, were analysed for enzymic reactions, cellular fatty acid (CFA) composition, electrophoretic mobility of malate and glutamate dehydrogenases, hybridization with *P. intermedia* and *P. nigrescens* species-specific oligonucleotide probes and, for genetic heterogeneity, by arbitrarily primed PCR (AP-PCR). *P. intermedia* ATCC 25611^T and *P. nigrescens* ATCC 33563^T were run in parallel for comparison. Twenty-nine isolates originated from the normal oral flora of 18 subjects (including five mother–child pairs), and four isolates from various infections. Except for a negative lipase reaction, enzymic profiles of the test isolates were similar to those of *P. intermedia* and *P. nigrescens*. Clustering of CFAs, electrophoretic mobility patterns, hybridization with DNA probes for *P. intermedia* and *P. nigrescens*, and AP-PCR band patterns of the test isolates differed from those of the type strains of *P. intermedia* and *P. nigrescens*, suggesting the existence, in humans, of a new anaerobic species of pigmented, moderately saccharolytic, indole-positive Gram-negative rods.

Keywords: *Prevotella intermedia/nigrescens*-like organism, fatty acid profiles, arbitrarily primed PCR, multilocus enzyme electrophoresis, oral micro-organisms

INTRODUCTION

During a recent study on the oral anaerobic microflora in young children and their mothers (17, 18) we occasionally isolated Gram-negative, faintly pigmented bacterial strains that did not clearly belong to any known species. The isolates shared several biochemical characteristics with *Prevotella intermedia* and *Prevotella nigrescens* (28). For instance, our isolates were indole-positive and displayed special-potency antimicrobial disk profiles, carbohydrate fermentation profiles and short-chain fatty acid profiles of metabolic end products similar to *P. intermedia* and *P. nigrescens*. However, some phenotypic characteristics consistently differed from those of *P. intermedia* and *P. nigrescens*: a negative lipase reaction on egg yolk agar and weak pigmentation (buff to tan) on rabbit laked blood

(RLB) agar. We tentatively called this new species/subspecies candidate a *P. intermedia/nigrescens*-like organism (PINLO).

The separation of the serologically and genetically heterogenic *P. intermedia* (previously *Bacteroides intermedius*) (3, 11, 13) into two species, *P. intermedia* and *P. nigrescens*, was confirmed based on differences in DNA–DNA homology and mobilities of the metabolic enzymes malate dehydrogenase (MDH) and glutamate dehydrogenase (GDH) in multilocus enzyme electrophoresis (MLEE) (28). To separate phenotypically closely related *P. intermedia* and *P. nigrescens* from each other we have successfully used genotypic methods, i.e. hybridization with species-specific oligonucleotide probes and the arbitrarily primed PCR (AP-PCR) (21, 22).

The *P. intermedia/nigrescens* group can be found in the oral cavity as well as extra-oral sites in both healthy and diseased conditions (6, 15, 16, 21, 30). However, some differences may exist within this group: *P. intermedia* has been reported in association with oral

Abbreviations: AP-PCR, arbitrarily primed PCR; CFA, cellular fatty acid; GDH, glutamate dehydrogenase; MDH, malate dehydrogenase; MLEE, multilocus enzyme electrophoresis; PINLO, *P. intermedia/nigrescens*-like organism.

infections, such as periodontitis, more frequently than *P. nigrescens* (3, 9, 10, 22), whereas *P. nigrescens* seems to be more common in children than *P. intermedia* (9, 22). According to our preliminary reports (17, 18) PINLO was detected from the oral cavities of adult women in whom its presence was associated with periodontal health, as well as from the oral cavities of young children. Certain enzymic characteristics among bacterial populations are of clinical interest. Both *P. intermedia* and *P. nigrescens* are capable of totally degrading human immunoglobulin A1 (IgA1); this may play a role in oral infections (8). We have also observed high frequencies of β -lactamase activity among oral pigmented *Prevotella* species isolated from young children (19).

The aim of the present study was to characterize our PINLO isolates and to clarify the taxonomic position of PINLO by using a comprehensive approach. This characterization included the semiquantitative determination of preformed enzymes, the determination of IgA1 degradation and β -lactamase production, cellular fatty acid (CFA) analysis, determination of MDH and GDH electrophoretic mobilities, and hybridization with *P. intermedia* and *P. nigrescens* species-specific oligonucleotide probes. In addition, AP-PCR was used in species identification as well as to examine the genetic heterogeneity among our PINLO isolates.

METHODS

Bacterial strains. Altogether 33 oral isolates were available from 23 mother-child pairs which had been previously examined (17, 18); 13 PINLO isolates from oral surfaces of nine young children and 16 PINLO isolates from saliva of nine mothers (in five cases PINLO was simultaneously isolated from the mother and her child). The samples were cultured as described previously (16). In addition, four suspected PINLO candidates originating from various infections of four unrelated subjects were included in the present study; two strains were from peri-implantitis (AHN 12313 and AHN 18349), one strain was from a patient with osteomyelitis of the mandible (AHN 18857), and one isolate was from a pulmonary aspirate (AHN 10991 = WAL 11083). *P. intermedia* ATCC 25611^T and *P. nigrescens* ATCC 33563^T were used for comparison. The isolates were revived from frozen stocks (-70°C) and subcultured for further analyses.

Enzymic profiling. The profiles of 19 preformed bacterial enzymes were semiquantitatively determined using commercial API ZYM kits (bioMérieux) according to the manufacturer's instructions. Colour reactions were interpreted after incubation of the kits in air at 37°C for 4 h. A negative α -fucosidase reaction was confirmed by using individual chromogenic Rosco Diagnostic tablets (Rosco Diagnostica) (5). To examine IgA1 degradation, human dimeric IgA1 (Kah) was isolated from the serum of a patient with multiple myeloma as previously described (7). Bacterial colonies from agar plates were suspended in $40\ \mu\text{l}$ of a $1\ \text{mg}\ \text{ml}^{-1}$ solution of IgA1 and tested for degradation of IgA1 after 1, 4, 24 and 48 h by immunoelectrophoresis (14). β -Lactamase activity was tested by using the chromogenic cephalosporin disk test (AB BIODISK) (25).

CFA analysis. CFA compositions were identified by using the computerized database library compiled by Microbial Identification System (MIS; Microbial ID, Inc.), Moore 3.8 or 3.9 version as a reference. Briefly, a loopful of isolated colonies was inoculated into tubes containing 10 ml pre-reduced peptone/yeast extract/glucose broth in an anaerobic chamber. The tubes were incubated at 37°C for 2–3 d. After centrifugation the supernatant was discarded and the sedimented cells were processed by saponification, methylation, extraction and basic wash as described by Moore *et al.* (24). The CFAs were separated by a HP 5890A gas chromatograph equipped with a $25\ \text{m} \times 0.2\ \text{mm}$ fused silica capillary column and flame ionization detector (Hewlett-Packard). In addition, a combined GLC-MS was used to confirm the identity of certain CFAs. A two-dimensional plot and a dendrogram in Euclidian Distance (ED) scale were generated by the cluster analysis of the quantitative CFA profiles included in the MIS database library.

Multilocus enzyme electrophoretic analysis. Electrophoretic mobilities of MDH and GDH were analysed using polyacrylamide gels in non-denaturing conditions with slight modifications of a previously described method (22). Bacterial cells from 2–3 d-old Brucella agar cultures suspended in HEPES buffer were disrupted by sonication and centrifuged. The supernatant was electrophoresed in polyacrylamide gel consisting of 4% stacking gel and 10% running gel for MDH or 7.5% running gel for GDH. The gels were run at 100 V for 2 h (MDH) or at 150 V for 3 h (GDH) in Tris base/glycine buffer using a Mini-Protein II electrophoresis apparatus (Bio-Rad). MDH was visualized in a solution containing malic acid ($2\ \text{mg}\ \text{ml}^{-1}$), and GDH in a solution containing glutamic acid ($20\ \text{mg}\ \text{ml}^{-1}$). All chemicals were from Sigma.

Hybridization with *P. intermedia* and *P. nigrescens* species-specific oligonucleotide probes. The chromosomal DNA from *P. intermedia*, *P. nigrescens* and PINLO cultures grown for 3–4 d was extracted using the method of Moncla *et al.* (23) with our modification (27). Hybridizations with *P. intermedia* and *P. nigrescens* species-specific oligonucleotide probes were performed as previously described (22). Briefly, target DNA was transferred to nylon membrane, fixed by UV irradiation and hybridized at 49°C separately with plasmid pKK3535 (1) and with *P. intermedia* and *P. nigrescens* species-specific oligonucleotide probes synthesized according to the 16S rRNA sequence data published by Dix *et al.* (4): *P. intermedia*, 5'-GGT CCT TAT TCG AAG GGT AAA TGC-3' (corresponding to 1Bi-1); and *P. nigrescens*, 5'-ATG AGG TAC ATG CAA TGG CGC ACA-3' (2Bi-1) (2). The probes were labelled with the DIG oligonucleotide tailing kit and the colorimetric detection was performed using the DIG DNA detection kit (Boehringer Mannheim).

AP-PCR analysis. Five microlitres of diluted chromosomal DNA (approx. 5 ng) were used as template DNA in a $50\ \mu\text{l}$ reaction volume consisting of $0.2\ \text{mM}$ each dNTP (Pharmacia Biotech), $0.5\ \mu\text{M}$ primer, $10\ \text{mM}$ Tris/HCl pH 8.3, $50\ \text{mM}$ KCl, $4\ \text{mM}$ MgCl_2 and $2.5\ \text{U}$ AmpliTaq DNA polymerase (Perkin Elmer Cetus). The random sequence oligonucleotides OPA-13 (5'-CAGCACCCAC-3') and OPA-03 (5'-AGTCAGCCAC-3') were chosen from the five tested primers (OPA-03, OPA-05, OPA-07, OPA-13, OPA-17) to be used as primers in AP-PCR. The temperature profile in a thermocycler (Perkin Elmer Cetus) was 35 cycles as follows: 94°C for 1 min, 32°C for 2 min, 72°C for 2 min. The initial denaturation was at 94°C for 5 min and final extension at

72 °C for 5 min. Amplification products were analysed electrophoretically in 1% agarose gel containing 0.5 µg ethidium bromide ml⁻¹ and photographed under UV illumination using the Polaroid MP4 system with 667 film. A DNA ladder (1 kb) (Gibco BRL, Life Technologies) was used as a molecular mass standard in gel electrophoresis.

RESULTS

Table 1 summarizes the key phenotypic characteristics of *P. intermedia* ATCC 25611^T, *P. nigrescens* ATCC 33563^T and our 33 PINLO isolates.

Enzymic reactions

API ZYM patterns of the examined PINLO isolates were indistinguishable from those of *P. intermedia* and *P. nigrescens* reference strains. In five cases α-fucosidase remained negative by the API ZYM system but a parallel α-fucosidase test by Rosco tablets confirmed positive reactions. All isolates induced total degradation of IgA1 as revealed by absence of precipitation lines in immunoelectrophoresis. For most isolates complete degradation was observed after incubation for 24 h and, for the remaining isolates, after incubation for 48 h (and for one isolate 72 h). β-Lactamase production was more frequent among the strains from children than among those from the mothers; β-lactamase-positive strains were found in 7/9 children but only in 3/9 mothers (Table 2).

CFA composition and cluster analysis

Numerical CFA analysis of the 33 PINLO isolates revealed anteiso-C_{15:0}, iso-C_{15:0}, iso-3-OH iso-C_{17:0} (summed feature 11; identity confirmed by GLC-MS) in quantitative order, and also an unknown CFA with an equivalent chain-length (ECL) of 13.570 (summed

feature 3), as major CFAs (> 10% of the total CFAs) (Table 3). The quantitative CFA pattern resembled the corresponding pattern of *P. nigrescens* ATCC 33563^T. The CFA analysis gave several alternatives for the identification of the examined PINLO isolates (Table 2). Despite this incoherent identification, most of the PINLO isolates formed a homogeneous group in the cluster analyses, differing from *P. intermedia* ATCC 25611^T and *P. nigrescens* ATCC 33563^T (Figs 1, 2). Five PINLO isolates (AHN 8792, 9778, 10006, 10991 and 12313; Fig. 1) clustering apart from the main cluster had a higher proportion of iso-C_{15:0} (range 20.3–26.5%) than the other PINLO isolates (range 7.7–17.6%) in the main cluster.

Electrophoretic migration of MDH and GDH

MLEE patterns of *P. intermedia* ATCC 25611^T, *P. nigrescens* ATCC 33563^T, and PINLO differed from each other (Table 2). *P. intermedia* showed faster MDH and GDH mobilities and *P. nigrescens* slower MDH and GDH mobilities than PINLO isolates. One PINLO isolate (AHN 18349) showed slightly different MDH mobility (M3; Table 2) from the other 32 PINLO isolates which had similar MDH mobilities. Two GDH groups with intermediate mobilities in relation to *P. intermedia* and *P. nigrescens* were found among the examined PINLO isolates: the faster GDH group of PINLO isolates (G2) differed only slightly from the GDH group of the type strain of *P. intermedia* (G1) as seen in Fig. 3.

Species-specific oligonucleotide probing

All isolates hybridized with the universal pKK3535 probe, and the type strains of *P. intermedia* and *P. nigrescens* hybridized with corresponding species-specific probes. The examined PINLO isolates did not hybridize with either *P. intermedia* or *P. nigrescens* species-specific oligonucleotide probes, thus supporting a separate species status for PINLO.

AP-PCR

AP-PCR with oligonucleotide OPA-13 revealed a common band in the 2.4 kb region for all 33 PINLO isolates, whereas 0.9 kb or 1.3 kb bands, typical for *P. intermedia* and *P. nigrescens*, respectively (21), were not found (Fig. 4). Among the 29 examined isolates from nine mothers and nine children, the AP-PCR amplification using primers OPA-03 and OPA-13 revealed 10 different AP-PCR profiles each. When the results with both primers were combined, a total of 14 clearly different AP-PCR banding patterns was obtained. In addition, two AP-PCR types, each in two unrelated subjects, were so closely related that they could not be definitely separated (banding patterns 1 + and 1 −, and 7 + and 7 −; Table 2). In six subjects, two to four PINLO isolates were available but only one AP-PCR type was found per subject. In the five related mother-child pairs from whom PINLO was simul-

Table 1. Key characteristics of 33 PINLO isolates from 22 subjects compared with the reference strains *P. intermedia* ATCC 25611^T and *P. nigrescens* ATCC 33563^T

	PINLO	ATCC 25611 ^T	ATCC 33563 ^T
Pigment on RLB agar*	Buff/tan	Black	Black
Colistin sensitivity	+	+	+
Lipase reaction*	−	+	+
Indole production	+	+	+
Sugar fermentation			
Glucose	+	+	+
Lactose	−†	−	−
Sucrose	+	+	+
Major metabolic end products in GLC	A, S‡	A, S	A, S

* Follow-up to 10 d.

† Except two lactose-fermenting isolates from the same mouth.

‡ A, Acetic acid; S, succinic acid.

Table 2. Summary of present results

Strain	Source*	Identification proposed by MIS (first choice)	Electrophoretic mobility group†		AP-PCR group	β-Lactamase activity
			MDH	GDH		
ATCC						
25611 ^T		<i>P. intermedia</i>	M1	G1		
33563 ^T		<i>P. nigrescens</i>	M4	G4		
AHN‡						
8418	m1	<i>P. nigrescens</i>	M2	G2	4	—
9153	m1	<i>P. intermedia</i>	M2	G2	4	—
10371	c2	<i>P. nigrescens</i>	M2	G3	13	+
8275	m3	<i>B</i> D28§	M2	G3	1—	—
8441	m6	<i>P. nigrescens</i>	M2	G2	3	+
8401	c6	<i>P. nigrescens</i>	M2	G2	3	+
8404	c6	<i>P. intermedia</i>	M2	G2	3	+
8431	c6	<i>P. veroralis</i>	M2	G2	3	+
8792	c8	<i>B</i> D28	M2	G3	9	+
8964	m11	<i>P. nigrescens</i>	M2	G2	2	—
8858	c11	<i>P. nigrescens</i>	M2	G2	6	+
8977	c11	<i>P. nigrescens</i>	M2	G2	6	+
9283	m14	<i>P. veroralis</i>	M2	G2	5	—
9301	c14	<i>B</i> D28	M2	G2	1+	+
9419	m15	<i>P. nigrescens</i>	M2	G2	7—	—
9423	m15	<i>P. nigrescens</i>	M2	G2	7—	—
9424	m15	<i>P. nigrescens</i>	M2	G2	7—	—
9425	m15	<i>P. nigrescens</i>	M2	G2	7—	—
9517	c15	<i>P. nigrescens</i>	M2	G3	11	+
9738	m19	<i>P. nigrescens</i>	M2	G2	10	—
9739	m19	<i>B</i> D28	M2	G2	10	—
9741	m19	<i>P. nigrescens</i>	M2	G2	10	—
9778	m19	<i>P. tannerae</i>	M2	G2	10	—
9761	c19	<i>P. nigrescens</i>	M2	G2	10	—
9793	c19	<i>P. nigrescens</i>	M2	G2	10	—
9899	m20	<i>P. nigrescens</i>	M2	G2	12	+
9954	c21	<i>B</i> D28	M2	G2	7+	—
10006	m22	<i>P. tannerae</i>	M2	G2	14	+
10290	c23	<i>P. veroralis</i>	M2	G3	8	+
12313	Peri-implantitis	<i>P. tannerae</i>	M2	G2		—
18349	Peri-implantitis	<i>P. nigrescens</i>	M3	G3		—
18857	Osteomyelitis	<i>P. intermedia</i>	M2	G2		+
10991	Pulmonary aspirate	<i>P. tannerae</i>	M2	G3		—

* m, Mother; c, child; numbers refer to the 23 mother–child pairs originally examined (16, 17).

† Mobility groups are presented in numerical order; the fastest mobility group = 1.

‡ AHN, Anaerobe Helsinki Negative.

§ *B* D28, *Bacteroides* D28 [currently *Prevotella tanneriae* (indole-negative)].

|| *Prevotella veroralis* (indole-negative).

taneously detected, two mother–child pairs each shared a similar AP-PCR type (Figs 4 and 5; Table 2).

DISCUSSION

In the present study using several biochemical and genetic methods, the examined 29 isolates from normal

oral flora and four isolates from various infections showed characteristics that clearly separated them from both *P. intermedia* and *P. nigrescens*. In fact, the differentiation of PINLO from the phenotypically similar *P. intermedia* and *P. nigrescens* was simpler than the differentiation between *P. intermedia* and *P. nigrescens*. The key phenotypic characteristics for this

Table 3. Main CFAs (> 3% of total CFAs) of PINLO isolates, *P. intermedia* ATCC 25611^T and *P. nigrescens* ATCC 33563^T

CFA	PINLO (n = 33)*	ATCC 25611 ^T †	ATCC 33563 ^T ‡
Iso-C _{14:0}	6.6 ± 2.1	11.1	10.0
Iso-C _{15:0}	14.4 ± 4.5	10.5	16.3
Anteiso-C _{15:0}	33.6 ± 2.0	27.6	31.6
C _{16:0}	3.8 ± 1.3	2.2	2.4
UN 13:570‡	10.8 ± 2.1	0.9	7.1
Iso-3-OH C _{17:0} §	13.5 ± 2.5	21.5	11.8

* Mean % ± SD.

† Mean % of two runs.

‡ Summed feature 3.

§ Summed feature 11 (CFA confirmed by GLC-MS).

Fig. 2. Two-dimensional plot of 33 PINLO isolates (●), and the reference strains *P. intermedia* ATCC 25611^T (◆) and *P. nigrescens* ATCC 33563^T (■), generated by principal component analysis of CFA profiles.

Fig. 1. Dendrogram of 33 PINLO isolates, and the reference strains *P. intermedia* ATCC 25611^T and *P. nigrescens* ATCC 33563^T generated by cluster analysis of CFA profiles.

Fig. 3. GDH mobilities of *P. intermedia* ATCC 25611^T, *P. nigrescens* ATCC 33563^T and PINLO. Lanes 1 and 2, PINLO (mobility group G2); lane 3, *P. intermedia* ATCC 25611^T (G1); lane 4, *P. nigrescens* ATCC 33563^T (G4); lanes 5 and 6, PINLO (G3); lanes 7 and 8, PINLO (G2).

Fig. 4. AP-PCR profiles obtained using primer OPA-13. Lanes 1–10, PINLO isolates from five mother–child pairs; lanes 11 and 12, PINLO isolates from two unrelated mothers; lane 13, *P. intermedia* ATCC 25611^T; lane 14, *P. nigrescens* ATCC 33563^T; M, molecular mass standard. Note a common band of 2.4 kb among the PINLO isolates.

Fig. 5. AP-PCR profiles of PINLO isolates obtained using primer OPA-03. Lanes 1–12 as in Fig. 4; M, molecular mass standard.

weakly pigmented Gram-negative anaerobic species are the positive indole reaction but negative lipase reaction. The reported negative lipase reaction for *P. nigrescens* ATCC 33563^T (= NCTC 9336) (26, 28) does not apply to clinical strains or to the type strain of *P. nigrescens* according to our own experience or other reports (9, 22). The discrepancy between the results may be due to a different follow-up time for the lipase reaction. It is notable that the inability to ferment lactose does not definitely discriminate *P. intermedia*, *P. nigrescens* or PINLO from other pigmented *Prevotella* species, since occasional lactose-fermenting strains belonging to the *P. intermedia/nigrescens* group (3, 9) as well as PINLO can be found.

PINLO shared an identical API ZYM enzyme pattern with *P. intermedia* and *P. nigrescens*. API ZYM enzyme reactions have no value in separating *P. intermedia* and *P. nigrescens* from each other, but a positive α -fucosidase reaction together with a positive indole reaction (spot test) reliably separates *P. intermedia* and *P. nigrescens* from other related species (5). It is notable that testing α -fucosidase activity with an individual tablet substrate revealed more positive reactions than testing with API ZYM.

CFA profiling has shown its value as a technically easy and reproducible method for taxonomic studies of several Gram-negative anaerobic species (24). In the present study, the quantitative CFA pattern of PINLO isolates resembled the CFA pattern of *P. nigrescens*, while *P. intermedia* produced a higher proportion of iso-3-OH-C_{17:0} but a lower proportion of an unknown CFA with an equivalent chain-length of 13.570 than *P. nigrescens* and PINLO. This result could refer to a closer relationship of PINLO to *P. nigrescens* than to *P. intermedia*. However, the more advanced methods, namely MLEE, species-specific oligonucleotide probes and AP-PCR, that have been used for differentiation of *P. intermedia* and *P. nigrescens* (21, 22, 26, 28), did not identify PINLO isolates as *P. intermedia* or as *P. nigrescens*. The enzymic mobility patterns of our PINLO isolates located between the fastest mobility group of *P. intermedia* and the slowest mobility group of *P. nigrescens*. Interestingly, two different GDH mobility groups were formed for the PINLO isolates

suggesting even greater heterogeneity among this group. The interpretation of MDH and, especially GDH mobilities can be difficult because of only slight differences between the bands of *P. intermedia/P. nigrescens*/PINLO. According to Frandsen *et al.* (8), the current use of only two enzymes (MDH and GDH) for the species differentiation between *P. intermedia* and *P. nigrescens* may not be sufficient, as some overlapping in electrophoretic mobilities can be observed, and therefore the use of MLEE analysis for identification should be based on several enzymes.

By using OPA-13 as a primer in AP-PCR, Mättö *et al.* (21) found a common band in the region 0.9 kb for *P. intermedia* and in the region 1.3 kb for *P. nigrescens*. In the present study, a common band was observed for the examined PINLO isolates in the region 2.4 kb, but more data are needed to show whether this band is a species-specific amplicon and applicable for routine identification of PINLO.

In the present study we combined the AP-PCR results revealed by two primers (OPA-03 and OPA-13), and thus greater heterogeneity was obtained than by using just one primer. Inter-individual genetic heterogeneity of PINLO was confirmed, as at least 14 different AP-PCR types were detected from oral samples from 18 subjects. The finding is consistent with the previously observed inter-individual heterogeneity of other oral pigmented *Prevotella* species (*Prevotella melaninogenica*, *P. intermedia*, *P. nigrescens*) (20–22). Among the five mother–child pairs that harboured PINLO in the oral cavity, in two cases a mother and her child shared an identical AP-PCR type, thus indicating a vertical transmission of this organism between these related subjects. On the other hand, the clonal diversity among PINLO seemed to be limited intra-individually; only a single AP-PCR type was found even when several isolates from the same mouth (two to four isolates in six subjects each) were available for analysis. According to our previous results revealed by ribotyping or AP-PCR, more than one strain of *P. nigrescens* and *P. intermedia* can simultaneously colonize the oral cavity (21, 22). The simultaneous colonization of the oral cavity by several strains of the same species was even more obvious with another frequently found pigmented Gram-negative anaerobic rod, *P. melaninogenica* (20). However, the limited number of PINLO isolates per subject may have influenced this unexpected finding.

Certain oral pigmented Gram-negative anaerobes (previously black-pigmented *Bacteroides* spp.) have been intensively studied during the past two decades because of their suspected connection with destructive periodontal diseases (3, 29, 30). Despite the research interest, hardly any observations of bacteria resembling PINLO are to be found in the pertinent literature. One explanation may be the selective lifting of dark-pigmented colonies for further identification in which case the faint pigmentation of PINLO may have been overlooked. Another possibility is the absence of PINLO in periodontally diseased sites (17, 18). Re-

cently Guillot & Mouton (12) described new PCR-DNA probe assays for the specific identification and detection of *P. intermedia* and *P. nigrescens*, and reported one strain that could not be assigned to either *P. intermedia* or *P. nigrescens* despite similar biochemical reactions. Pearce *et al.* (26) reported two atypical strains with intermediate electrophoretic mobilities of MDH and GDH among *P. intermedia* and *P. nigrescens* strains; one of these strains was lipase-negative resembling PINLO. Further characteristics that would allow better comparison were not provided and furthermore, no detailed clinical data or information on the origin of these strains were available. Despite the lack of information on PINLO in the literature, it seems to be a common colonizer of the oral cavity; our original material consisted of 23 mother-child pairs of whom nine mothers and nine children were culture-positive for PINLO (17, 18). Besides these PINLO isolates originating from the 'normal oral microflora' in the present material, we had some isolates from severe oral and extra-oral infections indicating the opportunistic pathogenic nature of PINLO. The ability to degrade human IgA1 totally may act as a potential virulence factor for PINLO as well as for *P. intermedia* and *P. nigrescens* (8). Our observation that oral PINLO isolates, especially from children, frequently produced β -lactamase may have clinical significance when treating paediatric anaerobic infections of oral origin.

In agreement with our previous observations on the weak colony pigmentation and negative lipase reaction, the present comprehensive investigation including CFA cluster analyses, MDH and GDH electrophoretic mobilities, AP-PCR amplification as well as negative hybridization with *P. intermedia* and *P. nigrescens* species-specific oligonucleotide probes, indicates that PINLO seems to form a new pigmented, moderately saccharolytic, indole-positive species different from *P. intermedia* and *P. nigrescens*. Other nucleic-acid-based methods, e.g. DNA-DNA hybridization or 16S rRNA sequencing coupled with more comprehensive enzyme mobility profiling are warranted to assess the exact taxonomic position of PINLO.

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