PCR-DNA Probe Assays for Identification and Detection of *Prevotella intermedia* Sensu Stricto and *Prevotella nigrescens*

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The purpose of this study was to construct PCR-DNA probe assays specific for *Prevotella intermedia* sensu stricto and *Prevotella nigrescens* based on the ability of randomly amplified polymorphic DNA (RAPD) fingerprinting to generate species-specific markers. The strategy included four steps: (i) construction of first-generation DNA probes from a 850-bp RAPD marker for *P. intermedia* sensu stricto and a 1,300-bp RAPD marker for *P. nigrescens*, (ii) cloning and sequencing of each RAPD marker, (iii) designing of primer pairs flanking specific internal sequences of 754 bp for *P. intermedia* sensu stricto and of ca. 1,100 bp for *P. nigrescens*, and (iv) synthesis (by PCR amplification) and digoxigenin labeling of quantities of DNA probes 754 and ca. 1,100 bp in size. The PCR-DNA probe assays combine either PCR amplification of a 754-bp specific sequence in the genomic DNA of strains of *P. intermedia* sensu stricto and hybridization with the 754-bp digoxigenin-labeled probe or amplification of a ca. 1,100-bp sequence of *P. nigrescens* and hybridization with the ca. 1,100-bp probe. Specific hybridization was observed with the amplified DNAs from 25 strains of *P. intermedia* and 24 strains of *P. nigrescens*, and no reaction was observed with the PCR products from 20 foreign species. The PCR-DNA probe assays described here should allow a highly specific and sensitive detection of *P. intermedia* sensu stricto and *P. nigrescens* in mixed infections.

Prevotella intermedia sensu lato (formerly *Bacteroides intermedius*) is a gram-negative anaerobic rod which has been associated with both oral and extraoral infections (16, 25, 33) and has also been reported as being part of the healthy gingival flora (18). Heterogeneity within *P. intermedia* sensu lato had long been demonstrated (9, 13, 17, 30) when Shah and Gharbia (31) proposed recognizing the two homology groups of Johnson and Holdeman (17) as two species: *P. intermedia* sensu stricto, corresponding to homology group VPI 4197, and *Prevotella nigrescens*, corresponding to homology group VPI 8944. As no discriminating tests were available, information from cultural studies concerning the ecology and respective roles of *P. intermedia* sensu stricto and *P. nigrescens* in periodontal disease was limited and partly controversial. An association between *P. intermedia* sensu stricto and periodontal disease (3, 10, 23) has been reported, whereas *P. nigrescens* appears to colonize healthy gingivae and endodontic sites (14), suggesting differences in pathogenic potential. These data contrast with those in a report by Moore et al. (26) in which homology group VPI 4197, now *P. intermedia* sensu stricto, was preferentially associated with healthy sites or shallow pockets whereas a statistically significant association between homology group VPI 8944, now *P. nigrescens*, and active, deeper periodontitis sites was observed, leading to the recognition of this species as one of the major pathogens of adult periodontitis. Both species share many phenotypic traits (20), making speciation difficult to perform. To separate the two species, one must resort to one of the following tests or techniques: DNA-DNA hybridization (17), serotyping with monoclonal antibodies (5, 12), enzyme electrophoresis of malate and glutamate dehydrogenases (31), sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of soluble cellular proteins (10), restriction fragment

length polymorphism analysis (8), hybridizations using 16S rRNA probes (6, 32), ribotyping (4, 28), or randomly amplified polymorphic DNA (RAPD) fingerprinting (22, 27).

Molecular methods, in particular those combining nucleic acid probes with PCR for added sensitivity, for detection of periodontopathic bacteria, including *P. intermedia* sensu stricto and *P. nigrescens*, would thus provide a significant advantage to the dental community, researchers and clinicians as well. In the present paper, we report on the construction of two PCR-DNA probe assays specific for *P. intermedia* sensu stricto and *P. nigrescens* based on the capacity of the arbitrarily primed PCR (AP-PCR) (35) to generate species-specific markers as RAPDs (36) according to a strategy that was previously developed in our laboratory (15). We also report on the biological specificities and sensitivities of these assays.

A total of 50 strains (Table 1) were used in the present study and included as references the type strains ATCC 25611 (VPI 4197) of the species *P. intermedia* sensu stricto and NCTC 9336 (= ATCC 33563, = VPI 8944 [31]) of the species *P. nigrescens*. The laboratory strains and clinical isolates, which were of diverse clinical and geographical origin, were classified as *P. intermedia* sensu lato in our laboratory (27) by using a short set of presumptive tests and those of the Rapid ID32 A system (bioMérieux, Marcy l'Étoile, France): colonies are brown to black on enriched blood agar and show a red fluorescence under long-wave UV light; they are indole positive and do not produce catalase; and they are α -glucosidase positive and tryptin negative. The strains were grown to mid-log phase in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, Md.) enriched with hemin (10 μ g/ml) and vitamin K (1 μ g/ml). Stock cultures were maintained by plating the organisms on the same medium containing 1.5% Noble agar, 2% horse serum, and 2% laked human erythrocytes. All cultures were grown in an anaerobic atmosphere (80% N₂, 10% H₂, and 10% CO₂) at 37°C. All bacteria used as negative controls (Table 2) were grown by using appropriate media and conditions.

Extraction and purification of genomic DNA were per-

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TABLE 1. Characterization of 50 strains of *P. intermedia* sensu lato by MDH analysis and by PCR-DNA probe assays specific for *P. intermedia* sensu stricto and *P. nigrescens*

Strain	Dot blot position ^a	MDH electro-morph ^b	Result of PCR-DNA probe assay for ^c :		Strain	Dot blot position ^a	MDH electro-morph ^b	Result of PCR-DNA probe assay for ^c :	
			<i>P. intermedia</i> sensu stricto	<i>P. nigrescens</i>				<i>P. intermedia</i> sensu stricto	<i>P. nigrescens</i>
ATCC 25611	A1	Pi	+	—	NCTC 9336	A1	Pn	—	+
A 15/3	A2	Pi	+	—	5W2	A2	Pn	—	+
A 9/3	A3	Pi	+	—	BH 18/23	A3	Pn	—	+
B 192	A4	Pi	+	—	Cg 1265	A4	Pn	—	+
BH 20/30	A5	Pi	+	—	MS/9b/918	A5	Pn	+	+
DNE	B1	Pi	+	—	R 102	B1	Pn	—	+
SUNYaB G8-9K3	B2	Pi	+	—	S19 g	B2	Pn	—	+
MM22-8	B3	Pi	+	—	YD22-4	B3	Pn	—	+
MS/5a/344	B4	Pi	+	—	VPI 8944-SA18	B4	Pn	—	+
MS/5b/367	B5	Pi	+	—	Dop 61	B5	Pn	—	+
NY 363	C1	Pi	+	—	Dop 2	C1	Pn	—	+
89A	C2	Pi	+	—	ALΣ	C2	?	—	—
R 22	C3	Pi	+	—	S1331 Lon	C3	Pn	—	+
SUNYaB 20-3	C4	Pi	+	—	S54 Gro	C4	Pn	—	+
VPI 4197-SA17	C5	Pi	+	—	S55 Gro	C5	Pn	—	+
SD	D1	Pi	—	+	S146 Dur	D1	Pn	—	+
S399 Char	D2	Pi	+	—	S289 Rot	D2	Pn	—	+
S1638 Bar	D3	Pi	+	—	MP31-1	D3	Pn	—	+
MS/5b/372	D4	Pi	+	—	MJMΣ1	D4	Pn	—	+
MS/7b/646	D5	Pi	+	—	MJMΣ2	D5	Pn	—	+
A 15/2	E1	Pi	+	—	S1297 Bla	E1	Pn	—	+
T2	E2	Pi	+	—	S1420 Bou	E2	Pn	—	+
MM22-5 brun	E3	Pi	+	—	T8	E3	Pn	—	+
MM22-5 noir	E4	Pi	+	—	T9	E4	Pn	—	+
JBΣ	E5	Pi	+	—	RSΣ	E5	Pn	—	+

^a Refers to the position in the dot blots of Fig. 6A and C.^b Pi, *P. intermedia* sensu stricto; Pn, *P. nigrescens*.^c +, positive; —, negative.^d Refers to the position in the dot blots of Fig. 6B and D.

formed by a standard miniprep procedure as described by Wilson (37). For production of DNA probes, quantities of each shared amplicon were prepared as follows. The AP-PCR products from four 25-μl amplifications (i.e., 100 μl) of the type strain *P. intermedia* ATCC 25611 or *P. nigrescens* NCTC 9336 were loaded in a single well of a preparative minigel electrophoresis apparatus and were electrophoresed as long as necessary to yield well-separated bands. The selected amplicon was excised from the agarose gel with a sterile razor blade on a UV transilluminator and then purified by using the Gene-Clean kit (Bio101) (BIO/Can, Mississauga, Ontario, Canada). The purified DNA was labelled by random priming with digoxigenin-dUTP (Boehringer, Mannheim, Germany) according to the manufacturer's instructions to yield the first-generation DNA probe.

Construction of PCR-DNA probe assays. The strategy that we used to construct the two PCR-DNA probe assays was identical to that previously described for the species *Bacteroides forsythus* (15) and included four steps.

(i) Construction of first generation DNA probes. The arbitrary primer OPA-13 (Operon Technologies, Alameda, Calif.) was used in the present work. This decamer (5'-CAGCACCC AC-3') was selected after a previous study (27), in which a total of 71 9- and 10-mer oligonucleotides were screened, showed that it had the capacity to reveal a genetic interspecific polymorphism among 69 strains of *P. intermedia* sensu lato. AP-PCR amplification reactions were done as described previously (15). The primer OPA-13 generated a ca. 900-bp amplicon which was present in the RAPD fingerprints of 13 *P. intermedia* sensu stricto strains and was absent from the fingerprints of 7

P. nigrescens strains and 20 strains of related and unrelated species that constituted the collection of negative controls (Fig. 1). Upon prolonged electrophoresis for 7 h in a 1% agarose gel, it appeared that the shared amplicon of ca. 900 bp could be

TABLE 2. Bacterial species used as negative controls in this study

Species	Strain	Lane ^a	Dot blot position ^b
<i>Actinobacillus actinomycetemcomitans</i>	ATCC 29522	21	D1
<i>Bacteroides fragilis</i>	ATCC 25285	22	D2
<i>Bacteroides levii</i>	ATCC 29147	23	D3
<i>Bacteroides merdae</i>	M-36	24	D4
<i>Bacteroides thetaiotaomicron</i>	ATCC 29741	25	D5
<i>Bacteroides vulgatus</i>	ATCC 8482	26	D6
<i>Campylobacter rectus</i>	ATCC 33238	27	D7
<i>Escherichia coli</i>	ATCC 33089	28	D8
<i>Fusobacterium nucleatum</i>	ATCC 10953	29	D9
<i>Porphyromonas asaccharolytica</i>	ATCC 25260	30	D10
<i>Porphyromonas circumdentaria</i>	3325	31	E1
<i>Porphyromonas endodontalis</i>	ATCC 35406	32	E2
<i>Porphyromonas gingivalis</i>	ATCC 33277	33	E3
<i>Porphyromonas salivosa</i>	3313	34	E4
<i>Prevotella oralis</i>	ATCC 33269	35	E5
<i>Prevotella oulori</i>	ATCC 43324	36	E6
<i>Pseudomonas aeruginosa</i>	ATCC 10145	37	E7
<i>Bacteroides macacae</i>	ATCC 33141	38	E8
<i>Streptococcus mutans</i>	ATCC 33534	39	E9
<i>Bacteroides forsythus</i>	ATCC 43037	40	E10

^a Refers to the lane of the gel shown in Fig. 1.^b Refers to the position in the dot blots of Fig. 5A and B.

FIG. 1. RAPD fingerprints obtained with primer OPA-13, generating shared amplicons of about 900 bp for *P. intermedia* sensu stricto strains (lanes 1 to 13) and of ca. 1,300 bp for *P. nigrescens* strains (lanes 14 to 20). The molecular size standard (M) is a 100-bp DNA ladder. Lanes: 1, ATCC 25611; 2, A 15/3; 3, A 9/3; 4, B 192; 5, BH 20/30; 6, DNE; 7, SUNYab G8-9K-3; 8, MM22-8; 9, R 22; 10, MS/5b/367; 11, NY 363; 12, 89A; 13, SUNYab 20-3; 14, NCTC 9336; 15, 5W2; 16, BH 18/23; 17, Cg 1265; 18, MS/9b/918; 19, R 102; 20, S19 g. Lanes 21 to 40 contain DNA from 20 strains of related and unrelated species (referred to in Table 2).

resolved into two adjacent bands of ca. 900 and 850 bp (Fig. 2). The same primer (OPA-13) yielded a fragment with an estimated size of 1,300 bp that appeared with strong intensity in the RAPD fingerprints of all seven of the *P. nigrescens* strains. This fragment was absent from the RAPD fingerprints of the 13 strains of *P. intermedia* sensu stricto and the 20 foreign species (Fig. 1). These three amplicons, with estimated sizes of 900 and 850 bp for *P. intermedia* sensu stricto and 1,300 bp for *P. nigrescens*, were retained as candidate species-specific markers. They were excised, purified, labelled, and used as first-generation DNA probes in dot blot hybridization tests as described previously (15) in order to verify their predicted specificities. The digoxigenin-labelled 850-bp amplicon hybridized with the genomic DNAs (1 µg of each spotted onto a Hybond N nylon membrane) from 13 strains of *P. intermedia* sensu stricto and was negative with the genomic DNAs from 7 *P. nigrescens* strains and 20 foreign species at the specified temperature of 67°C (data not shown). On the other hand, the digoxigenin-labelled 900-bp amplicon was not retained since it cross-hybridized with *P. nigrescens* strains. The DNA probe with an estimated size of 1,300 bp was specific for the species *P. nigrescens* and did not cross-hybridize with genomic DNAs

from 21 unrelated and related species, including 13 *P. intermedia* sensu stricto strains (data not shown).

(ii) **Cloning and sequencing of the species-specific markers.** The two species-specific amplicons of ca. 850 and 1,300 bp, respectively generated by AP-PCR from the reference type strains *P. intermedia* sensu stricto ATCC 25611 and *P. nigrescens* NCTC 9336, were cloned and sequenced as described previously (15). The sequence of the 831-bp species-specific marker for *P. intermedia* sensu stricto is listed in Fig. 3A. The sequence of the *P. nigrescens* species-specific marker was obtained as two small stretches (about 300 bp) at each end of the 1,300-bp cloned fragment (Fig. 3B). The GenBank and EMBL databases were searched for entries with homology to these nucleotide sequences and their derived amino acid sequences (1), but no significant degree of homology was shown.

(iii) **Designing of a primer pair flanking a specific internal sequence.** From the exact sequence obtained for each species-specific amplicon, the primer pair to be used in a PCR amplification was selected as described previously (15). The optimal upstream and downstream sequences were predicted with the help of the OLIGO primer analysis software (National Biosciences, Plymouth, Minn.), and selection of the efficient primers included an evaluation of their performance in PCR assays using a series of annealing temperatures to obtain the intended PCR product. The primer pair Pi 754-1 (5'-CAGCACCCAC AACGATATGA-3') and Pi 754-2 (5'-TTCCATCTTCTCTG CCTGTC-3'), flanking an internal sequence of 754 bp specific for *P. intermedia* sensu stricto, was selected. From the nucleotide sequences corresponding to each end of the 1,300-bp amplicon, we selected the primer pair Pn 1100-1 (5'-TTATG TTACCCGTTATGATGGAAG-3') and Pn 1100-2 (5'-ATGG CGAAATAGGAATGAAAGTTA-3'), which flanked an internal sequence with an estimated size of 1,100 bp specific for *P. nigrescens*. The optimal conditions for annealing were set at a temperature of 65°C for both *P. intermedia* sensu stricto and *P. nigrescens* PCR assays.

(iv) **Synthesis of the second-generation DNA probes by PCR amplification.** To obtain quantities of each second-generation DNA probe, 5-µl aliquots of the PCR products resulting from amplification of the genomic DNA of the *P. intermedia* type strain ATCC 25611 with the primer pair Pi 754-1–Pi 754-2 and from amplification of the genomic DNA of the *P. nigrescens* type strain NCTC 9336 with the primer pair Pn 1100-1–Pn 1100-2, respectively, were used. Labeling by random priming

FIG. 2. The shared amplicon of about 900 bp generated by AP-PCR with primer OPA-13 from *P. intermedia* sensu stricto strains can be separated by prolonged electrophoresis in 1% agarose as two adjacent bands of ca. 900 and 850 bp. M, 1-kb DNA ladder; lane 1, ATCC 25611.

A

5'CAGCACCCACAACGATATGATCTGCCACAGCTTTTGAACCATTTATGTTTCAGC
 ACTTACACTTGCCACAACATTATATCGTCCCATGGCAAATAGCCAACTTTTG
 NCTCAAGCCCGATATTGAACCTATCGTGCCCGTTATAGTGCAAGTCAAGACCT
 GTCAAAGAAGCTCCAAGTAGCCTTTACCTCTTGAAACTGTGCATGAATACC
 TAATGATAATGTTAATCCTAATAAAAGGAGGATAATTTCTTCATCATAATA
 ATACTGTTATGTAAAATCTATTATTGCTAAATTCATTGACAAAGGTAGT
 AGAAATATTGAATTACAAAAATTATGCTATCTTTGTGCTGATTGTCAA
 ATAAGTCTTTCGCTTCTTGCTTACAAATATGCTTTAAACGTATGAATAAAA
 AATTAATTACACTGATATTACTAAGCTGTCTTACGATAAATTGTTTGCACA
 AACGGGCGAAATATTATATAAATGGGATACAAAACCTTTCAAGAAACGTGA
 AACCCGTGCTGTTGGCTAACTACTTTGAACAATCTTGACTGGCCAAAGACAT
 TTGCAAACTCGGAGNAGGTATTGAAAGACAAAAGCAGGAACCTATTGATA
 TTTTATAGATAAATATGCTGCTGCAACATTAATACCGTTCTGTGCAACGCGT
 GTGCGTGCAGCCACCATTTATCCGTCCAACATAGAACCTTGGGACAAATGCTT
 GACAGGCAGAGAAGATGGAAATCCAACTACGACCCATTGGCATTTGCTGTT
 GAAGAATGCCATAAACGTGGGTGGAAATACATGCGTGGGTGCTG3'

B

5'CAGCACCCACACGGAACCGGATACGACCGGGGAAAGTTGGCTTTTATGTTAC
 CCGTTATGATGGAAGTAGTTGGCGTGTGTTGCTATAATCATGTGAATGCCT
 ACCGCACGCGCCAGCTGGCGGATACGGGTGATAGGCATTCAATTTCTTTTCCA
 GCGGTAAGGATAAGGCTCCGAACCTCATCAATGATGACAACGATGTACGGTA
 TGAATCGTGCTCTCCCGCAGGACTAAGCTGATGGCTAAGGAACTCTTGITA
 TACTCTTTTATATTGCGCGCTCTGTAG.....700bp.....AAATTAAGGGTGGATACT
 NCCAATTGGGAAAGGTTGCTGTGATTAAATAGGTGTTNCAAGGTCTGTCGT
 GCCTGCCACCCCTTACCTTGTGCTTTTCCAACCGACAGGACATTAACTTTC
 ATTCTATTTCGCCATTGGCAGCAGTCTCTTCATCGTTCTTTTCGCGGTGTTT
 TGTCTTAGCGTTAGGCTTTATCATGTTATTGGCAGGTATCGTCAAGAAAT
 TCCACCGTCTGGGACTCTGCATTCTGCAGCTTTTCTACTGCTTCTCTCCATTTC
 GTGGGTGCTG3'

FIG. 3. Nucleotide sequences of the specific RAPD fragments with an estimated size of 850 bp (A) and at both ends of the 1,300 bp RAPD fragment (B), which are identified by AP-PCR with primer OPA-13 in the species *P. intermedia* sensu stricto and *P. nigrescens*, respectively.

with digoxigenin-dUTP (Boehringer) was done according to the manufacturer's recommendations.

Biological specificities and sensitivities of the PCR-DNA probe assays. To assess the specificity of the PCR-DNA probe assay specific for *P. intermedia* sensu stricto, the primer pair Pi 754-1–Pi 754-2 was used to prime PCR amplification of genomic DNA from 13 strains of *P. intermedia* sensu stricto and 27 strains belonging to 21 unrelated and related species, including *P. nigrescens*. No amplification product from non-*P. intermedia* sensu stricto strains was detected by gel electrophoresis. A single PCR product of 754 bp was detected only in the 13 *P. intermedia* sensu stricto strains (Fig. 4A), indicating that the primer pair Pi 754-1–Pi 754-2 amplified a 754-bp DNA fragment specific for *P. intermedia* sensu stricto. The 754-bp second-generation probe was used to identify reactions yielding the intended PCR product after each PCR product (5 μ l) was immobilized on nylon membranes in dot blot formats for chemiluminescent detection as described previously (15). Strong and specific hybridization was observed with the amplified DNAs from 13 strains of *P. intermedia* sensu stricto. No cross-hybridization was found with the PCR products from 7 strains of *P. nigrescens* and 20 strains representing 20 related and unrelated species (Fig. 5A). To assess the specificity of the *P. nigrescens* PCR-DNA probe assay, the primer pair Pn 1100-1–Pn 1100-2 was used for PCR amplification of genomic DNA

from 7 strains of *P. nigrescens* and 33 strains belonging to 21 unrelated and related species, including *P. intermedia* sensu stricto. The expected 1,100-bp amplicon was present only in the 7 *P. nigrescens* strains (Fig. 4B) and was absent in all other strains belonging to 21 unrelated and related species, including *P. intermedia* sensu stricto. Surprisingly, a faint band adjacent to the 1,100-bp amplicon was observed. Hybridization of the second-generation probe derived from the 1,100-bp amplicon was species specific, giving a strong signal only with the amplified DNAs from seven strains of *P. nigrescens*. No hybridization signal was detected by dot blot analysis of DNAs from non-*P. nigrescens* strains (Fig. 5B).

To evaluate the sensitivity of each PCR-DNA probe assay, the lower detection limit of the template DNA was determined by dot blot hybridization. Genomic DNAs extracted from *P. intermedia* sensu stricto ATCC 25611 and *P. nigrescens* NCTC 9336 were serially diluted from 100 ng to 10 fg, respectively amplified with the primer pairs Pi 754-1–Pi 754-2 and Pn 1100-1–Pn 1100-2, and then dot blotted for hybridization with the species-specific second-generation probe. The limit of detection was 1 pg of template DNA for *P. intermedia* sensu stricto and 0.1 pg for *P. nigrescens* (data not shown), corresponding to genomic equivalents of about 100 and 10 bacteria, respectively.

Validation of the PCR-DNA probe assays. To confirm the capacities of the two PCR-DNA probe assays to discriminate *P. intermedia* sensu stricto from *P. nigrescens*, a total of 50 strains of *P. intermedia* sensu lato were typed for electrophoresis of the enzyme malate dehydrogenase (MDH) as described elsewhere (27). Two electrophoresis were observed: a fast-migrating MDH, characteristic of *P. intermedia* type strain ATCC 25611, and a slowly migrating band, characteristic of *P. nigrescens* type strain NCTC 9336 (data not shown). The MDH electrophoresis allowed the separation of our collection of 50 strains into two groups: 25 strains were typed as *P. intermedia* sensu stricto and 24 strains were typed as *P. nigrescens* (Table 1). A single strain, AL Σ , could not be typed by electrophoretic

FIG. 4. Specificity of PCR amplifications with the primer pairs specifically designed for *P. intermedia* sensu stricto and *P. nigrescens*. Specific amplifications of the 754-bp DNA fragment from *P. intermedia* sensu stricto strains (lanes 1 to 13) with the primer pair Pi 754-1–Pi 754-2 (A) and of the ca. 1,100-bp amplicon from *P. nigrescens* strains (lanes 1 to 7) with the primer pair Pn 1100-1–Pn 1100-2 (B) were performed. M, 100-bp DNA ladder.

FIG. 5. Dot blot analysis showing the specificity of the PCR-DNA probe assays for *P. intermedia* sensu stricto and *P. nigrescens*. (A) PCR products (5 μ l each) obtained with the primer pair Pi 754-1–Pi 754-2 and hybridized with the digoxigenin-labelled 754-bp second-generation probe; (B) PCR products (5 μ l each) obtained with the primer pair Pn 1100-1–Pn 1100-2 and hybridized with the digoxigenin-labelled 1,100-bp second-generation probe. Dots A1 to A10 and B1 to B3 contain PCR products from *P. intermedia* strains, dots C1 to C7 contain PCR products from *P. nigrescens* strains, and dots D1 to D10 and E1 to E10 contain PCR products from strains of related and unrelated species.

migration of MDH. All 50 strains were also subjected to the two PCR-DNA probe assays, one specific for *P. intermedia* sensu stricto (Fig. 6A and B), and the other specific for *P. nigrescens* (Fig. 6C and D). The dot blot experiments were conducted with a new material, Polymacron (NA Polymacron Rapitest kit; Kalyx Biosciences, Nepean, Ontario, Canada), as a solid phase for the rapid hybridization assays. We strictly followed the instructions in the manual supplied by the manufacturer and used the reagents furnished in the kit. Briefly, 5- μ l volumes of heat-denatured PCR products were spotted onto pre-cut, gridded sheets of Polymacron previously wetted in NA coating buffer and then fixed by exposure to UV light for 5 min. Each Polymacron sheet was prehybridized in 5 ml of universal blocking buffer for 30 min at 67°C and then hybridized with the denatured labelled DNA probe for 1 h in 5 ml of NA hybridization buffer. Three washes of 5 min each were performed at room temperature in wash buffer (10 mM Tris-HCl [pH 7.2], 0.85% NaCl, 0.05% [vol/vol] Tween 20). A colorimetric detection procedure was carried out following the instructions for the DIG nucleic acid detection kit (Boehr-

inger) except that the universal dilution buffer from Kalyx was used and that all washes were performed with the wash buffer. The macroporous nature of Polymacron increases the rapidity and ease of the assay; as a result, the procedure could be performed within 3 h instead of the 24 h required when using conventional nylon membranes.

With the exception of three strains, the two groups separated by the PCR-DNA probe assays matched the *P. intermedia*-*P. nigrescens* assignments resulting from electrophoretic migration of MDH (Table 1). Strain SD, which was typed as a *P. intermedia* sensu stricto electromorph, did not react with the PCR-DNA probe specific for *P. intermedia* sensu stricto (Fig. 6A, dot D1) but was detected by the PCR-DNA probe assay specific for *P. nigrescens* (Fig. 6C, dot D1). To further document this discordance, the RAPD fingerprint of strain SD, generated with the primer OPA-13, was rechecked; it was characteristic of a *P. nigrescens* pattern and did not reveal the shared amplicon with an estimated size of 900 bp specific for *P. intermedia* sensu stricto (27). Strain MS/9b/918, a *P. nigrescens* electromorph, reacted with both PCR-DNA probes (Fig. 6B

FIG. 6. Characterization of 50 strains by PCR-DNA probe assays specific for *P. intermedia* sensu stricto (A and B) or *P. nigrescens* (C and D) using Polymacron sheets. Dots on sheets A and B contained 5- μ l volumes of PCR products obtained with the primer pair Pi 754-1–Pi 754-2 and hybridized with the digoxigenin-labelled 754-bp second-generation probes from 25 *P. intermedia* strains (A) and 25 *P. nigrescens* strains (B). Dots on sheets C and D contained 5- μ l volumes of PCR products obtained with the primer pair Pn 1100-1–Pn 1100-2 and hybridized with the digoxigenin-labelled 1,100-bp second-generation probes from 25 *P. intermedia* strains (C) and 25 *P. nigrescens* strains (D). Dots F1 on sheets B and C contained are positive controls.

and D, dots A5). Strain ALΣ could not be assigned to any species by MDH migration or PCR-DNA probe assay (Fig. 6B and D, dots C2). It is apparent that further experiments are needed for accurate identification of strains SD, MS/9b/918, and ALΣ, which may belong to a third species distinct from *P. intermedia* sensu stricto and *P. nigrescens* (19).

Elucidation of the respective roles of *P. intermedia* sensu stricto and *P. nigrescens* in the etiology of periodontal diseases has awaited specific and sensitive methods of identification and detection which routine culture-based methods could not offer. This clearly calls for substitute bacteriological diagnostic procedures that are specific, sensitive, and fast, such as nucleic acid probe tests. The use of 16S rRNA oligonucleotide probes for the direct detection of *P. intermedia* and *P. nigrescens* in subgingival plaque samples has been reported by several groups (2, 6, 8, 23, 32). Although the hybridization techniques using oligonucleotide probes can be highly specific, their potential lack of practical sensitivity must be seen as a drawback (34), as target microbial populations of 10^4 bacteria or less in the sample usually cannot be detected accurately. The advent of PCR technology has provided new diagnostic opportunities, since the technique allows for sensitive detection of a given DNA fragment, present in a small number of copies, even in a complex mixture of molecules. Designing a diagnostic system that combines PCR amplification with subsequent probe detection can be seen as a desirable technological improvement to guarantee maximum sensitivity and specificity.

In this study, we propose the use of newly developed PCR-DNA probe assays for the specific and sensitive identification and detection of *P. intermedia* sensu stricto and *P. nigrescens* according to a strategy already applied to *B. forsythus* (15). This strategy can be seen as a universal method for designing detection assays for a multitude of microorganisms without the need for prior knowledge on the genetics of the target species. For this purpose, we have further exploited the potential offered by RAPD fingerprinting technology (35, 36) for the rapid design and construction of DNA probes that we (24) and others (7, 11, 21, 29) had explored. In its present format, the PCR-DNA probe assay which we propose consists of PCR amplification of a specific sequence in the genomic DNA of strains belonging to the target species followed by detection of the intended PCR product by dot blot hybridization with the species-specific second-generation probe. Enhanced specificity can be expected from this assay since it selects for a precise target DNA sequence at two stages: first, the target genomic DNA must have sequences complementary to the primers for PCR amplification under the selected stringency conditions, and second, the PCR product must contain the internal sequence for detection by hybridization. The results presented here demonstrate that the two PCR-DNA probe assays that we designed for *P. intermedia* sensu stricto and *P. nigrescens* are exquisitely specific as they do identify and detect their intended species selectively and thus discriminate between these two closely related species. The PCR amplification with the primer pair Pn 1100-1–Pn 1100-2 yielded a second, faint band adjacent to the 1,100-bp band. To account for this double band, we postulate that the target fragment is present in the genomic DNA as a duplicate and that each stretch differs in size slightly due to an insertion or deletion. Accordingly, AP-PCR with the short, 10-base primer OPA-13 would generate only one of the fragments, whereas longer PCR primers would amplify both of them. The 1,100-bp marker generated by PCR with the primer pair Pn 1100-1–Pn 1100-2 and used as a second-generation probe was specific for the species *P. nigrescens*.

Conclusion. Both PCR-DNA probe assays described here are rapid, sensitive, and specific detection procedures for ac-

curate discrimination between *P. intermedia* sensu stricto and *P. nigrescens*. Since the PCR-DNA probe assays allow the precise identification and detection of the species, they should constitute valuable tools for diagnostic and epidemiologic studies that would enhance the understanding of the respective roles of *P. intermedia* sensu stricto and *P. nigrescens* in the etiology of periodontal diseases and other oral infections.

Nucleotide sequence accession numbers. The GenBank accession number of the sequence of the 831-bp species-specific marker in *P. intermedia* ATCC 25611 is U66373. The GenBank accession numbers of the 5'-end sequence (292 bp) and the 3'-end sequence (300 bp) of the 1,300-bp species-specific marker in *P. nigrescens* NCTC 9636 are, respectively, U66374 and U66375.

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REFERENCES

- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- Conrads, G., and A. Brauner. 1993. Non-radioactively labelled DNA probes for the detection of periodontopathogenic *Prevotella* and *Porphyromonas* species. *FEMS Immunol. Med. Microbiol.* **6**:115–120.
- Dahlén, G., M. Wikström, S. Renvert, R. Gmür, and B. Guggenheim. 1990. Biochemical and serological characterization of *Bacteroides intermedium* strains isolated from the deep periodontal pocket. *J. Clin. Microbiol.* **28**:2269–2274.
- Dahlén, G. G., J. R. Johnson, and R. Gmür. 1996. *Prevotella intermedia* and *Prevotella nigrescens* serotypes, ribotypes and binding characteristics. *FEMS Microbiol. Lett.* **138**:89–95.
- Devine, D., M. A. Pearce, S. E. Gharbia, H. N. Shah, R. A. Dixon, and R. Gmür. 1994. Species specificity of monoclonal antibodies recognising *Prevotella intermedia* and *Prevotella nigrescens*. *FEMS Microbiol. Lett.* **120**:99–104.
- Dix, K., S. M. Watanabe, S. McArdle, D. I. Lee, C. Randolph, B. Moncla, and D. E. Schwartz. 1990. Species-specific oligodeoxynucleotide probes for the identification of periodontal bacteria. *J. Clin. Microbiol.* **28**:319–323.
- Fani, R., G. Damiani, C. Di Serio, E. Gallori, A. Grifoni, and M. Bazzicalupo. 1993. Use of random amplified polymorphic DNA (RAPD) for generating specific DNA probes for microorganisms. *Mol. Ecol.* **2**:243–250.
- Frandsen, E. V. G., K. Poulsen, and M. Kilian. 1995. Confirmation of the species *Prevotella intermedia* and *Prevotella nigrescens*. *Int. J. Syst. Bacteriol.* **45**:429–435.
- Fukushima, H., H. Moroi, J. Inoue, T. Onoe, T. Ezaki, E. Yabuuchi, K.-P. Leung, C. B. Walker, W. B. Clarck, and H. Sagawa. 1992. Phenotypic characteristics and DNA relatedness in *Prevotella intermedia* and similar organisms. *Oral Microbiol. Immunol.* **7**:60–64.
- Gharbia, S. H., M. Haapasalo, H. N. Shah, A. Kotiranta, K. Lounatmaa, M. A. Pearce, and D. A. Devine. 1994. Characterization of *Prevotella intermedia* and *Prevotella nigrescens* isolates from periodontic and endodontic infections. *J. Periodontol.* **65**:56–61.
- Giesendorf, B. A. J., A. van Belkum, A. Koeken, H. Stegeman, M. H. C. Henkens, J. van der Plas, H. Goossens, H. G. M. Niesters, and W. G. V. Quint. 1993. Development of species-specific DNA probes for *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* by polymerase chain reaction fingerprinting. *J. Clin. Microbiol.* **31**:1541–1546.
- Gmür, R. 1985. Human serum antibodies against *Bacteroides intermedium*. *J. Periodontol. Res.* **20**:492–496.
- Gmür, R., and B. Guggenheim. 1983. Antigenic heterogeneity of *Bacteroides intermedium* as recognized by monoclonal antibodies. *Infect. Immun.* **42**:459–470.
- Gmür, R., and B. Guggenheim. 1994. Interdental supragingival plaque: a natural habitat of *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, *Campylobacter rectus*, and *Prevotella nigrescens*. *J. Dent. Res.* **73**:1421–1428.
- Guillot, E., and C. Mouton. 1996. A PCR-DNA probe assay specific for *Bacteroides forsythus*. *Mol. Cell. Probes* **10**:413–421.
- Haffajee, A. D., and S. S. Socransky. 1994. Microbial etiological agents of destructive periodontal diseases. *Periodontology* **2000** **5**:78–111.
- Johnson, J. L., and L. V. Holdeman. 1983. *Bacteroides intermedium* comb. nov. and descriptions of *Bacteroides corporis* sp. nov. and *Bacteroides levii* sp. nov. *Int. J. Syst. Bacteriol.* **33**:15–25.
- Könönen, E. 1993. Pigmented *Prevotella* species in the periodontally healthy oral cavity. *FEMS Immunol. Med. Microbiol.* **6**:201–206.

19. **Könönen, E., M.-L. Väisänen, and H. Jousimies-Somer.** 1995. Cellular fatty acid and enzyme profiles of oral *Prevotella intermedia/nigrescens*-like organisms. World Congress on Anaerobic Bacteria and Bacterial Infections, San Juan, Puerto Rico.
20. **Maiden, M. F. J., A. Tanner, and P. J. Macuch.** 1996. Rapid characterization of periodontal bacterial isolates by using fluorogenic substrate tests. *J. Clin. Microbiol.* **34**:376–384.
21. **Martinez-Murcia, A. J., and F. Rodriguez-Valera.** 1994. The use of arbitrarily primed PCR (AP-PCR) to develop taxa specific DNA probes of known sequence. *FEMS Microbiol. Lett.* **124**:265–270.
22. **Mättö, J., M. Saarela, B. von Troil-Lindén, S. Alaluusua, H. Jousimies-Somer, and S. Asikainen.** 1996. Similarity of salivary and subgingival *Prevotella intermedia* and *Prevotella nigrescens* isolates by arbitrarily primed polymerase chain reaction. *Oral Microbiol. Immunol.* **11**:395–401.
23. **Mättö, J., M. Saarela, B. von Troil-Lindén, E. Könönen, H. Jousimies-Somer, H. Torkko, S. Alaluusua, and S. Asikainen.** 1996. Distribution and genetic analysis of oral *Prevotella intermedia* and *Prevotella nigrescens*. *Oral Microbiol. Immunol.* **11**:96–102.
24. **Ménard, C., P. Gosselin, J.-F. Duhaime, and C. Mouton.** 1994. Polymerase chain reaction using arbitrary primer for the design and construction of a DNA probe specific for *Porphyromonas gingivalis*. *Res. Microbiol.* **145**:595–602.
25. **Moore, W., and L. Moore.** 1994. The bacteria of periodontal diseases. *Periodontology 2000* **5**:66–77.
26. **Moore, W. E. C., L. H. Moore, R. R. Ranney, R. M. Smibert, J. A. Burmeister, and H. A. Schenkein.** 1991. The microflora of periodontal site showing active destructive progression. *J. Clin. Periodontol.* **18**:729–739.
27. **Paquet, C., and C. Mouton.** RAPD fingerprinting for the distinction of *Prevotella intermedia* sensu stricto from *Prevotella nigrescens*. Anaerobe, in press.
28. **Pearce, M. A., R. A. Dixon, S. E. Gharbia, H. N. Shah, and D. A. Devine.** 1996. Characterization of *Prevotella intermedia* and *Prevotella nigrescens* by enzyme production, restriction endonuclease and ribosomal RNA gene restriction analyses. *Oral Microbiol. Immunol.* **11**:135–141.
29. **Preus, H., and D. Russell.** 1994. Use of a nonradioactive genetic probe identified, synthesized, and labeled in the polymerase chain reaction. *Scand. J. Dent. Res.* **102**:161–167.
30. **Shah, H. N., and D. M. Collins.** 1990. *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. *Int. J. Syst. Bacteriol.* **40**:205–208.
31. **Shah, H. N., and S. E. Gharbia.** 1992. Biochemical and chemical studies on strains designated *Prevotella intermedia* and proposal of a new pigmented species, *Prevotella nigrescens* sp. nov. *Int. J. Syst. Bacteriol.* **42**:542–546.
32. **Shah, H. N., S. E. Gharbia, C. Scully, and S. M. Finegold.** 1995. Oligonucleotide probes to the 16S ribosomal RNA: implications of sequence homology and secondary structure with particular reference to the oral species *Prevotella intermedia* and *Prevotella nigrescens*. *Oral Dis.* **1**:32–36.
33. **Slots, J., and M. A. Listgarten.** 1988. *Bacteroides gingivalis*, *Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in human periodontal diseases. *J. Clin. Periodontol.* **15**:85–93.
34. **van Steenberg, T. J. M., M. F. Timmerman, F. H. M. Mikk, G. de Quincey, G. A. van der Weijden, U. van der Velden, and J. de Graaff.** 1996. Discrepancy between culture and DNA probe analysis for the detection of periodontal bacteria. *J. Clin. Periodontol.* **23**:955–959.
35. **Welsh, J., and M. McClelland.** 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18**:7213–7218.
36. **Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey.** 1990. DNA polymorphisms amplified arbitrarily by primers are useful as genetic markers. *Nucleic Acids Res.* **18**:6531–6535.
37. **Wilson, K.** 1991. Preparation of genomic DNA from bacteria, p. 2.4.1–2.4.2. In F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), *Current protocols in molecular biology*. Wiley-Interscience, Philadelphia, Pa.