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Polymerase Chain Reaction-Amplified Nonradioactive Probes for Identification of *Fusobacterium nucleatum*

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A polymerase chain reaction probe with 100% sequence identity to 120 deoxyribonucleotides of *Fusobacterium nucleatum* Fev1, coding for a part of the 40-kDa major outer membrane protein, was labeled with the steroid hapten digoxigenin. The probe was compared with various degenerate oligonucleotide probes and found to tolerate much more stringent washing conditions. It was therefore superior in distinguishing, by means of Southern blots and slot blots, *F. nucleatum* from other oral gram-negative bacteria in the periodontal pocket and from other fusobacterial species and in distinguishing among different strains of *F. nucleatum*. *F. periodonticum* was found to be more similar to *F. nucleatum* than the other fusobacterial species tested.

In most studies with bacteria, it is essential to identify and distinguish between different microorganisms. This is particularly important when dealing with a habitat such as the periodontal pocket, which houses more than 300 different species of bacteria.

Whole-chromosome DNA probes have been used to identify gram-negative bacterial strains within species (21). Probes have been developed from hypervariable segments of DNA encoding surface-exposed regions of outer membrane proteins (OMPs), with the assumption that such probes are species specific. These probes have been used to identify and differentiate between different genera and species of gram-negative bacteria (25). Others have used oligonucleotide probes complementary to the hypervariable regions of 16S rRNA sequences (18) or to the universally conserved 16S rRNA sequences to search for sequence identity (7, 16), but effective identity of 16S rRNA sequences is not necessarily sufficient to guarantee species identity (7).

Species and strains of *Fusobacterium* have been studied and compared in several ways, such as by DNA-DNA hybridization (20, 27), fatty acid analysis (14), composition of the peptidoglycan layer (28), and glutamate dehydrogenase electrophoretic patterns (8). OMPs have been compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (2), and their function as major antigens (1) has been elucidated. The different studies have underscored the heterogenous nature of the genus *Fusobacterium* and also of the species *Fusobacterium nucleatum*.

We have previously used synthetic oligonucleotide DNA probes to distinguish between different strains of *F. nucleatum* and other gram-negative bacteria, with special interest focused on the oral species connected with periodontal disease (5). Two of the three DNA probes used were based on the known amino acid sequence of a major 40-kDa OMP in strain Fev1 of *F. nucleatum* (2). Since the probes concerned were degenerate and did not have 100% sequence identity, they could only be handled under low-stringency conditions (12, 23) and were not particularly suitable for a closer study of strain variations. A third probe used, probe H2.1 (5), made from random cloning of a 2.1-kbp region of Fev1 DNA, was very specific but was not part of the gene coding for the 40-kDa protein. In the present work, we have made use of a polymerase chain reaction (PCR)-generated probe from a gene which encodes the 40-kDa major OMP of *F. nucleatum* Fev1 in addition to probe H2.1. High-stringency conditions can be used in the laboratory procedures with this new probe, and therefore there is less chance of unspecific binding of the probe on slot blots and Southern blots and the usefulness of the probe was thus greatly increased. We have also established a non-radioactive labeling procedure (13, 17) and included two additional oral gram-negative bacteria in our studies.

MATERIALS AND METHODS

Bacteria. The different bacterial strains and their sources are listed in Table 1. Strains were cultivated as described previously (5). *Fusobacterium periodonticum* was treated like the strains of *F. nucleatum*, and *Wolinella recta* was grown in accordance with the specifications of the supplier of this strain (American Type Culture Collection [10]).

Isolation of DNA from bacteria. DNA was isolated from the different bacteria as reported previously (5).

Digestion with restriction enzymes. *F. nucleatum* DNA was digested with the restriction enzymes *Hin*dIII, *Hin*cII, *Eco*RI, and *Eco*RV (Promega, Madison, Wis.), and *F. periodonticum* DNA was in addition treated with *Sau*3A. DNA from the other strains was treated with *Hin*cII only. Restriction endonuclease digestion of DNA was performed according to the specifications of the manufacturer.

Agarose gel electrophoresis. The digested DNA was run in 1% agarose gels containing 0.25 μg of ethidium bromide per ml. TAE (40 mM Tris-acetate, 1 mM EDTA [pH 8]) was used as the electrophoresis buffer in a Maxiphor electrophoresis unit (Pharmacia) (10 mA, overnight). *Hin*dIII-digested fragments of bacteriophage lambda DNA (Promega) and digoxigenin (DIG)-labeled molecular weight markers (Boehringer Mannheim) were used as size markers.

Southern transfer. The DNA was vacuum blotted to a positively charged nylon membrane (catalog no. 1209272; Boehringer Mannheim) recommended for the DIG labeling method, with a vacuum not exceeding 45 cm of H_2O, in a VacuGene vacuum-blotting unit (LKB, Bromma, Sweden). Transfer was performed for 2 h with 0.4 M NaOH as the transfer buffer. The filters were baked for 30 min at 120°C.

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TABLE 1. Strains used in this study

Species	Strain	Source[a]
F. nucleatum	F1	T. Hofstad
	F3	T. Hofstad
	F6	T. Hofstad
	ATCC 10953	ATCC
	ATCC 25586	ATCC
	Fev1	S. E. Mergenhagen
F. periodonticum	ATCC 33693	ATCC
F. necrogenes	2368	VPI
F. necrophorum	6161	VPI
F. mortiferum	0473	VPI
	5696	VPI
F. varium	0499A	VPI
F. russii	0307	VPI
Bacteroides fragilis	9343	NCTC
	11803	T. Hofstad
	12287	T. Hofstad
	12393	T. Hofstad
	25458	T. Hofstad
	30285	T. Hofstad
Leptotrichia buccalis	L11	T. Hofstad
Porphyromonas gingivalis	ATCC 33277	ATCC
Capnocytophaga sputigena	ATCC 33612	ATCC
Eikenella corrodens	23834	VPI
Actinobacillus actinomycetemcomitans	Y4	FDC
Haemophilus aphrophilus	ATCC 19415	ATCC
Wolinella recta	ATCC 33238	ATCC

[a] T. Hofstad, Bergen, Norway; ATCC, American Type Culture Collection, Rockville, Md.; S. E. Mergenhagen, Bethesda, Md.; VPI, L. V. Holdeman, Virginia Polytechnic Institute and State University, Blacksburg, Va.; NCTC, National Collection of Type Cultures, London, England; FDC, Forsyth Dental Center, Boston, Mass.

The Southern blots were hybridized only with the nonradioactive probe.

Slot blot. Slot blotting was performed as described earlier (5) for the radioactively labeled random-cloned probe H2.1; a nylon filter (catalog no. 1209272; Boehringer Mannheim) was used when the DIG-labeled PCR-generated probe was used. The nylon filters were baked at 120°C for 30 min, as for the Southern blots.

Amplification of a PCR-generated probe. Two different degenerate primers representing all possible coding combinations, a 27-mer primer called 214 and a 29-mer primer called 119, were constructed from the known N-terminal amino acid sequence of the 40-kDa major OMP in *F. nucleatum* Fev1 as described previously (5) and ordered from MedProbe A/S (Oslo, Norway). The region between the two primers was amplified in a Perkin-Elmer Cetus DNA Thermal Cycler or Programmable Thermal Controller (MJ Research Inc.) by using 50 ng of DNA from strain Fev1 of *F. nucleatum*, 1 pmol of each primer, 200 μM DIG-DNA labeling mix for random-primed DNA labeling with DIG-dUTP (Boehringer), 5 U of *Taq* polymerase (Boehringer), 10 μl of 10× *Taq* polymerase buffer (Boehringer), and water to a volume of 100 μl (13). The mixture was incubated for 3 min at 94°C and subjected to 25 cycles of PCR with the following cycle profile: 2 min at 94°C, 2 min at 45°C, and 3 min at 72°C. At the end of the last cycle, an extension step, 7 min at 72°C, was run. The PCR product was run in a 3% NuSieve GTG low-melting-point agarose gel (FMC, Rockland, Maine), and the fragment was cut out of the gel slices. When used for hybridizing, the gel was melted at 100°C for 10 min, and an adequate amount was added to the hybridization solution. The yield of DIG-labeled DNA was determined with a TKO-100 Mini-Fluorometer (Hoefer) and the fluorochrome HOECHST 33258 (6).

Hybridization. All Southern blots and those slot blots which were analyzed by the DIG hybridization method were prehybridized at 42 or 65°C for at least 20 min in hybridization buffer (50% [vol/vol] formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 2% [wt/vol] blocking reagent [Boehringer Mannheim], 0.1% [wt/vol] *N*-lauroylsarcosine, 0.02% [wt/vol] SDS). The same solution was used for hybridization, with the DIG-labeled probe added. Some slot blots, however, were prehybridized in BLOTTO (22) for 20 min and hybridized with the PCR-generated probe or the H2.1 probe labeled with [α-^{32}P]dCTP by random priming.

Washing and detection. Stringent washing and chemiluminescent detection were mainly carried out in accordance with the manufacturer's protocol for the detection of DIG-labeled nucleic acids with AMPPD [3-(2′-spiroadamantan)-4 methoxy-4-(3′-phosphoryloxy)-phenyl-1,2 dioxetan] (Boehringer). Briefly, the membranes were washed twice for 5 min each with 2× SSC–0.1% SDS at room temperature and then twice for 15 min each with 0.1× SSC–0.1% SDS at 65°C or washed only with 6× SSC twice for 15 min each at 42°C. Detection was performed as follows: a short wash (1 min) in washing buffer (0.03% [vol/vol] Tween 20 in autoclaved buffer 1 [0.1 M maleic acid, 0.15 M NaCl, pH 7.5]); incubation for 30 min in buffer 2, consisting of blocking stock solution (blocking reagent [10%, wt/vol] in buffer 1) diluted 1:10 in buffer 1; incubation for 30 min in diluted DIG-alkaline phosphatase conjugate (75 mU/ml, 1:10,000). The filters were washed twice for 15 min each in washing buffer, equilibrated for 2 min in buffer 3 (0.1 M diethanolamine, 1 mM $MgCl_2$, 0.02% sodium azide), and incubated for 5 min in AMPPD substrate solution diluted 1:100 in buffer 3. Excess liquid was allowed to drip off the membranes before they were sealed in a hybridization bag and incubated for 30 min at 37°C. The filters were exposed for 15 to 30 min at room temperature to Kodak X-OMAT film. The slot blots probed with radioactively labeled probes were washed at 65°C in 6× SSC or 0.1% SDS–0.1× SSC twice for 15 min each and exposed to the same type of film overnight.

Reprobing of the filters. The nylon membranes were washed in sterile H_2O, then twice in 0.2 M NaOH–0.1% SDS at 37°C for 15 min each, and then briefly in 2× SSC, as recommended by the manufacturer (Boehringer). The nitrocellulose filters were boiled for 5 min in 0.1% SDS–0.5× SSC. The filters were then ready for hybridization as described above.

Asymmetrical PCR and DNA sequencing. By running an unequal molar ratio of the two primers in the PCR, we obtained single-stranded DNA, which was sequenced with Sequenase version 2.0 (US Biochemicals). The PCR mixture contained 200 ng of DNA from *F. nucleatum* Fev1, 400 pmol of primer 119 plus 2 pmol of 214 or 6 pmol of primer 119 plus 50 pmol of primer 214, 200 μM each of the four deoxynucleoside triphosphates, 10 μl of 10× *Taq* polymerase buffer, 2.5 U of *Taq* polymerase, and water to bring the total volume to 100 μl. The thermal cycler was programmed for 94°C for 4 min; 50 cycles of 94°C for 1 min, 40°C for 30 s, and 72°C for 15 s; and finally elongation at 72°C for 5 min. The product was sequenced from both ends to verify that the sequence was correct. The sequencing primer was either primer 119 or 214. Sequencing was performed according to the protocol for

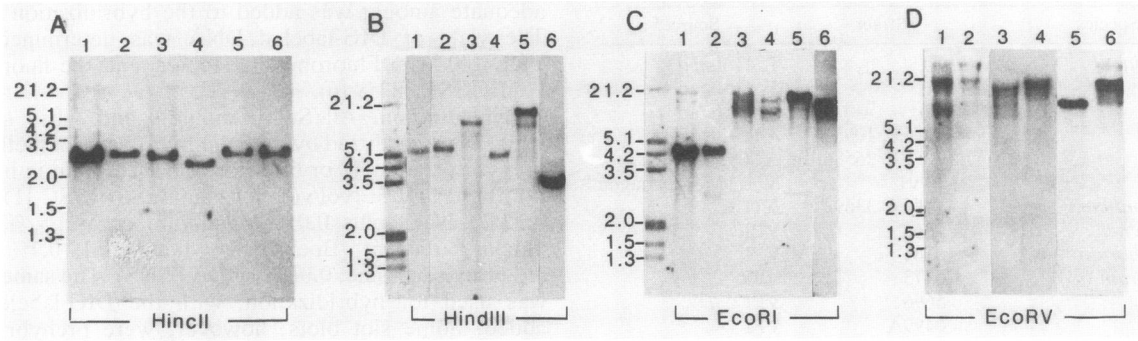

FIG. 1. Autoradiogram of Southern blots of DNA from six strains of *F. nucleatum* (lanes 1, F1; lanes 2, F3; lanes 3, F6; lanes 4, ATCC 10953; lanes 5, ATCC 25586; lanes 6, Fev1) digested with *Hin*cII (A), *Hin*dIII (B), *Eco*RI (C), or *Eco*RV (D). DNA from the other gram-negative bacteria listed in Table 1 was digested with *Hin*cII and Southern blotted but gave no signals when a washing temperature of 65°C was used (not shown). DIG-labeled DNA (molecular weight markers III; Boehringer) was used for size markers (shown in kilobase pairs), and the DIG-labeled DNA probe was used for hybridization.

Sequenase version 2.0 except that the labeling mix was omitted and replaced by H_2O. The samples were run both with and without manganese in the buffer.

The nucleotide sequence of the PCR-generated probe was consistent with the published N-terminal amino acid sequence of the 40-kDa major OMP of *F. nucleatum* Fev1 (2) except for three amino acids which were uncertain in the published sequence. The nucleotide sequence will be published elsewhere (4).

RESULTS

Southern blots hybridized with the PCR probe. We wanted to compare the PCR-generated probe, which was longer and had a 100% correct sequence, with probes 214 and 119, described earlier (5), which were degenerate and did not match the gene exactly. A considerably higher temperature could be used during hybridization and washing procedures with the PCR probe, and as expected, fewer bands were visible for each strain on Southern blots. In general, the bands observed were among those revealed by the degenerate probes (5).

DNA from different strains of *F. nucleatum* digested with *Hin*cII gave only one band for each strain (Fig. 1A). A band of about 3 kbp from strains F1, F3, F6, ATCC 25586, and Fev1 seemed to be recognized by the PCR-generated probe, while strain ATCC 10953 had a slightly smaller band of about 2.7 kbp.

*Hin*dIII digestion of the same strains (Fig. 1B) also gave a smaller number of bands than were obtained in previous work with degenerate probes (5). F1 and F3 again turned out to be similar, while all the other strains gave bands of different sizes.

Except for F1 and F3 digested with *Eco*RI (Fig. 1C), *Eco*RI and *Eco*RV gave very large fragments when used to cleave the DNA of different *F. nucleatum* strains (Fig. 1C and D), and the lower bands which we found previously with the degenerate probes for strains F6, ATCC 25586, and Fev1 (5) were not revealed when the PCR-generated probe was used. Again, F1 and F3 appeared to be similar.

The other gram-negative bacteria listed in Table 1 gave no reaction on Southern blots when cut with *Hin*cII and hybridized against the PCR-generated probe (not shown) at a washing temperature of 65°C. Under low-stringency conditions (42°C and 6× SSC), however, *F. periodonticum* DNA digested with *Hin*cII, *Hin*dIII, *Sau*3A, *Eco*RI, or *Eco*RV gave distinct bands on Southern blots (Fig. 2). A band similar in size to that found when the *F. nucleatum* strains (except for strain ATCC 10953) were digested with *Hin*cII was found for *Hin*cII-restricted *F. periodonticum* DNA (Fig. 1A and 2). The *Eco*RI and *Eco*RV bands of *F. periodonticum* (Fig. 2) were large, as for the *F. nucleatum* strains (Fig. 1C and D), and the *Hin*dIII band were within the variations of those of the *Hin*dIII-digested *F. nucleatum* strains. The *Sau*3A band of *F. periodonticum* (Fig. 2) was small, as were those of strains ATCC 25586 and F6 of *F. nucleatum* (3). The bands of *F. periodonticum* were significantly weakened when washing was done under the same conditions but at 50°C and disappeared gradually as the temperature rose to 60 and 65°C.

In conclusion, it was not difficult to differentiate between strains of *F. nucleatum* and other fusobacteria or between *F. nucleatum* and other gram-negative bacteria on a Southern blot. When washing was done at 65°C, there was no reaction with any bacteria but *F. nucleatum*.

Slot blots probed with DIG-labeled and radioactively labeled PCR-generated probe and H2.1 probe. Slot blots of the six different *F. nucleatum* strains, six other *Fusobacterium* species, and seven different oral gram-negative species are shown in Fig. 3. By using probe H2.1 and 6× SSC as the washing solution at 65°C, the fusobacterial strains could be

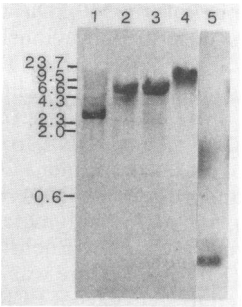

FIG. 2. Autoradiogram of Southern blot of DNA from *F. periodonticum* ATCC 33693 restricted with *Hin*cII, *Hin*dIII, *Eco*RI, *Eco*RV, and *Sau*3A (lanes 1 to 5, respectively), hybridized with the DIG-labeled DNA probe, and washed at 42°C with 6× SSC. *Hin*dIII-digested phage lambda DNA was used for size markers, and the numbers to the left indicate relative positions (in kilobase pairs).

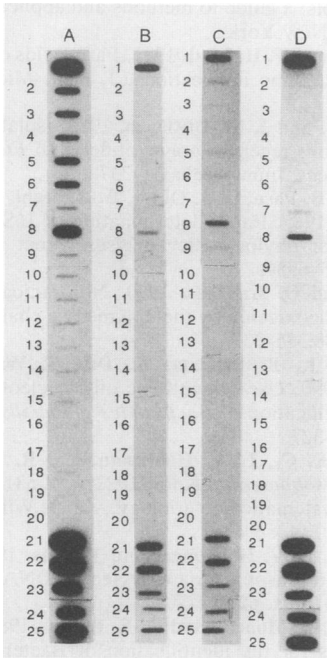

FIG. 3. Autoradiogram of slot blots hybridized with probe H2.1 (A and B) or the PCR-generated probe (C and D) all washed at 65°C. The washing solution was 6× SSC (A and C) or 0.1% SDS–0.1× SSC (B and D). Lanes: 1, *F. nucleatum* Fev1; 2, *F. mortiferum* 0473; 3, *F. mortiferum* 5696; 4, *F. necrophorum* 6161; 5, *F. necrogenes* 2368; 6, *F. varium* 0499A; 7, *F. russii* 0307; 8, *F. periodonticum* ATCC 33693; 9, *L. buccalis* L11; 10, *B. fragilis* 9343; 11, *B. fragilis* 11803; 12, *B. fragilis* 12287; 13, *B. fragilis* 12393; 14, *B. fragilis* 25458; 15, *B. fragilis* 30285; 16, *P. gingivalis* ATCC 33277; 17, *C. sputigena* ATCC 33612; 18, *E. corrodens* 23834; 19, *H. aphrophilus* ATCC 19415; 20, *A. actinomycetemcomitans* Y4; 21, *F. nucleatum* F1; 22, *F. nucleatum* F3; 23, *F. nucleatum* F6; 24, *F. nucleatum* ATCC 10953; 25, *F. nucleatum* ATCC 25586. *W. recta* ATCC 33238 was also slot blotted but gave no signals and is not shown.

easily separated from the other bacteria, with the possible exception of *F. russii*, which reacted weakly with probe H2.1 (Fig. 3A). A 0.1% SDS–0.1× SSC washing solution had to be used to differentiate *F. nucleatum* strains from other fusobacteria when probe H2.1 was used (Fig. 3B). The PCR-generated probe also showed somewhat unsatisfying results when 6× SSC was used as the washing solution (Fig. 3C), and the best results were obtained when 0.1% SDS–0.1× SSC was used for washing and hybridization was carried out at the same temperature as washing, namely 65°C (Fig. 3D). Like the Southern blots, however, the slot blots showed that *F. periodonticum* bound the PCR-generated probe more strongly than other fusobacteria and far more strongly than the non-*Fusobacterium* species of bacteria (Fig. 3C and D).

The best differentiation between *F. nucleatum* and *F. periodonticum* was obtained when Southern blots were washed with 0.1% SDS–0.1× SSC at 65°C, although 50°C was sufficient when the DIG-labeled PCR-generated probe was used (not shown).

DISCUSSION

Probes labeled with DIG have obvious advantages over radioactively labeled probes, including speed of detection, low hazardous potential in handling, and long-term storability. We sometimes found it difficult to obtain good signals under high-stringency conditions on Southern blots, especially from small fragments (not shown). This is probably because extensively fragmented DNA has a lower melting temperature than high-molecular-weight DNA (12). Another drawback with the method is that the hybridization, detection, and washing procedures must be repeated to change or repeat the washing conditions of one particular filter. This is very time-consuming compared with the washing procedure (twice for 15 min each) generally used with a radioactive probe.

Although degenerate probes cannot be used efficiently in hybridization experiments, they can be used to amplify parts of genomic DNA with high specificity (11). This is consistent with our findings that the nucleotide bases sequenced corresponded to the N-terminal amino acids of the 40-kDa OMP of *F. nucleatum* already published by Bakken et al. (2), and the sequencing of the probe was a satisfactory control for the region of DNA with which we were working. The sequence of the 40-kDa protein of *F. nucleatum* will be published elsewhere (4).

F. periodonticum has a guanine-plus-cytosine DNA content of 28 mol%, which is within normal values for the fusobacterial species (26 to 34%) (19, 20, 24). Very little DNA homology has been found between *F. periodonticum* and the type strains of *F. necrophorum*, *F. varium*, and *F. mortiferum* (6 to 8%), and only 38% homology was found between *F. periodonticum* and *F. nucleatum* ATCC 25586 in DNA-DNA hybridization experiments (20, 24). Our PCR-generated probe recognized this bacterial species under low-stringency conditions (Fig. 2 and 3) but distinguished it clearly from *F. nucleatum* on Southern blots (Fig. 1 and 2). The PCR-generated probe and the H2.1 probe also gave a rather strong reaction against *F. periodonticum* on slot blots (Fig. 3A and C), although it was not difficult to distinguish it from *F. nucleatum* under high-stringency conditions (Fig. 3B and D). This suggests a certain degree of sequence similarity between *F. nucleatum* and *F. periodonticum*, and the relationship between these species will be studied further.

In conclusion, we have improved our probes so that we are able to use high-stringency conditions, entailing high specificity. In addition, the nonradioactive labeling of the PCR-generated probe was found to be a useful alternative to radioactive labeling, especially when facilities for working with radioactive materials are poor or lacking. On the other hand, it is less convenient for testing and comparing the properties of a probe at different stringencies because the whole hybridization and detection procedure has to be repeated. It was often difficult to visualize small fragments on autoradiograms of Southern blots.

The results show that all *F. nucleatum* strains tested have DNA encoding the 40-kDa OMP. This is consistent with previous work on the DNA (5) and the protein (1). From the results, it may appear that the DNA sequence, and thus the protein sequence, varies considerably among the strains. The 40-kDa OMP is assumed to be involved in pore function (26) as well as in binding to various streptococci (15), a receptor function assumed to be closely related to the virulence of the strains. Such differences in primary sequence of the protein between strains may well explain the observed differences in the function and behavior of the strains.

F. nucleatum strains ATCC 10953 and ATCC 25586 may play different roles in the etiology of periodontitis (9). As shown, we have a PCR-generated probe which readily differentiates between these two strains, as well as others

strains tested, on Southern blots (Fig. 1). We are presently engaged in preparing a probe which is able to distinguish these strains on slot blots.

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