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Identification of *Bacteroides forsythus* in Subgingival Plaque from Patients with Advanced Periodontitis

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***Bacteroides forsythus* has been associated with destructive adult periodontitis. Up to now, detailed analysis by classical means was hampered by the fastidious nature of the organism. There is hope that the application of molecular detection methods such as indirect immunofluorescence or in situ hybridization (ISH) will allow for more rapid and accurate identification. Here we describe a *B. forsythus*-specific probe (BFV530), complementary to 16S rRNA, which correctly identified all *B. forsythus* isolates as confirmed by biochemical, protein, or fatty acid analysis. To assess whether this probe might be suitable for direct identification of *B. forsythus* in clinical specimens, a total of 92 subgingival plaque samples were analyzed. Fifty-five specimens were tested in parallel by culture, light microscopy, and filter hybridization. Unfortunately, the overall agreement between results of filter hybridization and conventional methods was 70.9% only. We therefore examined 37 new specimens by ISH and indirect immunofluorescence by using fluorescently labeled probe BFV530 or *B. forsythus*-specific monoclonal antibody 116BF1.2 (kindly provided by R. Gmür, Zurich, Switzerland), respectively. Agreement between these methods was 100%, indicating that ISH with probe BFV530 might be used to accurately identify *B. forsythus* directly in subgingival plaque samples.**

More than 300 bacterial species, up to 40 in one affected site, have been isolated from periodontal pockets (24). Despite this great microbial diversity only a few species have been found repeatedly in high numbers. Among these organisms associated with periodontal disease were *Actinobacillus* (*Haemophilus*) *actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas* (*Bacteroides*) *gingivalis*, *Prevotella intermedia*, *Peptostreptococcus micros*, *Capnocytophaga* spp., *Eikenella corrodens*, *Selenomonas* spp., *Treponema denticola*, and other bacteria (4, 7, 29, 33). Recently, a new species, *Bacteroides forsythus*, colonizing deep periodontal lesions, has been described and implicated in active destructive and recalcitrant adult periodontitis (15, 31). This gram-negative, fusiform organism is extremely fastidious and grows slowly on blood agar plates. Tiny colonies appear only after prolonged incubation under strict anaerobic conditions. However, coculture with feeder organisms, such as *P. gingivalis* or *F. nucleatum*, is required for optimal growth. It was therefore necessary to develop rapid and accurate methods to identify these organisms in culture or directly in subgingival plaque samples. Promising candidates for rapid detection and identification are monoclonal antibodies (MAbs) or probes (5, 6, 9–11, 22, 25, 28, 30, 32, 34, 35). Here we describe several methods, including nucleic acid hybridization and protein and whole-cell fatty acid analysis, to identify culture isolates accurately and to detect *B. forsythus* in clinical specimens by either filter or in situ hybridization (ISH) with radioactive and fluorescent probes.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All bacteria, except *Haemophilus* and *Actinobacillus* species, were grown under strict anaerobic conditions on prereduced yeast-cysteine-blood-agar (YCB) plates containing 1% (wt/vol) peptone, 0.5% (wt/vol) NaCl, 0.2% (wt/vol) meat extract, 0.5% (wt/vol) yeast extract, 0.03% (wt/vol) L-cysteine chloride monohydrate, 0.2% (wt/vol) glucose, 2% (wt/vol) Difco agar, and 10% (vol/vol) defibrinated sheep blood, supplemented with 0.001% (wt/vol) hemin and 0.0001% (wt/vol) vitamin K at 37°C for 7 to 10 days. The following reference strains were used: *B. forsythus* FDC 338 (ATCC 43037) (ATCC, American Type Culture Collection; FDC, Forsyth Dental Center, Boston, Mass.), *B. forsythus* FDC 331, *Porphyromonas gingivalis* OMZ 409 (OMZ, Oral Microbiology, Zurich, Switzerland), *Porphyromonas gingivalis* ATCC 33277, *Prevotella intermedia* (*Bacteroides intermedius*) NCTC 9336 (NCTC, National Collection of Type Cultures, London, England), *Fusobacterium nucleatum* ATCC 25586, *Porphyromonas* (*Bacteroides*) *endodontalis* ATCC 35406, *Prevotella* (*Bacteroides*) *corporis* ATCC 33547, *Prevotella* (*Bacteroides*) *denticola* DSM 20614, *Prevotella melaninogenica* (*Bacteroides melaninogenicus*) NCTC 11321, and *Porphyromonas asaccharolytica* (*Bacteroides asaccharolyticus*) NCTC 9337. All other bacteria were isolates from the Institut für Medizinische Mikrobiologie und Hygiene, Universität Freiburg, Freiburg, Germany.

Subgingival plaque samples. For the initial comparison of conventional techniques (culture, light microscopy) with filter hybridization, a total of 55 subgingival plaque samples was taken from 14 patients (age, 27 to 72 years). An additional 37 samples from 10 patients (age, 23 to 54 years) were analyzed to compare the performance of fluorescently labeled MAbs and probes. Sites were selected on the basis of bleeding on probing or pocket depth (≥4 mm). Subgingival plaque samples were removed with a calibrated (1 mg) Morse scaler and transferred into 500 μl of prereduced YCB medium. The suspensions were immediately frozen in liquid nitrogen and stored at −70°C up to 2 years. After thawing, the material was homogenized with a sterile pestle and then

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vortexed for 30 s. To roughly estimate the effect of freezing on the viability plaque bacteria, spirochete motility was tested by dark-field microscopy. About 20% of all specimens showing less than 50% of motile spirochetes were discarded. After dividing the suspension in half, one part was diluted and inoculated onto prereduced YCB plates and the other part was used for filter hybridization. *B. forsythus* was biochemically identified by the following criteria: presence of a trypsinlike enzyme as determined by the BANA (benzoyl-DL-arginine-naphthylamide) test, positivity for α-fucosidase and β-galactosidase activity, and failure to hydrolyze esculin or to produce indole or acid from glucose (3, 16, 17). All strains produced acetic acid and propionic acid as determined by gas chromatography (21).

Protein analysis of whole-cell extracts. Bacteria were grown on YCB plates to late-logarithmic phase. After about 10 colonies were harvested with a cotton swab, bacteria were placed in 1.5 ml of phosphate-buffered saline (PBS) and pelleted in a microcentrifuge at 13,000 × *g* for 5 min. Pellets were brought to 300 μl with sample buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% [wt/vol] sodium dodecyl sulfate [SDS], 5% [vol/vol] 2-mercaptoethanol, 20% [vol/vol] glycerol) containing 0.005% (wt/vol) bromphenol blue, and a small volume (5 μl) was loaded onto 7.5% (wt/vol) polyacrylamide gels. Gels were run at constant current of 40 mA per gel until the tracking dye was within 0.5 cm of the end of the gel. Gels were then stained in a solution containing 25% (vol/vol) methanol, 10% (vol/vol) acetic acid, and 0.1% (wt/vol) Coomassie brilliant blue R250 (Serva).

Whole-cell fatty acid analysis. *B. forsythus* whole-cell fatty acids were analyzed with the HP 5898A microbial identification system (Hewlett-Packard, Palo Alto, Calif.). Briefly, bacteria were cultivated at 37°C under strict anaerobic conditions on GC agar plates, consisting of 5.3% (wt/vol) brain-heart infusion agar with 0.5% (wt/vol) yeast extract, 0.001% (wt/vol) hemin, and 0.0001% (wt/vol) vitamin K. About 30 to 50 mg (wet weight) of bacteria grown to late-logarithmic phase was derivatized essentially as described by Miller (21). Extracts were injected and analyzed automatically. Fatty acids were identified by comparison to fatty acid methyl ester standards.

Sequencing of 16S rRNA and oligodeoxynucleotide synthesis. Bulk RNA was prepared as described previously (5). Colonies from about 20 to 30 YCB plates were harvested with a cotton swab, and bacteria were washed in PBS and lysed in LiCl-urea. The RNA was precipitated with ethanol and sequenced by reverse transcriptase (Pharmacia) and universal primer U5 (5′-TACCAGGGTATCTAATCCTG TT-3′), essentially as described before (5). Resulting sequences were compared with available 16S rRNA sequences (26) by the PcGene program (Intelligenetics, Mountain View, Calif.) to select regions suitable for probe construction. With the exception of the fluorescent probe, all oligodeoxynucleotides were synthesized on a Cyclone (Milligen/Biosearch, Bedford, Mass.) DNA synthesizer and purified by polyacrylamide gel electrophoresis (PAGE).

Filter hybridization. To assess the specificity of the *B. forsythus*-specific oligonucleotide probe, bacteria were diluted in PBS to a final concentration of about 10^7 to 10^8 cells ml^{-1}. Aliquots (100 μl) from 10-fold serial dilutions were blotted directly onto nylon membranes (Biodyne A; Pall) with Minifold I filtration manifolds (Schleicher & Schuell, Keene, N.J.). Air-dried filters were boiled in a solution containing 0.01× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.01% (wt/vol) SDS (14). Alternatively, bacteria were lysed in a solution containing 3 M NaCl, 0.3 M NaOH, and 2 M ammonium acetate (loading buffer [16]). The latter procedure has been used for the analysis of subgingival plaque materials. To determine the detection threshold of the probe, aliquots (100 μl) of 10-fold serial dilutions from a suspension containing 10^9 *B. forsythus* cells ml^{-1} were applied to positively charged nylon membranes (Biodyne B; Pall). Bacteria from subgingival plaque samples (250 μl) were pelleted by centrifugation in a microcentrifuge at 13,000 × *g* for 20 min. Pellets were dissolved in 300 μl of loading buffer and placed on ice for about 30 min. Aliquots (100 μl) were applied onto Biodyne B membranes, which were then rinsed in loading buffer and 0.5 M NaCl for 2 min per solution. Oligodeoxynucleotide probes (60 ng) AAC25 (5′-GCACCAGGGCTAAACCCCAATCCCC-3′), *A. actinomycetemcomitans* specific (5), and BFV530 (5′-GTAGAGC TTACACTATATCGCAAACTCCTA-3′), *B. forsythus* specific, were end labeled with 20 μCi [γ-^{32}P]ATP (3,000 Ci $mmol^{-1}$; Amersham) by using T4 polynucleotide kinase (Life Technologies) according to the supplier's recommendation. Free label was removed by gel filtration on Sephadex G50 (Pharmacia) columns. Hybridization was done at 70°C as described earlier, with 5 ng of radiolabeled oligonucleotide probe ml^{-1}. Filters were washed in 500 ml of 5× SSC with 0.2% (wt/vol) SDS at hybridization temperature for 3 min. Signals were detected by autoradiography using Kodak X-Omat film for 24 to 48 h (5, 12).

In situ hybridization (ISH) and indirect immunofluorescence (IIF). Fluorescent probe BFV530 was synthesized on a Gene Assembler Plus (Pharmacia) by PAC (Pharmacia) phosphoramidite chemistry and nonradioactively labeled at the 5′ end by automatically introducing a fluorescent amidite (Fluoreprime; Pharmacia), kindly provided by U. Schriek, Freiburg, Germany. To test the accessibility of target rRNA sequences, we used a eubacterium-specific oligodeoxynucleotide (5′-GCTGCCTCCCGTAGGAGT-3′; corresponding to positions 338 to 355 in the *Escherichia coli* 16S rRNA), labeled at the 5′ end with tetramethylrhodamine isocyanate (TRITC) (2). Subgingival plaque samples were centrifuged (see above). PBS (50 μl) was added to suspend the bacterial pellet. Specimens were processed for ISH essentially as described by Amann et al. (2). Briefly, 5 μl of a 37% (wt/vol) formaldehyde solution was added and specimens were fixed at 4°C for 16 h. Small drops (1 to 3 μl) of fixed material were spread onto glass slides coated with $CrKSO_4$-gelatin (0.01 and 0.1% [wt/vol] in water, respectively), allowed to air dry, and dehydrated in ethanol. Hybridization was done at 37°C for 5 to 16 h after addition of about 8 μl of hybridization buffer (5× SET with 0.2% [wt/vol] bovine serum albumin [Serva], 10% [wt/vol] dextran sulfate [Pharmacia], and 0.01% [wt/vol] polyadenylic acid [Sigma]; 5× SET is 750 mM NaCl, 100 mM Tris-HCl [pH 7.8], and 5 mM EDTA) containing about 2 ng of the fluorescently labeled oligonucleotide per ml. Slides were kept in 50-ml Falcon tubes containing paper towels soaked with 5× SET buffer to avoid evaporation. After hybridization, slides were washed repeatedly (three times, 10 min each) in 0.2× SET at 37°C, and samples were mounted in Citifluor (Citifluor) to prevent photobleaching. IIF analysis was done essentially as described earlier (10, 11, 32). Briefly, subgingival plaque samples were suspended in 500 μl of YCB medium and homogenized. Part of this suspension (10 μl per well) was distributed onto glass slides. Cells were fixed with methanol for 2 min at room temperature and incubated first with *B. forsythus*-specific MAB 116BF1.2 (5 μl per well; kindly provided by Rudolf Gmür, Zurich, Switzerland) in a humid chamber for 30 min at 37°C. After the slides were washed

with an excess of PBS with 0.05% Tween for about 10 min at room temperature, fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin G second antibody (1:100 dilution; Dianova, Hamburg, Germany) was added, and the slides were incubated for 30 min at room temperature. Final washes were done in the dark (see above), and slides were mounted in Citifluor. All specimens were examined by epifluorescence microscopy under oil immersion with a Neofluar (Zeiss, Oberkochen, Germany) 100× objective and a Zeiss Axioskop 20 equipped with a high-pressure mercury bulb (HBO 50) and filter set no. 487915 (Bandpass filter 546/12, Farbteiler 580, Langpass filter 590) for TRITC or no. 487909 (Bandpass filter 450-490, Farbteiler 510, Langpass filter 520) for FITC. Photomicrographs were taken on Fujichrome P1600 film (Fuji). Specifically stained bacteria from 10 randomly chosen high-power fields per well were counted by two independent examiners.

RESULTS

Phenotypic analysis. In a first set of experiments, 55 subgingival plaque samples from 14 patients with adult periodontitis were analyzed in parallel by culture, microscopy, and filter hybridization. *P. intermedia*, *P. gingivalis*, and *B. forsythus* were among the predominant flora of all sites, representing 4.9, 21.5, and 11.4% of the total cultivable bacterial count, respectively. The prevalence results were 71.4, 53.6, and 61.9%, respectively (20). *B. forsythus* grew best under strict anaerobic conditions, often surrounding *F. nucleatum* or *P. gingivalis* colonies. Shiny or dim grey colonies about 1 mm in diameter appeared on YCB agar plates after 5 to 6 days of cultivation, which later on developed central autolysis. To assess the phenotypic intraspecies homogeneity, 59 *B. forsythus* isolates were subjected to (PAGE) and gas chromatography to analyze whole-cell proteins and fatty acids. The polypeptide pattern of all isolates showed two prominent bands in the 200-kDa range, and was distinct from patterns of other oral bacteria included as controls (Fig. 1). Analysis of whole-cell fatty acids revealed a characteristic distribution of branched-chain hydroxy (C3OH-i-17, 3-hydroxy-15-methylhexadecanoic acid), branched-chain nonhydroxy (Ca-15, 12-methyltetradecanoic acid), and unbranched ($C_{16:0}$, hexadecanoic acid) fatty acids, a pattern which was similar for all *B. forsythus* isolates but different from fatty acid distributions in all other oral bacteria tested (Fig. 2).

Probe construction and analysis. Direct sequencing of *B. forsythus* 16S rRNA and subsequent sequence comparison allowed the design of a species-specific probe (BFV530: 5′-GTAGAGCTTACACTATATCGCAAACTCCTA-3′), complementary to variable region V5 of *B. forsythus* 16S rRNA (Fig. 3). Probe specificity was tested by hybridizing radiolabeled BFV530 to a variety of bacteria associated with periodontal disease and other organisms commonly found in the oral cavity. With the exception of a slight hybridization signal in blocks B7 and 8 and C10 to 12, which was due to unspecific trapping of the probe, only *B. forsythus* was recognized (Fig. 4). As microheterogeneity within 16S rRNA genes has been reported for some species, inclusivity of the probe was tested by hybridizing BFV530 to 59 *B. forsythus* isolates which had been identified on the basis of biochemical tests prior to hybridization. The identity of all but one strain was confirmed by hybridization (Fig. 5). The hybridization-negative strain (Fig. 5, G11) was subsequently identified as *Prevotella* (*Bacteroides*) *zoogleoformans*. The detection threshold of the radiolabeled probe was about 5×10^4 to 1×10^5 bacteria.

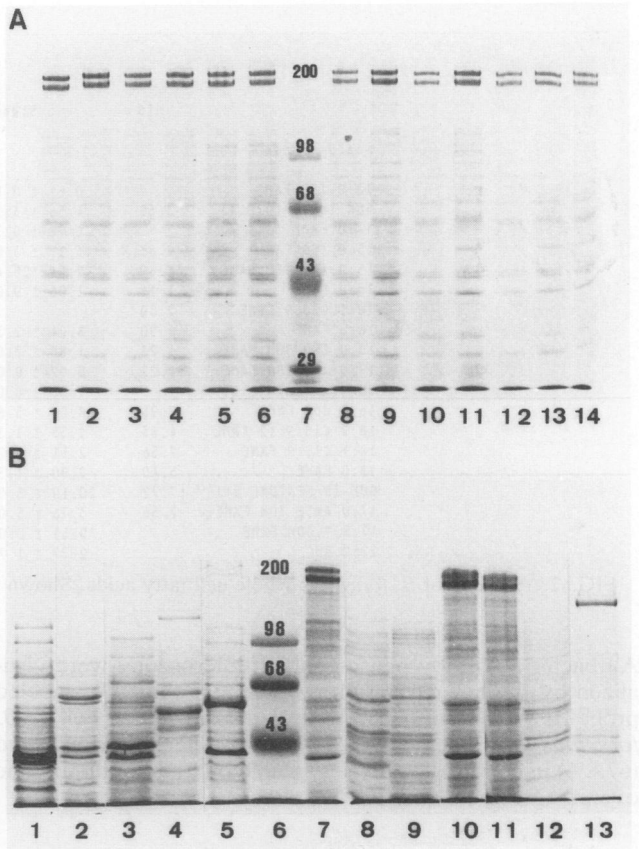

FIG. 1. SDS-PAGE of whole-cell extracts from *B. forsythus* and selected bacterial species. (A) *B. forsythus* clinical isolates (lanes 1 to 6 and 8 to 14); 10^3 molecular weight standards (lane 7). (B) *Selenomonas infelix* (lane 1); *Selenomonas noxia* (lane 2); *Selenomonas flueggei* (lane 3); *Selenomonas sputigena* (lane 4); *Centipeda periodontii* (lane 5); 10^3 molecular weight standards (lane 6); *Prevotella denticola* (lane 8); *P. melaninogenica* (lane 9); *Bacteroides fragilis* (lane 12); *Wolinella recta* (lane 13); *B. forsythus* (lanes 7, 10, and 11).

Direct detection of *B. forsythus* in subgingival plaque samples by filter hybridization and ISH. To assess the accuracy of the radiolabeled probe BFV530 for direct detection of *B. forsythus* in clinical specimens, 55 subgingival plaque samples from 14 patients were analyzed by filter hybridization, light microscopy, and culture (see above). A representative autoradiograph is shown in Fig. 6. Twenty-eight samples were positive by all methods. Eleven specimens were positive by culture only, while five culture-negative samples reacted with probe BFV530. Another 11 specimens were found negative by all assays. Hence, the agreement between filter hybridization and conventional techniques was 70.9%. Discouraged by these results, we analyzed in parallel a new set of clinical specimens from another 10 patients by ISH with fluorescently labeled BFV530 and IIF with a *B. forsythus*-specific MAB (116BF1.2). To test whether the 16S rRNA target molecules of all bacteria present in a given sample were accessible for the fluorescent probes, a TRITC-labeled eubacterium-specific oligonucleotide was hybridized to smears of formaldehyde-fixed subgingival plaque samples.

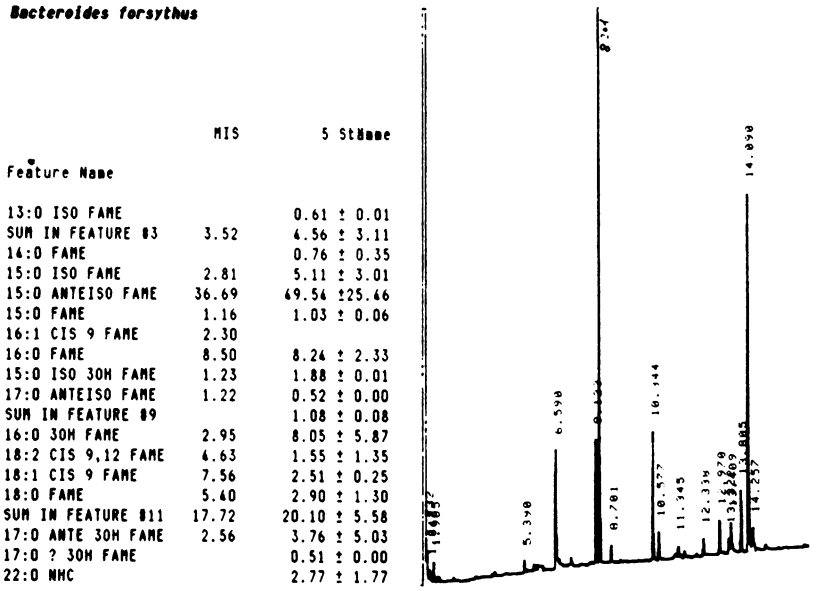

FIG. 2. Analysis of *B. forsythus* whole-cell fatty acids. Shown is a profile of fatty acid methyl esters from five representative strains.

All bacteria seen by phase-contrast microscopy were visualized by the eubacterial probe, whereas the FITC-labeled probe BFV530 identified *B. forsythus* exclusively (Fig. 7). *B. forsythus* was detected in 25 out of 37 specimens analyzed (67.8%) in counts of $<10^6$ per sample by both methods. The overall agreement was 100%.

DISCUSSION

Because of the fastidious nature of *B. forsythus*, its prevalence has certainly been underestimated. Earlier isolation frequencies varying from about 10 to 67% of samples from deep periodontal lesions have been described (7, 31). Apparent difficulties in culturing the organism prompted the development of fast and sensitive assays by producing *B. forsythus*-specific MAbs or probes. By using IIF with specific MAbs a higher prevalence of this organism was demonstrated (11). The IIF technique is sensitive but tedious and is hampered by the fact that some of the bacteria are nonreactive with the serological reagents available so far or that other bacteria express cross-reacting epitopes. Phylogenetic probes, however, targeting molecules conserved during evolution, e.g., probes complementary to 16S rRNA, would be good candidates for accurate detection of all members of a species (12). A variety of rRNA-based probes have been described and used with great success. However, heterogeneity within a species, interfering with accurate detection, has been described for some taxa (1, 27). It was therefore necessary to test the inclusivity of our probe against a large number of isolates. All *B. forsythus* strains included in this study were correctly identified by probe BFV530, an observation confirmed by others using similar probes (23). The detection threshold of the radiolabeled probe (about 10^4 to 10^5 bacteria) corresponded well to the average detection limit reported for other 16S rRNA-based oligonucleotide probes (13, 19, 22). Homogeneity of this species was further confirmed by phenotypic analysis showing identical protein and fatty acid profiles for all of the

B. fragilis	5'-AUACUCGCUGUUUGCGAUAUACAGU-AAGCGGCCAA-GCG-3'
P. intermedia	5'-AUGC-CGC-GUU-GCCCGGCGGCGGCGNAGCGA-AA-GCN-3'
P. gingivalis	5'-UUACUAGGAGUUUGCGAUAUACCGUCAAGCUUCCACAGCG-3'
P. endodontalis	5'-AUACUAGAUUUUUGCGAUAUACUGUAAGAG-UCUA-AGCG-3'
B. forsythus	5'-UUA**CUAGGAGUUUGCGAUAUAGUGUA--AGCUC**UACAGCG-3'

FIG. 3. Comparison of 16S-V5 rRNA sequences from *B. forsythus* and selected *Porphyromonas*, *Prevotella*, and *Bacteroides* species. The target region of probe BFV530 is printed in boldface and underlined.

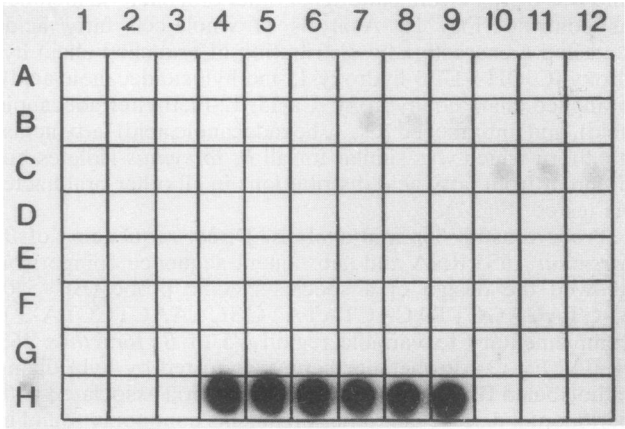

FIG. 4. Specificity of the *B. forsythus*-specific probe BFV530. The autoradiograph was made after filter hybridization with radiolabeled BFV530 (20 h of exposure). The following bacteria were applied: *P. gingivalis* (blocks A1 to 12, B1 to 6, and H10 to 12); *P. melaninogenica* (blocks B7 to 9); *Bacteroides thetaiotaomicron* (blocks B10 to 12); *B. fragilis* (blocks C1 to 3); *F. nucleatum* (blocks C4 to 6); *P. asaccharolytica* (blocks C7 to 9); *P. melaninogenica* (blocks C10 to 12); *Haemophilus aphrophilus* (blocks D1 to 12); *Haemophilus paraphrophilus* (blocks E1 to 6 and G4 to 6); *A. actinomycetemcomitans* (blocks E7 to 12, F1 to 12, and G1 to 3); *E. corrodens* (blocks G7 to 12); *P. endodontalis* (blocks H2 and 3); and *B. forsythus* (blocks H4 to 9).

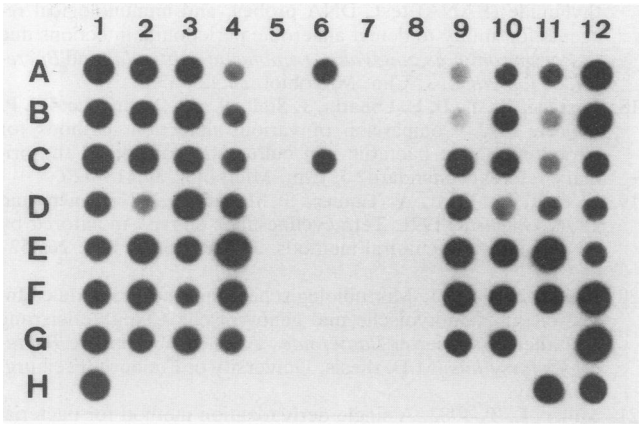

FIG. 5. Inclusivity of probe BFV530. Fifty-nine *B. forsythus* strains, isolated from subgingival plaque of 14 patients, and various obligate anaerobes were hybridized to radiolabeled BFV530 (autoradiograph, 20 h of exposure). The following bacteria were tested: *B. forsythus* clinical isolates (rows A to G, columns 1 to 4 and 9 to 12; blocks H1, 11, and 12); *B. forsythus* reference strains ATCC 43037 (block A6) and FDC 311 (block C6); *F. nucleatum* (blocks A7 and B7); *P. gingivalis* (block C7); *P. intermedia* (block D6); *P. endodontalis* (block D7); *P. corporis* (block E6); *P. melaninogenica* (block E7); *Veillonella parvula* (block F6); *Selenomonas* sp. (block F7); *P. denticola* (block G6); and *Clostridium* sp. (block G7). No bacteria were applied to all other positions.

organisms tested. This agrees well with earlier reports describing only one serotype (31) and the observation that 33 human-derived (group I) and 4 monkey-derived (group II) strains of *B. forsythus* were essentially identical for all genotypic and phenotypic traits with the exception of indole production (3). While performing well in identifying culture isolates (100% sensitivity and specificity), primary detection of *B. forsythus* in clinical specimens was unsatisfactory. The agreement between culture and hybridization was only about 70%. A similar value (75%) has been reported by Moncla et al. (23). Possible explanations for the discrepancy between the two methods include the reduction of the rRNA content in bacteria stored frozen under the above conditions (up to 2 years at −70°C), insufficient permeabilization of target bacteria, or the use of only one oligonucleotide probe labeled at the 5′ end, whereas other authors are using either a combination of oligonucleotides or larger probes, labeled to a higher specific activity (17–19, 22). Where the probe performed better than culture, the discrepancy may have resulted from a reduced detection limit of the culture method in those samples with a high viable count but a low proportion of *B. forsythus*. Recent studies comparing various methods for the detection of periodontopathic bacteria showed that culture cannot be considered the primary reference method to evaluate new tests (17–19). Among modern methods which in the future may complement and extend bacterial culture, the technique of ISH deserves particular attention. Like immunofluorescence, ISF can be applied to qualitatively and "quantitatively monitor subgingival plaque for specific bacteria" (11). At the level of single-cell resolution it combines rapid detection with accurate identification. Although both techniques are analogous in detecting bacteria in situ, there are several advantages of using fluorescently labeled phylogenetic probes, i.e., rRNA-based probes, rather than MAbs for in situ analysis: (i) rRNA-based probes can be generated quite easily, (ii) an increasing body of small subunit RNA sequences compiled in large data bases such as GenBank or EMBL (European Molecular Biology Laboratories) allows for rapid and accurate control of probe specificity, and (iii) as ribosome biosynthesis is under tight control and the number of ribosomes (and rRNA molecules) is strictly growth rate dependent, quantitation of bound probe may serve to determine the physiological state of the bacteria under study. This would be of utmost importance to assess microbial interactions in a complex ecosystem such as subgingival plaque, where qualitative and quantitative shifts in the bacterial population may determine the extent of periodontal destruction. Whether it may be useful to apply in vitro amplification of nucleic acids, e.g., the polymerase chain reaction, rather than hybridization techniques for rapid and accurate detection of periodontopathogenic organism remains to be shown (8).

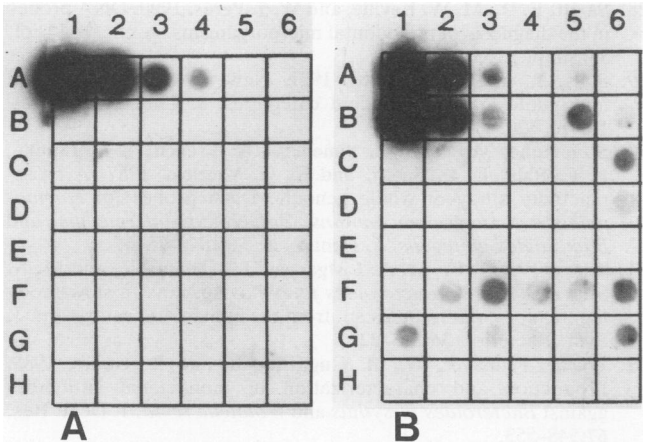

FIG. 6. Primary detection of *B. forsythus* in subgingival plaque samples. The autoradiograph was made after hybridization to radiolabeled probes AAC25 (A) and BFV530 (B). Hybridization to AAC25 was used to control nonspecific trapping of radiolabeled oligonucleotide probes. The membrane was used without removing probe AAC25 prior to hybridization with BFV530. The following strains were applied as controls: *A. actinomycetemcomitans*, each spot containing 6×10^8 to 6×10^5 organisms, respectively (blocks A1 to 4); *B. forsythus*, 9×10^7 to 9×10^4 bacteria (blocks B1 to 4); *P. gingivalis*, 5×10^8 to 5×10^5 bacteria (blocks C1 to 4); and *P. intermedia*, 2×10^8 to 2×10^5 bacteria (blocks D1 to 4). Subgingival plaque samples were applied to rows A to E, columns 5 and 6; row F, columns 1 to 6; row G, columns 1 to 6; and row H, columns 1, 2, and 5.

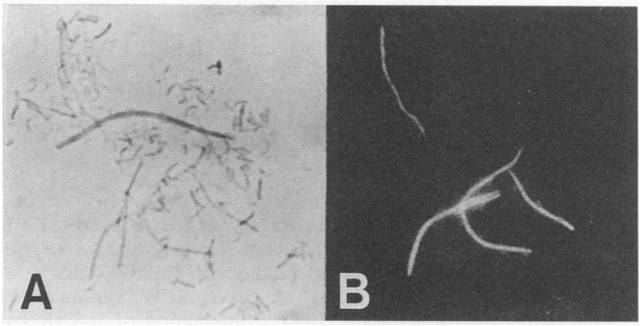

FIG. 7. Detection of *B. forsythus* in formaldehye-fixed subgingival plaque by in situ hybridization with FITC-labeled BFV530. A representative high-power field is shown by phase-contrast (A) or epifluorescence (B) microscopy.

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