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Rapid Presumptive Identification and Further Characterization of *Bacteroides forsythus*

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***Bacteroides forsythus* is a fastidious anaerobic gram-negative organism associated with various forms of periodontal disease. It is dependent on *N*-acetylmuramic acid for growth. A method for rapid presumptive identification of human-derived strains of *B. forsythus* is presented, based on the following eight criteria: (i) positive activity for α-glucosidase, (ii) positive activity for β-glucosidase, (iii) positive activity for sialidase, (iv) positive activity for trypsinlike enzyme, (v) negative indole production, (vi) requirement for *N*-acetylmuramic acid, (vii) colonial morphology, and (viii) gram stain morphology from blood agar medium deficient in *N*-acetylmuramic acid. Enzymes were assayed with rapid filter paper spot tests based on fluorogenic substrates (4-methylumbelliferone derivatives and *N*α-carbobenzoxy-L-arginine-7-amino-4-methylcoumarin hydrochloride). Gas-liquid chromatography analysis of the metabolic products of *B. forsythus* grown in peptone yeast extract broth supplemented with *N*-acetylmuramic acid and heat-inactivated horse serum revealed predominant amounts of acetate, propionate, butyrate, isovalerate, and phenyl acetate, with minor amounts of isobutyrate and succinate. The described presumptive identification scheme facilitated recognition of four strains of *B. forsythus* which were isolated from subgingival plaque samples from monkeys (*Macaca fascicularis*). With the exception of indole production, these organisms were essentially identical to the human strains of *B. forsythus* for all phenotypic and genotypic characteristics examined.**

Bacteroides forsythus, a gram-negative strictly anaerobic fusiform from the human oral cavity, was described by Tanner et al. in 1986 (29). The history of *B. forsythus* research dates back to 1979, when the organism, then referred to as "fusiform *Bacteroides*," was first isolated and associated with advancing periodontitis (28). In 1982, isolates designated *Bacteroides* D-10 and now recognized as *B. forsythus* were recovered from subgingival and supragingival plaque of young human adults with severe periodontitis. The numbers of *B. forsythus* organisms isolated from subgingival sites were in higher proportions than those isolated from supragingival sites (21, 27). These and other studies have demonstrated the association of *B. forsythus* with various forms of periodontal disease (2, 3, 10, 30). Like *Porphyromonas gingivalis*, another organism highly associated with periodontal disease, *B. forsythus* produces sialidase and a trypsinlike enzyme, which are thought to be involved with virulence (8, 16, 24, 31). Although *B. forsythus* has been implicated in the pathogenesis of periodontal disease, its role as a pathogen has not been established. While it has been detected in periodontally healthy human subjects by indirect immunofluorescence, the proportions of *B. forsythus* organisms in these subjects were lower than in patients with diseased sites; furthermore, most samples from the healthy subjects were negative for the organism (10).

Past difficulties with identification and culture of *B. forsythus* have probably been the main reasons that this organism has not been as extensively studied as other putative periodontal pathogens. On conventional blood agar plates, growth occurs either in the form of pinpoint colonies or in a satellite pattern near a feeder strain such as a staphylococcus or *Fusobacterium nucleatum*. In 1988, Wyss (33) determined that *B. forsythus* requires exogenous *N*-acetylmuramic acid (NAM), an acetylated amino sugar and component of peptidoglycan, as a growth factor. An unknown factor from blood or serum also seems to be required by most strains (33). The cells of *B. forsythus* (observed by indirect immunofluorescence) in direct plaque samples appear as medium to long rods or short filaments, whereas on NAM-deficient medium they appear pleomorphic (4). Presumably, in vivo the surrounding microflora provides a concentration of NAM adequate to maintain cell wall integrity.

In early studies, the identification and characterization of *B. forsythus* by conventional biochemical methods were impeded because of its fastidious growth. Enzymatic reactions and methods generally limited to a research setting, such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were used primarily to test for phenotypic traits reported in the original characterization (29).

The purpose of this study was to develop a scheme for rapid presumptive identification of *B. forsythus*. The resulting scheme consists of five filter paper spot tests (for indole, α-glucosidase, β-glucosidase, sialidase, and trypsinlike-enzyme determinations), Gram stain morphology from growth on culture medium deficient in NAM, colonial morphology, and requirement for NAM. Enzymes were assayed by using fluorogenic 4-methylumbelliferone derivatives and *N*α-carbobenzoxy-L-arginine-7-amino-4-methylcoumarin hydrochloride (CAMM), which have been proven to be rapid (15 min), reliable, and relatively inexpensive (17).

The presumptive identification scheme herein described facilitated the recognition of *B. forsythus* strains from monkeys (*Macaca fascicularis*). Further studies indicated that the monkey strains appear to be a biotype of *B. forsythus*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. forsythus* ATCC 43037^T was obtained from the American Type Culture

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Collection, Rockville, Md. All human strains were isolated from subgingival plaque samples from patients with various forms of periodontal disease. The 32 clinical isolates of *B. forsythus* were from 22 patients. The samples were obtained either at the University of Washington Graduate Periodontics Clinic or at a private Seattle-area dental practice. Collection and handling of specimens from human sites have been described previously (19). Four strains similar to *B. forsythus* were isolated from subgingival plaque samples of three monkeys (*M. fascicularis*) cared for in the Regional Primate Center at the University of Washington. The samples from monkeys were handled identically to the samples from humans, except that a prereduced salts solution was used for collection and transportation (18). The cultures were grown on heart infusion agar (Difco, Detroit, Mich.) plates containing 5% defibrinated sheep blood and incubated anaerobically at 37°C for 3 to 4 days with either a coculture of *Staphylococcus aureus* or a NAM disk prior to testing (see below).

NAM disks. Disks containing 300 μg of NAM were prepared by adding 20 μl of a filter-sterilized 1.5% aqueous solution of NAM (Sigma Chemical Co., St. Louis, Mo.) to sterile 0.25-in. (ca. 0.64-cm)-diameter paper disks (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.). The disks were air dried and stored at room temperature, at which they remain stable for several months. The application of one disk to the surface of a blood agar plate is sufficient to test this growth requirement.

Characterization. (i) Biochemicals. The benzoyl-DL-arginine-naphthylamide (BANA) hydrolysis test for the trypsinlike enzyme was performed as described by Laughon et al. (11). The tests for esculin hydrolysis, motility, and fatty acid analysis by gas-liquid chromatography (GLC) were performed by the methods of Holdeman et al. (6). Fatty acids were analyzed with a Hewlett-Packard model 5830A gas-liquid chromatograph. Phenylacetic acid at a concentration of 10 mM was included in the nonvolatile-fatty-acid standard. For most GLC analyses, peptone yeast broth (PY) and PY with glucose (PYG) were supplemented with 5% (vol/vol) heat-inactivated horse serum and 15 μg of NAM per ml (PY+SN and PYG+SN, respectively). All broth cultures were incubated anaerobically at 37°C for 5 days. Indole production was tested by adding several drops of Kovács reagent to PY or PY+SN. The catalase tests were performed with 3 and 15% hydrogen peroxide. A 1% aqueous solution of dimethyl-*p*-phenylenediamine was used for the oxidase test, and 1% paradimethylaminocinnamaldehyde in 10% (vol/vol) concentrated hydrochloric acid was used for the spot indole test as described by Sutter and Carter (26). Enzymatic reactions were determined with the API An-Ident (Analytab Products, Plainview, N.Y.) as specified by the manufacturers or with fluorogenic substrates (see below).

(ii) Reagents for fluorescent spot tests. For α-glucosidase and β-glucosidase assays, stock solutions were prepared by dissolving 10 mg of 4-methylumbelliferyl-α-D-glucoside or 4-methylumbelliferyl-β-D-glucoside (Sigma) in 7.4 ml of distilled water. If the substrate did not completely dissolve, the solution was allowed to stand for 5 or 10 min to allow particulate material to settle, and the liquid portion was decanted. The solutions were dispensed in 180-μl volumes and frozen at −20°C. Working solutions were prepared by adding 20 μl of 1 M sodium phosphate buffer (pH 7.0) to the thawed substrate and thoroughly mixing.

For the sialidase assay, a stock solution was prepared by dissolving 1 mg of 2′-4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (Sigma) in 6.6 ml of distilled water. The stock solution was dispensed in 180-μl volumes and frozen at −20°C. Working solutions were prepared by adding 20 μl of 1.0 M sodium acetate buffer (pH 4.6) to the thawed substrate and thoroughly mixing.

For the trypsinlike-enzyme assay, 10 mg of CAMM (Sigma) was dissolved in 4.16 ml of dimethyl sulfoxide. An equal volume of 0.1 M Tris buffer (pH 8.0) was added and the solution was thoroughly mixed. The solution was dispensed in 200-μl volumes and stored at −20°C until ready for use.

Filter paper spot tests. The filter paper spot tests were performed by smearing a loop of bacterial growth onto Whatman no. 2 filter paper strips (~6 by 60 mm) soaked with the substrates. The test strips were incubated at 37°C for 15 min in petri plates. Usually 12 to 20 isolates could be tested on a single strip. Positive and negative control organisms were run with each strip. The filters were examined under a long-wavelength (365-nm) handheld mineral lamp. Enzymatic activity was observed as a fluorescent blue spot on the filter paper.

(iii) DNA probe assay. Clinical isolates were identified by DNA hybridization with specific *B. forsythus* DNA probes (Bf2 and Bf8) by the method for pure bacterial strains which was previously described (18). The oligonucleotide DNA probes were provided by the MicroProbe Corporation of Bothell, Wash.

(iv) Gram stain morphology from NAM-deficient medium. Gram stains of organisms taken from blood agar plates with or without a feeder strain were made.

RESULTS AND DISCUSSION

Data for 33 human-derived strains (HDS) of *B. forsythus*, including the ATCC type strain 43037 (group I), and 4 monkey-derived strains (group II) are presented in Table 1. All strains developed circular, entire, slightly convex, pale speckled-pink, colonial morphology on blood agar plates and were easily seen at a magnification of ×8 or ×16 with a stereomicroscope after 6 to 7 days of anaerobic incubation of primary subgingival plaque samples. Other species were always present. On subculture, all strains grew in a satellite pattern near an *S. aureus* feeder strain, and Gram staining demonstrated characteristic bizarre fusiforms, usually with spheroids and centrally swelling forms (Fig. 1A).

The cellular morphology of *B. forsythus* is influenced by the concentration of NAM in the medium. Low NAM concentrations (0.1 mg/liter) in media result in increased proportions of spheroids compared with those in media with a higher NAM concentration, in which a more uniform morphology is observed (33). Figure 1A through C illustrates the cellular morphologies of HDS of *B. forsythus* grown under conditions in which the culture medium either contains an adequate concentration of NAM or is deficient in this growth factor. It is possible that traces of NAM are present in agar medium because of cellular constituents from nonviable bacteria retained during the commercial processing of agar and/or other components of medium in which bacteria may be present. This would explain the growth of minute colonies on blood agar plates after prolonged incubation. Feeder strains would provide an additional source of NAM.

Nine strains of *B. forsythus* from group I were tested for NAM dependence with 300-μg NAM disks; all strains yielded good growth after 3 to 4 days of incubation.

Twenty-one strains of *B. forsythus* from group I were tested with the API An-Ident. All strains gave positive results for *N*-acetylglucosaminidase, α-glucosidase, α-fu-

TABLE 1. Characteristics of HDS and *M. fascicularis*-derived strains of *B. forsythus*

Test or characteristic	HDS of *B. forsythus* (group I)			*M. fascicularis*-derived strains of *B. forsythus* (group II)		
	Result[a]	% Positive	No. positive/ no. tested	Result	% Positive	No. positive/ no. tested
Growth in CO_2	–	0	0/33	–	0	0/4
Stimulated by feeder strain	+	100	33/33	+	100	4/4
NAM dependence	+	100	9/9	+	100	4/4
Oxidase	–	0	0/21	–	0	0/3
Catalase	–	0	0/21	–	0	0/3
Esculin hydrolysis	+	100	21/21	+	100	3/3
Indole production						
Spot test	–	0	0/17	+	100	4/4
PY tube test	–	0	0/21	+	100	4/4
PY+SN tube test	–	0	0/5	+	100	4/4
API An-Ident tests						
Indole	–	0	0/21	+	100	4/4
N-Acetylglucosaminidase	+	100	21/21	+	100	4/4
α-Glucosidase	+	100	21/21	+	100	4/4
α-Arabinosidase	–	0	0/21	–	0	0/4
β-Glucosidase	V	62	13/21	V	25	1/4
α-Fucosidase	+	100	21/21	+	100	4/4
Phosphatase	+	100	21/21	+	100	4/4
α-Galactosidase	–	0	0/21	–	0	0/4
β-Galactosidase	V	81	17/21	V	50	2/4
Indoxyl acetate	+	95	20/21	+	100	4/4
Arginine utilization	–	0	0/21	–	0	0/4
Leucine aminopeptidase	–	0	0/21	–	0	0/4
Proline aminopeptidase	–	0	0/21	–	0	0/4
Pyroglutamic acid arylamidase	–	0	0/21	–	0	0/4
Tyrosine aminopeptidase	–	0	0/21	–	0	0/4
Arginine aminopeptidase	V	62	13/21	+	100	4/4
Alanine aminopeptidase	+	100	21/21	+	100	4/4
Histidine aminopeptidase	V	62	13/21	V	75	3/4
BANA trypsinlike enzyme	+	100	12/12	+	100	4/4
Fluorescent spot test						
MUB–α-glucosidase	+	100	17/17	+	100	4/4
MUB–β-glucosidase	+	100	17/17	+	100	4/4
MUB–sialidase	+	100	27/27	+	100	4/4
CAMM-trypsinlike enzyme	+	100	28/28	+	100	4/4
B. forsythus DNA probe	+	100	27/27	+	100	4/4
GLC analysis[b]	A, P, ib, B, IV, s, PAA		5/5[c]	A, P, ib, B, IV, s, PAA		3/3

[a] +, 90 to 100% positive; –, 0 to 10% positive; V, >10% but <90% positive.
[b] GLC of metabolic products of PY+SN. A, acetate; B, butyrate; ib, isobutyrate; IV, isovalerate; P, propionate; s, succinate; PAA, phenyl acetate. Capitals, predominant products; lowercase, minor products.
[c] Includes *B. forsythus* ATCC 43037.

cosidase, phosphatase, and alanine aminopeptidase. All strains failed to produce indole and were negative for α-arabinosidase, α-galactosidase, arginine utilization, and leucine aminopeptidase, proline aminopeptidase, pyroglutamic acid arylamidase, and tyrosine aminopeptidase activities. These results are in general agreement with previously published data, except the results for α-arabinosidase, which have been reported as variable (29, 31). Variable results were obtained for β-glucosidase, β-galactosidase, arginine aminopeptidase, and histidine aminopeptidase. All 21 strains hydrolyzed esculin and were negative for catalase, oxidase, and indole production in PY broth.

Twenty-seven *B. forsythus* HDS from group I were tested with a highly specific *B. forsythus* DNA probe assay (18). All 27 isolates demonstrated hybridization with the probes. Therefore, the identification of group I strains as *B. forsythus* is supported by both genotypic and phenotypic data.

The presence of the trypsinlike enzyme produced by *B. forsythus* is a key test for identification of the species, as most anaerobic gram-negative species from the human oral cavity lack this enzyme. Trypsinlike activity is also produced by *P. gingivalis*, *Treponema denticola*, and *Capnocytophaga* spp. All of these species have been associated with periodontal disease, and the enzyme has been implicated as a virulence factor (8).

Two commonly used substrates for the detection of the trypsinlike enzyme are BANA and CAMM. Loesche et al. (13) have developed a diagnostic test for anaerobic period-

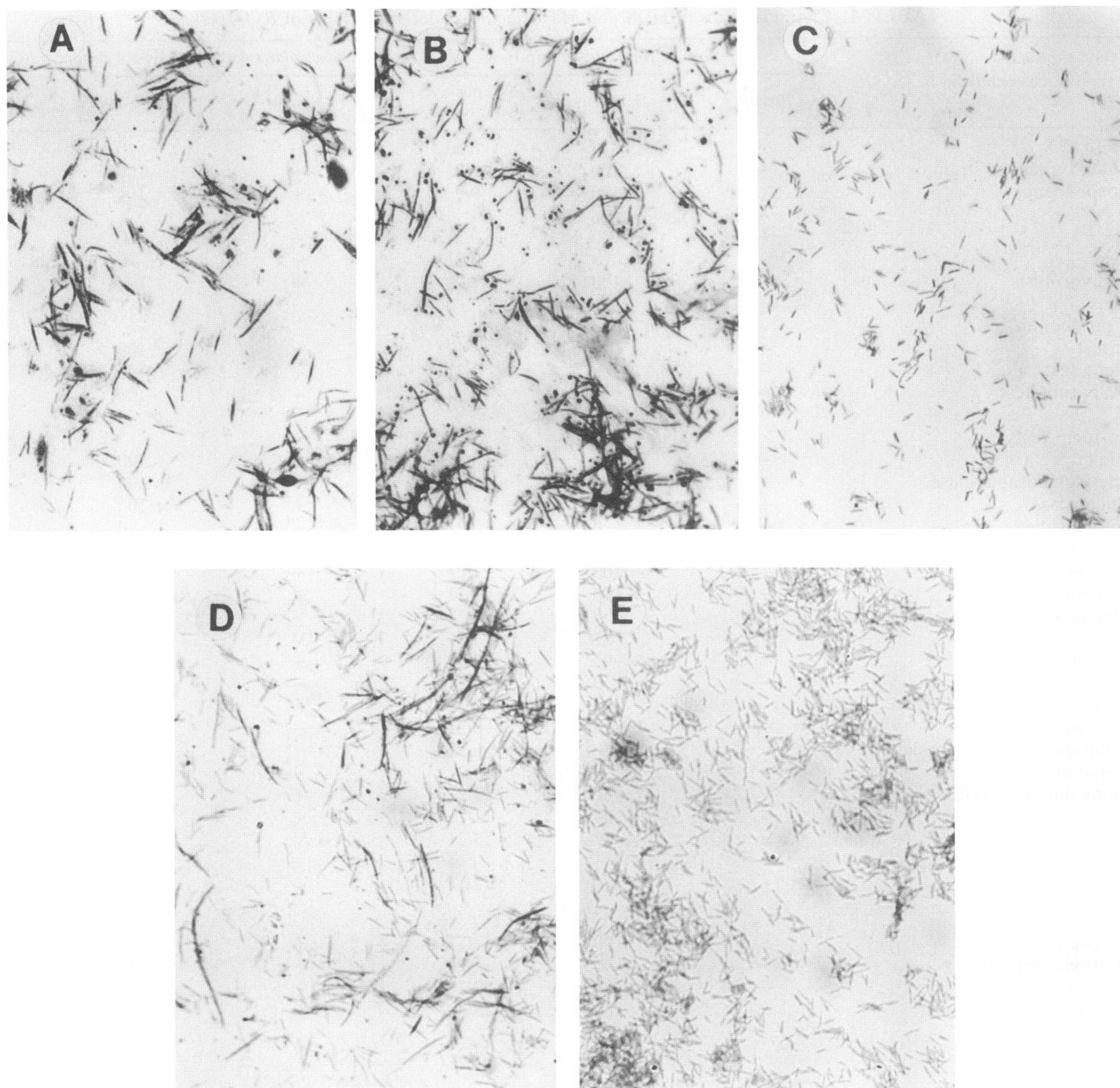

FIG. 1. Cellular morphology of *B. forsythus* from humans (BFH) and *B. forsythus* from *M. fascicularis* (BFM) in Gram stains from a blood agar (BA) plate deficient in NAM (with or without a feeder strain) and from BA with a NAM disk. Note bizarre fusiforms and spheroids from NAM-deficient medium, compared with a more homogeneous rodlike shape on medium with a NAM disk. (A) BFH on BA; (B) BFH on BA with feeder strain; (C) BFH on BA with a NAM disk; (D) BFM on BA with feeder strain; (E) BFM on BA with a NAM disk. Magnification, ×3,500.

ontal infections which is based on hydrolysis of the BANA substrate by strongly trypsinlike-enzyme-producing species (*P. gingivalis*, *T. denticola*, and *B. forsythus*). Slots used CAMM, a fluorogenic substrate, to detect colonies of *P. gingivalis* from mixed microflora (25). We adapted the latter method to a filter paper spot test. Twenty-eight *B. forsythus* strains from group I were examined with the CAMM substrate filter paper spot test; all were positive. Nine of the CAMM substrate-positive strains were also examined with the BANA substrate; all were positive. The two methods give comparable results for *P. gingivalis* and *B. forsythus*; however, we have observed some discrepancies between the two methods within the genus *Capnocytophaga* (data not shown).

Sialidase activity in *B. forsythus* has been previously reported when 44 strains (100%) gave positive reactions with the methylumbelliferone derivative (MUB) (16). Seventeen HDS of *B. forsythus* were also assayed for α-glucosidase by using 4-methylumbelliferyl-α-glucoside; all were positive. Indole production was not detected in any of 21 strains from group I with the PY tube indole test, nor was it detected in 5 strains grown in PY+SN. Spot indole tests performed on 17 strains from group I also gave negative test results. Although β-glucosidase activity in *B. forsythus* is variable with the API An-Ident test strip, there were no negative reactions observed when 4-methylumbelliferyl-β-glucoside was used as a substrate, probably because of the increased sensitivity of the 4-MUBs (1).

TABLE 2. Differentiation of HDS of *B. forsythus* from other fusiform and fusiformlike oral bacteria

Organism	No. of strains tested	Characteristic[a]							
		Spot indole	α-Glu[b]	β-Glu[b]	Sial[b]	Tryp[b]	CO_2 growth	Motility	Predominant fatty acid by GLC[c]
B. forsythus	12	−	+	+	+	+	−		A, B, IV, P, PAA
Capnocytophaga species	11	−[d]	+	+	V	V	+		A, S
E. saburreum	3	+	+	+	−	−	−	−	A, B
E. yurii	3	+[e]	−	−	−	−	−	+	A, B
F. nucleatum	17	+	−	−	−	−	−	−[f]	B
L. buccalis	1	−	+	+	−	−	V[f]	−[f]	L[f]

[a] +, 90 to 100% positive; −, 0 to 10% positive; V, >10% but <90% positive.
[b] Methylumbelliferone derivative used to assay α-glucosidase (α-Glu), β-glucosidase (β-Glu), and sialidase (Sial). CAMM was used to assay the trypsinlike enzyme (Tryp).
[c] A, acetate; B, butyrate; IV, isovalerate; P, propionate; S, succinate; L, lactate. PYG was used for GLC analysis for all organisms except *B. forsythus*, for which PY+SN was used.
[d] Four of four strains tested.
[e] Two strains of *E. yurii* gave weak reactions.
[f] Data from Holdeman et al. (7).

GLC analyses of volatile and nonvolatile fatty acids produced by *B. forsythus* were reported before NAM was recognized as a required growth factor. Accordingly, the organisms grew poorly, and the metabolic end products, acetate and succinate or acetate and propionate, were dependent on the formulation of the medium in which they were grown (29). In our studies, it was difficult to distinguish between the fatty acids present as background (acetate, lactate, and succinate) in uninoculated PYG and those that may have been produced by the organism, since growth was usually questionable. However, phenyl acetate (PAA) in trace amounts was detected in PYG in four of the group I isolates and was not detected in the uninoculated medium.

Since growth is improved by the addition of NAM and heat-inactivated horse serum to culture medium, fatty acid analyses were run with media containing these supplements. In PY+SN, all five strains of *B. forsythus* from group I which were examined (Table 1) produced acetate, propionate, butyrate, isovalerate, and PAA as the predominant metabolic end products, with smaller amounts of isobutyrate and succinate. Growth in PYG+SN was slight compared with growth in PY+SN, and it appears that 1% glucose may be inhibitory to *B. forsythus* (data not shown). The inhibitory effect of glucose and other monosaccharides on anaerobic species has been reported elsewhere (12).

The metabolic end products of *B. forsythus* are virtually identical to those produced by *P. gingivalis*, although the proportions do not appear to be the same. This may imply a closer relationship to the genus *Porphyromonas* than was previously suspected.

Butyrate and propionate have been shown to be toxic for human gingival fibroblasts, and butyrate has been shown to be cytotoxic for Vero cells (23, 32). These toxic metabolites may play a role in the pathogenesis of periodontal disease. The toxicity of PAA has not been fully elucidated, but the metabolite is produced by only a small minority of anaerobes studied to date. Mayrand and Bourgeau surveyed 17 genera of anaerobes for the production of PAA and found it in only two genera, *Bacteroides* (including the *Porphyromonas* species) and *Clostridium* (15). Among the species positive for PAA are several which can be of clinical significance, including *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Bacteroides ovatus*, *Clostridium difficile*, and *Clostridium perfringens* (15). More recently, PAA has been reported in the genus *Eubacterium*, in which it is produced by *Eubacterium timidum*, another organism associated with periodontitis (5, 20).

Group II of Table 1 consists of four strains of *B. forsythus* which were isolated from subgingival plaque samples from three monkeys (*M. fascicularis*) during screening for various putative periodontal pathogens and *B. forsythus*. Subcultures of colonies resembling *B. forsythus* failed to grow aerobically. Gram stains from anaerobically subcultured colonies near a *Staphylococcus* feeder strain revealed bizarre fusiforms typical of *B. forsythus* (Fig. 1D). Growth was markedly enhanced by NAM. Gram stains of growth from medium supplemented with NAM demonstrated uniform gram-negative bacilli (Fig. 1E). All four isolates gave results consistent with those for HDS of *B. forsythus* by both the spot tests and the API An-Ident test strips, except that the production of indole was detected by both methods. The indole test was also positive in PY+SN. All isolates hydrolyzed esculin and were negative for oxidase and catalase. Results of analyses of volatile and nonvolatile fatty acids from NAM- and serum-supplemented PY and PYG were essentially identical to those for HDS of *B. forsythus*. All four *B. forsythus* strains from *M. fascicularis* hybridized with the *B. forsythus* DNA probes.

These monkey-derived strains of *B. forsythus* appear to be a biotype of *B. forsythus*, but further analyses may be necessary to determine whether the similarity has a solid phylogenetic basis. If this proves to be the case, then *M. fascicularis* might provide a research model with which to study the role of *B. forsythus* in periodontal disease.

Differentiation of HDS of *B. forsythus* from other oral fusiforms or fusiformlike bacteria can be rapidly achieved by using the five filter paper spot tests. Five species from four genera, which may appear morphologically similar to *B. forsythus* in Gram stain preparations, are described in Table 2. HDS of *B. forsythus* most closely resemble the *Capnocytophaga* spp. by the five spot tests, but unlike the *Capnocytophaga* spp., *B. forsythus* will not grow in air plus CO_2. *Eubacterium saburreum* and *Eubacterium yurii* are gram-positive organisms which may appear gram negative in older cultures (14, 22). Three clinical isolates each of *E. yurii* and *E. saburreum* and seventeen clinical isolates of *F. nucleatum* were tested with the spot tests. In contrast to HDS of *B. forsythus*, both the *Eubacterium* spp. and *F. nucleatum* were positive for indole and negative for sialidase and the trypsinlike enzyme. The spot test results in Table 2 for the *Capno-*

cytophaga spp. and *F. nucleatum* are consistent with previously published data (6, 9, 16, 24). One clinical isolate of *Leptotrichia buccalis* was easily distinguished from *B. forsythus* by the lack of sialidase and the trypsinlike enzyme. Although the reactions for indole production and α-glucosidase activity for *L. buccalis* in Table 2 are in agreement with previously published data, it is necessary to test more strains for β-glucosidase, sialidase, and the trypsinlike enzyme (7). In any case, the requirement for NAM is the most striking feature of *B. forsythus*.

In summary, presumptive identification of HDS and monkey-derived strains of *B. forsythus* by the scheme presented here is reliable, rapid, and easy to perform. The filter paper spot tests may also aid in identification of other morphologically similar species. Additional tests such as analysis of metabolic products from PY+SN medium by using GLC or specific *B. forsythus* DNA probes are necessary for confirmation.

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