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Characterization of *Bacteroides forsythus* Isolates

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Fifteen *Bacteroides forsythus* strains freshly isolated from patients with periodontitis were used together with three collection strains and one type strain for characterization of growth on various media; determination of enzymatic profiles, antibiotic susceptibility profiles, 16S rRNA ribotypes, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) outer membrane protein profiles, and pathogenicity; and gas chromatography analysis by using a wound chamber model in rabbits. All strains were stimulated by *N*-acetylmuramic acid, while one strain needed a further supplement such as yeast extract for optimal growth. All strains showed trypsin-like activity. While 10 different ribotypes were found, the SDS-PAGE profiles revealed similar patterns for all strains. All strains were sensitive to penicillin G (MICs, <0.5 μg/ml), ampicillin (MICs, <1.0 μg/ml), amoxicillin (MICs, <0.38 μg/ml), metronidazole (MICs, <0.005 μg/ml), tetracycline (MICs, <0.19 μg/ml), doxycycline (MICs, 0.05 μg/ml), erythromycin (MICs, <0.4 μg/ml), and clindamycin (MICs, <0.016 μg/ml), while they were less sensitive to ciprofloxacin (MICs, <4 μg/ml). *B. forsythus* did not cause abscess formation by monoinoculation. *B. forsythus* coinoculated with *Fusobacterium nucleatum* ATCC 10953 caused abscess formation in 75% of rabbits, while it caused abscess formation in 100% of rabbits when it was coinoculated with *Porphyromonas gingivalis* FDC 381. In the case of the latter combination, four of six rabbits died of sepsis after 6 to 7 days, and *P. gingivalis* and *B. forsythus* were recovered from the heart blood at a proportion of 10:1. *B. forsythus* strains were highly virulent and invasive in combination with *P. gingivalis*.

More than 300 microorganisms are assumed to be harbored in the subgingival microbiota of the gingival sulcus and periodontal pocket of humans (25). One-third of them have been cultivated and identified, and only some of them are carefully examined as suspected periodontopathogens. Hence, one of the essential conditions for an organism to be recognized as a suspected periodontopathogen is to be cultivable in artificial medium. From this point of view, some bacterial species have come to be considered as suspected periodontopathogens within the last decade (10, 20, 26, 31).

Bacteroides forsythus is one of these more recently recognized periodontopathogens. This bacterium is a fastidious anaerobic gram-negative rod, first reported as a "fusiform *Bacteroides*" in 1979 by Tanner et al. (34) and later named *B. forsythus* (36). Although only a few reports have dealt with *B. forsythus*, it has been frequently isolated in large numbers from periodontal pockets of patients with active periodontitis and has also been reported to be related to early periodontal lesions (8, 10, 12).

B. forsythus is frequently isolated together with *Porphyromonas gingivalis*, which indicates an ecological relationship between these two. The need for some bacteria on which *B. forsythus* can feed, such as *Fusobacterium nucleatum* or *Streptococcus sanguis*, or *N*-acetylmuramic acid (NAM) to support the growth is reported (9, 38). *B. forsythus* is a well-known producer of a trypsin-like enzyme, which is assumed to be an important virulence factor in periodontal disease (21). *B. forsythus* is suggested to be associated with advanced and refractory periodontitis; however, due to the difficulty in cultivating *B. forsythus*, this bacterium has not been carefully examined.

The purpose of this study was to elaborate a supportive growth medium and to further characterize some of the biological activities of *B. forsythus.*

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MATERIALS AND METHODS

Bacteria. The laboratory strains used in this study were *B. forsythus* ATCC 43037 and *B. forsythus* OMZ 367, OMZ 408, and OMZ 471, kindly provided by R. Gmür, University of Zürich, Zürich, Switzerland.

B. forsythus OMGS 3073 to OMGS 3087 were our own isolates from deep periodontal pockets from 15 patients with untreated periodontitis. They were identified on the basis of their pinkish white speckled colonies, gram-negative fusiform micromorphology after growth on the brucella agar plates, and production of trypsin-like enzyme in API ZYM (API System; bioMérieux, Marcy-l'Etoile, France). No growth in 10% CO_2 in air also certified them as *B. forsythus.*

For tissue cage experiments, further strains were used: *F. nucleatum* ATCC 10953, *P. gingivalis* FDC 381, and *Streptococcus anginosus* OMGS 1902. Strain OMGS 1902 was our own isolate from a subgingival plaque sample from a patient with advanced periodontitis.

Comparison of growth media. Comparison of the growth of *B. forsythus* on various media was performed by streaking each strain onto agar plates, and the plates were incubated for 7 days at 36°C in jars with an atmosphere of 95% H_2 and 5% CO_2. After incubation, the thickness of the colonies and the maximal colony diameter were measured for each *B. forsythus* strain. Three basal media were tested; blood agar base (no. 2; Unipath Ltd., Basingstoke, England), brucella agar base (BBL Microbiology systems, Cockeysville, Md.), and Trypticase soy agar II (TSA II; BBL). The basal media were supplemented with yeast extract (4 g/liter; Difco Laboratories, Detroit, Mich.), phytone peptone (4 g/liter; BBL), and NAM (Sigma, St. Louis, Mo.). NAM was dissolved to 10 mg/ml in distilled water, stored at −20°C, and added to the medium to a final concentration of 10 mg/liter after autoclaving.

Enzymatic profiles. The enzymatic profiles of *B. forsythus* were examined by using the API ZYM system (36) as specified by the manufacturers (API System).

Gas-liquid chromatography. For analysis by gas-liquid chromatography (GLC), *B. forsythus* was inoculated into peptone yeast glucose (PYG) broth with 15 mg of NAM per liter and was incubated anaerobically at 36°C for 7 days. In addition, the strains were incubated in Huntoon broth medium, a special medium for fastidious bacteria, to which was added 15 mg of NAM per liter (27). Analysis of metabolic products was done by GLC (Sigma 2B; Perkin-Elmer, Norwalk, Conn.) with a chromatograph equipped with a flame ionization detector. GLC was performed as outlined in the Virginia Polytechnic Institute manual (17). The glass column for chromatography was packed with 5% AT 1000 (Altech Associates Inc., Deerfield, Ill.) on Chromosorb GHP 100/120 mesh (Johns-Manville, Denver, Colo.). The carrier gas was nitrogen (30 ml/min), the injection port temperature was 150°C, and the oven temperature was 120°C. One-microliter portions of the ether-extracted and methylated samples (17) were

used, and the results were compared with those obtained with standard solutions of volatile fatty acids.

Ribotyping. Methods for DNA extraction, restriction enzyme digestion, agarose gel electrophoresis, synthesis of probes, and analyses were essentially the same as those described in detail by Bowden et al. (2). Strains were grown anaerobically in jars containing 95% H_2 and 5% CO_2. DNA yield and purity were determined by measurement of the absorptions at 260 and 280 nm. Enzyme digestion with the endonuclease *Pst*I (Boehringer Mannheim, Scandinavia AB, Bromma, Sweden) was performed with DNAs from all strains. Electrophoresis was done in a horizontal agarose gel (Hoefer, San Francisco, Calif.) at a constant voltage (40 V) for 19 h at room temperature in 89 mM Tris-borate buffer (pH 8.0). A digoxigenin-labeled bacteriophage lambda DNA digest (Marker III; Boehringer Mannheim) was used as a molecular marker in the gels. The fragments were visualized after ethidium bromide staining under shortwave UV light and were photographed. DNA fragments were transferred to nylon membranes (Zeta-Probe GT; Bio-Rad, Solna, Sweden) with Southern blotting by using a vacuum blotter (Bio-Rad). The preparation of a digoxigenin-labeled probe was made by the random priming technique with a digoxigenin DNA-labeling kit (Genius Kit; 1093-657; Boehringer Mannheim), followed by hybridization and detection according to the manufacturer's instructions. The molecular weights of the major bands observed by ribotyping were compared with DNA molecular markers as a reference. Ribotype patterns, which were confirmed on independent gels, were estimated.

SDS-PAGE. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed in a Mini-Protean II (Bio-Rad) unit at 200 V for 45 min by using a vertical 0.75-mm-thick slab containing 7.5% (wt/wt) polyacrylamide. Bacterial samples were prepared by sonication of whole-cell suspensions at 50 W for 1 min. The preparations were heated with an SDS sample buffer at 100°C for 5 min. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. The following proteins were used as molecular size markers: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine plasma albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) (Bio-Rad standard marker proteins).

Antimicrobial susceptibility tests. The antimicrobial susceptibility of *B. forsythus* was determined by using the reference agar dilution method of the National Committee for Clinical Laboratory Standards (30) and the E-test (AB Biodisc, Solna, Sweden) as described by Citron et al. (5). Bacterial cells were harvested by scraping the agar plate and were suspended in dilution medium Viability Medium Göteborg (VMG) I (27) at a concentration of 0.5 (optical density at 605 nm). The suspension was then plated on TSA II medium supplemented with phytone peptone and yeast extract (TNPY medium) (volume, 30 ml) with the antibiotics, which were made up in a dilution series. After 72 h of anaerobic incubation, the MIC was estimated and was considered the lowest concentration of antibiotic yielding no growth. The E-test strips were put on the surface of the TPNY agar. After 72 h of anaerobic incubation, the MICs for the bacterial strains were determined. By the E-test, MICs were read at the point where the inhibition zone intersected the MIC scale on the strip, according to the manufacturer's instructions.

Tissue cage technique. The tissue cage technique was used as described previously (6, 7). Six tissue cages of Teflon net with a volume of 1.5 ml were implanted subcutaneously in the backs of New Zealand White rabbits (weight, 1.5 to 2.0 kg; both sexes; age, 6 to 9 months). After 10 days and proper healing, 0.1 ml of a bacterial suspension (approximately 10^9 CFU/ml) was inoculated into the tissue cages with a 1-ml graded syringe. Clinical and microbiological examinations were performed at 2 h and 1, 3, 7, and 14 days after inoculation. For microbiological examination, 0.1 ml of the content of each tissue cage at each time point was aspirated with a 1-ml graded syringe, diluted in series in VMG I, and inoculated anaerobically on a TNPY agar plate as described above. After 7 to 10 days of incubation, the numbers of colonies were counted. The infectivity was also estimated from visual inspection of the aspirated tissue cage material at the various time points, and the level of infectivity was classified in one of three categories (7).

RESULTS

Comparison of growth media and colony morphology. TSA II revealed the best growth with pinkish to yellowish white, speckled, sometimes donut-shaped colonies with a translucent zone. Two strains (strains OMGS 3078 and OMZ 471) demonstrated slightly, comparatively flat, roughly speckled colonies.

API ZYM test. The results of the API ZYM test for 19 strains of *B. forsythus* demonstrated strong trypsin-like activity. Strong alkaline phosphatase, acid phosphatase, and *N*-acetyl-β-D-glucosaminidase activities were demonstrated for all strains. All strains were negative for lipase, chymotrypsin, α-galactosidase, β-glucosidase, and α-mannosidase. Esterase lipase formation was generally weak; however, strains OMGS 3080 and OMGS 3081 were negative for esterase lipase formation, and OMZ 471 was strongly positive for esterase lipase formation. Leucine arylamidase reactions were negative for three strains (strains OMGS 3079, strain OMGS 3080, and OMGS 3081), and only strain OMGS 3084 was negative for β-glucuronidase.

TABLE 1. MICs for *B. forsythus* by the agar dilution method and E-test

Antibiotic	Agar dilution method MIC (μg/ml)		E-test MIC (μg/ml)	
	90%[a]	Range	90%	Range
Phenoxymethylpenicillin	0.5	<0.06–0.5	0.50	<0.016–0.5
Ampicillin	0.5	<0.06–0.5	1.0	<0.016–1.0
Amoxicillin	0.5	<0.06–0.5	0.38	<0.016–0.38
Metronidazole	<0.06	<0.06	0.0047	<0.002–0.125
Ciprofloxacin	2.0	0.12–>16	4	0.125–>32
Tetracycline	<0.06	<0.06–0.12	0.19	<0.016–0.19
Doxycycline	0.12	<0.06–0.12	0.38	0.016–0.38
Clindamycin	<0.06	<0.06	<0.016	<0.016–0.032

[a] 90%, MIC at which the growth of 90% of the strains was inhibited.

GLC analysis. The main volatile acid products in PYG broth determined by GLC analysis were propionate, isovalerate, and phenylacetate. Small amounts of isobutyrate and butyrate were also detected. The growth was, however, generally poor. In Huntoon medium, which revealed better growth, all strains also produced isobutyrate, butyrate, acetate, and succinate.

Ribotyping. Among the 19 strains tested, 10 different ribotype patterns were revealed by the use of *Pst*I restriction enzymes. Thus, the ribotype patterns could be used for strain identification in the wound chamber experiments (see below).

SDS-PAGE banding patterns. All 19 *B. forsythus* strains including the type strain ATCC 43037 showed similar banding patterns, and no subtypes were recorded.

Antibiotic susceptibility test. Table 1 summarizes the results estimating the susceptibilities of the strains to antibiotics by the agar dilution method and the E-test. Both methods provided similar results. The *B. forsythus* strains tested were most sensitive to clindamycin (MICs, <0.016 to 0.032 μg/ml) and metronidazole (MICs, <0.002 to 0.125 μg/ml). *B. forsythus* showed a comparatively lower susceptibility to ciprofloxacin (MICs, 0.125 to >32 mg/ml), while other antibiotics showed higher but more variable susceptibility patterns. All strains were sensitive to penicillin G (MICs, <0.5 μg/ml), ampicillin (MICs, <1.0 μg/ml), amoxicillin (MICs, <0.38 μg/ml), tetracycline (MICs, <0.19 μg/ml), doxycycline (MICs, <0.05 μg/ml), and erythromycin (MICs, <0.04 μg/ml).

Tissue cage technique. The results of experiments performed by the tissue cage technique with *B. forsythus* coinoculated with *P. gingivalis* FDC 381 or *F. nucleatum* ATCC 10953 are presented in Table 2. Neither *B. forsythus* ATCC 43037 nor OMGS 3073 was detected at day 1 when the strains were inoculated alone, the strains did not survive, and no abscess formation was recorded. In cages with either *B. forsythus* strain (ATCC 10953 and OMGS 3073) coinoculated with *F. nucleatum* FDC 381, abscess formation was observed. When either of the *B. forsythus* strains was coinoculated with *P. gingivalis* FDC 381, the *B. forsythus* strain was not detected at day 1 or 3 but was reisolated again after 7 and 14 days concomitantly with abscess formation.

P. gingivalis FDC 381 was used for coinoculation with four different *B. forsythus* strains (strains OMGS 3073, OMGS 3076, OMGS 3086, and OMZ 471). The four combinations showed

TABLE 2. Infectivity of *B. forsythus* alone or in combination with other strains in the wound chamber model in rabbits

Bacteria or bacterial combination	% Abscess formation after 7 days[a]
B. forsythus ATCC 43037 + *P. gingivalis* FDC 381	100
B. forsythus ATCC 43037 + *F. nucleatum* ATCC 10953	75
B. forsythus OMGS 3073 + *P. gingivalis* FDC 381	100
B. forsythus OMGS 3073 + *F. nucleatum* ATCC 10953	16
F. nucleatum ATCC 10953	0
P. gingivalis FDC 381	0
B. forsythus ATCC 43037	0
B. forsythus OMGS 3073	0

[a] Six rabbits were used for each bacterium or bacterial combination except that with *B. forsythus* ATCC 43037 and *F. nucleatum* ATCC 10953, for which four rabbits were used.

almost similar patterns initially, with abscess formation, purulent exudate, and swelling of the tissue cage area at day 6 or 7. Simultaneously, four rabbits died of sepsis. *P. gingivalis* and *B. forsythus* were recovered from the heart blood of three of them at a proportion of 10:1. The ribotype patterns obtained for three isolates from rabbit heart blood in the tissue cage experiment coincided with those of the inoculated strains OMGS 3085 (from one rabbit) and OMZ 471 (from two rabbits). All strains were inoculated simultaneously in monoculture and lost viability after 3 days and were not recovered from the cage after 7 days.

DISCUSSION

Many studies have been carried out to examine the subgingival plaque flora by use of cultivation; however, only a few studies have dealt with *B. forsythus* (8, 10, 14, 35). Those studies revealed an elevated proportion of this bacterium from active sites and initial lesions. It is apparent from more recent studies that the prevalence of *B. forsythus* in subgingival pocket flora has been underestimated due to its poor growth on culture media and its presence at levels below the levels of detection by the culture method (12). Higher prevalences have been obtained by noncultural methods (4, 12, 15, 22, 24). *B. forsythus* is recognized for its trypsin-like enzyme, which is also found in *P. gingivalis* and *T. denticola* (37) and disclosed in the benzoyl-DL-arginine-naphthylamide test used to monitor patients for periodontal disease (21). Further biological characterization, pathogenicity, and response to treatment have not been thoroughly examined due to the poor growth of *B. forsythus* in laboratory media. *B. forsythus* needs NAM for its growth (38, 39). When TSA II was supplied in the present study with only NAM and blood, the growth was, however, still poor after 7 days of incubation. To achieve better growth, we supplemented TSA II with phytone peptone and yeast extract. This medium, TNPY medium, was used for *B. forsythus* in all experiments and antibiotic susceptibility tests performed in this study. The colony morphology of *B. forsythus* on TNPY medium differed from strain to strain, and even for the same strain it changed with the growth conditions. That makes it difficult to detect *B. forsythus* on nonselective media in samples from the periodontal pocket, supporting the discussion of Gmür et al. (12), who suggested that *B. forsythus* is found less often in studies that use cultivation techniques than in those that use DNA probe or immunological techniques (12, 18, 22). *B. forsythus* showed a consistent enzymatic profile and produced metabolic acids and other products, thus confirming earlier reports (3, 37). While the SDS-PAGE profile was similar for all strains tested, ribotyping with *Pst*I disclosed 10 different patterns, confirming the existence of a heterogeneity on the genotypic level, similar to many other oral species (11).

Previously, Baker et al. (1) examined the susceptibilities of periodontal bacteria to several antibiotics. *B. forsythus* seems to exhibit a susceptibility pattern similar to that of *P. gingivalis* and many other oral anaerobic bacteria, being sensitive to many of the antibiotics tested (19, 29). Interestingly, in the present study *B. forsythus* was less susceptible to ciprofloxacin than to other antibiotics, which somewhat contradicts the report of Maiden et al. (23). It is not clear whether this reflects discrepancies in methodology or a true difference. Hence, peroral medication with ciprofloxacin may have an inadequate effect on *B. forsythus*. Ciprofloxacin has been introduced as an adjunct therapy in patients with periodontal diseases when opportunistic nonoral bacteria have become established in the subgingival region (32).

B. forsythus was not able to cause experimental infections in monoculture in the wound chamber method in rabbits, thereby revealing a pattern similar to those of many other anaerobes (33). A more successful infectivity was obtained by coinoculation with either *F. nucleatum* or *P. gingivalis*, which resulted in several significant conclusions. *B. forsythus* is pathogenic in an experimental infection model, thus fulfilling one important criterion as a putative periodontopathogen (16). The supporting bacteria do not necessarily have to be facultative anaerobes, and the need for low oxygen tension may be overcome by other mechanisms. *B. forsythus* showed invasiveness when it was coinoculated with *P. gingivalis*, a characteristic considered to be significant for bacterial pathogenicity. The invasiveness of *P. gingivalis* FDC 381, normally considered noninvasive (28), was an important finding. Coinoculation with *B. forsythus* alters the pathogenic capacity to a degree similar to that of invasive strains of *P. gingivalis* (13, 28). It means that the ability to invade is not only dependent on the characteristics of the bacterial species itself but may occur as a consequence of bacterial interaction. The invasion which is seen in the experimental wound chamber model and which may occur in periodontal disease may be caused by different mechanisms; however, the results of the present study show that strains cannot simply be divided into invasive and noninvasive variants.

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