API ZYM and API An-Ident Reactions of Fastidious Oral Gram-Negative Species

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API ZYM and API An-Ident enzymatic substrate tests were done on six oral species which are difficult to characterize with conventional biochemical tests. "*Bacteroides forsythus*," the "fusiform" *Bacteroides* species (A. C. R. Tanner, M. A. Listgarten, M. N. Strzempko, and J. L. Ebersole, manuscript in preparation), is difficult to cultivate in broth media, yet it gave 15 positive tests in these series. The tests were able to separate this new species from species of *Capnocytophaga* and *Fusobacterium*. "*B. forsythus*" reactions were similar but not identical to those of reference *Bacteroides* species. Positive reactions for α -glucosidase, β -glucosidase, α -fucosidase, and α -glucuronidase suggest that "*B. forsythus*" may be saccharolytic. It was the only species tested which was trypsin positive. *Wolinella* species, *Campylobacter concisus*, *B. gracilis*, and *Eikenella corrodens* are asaccharolytic, and characterization relies heavily on sensitivities to inhibitory agents. These species reacted weakly in the API ZYM and API An-Ident enzymatic substrate tests, and the reactions were not useful for separating these species. The enzyme reactions differentiated *Wolinella recta* and *C. concisus* from *Selenomonas sputigena*, another oral motile but saccharolytic organism.

Several species of oral microorganisms are difficult to characterize because they grow poorly in broth media. One such species, "*Bacteroides forsythus*" (the "fusiform" *Bacteroides* species), grows slowly and has pleomorphic, fusiform cells with long filaments and spheroids (5; A. C. R. Tanner, M. A. Listgarten, M. N. Strzempko, and J. L. Ebersole, manuscript in preparation). Cells grow minimally in broth media. "*B. forsythus*" is strictly anaerobic, with small white or pink speckled colonies, 1 to 3 mm in diameter. *Campylobacter concisus*, *Bacteroides gracilis*, and *Wolinella* species are small gram-negative asaccharolytic rods which grow in broth media supplemented with formate and fumarate. Because these last species have similar metabolisms, differentiation can be difficult and relies heavily on differences in sensitivity to dyes and chemical inhibitors.

The purpose of the present investigation was to evaluate the API ZYM and API An-Ident (Analytab Products, Plainview, N.Y.) enzymatic substrate tests as an alternate method of strain characterization which did not require broth growth. We were particularly interested in finding tests that would lead to rapid and accurate identification of "*B. forsythus*" and *Wolinella recta* because these two species show a strong relationship with active periodontal disease (5, 6; J. L. Dzink, A. C. R. Tanner, A. D. Haffajee, and S. S. Socransky, J. Clin. Periodontol., in press).

MATERIALS AND METHODS

Source and maintenance of strains. Most of the strains used were isolated from periodontally diseased sites (Table 1). Strains were maintained on Trypticase soy agar plates supplemented with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) and were incubated anaerobically. Cells of most species were harvested from plates after 4 to 6 days of anaerobic incubation. "*B. forsythus*" strains were maintained by streaking cells on one-half of each plate and *Fusobacterium nucleatum* cells on the other half. The latter species stimulates growth of "*B. forsythus*" (J. L.

Dzink, personal communication). "*B. forsythus*" strains were harvested from plates after 6 to 8 days of anaerobic incubation. All strains were tested in the API An-Ident system; most were tested in the API ZYM system.

Inoculation and reading of tests. Organisms for both test series were scraped from three to four Trypticase soy agar plates to provide a heavy inoculum, equal to a no. 5 McFarland standard. Lighter inocula led to inconsistent results. Each strain was examined by dark-field microscopy and was subcultured onto a plate incubated aerobically to check for culture purity. Cells were suspended in 2 ml of sterile water for each strip and were distributed into the wells with a sterile Pasteur pipette. Sterile water was put in wells of control strips. All tests were run at least twice.

Test strips were incubated in air at 37°C for 4 h in a humid atmosphere. Color development, indicating positive reactions, was graded with reference to the API ZYM and API An-Ident color charts.

API ZYM tests. The substrates used in the API ZYM tests have been previously reported (2-4). After incubation, 1 drop of each of developing reagents A (Tris, 250 g; 37% HCl, 110 ml; laurylsulfate, 100 g; distilled water to 1 liter) and B (fast blue BB, 3.5 g; 2-methoxyethanol to 1 liter) was added to each of the wells.

API An-Ident tests. The API An-Ident system consists of a series of 20 microcupules containing dehydrated substances. *N*-Acetyl- β -glucosaminidase, α -glucosidase, α -arabinosidase, β -glucosidase, α -fucosidase, phosphatase, α - and β -galactosidases, and indoxylacetate tests contain chromogenic substrates whose reactions are detected by the liberation of *p*-nitrophenol, *o*-nitrophenol, or indoxyl from the substrates. Leucine, proline, tyrosine, arginine, alanine, histidine, phenylalanine, and glycine aminopeptidases and pyroglutamic acid arylamidase microcupules require the addition of cinnamaldehyde to detect free β -naphthylamine (cinnamaldehyde per liter: *p*-dimethylaminocinnamaldehyde, 0.15 g; sodium dodecyl sulfate, 25 g; glacial acetic acid, 25 ml; 2-methoxyethanol, 50 ml; distilled water, 925 ml). Phenol red is used as the indicator in the arginine

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TABLE 1. Strains used for API ZYM and API An-Ident tests

Species	Strain ^a	Source
<i>Bacteroides forsythus</i>	42, 293, 334, 338, 148, 436, 2008, R145 BV16, BV19, DL12, DL38	Periodontal pocket Periapical tissue
<i>Wolinella recta</i> <i>Wolinella curva</i>	ATCC ^b 33238 ^T (371), 267, 285, 302, 303, 372, 1087 ATCC 35244 ^T (VPI ^c 9584) VPI 10659, 640 VPI 10296	Periodontal pocket Alveolar abscess Human clinical isolate Dental root canal
<i>Wolinella succinogenes</i>	ATCC 29543 ^T (602W) IB4 ^d	Bovine rumen Sewage
<i>Campylobacter concisus</i>	ATCC 33237 ^T (484), 483, 522, 569	Periodontal pocket
<i>Bacteroides gracilis</i> <i>Bacteroides ureolyticus</i>	ATCC 33236 ^T (1084), 406, 568, 1083 VPI 7814 VPI 7815	Periodontal pocket Anal fistula Human clinical isolate
<i>Eikenella corrodens</i>	373, 468, 469, 525, 1007, 1073, 1074	Periodontal pocket
Reference strains		
<i>Bacteroides buccae</i> (<i>Bacteroides capillus</i>)	ATCC 33574 ^T ATCC 33690, ATCC 33691	Gingival sulcus Gingival sulcus
<i>Bacteroides fragilis</i>	ATCC 25285 ^T ATCC 23745	Appendix abscess Pleural fluid
<i>Bacteroides distasonis</i>	ATCC 8503 ^T	Human feces
<i>Bacteroides thetaiotaomicron</i>	ATCC 29148 ^T ATCC 12290	Human feces Appendix abscess
<i>Bacteroides vulgatus</i>	ATCC 8482 ^T	Human feces
<i>Capnocytophaga sputigena</i>	ATCC ^b 33612 ^T (4)	Periodontal pocket
<i>Capnocytophaga gingivalis</i>	ATCC 33624 ^T (27)	Periodontal pocket
<i>Capnocytophaga ochracea</i>	6, 25	Periodontal pocket
<i>Fusobacterium nucleatum</i>	364, 40	Periodontal pocket
<i>Fusobacterium gonidiaformans</i>	ATCC 25563 ^T	Periodontal pocket
<i>Selenomonas sputigena</i>	1304	Periodontal pocket

^a Strains without specific designation were isolated at Forsyth Dental Center, Boston, Mass. ^T, Type strain of species.

^b American Type Culture Collection, Rockville, Md.

^c Virginia Polytechnic Institute and State University Anaerobe Laboratory, Blacksburg, Va.

^d Obtained from T. Yoshinari, Department of Health, Albany, N.Y.

cupules. Indole was detected by using Kovac reagent. Hydrogen peroxide is used in the α -glucosidase microcupule to detect catalase.

RESULTS

"*B. forsythus*" gave strong positive reactions in both API ZYM and API An-Ident enzymatic substrate tests (Table 2). The reactions of "*B. forsythus*" were specific to this species although were similar to those of the other *Bacteroides* species tested. Only "*B. forsythus*" had trypsin activity.

"*B. forsythus*" could be differentiated from the *Capnocytophaga* species by using the consistent positive aminopeptidase activity of the *Capnocytophaga* species. The fusobacteria were unreactive in both test series, with the exception of the indole and variable arginine aminopeptidase activities of *F. nucleatum*.

The formate- and fumarate-requiring species, *Wolinella* species, *Campylobacter concisus*, *B. gracilis*, and *Bacteroides ureolyticus*, reacted weakly in the API ZYM and An-Ident enzyme substrate tests (Table 2). *Eikenella corrodens* was acid phosphatase negative and proline aminopeptidase positive, which distinguished it from the formate- and fumarate-requiring species. *Selenomonas sputigena* was positive for α -galactosidase, β -galactosidase,

and α -glucosidase (API ZYM strip) and for α -glucosidase, β -glucosidase, and α -galactosidase (API An-Ident strip). These reactions separated this species from the other motile species tested (Table 2). The API An-Ident strip was more reactive than the API ZYM strip for the same test substrates, with the exception of β -galactosidase for *S. sputigena*.

DISCUSSION

In this investigation, we were able to get 15 positive reactions for "*B. forsythus*." Strains of this species do not grow well in broth media, including media supplemented with serum and media preconditioned with agar (A. C. R. Tanner, S. S. Socransky, and C. M. Smith, J. Dent. Res. 60A:485, 1981). By characterization with conventional tests, "*B. forsythus*" was esculin positive but was negative for other biochemical reactions, including carbohydrate fermentations. "*B. forsythus*" strains did not react in the API 20A or API 20E series. The positive reactions for α -glucosidase, β -glucosidase, α -fucosidase, and α -glucuronidase in the API enzyme substrate tests suggested that with sufficient broth growth the species may be saccharolytic. Thus, the API enzymatic substrate tests helped characterize this new species.

The enzyme reactions of "*B. forsythus*" were different

TABLE 2. API ZYM and API An-Ident test results

Test	Reaction ^a					
	" <i>B. forsythus</i> "	Other <i>Bacteroides</i> spp.	<i>Capnocytophaga</i> spp.	Formate and fumarate-requiring species ^b	<i>E. corrodens</i>	<i>S. sputigena</i>
Trypsin ^c	+	—	—	—	—	—
α-Fucosidase ^c	+	— ^e	—	—	—	—
α-Fucosidase and <i>N</i> -acetyl-β-glucosaminidase ^f	+	+	—	—	—	—
α-Glucosidase ^{c,f}	+	+	—	—	—	+
β-Glucuronidase ^c	+	V	—	—	—	—
<i>N</i> -Acetyl glucosaminidase ^f	+	+	—	—	—	—
α-Galactosidase ^{c,f}	—	+	W	—	—	+
β-Galactosidase ^c	—	+	—	—	—	+
α-Arabinosidase ^f	V	V ^e	—	—	—	—
Alanine aminopeptidase ^f	+	+	+	W	+	—
Arginine aminopeptidase ^f	+	+	+	+	+	—
Leucine aminopeptidase ^f	W	+	+	W/—	W	—
Valine and cystine aminopeptidase ^c	—	—	+	—	—	—
Proline aminopeptidase ^f	—	—	+	—	+	—
Tyrosine aminopeptidase ^f	—	W	+	—	W	—
Esterase and esterase lipase ^c	—	—	—	W	+	—
Phosphatase ^f	+	+	V	—	—	W

^a +, Positive reaction (color range of 3 to 5 on the API color charts); —, negative reaction; V, variable reaction; W, weak reaction (color range of 1 to 2 on the API color charts). Boxes indicate reactions which separate species.

^b *Wolinella* species, *Campylobacter concisus*, *B. gracilis*, and *B. ureolyticus*.

^c API ZYM reactions.

^d Of 12 strains, 10 were trypsin positive.

^e Except *B. fragilis*.

^f API An-Ident reactions.

^g Except *B. buccae*.

from those of any other species tested, and the reactions easily separated the fusiform species of the oral cavity. The *Capnocytophaga* species were consistently positive for all aminopeptidase reactions, whereas the *Fusobacterium* species were unreactive in the enzyme substrate tests.

The trypsin activity of *B. gingivalis* and *B. asaccharolyticus* has been implicated as a pathogenic mediator of these species (4). If so, it seems likely that a similar pathogenic mechanism could be possible for strains of "*B. forsythus*" that degrade trypsin.

In contrast, the reactivity of the formate- and fumarate-requiring species was disappointing and may reflect the asaccharolytic nature of these strains. The similar patterns of reactions of *Wolinella* species, *Campylobacter concisus*, and *B. gracilis* suggested that these tests are not useful to identify the individual species. Another disadvantage was that we needed at least three plates of these lightly growing species to get a sufficiently heavy inoculum for each test strip. *E. corrodens* and *S. sputigena* could be differentiated, but conventional biochemical tests can easily distinguish these species.

API ZYM and API An-Ident reactions for species of *Bacteroides*, *Capnocytophaga*, and *Fusobacterium* and for *W. recta* (API ZYM only) were similar to those previously reported (1–4). In this investigation, we extended the organisms tested so that reactions have now been reported in both systems for "*B. forsythus*," *F. gonidiaformans*,

Wolinella species, *Campylobacter concisus*, *B. gracilis*, *B. ureolyticus*, and *E. corrodens*. The API ZYM and An-Ident tests could neither distinguish between species of *Capnocytophaga* or *Fusobacterium* nor differentiate *Wolinella* species, *Campylobacter concisus*, and *B. gracilis* from each other.

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