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API ZYM System for Identification of *Bacteroides* spp., *Capnocytophaga* spp., and Spirochetes of Oral Origin

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A total of 80 oral strains of *Bacteroides gingivalis, B. asaccharolyticus, B. melaninogenicus* subsp. *intermedius, B. melaninogenicus* subsp. *melaninogenicus, Capnocytophaga, Treponema denticola*, and *T. vincentii* were characterized with the API ZYM system for 19 enzyme activities. Comparison of anaerobic and aerobic incubation with nine reference strains of these organisms showed no important differences. The key differential tests for black-pigmented *Bacteroides* strains and treponemes of oral origin were trypsin, α-glucosidase, and *N*-acetyl-β-glucosaminidase. All *Capnocytophaga* strains produced distinctive aminopeptidase activities but varied in their glycosidic capabilities. The presence of a trypsin-like activity in *B. gingivalis, T. denticola*, and a group of *Capnocytophaga* strains may contribute to tissue destruction in periodontal disease.

The API ZYM system is a rapid test procedure which uses chromogenic substrates to detect 19 enzyme activities. It has been examined by several investigators for its applicability to the differentiation of streptococci (8, 32), actinomycetes (10), peptococci (21), and gram-negative anaerobes of clinical origin (6, 29). This report concerns the enzyme profile of three groups of organisms which may be involved in the etiology of periodontal disease. The black-pigmented *Bacteroides* species, especially *Bacteroides gingivalis* (*B. asaccharolyticus*), have been recovered in elevated proportions from patients with advanced periodontitis (16, 23, 28). The recently described genus, *Capnocytophaga* (12), has been reported to produce alveolar bone loss in monoinfected gnotobiotic rats (5, 9) and to cause human neutrophil (22) and fibroblast (26) dysfunction in vitro. Oral spirochetes have long been observed to reach significantly high numbers in periodontitis lesions (15, 20) and to invade tissue in ulcerative gingivitis (14).

Because of the importance of these organisms in periodontal disease, the API ZYM system was employed to survey the enzyme profiles to determine whether any potentially tissue-destructive enzymes were present. As previously reported (B. E. Laughon, S. A. Syed, and W. J. Loesche, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, C29, p. 279), we observed activities such as alkaline phosphatase, aminopeptidase, trypsin, and *N*-acetyl-β-glucosaminidase which were consistently present in certain species.

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trypsin, and *N*-acetyl-β-glucosaminidase which were consistently present in certain species.

MATERIALS AND METHODS

Microorganisms. A total of 89 strains consisting of 9 reference strains, 72 isolates from human periodontal plaque, 8 isolates from the dental plaque of beagle dogs, and 23 isolates of oral spirochetes were used in this study. The 89 strains included representatives of the genera *Bacteroides, Capnocytophaga*, and *Treponema*. The following strains were obtained from the American Type Culture Collection: *Bacteroides asaccharolyticus* 25260, *B. melaninogenicus* subsp. *intermedius* 25611, and *B. melaninogenicus* subsp. *melaninogenicus* 25845. In addition, A. Tanner of the Forsyth Dental Center, Boston, Mass., kindly provided lyophilized cultures of *Capnocytophaga ochracea* strain 6, *C. gingivalis* strain 27, and *C. sputigena* strain 4. Jørgen Slots, State University of New York, Buffalo, generously provided the *B. gingivalis* strain 2561 (ATCC 33277), and the FM and N9 spirochetes were obtained from R. M. Smibert, Virginia Polytechnic Institute, Anaerobe Laboratory, Blacksburg, Va.

Growth conditions. All cultures were incubated at 35°C for 6 to 10 days in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) containing an atmosphere of 85% N_2, 10% H_2, and 5% CO_2. Bacteria other than spirochetes were cultured on enriched Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) (27). Spirochetes were grown in a broth medium containing (g/100 ml): Trypticase peptone (BBL Microbiology Systems), 1.0; yeast extract, 1.0; gelatin, 1.0; heart infusion broth (Difco Laboratories, Detroit, Mich.), 0.5; glucose, 0.1; cysteine-HCl, 0.1; $(NH_4)_2SO_4$, 0.05; $MgSO_4$, 0.088; K_2HPO_4, 0.113; KH_2PO_4, 0.09; NaCl, 0.1; and thiamine pyrophosphate, 0.00125. The medium also contained 10% (vol/vol) heat-inactivated rabbit serum.

API ZYM system. API ZYM is a registered trademark of Analytab Products (Plainview, N.Y.) and

consists of a series of microcupules containing dehydrated chromogenic substrates. The 19 enzyme activities and corresponding substrates are: alkaline phosphatase (2-naphthylphosphate), C-4 esterase (2-naphthylbutyrate), C-8 esterase lipase (2-naphthylcaprylate), C-14 lipase (2-naphthylmyristate), leucine aminopeptidase (L-leucyl-2-naphthylamide), valine aminopeptidase (L-valyl-2-naphthylamide), cystine aminopeptidase (L-cystyl-2-naphthylamide), trypsin (*N*-benzoyl-DL-arginine-2-naphthylamide), chymotrypsin (*N*-glutarylphenylalanine-2-naphthylamine), acid phosphatase (2-naphthylphosphate), phosphoamidase (naphthyl-AS-B1-phosphodiamide), α-galactosidase (6-bromo-2-naphthyl-α-D-galactopyranoside), β-galactosidase (2-naphthyl-β-D-galactopyranoside), β-glucuronidase (naphthyl-AS-B1-β-D-glucuronide), α-glucosidase (2-naphthyl-2-D-glucopyranoside), β-glucosidase (6-bromo-2-naphthyl-β-D-glucosaminide), *N*-acetyl-β-glucosaminidase (1-naphthyl-*N*-acetyl-β-D-glucosaminide), α-mannosidase (6-bromo-2-naphthyl-2-D-mannopyranoside), and α-fucosidase (2-naphthyl-α-L-fucopyranoside). Two reagents are used in the API ZYM system to develop the chromogenic substrates. Reagent A contains (per 100 ml): Tris, 25 g; HCl (37%), 11 ml; and sodium dodecyl sulfate, 10 g. Reagent B contains 0.35% Fast Blue BB salt in 2-methoxyethanol.

Test procedure. Stationary-growth phase bacteria other than spirochetes were removed from enriched Trypticase soy agar plates with an inoculating loop and suspended in 2 ml of sterile saline (0.85% NaCl) to a density approximating McFarland no. 5 or 6 turbidity standard. The spirochetes were harvested in the stationary phase of growth by centrifugation for 1 min at 15,000 × *g* in an Eppendorf microcentrifuge (Brinkmann Instruments Inc., Westbury, N.Y.), washed once in sterile saline, and resuspended in saline as above. The API ZYM strips were activated by adding 2 drops of bacterial suspension to each microcupule and were incubated aerobically or anaerobically for 4 to 6 h at 35°C in moist chambers.

To develop the color reactions 1 drop each of reagents A and B was added to each microcupule. The color was allowed to develop for 5 to 15 min, and the intensities were recorded on a scale of 0 to 5 by comparison with the color chart provided by the manufacturer.

RESULTS

The API ZYM system produced consistent results in duplicate testings of representative strains (17% of the isolates examined). No important qualitative differences were noted between aerobic and anaerobic incubation conditions, except that *B. gingivalis* (ATCC 33277) produced a weak reaction in the anaerobic C-4 esterase assay and no reaction under aerobic conditions. In addition, this strain also showed a more intense acid phosphatase activity under anaerobic conditions. Because the atmosphere for incubation did not affect the majority of enzymes, all subsequent tests were performed aerobically. The API ZYM activities elaborated by 80 oral isolates of black-pigmented *Bacte-*

TABLE 1. Enzymatic activities of oral black-pigmented *Bacteroides* strains

Enzyme	No. of strains[a] of:											
	B. gingivalis (human)			*B. asaccharolyticus* (beagle dog)			*B. melaninogenicus* subsp. *intermedius* (human)			*B. melaninogenicus* subsp. *melaninogenicus* (human)		
	Negative	Weak	Strong	Negative	Weak	Strong	Negative	Weak	Strong	Negative	Weak	Strong
Alkaline phosphatase	0	0	13	0	0	8	0	0	11	0	0	6
C-4 esterase	6	3	4	5	2	1	6	5	0	4	1	1
C-8 esterase lipase	8	5	0	5	3	0	6	5	0	5	1	0
C-14 lipase	13	0	0	8	0	0	11	0	0	6	0	0
Leucine aminopeptidase	13	0	0	8	0	0	11	0	0	6	0	0
Valine aminopeptidase	12	1	0	7	1	0	10	1	0	6	0	0
Cystine aminopeptidase	13	0	0	8	0	0	11	0	0	6	0	0
Trypsin	0	0	[13][b]	3	0	5	11	0	0	6	0	0
Chymotrypsin	13	0	0	8	0	0	10	1	0	6	0	0
Acid phosphatase	0	2	11	0	2	6	0	0	11	0	0	6
Phosphoamidase	0	9	4	0	4	4	0	0	11	0	0	6
α-Galactosidase	13	0	0	8	0	0	11	0	0	0	[5]	[1]
β-Galactosidase	13	0	0	8	0	0	11	0	0	0	0	[6]
β-Glucuronidase	13	0	0	8	0	0	11	0	0	5	1	0
α-Glucosidase	13	0	0	7	0	1	0	[1]	[10]	0	0	[6]
β-Glucosidase	13	0	0	8	0	0	11	0	0	5	0	1
N-Acetyl-β-glucosaminidase	12	1	0	2	5	1	11	0	0	0	0	[6]
α-Mannosidase	13	0	0	8	0	0	11	0	0	6	0	0
α-Fucosidase	13	0	0	8	0	0	4	7	0	3	1	2

[a] Number of strains exhibiting color intensities assigned a numerical value ranging from 0 to 5, with negative = 0, weak activity = 1 or 2, and strong activity = 3, 4, or 5.

[b] Boxes indicate enzyme activities that had diagnostic value.

roides spp., *Capnocytophaga* spp., and spirochetes are shown in Tables 1 to 3. All strains exhibited some acid phosphatase and phosphoamidase activity. The remaining tests produced differentiating patterns among the genera examined.

The trypsin-like activity of *B. gingivalis* was very consistent and distinctive. None of the human strains of either *B. melaninogenicus* subsp. were positive (Table 1). Moreover, the type strain of *B. asaccharolyticus* (ATCC 25260) failed to hydrolyze the trypsin substrate. The beagle dog isolates were variable for this characteristic.

The black-pigmented *Bacteroides* spp. (Table 1) showed a range of glycosidic activities. Human *B. gingivalis* strains were uniformly negative for hydrolysis of glycosidic bonds, whereas a few canine black-pigmented *Bacteroides* strains were weakly positive for *N*-acetyl-β-glucosaminidase. All *B. melaninogenicus* subsp. *intermedius* strains hydrolyzed only the α-glucosidase substrate, whereas *B. melaninogenicus* subsp. *melaninogenicus* hydrolyzed the substrates for α-glucosidase, β-galactosidase, and *N*-acetyl-β-glucosaminidase.

All 19 strains of *Capnocytophaga* possessed strong leucine, valine, and cystine aminopeptidase and α-glucosidase activities, but they appeared to segregate into three groups on the basis of four other enzymes (Table 2). Group I strains resembled the type strain of *C. gingivalis* in being negative for trypsin, negative or weakly positive for β-galactosidase, positive for β-glucosidase, and negative for *N*-acetyl-β-glucosaminidase. Group II strains included the *C. ochracea* and *C. sputigena* reference strains, 6 and 4, respectively. These were negative or weakly positive for trypsin and β-glucosidase and positive for β-galactosidase and *N*-acetyl-β-glucosaminidase. Group III consisted of seven strains which could not be associated with any described species. These organisms grew as spreading, rough-surfaced adherent colonies on enriched Trypticase soy agar blood agar plates and usually caused pitting of the agar. Trypsin and chymotrypsin were positive, β-galactosidase was weak or negative, β-glucosidase was variable, and *N*-acetyl-β-glucosaminidase was negative.

The two species of oral spirochetes examined, *Treponema denticola* and *T. vincentii*, both produced moderate amounts of C-8 esterase, acid phosphatase, and phosphoamidase activities (Table 3).

The *T. denticola* strains were consistently positive for trypsin and negative for *N*-acetyl-β-glucosaminidase, whereas the opposite was true for the *T. vincentii* strains. Very little hydrolytic activity was observed towards other glycosides.

A summary of the key differential enzymes for oral species of *Bacteroides* and *Treponema* is shown in Table 4. The enzyme profile of the *Capnocytophaga* strains was not sufficiently re-

TABLE 2. Enzymatic activities of human oral *Capnocytophaga* strains

Enzyme	No. of strains[a] of:								
	Group I (*C. gingivalis*)			Group II (*C. orchracea* and *C. sputigena*			Group III (rough surfaced)		
	Negative	Weak	Strong	Negative	Weak	Strong	Negative	Weak	Strong
Alkaline phosphatase	0	2	5	0	0	5	0	0	7
C-4 esterase	0	7	0	0	5	0	2	5	0
C-8 esterase lipase	0	7	0	0	1	4	0	7	0
C-14 lipase	4	3	0	2	3	0	6	1	0
Leucine aminopeptidase	0	0	[7][b]	0	0	[5]	0	0	[7]
Valine aminopeptidase	0	0	[7]	0	0	[5]	0	0	[7]
Cystine aminopeptidase	0	0	[7]	0	0	[5]	0	0	[7]
Trypsin	6	1	[0]	3	2	[0]	0	2	[5]
Chymotrypsin	4	3	0	0	5	0	0	4	[3]
Acid phosphatase	0	1	6	0	1	4	0	0	7
Phosphoamidase	0	5	2	0	2	3	0	6	1
α-Galactosidase	6	1	0	4	1	0	7	0	0
β-Galactosidase	2	5	0	0	2	3	3	4	0
β-Glucuronidase	6	1	0	4	1	0	7	0	0
α-Glucosidase	0	0	7	0	0	5	0	0	7
β-Glucosidase	0	[3]	[4]	3	2	0	4	1	2
N-Acetyl-β-glucosaminidase	7	0	0	0	[3]	[2]	7	0	0
α-Mannosidase	7	0	0	5	0	0	7	0	0
α-Fucosidase	7	0	0	5	0	0	7	0	0

[a] Number of strains exhibiting color intensities assigned a numerical value ranging from 0 to 5, with negative = 0, weak activity = 1 or 2, and strong activity = 3, 4, or 5.

[b] Boxes indicate enzyme activities that had diagnostic value.

TABLE 3. Enzymatic activities of human oral spirochetes

Enzyme	No. of strains[a] of:					
	T. denticola			*T. vincentii*		
	Negative	Weak	Strong	Negative	Weak	Strong
Alkaline phosphatase	10	10	0	3	0	0
C-4 esterase	2	17	1	0	3	0
C-8 esterase lipase	0	8	12	0	3	0
C-14 lipase	15	5	0	3	0	0
Leucine aminopeptidase	1	19	0	1	2	0
Valine aminopeptidase	15	5	0	2	1	0
Cystine aminopeptidase	11	9	0	3	0	0
Trypsin	0	0	[20][b]	3	0	0
Chymotrypsin	2	15	3	3	0	0
Acid phosphatase	0	9	11	0	3	0
Phosphoamidase	0	13	7	0	3	0
α-Galactosidase	8	12	0	3	0	0
β-Galactosidase	17	3	0	0	3	0
β-Glucuronidase	20	0	0	3	0	0
α-Glucosidase	19	1	0	3	0	0
β-Glucosidase	19	1	0	3	0	0
N-Acetyl-β-glucosaminidase	20	0	0	0	[2]	[1]
α-Mannosidase	20	0	0	3	0	0
α-Fucosidase	20	0	0	3	0	0

[a] Number of strains exhibiting color intensities assigned a numerical value ranging from 0 to 5, with negative = 0, weak activity = 1 or 2, and strong activity = 3, 4, or 5.
[b] Boxes indicate enzyme activity that had diagnostic value.

solved to make reliable identification at the species level.

DISCUSSION

The API ZYM system appears well-suited for the detection of enzymatic activity of the oral gram-negative bacteria examined in this study. The procedure is simple and convenient, does not require large cell masses, and may be performed under aerobic conditions. This system provides a rapid and reliable method for identification of the various black-pigmented *Bacteroides* and *Treponema* species (Table 4). The *Capnocytophaga* strains can be identified to the genus, but not to the species, level.

Human black-pigmented *Bacteroides* strains which did not hydrolyze glycosides but possessed an amidase activity against *N*-benzoyl-DL-arginine-2-naphthylamide were invariably isolates of *B. gingivalis*. These organisms, previously referred to as *B. asaccharolyticus* (4) differ from the type strain of *B. asaccharolyticus* in this trypsin-like activity toward *N*-benzoyl-DL-arginine-2-naphthylamide. Seven strains of *B. asaccharolyticus* isolated from nonoral sources were negative for the trypsin-like activity (29). These observations support the proposal of Coykendall et al. (4) for the recognition of a new species *B. gingivalis* and raise the possibility that this enzyme may contribute to the periodontal disease process.

The lack of activity against the chymotrypsin substrate suggests a specificity similar to that of the mammalian trypsin. None of the *B. melaninogenicus* strains hydrolyzed the *N*-benzoyl-DL-arginine-2-naphthylamide substrate. The two subspecies examined produced glycosidase activities in the API ZYM system which correlate with their individual fermentative capabilities (7).

The observation that all *Capnocytophaga* strains were strongly positive for leucine, valine,

TABLE 4. Key differential tests for oral species of *Bacteroides* and *Treponema*

Organism	Test result		
	Trypsin	α-Glucosidase	*N*-Acetyl-β-glucosaminidase
B. asaccharolyticus	−	−	−
B. gingivalis	+	−	−
B. melaninogenicus subsp. *intermedius*	−	+	−
B. melaninogenicus subsp. *melaninogenicus*	−	+	+
T. denticola	+	−	−
T. vincentii	−	−	+

and cystine aminopeptidases indicates more proteolytic potential than previously reported (25). Furthermore, the additional presence of trypsin and chymotrypsin activities in the rough-surfaced strains suggests a broad phenotypic diversity within the proposed genus. The API ZYM system also detected *N*-acetyl-β-glucosaminidase activity in *C. ochracea* and *C. sputigena*, but not in *C. gingivalis*. This activity, observed with *B. melaninogenicus* subsp. *melaninogenicus* and *T. vincentii*, may contribute to the breakdown of tissue ground substance or hyaluronic acid.

The 23 strains of *T. denticola* and *T. vincentii* produced reactions in the API ZYM system which were consistent with the available information on their physiology (3, 24). *T. denticola* is known to be proteolytic for fibrin and gelatin (17, 24) but had not previously been reported to have trypsin and chymotrypsin activities. The two species could be differentiated on the basis of trypsin and *N*-acetyl-β-glucosaminidase.

The presence of a trypsin-like activity in *B. gingivalis*, *T. denticola*, and the unidentified group of *Capnocytophaga* strains deserves further comment. *B. gingivalis* and the spirochetes reach high proportions in plaques associated with advanced periodontitis but are rarely detected in health (15, 16, 23). This trypsin-like activity seems to be unique to these bacteria as more than 25 other oral species are known, with the exception of *Bacterionema matruchotti* and *Rothia dentocariosa*, to be trypsin negative (6, 10, 29, 31, unpublished data). The presence of this trypsin activity primarily in periodontopathic organisms suggests that this enzyme may be an important determinant of their virulence in periodontal disease. Such proteolytic activity may have a direct effect upon the junctional epithelium in the periodontal pocket as trypsin has been shown in vitro to disrupt cell-cell or cell-substratum adhesions (2, 30). In addition, trypsin seems to activate latent gingival tissue collagenase by destruction of a collagenase inhibitor present in serum (1, 19). Finally, this bacterial enzyme could activate the alternate pathway of complement fixation causing the release of leukotactic factors C3a and C5a, as has been demonstrated with trypsin and certain bacterial proteinases (32). In fact, this trypsin-like enzyme could be the factor in periodontal plaques (11, 18) and pure cultures of *T. denticola* and *B. gingivalis* (13) which has been found to be chemotactic for polymorphonuclear leukocytes. These trypsin-like activities acting singly or in concert could effect significant pathology on the periodontium. The trypsin-like enzyme of these organisms should be further characterized to evaluate its relationship to pancreatic trypsin and its potential for periodontal pathogenesis.

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