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Rapid Identification of *Bacteroides gingivalis*

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Rapid colorimetric tests for trypsin and α-glucosidase are described for use in the identification of *Bacteroides gingivalis* from dental plaque.

Coykendall et al. (1) have recently proposed a new species, *Bacteroides gingivalis*, which consists of strains previously classified as *Bacteroides asaccharolyticus*. These organisms are found in the human oral environment, especially in patients with periodontal disease (5). They show little DNA-DNA homology with the type strain of *B. asaccharolyticus* (1), fail to react with a commercial fluorescent antibody prepared against *B. asaccharolyticus* (6; S. A. Syed, B. E. Laughon, and W. J. Loesche. Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, C26, p. 279), and produce phenylacetic acid as an end product (3). Furthermore, we have found that they produce a unique proteolytic activity which resembles the activity of trypsin and which differentiates them from *B. asaccharolyticus, Bacteroides melaninogenicus* subsp. *intermedius*, and *Bacteroides melaninogenicus* subsp. *melaninogenicus*.

B. gingivalis hydrolyzes *N*-benzoyl-DL-arginine-2-naphthylamide (BANA), a substrate commonly used to assay trypsin activity, and fails to hydrolyze *p*-nitrophenyl-α-glucoside (PNPG), a substrate used to assay α-glucosidase activity. We first observed these activities in testing the API ZYM system (Analytab Products, Plainview, N.Y.) for the rapid identification of gram-negative bacteria present in periodontal disease (4a). The API ZYM system consists of 19 enzyme reaction mixtures containing chromogenic substrates. Although we found this system to be very convenient and reliable, the rather high cost prohibited its use on a routine basis. We have, therefore, adapted the most important differential reactions (trypsin and α-glucosidase) for individual testing in a miniaturized system.

A stock solution of 44 mg of BANA (Sigma Chemical Co., St. Louis, Mo.) was prepared in 1 ml of dimethyl sulfoxide (DMSO) and stored at room temperature. The reaction mixture was prepared by diluting the BANA stock solution 1:100 in 0.1 M Tris-hydrochloride buffer (pH 8.5). This solution was then dispensed in 0.1-ml volumes into small plastic vials (catalog no. 640; Sarstedt, Inc., Princeton, N.J.), and either used immediately or stored in a refrigerator for 1 or 2 days. The vials were inoculated with an isolated colony or a loopful of growth from enriched blood agar plates (7) and incubated aerobically at 37°C for 18 h. The reactions were read by the addition of 1 drop each of the following two reagents: (i) 10 g of sodium dodecyl sulfate in 100 ml of 2.0 M Tris-hydrochloride buffer (pH 7.5); and (ii) 0.35 g of Fast Blue BB salt (Sigma) in 100 ml of ethylene glycol monomethyl ether. A positive test yielded an orange color within 5 min. The PNPG was prepared by dissolving 30 mg of PNPG in 1 ml of DMSO and diluting the mixture 1:10 in 0.1 M Sorensen phosphate buffer (pH 6.0). This reaction mixture was dispensed, inoculated, and incubated as described for the trypsin test. A positive reaction produced a bright-yellow color.

A total of 750 black pigment-producing *Bacteroides* strains were recovered from the dental plaque of patients with adult periodontitis, juvenile periodontitis, acute necrotizing ulcerative gingivitis, and gingivitis. Isolation and identification were performed as previously described (5, 7, 8) on the basis of colonial morphology, pigment, growth in air, Gram stain reaction, production of acid from glucose, production of indole and catalase, hydrolysis of esculin, and reduction of nitrate. All cultures were incubated for 7 to 10 days, and biochemical tests were performed on broth cultures with 3+ to 4+ turbidity. Identification of *B. gingivalis* or *B. melaninogenicus* was confirmed with representative strains by gas-liquid chromatography of volatile and nonvolatile acid end products (2) and by direct fluorescent-antibody staining with Fluoretec-M reagent (Pfizer Diagnostics, New York, N.Y.) (6).

The tests for trypsin and α-glucosidase correlated extremely well with conventional biochemical tests (Table 1). The identification of the 750 strains by anaerobic culturing was identical to that obtained in 18 h with these two rapid tests.

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TABLE 1. Comparison of trypsin and α-glucosidase activities with standard biochemical tests

Organism	No. of strains tested	% of strains positive for:				
		Trypsin	α-Glucosidase	Acid from glucose	Indole	Esculin
B. gingivalis[a]	191	98	0	0	100	0
B. melaninogenicus subsp. *intermedius*	539	0	100	100	99	0
B. melaninogenicus subsp. *melaninogenicus*	20	0	100	100	0	85

[a] *B. gingivalis* strains were gram-negative, anaerobic coccobacilli which produced indole and acetic, isobutyric, butyric, isovaleric, succinic, and phenylacetic acids. They were negative for nitrate reduction and catalase activity and failed to react with the Pfizer Fluoretec-M fluorescent-antibody reagent.

The trypsin test was often positive after only 5 min of incubation, but the α-glucosidase test sometimes took as long as 18 h. Since we did not observe strains positive for both, a positive trypsin test is a relatively consistent characteristic of *B. gingivalis*, but identification should be confirmed with a negative α-glucosidase test. The type strain of *B. asaccharolyticus* (ATCC 25260), an isolate of nonoral origin, is negative for both tests. The type strain of *Bacteroides macacae* (ATCC 33141), a species found in monkeys with periodontitis (1), is weakly positive for trypsin and negative for α-glucosidase. This organism can be distinguished from *B. gingivalis* by a simple catalase test (7). The advantages of these rapid procedures are as follows. (i) Identification can be obtained directly from colonies grown on blood agar plates; (ii) incubation is performed aerobically; (iii) results are obtained in 18 h or less; and (iv) the materials for the pair of tests cost approximately 5 cents.

The insolubility of BANA in water necessitates preparing a concentrated stock solution in DMSO. Since this solvent is known to rapidly penetrate the intact skin, care should be taken in handling it. The diluted trypsin reaction mixture will precipitate in the refrigerator over time, so it is best prepared immediately before use. The Fast Blue BB reagent should be protected from light and discarded if the solution becomes red.

This trypsin-like reaction of *B. gingivalis* is an amidase activity which releases β-naphthylamine from its linkage with the carboxyl group of arginine. We have not observed any activity of *B. gingivalis* against similar substrates whose amino acid moieties are phenylalanine, leucine, valine, or cysteine. This specificity resembles mammalian trypsin (4). Further characterization of this enzyme activity is in progress.

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