NOTES

Biochemical Properties of *Bacteroides melaninogenicus* Subspecies

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Three subspecies of *Bacteroides melaninogenicus* differ from one another in cell wall composition, malate dehydrogenase mobility, deoxyribonucleic acid base composition, and physiological tests.

Bacteroides melaninogenicus is commonly found in mixed infections (9) and has been shown to be an essential component of some artificially induced mixed anaerobic infections (16). By adolescence, *B. melaninogenicus* is of almost universal occurrence in the human mouth, although it is only occasionally isolated from the mouths of young children (2). However, strains called *B. melaninogenicus* have been isolated from many sites and allocated to three subspecies. If there are significant biochemical differences between these subspecies, it becomes important to know whether they occur in different ecological niches and to ensure that, for example, oral strains are used in studies which may implicate the appropriate type of *B. melaninogenicus* in oral infections.

In the present study, the biochemical properties of seven strains of the three subspecies of *B. melaninogenicus* have been compared (Table 1). One strain (T588) is typical of the dental plaque strains isolated so far in this laboratory and closely resembles NCTC 9338. This strain has been examined in the Anaerobic Laboratory, Virginia Polytechnic Institute (VPI 9144), and its status as *B. melaninogenicus* subsp. *intermedius* was confirmed (L. Holdeman, personal communication). The three strains representing *B. melaninogenicus* subsp. *asaccharolyticus* are all of intestinal origin. Strain B536, originally isolated by S. Finegold, was obtained from E. M. Barnes.

Strains were maintained on plates containing 5% defibrinated horse blood and 1% agar (preincubated anaerobically before use). Colonies of *B. melaninogenicus* subsp. *asaccharolyticus* took longer to become black than those of *B. melaninogenicus* subsp. *intermedius* or *B. melaninogenicus* subsp. *melaninogenicus* and also had a more mucoid appearance, although a

hematin pigment could be extracted from black cells of both types (15). Slight red fluorescence (12) was observed with young cultures of *B. melaninogenicus* subsp. *asaccharolyticus*, but not in the other subspecies where it may have been quenched by the rapidly forming black pigment. Liquid cultures contained 1% Trypticase (BBL), 1% proteose peptone (Oxoid), 0.5% yeast extract (Difco), 1% glucose, 0.5% sodium chloride, and 0.075% cysteine hydrochloride. The pH was adjusted to 7.4, and the medium was autoclaved at 121 C for 15 min. Filter-sterilized bovine serum (Oxoid) and menadione-hemin solution (7) were added aseptically to provide final concentrations of 2 and 1% (vol/vol), respectively, together with a few drops of sterile 10% sodium carbonate. Plates and bottles were incubated in anaerobic jars containing 95% hydrogen plus 5% CO₂. Larger liquid cultures were grown in 500-ml or 1-liter bottles which were completely filled with sterile medium after inoculation and tightly sealed. Fermentation tests were carried out using the above medium with 1% of the appropriate filter-sterilized carbohydrate in place of glucose. The physiological tests which most clearly distinguished *B. melaninogenicus* subsp. *asaccharolyticus* and *B. melaninogenicus* subsp. *intermedius* were the hydrolysis of starch and the fermentation of glucose and maltose. Only *B. melaninogenicus* subsp. *melaninogenicus* hydrolyzed esculin.

Glucose utilization and the production of metabolic end products were estimated using the above medium, with proteose peptone in place of Trypticase, after anaerobic incubation for 4 days. Cells were harvested, and the residual glucose in the medium was estimated by the hexokinase-glucose-6-phosphate dehydrogenase procedure (Boehringer-Mannheim GmbH),

TABLE 1. *Properties of strains of B. melaninogenicus*^a

Property	<i>B. melaninogenicus</i> subsp. <i>asaccharolyticus</i>			<i>B. melaninogenicus</i> subsp. <i>melaninogenicus</i>	<i>B. melaninogenicus</i> subsp. <i>intermedius</i>		
	NCTC 9337 Infected hemor- rhoids ^b	VPI 4199 Stools	B536 Stools	VPI 9085(2) (Gingival crevice)	NCTC 9336 Vincents gingivitis	NCTC 9338 Empyema	T588 Human dental plaque
Days taken to become black	8-10	8-10	8-10	5-8	3-4	3-4	3-4
Esculin hydrolysis	-	-	-	+	-	-	-
Starch hydrolysis	-	-	-	+	+	+	+
pH, 1% glucose	6.5	6.2	7.0	5.1	5.0	4.7	4.4
pH, 1% maltose	6.1	5.9	5.9	4.9	4.7	4.8	4.6
Utilization of 0.5% glucose (%)	28	34	24	27	44	36	90
Acid end products							
Acetic	+	+	+	+	+	+	+
Propionic	+	+	+	-	-	-	-
Iso-butyric	+	+	+	-	+	+	+
<i>n</i> -Butyric	+	+	+	-	-	-	-
Iso-valeric	+	+	+	-	+	+	+
Lactic	+	+	+	-	+	+	+
Succinic	-	-	-	+	+	+	+
Mobility of MDH (cm)	4.8	4.8	4.8	3.8	4.3	4.3	4.3
Basic amino acid of muco-peptide	Lysine	Lysine	Lysine	Lysine	DAP	DAP	DAP
Guanine + cytosine in DNA (%)	50.0	50.7	50.9	45.8	42.7	41.2	43.6

^a DNA, Deoxyribonucleic acid; DAP, diaminopimelic acid (DL isomer); MDH, malate dehydrogenase.^b Source.

with the exception that after deproteinization the supernatant was diluted fivefold. Utilization of glucose occurred to some extent in all subspecies, including *B. melaninogenicus* subsp. *asaccharolyticus* strains, which caused little fall of pH in fermentation tests.

Gas-liquid chromatography for volatile end products was carried out on ether extracts of medium acidified to pH 2 with sulphuric acid (4). Strains were also examined for volatile fatty acids together with lactic and succinic acids, using the chromatographic system described by Carlsson (3). The acid profiles were characterized by the lack of propionic and *n*-butyric acids among the metabolic end products of *B. melaninogenicus* subsp. *intermedius* strains, as noted previously (7), and by the absence of detectable succinic acid among the end products of *B. melaninogenicus* subsp. *asaccharolyticus*. Strain 9085 (2) produced acetic and succinic acids only.

Cell-free extracts were prepared either by shaking with Ballotini beads in a Mickle tissue disintegrator or by pressing once with a Biox pressure cell. After removing debris by centrifugation, extracts were electrophoresed on Cello-gel strips (14 by 7.5 cm; Reeve Angel Scientific

Ltd.) in barbitone-acetate buffer, 0.04 M, pH 8.6, for 1 h at a voltage gradient of 10 V/cm. Phenol red indicator migrated 8 cm under these conditions. Malate dehydrogenase was stained using 2 mg of malic acid per ml, 0.04 mg of phenazine methosulfate per ml, 0.2 mg of thiazolyl blue tetrazolium per ml, and 0.1 mg of nicotinamide adenine dinucleotide per ml in 0.08 M tris(hydroxymethyl)aminomethane buffer (pH 7.5). Single bands of enzyme were found in all strains, but those of *B. melaninogenicus* subsp. *asaccharolyticus* migrated significantly faster than those of *B. melaninogenicus* subsp. *intermedius*, which in turn were more mobile than *B. melaninogenicus* subsp. *melaninogenicus*. A mixture of extracts of three subspecies resolved into three bands, but mixtures of several strains of *B. melaninogenicus* subsp. *intermedius*, or of strains of *B. melaninogenicus* subsp. *asaccharolyticus*, did not so resolve. Cell walls were prepared by the rapid method of Schleifer and Kandler (14) and hydrolyzed overnight with 4 N HCl. Analysis was by paper chromatography in two dimensions (6), in the solvent (13) for the detection of diaminopimelic acid isomers, and by high voltage electrophoresis in the pH 5.2

pyridine-acetic acid buffer (1). The significant finding was the presence of two different dibasic amino acids in the three subspecies. The occurrence of diaminopimelic acid in *B. melaninogenicus* subsp. *intermedius* distinguishes this group from all the *Bacteroides* strains tested so far in this laboratory, including representatives of *B. oralis* and *B. fragilis*.

Cells possessing the lysine-mucopeptide type lysed readily with sodium dodecyl sulfate. The diaminopimelic acid-containing strains were rendered susceptible to lysis by growing them in the presence of 1% glycine (17). Deoxyribonucleic acid purification was based on the method of Marmur (10), except that the crude lysate was treated with proteinase K (Merck), 50 mg/ml, at 37 C for 16 h (5). This treatment reduced the number of deproteinization steps necessary to produce pure deoxyribonucleic acid and also improved the yield. Base composition was estimated by thermal denaturation (11). There is a significant difference between the mean base composition of *B. melaninogenicus* subsp. *intermedius* and that in *B. melaninogenicus* subsp. *asaccharolyticus*.

The results of this study indicate that there are substantial differences between strains of *B. melaninogenicus* subsp. *asaccharolyticus* and *B. melaninogenicus* subsp. *intermedius*, and it may in the future be considered inappropriate for them to be retained within the same species. Although only a small number of strains have been examined to date, it is possible that there may be an ecological difference between these subspecies, subsp. *asaccharolyticus* strains being derived from gut specimens and examples of *B. melaninogenicus* subsp. *intermedius* having been obtained mainly from oral samples.

Examples of *B. melaninogenicus* subsp. *melaninogenicus* are apparently difficult to obtain, and so far only one strain of this type has been examined. Further strains must be studied to confirm the difference in properties of this subspecies (Table 1). The ability to hydrolyze esculin (7) appears to offer the simplest method of detecting such strains.

The methods reported in this communication will be used to characterize a collection of black-pigmented *Bacteroides* strains isolated from oral specimens to establish which of the subspecies of *B. melaninogenicus* are prevalent in the mouth.

REPRINT REQUESTS

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