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Characterization of *Bacteroides melaninogenicus*

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Fifty-eight human isolates of *Bacteroides melaninogenicus*, 42 from a variety of clinical infections and the rest from normal flora, were studied for pigment production and ultraviolet light fluorescence and by forty biochemical and other tests, including end-product analysis by gas-liquid chromatography. In a number of instances, tests were repeated several times and the results were reproducible. Agar plate dilution susceptibility tests were also performed to 12 antimicrobial agents. These 58 strains could be reliably placed into three groups, corresponding to the three subspecies described, based on seven characteristics. These included acid production in peptone-yeast-glucose medium, production of *n*-butyric acid from peptone-yeast-glucose medium, esculin hydrolysis, starch hydrolysis, indole production, effect on milk, and lipase production. Production of hydrogen gas in peptone-yeast-fructose medium may be another distinguishing characteristic. In general there was not much difference in the susceptibility of the three groups to the various antimicrobial agents tested. Two strains had a minimal inhibitory concentration of penicillin G of 16 and 32 U/ml, respectively. Three strains did not produce a black pigment in spite of prolonged incubation on blood-containing media.

Oliver and Wherry initially described *Bacteroides*-like organisms which they noted produced black-pigmented colonies while growing on blood agar. They suggested the name *Bacterium melaninogenicum* for this organism in 1921 (5). The biochemical properties reported for *Bacteroides melaninogenicus* in the literature have been quite varied. Some of the confusion undoubtedly is related to difficulties in obtaining the organism in pure culture and in obtaining adequate growth. Some strains were noted to ferment a variety of carbohydrates (5, 6), whereas others were found to be completely non-saccharolytic (1, 6, 11). Similarly, Oliver and Wherry found that their strains did not liquefy gelatin (5), whereas Burdon reported that his strains were highly proteolytic, attacking gelatin, coagulated serum, egg albumin, and milk (1). Because of the major differences in the biochemistry of strains of *B. melaninogenicus*, three subspecies have been suggested by Holdeman and Moore (3). Production of a black pigment by *B. melaninogenicus* when growing on blood-containing media has been regarded as highly specific and is the sole basis for the differentiation from other gram-negative anaerobic bacilli used by most microbiology laboratories. Other workers have noted that other anaerobic organisms can produce a black pigment (3). However, it must be pointed out that the anaerobic cocci that produce a black pigment can produce it in the absence of blood, whereas *B. melaninogenicus* can only produce a black pigment on blood-containing media. There has been much confusion regarding the biochemical nature of the black pigment produced by *B. melaninogenicus* (2, 5, 7, 10). Strains of *B. melaninogenicus* that do not produce a black pigment have not been described. Myers et al., in 1969, pointed out that *B. melaninogenicus* on blood agar plates fluoresced a vivid red under ultraviolet light (4). Our study was designed to look at biochemical and other characteristics of *B. melaninogenicus* growing in pure culture in order to determine which characteristics were the most useful in characterization and identification of subspecies and at the same time to note whether these organisms had any other properties that were not described previously.

MATERIALS AND METHODS

Fifty-nine organisms that either produced a tan to black pigment while growing on blood-containing media or fluoresced brick red with ultraviolet light were initially studied. One organism, which pro-

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duced a brown pigment and did not fluoresce with ultraviolet light, was eventually identified as *Actinomyces naeslundii* and therefore was excluded from this study.

The 58 remaining human isolates included 42 from a variety of clinical infections and 16 from normal human flora. Organisms studied included the American Type Culture Collection (ATCC) strains 25260 and 25845 and the National Collection of Type Cultures (NCTC) strains 9336, 9337, and 9338 of *B. melaninogenicus*. The ATCC strain 25260 was initially isolated in the Wadsworth Anaerobic Laboratory (B440) and subsequently sent to the Virginia Polytechnic Institute Anaerobe Laboratory (VPI#4198). The ATCC strain 25845 was initially isolated in the Wadsworth Anaerobic Laboratory (B282) and subsequently sent to the Virginia Polytechnic Institute (VPI#2381). These two organisms were submitted by the Virginia Polytechnic Institute group to the ATCC.

All organisms were initially streaked onto a *Brucella* blood agar plate. *Brucella* agar (Pfizer) was supplemented, after autoclaving, with 10 μg of vitamin K_1 (Nutritional Biochemical Corp.) per ml and 5% sheep blood. They were also streaked onto a similar plate with laked blood. These were incubated anaerobically in GasPak jars (BBL). After 48 to 72 h of incubation, they were examined for purity with a hand lens or a dissecting microscope. If pure, they were subcultured onto an egg yolk agar plate containing 10 μg of vitamin K_1 and 5 μg of hemin per ml for subsequent testing for catalase and lipase production.

Cultures were examined at 48 to 72 h and then at 2-day intervals for fluorescence under ultraviolet light (365 nm). They were incubated for up to 21 days to detect pigment production. Each organism was also checked for aerotolerance. Smear and Gram stain from *Brucella* blood agar was done on all organisms studied. The Gram reaction, size, and arrangement of cells were noted and recorded. Motility was studied by the hanging-drop method or by observing growth in GAM semisolid media (Nissui).

Colonies from a pure surface growth on *Brucella* blood agar or, rarely, an inoculum from the growth in GAM semisolid medium were inoculated into tubes with 10 ml of fluid thioglycolate medium with dextrose and without indicator (BBL 135 C) to which hemin (5 μg/ml), vitamin K_1 (0.1 μg/ml), sodium bicarbonate (1 mg/ml), and Fildes enrichment (BBL) to a final concentration of 5% were all added. Preliminary studies with growth-stimulating additives (i) vitamin K_1 and hemin, (ii) vitamin K_1 and Fildes enrichment, and (iii) Tween 80 revealed that the vitamin K_1 and Fildes enrichment enhanced the growth best in peptone-yeast-glucose (PYG) medium. Hence, at the time of inoculation, these final concentrations of vitamin K_1 and Fildes enrichment were added to all tubes containing the prereduced, anaerobically sterilized media. When good growth was obtained in the maintenance thioglycolate broth, a Gram stain was again made and tubes containing prereduced, anaerobically sterilized media with various carbohydrates, etc., were inoculated under CO_2 with a Bellco inoculator, as outlined in the Virgina Polytechnic Institute *Anaerobe Laboratory Manual* (3).

Twenty-five carbohydrates and other substrates were added to the peptone-yeast (PY) basal medium containing resazurin, cysteine, and a balanced salt solution. After 21 days of incubation, acid production was checked by means of a Beckman pH meter. The pH after flushing with CO_2 and before incubation was usually 6.2 to 6.4. Analyses of metabolic products detected in PYG, PY-threonine, and PY-lactate media were carried out, using gas-liquid chromatography as outlined in the *Wadsworth Anaerobic Bacteriology Manual* (9). The volatile fatty acids, with the exception of formic acid, were determined with a flame ionization detector, using an aqueous injection. Formic acid was determined with a thermal conductivity detector, using an ether extract. The nonvolatile acids were determined with a thermal conductivity detector, using a methylated sample. Acid production in substrates was defined as a pH drop of at least 0.6 pH unit from that of the control PY medium. Hydrogen gas analyses were carried out on the final 20 strains studied after incubation of the organisms in the PY basal medium with fructose, using the technique outlined in the *Wadsworth Anaerobic Bacteriology Manual* (9).

The effects of the organisms on milk were also determined after 21 days of incubation. Reactions were read as digestion, clot formation, or no effect. Tests for catalase and indole production, esculin hydrolysis, starch hydrolysis, and urease production were performed as outlined in the *Wadsworth Anaerobic Bacteriology Manual* (9). Lipase production was looked for on the egg yolk agar plate. All organisms were studied for stimulation or inhibition of growth by 20% bile (2% oxgall) with 0.1% deoxycholate. The production of gas was looked for after incubation in both the maintenance thioglycolate broth and PYG agar.

Agar plate dilution susceptibility testing was performed on 50 of these strains with a variety of antimicrobial agents, including penicillin, carbenicillin, amoxicillin, clindamycin, erythromycin, chloramphenicol, thiamphenicol, tetracycline, doxycycline, minocycline, cefoxitin, and metronidazole, using the methods previously described from this laboratory (9).

RESULTS

As a group, *B. melaninogenicus* subsp. *asaccharolyticus* strains were the most difficult to grow on solid as well as in liquid media. The results of the various studies other than acid production that were carried out on all strains studied are outlined in Table 1. All 58 strains of *B. melaninogenicus* were obligately anaerobic, gram-negative, nonsporeforming coccobacilli. Fifty-five organisms produced a black pigment while growing on blood-containing media; three only became tan in spite of the prolonged incubation on blood and laked blood agar plates. Two of these latter organisms were subsequently identified as *B. melaninogenicus*

TABLE 1. *Characteristics of 58 strains of Bacteroides melaninogenicus*

Characteristic	No. positive/no. tested of strains of subspecies:		
	melaninogenicus	*intermedius*	*asaccharolyticus*
Obligate anaerobe	11/11	28/28	19/19
Black pigment	9/11	27/28	19/19
Tan pigment	2/11	1/28	0/19
Brick-red ultraviolet fluorescence	11/11	28/28	19/19
Nonmotile	11/11	28/28	19/19
Catalase produced	0/11	0/28	0/19
Growth in bile and deoxycholate	0/11	0/28	0/19
Gelatin liquefied	11/11	28/28	19/19
Indole produced	0/11	27/28	19/19
Effect of milk	C[a]11/11	D[a]28/28	D[a]19/19
Esculin hydrolyzed	11/11	0/28	0/19
Starch hydrolyzed	11/11	28/28	0/19
Lipase produced	0/11	20/28	0/19
Nitrate reduced	0/11	0/28	0/19
Urease production	0/11	0/28	0/19
Gas produced in PYG agar or thioglycolate broth	0/11	0/28	0/19
Meat digested	0/11	0/28	0/19
n-Butyric acid produced	0/11	0/28	19/19
Hydrogen gas produced[b]	0/1	0/6	13/13
Propionic acid produced from PY-threonine	0/11	0/28	0/19
Propionic acid produced from PY-lactate	0/11	0/28	0/19

[a] C, Clot; D, digested.
[b] Only the last 20 strains studied were tested.

subsp. *melaninogenicus*, and one was identified as subsp. *intermedius*. All 58 strains fluoresced brick red under ultraviolet light. All 58 strains were nonmotile and catalase negative and were inhibited by 20% bile with 0.1% deoxycholate. All 58 strains liquefied gelatin. Twenty-seven of 28 strains of subsp. *intermedius* and all 19 strains of subsp. *asaccharolyticus* produced indole. All 11 strains of subsp. *melaninogenicus* clotted milk, whereas all 28 strains of subsp. *intermedius* and 19 strains of subsp. *asaccharolyticus* digested milk. Esculin was hydrolyzed by only the 11 strains of subsp. *melaninogenicus*. All the strains of subsp. *melaninogenicus* and *intermedius* hydrolyzed starch. Twenty of the 28 strains of subsp. *intermedius* produced lipase; none of the 58 strains reduced nitrate. There was no urease produced by any of the strains studied. Only the 19 strains of subsp. *asaccharolyticus* produced *n*-butyric acid on fermentation of glucose. Also, all 13 strains of subsp. *asaccharolyticus* studied produced hydrogen gas.

Table 2 shows acid production by *B. melaninogenicus* strains in various substrates. All 11 strains of subsp. *melaninogenicus* produced acid in PYG medium, whereas only 16 of the 28 strains of subsp. *intermedius* and none of the 19 strains of subsp. *asaccharolyticus* produced acid. It should also be noted that all 11 strains of subsp. *melaninogenicus* produced acid in PY-mannose medium, whereas only one of the strains of subsp. *intermedius* and none of the strains of subsp. *asaccharolyticus* did.

TABLE 2. *Acid production[a] by Bacteroides melaninogenicus from various substrates in PY basal medium[b]*

Substrate	No. positive/no. tested of strains of subspecies:		
	melaninogenicus	*intermedius*	*asaccharolyticus*
Cellobiose	2/11	0/28	0/19
Esculin	1/11	0/28	0/19
Fructose	7/11	9/28	0/19
Glucose	11/11	16/28	0/19
Glycogen	10/11	12/28	0/19
Inositol	9/11	0/28	0/19
Lactose	9/11	1/28	0/19
Maltose	11/11	14/28	0/19
Mannose	11/11	1/28	0/19
Melibiose	3/11	0/28	0/19
Raffinose	10/11	9/28	0/19
Ribose	3/11	0/28	0/19
Salicin	1/11	0/28	0/19
Starch	10/11	16/28	0/19
Sucrose	11/11	13/28	0/19
Trehalose	0/11	13/28	0/19

[a] pH drop of at least 0.6 unit.
[b] No strains produced acid from adonitol, amygdalin, arabinose, erythritol, mannitol, melezitose, rhamnose, sorbitol, or xylose.

In Table 3 is a list of the characteristics we found most useful in identifying subspecies and

TABLE 3. *Characteristics most useful for clinical laboratories in subspeciation of Bacteroides melaninogenicus*

Characteristic	Reaction of strains of subspecies:		
	melaninogenicus	*intermedius*	*asaccharolyticus*
Esculin hydrolysis	+ (11/11)[a]	− (28/28)	− (19/19)
Starch hydrolysis	+ (11/11)	+ (28/28)	− (19/19)
Indole production	− (11/11)	+ (27/28)	+ (19/19)
Action on milk	C[b] (11/11)	D[b] (28/28)	D (19/19)
Lipase production	− (11/11)	+ (20/28)	− (19/19)
n-Butyric acid produced	− (11/11)	− (28/28)	+ (19/19)
Acid production[c] from PYG medium	+ (11/11)	+ (16/28)	− (0/19)

[a] Numbers in parentheses are number of strains giving indicated reaction/number tested.
[b] C, Clot; D, digested.
[c] pH drop of at least 0.6 unit.

the frequency with which they were found in the strains of *B. melaninogenicus* studied. If a strain of *B. melaninogenicus* hydrolyzes esculin or clots milk, it is a subsp. *melaninogenicus*. If a strain produces *n*-butyric acid on fermentation of PYG medium, it is a subsp. *asaccharolyticus*. Any strain that produces lipase is a subsp. *intermedius*. In a number of instances tests were repeated several times, and the results were always reproducible. Hydrogen gas production may be a useful test as well. Of the 20 strains studied, hydrogen gas production was noted only with the 13 strains of subsp. *asaccharolyticus*. Unfortunately, only one strain of subsp. *melaninogenicus* and six of subsp. *intermedius* were studied. Additional strains would have to be studied to ascertain whether this happens consistently.

In general there was not much difference in the susceptibility of these subspecies to the various antimicrobial agents tested. All 17 strains of subsp. *asaccharolyticus* studied had a minimal inhibitory concentration (MIC) of ≤4 U of penicillin G per ml. Twenty-three of the 24 strains of subsp. *intermedius* and 8 of the 9 strains of subsp. *melaninogenicus* were inhibited by 4 U or less of penicillin G per ml. One strain of subsp. *intermedius* had an MIC of 16 U/ml, and one strain of subsp. *melaninogenicus* had an MIC of 32 U/ml. All strains were susceptible to carbenicillin at concentrations of 8 μg or less per ml. Most of the strains tested were inhibited by amoxicillin at concentrations of 2 μg or less per ml. One strain of subsp. *asaccharolyticus* required 4 μg/ml; two strains of subsp. *intermedius* and one strain of subsp. *melaninogenicus* required 8 μg/ml.

All strains were inhibited by erythromycin and clindamycin at concentrations of 1 μg or less per ml. Chloramphenicol and thiamphenicol were active against all strains at 4 μg or less per ml.

With tetracycline, the majority of strains were inhibited by 2 μg or less per ml. One strain of subsp. *asaccharolyticus*, three strains of subsp. *intermedius*, and one strain of subsp. *melaninogenicus* required 4 μg/ml, whereas one strain of each subspecies required 8 μg/ml. Doxycycline and minocycline were slightly more active, with one one strain each of subsp. *asaccharolyticus* and *intermedius* requiring 4 μg of either drug per ml for inhibition; the remaining strains were inhibited by 2 μg or less per ml.

One strain of subsp. *intermedius* required 8 μg of cefoxitin per ml, whereas all other strains were inhibited by 2 μg or less per ml. All strains tested were inhibited by 2 μg of metronidazole or less per ml.

Table 4 shows the sources of the strains studied. Of the 19 strains of subsp. *asaccharolyticus*, seven were isolated from normal stool flora, five from soft-tissue infections, two from intra-abdominal infections, one from an infected hemorrhoidectomy, one from a urinary tract infection, two from patients with osteomyelitis, and one from a patient with an empyema (which was overlying a subphrenic abscess). Of the 28 strains of subsp. *intermedius*, seven were isolated from the upper respiratory tract normal flora, one each from a patient with maxillary sinusitis and Vincent's gingivitis, nine from lower respiratory tract infections, three from intra-abdominal infections, three from soft-tissue infections, one from a post-gastrectomy wound infection, and three from uterine infections. Of the 11 strains of subsp. *melaninogenicus*, two were isolated from the upper respiratory tract normal flora, five from lower respiratory tract infections, two from intra-abdominal infections, one from a case of osteomyelitis, and one from a blood culture from a drug addict who routinely licked his needle before injecting himself.

TABLE 4. *Sources of the 58 strains of Bacteroides melaninogenicus*[a]

Subspecies							
melaninogenicus		*intermedius*		*asaccharolyticus*	
Strain	Source	Strain	Source	Strain	Source
ATCC 25845	Sputum	WAL 170	Mouth	ATCC 25260	Empyema fluid overlying a subphrenic abscess
WAL 2849	TTA	WAL 2876	Tracheal suction		
WAL 2794	TTA	WAL 2719	Bronchial wash		
WAL 2730	Bronchial washings	WAL 2929[b]	Dental plaque		
WAL 2962	TTA	WAL 2930[b]	Dental plaque	WAL 1411	Normal stool
WAL 2724	TTA	WAL 2931[b]	Dental plaque	WAL 2966	Normal stool
WAL 2721	TTA	WAL 519	Mouth	WAL 1195	Normal stool
		WAL 485	Drainage maxillary sinus	WAL 1202	Normal stool
				WAL 930	Normal stool
WAL 2728	Peritoneal fluid	NCTC 9336	Vincent's gingivitis	WAL 536	Normal stool
WAL 2981	Intra-abdominal abscess aspirate			WAL 2031	Normal stool
WAL 2850	Osteomyelitis	WAL 2847	Pleural biopsy	WAL 2848	Fluid, perforated appendix
WAL 2971	Blood	WAL 2846	Lung aspirate		
		WAL 2731	TTA	WAL 2970	Intra-abdominal abscess aspirate
		WAL 2727	TTA		
		WAL 2968	TTA		
		WAL 2967	TTA	NCTC 9337	Infected hemorrhoidectomy
		WAL 2720	TTA		
		WAL 422	Empyema fluid	WAL 312	Urine
		NCTC 9338	Empyema fluid	WAL 2729	Wound infection, toe
		WAL 2748	Peritoneal fluid	WAL 2969	Decubitus ulcer, ischial tuberosity
		WAL 706	Aspirate, liver abscess		
		WAL 2717	Abdominal drainage	WAL 1047	Abscess, foot
		WAL 2845	Abdominal wound aspirate, post-gastrectomy	WAL 2972	Aspirate, thigh
		WAL 2963[c]	Cervical drainage	WAL 975	Exudate, toe
		WAL 2964[c]	Cervical drainage	WAL 2726	Osteomyelitis
		WAL 2965[c]	Cervical drainage	WAL 597	Osteomyelitis, femur
		WAL 2851	Ulcer, foot		
		WAL 2845	Aspirate, arm		
		WAL 2469	Aspirate, foot		

[a] Abbreviations: ATCC, American Type Culture Collection; WAL, Wadsworth Anaerobic Bacteriology Laboratory; NCTC, National Collection of Type Cultures; TTA, transtracheal aspirate.
[b] Obtained from J. Hardie.
[c] Obtained from W. Ledger.

DISCUSSION

B. melaninogenicus has been shown to play an essential role in the pathogenesis of experimental mixed anaerobic infections (8). Most microbiology laboratories report as *B. melaninogenicus* any black-pigmented anaerobe. A few investigators have noted that other anaerobes such as *Peptostreptococcus anaerobius* and *Peptostreptococcus micros* may produce a black pigment at times (3). Anaerobic cocci that produce a black pigment will do so in the absence of blood in the media, whereas *B. melaninogenicus* will only produce a black pigment on blood-containing media. In the current study, one strain of *Actinomyces naeslundii* produced a brown pigment. However, there has been no mention in the literature of strains of *B. melaninogenicus* that do not produce a black pigment. In our study we noted that three strains, two of which were subsp. *melaninogenicus* and the other subsp. *intermedius*, produced only tan pigment in spite of being incubated on laked blood agar and blood agar for at least 21 days. We have also noted colorless mutants of *B. melaninogenicus*. Therefore, we must be alert to the fact that not all strains of *B. melaninogenicus* produce a black pigment. At present, at least, there seems to be no reliable way to distinguish between *B. melaninogenicus* subsp. *melaninogenicus* and *B. oralis* except for black pigment production on blood-containing media by the former. However, some strains of *B. oralis* show tan or orange pigment, and some will fluoresce under ultraviolet light as well. Accordingly, these two organisms may well be identical and pigment production may not be a reliable indicator of the species.

The data from this study suggest that one explanation for the varied biochemical properties of *B. melaninogenicus* strains reported in the literature may be that different investiga-

tors were in fact reporting on different subspecies. In earlier studies a problem of contamination may also have existed. Lack of adequate growth may also have been a factor in the confusion.

The 58 strains of *B. melaninogenicus* studied could be reliably placed into three separate groups or subspecies, subsp. *melaninogenicus*, *intermedius*, and *asaccharolyticus*, based on seven characteristics. These included acid production from PYG medium, production of *n*-butyric acid from PYG medium, esculin hydrolysis, starch hydrolysis, indole production, effect on milk, and lipase production. Production of hydrogen gas in PY-fructose may be another distinguishing characteristic. However, this test is not usually performed in clinical microbiology laboratories. Williams et al. (12) recently showed that these three subspecies of *B. melaninogenicus* differ from one another in cell wall composition, malate dehydrogenase mobility, and deoxyribonucleic acid base composition. These tests, though definitive, are too complex to perform in clinical microbiology laboratories.

We agree with the view of Werner et al. (11) that it is difficult to have in the same species organisms that ferment carbohydrates and those that are non-saccharolytic. The Subcommittee on Gram-Negative Anaerobic Rods of the International Committee on Systematic Bacteriology, after a cooperative study on *B. melaninogenicus*, concluded that *B. melaninogenicus* subsp. *asaccharolyticus* should be placed in an entirely separate species and proposed that it be named *Bacteroides asaccharolyticus* (submitted for publication).

Williams et al. have suggested that there may be an ecological difference between these three subspecies, subsp. *asaccharolyticus* strains being derived from gut specimens and subsp. *intermedius* strains being derived mainly from oral samples (12). They had only studied one strain of subsp. *melaninogenicus* in their laboratory. Our data suggest that subsp. *asaccharolyticus* strains do not often reside in the oral cavity. Of the 26 strains isolated from upper respiratory tract normal flora or lower respiratory tract infections, only one was identified as a subsp. *asaccharolyticus*. This strain was isolated from a patient in whom the empyema fluid was overlying a subphrenic abscess. Therefore it is quite likely that the strain originated from an intestinal source. Strains of subsp. *intermedius* and *melaninogenicus* were isolated from sites both above and below the diaphragm, but were primarily associated with a respiratory tract source. Strains of subsp. *intermedius* were also isolated from patients with uterine infections.

There was no major difference in the susceptibility of the three groups to the various antimicrobial agents tested. There were two strains that were relatively resistant to penicillin G. We did not observe resistance in these organisms until recent years.

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