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Description of a Polyvalent Conjugate and a New Serogroup of *Bacteroides melaninogenicus* By Fluorescent Antibody Staining

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A polyvalent conjugate (fluorescein isothiocyanate-labeled antibody reagent) containing serogroups A, B, and C conjugates was prepared. This polyvalent conjugate gave a positive fluorescent antibody (FA) stain with 49 strains of *Bacteroides melaninogenicus* representing serogroups A, B, and C. When additional strains (92 strains) of the three subspecies of *B. melaninogenicus* were examined by the FA stain, with A, B, and C, and polyvalent conjugates, nine strains of *B. melaninogenicus* subsp. *intermedius* failed to give a positive stain with any conjugate. Therefore, an FA conjugate was prepared with the antiserum to one of these strains (532-70A); all nine strains stained positively with this conjugate. These nine strains were biochemically characteristic of *B. melaninogenicus* subsp. *intermedius;* thus, these strains were designated as a new serogroup, serogroup C-1. A new polyvalent conjugate containing serogroups A, B, C, and C-1 was prepared. This polyvalent conjugate stained positively with 23 representative strains from serogroups A, B, C, and C-1. The new conjugates failed to stain positively with other anaerobes or aerobes tested. The four individual conjugates, as well as the polyvalent conjugate, may be used for a more rapid identification of *B. melaninogenicus* than is possible by biochemical testing.

The recent application of fluorescent antibody (FA) techniques for the identification of *Bacteroides melaninogenicus* (3) provided support for the biochemical separation of this organism into the three subspecies proposed by Holdeman and Moore (2). The literature regarding the FA work on *B. melaninogenicus* has been reviewed previously (3).

The use of FA conjugates for the three subspecies of *B. melaninogenicus* provided a rapid identification of these organisms in the clinical laboratory as opposed to the slower method of biochemical identification. A polyvalent conjugate would provide an even shorter identification time by employing the use of one conjugate instead of three conjugates for the preliminary detection of *B. melaninogenicus* as a species. Therefore, this study was undertaken to develop a polyvalent conjugate for the FA detection of *B. melaninogenicus* in pure culture. During the study, a new serogroup of *B. melaninogenicus* subsp. *intermedius* was described.

MATERIALS AND METHODS

Strains. Strains of *B. melaninogenicus* included in this FA study were isolated from clinical specimens on laked blood agar plates (3). Strains of *B. melaninogenicus* from a previous study (3) and from this study were derived from a variety of clinical specimens. Strains of *B. melaninogenicus* subsp. *asaccharolyticus* were isolated from pelvic drainage, leg wound, peritoneal fluid, abdominal abscess, hip wound, penis abscess, pilonidal cyst, foot abscess, vulvar abscess, perianal abscess, and other sources. *B. melaninogenicus* subsp. *intermedius* was isolated from: an extradural mass, cervical swab, throat, neck drainage, chest incision, abdominal wound, maxillary sinus, vaginals, nasopharyngeal swab, rectal abscess, brain abscess, pleural cavity, and other sources. *B. melaninogenicus* subsp. *melaninogenicus* was isolated from: a neck wound, great toe, chest pus, bronchial washing, peritonsilar abscess, cranial fluid, and mouth.

B. melaninogenicus subsp. *intermedius* strains obtained from Lillian V. Holdeman, Anaerobe Laboratory, Virginia Polytechnic Institute, Blacksburg, Va. included: 722-74 (← VPI 4201 ← Finegold B511); 723-74 (← VPI 4202 ← Finegold B514); 724-74 (← VPI 4203 ← Finegold B515); 725-74 (← VPI 7791C ← Montgomery Co. Community Hospital 65J4); 726-74 (← VPI 7925F ← Montgomery Co. Community Hospital 98S); 727-74 (← VPI 7170 ← University of Kentucky Medical Center 1502); 728-74 (← VPI 8667 ← University of Virginia Medical School); 729-74 (← VPI 8944 ← NCTC 9336); 730-74 (← VPI 9042 ← Duke University ← CDC 1976); 731-74 (← VPI 9145 ← London Hospital Medical College T 584/72); 732-74

(← VPI 9146 ← London Hospital Medical College M 107/72); 733-74 (← VPI 9169 ← London Hospital Medical College T 588/72).

Other anaerobes and aerobes examined previously (3) were also included.

Identification. All strains of anaerobic bacteria were identified by the methods described by Holdeman and Moore (2). Biochemical tests used previously (3), gas chromatographic analyses (2), and pigment formation on laked blood agar plates comprised the identification scheme.

Immunological methods. One test strain for each of the three subspecies of *B. melaninogenicus* (3) was used for the production of antiserum. A strain of *B. melaninogenicus* subsp. *intermedius*, 532-70A, which failed to react serologically with the three previously described FA conjugates of *B. melaninogenicus* (3), was also used to immunize rabbits.

The methods for vaccine preparation, rabbit immunization, direct FA stains, and the agglutination method were identical to those described previously (3).

The globulin was precipitated from rabbit antiserum with 35% ammonium sulfate. The precipitate was dissolved in distilled water to the original serum volume, and the entire procedure was repeated twice. The precipitate was resuspended in distilled water and dialyzed against 0.066 M $NaHPO_4$-buffered saline (PBS), pH 7.2, at 4 C until the ammonium sulfate was no longer detected in the dialyzate. The total protein of the immunoglobulin was determined by the biuret method. Fluorescein isothiocyanate (FITC) dye was added to the globulin in the proportion of 1 mg of dye per 20 mg of protein; this mixture was incubated at room temperature for 2 h. The sample was dialyzed against 0.066 M PBS at 4 C with frequent changes for a period of 3 to 4 days until no FITC was detected in the dialyzate. The fluorescein/protein (F/P) ratio of each conjugate was determined by the method of Wells et al. (5). The FITC-labeled antibody (conjugate) was stored as previously described (3). Conjugates for four strains (138-71, 207-72C, 275-70A, and 532-70A) were prepared. FA smears were examined with a monocular Leitz fluorescence microscope equipped with a reflecting dark-field condenser and an Osram HBO 200 mercury lamp. A BG12 primary filter was used in combination with an OG1 secondary filter. Fluorescence was graded as: 4+, brilliant fluorescence with a well-defined peripherally stained edge; 3+, moderate fluorescence with a well-defined peripherally stained edge; 2+, faint fluorescence, no well-defined edge; 1+, bare fluorescence, no well-defined edge; and ±, doubtful staining. A 3+ fluorescence or greater was considered positive.

Preparation of polyvalent conjugate. The three specific *B. melaninogenicus* conjugates (138-71, 207-72C, 275-70A) were tested by the FA method with each homologous strain to determine the optimal dilution which should be used in the polyvalent conjugate. One dilution lower than the highest staining titer which gave a 3+ fluorescence was used as the specific staining titer (Table 1). The three conjugates were then pooled (10 ml, total volume) so that each conjugate was at its specific staining titer. Merthiolate was used as a preservative in a final concentration of 1:5,000. The polyvalent conjugate was stored at −65 C; small portions were stored at 5 C during test procedures.

TABLE 1. *Fluorescent antibody titers of FITC-labeled conjugate with homologous strains*

FITC-labeled antiserum (antigen strain no.)	Serogroup	Specific staining titer
Bacteroides melaninogenicus subsp. melaninogenicus (138-71)	A	1:80
B. melaninogenicus subsp. asaccharolyticus (207-72C)	B	1:20
B. melaninogenicus subsp. *intermedius* (275-70A)	C	1:160
B. melaninogenicus subsp. *intermedius* (532-70A)	C-1	1:40

A second polyvalent conjugate incorporating a new serogroup, serogroup C-1, was prepared; it contained FA conjugates 138-71, 207-72C, 275-70A, and 532-70A.

RESULTS

Strain identification. Strains of *B. melaninogenicus* were anaerobic, gram-negative bacilli that produced a dark brown or black pigmented colony on laked blood agar. A tan pigment at 10 days excluded classification of an organism as *B. melaninogenicus*. Smith (4) also classified dark brown to black colonies as *B. melaninogenicus*, although Duerden (1) classified only black colonies as *B. melaninogenicus*. The biochemical characteristics of each of the three subspecies of *B. melaninogenicus* conformed to the reactions described previously (3). Biochemical reactions for anaerobes other than *B. melaninogenicus* were characteristic of those reported by Holdeman and Moore (2).

Pigment production and colony description. In the pigment study, 94 strains of *B. melaninogenicus* were transferred weekly on laked blood agar plates at 37 C over a period up to 9 months: 8 strains were *B. melaninogenicus* subsp. *melaninogenicus*, 48 strains were *B. melaninogenicus* subsp. *asaccharolyticus*, and 38 strains were *B. melaninogenicus* subsp. *intermedius*. The incubation time required for production of the characteristic pigment depended upon the number of times that a particular strain had been transferred. Fresh clinical isolates usually required 2 to 7 days for pigment formation, although an occasional strain required 10 to 14 days before pigment was produced. Most strains that had been transferred for 1 month or longer usually produced pigment within 48 to 72 h.

Distinct differences in colony type were noted among the three subspecies of *B. melaninogenicus*. The following colonial description per-

tained to strains that had been transferred in the stock culture collection on laked blood agar for longer than 1 month. *B. melaninogenicus* subsp. *melaninogenicus*, serogroup A, colonies varied from 0.5 to 2.0 mm in diameter. The strains of this subspecies produced a dry colony which had either a dark brown or a brown-black pigment. The distinctive feature of the brown-black colony was a nucleated appearance, a brown-black center surrounded by a brown edge. The nucleated colony retained its nucleated characteristic even after incubation for 10 days; the colony never became black. *B. melaninogenicus* subsp. *melaninogenicus* is the most difficult of the three subspecies to maintain in stock culture since it is often nonviable upon transfer.

B. melaninogenicus subsp. *asaccharolyticus*, serogroup B, colonies were 1.5 to 2.0 mm in diameter. This subspecies produced a reddish-black colony at 1 to 2 days. After 3 days, the colonies tended to lose most of the red color and became black. A confluent growth, as well as individual colonies, of this subspecies had a moist appearance at 1 to 2 days, whereas confluent growth and individual colonies of *B. melaninogenicus* subsp. *melaninogenicus* and *B. melaninogenicus* subsp. *intermedius* were dry.

B. melaninogenicus subsp. *intermedius*, serogroups C and C-1, colonies were about 0.5 mm in diameter. At 1 to 2 days, an individual colony was dry and had a black center with a brown edge; after 3 days, the entire colony was black.

Agglutination. Sera obtained before injection of rabbits did not contain agglutinins to the immunizing antigens. After immunization, the agglutination titer for each of the three homologous strains was ≥1:640. Titers of heterologous strains did not exceed 1:20 (3).

F/P ratios of the conjugates. The F/P (in micrograms/milligram) ratios of the conjugates were as follows: 10 for the 138-71 conjugate, 9.5 for the 207-72C conjugate, 10.5 for the 275-70A conjugate, 10.5 for the 532-70A conjugate, and 10.0 for the polyvalent conjugates.

Direct FA staining of previous strains with *B. melaninogenicus* polyvalent conjugate (containing serogroups A, B, and C). Control smears were included each time that slides were examined by direct FA. The control slides included strains that stained either 2+, 3+, 4+, or negative. Comparison of the FA staining of test strains with the control slides minimized misinterpretation of the FA intensity.

Strains of *B. melaninogenicus* (46 strains) which stained previously with either serogroup A, B, or C (3) stained positively with the polyvalent conjugate containing these three serogroups (Table 2). One strain of *B. melaninogenicus* subsp. *melaninogenicus*, 427-70A, was nonviable. Two strains of *B. melaninogenicus* subsp. *intermedius* (532-70A and 854-70E) failed to fluoresce with the polyvalent conjugate. Strain 532-70A never reacted with serogroups A, B, or C (3), or with the polyvalent conjugate. Strain 854-70E originally gave a 4+ FA stain with serogroup C only; however, repeat testing of this strain showed no reaction with serogroups A, B, C, or the polyvalent conjugate. Apparently, 854-70E originally contained serogroup C colonies which were lost on subculture.

The polyvalent conjugate failed to give more than a 1+ fluorescent staining reaction with 192 strains of anaerobes, other than *B. melaninogenicus*, and aerobes. The 192 strains included: *Bacteroides fragilis* subsp. *fragilis* (15 strains); *B. fragilis* subsp. *thetaiotaomicron* (19 strains); *B. fragilis* subsp. *distasonis* (11 strains); *B. fragilis* subsp. *vulgatus* (11 strains); *Bacteroides corrodens* (4 strains); *Bacteroides capillosus* (1 strain); *Bacteroides praeacutus* (1 strain); *Bacteroides putredinis* (6 strains); *Bacteroides oralis* (22 strains); *Bacteroides ochraceus* (3 strains); *Bacteroides* group I (7 strains) (2); *Bacteroides* group PS (proteolytic, saccharolytic (2) (20 strains); *Bacteroides ruminicola* subsp. *brevis* (7 strains); *B. ruminicola* subsp. *ruminicola* (6 strains); *Fusobacterium fusiforme* (5 strains); *F. naviforme* (6 strains); *F. necrophorum* (5 strains); *F. nucleatum* (6 strains); *F. russii* (4 strains); *F. varium* (5 strains); *Eubacterium lentum* (9 strains); *Bifidobacterium adolescentis* (6 strains); *B. bifidum* (1 strain); *Proteus morganii* (2 strains); *P. mirabilis* (3 strains); *Escherichia coli* (6 strains); *Serratia marcescens* (1 strain); and *Staphylococcus aureus* (1 strain).

Direct FA staining of new clinical isolates with three *B. melaninogenicus* conjugates

TABLE 2. *Strains tested by the direct fluorescent antibody staining method with a Bacteroides melaninogenicus polyvalent conjugate*[a]

Organism and strain no.	Polyvalent conjugate (serogroups A, B, C)
B. melaninogenicus subsp. *melaninogenicus* (6 strains)	+[b]
B. melaninogenicus subsp. *asaccharolyticus* (30 strains)	+
B. melaninogenicus subsp. *intermedius* (10 strains)	+
(2 strains) 532-70A, 854-70E	−[c]

[a] FITC-labeled rabbit antisera.
[b] 3 to 4+ fluorescence.
[c] 0 to 1+ fluorescence.

and with the polyvalent conjugate (containing serogroups A, B, and C). A total of 92 clinical isolates were examined for the first time by FA with conjugates to serogroups A, B, and C as well as with the polyvalent conjugate (Table 3). The 83 strains stained 3 to 4+ with the homologous conjugate, as well as with the polyvalent conjugate containing serogroups A, B, and C.

Nine strains of *B. melaninogenicus* subsp. *intermedius* failed to stain with conjugates of serogroups A, B, and C as well as with the polyvalent conjugate. These strains had the biochemical characteristics and gas chromatographic products typical of *B. melaninogenicus* subsp. *intermedius*.

Certain strains (276-74E, 335-74E, 724-74, 726-74, 727-74, 729-74, 731-74, and 732-74) presented a special problem since they gave a 3 to 4+ FA stain with serogroups A and C conjugates. These strains were initially called *B. melaninogenicus* subsp. *intermedius*, although the biochemical reactions of certain of these strains did not delineate between *B. melaninogenicus* subsp. *melaninogenicus* and *B. melaninogenicus* subsp. *intermedius*.

These strains were studied further to try to determine whether these particular cultures contained a mixture of serogroups A and C or whether they might represent a new serogroup, serogroup A-C, which had antigens of both serogroups. Results presented below indicate that the cultures that gave the A-C reaction were mixtures of *B. melaninogenicus* subsp. *melaninogenicus* (serogroup A) and *B. melaninogenicus* subsp. *intermedius* (serogroup C), and that it was difficult to recover the *B. melaninogenicus* subsp. *melaninogenicus* from this mixture. *B. melaninogenicus* subsp. *intermedius* was recovered in pure culture from seven cultures, although *B. melaninogenicus* subsp. *melaninogenicus* was recovered in pure culture from only one of these seven cultures.

After repeated streaking and careful examination of the colonies, two colony types were seen in 727-74; a nucleated colony with a brown-black center and a brown edge (724-74A1) and a black colony (724-74A2). The nucleated colony (727-74A1) was biochemically typical of *B. melaninogenicus* subsp. *melaninogenicus* and gave a positive FA reaction with serogroup A conjugate only. The black colony (727-74A2) was biochemically typical of *B. melaninogenicus* subsp. *intermedius* and stained with serogroup C only.

From two other strains (276-74E and 335-74E), only one colony type could be detected in pure culture for several months. Five colonies selected from each culture gave a positive FA stain with both A and C conjugates. However, after repeated transfers of broth cultures for 7 months, two colony types growing closely together were noted. A pure culture of the black colony from each strain was biochemically characteristic of *B. melaninogenicus* subsp. *intermedius* and gave a positive FA stain with serogroup C conjugate only. However, when the nucleated colony from each culture was transferred, only the black colony type grew, and these cultures were biochemically like *B. melaninogenicus* subsp. *intermedius* and stained only with serogroup C conjugate. Thus, we were unsuccessful in purifying *B. melaninogenicus* subsp. *melaninogenicus* (serogroup A) from these cultures. Similarly, only serogroup C (*B. melaninogenicus* subsp. *intermedius*) was purified from strains 724-74 and 729-74. From these two cultures, the nucleated colony that was observed failed to grow on transfer.

Strains 731-74 and 732-74 also both originally stained as serogroup A and C, but repeated subcultures of both strains yielded a pure culture of a black colony that gave a positive stain with serogroup C conjugate only; the black colony was biochemically characteristic of *B. melaninogenicus* subsp. *intermedius*. The serogroup A colony was never seen in either strain.

Even after repeated subculture for 7 months, strain 726-74 continued to show only one colony type and gave a 3 to 4+ FA stain with A and C conjugates. This strain was brown and never produced a black pigment, even after prolonged incubation. Biochemically, the strain was typical of *B. melaninogenicus* subsp. *intermedius*.

Some strains, for example 1282-74G, produced black and brown pigmented colonies in the same culture at 48 to 72 h. The black, as well as the brown, colony may yield either the black or the brown and black colonies on transfer; we have not noted pure cultures of the brown colony from these strains. Both the black and brown colonies from 1282-74G gave a positive FA stain with serogroup C only. When 1282-74G, and other strains showing black and brown colonies at 48 to 72 h, are held for a total of 72 to 96 h only black colonies are seen. Apparently, the development of the black pigment in 1282-74G was a function of age.

Description of a new serogroup (C-1). The nine strains of *B. melaninogenicus* subsp. *intermedius* that failed to stain positively with serogroups A, B, C, and polyvalent conjugates (Table 3) were biochemically typical of *B. melaninogenicus* subsp. *intermedius* and all nine strains produced a black pigment. To determine if these strains represented another, or multiple, new serogroups, antiserum was produced to 532-70A and the globulin from this

TABLE 3. *B. melaninogenicus strains tested with four FA conjugates[a] by the direct fluorescent antibody staining method*

Organism and strain no.	*B. melaninogenicus* conjugate			
	Serogroup A (anti-138-71)	Serogroup B (anti-207-72C)	Serogroup C (anti-275-70A)	Polyvalent (serogroups A, B, C)
B. melaninogenicus subsp. *melaninogenicus* (3 strains) 727-74A1, 1451-74D2, 1653-74	+[b]	−[c]	−	+
B. melaninogenicus subsp. *asaccharolyticus* (52 strains) 57-73F, 187-73D, 251-73E, 484-73D, 510-73D, 548-73E, 549-73E, 987-73D, 1217-73A, 1259-73A, 1394-73F, 19-74C, 33-74D, 156-74D, 169-74F, 304-74D, 356-74H, 376-74F, 383-74D, 393-74E, 717-74E, 858-74B, 873-74F, 885-74G, 899-74F, 978-74E, 1005-74F, 1027-74E, 1027-74F, 1041-74E, 1120-74B, 1153-74F, 1158-74I, 1228-74E, 1236-74E, 1272-74F, 1340-74G, 1352-74F, 1355-74E, 1478-74E, 1502-74D, 1631-74F, 1646-74, 1695-74, 1718-74, 741-75A, 750-75G, 783-75A, 785-75C, 824-75H, 1006-75D, 1016-75A	−	+	−	+
B. melaninogenicus subsp. *intermedius* (28 strains) 276-74EA2, 292-74F, 297-74G, 302-74, 335-74EA2, 358-74H, 360-74G, 476-74G, 722-74, 723-74, 724-74A2, 725-74, 726-74A, 727-74A2, 729-74A2, 730-74, 731-74, 732-74, 733-74, 1054-74F, 1282-74G, 1487-74A, 1645-74, 1685-74, 1686-74, 1694-74, 1698-74E, 1701-74E	−	−	+	+
(9 strains) 532-70A, 574-70B, 854-70E, 2-71I, 237-73C, 728-74, 57-75H, 114-75, 817-75E	−	−	− to 2+	−

[a] FITC-labeled rabbit antisera.
[b] 3 to 4+ fluorescence.
[c] 0 to 1+ fluorescence.

antiserum was labeled with FITC. The nine strains were tested with the 532-70A conjugate (Table 4). All nine strains stained 3 to 4+ with 532-70A conjugate. Because these nine strains were biochemically typical of *B. melaninogenicus* subsp. *intermedius* and were serologically distinct from other *B. melaninogenicus* strains and other anaerobes tested, we are designating these strains as serogroup C-1. This conjugate failed to give a positive FA stain with 49 strains selected at random from the other three serogroups of *B. melaninogenicus*.

The conjugate prepared with anti-532-70A failed to give a positive FA stain with 35 strains of anaerobes other than *B. melaninogenicus*. These strains included: *B. oralis* (seven strains); *B. putredinis* (five strains); *B. ochraceus* (two strains); *Bacteroides* group I (six strains) (2); *Bacteroides* group PS (five strains) (2); *B. corrodens* (one strain); *B. ruminicola* subsp. *brevis* (four strains), and *B. ruminicola* subsp. *ruminicola* (five strains).

TABLE 4. *Bacteroides melaninogenicus subsp. intermedius strains tested by the direct fluorescent antibody staining method with 532-70A conjugate[a]*

Organism and strain no.	*B. melaninogenicus* subsp. *intermedius* conjugate serogroup C-1 (anti-532-70A)
B. melaninogenicus subsp. *intermedius* (9 strains) 532-70A, 574-70B, 854-70E, 2-71I, 237-73C, 728-74, 57-75H, 114-75, 817-75E	+[b]
B. melaninogenicus subsp. *melaninogenicus* 8 strains	−[c]
B. melaninogenicus subsp. *asaccharolyticus* 22 strains	−
B. melaninogenicus subsp. *intermedius* 19 strains	− to 2+

[a] FITC-labeled rabbit antisera.
[b] 3 to 4+ fluorescence.
[c] 0 to 1+ fluorescence.

Direct FA staining of clinical isolates with polyvalent conjugate (containing serogroups A, B, C, and C-1). Control smears, as described previously, were included each time that slides were examined by direct FA. Since a new serogroup was described, serogroup C-1, this serogroup had to be included in the polyvalent conjugate. The new polyvalent conjugate contained the four previously described serogroups of *B. melaninogenicus:* serogroups A, B, C, and C-1. Representative strains (23 strains) of the four serogroups of *B. melaninogenicus* stained positively with the new polyvalent conjugate. The 23 strains included *B. melaninogenicus* subsp. *melaninogenicus* (five strains of serogroup A); *B. melaninogenicus* subsp. *asaccharolyticus* (six strains of serogroup B); *B. melaninogenicus* subsp. *intermedius* (six strains of serogroup C); and *B. melaninogenicus* subsp. *intermedius* (six strains of serogroup C-1). Therefore, a *B. melaninogenicus* polyvalent FA conjugate should contain serogroups A, B, C, and C-1.

DISCUSSION

Immunofluorescence using specific conjugates of *B. melaninogenicus* has several important uses in clinical and research laboratories: rapid identification of *B. melaninogenicus* even before pigmentation develops; delineation of specific serogroups of *B. melaninogenicus;* as an aid in distinguishing *B. melaninogenicus* from certain organisms which are biochemically similar; and separation of a specific serogroup of *B. melaninogenicus* in cultures composed of more than one serogroup of *B. melaninogenicus*.

On initial isolation from clinical specimens, several species of *Bacteroides* which produced tan to brown pigment at 48 to 96 h, were confused with some strains of *B. melaninogenicus* which also produced a tan to brown pigment at 48 to 96 h. Tan pigment was noted in certain cultures of *B. oralis* and *B. ochraceus*. Tan to brown pigment has been noted in certain strains of *B. ruminicola*. In addition, *B. oralis* has similar biochemical reactions to *B. melaninogenicus* subsp. *melaninogenicus*, and both of these organisms produce the same metabolic products from glucose (2). Thus, the FA stain provided a rapid method to separate *B. melaninogenicus* subsp. *melaninogenicus* and *B. melaninogenicus* subsp. *intermedius* from other saccharolytic *Bacteroides*.

B. putredinis and *B. melaninogenicus* subsp. *asaccharolyticus* are similar biochemically; both are asaccharolytic and both produce the same metabolic products from glucose (2). At 24 to 48 h on laked blood agar, *B. putredinis* colonies may be tan or translucent; the translucent colony may appear red because the medium underneath the colony is red, but the colony does not have a red pigment. *B. melaninogenicus* subsp. *asaccharolyticus* colonies are reddish-black to black at 24 to 48 h. Although pigmentation may be helpful in separating these two species, the presence or absence, or the intensity, of pigmentation may be difficult to interpret. FA staining definitively separated *B. melaninogenicus* subsp. *asaccharolyticus* from *B. putredinis*.

Formation of pigment is dependent upon cultural conditions such as age of the culture, presence of heme or heme-containing compounds in the medium (4), and other factors; therefore, final identification of *B. melaninogenicus* should not be based on pigmentation characteristics alone. In addition, brown pigmented colonies of *B. melaninogenicus* subsp. *intermedius* became black with prolonged incubation, whereas the brown pigmented strains of *B. melaninogenicus* subsp. *melaninogenicus* remained brown even after 10 days of incubation. Tan to brown pigmented colonies of *B. oralis*, *B. ruminicola*, and *B. putredinis* did not become black with incubation up to 10 days.

A new serogroup composed of nine strains of *B. melaninogenicus* subsp. *intermedius*, serogroup C-1, was described. This makes four serogroups of *B. melaninogenicus* thus far reported.

A new polyvalent FA conjugate containing the four serogroups of *B. melaninogenicus* (serogroups A, B, C, and C-1) was developed and evaluated. This polyvalent conjugate may be used to identify *B. melaninogenicus* isolates which occur in mixed culture from clinical specimens. Immunofluorescence for identification of *B. melaninogenicus* is rapid, since the direct FA stain may be performed as soon as a colony appears on the isolation medium; in contrast, biochemical identification requires several days to obtain a pure culture, and for inoculation, incubation, and reading of biochemical tests. After use of a polyvalent conjugate for identification of *B. melaninogenicus*, the individual conjugates of serogroups A, B, C, and C-1 may be used to define the specific serogroup of *B. melaninogenicus*.

Certain cultures (eight strains) were mixtures of *B. melaninogenicus* subsp. *melaninogenicus* (serogroup A) and *B. melaninogenicus* subsp. *intermedius* (serogroup C) as detected by the FA stain. Biochemical reactions did not discern this mixture of the two subspecies. Serogroup C alone subcultured easily, although serogroup A was successfully separated in pure culture from only one culture. This phenomenon suggested a possible symbiotic relationship

between certain strains of serogroups A and C: *B. melaninogenicus* subsp. *intermedius* (serogroup C) may produce a factor necessary for the growth of certain strains of *B. melaninogenicus* subsp. *melaninogenicus* (serogroup A). If this symbiotic relationship exists, *B. melaninogenicus* subsp. *melaninogenicus* (serogroup A) may occur more commonly in clinical specimens than we thought previously (3, 6).

In 59 strains of what appeared to be pure cultures of either *B. melaninogenicus* subsp. *melaninogenicus* (serogroup A) or *B. melaninogenicus* subsp. *intermedius* (serogroup C or C-1), we have never detected *B. melaninogenicus* subsp. *asaccharolyticus* (serogroup B). Apparently, *B. melaninogenicus* subsp. *asaccharolyticus* strains grow quite independently of the other two subspecies of *B. melaninogenicus*. However, more than one subspecies of *B. melaninogenicus* may occur in direct culture of clinical specimens.

Williams et al. (6) suggested a possible ecological difference between the three subspecies of *B. melaninogenicus:* for example, that *B. melaninogenicus* subsp. *asaccharolyticus* strains were derived from the gut, and strains of *B. melaninogenicus* subsp. *intermedius* were isolated mainly from oral samples. In our experience, *B. melaninogenicus* subsp. *asaccharolyticus* isolates have been obtained from gut, genital, and other sources; *B. melaninogenicus* subsp. *intermedius* was recovered from gut, genital, upper respiratory, and other locations; and *B. melaninogenicus* subsp. *melaninogenicus* was isolated primarily from upper respiratory specimens, as well as from a great toe infection. Thus, it would appear that there was no definite ecological relationship of these three subspecies of *B. melaninogenicus* isolated from human sources.

It has been suggested (6) that since a significant difference in deoxyribonucleic acid mean base composition exists between *B. melaninogenicus* subsp. *intermedius* and *B. melaninogenicus* subsp. *asaccharolyticus* it may be inappropriate to retain both organisms in the same species. If *B. melaninogenicus* subsp. *asaccharolyticus* is removed from *B. melaninogenicus*, this would still represent a specific serogroup within the *Bacteroides*.

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