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Attachment of *Bacteroides melaninogenicus* subsp. *asaccharolyticus* to Oral Surfaces and Its Possible Role in Colonization of the Mouth and of Periodontal Pockets

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This investigation examined the ability of cells of *Bacteroides melaninogenicus* subsp. *asaccharolyticus* 381 to adhere to surfaces that might be important for its initial colonization of the mouth and its subsequent colonization in periodontal pockets. Of 48 asaccharolytic strains of *B. melaninogenicus*, 47 agglutinated human erythrocytes, whereas none of 20 fermentative strains, which included reference cultures of the subspecies *intermedius* and *melaninogenicus*, were active. Electron microscopy indicated that both asaccharolytic and fermentative strains possessed pili; hence, the presence of pili did not correlate with the hemagglutinating activities of *B. melaninogenicus* strains. Both asaccharolytic and fermentative *B. melaninogenicus* strains suspended in phosphate-buffered saline adhered in high numbers to buccal epithelial cells and to the surfaces of several gram-positive bacteria tested, including *Actinomyces viscosus*, *A. naeslundii*, *A. israelii*, *Streptococcus sanguis*, and *S. mitis*. *B. melaninogenicus* subsp. *asaccharolyticus* 381 also attached, but in comparatively low numbers, to untreated and to saliva-treated hydroxyapatite. Addition of clarified whole saliva to suspensions of strain 381 almost completely eliminated adherence to buccal epithelial cells and to hydroxyapatite surfaces, but saliva had no detectable effect on attachment to gram-positive plaque bacteria. Both fermentative and nonfermentative strains of *B. melaninogenicus* also attached in high numbers to crevicular epithelial cells derived from human periodontal pockets, but normal human serum strongly inhibited attachment. Serum also inhibited attachment of strain 381 to saliva- and serum-treated hydroxyapatite, but it had little effect upon attachment to gram-positive bacteria. These observations suggested that salivary and serum components would strongly inhibit the attachment of *B. melaninogenicus* cells to several oral surfaces, but not to the surfaces of certain gram-positive bacteria commonly present in human dental plaque. This was confirmed by an in vivo experiment in which streptomycin-labeled cells of *B. melaninogenicus* 381-R were introduced into the mouths of two volunteers. After 10 min, several hundred-fold higher numbers of the organism were recovered from preformed bacterial plaque present on teeth than from clean tooth surfaces or from the buccal mucosa and tongue dorsum. High numbers of *B. melaninogenicus* cells were also recovered from preformed plaque after 150 min, but virtually no cells of the organism were recovered from the other surfaces studied. These data suggest that the presence of dental plaque containing *Actinomyces* and other gram-positive bacteria may be essential for the attachment and colonization of *B. melaninogenicus* cells after their initial introduction into the mouth. Similarly, the presence of subgingival plaque containing gram-positive bacteria may be necessary for its secondary colonization in periodontal pockets.

Gram-negative anaerobic rods have been recently shown to comprise approximately 75% of the cultivable bacteria in plaque removed from the base of deep gingival pockets of adults (21). *Bacteroides melaninogenicus* constituted almost half of these gram-negative isolates, and most of the *B. melaninogenicus* strains were nonfermentative and appeared to belong to the subspecies *asaccharolyticus* (21). *B. melaninogenicus* strains comprise a much lower percentage of the cultivable bacteria in samples of supragingival plaque, and they are present on the tongue dorsum and vestibular mucosa in low proportions, if they are detected at all (5, 10,

13). These organisms are infrequently isolated from humans before puberty (1, 10). The relatively high proportions of *B. melaninogenicus* subsp. *asaccharolyticus* found in periodontal pockets and the potential of this organism to synergistically produce mixed anaerobic infections in experimental animals (5, 14, 23) suggest that it may play an important role in the etiology and pathogenesis of periodontitis.

Little is known about the parameters that influence the colonization of *B. melaninogenicus* in periodontal pockets or in other sites of the mouth. Factors that have been assumed to be important for the establishment and regulation of oral microbial populations include nutrition, pH, Eh, the presence of antibacterial secretions, and microbial interactions (8, 12). Although certain nutritional, physical, and chemical conditions must be met to permit growth of oral organisms, these factors do not appear to be sufficiently selective to explain adequately why only distinct types of gram-negative anaerobic rods predominate in deep periodontal pockets and why differences exist in the types of bacteria present in gingival pockets of patients with different clinical types of periodontal disease (16, 17, 20, 21).

Several recent studies have indicated that the ability of bacteria to attach to surfaces is an important ecological determinant which influences their colonization in environments that contain a fluid flow (8). Oral streptococci and other bacteria attach with a high degree of specificity to teeth and to oral epithelial surfaces, and the relative adherence of each species studied to date correlates with their natural patterns of intraoral colonization (8). However, nothing is known about the ability of *B. melaninogenicus* to adhere to surfaces present in the mouth. Okuda and Takazoe (18) noted that some *B. melaninogenicus* strains could attach to and agglutinate erythrocytes, and they reported the presence of pilus-like structures on such strains. Based upon these observations, they suggested that pili might mediate the attachment of *B. melaninogenicus* cells to mucous membranes.

The present study was initiated to determine the ability of a strain of *B. melaninogenicus* subsp. *asaccharolyticus* to attach to various oral surfaces, and to evaluate the role that adherence might play in its oral and subgingival colonization.

MATERIALS AND METHODS

Cultures and cultural conditions. *B. melaninogenicus* subsp. *asaccharolyticus* 381 and 2015, *B. melaninogenicus* subsp. *intermedius* 1223 and 2203, *B. melaninogenicus* subsp. *melaninogenicus* 581, *Fusobacterium nucleatum* 2056 and 2125, *Capnocytophaga* (*B. ochraceus*) 2010 and 2159, *Eikenella corrodens* 1073 (rough colony), *E. corrodens* 374 (smooth colony), "corroding" *Bacteroides* strains 401 and 402, *Campylobacter* strain 288, *Vibrio* strain 371, *A. israelii* 1503, and *Clostridum sporogenes* 2171 were isolated from the base of deep periodontal pockets in humans; these reference strains were kindly provided by S. S. Socransky, Forsyth Dental Center. *B. melaninogenicus* subsp. *asaccharolyticus* BMD 4 was isolated from a periodontal pocket in a beagle dog at J. Lindhe's laboratory (Göteborg, Sweden). *B. melaninogenicus* subsp. *asaccharolyticus* 536-B was isolated from a human perirectal abscess and was obtained from S. M. Finegold, Los Angeles. *A. naeslundii* C2 was a fresh isolate that possessed pili, provided by R. P. Ellen, Toronto, Canada. Laboratory strains (*A. naeslundii* 12104 and I, *A. viscosus* T14, *S. sanguis* 34, *S. salivarius* CM 6, *S. mitis* 26, and *S. mutans* GS 5) were obtained from the culture collection at the Forsyth Dental Center. Other strains of *B. melaninogenicus* studied were freshly isolated from samples of subgingival plaque and from swabbings of tonsillar crypts obtained from 15 individuals.

All organisms were maintained by weekly transfer on plates containing Todd-Hewitt agar (BBL) supplemented with 10% sheep blood, 5 μg of hemin per ml, and 0.2 μg of menadione per ml. Bacterial cells used for each experiment were grown in Todd-Hewitt broth containing corresponding supplements of hemin and menadione. All cultures were incubated at 37°C in Brewer jars containing 80% N_2, 10% H_2, and 10% CO_2.

Tritium-labeled cells of *B. melaninogenicus* subsp. *asaccharolyticus* 381 were prepared by growing the organisms in Todd-Hewitt broth containing 10 μCi of [^{3}H]thymidine per ml (New England Nuclear Corp., Boston, Mass.). A mutant of *B. melaninogenicus* subsp. *asaccharolyticus* 381 that was resistant to 2,000 μg of streptomycin per ml in Todd-Hewitt broth was prepared as previously described (26); this strain was designated 381-R.

Hemagglutination studies. Bacterial cells were washed twice with 0.01 M phosphate-buffered saline (PBS) at pH 7.2 and suspended in PBS at a concentration of approximately 5×10^8 organisms per ml. Hemagglutination activity was determined by direct slide agglutination and by use of microtitration plates as previously described (6). A 2% suspension of washed human type A erythrocytes was used for testing.

Hemagglutination inhibition studies were also carried out in microtitration plates. Samples (0.025 ml) of substances examined for hemagglutination-inhibition activity were serially twofold diluted with PBS, and 0.025 ml of the highest dilution of the bacterial cell suspension that still gave strong hemagglutination was added to each well. The mixtures were gently shaken for 1 h at room temperature, except in the case of trypsin, which was incubated for 3 h at 37°C, and then 0.025 ml of the washed erythrocyte suspension was added. Following 1 h of shaking at room temperature, the mixtures were stored overnight at 4°C. Agglutination was determined with a dissecting microscope; wet-mount preparations of each sample were also examined at ×400 magnification.

Preparation of partially purified pili. Partially purified pili were prepared from PBS-washed cells of

B. melaninogenicus 381 harvested from 1-liter cultures grown in Todd-Hewitt broth. The organisms were treated with sonic oscillation (MSE sonicator) for 20 s at 8 μm amplitude to remove pili. The suspension was then centrifuged at 15,000 × *g* for 15 min to remove intact cells and large debris. The clear supernatant liquor, which possessed strong hemagglutinating activity, was lyophilized, and the dried material was suspended in 10 ml of PBS; sonic oscillation was used to facilitate dispersion. Samples of the suspension were applied to columns (2.5 by 80 cm) of Bio-gel A-15, which were eluted with PBS. Strong hemagglutinating activity was only detected in the void volume fractions of the column. Negatively stained samples of these active fractions were examined by electron microscopy.

Preparation of saliva. Samples of whole nonstimulated saliva (2- to 5-ml) were clarified by centrifugation at 12,000 × *g* for 10 min and heated at 60°C for 30 min to inactivate degradative enzymes. Saliva fractions were prepared from whole, nonstimulated saliva obtained from an adult of blood type AB. In this instance, the saliva was collected in a tube chilled in an ice bath. It was heat inactivated at 60°C for 30 min, and then ethylenediaminetetraacetic acid (pH 8.0) was added to a final concentration of 0.1 M. The saliva was clarified by centrifugation, dialyzed against distilled water for 24 h, and lyophilized. The material was dissolved in PBS at 10× concentration and fractionated on a column (2.5 by 80 cm) of Bio-gel A-15 as previously described (9). Three pooled fractions were obtained: (i) the high-molecular-weight mucin-containing fraction eluting in the void volume; this material was strongly blood group-substance reactive when assayed as described previously (6); (ii) intermediate-molecular-weight fractions; and (iii) the low-molecular-weight fractions. Immunoglobulin A was present in fraction ii, but none was detected in fractions i and iii. The pooled fractions were dialyzed against distilled water for 24 h and lyophilized. Each fraction was dissolved in 0.05 M KCl containing 1 mM potassium phosphate (pH 6.0) and 1 mM $CaCl_2$ at a concentration comparable to that present in the original unfractionated saliva.

In vitro adherence to epithelial cells. The ability of *B. melaninogenicus* strains and certain other gingival bacteria to adhere to washed epithelial cells was determined by using an in vitro system previously described (7). Buccal epithelial cells were collected from volunteers by scraping their cheek surfaces with wooden applicator sticks. Periodontal pocket epithelial cells were obtained from deep pockets in adults; they were collected by scraping the crevicular surface with a periodontal scaler. The mean number of bacterial cells that attached per epithelial cell after 30 min of incubation at 37°C was determined by direct light microscopic enumeration of 25 epithelial cells. Bacterial counts of control epithelial cells incubated only with PBS were performed in a similar manner to establish the number of indigenous bacteria present before exposure to the test organisms. These values were subtracted to obtain the mean number of test organisms that attached.

The effect of whole saliva and of crude saliva fractions on the attachment of *B. melaninogenicus* subsp. *asaccharolyticus* 381 to human buccal epithelial cells was determined by incubating 0.5 ml of each saliva sample with 0.5 ml of the epithelial cell suspension for 30 min at 37°C. Washed bacterial cells (0.5 ml) were then added, and the mixture was again incubated for 30 min at 37°C. The effect of serum on the attachment of *B. melaninogenicus* 381 to crevicular epithelial cells was studied in a similar manner.

In vitro adherence of ^{3}H-labeled *B. melaninogenicus* subsp. *asaccharolyticus* 381 to HA surfaces. The ability of *B. melaninogenicus* 381 to attach to hydroxyapatite (HA) surfaces was examined using a technique recently described (W. Clark and R. J. Gibbons, Abstract #13, J. Dent. Res. **56**, special issue B, 1977). Samples of 40 mg of spheroidal HA beads (BDH Chemicals), which possessed a total surface area of 10.9 cm^2, were equilibrated overnight by continuous inversion in 0.05 M KCl containing 1 mM phosphate (pH 6.0), 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$. The buffered KCl was removed and replaced with whole-clarified saliva obtained from a subject of blood type A. The HA beads were continuously inverted for 1 h and then washed three times in the phosphate buffer. Tritium-labeled washed *B. melaninogenicus* 381 cells were suspended in buffered KCl or in whole saliva or serum at a concentration of 10^8 cells per ml. Samples of the bacterial suspensions (1.0 ml) were added to the pretreated HA beads, and the mixtures were continuously inverted for 2 h; preliminary experiments indicated that this time period ensured equilibrium. The beads were then washed three times with buffered KCl to remove unattached bacterial cells. Samples of the original bacterial suspensions were counted with a Packard Tri-Carb liquid scintillation spectrometer so that counts per minute could be related to bacterial cell number. The number of bacteria that attached to the HA was also determined by scintillation counting; these values were corrected for quench caused by the HA. The number of bacterial cells attached per square centimeter of the saliva- or serum-treated HA beads was then calculated.

In vitro adherence between bacterial species. Organisms tested for interspecies adherence were washed twice and suspended in PBS to yield suspensions having an optical density at 550 nm of 1.0. Mixtures containing 0.1 ml of each bacterial suspension and 0.1 ml of PBS were prepared and incubated at 37°C for 60 min in a shaking water bath. Because several of the strains studied autoagglutinated, it was not possible to evaluate interspecies adherence by the formation of macroscopic aggregates. Consequently, the attachment of gram-negative organisms to gram-positive species was determined by examination of Gram-stained smears for the presence of aggregates composed of complexed gram-positive and gram-negative organisms; attachment was scored from 0 (little or no evidence of mixed aggregates) up to 2+ (abundant mixed aggregates).

The effect of whole saliva and serum (from a subject of blood type A) on interspecies adherence was tested by adding 0.1 ml of the test solution to 0.1 ml of a washed suspension of one gram-negative organism. After incubation for 1 h at 37°C, 0.1 ml of washed cells of a gram-positive species was then added. The mixtures were incubated for an additional 1 h at 37°C,

and interbacterial adherence was determined as described above.

In vivo adherence of streptomycin-resistant *B. melaninogenicus* subsp. *asaccharolyticus* 381-R to dental plaque and to clean tooth, buccal, and tongue surfaces. Cells from a 48-h broth culture of *B. melaninogenicus* 381-R were washed twice and suspended in PBS at a concentration of 10^7 organisms per ml. Samples (1.0 ml) of the bacterial suspension were introduced into the mouths of two volunteers (J.S. and R.J.G.), who distributed it throughout their mouths for 5 min before expectorating. Calgiswab samples were taken from clean molar surfaces and from buccal and tongue surfaces 10 and 150 min later. Samples of supragingival plaque were also collected with a periodontal scaler. The samples were dispersed in prereduced Ringer solution using continuously anaerobic techniques as described by Manganiello et al. (15). Reference samples of appropriate dilutions were plated in duplicate on Todd-Hewitt blood agar plates with and without 250 μg of streptomycin per ml. After 14 days of anaerobic incubation, total cultivable colony counts were determined on the blood agar plates, and the numbers of colonies of *B. melaninogenicus* 381-R were enumerated on the plates that contained streptomycin.

RESULTS

Hemagglutinating activities of strains of *B. melaninogenicus*. A total of 68 strains of *B. melaninogenicus*, which represented fresh isolates from subgingival plaque and tonsillar swabbings as well as reference cultures, were examined for hemagglutinating activity. Of the 68 strains, 47 strongly agglutinated human type A erythrocytes, whereas the remaining 21 strains were inactive. All strains that possessed hemagglutinating activity failed to ferment glucose and apparently were representatives of the subspecies *asaccharolyticus*. In contrast, 20 of the 21 nonhemagglutinating strains were fermentative, the exception being *B. melaninogenicus* subsp. *asaccharolyticus* 536-B, which had originally been isolated from a perirectal abscess.

Six representative asaccharolytic strains which possessed hemagglutinating activity and six saccharolytic strains lacking this activity were examined for the presence of pili in negatively stained preparations by electron microscopy by Z. Skobe, Forsyth Dental Center. Pili were present on all strains examined, including strain 536-B and others lacking hemagglutinating activity (Fig. 1). It is apparent from these observations that the presence of surface pili does not directly correlate with the hemagglutinating activities of *B. melaninogenicus* strains. Rather, it would appear that hemagglutinating activity is mainly associated with nonfermentative strains belonging to the subspecies *asaccharolyticus* and not to fermentative subspecies strains.

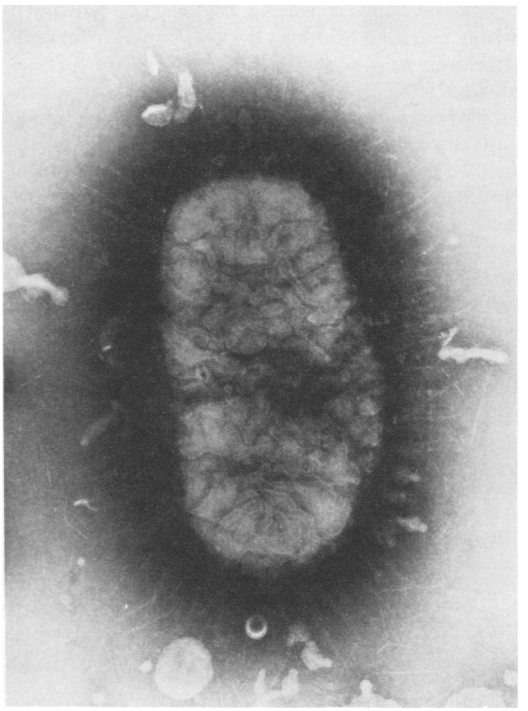

FIG. 1. *Negatively stained preparation of B. melaninogenicus subsp. intermedius 377 showing the presence of surface pili. This organism and other piliated fermentative strains did not agglutinate human erythrocytes. Photo kindly provided by Z. Skobe, Forsyth Dental Center.*

Partially purified pili preparations derived from *B. melaninogenicus* subsp. *asaccharolyticus* 381 possessed strong hemagglutinating activity. When examined by electron microscopy, these preparations were found to consist of large numbers of aggregated pili mixed with globular material. Attempts to eliminate the globular particles by ammonium sulfate precipitation (4) or by isoelectric precipitation at pH 3.8 as described by Brinton (3) were unsuccessful. The strong hemagglutinating activity possessed by partially purified pili preparations derived from strain 381 is consistent with the contention of Okuda and Takazoe (18) that pili are responsible for the hemagglutinating activities of certain *B. melaninogenicus* strains.

Hemagglutinating activity of intact *B. melaninogenicus* subsp. *asaccharolyticus* 381 cells and of partially purified pili was completely destroyed by heating at 60°C for 15 min. Hemagglutinating activity was unaffected by 0.1 M ethylenediaminetetraacetic acid (pH 8.0), by treatment with 1 mg of trypsin per ml, or by the presence of several hexoses, disaccharides,

or hexosamines tested (Table 1). However, whole clarified human saliva obtained from children and adults strongly inhibited the hemagglutinating activity of strain 381. The high-molecular-weight mucinous fraction of saliva, which contained blood group-reactive components, appeared to be primarily responsible for the inhibition, though some inhibitory activity was observed in the intermediate- and low-molecular-weight saliva fractions. No differences were observed in the hemagglutination-inhibition properties of saliva obtained from children or from adults with and without periodontitis (Table 1).

Human sera strongly inhibited hemagglutinating activity of *B. melaninogenicus* 381, but, on the basis of the limited number of samples examined, no relationship was observed between hemagglutination inhibition and the presence of periodontitis or juvenile periodontitis (periodontosis) (Table 1). Normal rabbit serum was moderately inhibitory.

TABLE 1. *Inhibition of hemagglutinating activity of B. melaninogenicus subsp. asaccharolyticus 381*

Test material	Hemagglutination inhibition titer	
	Mean	Range
PBS	0	
Whole saliva		
5 children, 3 to 5 years of age	16	8–32
5 adults without periodontitis	16	8–32
5 adults with periodontitis	32	16–64
Saliva fractions		
High-molecular-weight mucin fraction	8	
Intermediate-molecular-weight fraction including immunoglobulin A	1	
Low-molecular-weight fraction	1	
Serum		
1 healthy adult without periodontitis	256	
2 juvenile periodontitis (periodontosis) patients	256, 256	
2 periodontitis patients	256, 256	
Normal rabbit	64	
Sugars (0.1 M)		
D-Fructose	0	
L-Fucose	0	
D-Galactose	0	
D-Mannose	0	
D-Rhamnose	0	
Lactose	0	
Maltose	0	
Melibiose	0	
Raffinose	0	
D-Galactosamine	0	
D-Glucosamine	0	
D-Mannosamine	0	
N-acetyl-D-galactosamine	0	
N-acetyl-D-glucosamine	0	

Attachment of *B. melaninogenicus* and other bacteria to crevicular epithelial cells. The ability of strains representing the three subspecies of *B. melaninogenicus*, as well as of other bacteria found in sub- and supragingival plaque, to attach to crevicular epithelial cells derived from periodontal pockets was studied. With the exception of strain 536-B, *B. melaninogenicus* strains representative of all subspecies attached in high numbers to human crevicular epithelial cells (Table 2). Thus, although fermentative strains of *B. melaninogenicus* failed to attach to erythrocytes, they appeared to attach comparably with asaccharolytic strains to crevicular epithelial cells. This suggests that crevicular epithelial cells possess receptors for these organisms that are absent on human erythrocytes. *F. nucleatum* 2056, *E. corrodens* 1073, and strains of *S. sanguis*, *S. salivarius*, and *S. mitis* also attached in moderate or high numbers to crevicular epithelial cells. The remaining organisms tested possessed only feeble adherent properties to crevicular epithelium under the in vitro conditions tested.

In in vivo situations, crevicular epithelial cells would be expected to be exposed to the flow of crevicular fluid, which could influence bacterial attachment. This fluid is thought to be a serum transudate. Consequently, the influence of pooled human serum on the attachment of *B. melaninogenicus* 381 to human crevicular epithelial cells was studied. In two separate experiments, pooled human serum virtually completely inhibited attachment of this organism to crevicular epithelial cells (mean net number per cell 1 ± 3; 1 ± 2), thus suggesting that serous components present in crevicular fluid would also inhibit the attachment of this organism.

Attachment of *B. melaninogenicus* subsp. *asaccharolyticus* 381 to human buccal epithelial cells in vitro. The ability of *B. melaninogenicus* subsp. *asaccharolyticus* 381 to attach to human buccal mucosal epithelial cells collected from three children 3 to 5 years of age and from three adults was determined. Bacterial attachment, expressed as the mean number of *Bacteroides* cells attached per epithelial cell, plus or minus the standard error of the mean, was 96 ± 20 for epithelial cells from adults and 78 ± 20 for cells from children. The somewhat lower number of *B. melaninogenicus* 381 cells that attached to epithelial cells from children was not statistically different from the number that attached to adult buccal epithelial cells.

Clarified whole saliva completely inhibited the attachment of *B. melaninogenicus* to buccal epithelial cells derived from adults (Table 3).

TABLE 2. *Bacterial attachment to human crevicular epithelial cells in vitro*

Organisms	Mean net no. of bacteria attached per epithelial cell ± SE[a]
B. melaninogenicus subsp. *asaccharolyticus*	
381	99 ± 20
536B	24 ± 7
BM D 4	134 ± 17
B. melaninogenicus subsp. *intermedius*	
1223	84 ± 23
2203	78 ± 16
B. melaninogenicus subsp. *melaninogenicus* 581	110 ± 18
F. nucleatum	
2056	65 ± 10
2156	20 ± 3
Capnocytophaga (*B. ochraceus*)	
sp. 2010	23 ± 7
2159	18 ± 5
E. corrodens	
374	28 ± 8
1073	46 ± 12
Corroding *Bacteroides*	
sp. 401	27 ± 5
402	12 ± 5
Campylobacter sp. 288	24 ± 6
Anaerobic *Vibrio* sp. 371	12 ± 3
A. israelii 1503	19 ± 3
A. naeslundii	
12104	17 ± 7
I	8 ± 3
C2	24 ± 6
A. viscosus T14	17 ± 3
S. sanguis 34	68 ± 16
S. salivarius CM 6	54 ± 11
S. mitis 26	188 ± 22
S. mutans GS 5	22 ± 5
C. sporogenes 2171	0 ± 1

[a] Each figure denotes the mean of at least two experiments. SE, Standard error.

The high-molecular-weight mucinous components of saliva exerted an inhibitory effect upon *Bacteroides* attachment essentially equal to that of whole saliva, but intermediate- and low-molecular-weight salivary fractions also caused significant adherence inhibition.

Attachment of *B. melaninogenicus* subsp. *asaccharolyticus* 381 to saliva- and serum-treated HA. When suspensions containing 10^8 *B. melaninogenicus* 381 cells per ml were incubated with untreated HA beads, 64×10^4 *Bacteroides* cells adsorbed per cm^2 of surface area (Table 4). This is 50- to 100-fold lower than the number of cells of *S. mutans* or *A. viscosus* that adsorbed when tested similarly (W. B. Clark and R. J. Gibbons, unpublished data). Pretreatment of the HA with clarified human saliva or with human serum to form a film of selectively adsorbed components reduced the number of *Bacteroides* cells attached (Table 4). Additional inhibition of *Bacteroides* attachment occurred when saliva or serum was used as a suspension fluid in place of buffered KCl. These in vitro data suggest that cells of *B. melaninogenicus* in the saliva or serum-containing environments of the mouth would possess a relatively feeble capacity to attach to tooth surfaces containing adsorbed salivary or serum components.

Attachment of strains of *B. melaninogenicus* and other gram-negative subgingival bacteria to the surfaces of gram-positive organisms. *B. melaninogenicus* strains representative of the subspecies *asaccharolyticus*, *intermedius*, and *melaninogenicus* were found to attach well to the surfaces of several gram-positive bacteria studied, including strains of *A. israelii*, *A. naeslundii*, *A. viscosus*, *S. sanguis*, *S. mitis*, *S. mutans*, and *S. salivarius*. *B. melaninogenicus* 536-B again was an exception; it did not attach to any of the gram-positive organisms studied. The attachment of *B. melaninogenicus* strains exhibited some specificity, for none of the strains tested attached to cells of *C. sporogenes*. Strains of *F. nucleatum* and *Capnocytophaga* (*B. ochraceus*) also attached to most of the gram-positive organisms studied, whereas the attachment of strains of *E. corrodens* and "corroding" *Bacteroides* exhibited more selectivity (Table 5).

In contrast to the adherence-inhibiting effects of saliva and serum on the attachment of *B. melaninogenicus* 381 to erythrocytes, to buccal epithelial cells, and to serum- and saliva-coated HA, these fluids did not strongly inhibit the attachment of *B. melaninogenicus* 381 or 1223 or *Capnocytophaga* (*B. ochraceus*) 2159 to any of the gram-positive bacteria studied. These observations suggest that the surfaces of gram-positive bacteria may serve as important recep-

TABLE 3. *Effect of salivary components on the attachment of B. melaninogenicus subsp. asaccharolyticus 381 to human buccal epithelial cells*

Additive	Epithelial cell donor 1		Epithelial cell donor 2		Epithelial cell donor 3	
	Mean net no. of bacteria/cell ± SE	% of control	Mean net no. of bacteria/cell ± SE	% of control	Mean net no. of bacteria/cell ± SE	% of control
PBS (control)	82 ± 15	100	81 ± 18	100	122 ± 23	100
Whole saliva	−1 ± 2	0	1 ± 12	1.2	0 ± 6	0
High-molecular-weight mucinous components of saliva	0 ± 4	0	4 ± 10	4.9	3 ± 8	2.5
Intermediate-molecular-weight fraction of saliva	20 ± 11	24.4	23 ± 16	28.4	40 ± 18	38.8
Low-molecular-weight fraction of saliva	30 ± 14	36.6	15 ± 15	18.5	8 ± 6	6.6

TABLE 4. *Attachment of B. melaninogenicus subsp. asaccharolyticus 381 to HA surfaces*

Bacterial suspension fluid	Prior treatment of HA	Total no. of bacteria attached per cm^2 of HA surface × 10^4	% Attachment of untreated control
0.001 M phosphate-buffered KCl	None (control)	64	100
0.001 M phosphate-buffered KCl	Saliva	38	59
0.001 M phosphate-buffered KCl	Serum	20	31
Saliva	Saliva	11	17
Saliva	Serum	8	12
Serum	Saliva	2	3
Serum	Serum	12	19

tors for the attachment of *B. melaninogenicus* strains and certain other gram-negative bacteria in the oral environment.

Adherence of *B. melaninogenicus* subsp. *asaccharolyticus* 381-R to oral surfaces in humans. When suspensions of streptomycin-resistant cells of *B. melaninogenicus* 381-R were introduced into the mouths of two volunteers, the highest numbers of labeled organisms were recovered from the surfaces of preformed supragingival dental plaques (Table 6). In contrast, virtually no *Bacteroides* cells were recovered from clean tooth surfaces or from swabbings of the tongue dorsum or buccal mucosa 150 min after introduction of the mixture, even though the surface area of the tongue and buccal mucosa samples was far greater than that of dental plaque (Table 6). Labeled *B. melaninogenicus* cells were repeatedly recovered from samples of plaque from both individuals up to 3 weeks later. At that time, the labeled strain was eliminated from one subject (J.S.) during the course of tetracycline therapy; it persisted in the second individual (R.J.G.) for 8 weeks, but then became undetectable. These observations, therefore, indicate that colonization had occurred, and they are consistent with the in vitro studies that suggest that *B. melaninogenicus* subsp. *asaccharolyticus* strains primarily attach to receptors present on the surfaces of other bacteria in dental plaque.

DISCUSSION

The eventual colonization of a bacterial species in a periodontal pocket may be theorized to follow several steps. Upon initial introduction into the mouth, some cells of the species probably must attach to an oral surface exposed to saliva to initiate colonization. If this initial event occurred on a tooth surface near the gingival margin, progeny of the organism could enter the gingival crevice or a periodontal pocket by spreading proliferation of the microbial plaque. If initial colonization occurred on an oral mucosal surface, dislodged progeny present in saliva must then attach to the tooth near the gingiva so as to proliferate into subgingival sites. Rather than being dependent upon apical growth of attached cells, motile organisms might be able to enter subgingival environments by using their locomotion. It is also conceivable that organisms could be introduced into periodontal pockets by the forces of mastication, by oral hygiene procedures such as toothbrushing, or by use of dental instruments, etc. Such procedures might also transfer organisms from one periodontal pocket to another.

The present study investigated the ability of *B. melaninogenicus* subsp. *asaccharolyticus* 381 to attach to surfaces that might be important for its initial oral colonization and for its subse-

TABLE 5. *Attachment of gram-negative organisms to the surfaces of gram-positive bacteria*[a]

Gram-negative organisms tested	Suspension fluid	*A. israelii* 1503	*A. naeslundii* 12104	*A. naeslundii* I	*A. viscosus* T14	*S. sanguis* 34	*S. salivarius* CM6	*S. mitis* 26	*S. mutans* GS 5	*C. sporogenes* 2171
B. melaninogenicus subsp. *asaccharolyticus*										
381	PBS	2+	2+	2+	2+	2+	2+	2+	2+	0
	Saliva	2+	ND	ND	2+	2+	ND	2+	ND	ND
	Serum	2+	ND	ND	2+	+	ND	+	ND	ND
B. melaninogenicus subsp. *intermedius*										
1223	PBS	2+	2+	+	2+	2+	2+	2+	+	0
	Saliva	2+	ND	ND	2+	2+	ND	2+	ND	ND
	Serum	2+	ND	ND	2+	+	ND	+	ND	ND
Capnocytophaga (*B. ochraceus*)										
sp. 2159	PBS	2+	+	2+	2+	2+	2+	2+	+	0
	Saliva	+	ND	ND	2+	2+	ND	2+	ND	ND
	Serum	2+	ND	ND	2+	+	ND	+	ND	ND
B. melaninogenicus subsp. *asaccharolyticus*										
2015	PBS	2+	2+	+	+	2+	2+	2+	2+	0
BM D 4	PBS	2+	2+	2+	2+	2+	2+	2+	2+	0
536 B	PBS	0	0	0	0	0	0	0	0	0
B. melaninogenicus subsp. *intermedius*										
2203	PBS	2+	2+	2+	2+	2+	2+	2+	2+	0
B. melaninogenicus subsp. *melaninogenicus*										
581	PBS	2+	2+	2+	2+	2+	2+	2+	2+	0
F. nucleatum										
2056	PBS	+	2+	+	2+	+	2+	2+	+	0
2125	PBS	+	+	+	+	+	+	+	+	0
Capnocytophaga (*B. ochraceus*)										
sp. 2010	PBS	+	+	+	2+	+	+	2+	+	0
E. corrodens										
374	PBS	0	2+	0	2+	+	2+	+	2+	0
1073	PBS	0	2+	0	2+	+	2+	+	2+	0
Corroding *Bacteroides*										
sp. 401	PBS	2+	0	2+	0	0	+	0	+	0
sp. 402	PBS	2+	+	2+	0	+	+	0	0	0

[a] Adhesion scored from 0 (no adherence) to 2+ (marked adherence). ND, Not determined.

TABLE 6. *In vivo adherence of streptomycin-labeled B. melaninogenicus subsp. asaccharolyticus 381-R to oral surfaces in humans*

Sample sites	Approximate sampling area (mm^2)	Total organisms/mm^2 (ml) recovered[a]	No. of *B. melaninogenicus*/mm^2 (ml)			
			Subject 1		Subject 2	
			10 min	150 min	10 min	150 min
Dental plaque	25	1,228,000	200	468	37,200	1,044
Clean tooth	25	1,876	0	0	9	0
Tongue dorsum	350	48,143	10	1	857	1
Buccal mucosa	250	2,756	0	0	4	0
Saliva		4.9×10^8	5.0×10^5	ND[b]	5.6×10^6	3.4×10^4

[a] Recovered on nonselective blood agar plates. Figures denote the geometric means of the four samples obtained from the two subjects studied.

[b] ND, Not done.

quent colonization in periodontal pockets. *B. melaninogenicus* 381 cells suspended in PBS attached well to buccal epithelial cells obtained from children and adults and to the surfaces of certain gram-positive bacteria that are prominent in human dental plaque. *B. melaninogenicus* cells also attached, but less well, to saliva-treated HA surfaces thought to mimic teeth. However, when adherence of the organism was tested in the presence of clarified human saliva, the number of *Bacteroides* cells that attached to buccal epithelial cells and to saliva-treated HA was markedly reduced. The high-molecular-weight mucinous glycoprotein fraction of saliva, which possessed blood group-substance reactivity (6), exerted the strongest inhibitory effect, though intermediate- and low-molecular-weight salivary components also possessed adherence-inhibiting properties. However, clarified whole saliva did not affect the attachment of *B. melaninogenicus* 381 cells to the surfaces of any of the *Actinomyces* and *Streptococcus* strains studied. This indicates that certain gram-positive organisms found in dental plaque possess receptors for the attachment of *B. melaninogenicus* cells and that these receptors are different from those present on buccal epithelial cells, on erythrocytes, and on saliva-treated HA.

Observations of the attachment of a streptomycin-resistant mutant of *B. melaninogenicus* 381 to oral surfaces in vivo agreed well with these in vitro findings. *Bacteroides* cells introduced into the mouths of volunteers attached in high numbers to preexisting dental plaque, but virtually no organisms attached to clean tooth surfaces or to buccal mucosa, and only a few cells were recovered from swabbings of the tongue dorsum. Since tongue epithelial cells are known to contain much higher numbers of indigenous bacteria than those of buccal mucosa, the few *Bacteroides* cells that did become associated with the tongue might have attached to these preexisting organisms. Collectively, therefore, both the in vitro and in vivo observations imply that preexisting dental plaques containing high proportions of *Actinomyces* and *Streptococcus* species would greatly foster the initial attachment and colonization of *B. melaninogenicus* subsp. *asaccharolyticus* cells after their introduction into the mouth.

Studies of microbial changes occurring during the development of supragingival plaque support this hypothesis. Several investigators have noted that plaques developing on initially clean tooth surfaces are composed primarily of gram-positive bacteria for the first few days, and that the proportions of gram-negative organisms increase over time (19, 24, 25). Similarly, Bladen et al. (2) found that preexisting accumulations of *Actinomyces* cells on wires greatly enhanced the colonization of *Veillonella* cells. These observations and the finding that strains of *Fusobacterium*, *Capnocytophaga* (*B. ochraceus*), *Eikenella*, and "corroding" *Bacteroides* also attached well to certain *Actinomyces* and *Streptococcus* species suggest that the surfaces of gram-positive organisms may play an important role in the colonization of several gram-negative species on teeth.

Okuda and Takazoe (18) reported that certain strains of *B. melaninogenicus* possessed hemagglutinating activity that seemed to be mediated by surface pili. They suggested that these structures might also mediate the attachment of *B. melaninogenicus* cells to oral mucosa. In the present study, both reference strains of *B. melaninogenicus* subsp. *asaccharolyticus* and freshly isolated nonfermentative strains possessed strong hemagglutinating activity, but none of the fermentative strains tested, including reference cultures of subspecies *intermedius* and *melaninogenicus*, were active. *B. melaninogenicus* 536-B proved an exception. This nonfermentative strain, which has been maintained in the laboratory for several years, did not possess hemagglutinating activity; in fact, it did not attach well to any of the surfaces studied. It is possible that prolonged cultivation resulted in a loss of adherence of this strain; alternatively, this strain was not derived from the oral cavity, and it may represent a different subtype within the subspecies *asaccharolyticus*.

Partially purified pili preparations derived from *B. melaninogenicus* 381 possessed strong hemagglutinating activity, thus supporting the suggestion of Okuda and Takazoe that pili mediate this reaction. Non-hemagglutinating strains of *B. melaninogenicus* also contained pili, and these organisms attached well to buccal epithelial cells. Thus, no clear relationship exists between the hemagglutinating activity of strains of *B. melaninogenicus* and their ability to attach to buccal epithelial cells. The observations further suggest that several types of pili exist on different strains and subspecies of *B. melaninogenicus*.

It is reasonable to assume that bacteria are constantly being removed from periodontal pockets by the cleansing actions present; otherwise, even a slow rate of bacterial proliferation in subgingival sites would overwhelm the periodontium. The flow of crevicular fluid is thought to play an important role in the cleansing of gingival pockets (8). Consequently, the ability of nonmotile organisms to attach to a subgingival surface so as to resist removal probably plays an important role in the colonization of periodontal pockets. Motile organisms, on the other

hand, conceivably could resist removal by swimming against the fluid flow.

Crevicular epithelial cells, subgingival plaque, and teeth provide surfaces that are potentially available for the attachment of nonmotile organisms such as *B. melaninogenicus* in periodontal pockets. *B. melaninogenicus* 381 cells suspended in PBS were found to attach well to crevicular epithelial cells derived from periodontal pockets and weakly to serum-treated HA. Normal human serum markedly inhibited attachment to these surfaces, but it did not prevent *B. melaninogenicus* cells from attaching to the gram-positive bacteria studied. This suggests that *B. melaninogenicus* cells would also primarily colonize the surface of gram-positive bacteria in subgingival environments. Ultrastructural studies of subgingival plaque support this contention. Listgarten (11) observed that a mat of gram-positive bacteria appeared to be attached to the tooth surface and a band of gram-negative organisms was present on their surface. In deep areas, where gram-positive bacterial plaque was absent, loosely attached or unattached gram-negative forms were associated with the root surface. A relatively loose attachment also appeared to exist between gingival bacteria and the crevicular epithelium. Motile organisms and polymorphonuclear leukocytes were typically found lining the epithelium. Collectively, these observations suggest that the subgingival colonization of *B. melaninogenicus*, and perhaps of other nonmotile gram-negative forms, is dependent upon the prior presence of certain gram-positive bacteria. The predominance of gram-positive organisms in healthy gingival crevices (22) also suggests that the subgingival area is commonly colonized by gram-positive organisms before the establishment of gram-negative species.

The findings of the present investigation have some direct clinical applicability. The observation that *B. melaninogenicus* and probably certain other gram-negative organisms appear to preferentially colonize gram-positive bacteria present in dental plaque underscores the necessity of supragingival plaque removal for reducing the colonization of gram-negative bacteria. The data further point to the need to control adherent gram-positive bacteria present in both supra- and subgingival sites, for they appear to serve as attachment sites that promote colonization of *B. melaninogenicus* cells and possibly other gram-negative organisms.

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LITERATURE CITED

1. **Bailit, H. L., D. C. Baldwin, and E. E. Hunt, Jr.** 1964. Increasing prevalence of gingival *Bacteroides melaninogenicus* with age in children. Arch. Oral Biol. **9**:435–438.
2. **Bladen, H., G. Hageage, F. Pollock, and R. Harr.** 1970. Plaque formation *in vitro* on wires by gram negative oral microorganisms (*Veillonella*). Arch. Oral Biol. **15**:127–133.
3. **Brinton, C. C., Jr.** 1965. The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram-negative bacteria. Trans. N.Y. Acad. Sci. **27**:1003–1054.
4. **Buchanan, T. M.** 1975. Antigenic heterogeneity of gonococcal pili. J. Exp. Med. **141**:1470–1475.
5. **Gibbons, R. J.** 1974. Aspects of the pathogenicity and ecology of the indigenous oral flora of man. *In* A. Barlows, R. D. Dehann, V. R. Dowell, and L. B. Guze (ed.), Anaerobic bacteria: role in disease. Charles C Thomas, Publisher, Springfield, Ill.
6. **Gibbons, R. J., and V. Qureshi.** 1976. Interactions of *Streptococcus mutans* and other oral bacteria with blood group reactive substances, p. 163–181. *In* W. J. Loesche and H. M. Stiles (ed.), Microbial aspects of dental caries. Information Retrieval Inc., Washington, D.C.
7. **Gibbons, R. J., and J. van Houte.** 1971. Selective bacterial adherence to oral epithelial surfaces and its role as an ecological determinant. Infect. Immun. **3**:567–573.
8. **Gibbons, R. J., and J. van Houte.** 1975. Bacterial adherence in oral microbial ecology. Annu. Rev. Microbiol. **29**:19–44.
9. **Hay, D. I., R. J. Gibbons, and D. M. Spinell.** 1971. Characteristics of some high molecular weight constituents with bacterial aggregating activity from whole saliva and dental plaque. Caries Res. **5**:111–123.
10. **Kelstrup, J.** 1966. The incidence of *Bacteroides melaninogenicus* in human gingival sulci, and its prevalence in the oral cavity at different ages. Periodontics **4**:14–18.
11. **Listgarten, M. A.** 1976. Structure of the microbial flora associated with periodontal health and disease in man. A light and electron microscopic study. J. Periodontol. **47**:1–18.
12. **Loesche, W. J.** 1968. Importance of nutrition in gingival crevice microbial ecology. Periodontics **6**:245–249.
13. **Loesche, W. J., and R. J. Gibbons.** 1966. Influence of nutrition on the ecology and cariogenicity of the oral microflora, p. 305–317. *In* A. E. Nizel (ed.), The science of nutrition and its application in clinical dentistry. W. B. Saunders Co., Philadelphia.
14. **Macdonald, J. B., S. S. Socransky, and R. J. Gibbons.** 1963. Aspects of the pathogenesis of mixed anaerobic infections of mucous membranes. J. Dent. Res. **42**:529–544.
15. **Manganiello, A. D., S. S. Socransky, C. Smith, D. Propas, V. Oram, and I. L. Dogan.** 1977. Attempts to increase viable count recovery of human supra-gingival dental plaque. J. Periodontal Res. **12**:107–119.
16. **Newman, M. G., and S. S. Socransky.** 1977. Predominant cultivable microbiota in periodontosis. J. Periodontal Res. **12**:120–128.
17. **Newman, M. G., S. S. Socransky, E. D. Savitt, D. A. Propas, and A. Crawford.** 1976. Studies of the microbiology of periodontosis. J. Periodontol. **47**:373–379.
18. **Okuda, K., and I. Takazoe.** 1974. Hemagglutinating activity of *Bacteroides melaninogenicus*. Arch. Oral Biol. **19**:415–416.
19. **Ritz, H. L.** 1967. Microbial population shifts in developing human dental plaque. Arch. Oral Biol. **12**:1561–1568.
20. **Slots, J.** 1976. The predominant cultivable organisms in juvenile periodontitis. Scand. J. Dent. Res. **84**:1–10.
21. **Slots, J.** 1977. The predominant cultivable microflora of advanced periodontitis. Scand. J. Dent. Res. **85**:114–121.

22. **Slots, J.** 1977. Microflora in the healthy gingival sulcus in man. Scand. J. Dent. Res. **85**:247-254.
23. **Socransky, S. S., and R. J. Gibbons.** 1965. Required role of *Bacteroides melaninogenicus* in mixed anaerobic infections. J. Infect. Dis. **115**:247-253.
24. **Socransky, S. S., A. D. Manganiello, D. Propas, V. Oram, and J. van Houte.** 1977. Bacteriological studies of developing supragingival dental plaque. J. Periodontal Res. **12**:90-106.
25. **Theilade, E., W. H. Wright, S. B. Jensen, and H. Löe.** 1966. Experimental gingivitis in man. II. A longitudinal clinical and bacteriological investigation. J. Periodontal Res. **1**:1-13.
26. **van Houte, J., R. J. Gibbons, and A. J. Pulkkinen.** 1971. Adherence as an ecological determinant for streptococci in the human mouth. Arch. Oral Biol. **16**:1131-1142.