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Immunochemical Differences Between Oral and Nonoral Strains of *Bacteroides asaccharolyticus*

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We undertook a morphological and immunochemical comparison of purified outer membrane antigens from oral and nonoral isolates of *Bacteroides asaccharolyticus*. Electron micrographs of thin sections of whole bacteria revealed a compact, electron-dense capsule external to the outer membrane of oral strains. A loose, web-like material was noted on the surface of several nonoral strains that was distinct from the dense capsule seen on oral strains. Polyacrylamide gel electrophoresis showed distinct differences in the protein band pattern between oral and nonoral isolates; sugar composition was similar with a few exceptions. An indirect fluorescent-antibody test utilizing antiserum to a purified capsular antigen from a single oral strain cross-reacted with all of numerous oral and nonoral strains of *B. asaccharolyticus*, thereby demonstrating a shared antigen that is species specific for *B. asaccharolyticus*. However, antibodies to an oral strain-derived capsular antigen were detectable by enzyme immunoassay only in serum from rabbits immunized with oral strains. Thus, definite morphological and immunochemical differences were found between oral and nonoral isolates of *B. asaccharolyticus*.

The role of various microbes in the pathogenesis of periodontal disease has been the subject of intensive investigation in recent years. Despite the complexity of the gingival microflora, several pathogens have been implicated as major offenders in the destruction of supporting soft tissues and alveolar bone which characterizes this common disease process. These pathological changes are ultimately responsible for periodontal disease being the major cause of tooth loss in adults. *Bacteroides asaccharolyticus*, formerly *Bacteroides melaninogenicus* subspecies *asaccharolyticus*, has emerged as one of the most significant periodontal pathogens in a subset of periodontal disease known as rapidly advancing adult periodontitis (16). The extent of its association with other forms of periodontitis is not yet completely defined.

B. asaccharolyticus is a component of the indigenous mouth flora and the vaginal flora, but is not considered part of the bowel flora (3), a finding which may be partly attributable to its bile sensitivity (18). Despite this fact, *B. asaccharolyticus* has been isolated from fecal flora on occasion and has been associated with infections that do not appear to be caused by microbes from the oral cavity, such as perirectal abscess and diabetic foot ulcers (9).

Some preliminary data exist which suggest that strains of *B. asaccharolyticus* isolated from the oral cavity are immunochemically different from those strains which apparently colonize ecological niches of the body other than the mouth, specifically the lower gastrointestinal tract. This report is directed toward a further understanding of this unique phenomenon.

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MATERIALS AND METHODS

Bacterial strains. Oral strains of *B. asaccharolyticus* taken from our collection were obtained originally from A. Tanner (Forsyth Dental Laboratory, Boston, Mass.). Other strains of *B. asaccharolyticus* were graciously provided by Rial Rolfe (Wadsworth Veterans Administration Hospital, Los Angeles, Calif.) and Lillian Holdeman (Virginia Polytechnic Institute and State University, Blacksburg, Va.) and were derived from both oral and nonoral sites. All other bacterial strains were taken from our collection.

Media and growth conditions. Bacteria were stored frozen at −70°C in peptone-yeast broth (Scott Laboratories, Fiskeville, R.I.) and were grown on Todd-Hewitt base agar and 5% sheep blood, supplemented with hemin (5 μg/ml) and menadione (0.5 μg/ml) in an anaerobic jar (GasPak, BBL Microbiology Systems, Cockeysville, Md.) at 37°C before use. Bacteria were grown in Trypticase (BBL Microbiology Systems) yeast extract basal medium in 6-liter broth cultures for antigen purification (11).

Isolation of outer membrane antigens. Pelleted organisms from 6-liter broth cultures grown for 24 to 48 h were suspended in a buffer composed of 0.05 M sodium phosphate, 0.15 M NaCl, and 0.001 M ethyl-

enediaminetetraacetic acid and heated for 30 min in a 60°C water bath. The suspension was then sheared manually through a 25-gauge needle. The outer membranes of the bacteria were isolated by differential centrifugation as described previously (11). Homogeneity of the outer membrane preparation was shown previously by electron microscopy and by banding on a sucrose density gradient (11). The purified outer membrane material was lyophilized and stored at −70°C before use in immunochemical studies. For some experiments the outer membrane was suspended in a buffer containing 0.05 M glycine, 0.001 M ethylenediaminetetraacetic acid, and 0.5% sodium deoxycholate, a lipopolysaccharide disaggregating detergent, and the pH was adjusted to 9.5. The material was then chromatographed on a column (1.6 by 82 cm) of Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated in the same buffer; two major antigenic fractions were collected, as detailed previously (11). The first fraction eluted at the void volume (V_0) and was purified further by enzyme digestion with pronase (Calbiochem, San Diego, Calif.); this material was then chromatographed on a column of Sepharose 4B (Pharmacia) equilibrated with 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 7.3. The V_0 material was concentrated, precipitated with cold 80% ethanol, and stored in a lyophilized state until used as described previously (11). This material is considered to be a capsular antigen, as detailed elsewhere (11). The second antigen fraction from the Sephadex G-100 column was purified and characterized in a separate study (12) and was not used in the present studies.

Chemical studies. Protein was determined by the Folin-phenol method (10) using bovine serum albumin as a standard. Total sugars were measured by the phenol sulfuric acid method (1) with glucose as a standard. The method of Karkhanis et al. (6) was used for the determination of 2-keto-3-deoxyoctonate (KDO) with purified KDO (Sigma Chemical Co., St. Louis, Mo.) as the standard. Uronic acids were determined by the Dische carbazole method (5) with glucuronic acid as a standard. Nucleic acid content was estimated by the ratio of ultraviolet light absorbance at 260 nm compared to that at 280 nm (8).

Carbohydrate analysis by GLC. Sugars of the various antigens of *B. asaccharolyticus* were determined by gas-liquid chromatography (GLC) of their alditol acetates, prepared by the method of Griggs et al. with minor modification (4). Xylose was used as an internal standard. The samples were analyzed on an Antek 300 gas chromatograph (Antek Instruments, Inc., Houston, Tex.) fitted with a (6 feet by ⅛ inch [ca. 183 cm by 29 mm]) stainless-steel column and packed with 3% OV-225 on Supelcoport 80/100 mesh (Supelco, Bellefonte, Pa.). The temperature was programmed between 160 and 240°C at a rate of 2°C/min. The injector temperature was 250°C, and the flame ionization detector temperature was 275°C. Quantitation was achieved with a Minigrator (Spectra-Physics, Santa Clara, Calif.) connected to the chromatograph. The chromatograph pattern was recorded on a Rikadenki multipen recorder (Soltec Corp., Encino, Calif.). Cochromatography was performed with the alditol acetates of standard sugars prepared similarly to the unknown samples for positive identification.

PAGE. Continuous polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was performed on the purified outer membranes of various strains of *B. asaccharolyticus* by the method of Fairbanks et al. (2). Samples containing 50 μg of protein (20 μl) were suspended in 25 μl of a buffer containing 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8), 1 mM ethylenediaminetetraacetic acid, 7.5% sucrose, and 4% SDS. Bromophenol blue (0.05% [5 μl]) and 1 μl of 2-mercaptoethanol were added. The samples were heated at 50°C for 20 min in a water bath; a standard solution of proteins (Bio-Rad Laboratories, Richmond, Calif.) was treated similarly. The samples were added to gels containing 5.6% acrylamide and were electrophoresed at 5 mA/gel for 1.5 to 2 h in a tris(hydroxymethyl)aminomethane-acetate buffer (pH 7.4) containing 1% SDS. The gels were stained with Coomassie brilliant blue by the method described by Fairbanks et al. (2).

Serological methods. Rabbit antisera were prepared as described previously (11) by using 1 ml of saline suspension of ~5×10^8 viable bacteria as the immunogen. All sera had been obtained within the past 1 to 2 years, including rabbit antiserum to a specific capsular antigen (11), and were stored frozen at −70°C.

An enzyme-linked immunosorbent assay (ELISA), as described elsewhere (13), was used to detect rabbit antibodies (immunoglobulin G [IgG]) directed against the purified capsular antigen of a single strain of *B. asaccharolyticus* derived from an oral source. Briefly, 500 ng of purified capsular antigen (strain 381), containing glucose, galactose, and mannose and ~10% nonenzyme digestible protein, suspended in 0.05 M carbonate buffer (pH 9.5), was adsorbed to polystyrene tubes (Falcon Plastics, Los Angeles, Calif.) at 37°C for 3 h. The tubes were washed thoroughly with 0.9% NaCl–0.05% Tween-20 (ST). Rabbit antisera to various strains of *B. asaccharolyticus* (oral and nonoral isolates), *B. melaninogenicus* subspecies *intermedius*, *B. melaninogenicus* subspecies *melaninogenicus*, and various strains from the *B. fragilis* group, as well as nonimmune sera, were added to the tubes at 1:100 dilution in phosphate-buffered saline in 0.05% Tween-20, pH 7 (PBS-T). The tubes were incubated for 5 h at room temperature, and the tubes were again rinsed thoroughly with ST. Alkaline phosphatase conjugated to goat anti-rabbit IgG was added to the tubes at a 1:100 dilution in PBS-T and incubated overnight at room temperature. The tubes were rinsed again with ST; and *p*-nitrophenylphosphate, an alkaline phosphatase substrate, 1 mg/ml in 0.05 M carbonate buffer (pH 9.5) containing 1 mM $MgCl_2$, was added. After incubation for 60 min, the enzyme reaction was stopped with 1 N NaOH, and the optical density (OD) of the solution was measured at 400 nm in a spectrophotometer.

An indirect fluorescent-antibody (IFA) test was performed by using rabbit antiserum to a purified capsular antigen from a single strain of *B. asaccharolyticus*. The technique has been detailed elsewhere (14). Overnight broth cultures of *B. asaccharolyticus* from oral and nonoral sources, as well as several strains of other bacterial species, were tested. Anti-capsular antiserum

to a single oral strain of *B. asaccharolyticus* was incubated with each bacterial strain in a 1:100 dilution. In separate experiments, fluorescein-conjugated goat antiserum to rabbit globulin, IgG, or IgM was then added to the bacteria-antiserum complex, and the degree of fluorescence was recorded by use of a Zeiss fluorescence microscope (Carl Zeiss, New York, N.Y.). Fluorescence was graded from 0 to 4+ by two observers. Nonimmune rabbit serum was incubated with each bacterial strain for comparison.

Electron microscopy. Suspensions of whole bacteria in broth were prefixed for 2 h at 4°C by the addition of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) to a final concentration of 0.5%, and 0.05% $CaCl_2$ was added according to the method of Kasper (7). After centrifugation for 10 min at 12,000 × *g*, the pelleted cells were embedded in 2% Ionagar (Oxoid) in 0.1 M cacodylate buffer (pH 7.2), cut into 1- to 2-cm squares, and fixed for 2 h at 4°C in 2.5% glutaraldehyde in the same buffer. After washing the blocks, we fixed them overnight at 4°C in buffered 2% OsO_4 and washed, dehydrated, and embedded them in Spurr low-viscosity embedding medium (17). Thin sections were stained with uranyl acetate and lead citrate (15) and examined with a JEM 100-CX electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan).

RESULTS

Electron microscopy. The morphology of six strains of *B. asaccharolyticus* isolated from nonoral sources was compared to that of strains from an oral source. The ultrastructure is shown in Fig. 1. The oral isolates demonstrated an electron-dense layer external to the outer membrane when fixed with glutaraldehyde and stained with uranyl acetate (Fig. 1A and B). This finding has been demonstrated previously (19); this electron-dense layer, or capsule, has also been shown by ruthenium red staining (11) and ferritin labeling (13). In contrast, the nonoral strains of *B. asaccharolyticus* prepared similarly demonstrated a much finer, less compact external layer (Fig. 1C and D). A similar appearance of a single nonoral strain has been demonstrated in an earlier study by ruthenium red staining (11). These surface characteristics were noted uniformly among organisms as shown by low-power magnification (Fig. 2A and B).

Chemical studies. The outer membrane of several strains of *B. asaccharolyticus* derived from nonoral sources was isolated as described previously (11). Chemical studies of these preparations were compared to purified outer membranes from oral isolates of *B. asaccharolyticus*. The results are summarized in Table 1. Certain differences in chemical composition were noted among the strains tested. The percentage of total protein varied between 34 and 60%. Total sugars ranged between 9 and 34% of the dry weight. Uronic acid was measurable in outer membrane preparations from two of three oral isolates of *B. asaccharolyticus*. None was detected in the outer membranes of three nonoral strains. KDO was not detectable in any of the outer membrane preparations, confirming previous observations (11). No nucleic acids were detected in any of the outer membranes.

GLC. The carbohydrate composition of several outer membrane preparations of *B. asaccharolyticus* was examined by GLC. The sugars were measured as their alditol acetates and compared to similarly derived standard sugars. Results are summarized in Table 2. Among the oral isolates of *B. asaccharolyticus*, glucose and galactose were found to be the predominant neutral sugars, with lesser amounts of rhamnose, glucosamine, and galactosamine (in some strains). These data corroborate previous GLC studies with trimethylsilyl derivatives (11). One major difference was noted in the sugar composition of the outer membranes of nonoral strains of *B. asaccharolyticus*. Mannose was detected in trace amounts in one of four outer membrane preparations from nonoral isolates; it was absent in the other three. Mannose was uniformly present in the outer membranes from oral strains. The remainder of the sugar composition was variable, but generally similar to that of the oral isolates. Two unknown peaks were uniformly detected, as shown in a typical GLC profile (Fig. 3).

SDS-PAGE. The outer membrane proteins of several oral and nonoral strains of *B. asaccharolyticus* were analyzed by SDS-PAGE. Results are shown in Fig. 4. The six oral strains had remarkably similar peptide band patterns (Fig. 4, 01 to 06). Four major bands in variable concentrations were noted in all six strains. The molecular weights of these four major peptide bands, as compared to standards (Fig. 4, a to e), were measured as 117,000, 107,000, 62,000, and 53,000. Among three nonoral strains of *B. asaccharolyticus*, four major peptide bands and several minor bands were identified in the outer membrane (Fig. 4, N1 to N3). None of the band patterns was shared with those of the oral strains; several similar bands were noted between strains of nonoral origin, but no uniform pattern was noted. The molecular weights of the four major peptide bands were 130,000, 102,000, 68,000 and 27,000.

IFA. An IFA test was applied by using rabbit antiserum to the purified capsular antigen from a single strain of *B. asaccharolyticus* from an oral source (14). It was shown previously that all of twenty-three strains of *B. asaccharolyticus* cross-reacted and that the IFA was species specific. Only 1 of the 23 isolates tested originally was derived from a nonoral source. In the pres-

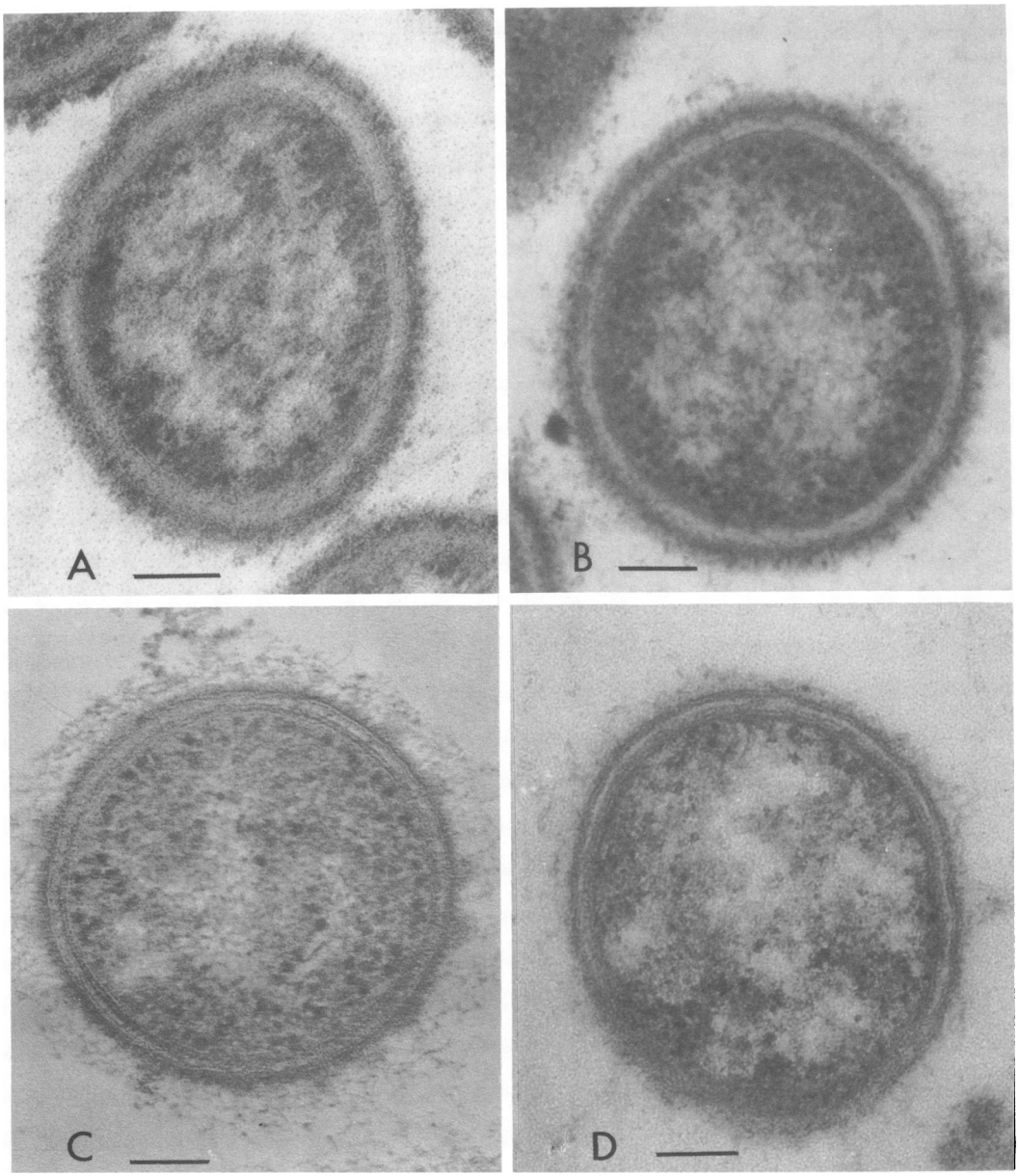

FIG. 1. *Electron micrographs of thin sections of B. asaccharolyticus. Bars, 0.1 μm. Oral strains (A) 381 and (B) BMD-1 have well-developed capsules; nonoral strains (C) 536 and (D) 1047 have a loose, web-like extracellular material rather than a discrete capsule.*

ent study 11 nonoral strains of *B. asaccharolyticus* were tested in the same IFA assay. Table 3 lists the source of each strain and the IFA result. All 11 strains were positive in the IFA (3 to 4+), thus demonstrating that a surface antigen exists in both oral and nonoral strains of *B. asaccharolyticus* which cross-reacts with rabbit antiserum to a purified capsular antigen from an oral isolate of *B. asaccharolyticus*. These findings were noted with antiglobulin and anti-IgG and less so with an anti-IgM fluorescein conjugate.

ELISA. An ELISA was developed as described previously (13). The assay was devised to detect the presence of antibody (IgG) in rabbit serum that reacts with a purified capsular antigen from a single oral strain of *B. asaccharolyticus*. Rabbit antisera to five strains of *B. asaccharolyticus* derived from nonoral sources were

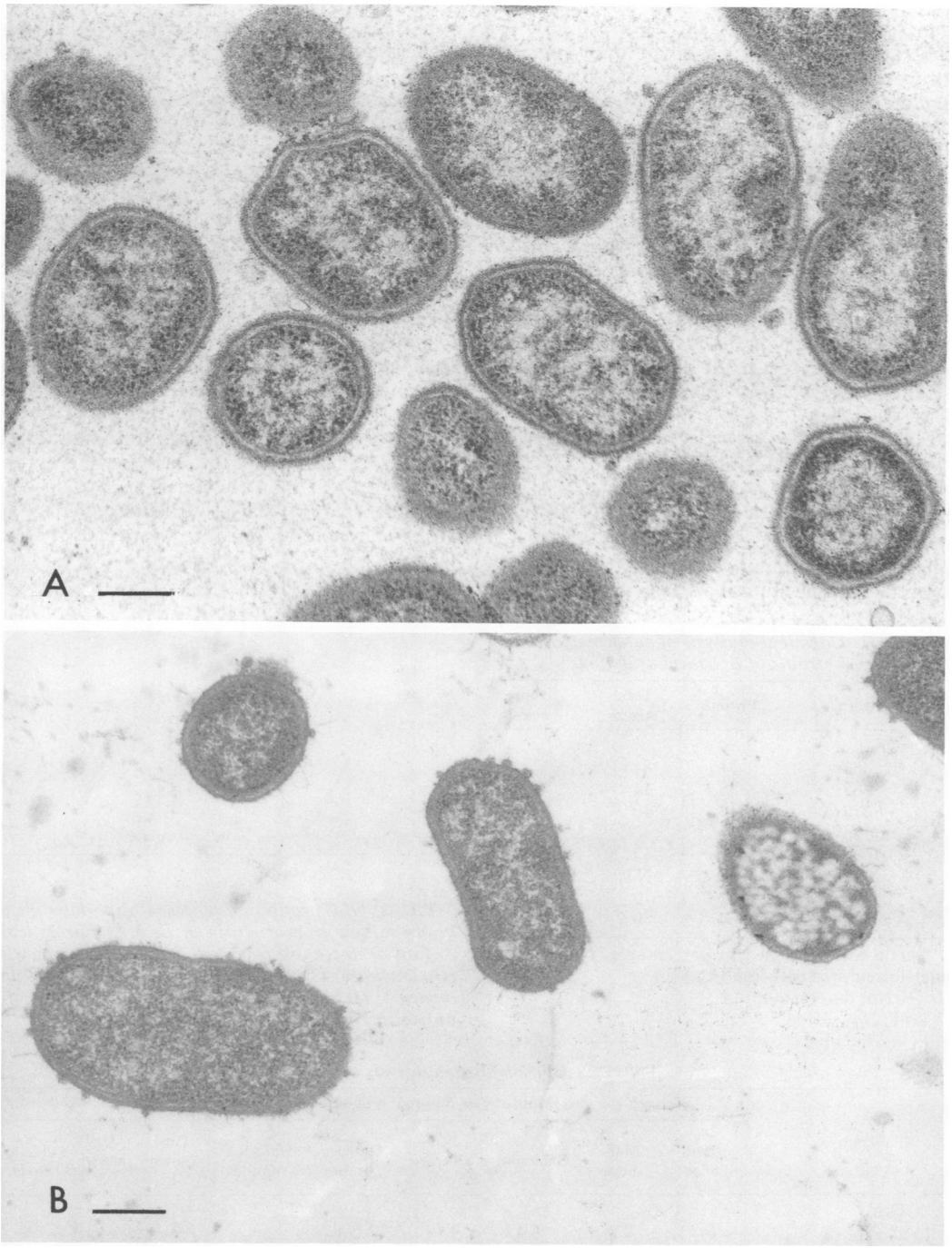

FIG. 2. *Thin sections of B. asaccharolyticus. Bars, 0.2 μm. (A) Oral strain BMD-2 has a prominent capsule, whereas (B) nonoral strain 11130 has no capsular structure on any of the cells.*

tested, along with antisera to three strains of *B. melaninogenicus* subspecies *intermedius*, one strain of *B. melaninogenicus* subspecies *melaninogenicus*, three strains of *B. asaccharolyticus* from an oral source, and two strains of *B. fragilis*. The results are displayed in Table 4. As was previously shown, the sera from strains of *B. asaccharolyticus* (all from an oral source)

had significantly higher IgG titers specific for the capsular antigen of a single orally derived strain than rabbit sera to strains of other species as determined by OD. The range of OD varied between 3.0 and 7.0 for sera from oral strains of *B. asaccharolyticus*. Serum from one of the nonoral *B. asaccharolyticus* strains fell within that range (5.4). The remaining four sera from nonoral strains of *B. asaccharolyticus* had ODs between 0.07 and 0.31, which were not significantly higher than the ODs of sera from heterologous species of *Bacteroides*. Thus, only a small amount of IgG specific for the capsular antigen of an oral strain of *B. asaccharolyticus* was detected in sera made to four of five nonoral strains of *B. asaccharolyticus* by means of the ELISA.

DISCUSSION

Despite classification into the same species by biochemical tests, it is apparent that oral and nonoral strains of *B. asaccharolyticus* have morphological and immunological differences. Electron microscope studies disclose the presence of a dense, extracellular capsule on the surface of oral isolates of *B. asaccharolyticus*, a finding that has been shown in other studies (13, 19). In contradistinction, the surface of isolates of *B. asaccharolyticus* derived from fecal flora has a loose, web-like layer external to the outer membrane.

Antigenic differences between oral and nonoral strains of *B. asaccharolyticus* also exist. Antigenic cross-reactivity among oral strains of *B. asaccharolyticus* based on a shared capsular antigen (14) has been described previously. An IFA test was used to study the immunological relationship of oral and nonoral isolates of *B. asaccharolyticus*. By using rabbit antiserum to the purified capsular antigen of a single oral strain of *B. asaccharolyticus*, we have shown that all oral strains of *B. asaccharolyticus* tested (and one nonoral strain) are strongly cross-reactive (13) and that the test is species specific for *B. asaccharolyticus*. In the present study all of the 11 nonoral isolates of *B. asaccharolyticus*

TABLE 1. *Chemical analysis of outer membranes from strains of B. asaccharolyticus*[a]

Strain	Protein	Total sugars	Uronic acid
Oral			
381	34	34	7
382	58	14	6
376	45	10	–[b]
Nonoral			
1047	37	13	–
2031	36	NT[c]	–
975	52	10	–
536	60	9.5	–

[a] Data are expressed in percentages. No KDO or nucleic acid was detected (<0.5%).
[b] –, Not detectable.
[c] NT, Not tested.

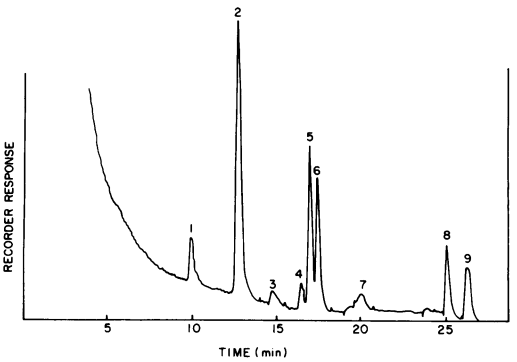

FIG. 3. *GLC profile of a representative outer membrane sample from a strain of B. asaccharolyticus. Alditol acetates are from the following compounds: (1) rhamnose; (2) xylose (internal standard); (3) unknown I; (4) mannose; (5) galactose; (6) glucose; (7) unknown II; (8) glucosamine; (9) galactosamine.*

TABLE 2. *Carbohydrate analysis by GLC*[a]

Strain	Quantity (μg/mg) found in outer membrane prepn								
	Rhamnose	Mannose	Galactose	Glucose	Glucosamine	Galactosamine	Unknown 1	Unknown 2	Heptose
Oral									
381	3.2	2.2	6.4	44.4	29.8	–	+	+	–
376	5.3	2.3	15.7	13.3	28.1	11.4	+	+	–
BMD-1	6.9	4.0	4.0	1.7	39.9	14.9	+	+	–
Nonoral									
3754	7.9	3.2	32.4	22.8	11.0	8.1	+	+	–
975	2.5	–	20.9	7.7	1.4	–	+	+	–
1047	–	–	13	18	±	±	+	+	–
536	–	–	±	8	25	–	+	+	–

[a] –, Not detectable; ±, trace amount detected; +, detectable, but unable to quantitate.

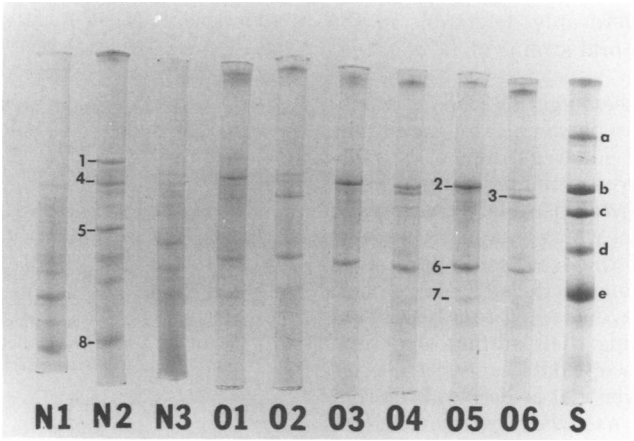

FIG. 4. *SDS-PAGE of outer membranes from oral strains (01-06) and nonoral strains (N1-N3) of B. asaccharolyticus. A standard mixture of proteins (S) is included to calculate molecular weights of unknown peptide bands. Gels are stained with Coomassie brilliant blue. Molecular weights are: (a) myosin, 200,000; (b) β-galactosidase, 130,000; (c) phosphorylase b, 94,000; (d) BSA, 68,000; (e) ovalbumin, 45,000; (1) 130,000; (2) 117,000; (3) 107,000; (4) 102,000; (5) 68,000; (6) 62,000; (7) 53,000; (8) 27,000.*

TABLE 3. *Results of IFA test of isolates of B. asaccharolyticus*

Strain	Source	Result
381	Mouth	4+[a]
2726	Foot (osteomyelitis)	4+
2729	Toe wound	3+
3754	Abdominal wound	4+
4157	Rectal abscess	3+
4165	Transtracheal aspirate	3+
536	Fecal flora	4+
975	Toe exudate	3-4+
2031	Fecal flora	3-4+
1047	Foot abscess	2-3+
11365G	Bacterial vaginitis	4+
10177	Scrotal abscess	4+

[a] Positive IFA results were recorded as 3+ or 4+; negative titers were 0, 1+, or 2+.

TABLE 4. *Results of an ELISA measuring anti-capsular IgG to a strain of B. asaccharolyticus*

Antiserum to strain	OD
B. asaccharolyticus	
381[a]	7.0
BMD-1	3.5
BMD-2	3.0
3754(N)[b]	5.4
536(N)	0.33
2031(N)	0.31
1047(N)	0.27
975(N)	0.07
B. melaninogenicus subsp. intermedius	
576	0.19
BLI-37	0.09
581	0.04
B. melaninogenicus subsp. melaninogenicus	
379	0.21
B. fragilis	
8503	0.13
2429	0.12

[a] Strain 381 is the homologous strain.

[b] (N), Nonoral source. Remainder of unlabeled *B. asaccharolyticus* sera is from oral strains.

reacted strongly in the same IFA test, confirming the existence of a surface antigen that is cross-reactive among strains of *B. asaccharolyticus* derived from both oral and nonoral sources. In an attempt to identify further the immunological relationship between oral and nonoral strains of *B. asaccharolyticus*, a sensitive immunoassay, ELISA, was employed. Rabbit antisera to four out of five nonoral strains were shown to have very little antibody (IgG) directed against a capsular antigen from a single oral strain. In contrast, all 14 antisera to oral strains of *B. asaccharolyticus* had significant amounts of anti-capsular IgG as demonstrated in a previous study (B. J. Mansheim, D. L. Kasper, J. Infect. Dis., in press).

Chemical analysis of outer membrane preparations also revealed differences between oral and nonoral isolates of *B. asaccharolyticus*. Most striking were the peptide band patterns seen on SDS-polyacrylamide gels. Whereas the outer membranes from oral isolates had remarkably similar band patterns, the nonoral isolates had completely different strain-specific patterns. The chemical composition was found to be strain variable; of particular interest is the finding that mannose, with one exception in a nonoral strain,

and uronic acids were only detectable in the outer membrane of oral strains of *B. asaccharolyticus*.

Reasons for the seemingly discrepant immunological relationship between outer membrane antigens of oral and nonoral strains of *B. asaccharolyticus* are currently limited to hypothesis. That *B. asaccharolyticus* is widely known to be bile sensitive is a fact which should preclude its survival as part of the fecal microflora. It is possible that the nonoral (fecal) isolates of *B. asaccharolyticus* have adapted to a hostile environment by altering their surface structure. The electron microscope data suggest the presence of a surface layer that is distinct from that of oral isolates of *B. asaccharolyticus*. The IFA test shows the presence of cross-reactive antigen common to both oral and nonoral strains. It is quite possible that rabbits immunized with nonoral strains produce antibodies mainly to the most external surface antigens and less so to deeper antigens. Thus, when tested by ELISA, this antiserum would show only trace amounts of antibody specific to the capsular antigen which is present in the nonoral strains but may be "hidden" beneath the surface. This hypothesis could explain the low levels of antibody that we found by ELISA.

Immunochemical dissimilarity between strains of the same species of bacteria is far from unusual and often provides the basis for serogroups and serotypes. We have shown the presence of a surface antigen that is common to all strains of *B. asaccharolyticus*, whether from oral or nonoral sources. However, we have also observed that distinct immunochemical differences exist between oral and nonoral isolates. This finding of dissimilarity within a bacterial species based completely on difference in ecological habitat is unique.

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