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Characterization of Tufted Streptococci Isolated from the "Corn Cob" Configuration of Human Dental Plaque

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Streptococci isolated from "corn cob" configurations of human dental plaque possess a polar fibrillar tuft extending 100 to 150 nm from one pole of the cell. The two strains studied were physiologically related to the *Streptococcus sanguis-Streptococcus mitior* group and were most similar to *Streptococcus mitis* ATCC 903. The corn cob streptococci were serologically related to *S. sanguis* serotype 1. The polar tuft contained at least two antigenically distinct components, one serologically related to the glycerol phosphate backbone of teichoic acid. The other was an electrophoretically slow-moving antigen similar to a component of *S. mitis* ATCC 903. It is suggested that the corn cob streptococci in vivo adhere to *Bacterionema matruchotii* by means of the polar tuft.

Cell-to-cell binding is a major determinant of bacterial accumulation on oral surfaces (27). The "corn cob" configuration (CCC) of human dental plaque, which consists of coccoid organisms epiphytic on a filamentous bacterium, is a readily identifiable example of an in vivo bacterial adhesive interaction. Since the mechanisms of such interactions are still largely unknown, CCC provides a potentially useful model for in vitro studies of interbacterial adhesion. The identification of the organisms comprising CCC is a prerequisite for such studies.

The filamentous component of CCC has been identified previously by immunofluorescent procedures as *Bacterionema matruchotii* (C. Mouton, R. J. Nisengard, P. A. Mashimo, R. T. Evans, and R. J. Genco, J. Dent. Res. **56B:** abstr. 286, B123, 1977). Also, two coccal strains designated CC5A and CC6 have been isolated from CCC (20). The coccal isolates were obtained from human dental plaque by micromanipulation and culture methods. Their presence as components of CCC was verified by using immunofluorescence techniques. The present study characterizes the physiological and serological properties of CCC coccal isolates and describes a novel "tuft" localized to one pole of these cocci.

MATERIALS AND METHODS

Strains. Strains CC5A and CC6 were isolated by micromanipulation from two different CCC of human dental plaque samples collected on the lingual aspect of the lower left first molar from a single subject (20). The two coccal strains were grown in pure culture, and samples were stored at −196°C in liquid nitrogen and by lyophilization. Subcultivation took place aerobically at 37°C in Todd-Hewitt broth (THB), on Trypticase soy agar (BBL Microbiology Systems) supplemented with 5% defibrinated sheep blood (SBA), or on mitis salivarius agar (MSA). Stock strains of microorganisms used as controls included the following: *Streptococcus mutans* 6715, *Streptococcus mitis* ATCC 903, and *Streptococcus sanguis* ATCC 10556, ATCC 10557, and M-5 (the latter obtained from B. Rosan, Center for Oral Health Research, University of Pennsylvania, Philadelphia).

Cellular and colony morphology. Cell morphology was determined from Gram-stained smears and from wet mounts by phase-contrast microscopy. Colony morphology and hemolysis were recorded on SBA after 48 h of incubation both aerobically and in an atmosphere of 95% N_2–5% CO_2. Colony morphology on MSA was recorded after a 3-day aerobic incubation at 37°C.

Sensitivity testing. Optochin and bacitracin sensitivity tests were carried out by applying disks (Taxo P and Taxo A; BBL Microbiology Systems) to SBA, with a 30-μg neomycin disk in the center.

Oxidase and catalase activities. Oxidase activity was determined on 24-h cultures in brain heart infusion by the method of Gaby and Hadley (11). The catalase test was performed on smears by flooding bacterial specimens with 3% hydrogen peroxide.

Production of extracellular polysaccharide from sucrose and glucose. The production of dextran and levan precipitates was tested by adding 1, 2, and 2.5 volumes of absolute ethanol to 1-ml samples of clear culture supernatant obtained from cultures grown for 48 h in phenol red broth base supplemented with 1, 2.5, and 5% glucose or sucrose. Inverted Durham tubes were included to test for gas production.

Iodophilic intracellular polysaccharide. The sedimented cells of cultures grown in phenol red broth base containing 2.5% glucose or sucrose were flooded with dilute Lugol iodine solution (14). Color was recorded microscopically after 30 min; cells staining light brown indicated a weak production of intracellular glycogen-amylopectin-type polysaccharide.

Arginine hydrolysis. The method of Niven et al. (11) was used to test for the production of ammonia from L-arginine with Nessler reagent; cultures were incubated for 3 days.

Esculin hydrolysis. The medium described by Carlsson (5) was used for testing esculin hydrolysis. The esculin tubes were examined daily for up to 7 days for blackening of the broth.

Starch hydolysis. The method of Carlsson (4), using starch broth, was employed for testing starch hydrolysis. After 2, 4, 6, and 7 days of incubation, 1 drop of culture was mixed with 1 drop of iodine solution, and the resulting color was recorded. A blue color indicated no hydrolysis; a purple-to-brown color indicated partial hydrolysis; no color indicated complete starch hydrolysis.

Acetoin production from glucose. The organisms were grown for 2 days at 37°C in 1% peptone broth containing 0.5% glucose. The test for acetoin production from glucose was performed by the method of Barrit (11).

Benzidine test. The test for the detection of cytochrome oxidase was performed after 48 h of incubation on Trypticase soy agar by the method of Deibel and Evans (12).

Hydrogen peroxide production. Heated blood-benzidine plates were made by the method of Whittenbury (29), using defibrinated sheep blood and nutrient agar (BBL Microbiology Systems). After 48 h of incubation in an atmosphere of 95% N_2–5% CO_2 at 37°C, the production of H_2O_2 was indicated by the medium around and under the colonies becoming dark brown.

Sulfide production. Tubes containing SIM (BBL Microbiology Systems) medium were stabbed in the center and incubated for up to 7 days; they were checked daily for blackening of the medium, indicating sulfide reaction.

Growth at 45°C. Tubes of THB were inoculated and incubated for 7 days in a thermostatically controlled incubator; the tubes were checked daily for turbidity.

Tolerance to potassium tellurite. A 10% filter-sterilized aqueous solution of potassium tellurite was added to THB to give final concentrations of 0.1 and 0.04%. The tubes were incubated at 37°C and checked daily for turbidity.

Tolerance to methylene blue. A filter-sterilized 10% aqueous solution of methylene blue was added to sterile skim milk to give final concentrations of 0.1 and 0.01%. Reduction of the dye and curdling of the milk were recorded after 24 and 48 h of incubation at 37°C.

Tolerance to NaCl. NaCl was added to Trypticase soy broth (BBL Microbiology Systems) to give final concentrations of 4 and 6.5%. The tubes incubated at 37°C were checked daily for up to 7 days for turbidity.

Growth at pH 9.6. A glucose-phenolphtalein broth, pH 9.6, prepared by the method of Cowan and Steel (11), was inoculated and incubated at 37°C. Tubes were checked after 24 and 48 h for turbidity and decolorization.

Bile tolerance. Oxgall was added to Trypticase soy agar supplemented with 5% defibrinated sheep blood to give final concentrations of 7.5, 10, and 40%. Positive growth on plates incubated for 48 h at 37°C was checked by phase-contrast microscopy, and, when growth occurred, hemolysis was recorded.

Tolerance to sulfisomidine. To MSA was added 0.1% sulfisomidine (United States Biochemical Corp., Cleveland, Ohio) to obtain a medium similar to the MC agar of Carlsson (6) used by Mejàre and Edwardsson (19). Growth and colony size were recorded.

Acid production from carbohydrates. Phenol red broth base was used as a base medium to which 10% solutions of carbohydrates were added to give a final concentration of 1% (0.2% in the case of esculin and starch). The substrates were autoclaved with the medium, except for esculin, salicin, and inulin, which were filter sterilized. The fermentation tubes and non-inoculated controls were incubated for 3 days at 37°C. A decrease in pH equal to or more than one unit indicated acid production.

Antisera. Antisera were produced by intravenous injection of rabbits with washed whole cells of strains CC5A, CC6, and *S. sanguis* M-5, as described previously (20).

An antiserum specific for the polyglycerol phosphate (PGP) of lipoteichoic acids (LTA) produced against *Lactobacillus casei* L324M (22–24) and an antiserum to *S. mitis* ATCC 903 were generous gifts of B. Rosan. The specificity of antiserum to *L. casei* L324M was assessed by showing that it reacted by agar gel double diffusion with a reference LTA extracted from *Lactobacillus fermenti* NCTC 6991 (obtained from A. J. Wicken, School of Microbiology, University of New South Wales, Kensington, Australia). To confirm the specificity of antiserum to *L. casei* L324M for the PGP backbone of teichoic acids, we prepared glycerol-phosphoryl-glycerol-phosphoryl-glycerol (G_3P_2) (18) from cardiolipin by mild deacylation (30) and examined it in a passive hemagglutination assay for its ability to inhibit the hemagglutination reaction. The hemagglutination titer of 3,200 by antiserum to *L. casei* L324M reacting with a 1% suspension of sheep erythrocytes sensitized with 1 μg of LTA from *L. fermenti* NCTC 6991 per ml was reduced to a titer of <50 by 1 μmol G_3P_2. In contrast, hemagglutination at a low titer (titer, 400) by antiserum to *S. mitis* ATCC 903 was not inhibited by G_3P_2. This indicates that high serological reactivity in antiserum to *L. casei* L324M is dependent upon antibodies specific for a glycerol phosphate chain, whereas low reactivity for LTA (as detected by the sensitive hemagglutination assay) in antiserum to *S. mitis* ATCC 903 is not directed to a glycerol phosphate polymer.

Rabbit antisera to *S. sanguis* M-5 contain antibodies directed mainly to the LTA molecule. They are reactive specifically with a carbohydrate substitute on the teichoic acid moiety (B. Rosan, personal communication) which has been designated as the Lancefield group H antigen (24). Antisera to *S. sanguis* M-5 do not react with LTA on the basis of the PGP backbone.

Antiserum to *S. mitis* ATCC 903 reacted strongly with at least three components in autoclave extracts of *S. mitis* ATCC 903 when assessed by immunoelectrophoresis (data not shown).

Antigenic extracts. Extracts of strains CC5A, CC6, and *S. mitis* ATCC 903 were made from 16- to 18-h cultures grown in 1 liter of THB. The bacterial cells were washed three times in saline and suspended

at 200 mg (wet weight) per ml in a 0.9% NaCl solution. The suspensions were autoclaved for 15 min at 121°C (21) and centrifuged at 12,000 × *g* for 10 min to obtain a cell-free supernatant. The autoclave extracts were then dialyzed against three changes of water and lyophylized.

Serological methods. Antisera for streptococcal groups H, K, L, MG, N, and O were obtained from Difco Laboratories and tested with their corresponding antigen as positive controls. The cell-free supernatants of autoclave preparations of strains CC5A and CC6 were used in an inverted capillary precipitin test to determine group reactions; readings were made after 2 to 5 min and 30 min.

Agar gel double diffusion was performed in 1.2% agarose (Seakem; Marine Colloids Inc.) in Veronal buffer (pH 8.2) by the slide micromethod. Immunoelectrophoresis was performed by a slide micromethod in a 1.2% agarose gel in Veronal buffer (pH 8.2, ionic strength = 0.05) at 6 V/cm for 45 min. The lyophilized autoclave extracts were dissolved in phosphate-buffered saline (pH 7.2) at a concentration of 20 mg/ml and used at this concentration in gel diffusion and immunoelectrophoresis. Photographs were taken after 24 h of diffusion at room temperature.

Indirect fluorescent-antibody tests were performed on smears made of bacterial cells suspended at 10^8/ml as previously described (20).

Electron microscopy. A culture of strain CC5A grown in THB for 18 h was fixed by adding formaldehyde to a final concentration of 1%. The cells were washed three times in phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20. After the last centrifugation, the pellet was suspended in distilled water at a concentration of approximately 10^8 cells per ml. Samples of the bacterial suspension were placed for 1 min on copper grids coated with Formvar to allow adhesion of the cells to the film, and excess fluid was removed with filter paper. Serum treatment was carried out on some grids with 1 drop of antiserum to *S. mitis* ATCC 903 diluted 1:50 in phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20. The serum was incubated on the grid for 1 to 5 min. The excess serum was removed with filter paper, and the grids were washed one to three times with distilled water or phosphate-buffered saline. The grids were shadowed with a palladium-platinum alloy (80:20; Fullam) at an angle of 20° and a distance of 11 cm from the source. The specimens were examined in an Hitachi HS-8 transmission electron microscope (50 kV, 50-μm-objective aperture).

Specimens for scanning electron microscopic observation were processed by the method of Amako and Umeda (1). A drop of sterile 3% agarose and 0.1% gelatin in THB was allowed to gel on small pieces of glass fiber filter in a petri dish. Each agar-coated filter piece was inoculated with one loopful of an exponential-phase growth of strain CC5A in THB and incubated at 37°C for 2 h. Each preparation was then fixed overnight at 4°C in 1% glutaraldehyde in 0.15 M sodium cacodylate buffer, pH 7.4, and washed three times in the cacodylate buffer. The specimens were dehydrated in a graded series of acetone and amyl acetate before critical-point drying with CO_2 in a Denton apparatus. They were coated with a gold-palladium alloy (60:40; Fullam) in a rotating and tilting vacuum evaporator and examined in an ETEC Autoscan U100 electron microscope with a tilt angle of ~45° at an accelerating voltage of 20 kV.

RESULTS

Physiological tests. Strains CC5A and CC6 are gram-positive facultative cocci. They exhibit alpha hemolysis under aerobic and anaerobic conditions. In 18-h broth cultures, the cells occur as diplococci arranged in short chains. The diplococcal unit consists typically of ovoid cells with their long axis perpendicular to the axis of the chain. The long axis of the cell is 0.8 to 1.0 μm, with CC6 usually being larger and slightly more rounded than strain CC5A. Colonies on MSA are 0.5 to 1 mm in diameter, circular, regular, low convex, smooth, opaque, light blue, and soft, leaving the surrounding agar surface smooth.

Both strains were catalase and oxidase negative. Acetoin was not produced. Esculin was not hydrolyzed, but ammonia was produced from arginine. Strain CC6 partially hydrolyzed starch, whereas strain CC5A did not. No extracellular polysaccharide was produced in sucrose or glucose broth at 2.5 and 5%; however, a slight precipitate appeared when 2.0 and 2.5 parts of absolute ethyl alcohol were added to the supernatant of cultures in a 1.0% sucrose broth. Hydrogen peroxide was produced by both strains. Growth was inhibited at 45°C, by 4% NaCl, and by 0.1% methylene blue. Both strains grew on bile-blood agar at 7.5%; only strain CC6 tolerated 40% bile. Only minute colonies in areas of heavy inoculation appeared on MC agar (0.1% sulfisomidine). Both strains were tolerant to 0.1% potassium tellurite. Sucrose, glucose, lactose, and galactose were fermented without gas production; the final pH in broth containing 1.0% of these carbohydrates was not lower than 5.3. Only strain CC5A fermented salicin and trehalose. Mannitol, sorbitol, cellobiose, esculin, inulin, melibiose, raffinose, and starch were not fermented. Strains CC5A and CC6 were found to be physiologically most similar to *S. mitis* ATCC 903. However, strain ATCC 903 fermented raffinose, whereas strains CC5A and CC6 did not. These and other physiological characteristics are listed in Table 1.

Antigenic studies. Autoclave extracts of strains CC5A and CC6 reacted in the capillary precipitin test with Difco streptococcal group K antiserum but not with the other grouping antisera tested.

In general, similar immunoelectrophoretic patterns were obtained when extracts of strain CC5A or CC6 were tested with any of the antisera used in this study. This suggests the pres-

TABLE 1. *Comparison between physiological characteristics of reference strain S. mitis ATCC 903 and of CCC isolates CC5A and CC6*[a]

Characteristic	*S. mitis* 903	CC5A	CC6
Cell size (μm)	0.8–1.0	0.8–1.0	0.8–1.0
Mean no. of cells in the chains	<10	<10	<10
Consistency of colony on MSA	Soft	Soft	Soft
Appearance of MSA surface around colony	Smooth	Smooth	Smooth
Hemolysis			
Aerobic	Alpha	Alpha	Alpha
Anaerobic	Alpha	Alpha	Alpha
Final pH in broth containing sucrose	4.9	5.3	5.4
Final pH = or <5.6 in broth containing:			
Cellobiose	No	No	No
Esculin	No	No	No
Glucose	+	+	+
Inulin	No	No	No
Lactose	+	+	+
Mannitol	No	No	No
Melibiose	No	No	No
Raffinose	+	No	No
Salicin	+	+	No
Sorbitol	No	No	No
Sucrose	+	+	+
Starch	No	No	No
Trehalose	No	+	No
Hydrolysis of:			
Esculin	No	No	No
Starch	No	No	±
Ammonia produced from L-arginine	+	+	+
Hydrogen peroxide production	+	+	+
Oxidase reaction	No	No	No
Benzidine test	No	No	No
Catalase test	No	No	No
Optochin sensitivity	No	No	No
Acetoin from glucose	No	No	No
Precipitate in 1% sucrose broth with:			
1 Part ethyl alcohol	No	No	No
2 Parts ethyl alcohol	±	±	±
2.5 Parts ethyl alcohol	±	±	±
Precipitate in 2.5% sucrose broth with:			
1 Part ethyl alcohol	No	No	No
2 Parts ethyl alcohol	No	No	No
2.5 Parts ethyl alcohol	No	No	No
Iodophilic cells in broth containing:			
Glucose	NT[b]	Brown	Yellow
Sucrose	NT	Yellow	Yellow
Growth at pH 9.6	No	No	No
Growth at 45°C	No	No	No
Growth in medium containing:			
NaCl, 4%	No	No	No
Potassium tellurite, 0.1%	+	+	+
Potassium tellurite, 0.04%	+	+	+
Methylene blue, 0.1%	No	No	No
Methylene blue, 0.01%	+	+	+
Growth on:			
MC agar (sulfisomidine)	±	±	±
Bile blood agar			
7.5%	±	±	±
10%	±	±	±
40%	No	No	±

[a] Symbols: +, Positive reaction; ±, weak reaction; No, Negative reaction.
[b] NT, Not tested.

ence of antigens common to both strains (Fig. 1). Strains CC5A and CC6 reacted with antiserum to *S. sanguis* M-5 by forming a single arc characteristic of a negatively charged antigen (Fig. 1c). A single arc characteristic of an uncharged antigen was observed upon reaction of extracts of strains CC5A and CC6 with antiserum to *S. mitis* ATCC 903 (Fig. 1d). Extracts of both strains CC5A and CC6 gave a single band with an electrophoretic-mobility characteristic similar to that of teichoic acid when reacted with antiserum to *L. casei* L324M (Fig. 1e).

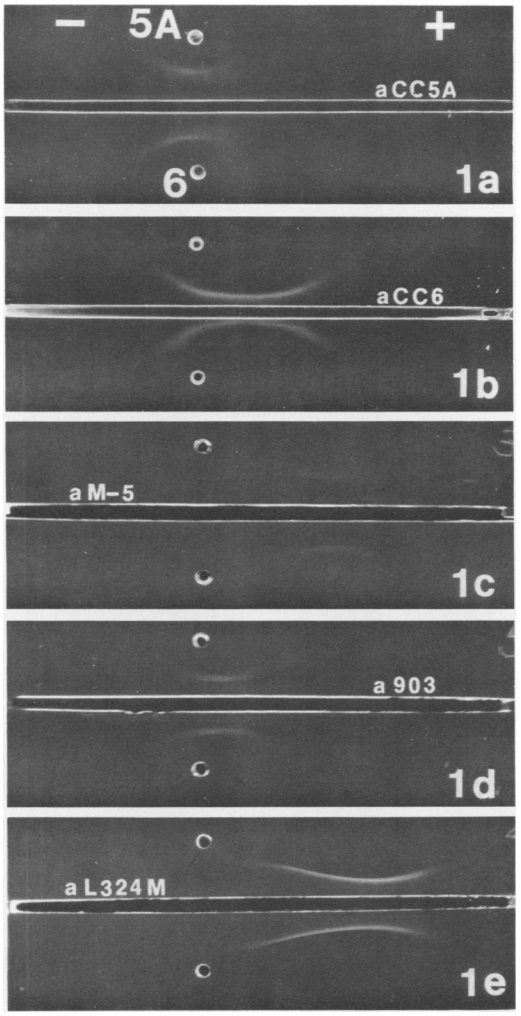


FIG. 1. *Immunoelectrophoretic analysis of autoclave extracts from strain CC5A (upper wells) and strain CC6 (lower wells) showing their antigenic relationship to the bacterial strains used for the production of the following antisera: (a) anti-CC5A, (b) anti-CC6, (c) anti-S. sanguis M-5, (d) anti-S. mitis ATCC 903, and (e) anti-L. casei L324M or PGP specific. The reaction in (e) indicates the presence of a teichoic acid in strains CC5A and CC6. In (c), it can be seen that a determinant in this teichoic acid has the S. sanguis specificity.*

A single band of precipitate showing deviation with complete fusion was observed when extracts of strains CC5A, CC6, and *S. mitis* ATCC 903 and the reference LTA were reacted in a gel diffusion experiment with antiserum to L324M, and antiserum directed to the PGP backbone of LTA (Fig. 2a). This indicates that a PGP antigen is common to strains CC5A, CC6, and *S. mitis* ATCC 903. A single precipitin band showing identity between extracts of strains CC5A and CC6 was demonstrated by reaction with antiserum to *S. mitis* ATCC 903 (Fig. 2b). The same antiserum failed to form any precipitin band with the reference LTA, indicating the absence of antibodies to the PGP antigen in antiserum to *S. mitis* ATCC 903 and therefore suggesting the non-PGP nature of the antigen detected. The antigen(s) in CC5A and CC6 extracts detected by antiserum to *S. mitis* ATCC 903 is also present in the extract of strain ATCC 903; however, the fusion of these precipitin bands is obscured by a superimposition of several precipitin bands in the homologous reaction (Fig. 2b).

A reaction of nonidentity (no deviation and no fusion) was observed between the immunoprecipitates of CC5A and CC6 extracts reacted

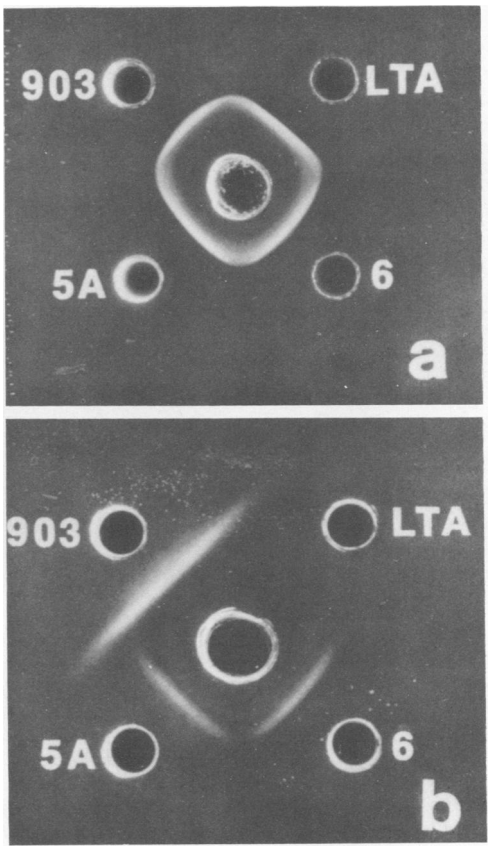


FIG. 2. *Gel double-diffusion analysis of extracts from strains CC5A, CC6, and S. mitis ATCC 903 and a reference LTA extracted from L. fermenti NCTC 6991 reacted with the PGP antiserum (a) and with antiserum to S. mitis ATCC 903 (b). A PGP antigen common to strains CC5A, CC6, and S. mitis ATCC 903 and to LTA is detected by the PGP antiserum (a). An antigen common to strains CC5A, CC6, and S. mitis ATCC 903, but not present in LTA and therefore distinct from PGP, is detected by antiserum to S. mitis ATCC 903 (b).*

with the antiserum to *L. casei* L324M on the one hand and with antiserum to *S. mitis* ATCC 903 on the other hand (Fig. 3). Both the gel diffusion and the immunoelectrophoretic studies showed that extracts of strains CC5A and CC6 contain at least two antigenic components: one with LTA antigenic reactivity and a second one antigenically distinct from LTA, which is also found in *S. mitis* ATCC 903.

The serological relationships of the CCC cocci CC5A and CC6 with related bacterial species were further assessed by the indirect immunofluorescence procedure. Homologous titers were highest among the reactions tested, each indicating specificity for the immunizing strain.

An unusual distribution of fluorescence on the cell surface of CC5A was observed at high dilutions of the homologous antiserum, at all dilutions of antisera to *S. mitis* ATCC 903 and to *L. casei* L324M, and at most dilutions of antiserum to *S. sanguis* M-5 (Table 2). This pattern consisted of polar fluorescent caps on single cells or scalloped festoons on chains (Fig. 4).

As in strain CC5A, fluorescent caps stained on cells of strain CC6; however, the latter also exhibited patches scattered over the cell surface

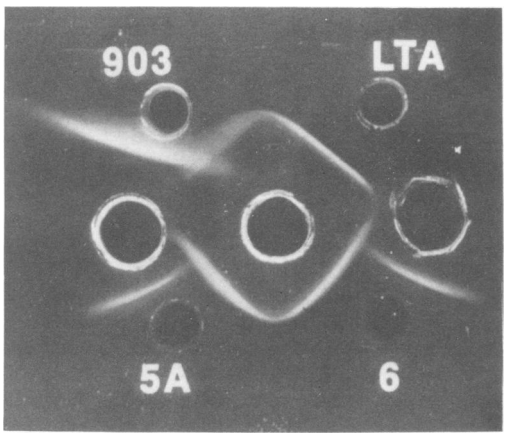


FIG. 3. *Gel double-diffusion analysis showing a reaction of nonidentity between the PGP antigen in CC5A, CC6, S. mitis ATCC 903, and LTA reacting with the PGP antiserum (large center well) on the one hand and the antigen of CC5A, CC6, and S. mitis ATCC 903 reacting with antiserum to S. mitis ATCC 903 (left and right large wells) on the other hand.*

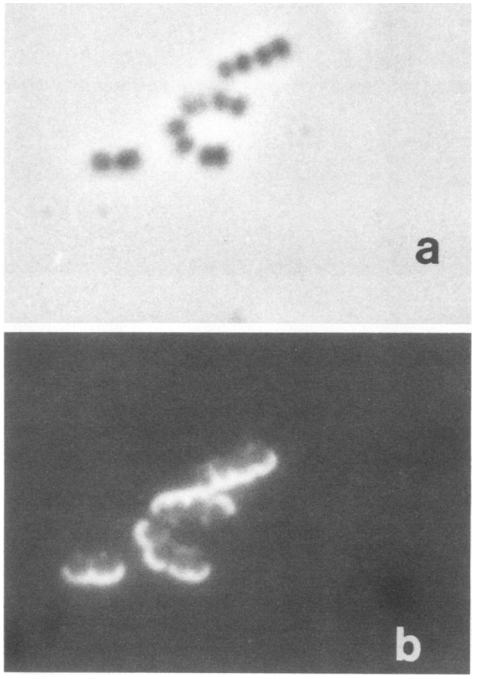


FIG. 4. *Indirect fluorescent-antibody test on cells of strain CC5A with antiserum to S. mitis ATCC 903. ×3,300. (a) Short chains and diplococci; phase-contrast illumination. (b) Same field as in (a); ultraviolet light illumination. A typical fluorescent pattern of scalloped festoons indicates that an antigen(s) present only at one end of the cells (polar cap) reacts with antibodies from antiserum to S. mitis ATCC 903 (diluted 1:1,600). A similar fluorescent pattern was also observed with the PGP antiserum when tested at 1:1 to 1:640 dilutions, suggesting that the polar structure of CC5A cells contains the glycerol phosphate backbone common to teichoic acids. The polar cap staining was also seen when antiserum to CC5A (diluted > 1:200), antiserum to CC6 (diluted ≥ 1:50), and antiserum to S. sanguis M-5 (diluted ≥ 1:50) were used.*

TABLE 2. *Ranges of titers[a] of selected antisera resulting in fluorescence restricted to a polar cap (C) or as patches (P) or both (C/P) on cells of strains CC5A and CC6 by indirect fluorescent-antibody staining*

Bacterial strain	Range of titers of antiserum to:				
	CC5A	CC6	*S. mitis* ATCC 903	*S. sanguis* M-5	*L. casei* L324M
CC5A	200–3,200 (C)	50–3,200 (C)	1–3,200 (C)	50–800 (C)	1–640 (C)
CC6	50–1,600 (C/P)	400–3,200 (P)	1–3,200 (C/P)	100–400 (P)	1–640 (P)

[a] Titers are expressed as the reciprocal of highest dilutions giving definite staining reactions.

with antiserum to strain CC5A and antiserum to *S. mitis* ATCC 903. With the other antisera, only patches were stained on the cells of strain CC6 (Table 2).

In contrast, no unusual distribution of fluorescence, but rather a uniform fluorescent cell wall definition of several strains representative of *S. sanguis* serotype 1 (ATCC 903), serotype 2 (M-5 and ATCC 10556), and heterologous serotype (ATCC 10557), was observed when these strains were reacted with the five antisera tested (data not shown).

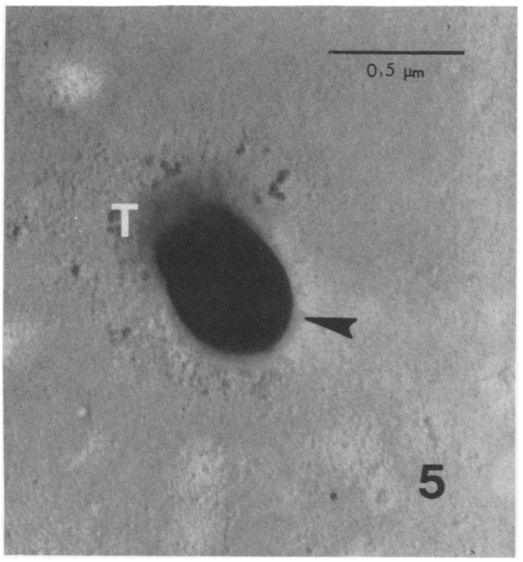
Electron microscopy. The typical morphology of metal-shadowed cells of strain CC5A is shown in Fig. 5. Cells of strain CC5A appeared as ovoid cells, 0.60 to 0.80 μm in their largest diameter. A structural component consistently observed on these cells was a polar tuft, extending 100 to 150 nm from one end of the cell; it can be seen on a single cell in Fig. 5. Continuous with the tuft was a structure approximately 50 nm thick, coating the entire surface of the cell. The tuft of strain CC5A was striated, suggesting that it consisted of a finely fibrillar material spreading outward from the tip of the bacterium.

Cells of strain CC5A, exposed for 1 to 5 min to antiserum to *S. mitis* ATCC 903, exhibited a more prominent polar tuft. The increased shadowing observed presumably was a result of the increase in thickness of the outer surface resulting from the binding of antibodies from antiserum to *S. mitis* ATCC 903 specific for an antigen(s) present in the tuft. Such reactions on cells of strain CC5A are shown in Fig. 6.

Scanning electron microscopic observation of cells of strain CC5A confirmed the basic morphological characteristics seen in the light and transmission electron microscopes. In the scanning electron microscope, the cells appeared as elongated diplococci with prominent polar tufts. Typical scanning electron microscopic evaluation (Fig. 7) revealed an indentation running along the long axis of the cells, in the plane of the tufts, which likely corresponded to the plane of cell division. The constant finding of equatorial constrictions orthogonal to the axis of chains of diplococci suggests that the cells were dividing as prolate spheroids. The polar tuft material appeared to link the cells in the chains by lateral or side-to-side connections. In contrast to the tufted end of the cocci, the rest of the cell appeared to be smooth in the scanning electron micrographs.

DISCUSSION

Strains CC5A and CC6 appeared to physiologically fit into the group V streptococci of Carlsson (7) since they exhibited the following phe-



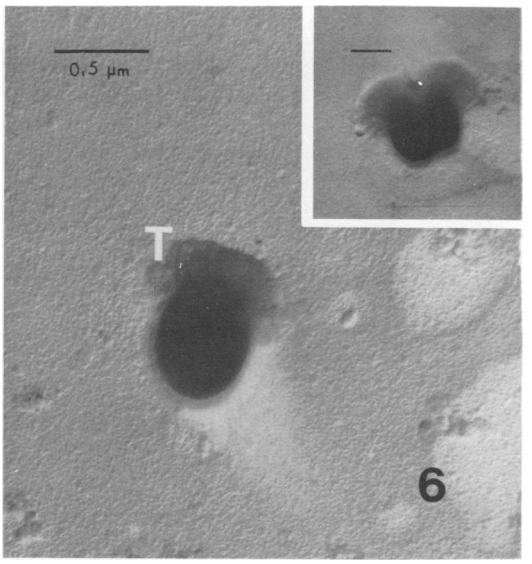


FIG. 5. *Electron micrograph of a typical cell of strain CC5A; metal shadowing. A polar tuft (T), consisting of a finely fibrillar material can be seen spreading outward up to 150 nm from the cell surface at one end of the bacterium. The outer surface of the cell consists of a layer 50 nm thick (arrow). All of the cells observed in the electron microscope with recognizable morphology showed such a polar tuft.*

FIG. 6. *Shadow-cast preparations after 1 min of incubation of CC5A cells with antiserum to S. mitis ATCC 903. The resulting reaction, possibly an antigen-antibody complex localized at the polar tuft (T) of the bacterium, enhances visualization of the polar structure typical of CC5A cells.*

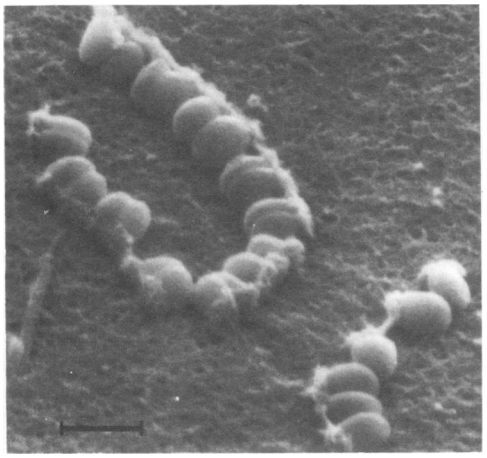


FIG. 7. *Scanning electron micrograph of a 2-h growth of strain CC5A on thin agar layer. Various stages of cell division, as determined by the presence of constrictions indicative of septum formation, can be seen. The equatorial plane of division of the prolate spheroid cocci determines two poles, one of which bears the external tuft typical of strain CC5A. Bar = 1.0 μm.*

notypic characteristics: a soft colony on aerobic MSA; no formation of precipitate by the addition of ethanol to sucrose broth cultures; alpha hemolysis; hydrogen peroxide production; a final pH in sucrose broth of not lower than 5.3; fermentation of lactose; and no fermentation of mannitol, sorbitol, and esculin. However, it was impossible to further assign strains CC5A and CC6 to either group V:A or group V:B of Carlsson as they showed characteristics of both groups; for example, they produced ammonia from arginine, did not ferment inulin, and grew in 0.1% tellurite broth. For Carlsson (7), strains in group V resemble *S. mitis* of Sherman et al. (25). Colman and Williams (9), reviewing the data of Carlsson, consider that group V:A is better allocated to *Streptococcus mitior* and that group V:B is better allocated to *S. sanguis*.

Although strains of *S. sanguis* usually produce extracellular dextran from sucrose and hydrolyze esculin, Colman and Williams (9) suggest that strains which do not produce dextran but which are otherwise similar to *S. sanguis* in properties such as cell wall composition should be classified as *S. sanguis*. However, they remain undecided about the taxonomic position of five strains in their study which reacted with group K antisera, did not produce dextran, and did not hydrolyze esculin. If brought into *S. sanguis*, these five strains make the species more heterogeneous. Strains CC5A and CC6 appear to resemble the five strains excluded from *S. sanguis* by Colman and Williams (9) since they carry the K antigen, do not produce dextran, and do not hydrolyze esculin, but otherwise exhibit physiological properties of *S. sanguis*.

Strains CC5A and CC6 also correspond to the description by Mejàre and Edwardsson (19) of an *S. mitior* group of strains that produced ammonia from arginine, in contrast to the *Streptococcus viridans* reference strain that they used (NCTC 10712, similar to *S. mitior* of Colman and Williams [9]). Precise allocation of strains CC5A and CC6 to either *S. sanguis* or *S. mitior* species requires between schemes for the taxonomic differentiation between *S. sanguis* and *S. mitior*. Further characterization, including cell wall composition analysis and deoxyribonucleic acid base composition and deoxyribonucleic acid base sequence homology studies, may also aid in their taxonomy.

The extracts of strains CC5A and CC6 gave a precipitin reaction with Difco streptococcal group K antiserum. All extracts of *Streptococcus salivarius* tested by Facklam (13) gave this reaction, as did strains *S. sanguis* II, *S. mitis*, *Streptococcus* MG, and also four strains of *S. mitior* studied by Colman and Williams (9). Although they react with group K antisera, strains CC5A and CC6 are not physiologically similar to *S. salivarius*. The relationship to group K must be interpreted with caution since the serological group K is not clearly defined (9) and includes physiologically different strains of viridans streptococci reacting with K grouping sera.

Extracts of strains CC5A and CC6 did not react with Difco group H antiserum, but did react with antisera to *S. sanguis* M-5. This was not totally unexpected since various commerical producers of streptococcal grouping sera use antigenically different immunizing strains of group H (2, 17). The Difco group H antiserum produced against strain ATCC 12396 (identical to strain Perryer) recognizes a different group H antigen than does the Burroughs Wellcome group H antiserum produced against strain Blackburn (NCTC 10231), which in turn is similar to strains Challis and M-5 (8). The immunoelectrophoretic and gel double-diffusion patterns observed when extracts of strains CC5A and CC6 are reacted with antiserum to *L. casei* L324M which has specificity for the glycerol phosphate backbone common to teichoic acids (23) suggests the presence of a teichoic acid in these strains. This presence was further substantiated by the immunoelectrophoretic pattern observed upon reaction with antiserum to *S. sanguis* M-5. The latter, however, is not PGP or backbone specific, but rather has specificity for a carbohydrate of the *S. sanguis* teichoic acid (Rosan, personal communication). These results suggest that strains CC5A and CC6 carry a glyc-

erol teichoic acid antigen with an *S. sanguis* group-specific antigen determinant also designated as the Lancefield group H antigen (24).

The serological relationship of strains CC5A and CC6 to the *S. sanguis* group was further assessed by testing their relationship to strain *S. mitis* ATCC 903, a representative of the serotype 1 of Rosan (22). It was found that antiserum to *S. mitis* ATCC 903, which did not react with the reference LTA from *L. fermenti* NCTC 6991, did react with an antigen(s) common to *S. mitis* ATCC 903 and strains CC5A and CC6.

The failure of antiserum to *S. mitis* ATCC 903 to detect the PGP antigen in our experiments, suggesting the absence of anti-lipoteichoic acid antibodies in this antiserum, is in apparent contradiction to the fact that *S. mitis* ATCC 903 possesses glycerol teichoic acid (22, 24). This antigen is characteristic of the *S. sanguis* strains in serotype 1 (22, 24). This apparent inconsistency may by explained by the observation that LTA varies in its immunogenicity since variable antibody responses to LTA have been documented in *Lactobacillus* species (26). For example, intravenous injection of *L. fermenti* NCTC 6991 whole organisms into rabbits induces anti-lipoteichoic acid antibodies, whereas a similar immunization with *L. casei* NCTC 6375 fails to do so (26). Isolated LTA from both species, however, are immunogenic and cross-react serologically. The difference in serological behavior of the LTA in these two *Lactobacillus* strains was explained by a proportion of the teichoic acid being exposed, and hence immunogenic, on or near the cell surface of *L. fermenti* but not of *L. casei*. Labeling of *L. casei* cells by anti-lipoteichoic acid antibodies and a ferritin conjugate was, however, achieved on *L. casei* NCTC 6375 and supported the evidence for LTA being a membrane component of these cells (26). It is likely that the location of LTA in *S. mitis* ATCC 903 is similar to that in *L. casei* NCTC 6375, i.e., at the cell membrane level and thus not likely to be immunogenic upon intravenous injection of whole cells. However, the LTA of *S. mitis* ATCC 903 is readily extracted by autoclaving and labeled by indirect fluorescent-antibody tests.

The immunofluorescence experiments revealed an unusual distribution of antigens on the cell surface of strain CC5A. The two distinct major antigens observed in the gel diffusion experiment (Fig. 3) appear to be located in the tuft structure of strain CC5A. In the immunofluorescence experiments, both the antiserum to *S. mitis* ATCC 903 and the PGP antiserum reacted with the tufts only. The patches observed on CC6 cells suggest that this strain, in addition to the polar tuft, possesses minute amounts of tuft components scattered over the whole cell envelope. Antigen localization restricted to a polar area was not observed on any of the reference bacterial strains tested (*S. sanguis* 10556, 10557, and M-5 and *S. mitis* ATCC 903). Hence, it is likely that strains CC5A and CC6 are respresentative of a unique group of oral streptococci possessing this topographical feature.

Antigenically, the tuft consists of components shared with other related species, two of which could be detected in the present study. One component presumably is the group glycerol teichoic acid common to all *S. sanguis* species as suggested by the indirect fluorescent-antibody test reactivity of cell walls, including the tufts, with antiserum to *S. sanguis* M-5. Further evidence for glycerol teichoic acid in the tuft comes from the strong indirect fluorescent-antibody test reaction of the tuft, and of the tuft only, with antiserum to *L. casei* L324M whose specificity is for the PGP backbone of LTA. Another antigenic component present in the tuft only was also detected with antiserum to *S. mitis* ATCC 903. This component is presumably distinct from the PGP backbone of glycerol teichoic acid for the following reasons: (i) antiserum to *S. mitis* ATCC 903 does not react by gel diffusion with LTA; (ii) immunoelectrophoresis of an autoclave extract of *S. mitis* ATCC 903 reacted with the homologous antiserum shows no band with an electrophoretic mobility similar to that of teichoic acid (data not shown); and (iii) the weak hemagglutination of LTA-sensitized erythrocytes by antiserum to *S. mitis* ATCC 903 is not inhibited by G_3P_2. Thus, the absence (or low level) of antibodies to the PGP backbone of LTA in the antiserum to *S. mitis* ATCC 903 facilitated the detection of the second antigenic determinant in the tufts of strains CC5A and CC6. It is not excluded, however, that the specificity of antiserum to *S. mitis* ATCC 903 might be for substitutes on the glycerol phosphate chain. If so, antisera to *L. casei* L324M and *S. mitis* ATCC 903 would each detect different antigenic determinants on the same PGP-containing polymer. The immunoelectrophoretic data, however, do not support this view since the antigens detected in autoclave extracts of strains CC5A and CC6 by each antiserum have distinct electrophoretic mobilities. An alternative explanation is that the specificity of antiserum to *S. mitis* ATCC 903 on the tuft of strains CC5A and CC6 is directed to an antigen distinct from a teichoic acid molecule, possibly a protein or a glycoprotein. Further studies, including a serological characterization of *S. mitis* ATCC 903, are needed to elucidate the nature of the tuft antigens of strains CC5A and CC6.

The metal-shadowed samples of CC5A cells

observed by electron microscopy further showed the presence of a tuftlike structure. This tuft appeared fibrillar, composed of hairlike projections extending outward from the end of the cell. The presence of such a structure had been suggested by the polar fluorescent reactions observed. In addition, the polar tuft of CC5A cells was even better visualized in electron micrographs of whole-cell preparations treated with antiserum to *S. mitis* ATCC 903. This serum was shown by the immunofluorescent experiments to react only with the polar cap of CC5A cells.

The polar tuft typical of the CCC cocci may consist of pili (fimbriae). Pili have been described in some strains of *S. sanguis* and *S. mitis* and were seen most often on the older hemisphere of dividing cells (8). Localized structures other than flagellae appear to be rare among bacteria; however, several organisms with polar tuftlike structures have been described. For example, Halhoul and Colvin, in ultrastructural studies of human gingival plaque (16), described a bacillus-like organism, not associated with CCC, with a tuft of thin fibers at only one end of the cell. Polar pili have been described in many species of *Pseudomonas* (28). Breznak and Pankratz (3), in studies of the gut of termites, observed bacteria secured to other bacterial cells by means of a fibrous holdfast material. There are other examples of organisms in the genera *Blastobacter* and *Asticcacaulis* (10) which secrete holdfast material from a specific area on the cell surface. It has also been suggested that a large portion of the bacteria epiphytic on aquatic plants is composed of groups which possess holdfast structures (10).

The polar structures observed by immunofluorescence and transmission electron microscopy were always located at one pole of the single cocci and in a scalloped fashion in chains of these streptococci. They did not extend peripherally or centripetally from the coccal equator at sites of new cell wall and septum formation, as is the case for protein M on group A streptococci (15). Hence, it is likely that the polar tufts represent discrete differentiated structures.

The surface topography study by scanning electron microscopy established the location of the tuft at a polar end determined by the plane of division of the streptococci. Adjacent mother-daughter cells at various stages of division, as well as single mature cells, were seen to bear identical monopolar tufts. This observation indicates that the polar tufts are not transitory surface structures seen only during cell division or autolysis. Studies during the various stages of growth of these bacteria should provide further understanding of the relationship of the polar surface structure to cell division.

Because of its high density on a restricted area of the cell surface, the tuft structure of strains CC5A and CC6 presumably contributes to promote adhesive interaction with attachment sites on the *B. matruchotii* cell surface. This results in CCC of human dental plaque. Further studies are in progress to determine the chemical nature of the interaction of this tuft with *B. matruchotii* cells.

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