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Biochemical and Immunological Differences Between Hydrophobic and Hydrophilic Strains of *Streptococcus mutans*

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Hydrophobic strains of *Streptococcus mutans* were compared with paired variants showing reduced hydrophobicity. Extracts of hydrophobic cells contained a number of high-molecular-weight proteins which were not present on cells with decreased hydrophobicity. The proteins were found in purified cell walls, suggesting that they are located on the bacterial surface. Trypsin treatment of whole cells destroyed the proteins and reduced the hydrophobicity. Chemical analysis did not reveal any marked differences in the proportion of cell wall constituents. The amino acid compositions and lipoteichoic acid contents of hydrophobic and hydrophilic cell walls were similar. Culture supernatants from the hydrophilic variants contained high-molecular-weight proteins similar to those extracted from the cell walls of the hydrophobic parent strains, indicating that the variants were impaired in their ability to incorporate the hydrophobicity-associated proteins into the cell wall. The dominant protein had a molecular weight of 190,000, similar to that of antigen I/II (B) of *S. mutans*.

A series of observations indicate that hydrophobic forces are involved in the interaction between microorganisms and host surfaces (11, 13, 24, 27, 35). Freshly isolated strains of oral streptococci have a high degree of hydrophobicity, but *Streptococcus mutans* is unique in that it loses its hydrophobic character when it is subcultured on laboratory medium (27). Upon repeated subculture, the adhesive properties are diminished (28), and coincident with the loss in hydrophobicity, there is a decrease in the ability to bind to saliva-coated hydroxyapatite (36) and to become implanted in the oral cavity in humans (M. Svanberg, G. Westergren, and J. Olsson, manuscript in preparation) and in hamsters (J. Olsson and C. G. Emilson, manuscript in preparation). The nature of the molecules conferring hydrophobicity on the streptococcal cell surface have not yet been identified.

A relationship between M protein and hydrophobic properties of group A streptococci has been suggested (33), but recently Miörner et al. (22) have stated that lipoteichoic acid (LTA) is the major cell wall component responsible for surface hydrophobicity of these bacteria. Both M protein and LTA have been associated with adherence of group A streptococci to epithelial surfaces (12). Beachey (1) has suggested that the LTA in the cell wall may be oriented with the hydrophobic lipid moiety extending from the cell surface. The lipid would be available to react with hydrophobic receptors on epithelial cells.

For the initial attachment of *S. mutans* to tooth surfaces, various cell surface components have been implicated. Thus, it has been suggested that LTA, because of its calcium-binding ability, is a major factor in the adherence of *S. mutans* to enamel (29). Hamada and Slade (15) have proposed that cell-bound glucosyl transferase can bind to a specific protein receptor on the tooth surface, and Staat et al. (32) have found that cell surface proteins mediate the initial adherence of *S. mutans* in vitro.

Numerous proteins exists within the cell wall (25, 30) and extend from the cell surface in structures which have been called fibrillar or fuzzy coats. Some of these proteins may be hydrophobic or may possess domains which are rich in hydrophobic amino acids (13, 34).

In this study, we demonstrate that hydrophobic and adherent strains of *S. mutans* differ from hydrophilic strains with regard to biochemical and immunological characteristics.

MATERIALS AND METHODS

Organisms. *S. mutans* strains LK, LK36, GW, and GW36 were described previously (36). *S. mutans* C6715 was obtained from Hans van der Hoeven, Nijmegen, The Netherlands. Unless indicated otherwise, all organisms were grown overnight at 37°C in tryptic soy broth. To ensure that the organisms were subcultured a minimal number of times, the original freeze-dried cultures were grown in liquid medium, divided into a number of 1-ml aliquots, and stored at −70°C in 7% sterile dimethyl sulfoxide. Each experiment was started from a frozen inoculum. Cells were harvested by centrifugation and washed three times in phosphate-buffered saline (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl [pH 7.0]) for hydrophobicity assays or three times with 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.2) for preparation of cell walls.

Hydrophobicity. Hydrophobicity was assayed as described by Westergren and Olsson (36). Three milliliters of a washed suspension of cells (absorbance at 436 nm, 0.5; 1-cm lightpath) was mixed for 60 s with 100 μl of hexadecane. The mixture was allowed to stand for 15 min, and then a sample of the aqueous phase was removed and the absorbance at 436 nm was determined.

Preparation of cell walls. *S. mutans* cell walls were prepared by a modification of a procedure described previously (21); 2 liters of an 18-h culture was harvested by centrifugation, washed three times with phosphate-buffered saline, and broken with glass beads (75 to 150 μm in diameter) in a Mini-Mill (Gifford-Wood Co., Hudson, N.Y.). The crude walls were separated from the unbroken cells by repeated differential centrifugation for 15 min at 3,000 × *g* until the cell walls were free of whole cells as judged by phase-contrast micros-

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copy, and then the walls were washed several times with 0.15 M NaCl.

When required, walls were suspended in 0.05 M HEPES (pH 7.2) containing 4% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) at room temperature for 20 min and centrifuged and the extraction procedure was repeated. The extracted walls were washed five times with distilled water. In some cases, walls were extracted with 3% sodium dodecyl sulfate (SDS) at room temperature for 1 h. The walls were then washed exhaustively to remove detergent. Walls required for chemical analysis were incubated with RNase (20 μg/ml) and DNase (20 μg/ml) for 3 h at 37°C and then washed three times with distilled water.

Digestion of cell walls. Cell walls, prepared as described previously, were suspended in 0.05 M HEPES buffer (pH 7.2) to give an absorbance at 660 nm (1-cm light path) of 0.42 when diluted 30-fold. The walls were mixed with 725 U of mutanolysin (Sigma) and incubated at 37°C overnight. Undigested walls were removed by centrifugation. The absorbance at 660 nm of the mutanolysin-treated walls was reduced by 80%. Protease activity was not detected when mutanolysin was incubated overnight at 37°C with azocoll.

Analytical procedures. Protein concentrations were measured by the method of Lowry et al. (20), with bovine serum albumin as the standard; total hexoses were measured by the phenol sulfuric acid method (10), with glucose as the standard; and rhamnose was measured by the method of Dische and Shettles (8). Phosphorus determinations were performed by the method of Chen et al, (6), with KH_2PO_4 as the standard. Amino acid analyses of cell walls were performed on a Dionex D550 amino acid analyzer after hydrolysis of the walls with 6 N HCl in vacuo for 16 h at 100°C. In all cases, standards were treated identically to cell walls.

PAGE. Nondenaturing gels containing 7.5% polyacrylamide were run in the absence of SDS and 2-mercaptoethanol. SDS-polyacrylamide gel electrophoresis (PAGE) was run with 7.5% polyacrylamide (19), and 2 μg of protein was applied to the gels. Molecular weight standards were: myosin, 200,000; β-galactosidase, 116,250; phosphorylase B, 92,500; bovine serum albumin, 66,200; and ovalbumin, 45,000.

Gels were stained for protein with Coomassie blue and for carbohydrate with periodic acid-Schiff reagent (38) or were silver stained (26).

Electron microscopy. Cells were negatively stained with a 2% (wt/vol) phosphotungstic acid that was adjusted to pH 7.2 with KOH. Observations were made with a Philips EM 300 electron microscope.

LTA assay. LTA was extracted from 1 g of freeze-dried cells by the procedure of Wicken et al. (37). LTA was quantitated by rocket immunoelectrophoresis (17). Reference LTA was kindly supplied by J. E. Ciardi, National Institute of Dental Research, Bethesda, Md., and anti-LTA was a gift from Michael Cole, National Institute of Dental Research.

Extraction of cell wall proteins. Whole cells (1 mg/ml, dry weight) or cell walls (1 mg/ml, dry weight) suspended in distilled water or 2% SDS–5% 2-mercaptoethanol were placed in a boiling-water bath for 30 min. The extract was clarified by centrifugation and stored at −20°C until required. In some cases, the procedure was modified by including salts or detergents in the extraction medium; these compounds were removed by exhaustive dialysis of the extract against distilled water. When required, extracts were freeze-dried and then dissolved in distilled water to give the desired concentration of protein.

Immunological procedures. (i) Immunization. Rabbits were immunized with formalinized whole cells (absorbance at 660 nm, 1.0) by intravenous injection of 0.2 ml on three consecutive days, and an additional 0.3 ml was injected on days 6, 7, 8, 9, and 10 after the initial injection. Whole, nonformalinized cells were injected on days 19 through 23. Blood was collected 1 week after the final injection. A specific antiserum was prepared by extracting anti-LK serum with strain LK36 (10 mg of cells [dry weight] per ml of antiserum).

(ii) Western blots. Proteins were transferred (3) onto nitrocellulose paper (Bio-Rad Laboratories, Richmond, Calif.) in 25 mM Tris–192 mM glycine–20% MeOH buffer (pH 8.3) at 9°C. A current of 4 mA was applied for 18 h in a Bio-Rad Trans-Blot cell.

Antigens transferred and bound to nitrocellulose were visualized by staining with anti-LK serum, followed by goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate, by the procedure outlined in the Bio-Rad technical bulletin supplied with the Bio-Rad Immun-Blot (GAR-HRP) assay kit. Goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate, gelatin, Tween-20, and the HRP color development reagent were purchased from Bio-Rad Laboratories.

(iii) Immunoelectrophoresis. Standard immunoelectrophoretic techniques were performed with agarose gels in 0.08 M Tris–0.02 M tricine buffer (pH 8.6).

Cell modification. (i) Trypsin. Freeze-dried cells (0.1 g) were suspended in 5 ml of phosphate-buffered saline (pH 7.2) containing trypsin (1.2 mg/ml) and incubated for 2 h at 37°C. The cells were removed by centrifugation and washed three times with phosphate-buffered saline. The supernatant was dialyzed against distilled water and freeze-dried.

(ii) Sonication. A 5-ml suspension of cells (absorbance at 660 nm, 5) was sonicated for 6 min at an output of 7 on a model 35 sonifier (Branson Sonic Power Co., Danbury, Conn.).

Chemicals. All chemicals were obtained from Fisher Scientific Co., Pittsburgh, Pa., unless specified otherwise. Trypsin type XI from bovine pancreas, RNase, and DNase were obtained from Sigma Chemical Co. Molecular weight standards were obtained from Bio-Rad Laboratories.

RESULTS

Hexadecane binding. The hydrophobic characteristics of the strains studied are shown in Table 1. As reported by Westergren and Olsson (36), the serotype c strains, LK36 and GW36, which they had repeatedly subcultured, were less hydrophobic than the parent strain. Such an instability was not shared by the serotype d strain, C6715, which retained its hydrophobicity despite having been cultivated on laboratory medium for many years.

Cells grown in brain heart infusion broth were less hydrophobic than cells grown in tryptic soy broth. The difference in hydrophobicity was not great but was observed in all experiments. Cells grown in defined medium (5) had hydrophobic properties similar to those of tryptic soy broth-grown cells. Fibrillar structures were not observed in negatively stained preparations of LK and LK36.

Cell modification. Whole cells of *S. mutans* LK were subjected to a number of modification reactions in an attempt to characterize the cell surface components which contributed to hydrophobicity (Table 2). Cells which had been shaken in 3% SDS at room temperature for 1 h and then washed exhaustively remained hydrophobic. Extraction of cells in 3% SDS at 100°C for 15 min reduced hydrophobicity

TABLE 1. Hydrophobicity of *S. mutans*

Strain	% Adsorbed to hexadecane
LK	78 ± 3
LK36	22 ± 5
GW	76 ± 2
GW37	21 ± 7
C6715	78 ± 4
LK[a]	64 ± 2

[a] Organisms were grown in brain heart infusion broth; all other organisms were grown in tryptic soy broth.

TABLE 3. Cell wall composition of *S. mutans* LK and LK36[a]

Component	Concn (μg/mg [dry wt]) in strain:	
	LK	LK36
Rhamnose	390	377
Hexoses[b]	401	381
Phosphate	8	8
Protein	198	229

[a] Cell walls prepared from cells grown in tryptic soy broth were extracted for 1 h with 3% SDS at room temperature and then washed exhaustively with distilled water and freeze-dried before analysis.
[b] As glucose.

to zero. Incubating cells at 100°C for 30 min in HEPES buffer also reduced hydrophobicity to zero. Cells subjected to sonication for 5 min exhibited more hydrophobicity than did the nonsonicated control. Cells treated with trypsin and then washed free of the enzyme were unable to bind to hexadecane, whereas cells treated with heat-inactivated trypsin were unaffected. Trypsin treatment of LK36 reduced hydrophobicity to zero. Phenol-extracted cells self-aggregated and could not be assayed in the hexadecane binding assay.

Cell wall analysis. The chemical compositions of purified cell walls of strains LK and LK36 are shown in Table 3. Rhamnose, hexose, phosphate, and protein contents were essentially the same for both strains. The amino acid content of the cell walls is shown in Table 4. The high levels of alanine, glutamic acid, and lysine are consistent with a preparation containing a significant proportion of peptidoglycan. LK cell walls have higher levels of the nonpolar amino acids valine, leucine, and isoleucine and the polar amino acids threonine and serine. A small amount of methionine was present in LK36 but was not found in LK. The LTA contents of LK and LK36 were similar when hot-water–phenol extracts were analyzed by rocket immunoelectrophoresis.

SDS-PAGE analysis of whole cells. Hot water and SDS extracts of *S. mutans* cells were analyzed by SDS-PAGE. Coomassie blue-stained gels revealed that the hydrophobic strains LK, GW, and C6715 all possessed a large protein which was absent in the hydrophilic variants. Comparison with standard molecular weight markers indicated that the protein had a molecular weight of 190,000. The protein stained with periodate-Schiff reagent.

Gels stained with silver nitrate revealed a number of differences which were not visible in gels stained with Coomassie blue. SDS-PAGE analysis of SDS (100°C) extracts of a number of *S. mutans* strains is shown in Fig. 1. Extracts of the hydrophobic strains LK, GW, and C6715 (Fig. 1, lanes 1, 3, and 5, respectively) contained large quantities of the 190,000-molecular-weight protein (protein a), whereas the extracts from hydrophilic strains LK36 and GW36 (lanes 2 and 4) had either none or very little of the protein. In addition, there were a number of bands that stained with $AgNO_3$ in the hydrophobic strains that were not present in the hydrophilic strains (e.g., proteins b and c). Protein d was found in extracts from both hydrophilic and hydrophobic strains. Other quantitative and qualitative variations can be observed in Fig. 1, but unlike the differences noted above, these differences were not consistent from one preparation to another. In general, the hydrophilic variants appeared to possess less extractable material than did the parent strain. In addition to the differences between hydrophilic and hydrophobic strains, there were distinct variations in the staining patterns of strains LK and GW. For example, band f was a prominent component of GW but was present only as a small band in LK. Conversely, bands g and h were present in a much higher concentration in LK than in GW.

The high-molecular-weight (HMW) proteins a, c, and e were extracted by boiling strain LK in distilled water (Fig. 2A, lane 1). The quantity of the proteins extracted by this procedure was much less than when cells were extracted with SDS at 100°C. Only protein e was found in hot-water

TABLE 2. Effect of cell modification on hydrophobicity

Treatment	% Adsorbed to hexadecane
None	78 ± 3
SDS (room temp)	76 ± 4
100°C, 20 min	0
SDS-BME[a] (100°C, 10 min)	0
Trypsin	0
Heat-denatured trypsin[b] (boiled)	78 ± 4
Sonication[c]	90 ± 6

[a] BME, 2-Mercaptoethanol.
[b] Trypsin was placed in a boiling water bath for 30 min before being added to the cell suspension.
[c] Sonicated 6 min.

TABLE 4. Amino acid analysis of LK and LK36 cell walls[a]

Amino acid	Concn (nmol/mg [dry wt]) in strain:	
	LK	LK36
Aspartic	33.0	30.6
Glutamic	477.3	352.0
Histidine	5.3	6.8
Lysine	443.4	331.4
Arginine	10.4	13.6
Threonine	32.9	16.7
Serine	60.6	33.8
Proline	13.0	11.6
Glycine	23.7	31.5
Alanine	1,520.0	1,150.0
Methionine	0.0	4.0
Valine	25.7	19.2
Leucine	75.1	52.4
Isoleucine	64.9	38.4
Tyrosine	8.8	8.1
Phenylalanine	11.3	12.0

[a] Cell walls prepared from cells grown in tryptic soy broth were extracted for 1 h with 3% SDS at room temperature and then washed exhaustively with distilled water and freeze-dried before analysis.

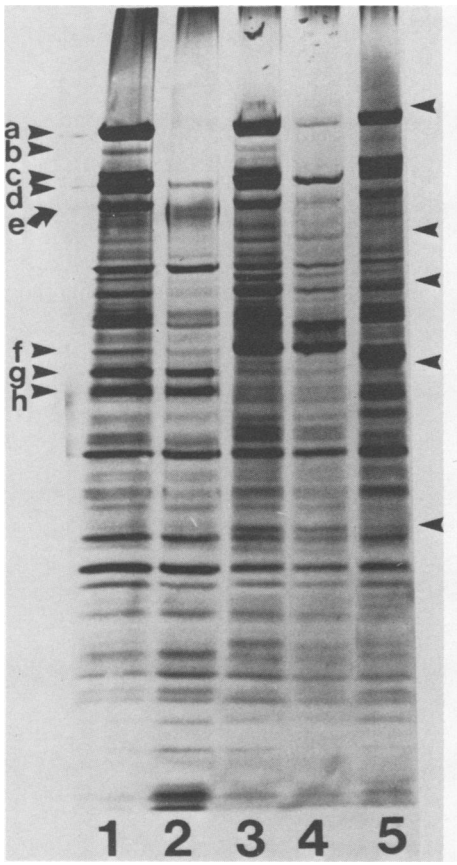

FIG. 1. SDS-PAGE analysis of SDS extracts of hydrophobic and hydrophilic *S. mutans* strains. Whole cells were suspended in 2% SDS–5% 2-mercaptoethanol and placed in boiling water for 30 min. The particulate matter was removed by centrifugation, and the supernatant was analyzed by SDS-PAGE. Lane 1, *S. mutans* LK; lane 2, *S. mutans* LK36; lane 3, *S. mutans* GW; lane 4, *S. mutans* GW36; lane 5, *S. mutans* 6715. Molecular weight standards: myosin, 200,000; galactosidase, 116,250; phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000.

extracts of LK36. The HMW proteins were not extracted with 3% SDS at room temperature. Physical procedures such as sonication, passage through a French pressure cell, or grinding in a Mini-Mill did not release the proteins. The HMW proteins were not found in SDS extracts of cells which had previously been incubated with trypsin. The supernatants from trypsin-treated cells did not possess any silver nitrate-staining bands which exhibited migratory patterns similar to those of the hot-water extracts of untreated cells. Extraction of whole LK cells with LiCl, urea, β-mercaptoethanol, dilute acid, dilute base, or NaCl at room temperature did not remove the HMW proteins. Urea extracts (100°C) of whole cells presented a different gel profile; the 190,000-molecular-weight protein was absent, but a larger protein was present (Fig. 2B, lane 4).

Culture supernatants obtained from overnight cultures were concentrated by ultrafiltration on Diaflo XM-50 membranes and analyzed by gel electrophoresis. Supernatant from the hydrophilic strain LK36 contained proteins with molecular weights similar to those of proteins a, b, c, d, and e extracted from the cell walls of the hydrophobic strains (Fig. 2A, lane 4). Proteins a, c, d, and e were also present in LK culture supernatant, but in much lower concentrations. The 190,000-molecular-weight protein stained with periodate-Schiff reagent. The uninoculated-medium control did not contain any high-molecular-weight, Coomassie blue-, or $AgNO_3$-stained bands.

When analyzed by nondenaturing PAGE, the HMW proteins from the concentrated LK36 supernatant migrated into the gel, whereas the protein from the hot-water extract of LK did not enter the gel.

SDS-PAGE analysis of cell walls. To provide more evidence for the location of the HMW proteins, cell walls were isolated from physically disrupted cells. Crude cell wall preparations isolated by differential centrifugation were extracted with SDS (100°C), and the extracts were analyzed by 7.5% SDS-PAGE. Crude cell wall preparations of LK (Fig. 2B, lane 1) possessed the proteins found in whole cell extracts. Silver nitrate-staining bands corresponding to proteins c, d, and e appeared in SDS extracts of LK36. To see these bands, it was necessary to heavily overload the gel, indicating that, in contrast to those of strain LK, these bands are present at a low level. The large number of proteins in the extracts indicates that the crude wall preparations are probably contaminated with ribosomes and cytoplasmic membranes. Extraction of the cell walls with 3% SDS at room temperature before hot-SDS extraction did not remove the HMW proteins but did reduce the amount of low-molecular-weight proteins. Digestion of walls with RNase and DNase did not alter the protein profile of the hot-SDS extracts.

Purified cell walls extracted with 3% SDS at room temperature were incubated overnight with mutanolysin, and the material solubilized from this treatment was then analyzed by SDS-PAGE. The digest of LK (Fig. 2A, lane 5) contains the 190,000-molecular-weight protein and a protein migrating a similar distance to protein e. A number of other bands can be seen, but they do not correspond to the bands extracted with SDS. Mutanolysin digests of LK36 walls contained protein e.

Immunological analysis. Hot-water extracts and concentrated supernatants of LK and LK36 were electrophoresed in an SDS-polyacrylamide gel and then transferred electrophoretically to nitrocellulose. The nitrocellulose was reacted with anti-LK serum, and antigen-antibody complexes were stained with antibody conjugated to immunoperoxidase.

Extracts of whole cells of LK contained an antigen reactive with anti-LK serum which migrated to the same position as the 190,000-molecular-weight protein (Fig. 3A, lane 1). This antigen was not present in extracts of LK36 (Fig. 3A, lane 2). In addition, LK extracts contained an antigen corresponding to protein d. Antigens corresponding to proteins b and c were found as weakly staining bands in extracts of LK. LK36 extracts contained only the antigen corresponding to protein e. Extracts of hydrophobic strain GW were similar to those of LK. Mutanolysin digests of SDS-extracted LK cell walls contained antigens corresponding to proteins a (molecular weight, 190,000), b, and e (Fig. 3A, lane 5). A number of antigens reactive with anti-LK serum were seen in the mutanolysin digest of LK walls (Fig. 3A, lane 5); one of these antigens migrated to a point midway between protein a and d, whereas the remainder had a much lower molecular weight. Corresponding digests of LK36 were found to possess only the antigen corresponding to protein e (Fig. 3A, lane 6).

Culture supernatant prepared from LK36 possessed antigens reactive with anti-LK serum; these corresponded to proteins a, b, c, and e found in the extracts of LK (Fig. 3A, lane 3). Culture supernatant from LK contained antigens

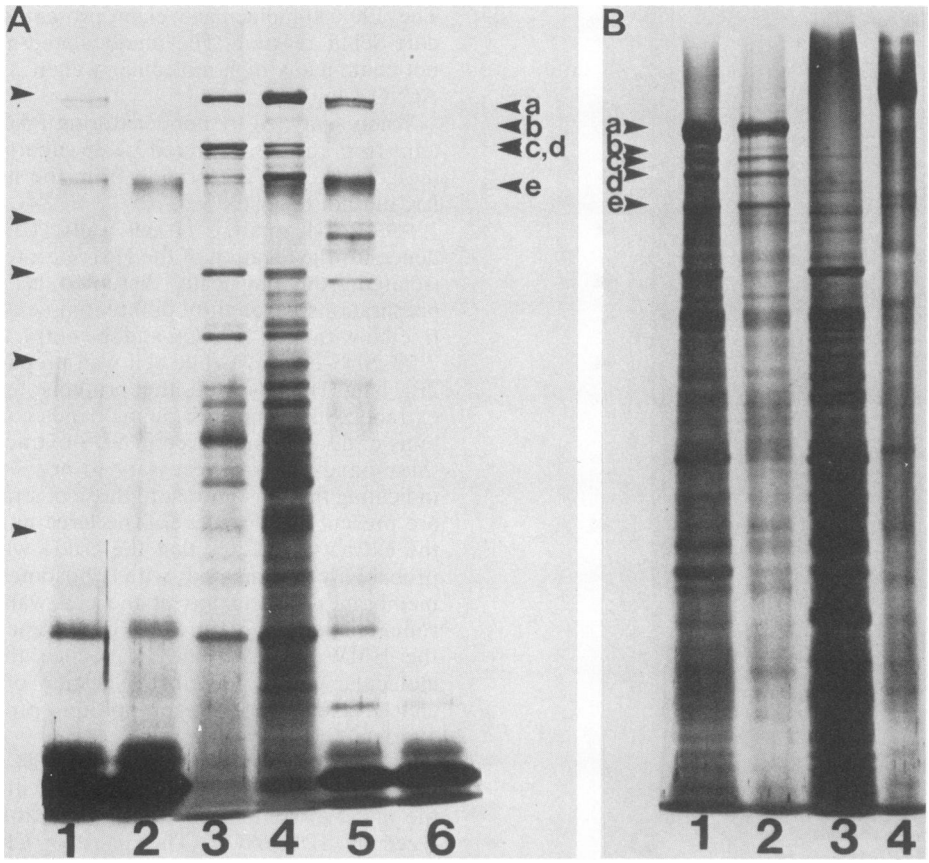

FIG. 2. SDS-PAGE analysis of whole cells and cell walls of *S. mutans* LK and LK36 whole cells. (A) Lane 1, extract of LK prepared by heating cells in distilled water at 100°C for 30 min; lane 2, extract of LK36 prepared by heating cells in distilled water for 30 min; lane 3, LK culture supernatant concentrated 50-fold by filtration on an Amicon XM-50 membrane; lane 4, LK36 culture supernatant concentrated as described for strain LK; lane 5, mutanolysin-solubilized cell walls of LK; lane 6, mutanolysin-solubilized cell walls of LK36. (B) Lane 1, extract of LK cell walls prepared by boiling with SDS and 2-mercaptoethanol for 30 min; lane 2, extract of LK cell walls previously extracted with 3% SDS at room temperature before being boiled with SDS and 2-mercaptoethanol for 30 min; lane 3, extract of LK36 cell walls prepared by boiling with SDS and 2-mercaptoethanol for 30 min; lane 4, extract of LK cell walls prepared by boiling with 8 M urea for 30 min.

corresponding to proteins a, c, and e but were lacking the antigen corresponding to protein b. Supernatants from LK36 contained considerably more of the antigens than did LK supernatants. In other preparations, culture supernatants of both LK and LK36 were found to contain equivalent amounts of an antigen with a molecular weight of approximately 43,000. This antigen was found in extracts of whole cells of LK but not LK36.

Anti-LK serum adsorbed with LK36 possessed antibody reactive with the 190,000-molecular-weight protein extracted from LK (Fig. 3B, lane 1); there was no reaction with LK36 extracts (lane 2). The adsorbed antiserum reacted strongly with the 190,000-molecular-weight antigen found in culture supernatant obtained from LK36 (Fig. 3B, lane 3).

Immunoelectrophoretic analysis showed that LK36 supernatant possessed three antigens reactive with anti-LK serum. Extracts of LK possessed similar antigens, whereas extracts of LK36 had only two antigens.

DISCUSSION

The discovery of *S. mutans* strains which had lost their hydrophobic properties created the opportunity to compare the cell surface of these strains with that of their wild-type parents.

In this study, we have shown that extracts of whole cells of hydrophobic strains possess a family of HMW proteins which are not present on cells with greatly decreased hydrophobic properties. The proteins can be found in purified cell walls, suggesting that they are located on the bacterial cell surface. This conclusion is supported by the observations that antiserum raised against whole cells reacts with proteins (Fig. 3) and that trypsin treatment of whole cells destroys the proteins. The susceptibility of the proteins to proteolytic attack suggests that the molecules are located on the external surface of the walls; however, it cannot be ruled out that the proteins are buried in the wall and that the trypsin acts indirectly by hydrolyzing a molecule which is responsible for anchoring these proteins to the cell wall.

According to Westergren and Olsson (36), serotype d *S. mutans* strains maintain their hydrophobic properties when subcultured on laboratory medium. In our study, extracts of *S. mutans* C6715 (serotype d) look similar to those of the fresh isolates LK and GW, despite the fact that C6715 has been cultivated on laboratory medium for many years. The implication of these findings is that it is necessary to characterize the hydrophobic properties of organisms that are being used in adherence studies.

The influence of growth conditions on the adherence properties (4, 16) and chemical characteristics (18) of oral

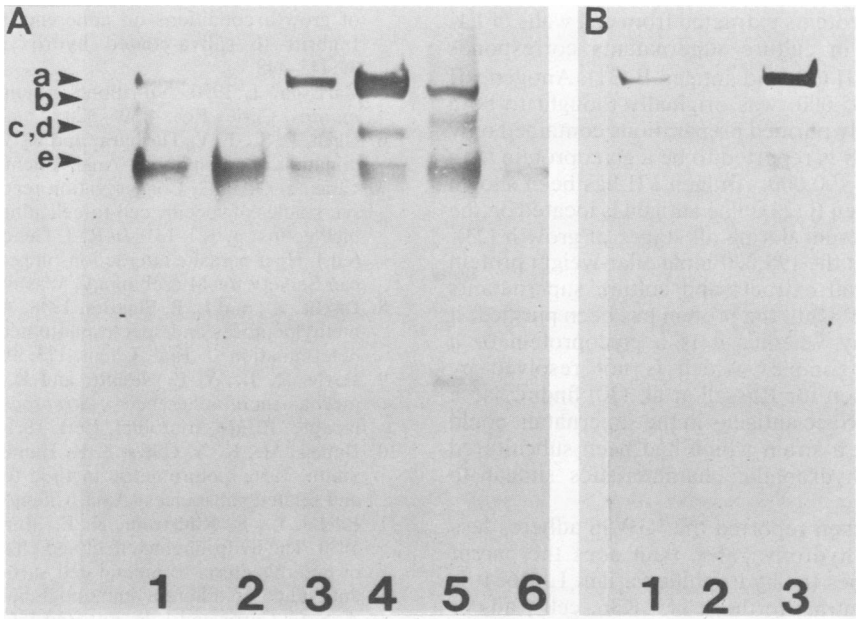

FIG. 3. Western blots of cell extracts and culture supernatants of LK and LK36 separated by SDS-PAGE. (A) Antigens adsorbed to nitrocellulose were reacted with rabbit anti-LK serum. Lane 1, SDS–2-mercaptoethanol extracts of whole cells of LK; lane 2, SDS–2-mercaptoethanol extracts of whole cells of LK36; lane 3, concentrated culture supernatant from LK; lane 4, concentrated culture supernatant from LK36; lane 5, mutanolysin-solubilized LK cell walls; lane 6, mutanolysin-solubilized LK36 cell walls. (B) Antigens adsorbed to nitrocellulose were reacted with rabbit anti-LK serum which had been adsorbed with LK36. Lane 1, SDS–2-mercaptoethanol extract of LK; lane 2, SDS–2-mercaptoethanol extract of LK36; lane 3, concentrated culture supernatant from LK36.

streptococci has been well documented. The observation that cells grown in brain heart infusion broth are less hydrophobic than cells grown in tryptic soy broth supports the concept that environment influences the surface properties of microorganisms. In this case, the effect of brain heart infusion broth may be to reduce the synthesis of molecules conferring hydrophobicity, or it may simply be that hydrophobicity is masked by the adsorption of factors from the growth medium onto the cell surface (14).

Although the proteins associated with hydrophobicity were not present in the cell walls of the hydrophilic variant, they appeared in the culture supernatant of the organism, suggesting that our inability to find them in the cell wall was not due to inhibition of synthesis but, rather, to their inability to be incorporated into the cell wall.

The observation that protein obtained from culture supernatant was able to enter a nondenaturing gel, whereas the protein extracted from cell walls was not, suggests that the material extracted from the walls might exist in the form of large complexes which cannot enter the gel until they are reduced to their component parts by being boiled in SDS. Protein released by the hydrophilic strains may exist as subunits that have not been incorporated into the complex that forms within the cell wall. These complexes could exist as multimers of a single subunit or as complexes containing a number of different subunits. In either case, there could be a number of functionally unique structural components on the cell surface of gram-positive organisms. Cisar (7) has shown that *Actinomyces* spp. possess fibrillar structures with different adherence and aggregation properties. Recently, Weerkamp and Jacobs have speculated that surface antigens of *S. salivarius* may exist as discrete fibrillar structures (34). The inability to physically remove the protein by sonication or grinding indicates that the proteins are tightly bound to the cell wall. It is unlikely, however, that they are covalently bound to the structurally essential peptidoglycan because of the ease with which they can be extracted in hot water.

Chemical analysis did not indicate any marked alterations in the proportion of cell wall constituents. The amino acid compositions of hydrophobic and hydrophilic cell walls were similar, but this does not rule out the possibility that there are unique hydrophobic regions within some of the cell wall proteins. If these hydrophobic domains were oriented towards the cell surface, hydrophobicity would be enhanced.

Miörner et al. (22) have suggested that LTA is responsible for the hydrophobicity of *S. pyogenes*, whereas the work reported here supports the concept that cell surface proteins are associated with the hydrophobicity of *S. mutans*. In the case of *S. mutans*, the LTA content of the hydrophobic strain was found to be similar to that of the hydrophilic variant. Digestion with trypsin or extraction with hot water removed a number of cell wall proteins and concurrently reduced hydrophobicity to zero. Despite these observations, it is possible that LTA exists in tight association with the protein and that it is removed from the cell coincident with protein degraded by enzymatic hydrolysis or with protein released by hot-water extraction. It is also possible that LTA may be oriented in a different manner when certain cell wall proteins are missing.

The hydrophilic variants isolated by Westergren and Olsson (36) demonstrated reduced hydrophobicity, but they did not completely lose the ability to bind to hexadecane. This observation may be the result of the partial loss of a hydrophobic component or may mean that there is more than one component, only one of which is lost during laboratory culture. Regardless of which explanation is correct, the residual hydrophobicity observed in LK36 is lost when cells are treated with trypsin.

The largest of the proteins extracted from cell walls of LK and which is found in culture supernatants corresponds closely with antigen I/II (30) and antigen B (31). Antigen I/II (molecular weight, 185,000) was originally thought to be a glycoprotein, but highly purified preparations contained only protein (39). Antigen B is reported to be a glycoprotein with a molecular weight of 190,000. Antigen I/II has been shown to be identical to antigen B (23). The antigen is located on the cell surface and is present during all stages of growth (23). We have observed that the 190,000-molecular-weight protein band found in cell wall extracts and culture supernatants contains carbohydrate. Until the protein has been purified, it is not possible to say whether it is a glycoprotein or a protein-carbohydrate complex which is not resolved by SDS-PAGE. The reason for Russell et al. (30) finding large amounts of the cell surface antigens in the supernatant could be because they used a strain which had been subcultured frequently and had hydrophilic characteristics similar to those of LK36.

Westergren and Olsson reported that GW36 adheres less well to saliva-coated hydroxyapatite than does the parent GW but better than does the hydrophilic variant LK36. It is interesting that, in contrast to those of LK36, cell walls of GW36 retained a small amount of the 190,000-molecular-weight protein. In light of the relationship among hydrophobicity, adherence, and the presence of the 190,000-molecular-weight protein in the cell wall, it is tempting to speculate that antigen I/II may be an adhesin involved in forming hydrophobic bonds with hydrophobic regions of the salivary pellicle. Antibody combining with the adhesin would impair the ability of *S. mutans* to colonize and would provide an explanation for the protection against caries conferred by immunization with this hydrophobicity-associated antigen. Interference with hydrophobic bonding would preclude the stabilizing effect that is postulated to be essential for forming bacteria-pellicle bonds (9).

In addition to the HMW protein, there are a number of other cell wall proteins absent in the poorly adhering hydrophilic strains. Each of these molecules may be the hydrophobic moiety conferring adhesive properties on the cell. The adhesin is possibly a complex of a number of these molecules. The binding activity may be dependent on having all the subunits organized into a structure with hydrophobic adhesin properties. Antibodies might be able to block binding by interacting with subunits, but binding would be dependent on having an intact adhesin. Subunit I/II may act as an anti-adhesin-inducing antigen. The biological function of the proteins released by hydrophilic variants is under study.

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