Cell-surface Proteins of *Streptococcus sanguis* Associated with Cell Hydrophobicity and Coaggregation Properties

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Incubating cells of *Streptococcus sanguis* with sodium lauroyl sarcosinate, under conditions that did not cause lysis, solubilized material comprising 5–8% of the cell dry weight. The treatment reduced cell hydrophobicity, and reduced the ability of the cells to coaggregate with *Actinomyces* spp. The extract contained about 20 polypeptides and these were identified as being cell-surface components on the basis of one or more of the following criteria: (a) being degraded when cells were incubated with protease; (b) being labelled when cells were iodinated using a lactoperoxidase-catalysed reaction; (c) reacting with antibodies raised to fixed whole cells. Eight of the polypeptides accounted for more than 70% of the total protein extracted, and one component (molecular mass 16 kDa) was hydrophobic. The cell-surface proteins described are implicated in cell hydrophobicity and coaggregation.

INTRODUCTION

Streptococcus sanguis is one of the first colonizers of the tooth surface and is a major component of human dental plaque (Gibbons & van Houte, 1975; van Houte *et al.*, 1970; Hamada & Slade, 1980). Adherence of the organism to the tooth surface involves electrostatic and hydrophobic interactions between surface components of the *Streptococcus* and adsorbed salivary glycoproteins comprising the pellicle (Murray *et al.*, 1982; Nesbitt *et al.*, 1982a, b; Rosenberg *et al.*, 1983; Eifert *et al.*, 1984). *S. sanguis* also coaggregates with other oral bacteria (Gibbons & Nygaard, 1970) and the specific aggregations, such as those with *Actinomyces viscosus* and *A. naeslundii* (Cisar *et al.*, 1979; Kolenbrander & Williams, 1981; Sato *et al.*, 1984) contribute to the formation of dental plaque.

It appears that proteins present in the cell envelopes of streptococci mediate attachment of the cells to various surfaces including epithelia (Ellen & Gibbons, 1972) and salivary pellicle (Liljemark & Bloomquist, 1981), and to other bacteria (Weerkamp & McBride, 1981). Following the observation by Weerkamp & Jacobs (1982) that adherence-defective mutants of *S. salivarius* lacked several of the normal cell-wall proteins, considerable interest has centred on the high molecular mass proteins present in the cell wall of *S. mutans* (Russell, 1979; Nesbitt *et al.*, 1980; Ogier *et al.*, 1984; Smith *et al.*, 1984) and their possible role in adherence. Recently, McBride *et al.* (1985) have suggested that various proteins present in the cell wall of hydrophobic strains of *S. sanguis* may be involved in adherence of cells to saliva-coated hydroxyapatite.

To identify components at the cell surface of *S. sanguis* involved in adhesion, we have looked first at ways of removing proteins specifically from the surface of the cells. This paper describes the isolation of a number of previously unidentified proteins that are present at the cell surface of *S. sanguis* and that may confer hydrophobicity and coaggregation properties.

Abbreviations: BSA, bovine serum albumin; 2-MeH, 2-mercaptoethanol; PAS, periodic acid-Schiff; PMSF, phenylmethylsulphonyl fluoride; SLS, sodium lauroyl sarcosinate.

METHODS

Chemicals. *N*-Lauroylsarcosine, Nonidet P-40, egg-white lysozyme (EC 3.2.1.17), mutanolysin, chymotrypsin, *Streptomyces griseus* protease type XIV (pronase), *Staphylococcus aureus* protease type XVII (V8 protease), lactoperoxidase (EC 1.11.1.7), amino acids, vitamins, and molecular mass markers for gel electrophoresis were purchased from Sigma. Zwittergents were obtained from Calbiochem-Behring. All other chemicals were from BDH unless otherwise stated.

Bacteria. *S. sanguis* strain OB11 is a Challis strain (serotype 2; Rosan, 1973) kindly provided by Dr F. L. Macrina, Virginia Commonwealth University, Va, USA, and was used throughout this work. Other strains used were FW35 (serotype 1; Nugent & Cole, 1977) and Channon (NCTC 7869) (serotype 2; Cole *et al.*, 1976), which were kindly supplied by Dr J. Ranhand, National Institutes of Health, Frederick, Md, USA. Stocks were kept as freeze-dried suspensions in horse serum (Difco); the strains conformed to the physiological description of *S. sanguis* (Hardie & Bowden, 1976). *Actinomyces viscosus* strain T14V and *A. naeslundii* strain W1544 were gifts from Dr J. O. Cisar, National Institute of Dental Research, Bethesda, Md, USA.

Media. Bacteria were cultured on BHYN agar, which contained per litre brain heart infusion broth (Difco), 37 g; yeast extract (Difco), 5 g; neopeptone (Difco), 5 g; and agar, 15 g. Plates were incubated at 37 °C in a Gas-Pak (BBL) System for 24–36 h. Liquid cultures were grown at 37 °C in screw-capped bottles or tubes as stationary cultures in BHY medium (brain heart infusion broth, 37 g l⁻¹; yeast extract, 5 g l⁻¹). *S. sanguis* was also grown in a defined medium containing glucose, sucrose or lactose as carbon and energy source. The defined medium was based on that described by Carlsson (1972) and contained per litre K₂HPO₄, 10 g; KH₂PO₄, 2 g; (NH₄)₂SO₄, 1.3 g; NaCl, 2 g; MgSO₄·7H₂O, 0.5 g; CaCl₂·2H₂O, 0.15 g; L-glutamate, 0.5 g; L-arginine·HCl, 0.2 g; L-cysteine·HCl, 0.2 g; L-tryptophan, 0.02 g; Casamino acids (Difco), 2 g; MnCl₂·4H₂O, 20 mg; FeCl₃·6H₂O, 1 mg; pyridoxine·HCl, 24 mg; nicotinic acid, 4.6 mg; pantothenic acid, 2.4 mg; riboflavin, 0.4 mg; thiamin·HCl, 0.1 mg; D-biotin, 12 µg; glucose (sucrose or lactose), 8.0 g; initial pH 7.4. Sterile stock solutions of vitamins, MgSO₄, CaCl₂, glutamate, tryptophan, arginine and cysteine were added to the bulk medium after autoclaving.

Extraction of whole cells. An exponential-phase culture (10 ml) of *S. sanguis* strain OB11 at OD₆₀₀ 0.7 (1.5 × 10⁹ c.f.u. ml⁻¹) was centrifuged for 10 min at 6000 g at 20 °C, the supernatant was discarded and the cells were washed with 10 ml 0.15 M-NaCl. To the washed-cell pellet was added 0.2 ml of a 1% (w/v) solution of sodium lauroyl sarcosinate (SLS) (or other detergent, see Results) in 10 mM-Tris/HCl (pH 7.5) containing 1 mM-Na₂EDTA (TE buffer). The cells were vortex-mixed and the suspension was transferred to an Eppendorf microfuge tube. For extraction of cells with other agents, e.g. 1 M-NaCl or 8 M-urea, 0.1 ml was used in place of the 0.2 ml detergent solution. After 20 min at 20 °C, the cell suspension was centrifuged (6000 g, 10 min, 20 °C) and a portion of the supernatant (0.15 ml) was carefully removed from above the pellet into a fresh tube and centrifuged as before. A portion of the supernatant (0.10 ml) was removed, bromophenol blue (0.01%) in 70% (v/v) glycerol was added (0.01 ml) and the extract was then either heated at 70 °C for 10 min and subjected to electrophoresis (see below) or stored at -23 °C. To extract a larger quantity of cells, the volume of detergent solution was increased proportionately, and then the extract was diluted with 8 vols 80% (v/v) ethanol, left overnight at -23 °C, and the precipitate was collected by centrifugation (15000 g, 20 min, 4 °C). The pellet was suspended in distilled water (0.5–1.0 ml), and stored at -23 °C.

Protease treatment of cells. Exponential-phase cells were harvested and washed as described above, and the pellet was suspended in 10 mM-Tris/HCl, pH 7.5 (1 ml). Pronase (stock solution 10 mg ml⁻¹ in 10 mM-Tris/HCl, 0.15 M-NaCl, pH 7.5, self-digested for 2 h at 37 °C), chymotrypsin or V8 protease (stock solutions 10 mg ml⁻¹ also in Tris/NaCl) was added, and the suspension was incubated at 20 °C for 20 min. Distilled water was added to 10 ml, the suspension was centrifuged (6000 g, 5 min), and the pellet was suspended in 10 mM-Tris/HCl, pH 7.5 containing 1 mM-phenylmethylsulphonyl fluoride (PMSF). The cells were pelleted by centrifugation as before, washed again and then extracted with SLS as described above.

Electron microscopy. Cells were fixed with 1% (w/v) osmium tetroxide, embedded in Epon 812 resin, then thin-sectioned, stained with uranyl acetate and examined. For negative-staining, one drop of turbid cell suspension was placed on a Formvar grid, excess liquid was removed with filter paper, and the grid was then allowed to dry for 5 min at 20 °C. Phosphotungstic acid (1%, w/v; 5 µl) was added for 10 s and excess was dried off with filter paper.

Cell fractionation. Exponential-phase cells (100 ml culture) were harvested by centrifugation (6000 g, 10 min, 4 °C), the supernatant was discarded and the pellet was washed with 50 ml 0.15 M-NaCl. The pellet was suspended in 10 mM-Tris/HCl, pH 7.5, containing 1 mM-EDTA and 1 mM-PMSF (TEP buffer) and the cells were broken by shaking them with glass beads (0.10–0.11 mm diam.; 7 ml beads plus 10 ml cells) in a Braun homogenizer run at full speed for 4 min with carbon dioxide cooling to 4 °C. The beads were allowed to settle and the suspension was decanted. The beads were washed with TEP buffer (5 ml), the washing was combined with the broken cell suspension, and the suspension centrifuged (3000 g, 5 min, 4 °C). The supernatant was decanted off the pellet into a fresh tube and re-centrifuged as before, and the process was repeated a further time. Finally, the supernatant was removed carefully from the pellet leaving approximately 1 ml behind with the pellet, and the supernatant was centrifuged for 90 min at 100000 g (4 °C). The supernatant from this centrifugation is referred to as the 'high-speed

supernatant', and the pellet is referred to as the 'cell-envelope fraction'. To extract proteins for electrophoresis, the cell-envelope fraction was suspended in 10 mM-Tris/HCl, pH 8.8, containing 1% (w/v) SDS and 0.5% (v/v) 2-mercaptoethanol (2-MeH), heated at 70 °C for 10 min, and then centrifuged (5000 g, 5 min, 20 °C). The clarified supernatant was mixed with bromophenol blue and glycerol as described above and subjected to SDS-PAGE.

Solubilization of cell wall. After extraction of the envelope fraction with SDS/2-MeH, the pellet was suspended in distilled water (0.2 ml) and boiled for 10 min. The suspension was centrifuged (5000 g, 10 min, 20 °C), the pellet was suspended in 0.5 ml distilled water and heated again to 100 °C for 10 min, and then centrifuged as before. The pellet was suspended in 0.2 ml 10 mM-Tris/HCl, pH 7.5, containing 0.1 M-NaCl, lysozyme (1 mg ml⁻¹) and mutanolysin (20 units), a drop of toluene was added and the mixture was incubated for 14 h at 37 °C. After adding SDS to a final concentration of 1% and 2-MeH to 0.5%, the suspension was heated at 70 °C for 10 min and centrifuged, and the supernatant was mixed with glycerol and bromophenol blue for electrophoresis.

Concentration of exocellular culture fluid. An exponential-phase culture (5 l) of *S. sanguis* in defined medium containing glucose was centrifuged at 5000 g for 10 min (4 °C) and the supernatant (exocellular fluid) was filtered under vacuum through a Whatman GF/C glass fibre filter. The filtrate was concentrated to 200 ml by ultrafiltration through an Ulvac membrane (10 kDa cut-off). The concentrate was brought to 70% saturation with ammonium sulphate at 4 °C and, after several hours of stirring the suspension, the precipitate was collected by centrifugation at 7000 g for 20 min (4 °C). The pellet was suspended in TEP buffer and dialysed against deionized water (5 l) for 16 h at 4 °C. The dialysed suspension contained about 2 mg protein ml⁻¹, as measured by the Lowry method. For electrophoresis, a portion was mixed with an equal volume of 10 mM-Tris/HCl, pH 8.8, containing 2% (w/v) SDS and 1% (v/v) 2-MeH, heated to 70 °C for 10 min, and mixed with tracking dye and glycerol as described above.

SDS-PAGE. Samples were electrophoresed through 13% (w/v) acrylamide gels containing 0.1% SDS by the system of Laemmli & Favre (1973). Gels were stained with Coomassie blue, de-stained, scanned with a densitometer, and autoradiographed as described by Jenkinson *et al.* (1981). Gels were silver-stained by the method of Merrill *et al.* (1981) and stained for carbohydrate by the periodic acid-Schiff (PAS) procedure of Fairbanks *et al.* (1971). Molecular masses of proteins were estimated from their distances of migration by reference to a plot relating migration distances for six marker proteins to log molecular mass. The markers were bovine serum albumin (BSA) (66 kDa), chicken egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa) and bovine milk α -lactalbumin (14.2 kDa). Proteins were transferred from acrylamide gels to nitrocellulose by electroblotting (Towbin *et al.*, 1979) in 25 mM-Tris, 192 mM-glycine, 20% (v/v) methanol, pH 8.3, at 30 V for 16 h.

Phenol extraction of cells. Cells in exponential phase were harvested by centrifugation (6000 g, 10 min, 4 °C), washed with 0.15 M-NaCl and suspended to 5 mg ml⁻¹ in 0.01 M-MgSO₄. A crude lipoteichoic acid preparation was obtained by hot (68 °C) phenol extraction as described by Kessler & Shockman (1979).

Antiserum and immunodetection. Antibodies to glutaraldehyde-fixed cells were raised in New Zealand male rabbits. Cells (70 mg dry wt) were fixed with 0.25% (v/v) glutaraldehyde in 0.1 M-KH₂PO₄/KOH, pH 7, containing 0.15 M-NaCl (PBS) at room temperature for 40 min. They were then diluted to 10 ml with PBS, washed three times with PBS, suspended in PBS and portions (0.5 ml containing approx. 8×10^{10} cells) were injected intramuscularly on four separate occasions at intervals of 3 d. Then 20 d after the last of these injections, the rabbits were boosted with a further 5×10^{10} cells in PBS (0.5 ml), and bled after 5–7 d. Antibodies to extracellular material were raised as follows: a portion (0.25 ml) of suspension containing approximately 2 mg protein ml⁻¹ in PBS was mixed with an equal volume of Freund's complete adjuvant (Gibco Laboratories) and injected intramuscularly. After 21 d, the rabbits were boosted with an identical injection to the first, and bled after a further 5–7 d. Six weeks later, they were given another booster and bled again 5–7 d later. Proteins blotted on to nitrocellulose were detected by antibody-binding as follows: the blot was blocked with 3% (w/v) BSA in PBS (pH 7.5) for 1 h at 20 °C, and then incubated with antiserum diluted 200-fold in 1% (w/v) BSA in PBS (pH 7.5) for 2–3 h at 20 °C. The blot was washed twice with 0.1% Tween 20 in PBS (50 ml, 10 min each time), and then incubated in 100 ml 1% (w/v) BSA in PBS containing 0.2 μ g [¹²⁵I]Protein A labelled by the chloramine-T method (Greenwood *et al.*, 1963) to a specific activity of 10–20 μ Ci μ g⁻¹ (370–740 Bq μ g⁻¹). After 1–2 h incubation at 20 °C the blots were washed with 0.1% Tween 20 in PBS (five times, 50 ml each) and then with PBS until the spent wash fluid was virtually free of radioactivity. The blots were dried at 60 °C for 10 min, then exposed to Kodak medical X-ray film at room temperature for 1–5 d as necessary. To check that the proteins had blotted on to the nitrocellulose paper, the blot was stained with India ink (Hancock & Tsang, 1983) either before or after autoradiography. A lane of molecular mass marker proteins was included in each blot and by staining the blot with ink after autoradiography an accurate estimation of the molecular masses of the reactive antigens was obtained.

Surface iodination of cells. Cells (approximately 70 mg dry wt) were harvested from exponential-phase cultures by centrifugation (6000 g, 10 min, 4 °C), washed once with 0.15 M-NaCl and suspended to a density of 30 mg dry wt ml⁻¹ in PBS. A portion of the cell suspension (1 ml) was mixed with ¹²⁵I (0.5 mCi; Amersham), and the cells were surface-labelled using lactoperoxidase and hydrogen peroxide by the method of Marchalonis (1969) and the procedure described by Swanson *et al.* (1975). The reaction was terminated by adding 5 mM-L-cysteine. HCl in PBS

containing 5 mM-KI. The cells were harvested by centrifugation (5000 g, 10 min, 4 °C) and then washed twice with 10 mM-Tris/HCl, pH 7.5, containing 1 mM-PMSF. The specific activity of the radio-iodinated cells was 0.2–0.5 μCi (mg cell dry wt)⁻¹. To measure radioactivity in various fractions from broken cells, portions (0.01–0.02 ml) were added to 5 ml 5% (w/v) TCA at 4 °C. BSA solution was added (1 ml, 50 μg ml⁻¹), mixed, and after 10 min the precipitate was collected by filtration under vacuum through a Whatman GF/C fibre filter. The filters were washed under vacuum with 5% (w/v) TCA (10 ml) and with absolute ethanol (10 ml), and then counted for radioactivity in an LKB 1271 Riagamma counter.

Hydrophobic interaction chromatography. A sample of SLS extract (0.2 ml) containing about 20 μg protein was made to 1 M in ammonium sulphate and applied to a column of Octyl-Sepharose CL-4B (Pharmacia), bed volume 2 ml. The column was eluted (flow rate 20 ml h⁻¹) first with 10 ml 0.01 M-NaH₂PO₄/NaOH buffer, pH 7, to remove unbound material, and then with a gradient of decreasing ammonium sulphate concentration and increasing SLS concentration (final concentrations 0 g l⁻¹ and 5 g l⁻¹ respectively). Fractions (0.2 ml) were collected, mixed with 1.3 ml 4% (w/v) TCA and, after they had stood at 4 °C for 1 h, the precipitated protein was pelleted by centrifugation, dissolved in sample buffer and subjected to SDS-PAGE.

Cell hydrophobicity. Exponential-phase cells were washed twice with PUM buffer (containing per litre 22.2 g K₂HPO₄·3H₂O, 7.2 g KH₂PO₄, 1.8 g urea, 0.2 g MgSO₄·7H₂O, pH 7.1; Rosenberg *et al.*, 1980) and suspended to an OD₆₀₀ of approx. 0.6 (1×10^9 c.f.u. ml⁻¹) in PUM buffer (1.5 ml) and vortex-mixed with hexadecane or xylene for 2 min (Rosenberg *et al.*, 1980). After allowing the suspensions to settle for 15 min to permit phase separation, the decrease in OD₄₅₀ of the aqueous lower phase was used as a measure of the cell-surface hydrophobicity.

Coaggregation assay. *A. viscosus* T14V and *A. naeslundii* W1544 were grown in BHY medium and harvested in mid-exponential phase of growth (OD₆₀₀ approx. 0.7). The cells were washed by three cycles of centrifugation (6000 g, 10 min, 4 °C) and suspension of the pellet in coaggregation buffer (1 mM-Tris/HCl, pH 8.0, containing 0.1 mM-MgCl₂, 0.1 mM-CaCl₂ and 0.15 M-NaCl; Cisar *et al.*, 1979). Cells of *S. sanguis* were grown in defined medium with glucose and prepared for coaggregation in an identical fashion. Actinomycete cell suspension (1 ml; OD₆₀₀ approx. 0.5, equivalent to 5×10^8 c.f.u. ml⁻¹) was vortex-mixed for 10 s with an equal volume of *S. sanguis* cell suspension containing about 1×10^9 c.f.u. ml⁻¹ (OD₆₀₀ approx. 0.6). Controls of actinomycete or streptococcal cell suspensions alone were included in every assay. The suspensions were allowed to stand at room temperature for 3 h, then scored for coaggregation. They were then allowed to stand at room temperature overnight, mixed the next day, and scored again after 2 h. The degree of coaggregation was scored as 0 (negative), 1 (questionable), 2 (weak) or 4 (marked). The extent of coaggregation between bacterial pairs was also determined spectrophotometrically and expressed as a percentage, as described by Cisar *et al.* (1979), but using the coaggregation buffer described above.

Thin-layer chromatography (TLC). Cell extracts were made to 1 M-HCl and heated to 100 °C in sealed vials for 60 min (partial hydrolysis) or for 10 h (complete hydrolysis). The hydrolysates were freeze-dried, suspended in distilled water and portions were applied to the origin of a strip of TLC aluminium roll pre-coated with silica gel 60 F₂₅₄ (Merck). The sugars were separated by ascending chromatography, using a solvent containing propan-1-ol/acetone/1 M-lactic acid (7:1:1, by vol.). The chromatogram was sprayed with aniline/diphenylamine (Hansen, 1975), and the blue sugar spots were developed by heating the chromatogram at 110 °C for 30–60 min. Sugars were identified by comparison of their *R_F* values with those of standard sugars run on the same chromatogram.

Amino acids and phosphate esters were separated by TLC in a solvent containing propan-1-ol/ammonia/water (6:3:1, by vol.) (Archibald, 1972). The chromatogram was sprayed first with ninhydrin (0.2%, w/v) in acetone and heated briefly at 100 °C to detect amino compounds. It was then sprayed with ammonium molybdate (Mann *et al.*, 1979) to detect phosphate. Suitable standards were run with each set of samples.

Estimation of hexose, phosphorus and protein. Hexose was determined by the phenol/sulphuric acid method of Dubois *et al.* (1956), using D-glucose as standard, and phosphorus was estimated as inorganic phosphate by the method of Chen *et al.* (1956), using K₂HPO₄ as standard. Protein concentration was measured by the Lowry method with BSA as standard.

RESULTS

Extraction of cell-surface proteins

Experiments were initially done to find a suitable method for removing cell-surface proteins of *S. sanguis* strain OB11 without disrupting the cells. Cells were treated with a variety of agents, e.g. high salt, EDTA, chaotropic salts and detergents, the extracts obtained were subjected to SDS-PAGE and the fractionated proteins were identified by staining the gels with silver nitrate.

No significant amount of protein (as detected by silver-staining of extracts subjected to SDS-PAGE) was removed from the cells when they were treated with 1 M-NaCl, 1 M-MgCl₂ or 0.1 M-EDTA at 20 °C for 20 min. On the other hand, extracts from cells incubated at 20 °C for 20 min with 1% (w/v) SDS contained 15 to 20 polypeptides (Fig. 1, lane A). A similar gel pattern was

Fig. 1

Fig. 1. SDS-PAGE pattern of proteins (stained with silver nitrate) extracted from cells of *S. sanguis* strain OB11 with SDS (lane A), SLS (lane B), and with SLS from cells incubated for 20 min at 20 °C with various concentrations of pronase ($\mu\text{g ml}^{-1}$): zero (lane C), 20 (lane D), 50 (lane E) and 100 (lane F). The positions of molecular mass markers are indicated for reference on the right and the protein bands in the detergent extracts are labelled according to their apparent molecular masses on the left. Samples each contained about 5 μg protein (Lowry).

Fig. 2

Fig. 2. Autoradiogram of ^{125}I -labelled proteins extracted from *S. sanguis* cells with SLS and separated by SDS-PAGE. Positions of molecular mass marker proteins are shown on the left and the prominent iodinated protein bands are labelled according to their approximate molecular masses on the right.

obtained for extracts with 8 M-urea, although the amount of protein extracted was estimated to be at least 10-fold less (results not shown). Since detergent extraction seemed the most promising method for removing protein, cells were incubated with solutions of various detergents to see if these would extract different proteins. However, the SDS-PAGE profiles of proteins extracted with 1% solutions of the following detergents were identical: SLS, Nonidet P-40, Zwittergent 3:12, Zwittergent 3:16. The protein profile of the SLS extract was representative of the different detergent extracts (Fig. 1, lane B); interestingly, these detergents all removed a 16 kDa

protein from the cells which SDS effectively did not (see Fig. 1, lane A). Extraction with SLS removed about 2.7% of total cell protein as estimated by the Lowry method. The proteins extracted with SLS ranged in molecular mass from about 130 kDa to 12 kDa (Fig. 1, lane B). Some of the more prominent components are labelled in Fig. 1 according to their approximate molecular masses (kDa); the designations are intended as a guide to protein identification rather than accurate molecular mass determinations. Densitometry of the silver-stained gel (Fig. 1, lane B) showed that seven polypeptides in the range 20–24 kDa accounted for 50–60% of the total stained extract and, also, that the 16 kDa polypeptide comprised about 15% of the total. The other polypeptide bands identified were relatively minor components, but were always present. Increasing the temperature of extraction to 40 °C resulted in a smeared SDS-PAGE profile indicative of a complex mixture of proteins. The pattern in Fig. 1 (lane B) was not altered either by washing the cells up to four times (with water, 0.15 M-NaCl or 10 mM-Tris/HCl, pH 7.5) before extraction, or by including 1 mM-PMSF (serine protease inhibitor) in the washing and extraction solutions. Neither the phase of growth (exponential or stationary) nor the carbon source (glucose, lactose or sucrose) in a semi-defined medium affected the SDS-PAGE pattern as shown in Fig. 1 (lane B). Also, cells of strain Channon and strain FW35 (see Methods) were extracted with SLS and the protein profiles after SDS-PAGE were identical to that for the Challis serotype 2 strain OB11 (results not shown).

Protease treatment of cells

To determine if the polypeptides extracted with SLS were surface proteins, cells were incubated with different concentrations of various proteases and then treated with SLS. Proteins normally present in the SLS extract and either diminished or absent in SLS extracts of protease-treated cells were likely to be accessible at the cell surface. Pre-treatment of cells with chymotrypsin (100 µg ml⁻¹) as described in Methods had no effect on the SDS-PAGE pattern of proteins extracted with SLS (results not shown). In contrast, a number of the proteins normally present in the SLS extract were absent in extracts of cells that had been treated with either *S. aureus* V8 protease or with *S. griseus* protease (pronase E). Fig. 1 (lanes C, D, E and F) shows the effect of incubating the cells with pronase on the SDS-PAGE pattern of proteins extracted with SLS. With the exception of those polypeptides in the 20–26 kDa range, all the proteins normally present in the SLS extract were either absent, or their amounts were reduced, in the SLS extract of pronase-treated cells. To show that the protease was only acting before SLS treatment (and not during the subsequent extraction), pronase (100 µg ml⁻¹) was added to cells which were then immediately washed, as described in Methods, and incubated with SLS. The SDS-PAGE pattern of proteins in this extract (Fig. 1, lane C) was identical to that of the untreated cells (Fig. 1, lane B). All of the proteins in the SLS extract were susceptible to pronase because they were degraded when a portion of an extract (40 µl) was incubated with the enzyme (1 µg, 10 min, 20 °C), and then subjected to SDS-PAGE (results not shown). These results suggest that with the exception of the proteins in the 20–24 kDa group (which either are not surface proteins or, *in situ*, are not susceptible to pronase), the protein components of the SLS extract are at the cell surface.

Lactoperoxidase-catalysed iodination of cell-surface proteins

Confirmation that SLS extracted surface proteins was sought by surface-labelling cells of *S. sanguis* with ¹²⁵I, using a lactoperoxidase-catalysed reaction. The iodinated cells were extracted with SLS and the labelled proteins, i.e. those exposed on the surface, were separated by SDS-PAGE and identified by autoradiography.

Treatment of iodinated cells with SLS removed 37% of the TCA-precipitable radioactivity. The extract contained about 30 iodinated polypeptides (Fig. 2), a number of which were not previously visualized by silver-staining (see Fig. 1). The major labelled components were the 63 kDa and 16 kDa polypeptides (Fig. 2). The 26 kDa, 24 kDa and 20 kDa proteins that were not degraded by pronase treatment of cells were nonetheless iodinated (Fig. 2), indicating that they are surface-exposed. The remaining five polypeptides in the 20–24 kDa group were not surface-labelled.

Table 1. *Composition of SLS extract from cells of S. sanguis*

The values are means of triplicate samples, none of which varied by more than 7% from the mean.
ND, Not determined.

	Before acid hydrolysis (μ g)	After acid hydrolysis (μ g)	Percentage of extract dry wt
Dry weight	360	ND	—
Hexose*	32	37	10.2
Protein (Lowry)	25	ND	6.9
Phosphorus	< 3	40	11.1

* Measured as glucose.

Effect of protease or SLS treatments on cell morphology

Cells that had been incubated with SLS and then plated out on to BHY agar did not form colonies. Pronase treatment, on the other hand, did not affect viability. Neither treatment caused gross changes in cell morphology as ascertained by phase-contrast microscopy. However, there was an indication in electron micrographs of thin sections of SLS-treated cells that the cell surface was disturbed, so cells were negatively-stained to reveal fibrillar surface structures (Handley *et al.*, 1985). Pronase treatment of the cells did not significantly alter the fibrillar surface, but SLS treatment appeared to reduce the numbers of fibrils (results not shown).

Analysis of SLS-extracted material

Approx. 500 mg equiv. dry wt cells of *S. sanguis* were incubated with SLS, and the extracted material was precipitated with ethanol and collected as described in Methods. Portions of it were dried to constant weight at 85 °C, and other samples were assayed before and after acid hydrolysis for protein, phosphorus and carbohydrate. The SLS-extracted material comprised 5–8% of the cell dry weight, and contained (by weight) 10.2% hexose, 11.1% phosphorus and 7% protein (Lowry method) (Table 1). Phosphorus was only detected after acid hydrolysis of the extract (the assay measures inorganic phosphate).

To determine if the SLS extract contained nucleic acids, portions were subjected to gel electrophoresis through 1% (w/v) agarose, and the gel was incubated with ethidium bromide solution (0.5 μ g ml⁻¹) to stain nucleic acids. The extract contained some high molecular mass DNA, which was susceptible to degradation by DNAase I, but no RNA. It was estimated that < 5% of the total extract was DNA and, therefore, this would not contribute significantly to the phosphorus estimation.

Partial and complete acid hydrolysates of SLS-extracted material were analysed for component sugars by TLC. Glucose, galactose and rhamnose were present and the pattern of sugars was identical to that obtained when an acid hydrolysate of a crude lipoteichoic acid preparation (extracted from cells with phenol; Wicken *et al.*, 1973) was subjected to TLC.

Hydrolysates of the SLS and phenol extracts were also subjected to TLC in a different solvent to that used for the sugars, and the plates were sprayed first to detect ninhydrin-positive compounds, and then to detect phosphorus. The hydrolysate of the SLS extract, but not that of the phenol extract, contained ninhydrin-positive spots. Similar patterns of phosphate esters were present in both extracts. Thus, the SLS extract contained, in addition to protein, material similar in composition to that extracted from cells with aqueous phenol, (that is, mainly lipoteichoic acid).

SDS-PAGE patterns of proteins from extracellular and subcellular fractions

Cells of *S. sanguis* secreted 30 or so polypeptides into the culture fluid during growth (Fig. 3, lane B), the major proteins having molecular masses of 44 kDa and 66 kDa. While the SLS extract from cells contained several of the culture fluid proteins, e.g. 44 kDa, 31 kDa, 28 kDa, the SDS-PAGE patterns of proteins in the two extracts were, in general, quite different (Fig. 3,

Fig. 3. SDS-PAGE patterns of proteins (stained with silver nitrate) from various subcellular fractions. Lanes: A, molecular mass markers; B, culture supernatant; C, high-speed supernatant (soluble) fraction; D, SLS extract; E, cell-envelope fraction; F, cell-wall fraction. The derivations of the various fractions are described in Methods. Protein bands are labelled according to their approximate molecular masses. L, lysozyme. Each lane contained 5–10 μ g protein.

lanes B and D). The 16 kDa cell-surface protein extracted with SLS was present in a high-speed supernatant fraction prepared from broken cells (Fig. 3, lane C). This polypeptide appears to be solubilized from the cell surface upon breakage of the cells.

The cell-envelope fraction was dominated by bands at 35 kDa, 31 kDa and 28 kDa, and a group of proteins between 16 and 20 kDa (Fig. 3, lane E). After extracting the envelopes with SDS/2-MeH, the residue was digested with a mixture of lysozyme and mutanolysin, and then re-extracted. The SDS-PAGE profile of this wall extract contained no major new bands (Fig. 3, lane F). The polypeptide band at about 37 kDa in the wall extract stained with PAS reagent; no other protein bands in any of the gels stained for carbohydrate with PAS.

Comparison of the gel patterns shows that several of the dominant bands in the cell-envelope and cell-wall extracts, e.g. 31, 28 and 23 kDa bands, were minor components in the SLS extract. In general, though, the SDS-PAGE pattern of proteins in the SLS extract bore little similarity to the pattern of proteins in the envelope or wall extracts. Thus, incubation of cells with SLS provides an extract containing, as major components, proteins that are not easily identified in other subcellular fractions.

Fig. 4. Western blots of proteins separated by SDS-PAGE and extracted from whole cells with SLS (lanes A, C and E) or from the cell-envelope fraction with SDS/2-MeH (lanes B, D and F). Lanes A and B are India ink stains of the transferred proteins; lanes C and D are autoradiograms of blots reacted with whole-cell antiserum and then [125 I]Protein A; lanes E and F are autoradiograms of blots reacted with antiserum to culture supernatant proteins and then [125 I]Protein A. The molecular mass marker proteins of 66 kDa and 29 kDa were electroblotted and stained with ink (left). Other protein bands are labelled according to their approximate molecular masses. Each gel lane contained 30 μ g protein (Lowry).

Characterization of antigens by immunoblotting

To characterize further the proteins present in the SLS and envelope extracts, and to attempt to relate some of these to the wall antigens of *S. sanguis* described by others (Schöller *et al.*, 1982; Rosan & Appelbaum, 1982; Liljemark & Bloomquist, 1981), proteins were reacted with either whole-cell antibodies or antibodies made to culture supernatant proteins. The proteins that had been separated by SDS-PAGE were electrolytically transferred to nitrocellulose and reacted with the antisera. The antigen-antibody complexes were detected by incubating the blots with

Fig. 5. Adsorption of *S. sanguis* cells to hexadecane. Cells were vortex-mixed with hexadecane, allowed to settle and the OD₄₅₀ of the aqueous phase was measured. ○, Control; ●, SLS-treated cells; □, pronase-treated cells. Details of the treatments are described in Methods. The experiment was repeated four times and data are individual values from a representative experiment.

[¹²⁵I]Protein A and then autoradiographing them. The transfer of the proteins to nitrocellulose was monitored by staining the blots with India ink (Fig. 4, lanes A and B). Proteins of molecular mass <20 kDa were not efficiently transferred. A comparison of the blots reacted with whole-cell antibodies suggests a number of antigens (e.g. 130 kDa, 90 kDa and 63 kDa proteins) are common to both the SLS and envelope extracts (Fig. 4, lanes C and D). The major reactive antigens in the envelope extract (about 10 between 26 and 35 kDa; Fig. 4, lane D) were not present in the SLS extract. The 20–24 kDa group of proteins in the SLS extract reacted with the whole-cell antibodies (Fig. 4, lane C).

Fewer of the polypeptides in the SLS extract reacted with antibodies to supernatant proteins (Fig. 4, lane E). A group of proteins (130–90 kDa) and the 63 and 35 kDa proteins reacted, suggesting these antigens are secreted into the culture fluid; the 20–24 kDa group of proteins did not react. Most of the major envelope polypeptides reacted with antibodies raised to culture supernatant proteins (Fig. 4, lane F), showing that these are secreted during growth of the cells. There was no reaction of any of the antigens on the blots with rabbit pre-immune serum (1/100 dilution).

Effect of protease and SLS treatments on cell hydrophobicity

To determine if the components of the SLS extract conferred hydrophobicity to the cell surface (and might therefore be involved in cell adhesion), cells treated with SLS or protease were tested for their ability to adsorb to the hydrophobic hydrocarbon hexadecane. Cells that were treated with SLS or pronase (100 μg ml⁻¹, 20 min, 20 °C) had reduced hydrophobicity (Fig. 5), as shown by lowering of affinity of the cells for hexadecane. Initially, adsorption to xylene (Rosenberg *et al.*, 1983) was also used as a measure of cell hydrophobicity, but this did not give consistent results between experiments as it caused lysis.

Since both protease and SLS treatments reduced cell hydrophobicity, components of the SLS extract were probably hydrophobic. To test this, a portion of SLS extract containing about 20 μg protein was subjected to hydrophobic interaction chromatography on Octyl-Sepharose. After applying the extract to the column (Fig. 6, lane A) and washing out unbound materials (see Methods), adsorbed proteins were eluted with a gradient of decreasing ammonium sulphate concentration and simultaneously increasing SLS concentration. The polypeptides in the 20–24 kDa range were not retained in the column, but the 16 kDa protein was (Fig. 6), showing that it was the major hydrophobic protein component of the SLS extract.

*Effect of SLS or protease treatments on coaggregation of *S. sanguis* with *A. viscosus* T14V and *A. naeslundii* W1544*

Glucose-grown cells of *S. sanguis* strain OB11 aggregated with cells of both *A. viscosus* and *A. naeslundii*, forming clumps that settled over several hours (Table 2). Pronase-treated cells of

Fig. 6. Separation of proteins in SLS extract by hydrophobic interaction chromatography. Approximately 20 μg protein in 1 M- $(\text{NH}_4)_2\text{SO}_4$ was applied to a column of Octyl-Sepharose CL-4B. Lane A is an SDS-PAGE profile (stained with silver nitrate) of a portion of the extract applied to the column. The column was eluted with a gradient of decreasing $(\text{NH}_4)_2\text{SO}_4$ concentration and simultaneously increasing SLS concentration (final concentrations 0 and 5 g l^{-1} respectively). The SDS-PAGE profile of proteins (stained with silver nitrate) in every fifth fraction is shown, and the position of the 16 kDa protein is indicated.

Table 2. Effect of pronase or SLS treatments on coaggregation properties of *S. sanguis*

Percentage coaggregation was estimated spectrophotometrically (see Methods). Numbers in parentheses are visual coaggregation scores on a scale from zero (none) to four (maximum). Experiments were repeated twice; results are mean values for replicates within one experiment.

Treatment	Percentage coaggregation with:	
	<i>A. viscosus</i> T14V	<i>A. naeslundii</i> W1544
None	49 (4)	43 (4)
Pronase	38 (2)	41 (4)
SLS	22 (1)	28 (1)

S. sanguis did not aggregate as well with *A. viscosus* as did untreated cells (Table 2), but their aggregation with *A. naeslundii* was not significantly affected (Table 2). Treatment of *S. sanguis* cells with SLS considerably reduced their ability to coaggregate with both *A. viscosus* and *A. naeslundii* (Table 2). Thus, the SLS-extracted material contained components necessary for coaggregation, as well as for cell hydrophobicity.

DISCUSSION

In studying the relationship between cell-surface proteins and adherence of *S. sanguis*, interest has been recently centred on a class of high molecular mass (> 80 kDa) proteins present in the cell wall (McBride *et al.*, 1985). It was suggested that, since mutants of *S. sanguis* that are less hydrophobic than the parent strain also lack some of these polypeptides, the proteins are associated with cell hydrophobicity (McBride *et al.*, 1985). Although the proteins were present in isolated cell walls, McBride *et al.* (1985) provided no data to show that the polypeptides were exposed at the cell surface. Furthermore, since fimbriae and other surface antigens can be removed from cells of *S. sanguis* without breaking them (for example, by pulse-blending; see Fachon-Kalweit *et al.*, 1985), surface components conferring hydrophobic or adherence properties may be absent from cell wall preparations. The work described in this paper identifies proteins present at the cell surface, probably within the fibrillar coat, by gently extracting whole cells and then subjecting the extracts to SDS-PAGE on gels that separate proteins over a wide range of molecular mass (about 150 kDa to 12 kDa).

Extraction of cells with SLS at room temperature removed proteins from the cell surface and also material similar in composition to that extracted from cells with hot aqueous phenol; this includes lipoteichoic acid (Wicken & Knox, 1975; Rosan, 1978), polynucleotides and polysaccharides (Chiu *et al.*, 1974; Emdur *et al.*, 1974). The protein composition of the extract was not phenotypically variable, and was similar for serotype 1 and serotype 2 strains of *S. sanguis* that were tested. The major protein components (seven proteins in a group between 20 and 24 kDa, and a 16 kDa protein) have not previously been identified, and they are not prominent components of either the extracellular culture fluid or the cell-envelope fraction prepared from mechanically broken cells. All the constituents of the SLS extract were shown to be accessible at the cell surface except for five of the 20–24 kDa group of proteins, which were not surface-iodinated or degraded by pronase-treating cells. These experiments do not rule out the possibility that the 20–24 kDa proteins may be surface components, and the observation that they reacted with antibodies raised to fixed cells indeed suggests that they are.

The 16 kDa protein is hydrophobic, exposed at the cell surface and constitutes at least 0.3% of total cell protein. The protein is degraded when cells are treated with pronase, and is extracted with SLS, both treatments that reduce cell hydrophobicity. This suggests that the 16 kDa polypeptide may confer hydrophobic properties to the cell surface. A similar property has been attributed to the M protein present on the surface of group A streptococcal cells. Protease treatment of *S. pyogenes* degrades the M protein leaving the fibrillar coat intact (Beachey & Ofek, 1976), while M⁻ strains are less hydrophobic than M⁺ strains (Wadström *et al.*, 1984).

It is apparent from surface-labelling cells with ¹²⁵I that at least 30 polypeptides extractable with SLS are exposed at the cell surface. A somewhat similar SDS-PAGE profile to that in Fig. 2 was obtained by Appelbaum & Rosan (1984) when iodinated cells of *S. sanguis* were extracted with boiling SDS. Surface proteins on *S. sanguis* have been implicated by various authors in conferring hydrophobicity and adherence properties. For example, Rosan & Appelbaum (1982) described the loss of 86, 92 and 160 kDa proteins from the surface of *S. sanguis* on protease treatment, Liljemark & Bloomquist (1981) partly purified an 86 kDa adhesin from the cell surface, and Schöller *et al.* (1982) isolated a soluble wall-associated common protein antigen of about 60 kDa from various oral bacteria including *S. sanguis*. Proteins in the wall of higher molecular mass (> 130 kDa) have also been described (McBride *et al.*, 1985; Knox *et al.*, 1985); some of these had glucosyl transferase activity (Knox *et al.*, 1985) and others cross-reacted with antiserum to *S. mutans* wall antigens B and I/II (Forester *et al.*, 1983).

Hydrophobic interactions are involved in adhesion of *S. sanguis* (Nesbitt *et al.*, 1982a; Gibbons & Etherden, 1983) but Knox *et al.* (1985) have recently shown that there is no direct correlation between cell hydrophobicity and the ability of *S. sanguis* to adhere to saliva-coated hydroxyapatite. Thus, a measurement of hydrophobicity is not necessarily an index of the ability of cells to adhere; hydrophobicity should be considered a phenotypic property of the cells, which can be changed by altering the composition of the cell surface. The 16 kDa polypeptide is on the cell surface of *S. sanguis* and is hydrophobic, but it may not necessarily be involved in cell adherence. In more general terms, though, treating the cells with SLS reduced their

hydrophobicity and appeared to remove fibrillar surface structures. This observation is in keeping with the suggestions of Gibbons *et al.* (1983) and Fives-Taylor & Thompson (1985) that components of the fibrillar coat confer hydrophobicity to the cell. In addition, treating the cells with SLS reduced their ability to coaggregate with both *A. viscosus* and *A. naeslundii*. The extraction method is thus effective in removing, from the cell surface, molecules determining both hydrophobic and coaggregation properties. Components of the SLS extract are currently being purified in order to determine precisely which ones are involved in cell adherence.

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