Polypeptides associated with tufts of cell-surface fibrils in an oral Streptococcus

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INTRODUCTION

Streptococci are found at most sites within the human oral cavity (Frandsen et al., 1991) and are amongst the predominant bacteria in human dental plaque (Socransky et al., 1977). Successful colonization is due, at least in part, to the abilities of streptococcal cells to bind to salivary components within the pellicle formed on hard surfaces, to host epithelial cells, and to other oral bacteria (Kolenbrander & London, 1993; Ofek & Doyle, 1994). Streptococcal cell-surface proteins that function as adhesins may be considered to fall into two structural groups. The first group comprises high molecular mass cell-wall-associated polypeptides, the best-characterized being the antigen I/II polypeptides found on a variety of streptococcal species and involved in binding of cells to salivary glycoproteins (Bleiweis et al., 1992; Jenkinson, 1994). Other wall-associated polypeptide adhesins include antigen B (AgB) and antigen C (AgC) on Streptococcus salivarius mediating binding to Veillonella atypica and host epithelial cells, respectively (Weerkamp et al., 1986a), and the CshA polypeptide of Streptococcus gordoni involved in adherence to, or coaggregation with, the oral bacterium Actinomyces naeslundii (McNab & Jenkinson, 1992a, 1994). The second group of proteins comprises cell-surface-associated lipoproteins. This group includes SarA (Jenkinson & Easingwood, 1990; Jenkinson, 1992) and ScaA (Andersen et al., 1993; Kolenbrander et al., 1994) of S. gordoni, which are involved in coaggregation with A.
naeslundii, and SsaB of *Streptococcus sanguis* (Ganeshkumar et al., 1991) and FimA of *Streptococcus parasanguis* (Fenno et al., 1989; Oligino & Fives-Taylor, 1993), which are salivary-pellicle adhesins.

A variety of cell-surface structures are elaborated by different strains of oral streptococci but their functions in adhesion generally are not well understood (Handley, 1990). Binding of streptococci to experimental salivary pellicle may be influenced by the presence of cell-surface fibrils (Gibbons et al., 1983; Morris et al., 1987) or fimbriae (Fives-Taylor & Thompson, 1985), and ability to coaggregate with other oral genera was found to correlate with surface structure morphology (Handley et al., 1985).

For strains of *Streptococcus crista* producing polur tufts of fibrils (Handley et al., 1991), electron microscopic evidence suggests that these structures are involved in the formation of ‘corn-cob’ coaggregates with *Corynebacterium matruchotii* (Lancy et al., 1980; Mouton et al., 1980) or *Fusobacterium nucleatum* (Lancy et al., 1983). A functional role for surface fibrils in cell adhesion has been demonstrated unequivocally only in *S. salivarius* HB, where glycoprotein adhesin AgB and polypeptide AgC demonstrated were shown to be separate classes of cell-surface fibril (Weerkamp et al., 1986b).

Handley et al. (1984) described cells of an oral *Streptococcus* (strain CN3410) with the Lancefield group K antigen that carried lateral tufts of long and short fibrils with further peritrichous fibrils covering the rest of the cells. This strain conforms to the biochemical description of the species *Streptococcus oralis* (Kilian et al., 1989). Previous studies showed that what appeared to be the long fibril components of the tufts could be released, morphologically intact, from the surface of *S. oralis* CN3410 by incubating the cells with trypsin (Handley et al., 1984; Hesketh et al., 1987). To try to define better the structural components and function of the fibrils in these tufts, we have extended previous work by isolating fibrils following treatment of *S. oralis* CN3410 cells with trypsin and characterizing biochemically and immunologically the fibril-associated polypeptides. This paper describes these trypsin-extracted high molecular mass polypeptides, which appear to be components of long fibrils but not determinants of *S. oralis* CN3410 cell adhesion to parotid salivary pellicle or to host epithelial cells.

**METHODS**

**Microbial strains and growth conditions.** Bacterial strains utilized in this study were *S. oralis* CN3410 (previously denoted *S. sanguis* biotype II by Hesketh et al., 1987). *S. oralis* KP34V (a variant of CN3410 isolated during the course of this study), *S. crista* NCTC 12479 and AK1 (Handley et al., 1991), *A. naeslundii* T14V, BE64, WVU627, W1544, EF1006, TF11 and ATCC 12104, and *C. matruchotii* ATCC 14266. Bacteria were grown on TSBY agar containing trypsin case soy broth (BBL) supplemented with 5 g yeast extract (Difco) l⁻¹ and 15 g agar l⁻¹ at 37°C under reduced oxygen concentration. Liquid cultures were grown at 37°C in screw-capped bottles or tubes as stationary cultures in TSBY medium or, for streptococci only, TY-glucose medium, pH 7-5, containing 5 g tryptone (Difco) l⁻¹, 5 g yeast extract l⁻¹, 4 g K₂HPO₄ l⁻¹ and 8 g glucose l⁻¹.

**Trypsin treatment of cells.** Cells in the late exponential phase of growth (10 ml culture) at an OD₆₀₀ of 1-3 (measured using a Shimadzu model UV-120 spectrophotometer) (approximately 8 x 10⁸ cells ml⁻¹) were harvested by centrifugation (5000 g, 10 min, 4°C). The supernatant was discarded, the cells were washed twice by suspension in distilled water and centrifugation, and were then suspended in 10 mM Tris/HCl, pH 7-0 (0-6 ml), containing trypsin (type III from bovine pancreas, Sigma; 0-2 mg ml⁻¹). Suspensions were incubated for 1 h at 37°C, centrifuged (5000 g, 10 min, 4°C) and the supernatants were carefully removed and centrifuged as before. In some experiments trypsin inhibitor (Sigma type 1-S, 0-1 mg ml⁻¹ final concentration) was added immediately following incubation of cells with trypsin. For subsequent separation of components by electrophoresis, a portion of cell-free trypsin extract (containing fibrils) was mixed with an equal volume of sample buffer (0-125 M Tris/HCl, pH 6-8, containing 0-02% 2-mercaptoethanol and 2%, w/v, SDS) and heated for 10 min at 70°C. For larger-scale purification of trypsin-extracted material, cells from 1 l culture were collected and washed as described above, suspended in 60 ml 10 mM Tris/HCl, pH 7-0, containing 0-2 mg trypsin ml⁻¹, and incubated for 1 h at 37°C. Cells were pelleted by centrifugation (5000 g, 10 min, 4°C), the supernatant was removed and recenterfuged, then the supernatant was subjected to centrifugation at 12000g for 4 h at 4°C. The pellet was suspended in 60 ml deionized water, centrifuged as before, and the pellet was then suspended in deionized distilled water (0-3 ml). The suspension contained fibrill-like material as visualized by negative staining and electron microscopy.

**Preparation of cell-surface or culture-fluid proteins.** Cells in late exponential phase of growth were collected and washed as described earlier and were suspended at a density of approximately 2 x 10⁸ bacteria ml⁻¹ in one of the following: 0-2 mg pronase ml⁻¹ in 10 mM Tris/HCl, pH 7-5, for 1 h at 37°C; 0-1 M NaOH for 30 min at 4°C, then neutralized with HCl (McNab & Jenkinson, 1992b); 5 M urea in 10 mM Tris/HCl, pH 7-5, containing 1 mM EDTA for 15 min at 4°C; sample buffer containing SDS (see earlier) for 10 min at 70°C. Suspensions were then centrifuged (6000 g, 10 min, 4°C) and a portion of each supernatant was mixed with an equal volume of sample buffer in preparation for gel electrophoresis. Culture fluid polypeptides were precipitated from cell-free culture supernatant with 80% (w/v) acetone at -20°C, collected by centrifugation and solubilized as described previously by McNab & Jenkinson (1992b).

**Electron microscopy.** Streptococcal cells were prepared for electron microscopy and visualized after staining with 1% (w/v) methylene tungstate as described by Handley et al. (1985). To visualize fibrillar material, a 25 μl suspension of fibrils was applied to an ionized Formvar-coated copper grid, blotted dry, and then stained with 1% (w/v) methylene tungstate.

**SDS-PAGE and electroblotting.** Proteins were separated by SDS-PAGE according to the method of Laemmli & Favre (1973) and were stained with silver nitrate (Morrissey, 1981) or with Coomassie brilliant blue. The molecular masses of proteins were determined by reference to a plot relating distance migrated to log molecular mass for marker proteins in the range 210–15 kDa (Life Technologies). Polypeptides were transferred from polyacrylamide gel to nitrocellulose membrane by electroblotting in 25 mM Tris, 192 mM glycine, 20% (w/v) methanol, pH 8-3 (Towbin et al., 1979), at 20 V cm⁻¹ for 1 h at 4°C, without prior equilibration of the gel in the electro-transfer buffer.

**Antisera, immunoblot analysis and ELISA.** Antisera to trypsin-extracted polypeptides (TEPs) were prepared following pre-
parative SDS-PAGE and visualizing bands by immersing the gel in 10 vols 4 M sodium acetate (Higgins & Dahmur, 1979). The required polypeptide bands were excised, electroeluted from the gel with a Bio-Rad model 422 apparatus and dialysed against 5 l distilled water for 16 h at 4 °C with three changes. Samples were freeze-dried and suspended in 0.15 M NaCl for inoculation into rabbits. Fibrils were prepared from cells following trypsin treatment as described above and collected by high-speed centrifugation. The preparation was incubated with 10 μg pronase ml⁻¹ in 10 mM Tris/HCl, pH 7.0, for 1 h at 37 °C, which degraded TEP components (see Results) but not the fibril structures as visualized by electron microscopy of negatively stained preparations of fibrils. The fibril suspension was then dialysed against deionized water (10 l) for 16 h at 4 °C, freeze-dried, and the residue was suspended in 0.15 M NaCl. Antibodies were raised in New Zealand White rabbits by intramuscular injection of approximately 20 μg protein or fibril preparation, followed by two boosters of 4 μg protein or fibrils after 3 and 10 weeks.

Antiserum reactivity was determined on nitrocellulose blots of streptococcal proteins incubated with antiserum diluted 1:500 or 1:1000. Antibody binding was detected with peroxidase-conjugated swine antibodies to rabbit immunoglobulin G (Dako) as described by Jenkinson & Easingwood (1990). Reactivity with whole cells was determined by ELISA. Briefly, streptococcal cells from the late exponential phase of growth were suspended at an OD₅₀₀ of 0.5 in PBS (15 mM NaH₂PO₄, 0.15 M NaCl) and 0.2 ml per well were added to the wells of Maxisorp (Nunc) flat-bottom 96-well polystyrene plates (0·05 ml per well) and the plates were centrifuged at 800 g for 5 min to deposit the cells. Cells were fixed to the plastic surface by addition of 0·25 % glutaraldehyde (0·1 ml per well) for 30 min, washed twice with PBS containing 0·05 % Tween 20 (PBS-Tween), then wells were blocked with PBS containing 0·25 % gelatin (PBS-gelatin) for 16 h at 4 °C. Serial twofold dilutions of immune sera or preimmune sera in PBS-gelatin were added to wells, plates were incubated for 1 h at 37 °C, well contents were discarded and wells were washed four times with PBS-Tween. Antibody binding was detected with peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins (Dako) diluted 1:1000 in PBS-gelatin (0·05 ml per well). Plates were developed with 1,2-phenylenediamine as enzyme substrate and the A₄₉₀ was measured.

Estimation of protein and carbohydrate. Protein concentration was determined using Folin reagent with BSA as standard. Carbohydrate determination was determined by phenol-sulphuric acid method (Dubois et al., 1956) with glucose as standard.

Adhesion to saliva-treated hydroxylapatite beads. Streptococci were grown to late exponential phase in TY-glucose medium with [methyl-³H]thymidine [0·22 MBq (6 μCi) ml⁻¹; 85 Ci mmol⁻¹], harvested by centrifugation (5000 g for 10 min), washed twice with distilled water and suspended at an OD₅₀₀ of 1·0 in KCl buffer (2 mM NaH₂PO₄, 5 mM KCl, 1 mM CaCl₂, adjusted to pH 6·4 with NaOH) (Eifert et al., 1984). The numbers of cells adhering to hydroxylapatite beads (BDH) treated with whole or parotid saliva (diluted 1:5 in KCl buffer) were measured as described by Hawkins et al. (1993). Unstimulated whole saliva was collected from five individuals, pooled, clarified by centrifugation (10000 g, 20 min, 4 °C) and diluted 1:5 in KCl buffer. Stimulated parotid saliva was obtained from one individual using a modified Carlson-Crittenden device (Shannon et al., 1962). In some experiments, cells were suspended in 10 mM Tris/HCl buffer, pH 7·0, and treated with trypsin for 60 min at 37 °C, or in PBS containing appropriate dilution of antiserum with end-over-end mixing for 90 min at 20 °C, collected by centrifugation, washed three times by alternate suspension in KCl buffer and centrifugation, and then assayed for adhesion.

Adhesion to buccal epithelial cells (BECs). BECs were collected from 20 individuals by gently scraping their inside cheek surfaces with wooden spatulas. Cells were pooled in TSMC buffer (5 mM Tris/HCl, pH 7·4, containing 100 mM NaCl, 50 mM KCl, 1 mM MgCl₂ and 1 mM CaCl₂), allowed to settle and the supernatant was decanted from the cells. The BECs were suspended in TSMC buffer at a density of 1·0 × 10⁶ cells ml⁻¹ (assessed by direct microscopic count) and 0·05 ml portions were distributed into Maxisorp microtitre plate wells. Plates were centrifuged (800 g, 3 min), incubated for 12 h at 4 °C, and then the TSMC buffer was aspirated and replaced with TSMC buffer containing 0·2 % Tween 20 (TSMC-Tween) (0·02 ml per well) for 16 h at 4 °C to block any remaining binding sites on the plastic. Microscopic examination of the wells revealed that the BECs formed a monolayer with approximately 80 % coverage of the plastic surface. The wells were washed twice with TSMC buffer, 0·05 ml suspension containing 1·25 × 10⁶ cells of ³H-labelled streptococci in TSMC buffer was added to each well and the plates were incubated for 2 h at 20 °C with shaking. The liquid contents of the wells containing unattached streptococcal cells were discarded; the wells were washed once with TSMC-Tween and then twice with TSMC buffer. Bound streptococcal cells were then removed by suspension in 0·1 M NaOH containing 0·2 % SDS and transferred to scintillation vials for radioactivity counting as previously described by Jenkinson et al. (1993). In some experiments, bacterial cells were incubated with trypsin or with antisera as described above, and washed with TSMC buffer before assaying for adhesion.

Cell-surface hydrophobicity. Late exponential growth phase cells in TY-glucose medium were collected by centrifugation, washed twice with PUM buffer (Rosenberg et al., 1980) and suspended at an OD₅₀₀ of 0·5 (approximately 4 × 10⁹ bacteria ml⁻¹). Streptococcal cell suspension (3 ml) was mixed with hexadecane (0·3 ml), the phases were allowed to settle, and the percentage of input cells associated with the organic phase (a measure of the net surface hydrophobicity of the cell population) was calculated from the decrease in OD₅₀₀ of the aqueous phase.

Coaggregation. The ability of streptococcal cells to coaggregate with A. naelundii cells was assessed by light microscopic examination following mixing of equal volumes of partner cell suspensions in TSMC buffer each containing about 1 × 10⁸ bacteria ml⁻¹ and incubating for 1 h at 37 °C with shaking. The ability of streptococci to form corn-cob coaggregates with C. mutans was investigated by mixing a 10 ml culture of streptococcal cells in exponential growth phase with a 40 ml exponential growth phase culture of C. mutans in TSBY medium and incubating with gentle shaking for 12 h at 37 °C. Coaggregation and the presence of corn-cob formations was investigated by light microscopy.

RESULTS

Extraction of cell-surface polypeptides and effects on cell morphology

To begin to characterize the components of surface fibrils on S. oralis CN3410, a number of agents were examined for their effectiveness in solubilizing polypeptides and removing concomitantly the tufts of fibrils from the cell surface. Incubation of cells with 0·1 M NaOH, which has been shown to extract surface proteins and polysaccharides from S. gordonii (McNb & Jenkinson, 1992b),

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did not effect significant release of polypeptides from strain CN3410 cells as determined by SDS-PAGE. A 5 M urea extract of cells contained approximately 20 polypeptide bands in the range 15-70 kDa (results not shown). Neither NaOH nor urea treatment significantly affected cell-surface fibril morphology. Incubation of cells with sample buffer containing SDS (see Methods) solubilized a complex mixture of > 50 polypeptides and also high molecular mass material that did not enter the separating gel (Fig. 1d, lane 1). Cells that had been treated with SDS showed partial disruption of cell wall layers and of fibril tufts (results not shown). Incubation of cells with pronase completely removed the long and short fibril components of the tufts (Handley et al., 1984) but no polypeptide bands were discernible following SDS-PAGE of pronase extracts (Fig. 1c, lane 1). Incubation of cells with trypsin resulted in release of intact fibrils (see below) and upon SDS-PAGE of trypsin extracts several high molecular mass polypeptides were identified (Fig. 1a, lane 1) which we called TEPs. The polypeptides present in the trypsin extracts were divided into two groups based on their molecular mass and staining characteristics. Group A comprised three bands (estimated molecular masses 246 kDa, 238 kDa and 227 kDa) which stained with silver nitrate but only weakly with Coomassie blue. Group B comprised four polypeptide bands (estimated molecular masses 208 kDa, 195 kDa, 183 kDa and 175 kDa) which stained well with both silver nitrate and Coomassie blue. These gel patterns were highly reproducible and were not affected by the addition of trypsin inhibitor immediately following trypsin digestion of cells. Little or no material of molecular mass < 100 kDa was visible on SDS-PAGE of trypsin extracts suggesting that other trypsin-susceptible cell-surface proteins were extensively degraded. Further incubation of extracts with fresh trypsin (up to 6 h) did not result in significant degradation of the high molecular mass TEPs. Trypsin extracts of cells were estimated to contain about 8% of total cell protein.

Cells of strain CN3410 carried lateral tufts of short very fine densely packed fibrils through which longer sparser clumped fibrils protruded, as well as a peritrichous fringe of much shorter indistinct fuzzy material which could also be fibrillar (Fig. 2a). Frequently visible were loose fibrils around the cell periphery. Treatment of cells with trypsin for 60 min at 37 °C removed the long and short fibril components of the tufts from all cells leaving loose fibrils associated with the cell surface (Fig. 2b). Released fibrils were collected by high-speed centrifugation of trypsin extracts, and they appeared by electron microscopy as a tangled mass of clumped fibres (Fig. 2c). Occasionally, very thin, possibly single, fibrils could be resolved. The fibrillar material contained protein and carbohydrate in the ratio 3:6:1 based on colorimetric assays. The SDS-PAGE profile of proteins extracted with sample buffer from these fibrils (Fig. 1b, lane 1) was similar to the protein profile of the trypsin extract of cells (that contained fibrils before high-speed centrifugation), with some slight differences in migration and staining intensity of group B bands. These results suggested that the TEPs were associated with the cell-surface fibrils.
**Fig. 2.** Transmission electron micrographs of cells or fibrils negatively stained with methylamine tungstate. (a) *S. oralis CN3410* cells showing tufts comprising short fibrils (large arrows) through which long fibrils protrude (small arrows), and peritrichous fringe material. (b) *S. oralis CN3410* following incubation with trypsin for 60 min at 37 °C; cells are stripped of their tufts and loose fibrils surround the cells (arrows). (c) Loose fibrils following trypsin treatment as in (b) comprising material collected by high-speed centrifugation as described in the text (arrows show possible separate fibrils). (d) *S. oralis KP34V* cells showing tufts composed of long fibrils only. Bars, 200 nm.
Table 1. ELISA of immune or preimmune sera activities with immobilized cells of \textit{S. oralis} CN3410 or KP34V

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Group A TEPs</th>
<th>Group B TEPs</th>
<th>Fibrils</th>
<th>Preimmune</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{S. oralis} CN3410</td>
<td>0.58†</td>
<td>0.34</td>
<td>0.42</td>
<td>0.05</td>
</tr>
<tr>
<td>\textit{S. oralis} CN3410 + trypsin‡</td>
<td>0.32</td>
<td>0.16</td>
<td>0.20</td>
<td>0.07</td>
</tr>
<tr>
<td>\textit{S. oralis} KP34V</td>
<td>0.46</td>
<td>0.42</td>
<td>0.53</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Diluted $6.4 \times 10^4$.
† Means of duplicate samples from a representative experiment that differed by $< \pm 5\%$.
‡ Cells incubated with 0.2 mg trypsin ml$^{-1}$ for 1 h at 37°C, harvested by centrifugation, washed and deposited onto microtitre plate wells as described in Methods.

Antigenic relatedness of TEPs and fibril proteins

To investigate the antigenic relatedness of group A TEPs, group B TEPs, and fibrils, antibodies were raised in rabbits to each of these antigens. Antiserum from rabbits immunized with group A TEPs reacted most strongly with group A bands, as might be expected, and also with group B bands (Fig. 1a, lane 2). Antibodies to group B TEPs reacted more strongly with group A bands than with group B bands (Fig. 1a, lane 1). The reactions of the various antisera with blots of polypeptides extracted from purified fibrils mirrored the results just described (Fig. 1b). Antibodies to fibrils, but not to group A or group B polypeptides, reacted weakly with a diffuse band on nitrocellulose blots of pronase-extracted material separated by SDS-PAGE (Fig. 1c). Each antiserum preparation reacted with high molecular mass material (> 300 kDa) on blots of proteins extracted from cells with sample buffer (Fig. 1d).

The reactivities of sera with intact cells were determined by ELISA in microtitre plate wells coated with streptococcal cells. Similar high titres were obtained for each antiserum, with the anti-group-A polypeptides serum being most reactive, while preimmune serum was not reactive (Table 1). The ELISA titres for each of the sera were > 50% reduced with streptococcal cells that had been preincubated with trypsin (Table 1). These data confirmed the antigenic relatedness of TEPs and fibril components, and showed further that these antigens were accessible on the cell surface.

Fibril and TEP production by a variant of strain CN3410

Following routine subculture of \textit{S. oralis} CN3410 on TSBY agar a single smaller-colony variant was identified. This strain was purified as a spontaneously derived mutant strain that formed marginally smaller colonies than the parent strain, but which was otherwise identical to the taxonomic description of \textit{S. oralis} CN3410. Electron microscopic examination of cells of the mutant strain (denoted KP34V) showed that they carried peritrichous fringe material similar to the parent strain; however the short fibrils were missing from the lateral tufts of fibrils which, in the mutant, consisted of the long fibrils only (Fig. 2d). Trypsin-released fibrils from strain KP34V collected by high-speed centrifugation appeared identical by electron microscopic observation to those collected from the parent strain CN3410 and shown in Fig. 2(c).

The SDS-PAGE profiles of TEPs extracted from KP34V were identical to those from the wild-type strain CN3410 (Fig. 3a). On immunoblots, the TEPs from KP34V reacted with anti-group-B TEPs serum identically to the wild-type TEPs (Fig. 3a). They also reacted similarly to the TEPs from wild-type cells with group A TEPs and fibril antisera (not shown). In ELISA, anti-serum to the group A TEPs reacted somewhat less well with KP34V cells than with wild-type cells (Table 1). Conversely, antibodies to the group B TEPs and to fibrils both reacted better with KP34V cells than with the wild-type cells (Table 1).

Since there were no obvious protein differences between trypsin extracts of cells of CN3410 and KP34V, we investigated the profiles of proteins secreted into the culture fluid. Both strains secreted similar polypeptides as revealed by SDS-PAGE and staining with silver nitrate (results not shown) but there was a considerably larger amount of high molecular mass (> 300 kDa) material present in the culture fluid of KP34V. This material was the predominant component on SDS-gels of culture fluid proteins from KP34V stained with Coomassie blue (Fig. 3b). Nitrocellulose blots of culture fluid proteins from the two strains gave slightly different reactions with anti-group-B TEPs serum (Fig. 3b) with the high molecular mass material from strain KP34V reacting comparatively weakly. This material was therefore possibly not antigenically closely related to the TEPs. Culture fluid polypeptides from each strain were then prepared, incubated with trypsin, separated by SDS-PAGE, blotted and reacted with group B antiserum. The resulting immunoblot patterns were identical for each strain with two
Streptococcal cell-surface fibril polypeptides

Comparison of SDS-PAGE and immunoblot profiles of trypsin-extracted or culture-fluid polypeptides of *S. oralis* CN3410 and KP34V. (a) TEPs from CN3410 cells (lane 1) or KP34V cells (lane 2) stained with Coomassie blue, and corresponding nitrocellulose blots (lanes 3 and 4) reacted with anti-group-B TEPs serum (1:500). (b) Cell-free culture-fluid polypeptides from CN3410 (lane 1) or KP34V (lane 2) stained with Coomassie blue and corresponding blots reacted with anti-group-B TEPs serum (lanes 3 and 4). (c) Culture-fluid polypeptides corresponding to those in panel (b) but incubated with trypsin for 60 min at 37 °C before SDS-PAGE, electroblotting and reaction of blots with anti-group-B TEPs serum (lane 1, CN3410; lane 2, KP34V). The group A TEPs stained relatively weakly with Coomassie blue (a, lanes 1 and 2). The molecular masses of marker proteins are indicated (kDa).

Table 2. Comparison of cell adhesion properties of *S. oralis* CN3410 and KP34V and effects of trypsin or antibodies to fibrils on adhesion of CN3410 cells

<table>
<thead>
<tr>
<th>Strain and treatment*</th>
<th>Percentage adhesion (±SD, n = 4) to hexadecane (hydrophobicity)</th>
<th>10^−3 × No. of cells adhering (±SD, n = 4) to:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Parotid saliva-treated hydroxylapatite beads†</td>
</tr>
<tr>
<td>CN3410</td>
<td>56.2±6.10</td>
<td>287.7±2.40</td>
</tr>
<tr>
<td>KP34V</td>
<td>57.4±3.10</td>
<td>35.7±1.70</td>
</tr>
<tr>
<td>CN3410 + trypsin</td>
<td>1.06±0.06</td>
<td>32.2±2.20</td>
</tr>
<tr>
<td>+ anti-fibril serum</td>
<td>ND</td>
<td>26.0±2.30</td>
</tr>
<tr>
<td>(1:100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ anti-fibril serum</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>(1:1000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ preimmune serum</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>(1:100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>28.75±1.10</td>
</tr>
</tbody>
</table>

ND, Not determined.

* Conditions of incubation with trypsin or serum are described in Methods.
† Input number of streptococcal cells 2.5 × 10⁷.
‡ Input number of streptococcal cells 1.25 × 10⁷.

Closely migrating antigenic bands similar in size to the group A TEPs (Fig. 3c).

Cell-surface hydrophobicity and cell adherence properties

Cell-surface hydrophobicity is a factor that has been shown to influence adhesion properties of streptococci, particularly to salivary pellicle (Doyle et al., 1990). Surface hydrophobicities of *S. oralis* CN3410 and KP34V cells, as measured by the numbers of bacteria adsorbing to hexadecane, were found to be virtually identical (Table 2). Cell-surface hydrophobicity was abolished by treating cells with trypsin under the standard conditions that removed TEPs and fibril tufts (Table 2). Cells of *S. oralis* CN3410 and KP34V showed similar adhesion abilities to hydroxylapatite beads treated with human parotid salivary proteins (Table 2). Tenfold less numbers of cells of both strains bound to beads treated with human whole saliva (results not shown). Treatment of strain CN3410 cells with trypsin had no effect on their adhesion to parotid-saliva-treated beads (Table 2). Incubation of streptococcal cells with preimmune serum or with antibodies to fibrils had no effect on their ability to adhere to parotid-saliva-treated hydroxylapatite beads (Table 2). Incubation of cells with antibodies to group A or group B TEPs also had no effect on their adhesion to beads (not shown in Table 2). Both strains adhered equally well to human saliva-treated hydroxylapatite beads.
BECs and binding was > 90% reduced following incubation of streptococcal cells with trypsin (Table 2). However, streptococcal cell binding was unaffected by incubation of cells with preimmune serum or with antiserum to fibrils (Table 2), or with antiserum to group A or group B TEPs (not included in Table 2). Neither of seven strains of A. naeslundii that were tested (14V, BE64, WUV627, W1544, EF1006, TF11, ATCC 12104). This suggests that, unlike many strains of S. oralis, cells of strain CN3410 and KP34V do not carry a surface polysaccharide receptor for A. naeslundii (Kolenbrander & London, 1993). In addition, CN3410 or KP34V cells did not coaggregate with C. matruchoti, whereas under similar experimental conditions cells of S. crista NCTC 12479 and AK1 formed corn-cob coaggregates with C. matruchoti.

**DISCUSSION**

Fibril tufts are some of the largest extracellular structures produced by streptococci, yet their structural and functional components are unknown. Therefore, we set out to determine the protein composition and function in adhesion of the fibril tufts produced by S. oralis CN3410. This strain was chosen because previous work had suggested that the fibril tufts of strain CN3410, unlike those of several other tufted strains, could be removed morphologically intact by incubation of cells with pronase (Hesketh et al., 1987). When these experiments were repeated and the polypeptide components of the fibrils were analysed by SDS-PAGE, we detected no polypeptide bands. One explanation for this was that while the fibrils were morphologically intact, the pronase had nevertheless substantially cleaved the protein components which remained ligated within the fibril structures until solubilized with a denaturant (SDS).

Treatment of CN3410 cells with trypsin also resulted in release of long fibrils and loss of tufts from the cells. Trypsin extracts of cells, unlike pronase extracts, contained reproducible SDS-PAGE patterns of proteins and these were divided conveniently into two groups. The group of higher molecular mass bands, termed group A TEPs, stained only weakly with Coomassie blue, which is a characteristic of some glycosylated polypeptides. When fibrils were collected by high-speed centrifugation of the trypsin extracts, and the proteins associated with the fibrils were solubilized by SDS, an almost identical SDS-PAGE pattern of polypeptides was obtained to that from the crude trypsin extracts of whole cells that contained loose fibrils. This suggested that the TEPs might be components of the fibrils. In support of this notion, antibodies raised to pronase-treated fibrils, which contained no detectable proteins by SDS-PAGE, reacted with the group A and group B TEPs more or less identically to the antibodies that had been raised to the purified TEPs. Thus the pronase-treated fibrils contained peptide fragments that had common epitopes with the TEPs. The group A TEPs and group B TEPs were shown to be immunologically cross-reactive. Therefore the TEPs are possibly proteolytic fragments of a single gene product, alternatively they could be derived from the products of expression of more than one related gene.

Tufts on CN3410 cells were composed of two structurally distinct types of fibrils, the short fibrils were 159 ± 50 nm and the long fibrils were 289 ± 150 nm in length (Handley et al., 1984). It was not possible to deduce from electron micrographs whether the isolated fibrils contained long and short fibrils, or long fibrils only. When removed from the cells and collected by high-speed centrifugation, the fibrils became aggregated and structures up to a few microns in length with tapered ends were visible (Fig. 2c). However, it is likely that the isolated fibrils comprised the long fibrils only, for two reasons: firstly, short tuft fibrils are removed and/or degraded by pronase treatment of strain CN3410 cells before the long tuft fibrils are lifted-off (Hesketh et al., 1987); secondly, isolated fibrils from strain KP34V, which lacked the short fibril components of the tufts, appeared identical in electron micrographs to those isolated from strain CN3410. The anti-fibril serum reacted better with KP34V cells than with wild-type cells, which may indicate that in the absence of the short fibrils on KP34V cells, long fibril epitopes were more accessible. The isolation of strain KP34V was also crucial in establishing that the TEPs were not components of the shorter fibrils, since SDS-PAGE profiles of TEPs extracted from wild-type or mutant cells were identical. Taken together, these results suggest strongly that the TEPs are components of the long fibrils. We have attempted to confirm this by electron microscopic observations of cells reacted with anti-TEPs sera and gold-labelled secondary antibody. However these experiments have been unsuccessful to date because the streptococcal cells appear to bind avidly conventional blocking agents such as ovalbumin or bovine albumin, as well as non-specifically bind the gold-labelled secondary antibody.

In S. crista NCTC 12479 the long fibril components of tufts have been implicated in conferring cell-surface hydrophobicity (Busscher et al., 1991) while the short fibrils may confer a localized surface negative charge (Handley, 1991). Trypsin treatment of S. oralis CN3410 cells abolished their hydrophobicity, which corroborates numerous other findings that polypeptides are determinants of cell-surface hydrophobicity in streptococci (Doyle et al., 1990). However, since trypsin will degrade many surface polypeptides these data do not imply that the long fibrils are necessarily hydrophobins. Clearly though the short fibrils are not determinants of cell-surface hydrophobicity in strain CN3410 since mutant cells lacking short fibrils were unaffected in their ability to bind hexadecane. On the other hand, treatment of strain CN3410 cells with trypsin did not affect their adhesion to parotid salivary pellicle. This observation is in contrast to many others which have shown that protease treatment of streptococcal cells reduces not only hydrophobicity (Jenkinson, 1986) but also adhesion to salivary pellicle (Oakley et al., 1985; Hesketh et al., 1987). Despite the overwhelming evidence that hydrophobic interactions are important for adhesion of streptococci (Doyle et al., 1990), these experiments reveal that for S. oralis CN3410 hydrophobicity is not an essential attribute for cell
adhesion to pellicle. This has also been shown recently for isogenic mutants of S. gordonii deficient in production of surface polypeptides CshA and CshB. These mutants had much-reduced cell-surface hydrophobicity but nevertheless adhered normally to experimental salivary pellicles (McNab et al., 1994). In summary, adhesion of strain CN3410 cells to parotid salivary pellicle is not sensitive to trypsin under the conditions employed, is independent of cell-surface hydrophobicity, and does not involve the long or short components of the fibril tufts since these were wholly removed by trypsin treatment of cells.

Binding of strain CN3410 cells to BECs involves different adhesins from those necessary for binding to parotid salivary proteins since trypsin treatment of cells abolished their ability to bind to BECs. However, antibodies to TEPs or fibrils did not inhibit adhesion of strain CN3410 cells to BECs. This suggests that the TEPs are distinct from the receptor-binding sites of the adhesins. By analogy, it has been shown for the Gram-positive oral bacterium A. naeslundii that antibodies directed to the type 1 fimbrial structural subunit do not react with the receptor-binding sites of the type 1 fimbriae (Cisar et al., 1991). The involvement of surface structures in adhesin presentation by Escherichia coli is well-documented. In uropathogenic strains expressing P pili the papG gene product encodes the lectin adhesin which is located at the tip of the pilus rod (Kuehn et al., 1992), while in E. coli expressing type 1 fimbriae the FimH protein is thought to be responsible for the adhesiveness of the fimbriae, which are composed of polymers of FimA (Sokurenko et al., 1994; Ofek & Doyle, 1994). To elucidate better the structure and function of fibrillar tufts in S. oralis CN3410, we are attempting to isolate the gene(s) encoding the TEPs. By insertionally inactivating the coding sequence(s) on the S. oralis chromosome it should be possible to determine more precisely how the TEPs are involved in production of fibrillar tufts and in adhesin presentation.

ACKNOWLEDGEMENTS

We are most grateful to R. Whiley, The London Hospital Medical College Dental School, London, UK, for performing definitive experiments that enabled speciation of strain CN3410. We thank R. McNab and other colleagues in the Experimental Oral Biology Laboratory, University of Otago, for helpful discussions and R. A. Baker for technical assistance. M. W. J. was in receipt of a University of Otago Postgraduate Studentship.

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Received 26 April 1995; revised 28 June 1995; accepted 5 July 1995.