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Labeling of Binding Sites for β_2-Microglobulin (β_2m) on Nonfibrillar Surface Structures of Mutans Streptococci by Immunogold and β_2m-Gold Electron Microscopy

DAN ERICSON, RICHARD P. ELLEN,* AND ILZE BUIVIDS

Faculty of Dentistry, University of Toronto, Toronto, Canada M5G 1G6

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As little detail is known about the surface structure of streptococci in the mutans group and the relationship of surface structure to host ligand-binding functions, the twofold purpose of this investigation was to examine in detail, by a range of electron microscopic techniques, the surface structures of streptococci in the different species of the mutans group and to investigate the distribution of β_2-microglobulin (β_2m)-binding sites on such structures. Strains representing *Streptococcus mutans*, *S. cricetus*, *S. rattus*, *S. sobrinus*, and four fresh isolates were studied by shadowcasting and histochemical staining of whole-mounted cells as well as by ultrathin and thick sectioning of embedded specimens. β_2m-binding site distribution was visualized by indirect immunogold electron microscopy and by direct bacterial binding of β_2m-conjugated gold probes. Shadowcast preparations revealed binding of gold probes to the cell surface of known β_2m-binding strains but not to their polar fibrillar appendages. These long fibrils, common to all strains, were trypsin and sonication sensitive and stained with lead citrate but not with uranyl acetate or ruthenium red. More gold particles were bound by the indirect technique. For grid-mounted bacteria, the gold was mostly bound in clusters at the periphery of the cells. When gold probes were reacted in suspension with bacteria before mounting onto grids, a more even distribution of the gold was seen, but the bacteria were aggregated. Heating the bacteria eliminated β_2m-gold binding but had no effect on the morphology of the fibrils. Thick sections of embedded bacteria prereacted with β_2m-conjugated gold probes were analyzed by stereo imaging. A wispy, uranyl acetate-stained fuzzy layer, distinct from the fibrils seen by shadowcasting and extending up to one cell diameter from the cell wall, contained the gold probes. These findings introduce a concept that binding sites for some salivary ligands on mutans streptococci may be clustered on very delicate, nonfibrillar structures extending much further from the cell wall than previously appreciated. As for β_2m, which composes part of the human histocompatibility antigens, part of the bacterial surface would be coated at a distance from its body with a protein not necessarily recognized as foreign by the host.

Indigenous oral bacteria bind various host molecules soluble in saliva, on epithelial surfaces, and on tooth pellicles (for a review, see reference 12). Such interactions govern whether a bacterium will adhere to host surfaces or be cleared from the mouth (9, 12, 29). Nonimmune binding of host molecules by bacteria may be of significance in modulation or evasion of immune recognition (C. L. Greenblatt, ASM News **49**:488–493, 1983) as well as in uptake of nutrients.

Although immunoelectron microscopy has been used to localize purified bacterial adhesions on bacterial surface structures, the distribution of oral bacterial binding sites for host ligands in secretions has not been addressed. Therefore, the overall objective of this research program is to link structure to function by studying the degree of coverage and pattern in which ligand-binding sites are displayed on oral bacteria as well as defining the type of structures on which they are located. By virtue of their projecting surface position and through extensive supporting experimental data, structures such as fimbriae, fibrils, and fuzzy coats have been ascribed adhesion functions for several oral bacteria, including actinomycetes and streptococci (5, 10, 15, 17, 18, 32, 33). For example, three different antigenic types of fimbriae have been found on *Streptococcus salivarius*, and the mucosal attachment adhesin has recently been localized to one of these (31).

Because of their cariogenicity, members of the mutans group of streptococci have probably been studied more extensively than other bacteria indigenous to the oral cavity, yet very little detailed information about their surface structure is available. Only a few investigators have even mentioned the presence of long appendages, most attention being paid to the shorter "fuzzy coat" (16, 19, 24, 26). Long, 20- to 30-nm-thick surface projections that link *S. mutans* cells together have also recently been reported by Moro and co-workers (23) in a scanning electron microscopy study. Mutans streptococci are capable of binding various human salivary ligands, such as high-molecular-weight agglutinins (9, 14), amylase (4, 28), lysozyme (25), secretory immunoglobulin A (IgA) (3), fibronectin (8a, 21), and β_2-microglobulin (β_2m) (6–8). As binding of β_2m to strains of *S. mutans* has been studied in detail (6–8) but not yet related to their structure, electron microscopy (EM) studies demonstrating the array of β_2m-binding sites on the surfaces of the mutans group of streptococci seemed an appropriate experimental progression.

The purpose of this investigation was twofold: to examine by a wide range of EM methods the surface structures of streptococci in the mutans group and to determine the presence, distribution, and surface coverage of receptors for

* Corresponding author.

β_2m as they relate to fibrils, fuzzy coats, and other surface appendages observed.

MATERIALS AND METHODS

Strains and culture conditions. *S. mutans* strains NCTC 10449 and KPSK2 (serotype c), LM7 (serotype e), and OMZ175 (serotype f); *Streptococcus cricetus* AHT and 3720 (serotype a); *Streptococcus rattus* Fa1 and BHT (serotype b); *Streptococcus sobrinus* ME1 (serotype d) and OMZ65 (serotype g); and four fresh isolates yielding typical frosted-glass colonies on MSB agar and glass-adherent growth in sucrose broth were maintained on blood agar plates and subcultured monthly. The fresh isolates were subcultured twice to check purity. Strains 10449, KPSK2, ME1, and OMZ65 are known to bind β_2m; strain BHT is an established nonbinder (6, 7). For experiments, cultures were grown overnight in an atmosphere of 10% CO_2, 10% H_2, and 80% N_2 in either Todd-Hewitt broth (THB; Difco Laboratories, Detroit, Mich.), tryptic soy broth (TSB; Difco), brain-heart infusion with 5% sucrose (BHI; Difco), or a chemically defined sucrose-free medium (27). Cultures were washed thrice in 0.01 M phosphate buffer, pH 6.0, containing 0.05 M KCl and 1.0 mM $CaCl_2$ (KCl) and suspended in KCl to an OD_{550} of 1.0 (model 35 spectrophotometer, 1.0-cm path length; G. K. Turner Associates, Palo Alto, Calif.).

Surface structure of whole-mounted bacteria. Formvar- and carbon-coated Ni grids were floated on droplets of bacterial suspensions and dried by gently absorbing excess fluid with filter paper. Specimens were then shadowcast with Pt-Pd at a 45° angle in a Balzers MBA3 (Balzer AG., Lichtenstein) vacuum evaporator. Grid-mounted bacteria were also stained with 2% phosphotungstic acid (PTA) at pH 0.3, 1.2, or 7.0; with 14.6% uranyl acetate in 50% methanol (UA); with Reynolds lead citrate at pH 11.0; with 0.1% ruthenium red at pH 6.0; or with 1% ammonium molybdate at pH 7.0. In some cases, the staining solution was mixed with an equal volume of bacterial suspension for 1 to 10 min, and a droplet was air-dried onto a grid.

Heat treatment and sonication. To determine the effect of heat on the surface structures, suspensions of strain OMZ65 were heated in a water bath for 10 min at 50, 60, 70, 80, and 100°C. Suspensions were also sonicated for 30 s in a Kontes sonicator (Mandel Scientific, Brockville, Ontario) at maximum power and were then centrifuged at 12,000 × *g* for 5 min. The bacterial pellet and the supernatant were examined after shadowcasting.

Protease treatment. Pellets of 200-μl suspensions of OMZ65 were suspended in 200 μl of trypsin (T-8253, lot 16F-0249; Sigma Chemical Co.), 1 mg/ml in 0.15 M phosphate-buffered saline, pH 7.6 (PBS), or in protease K (P-0390, lot 125F-0783; Sigma), 1 mg/ml in PBS. Controls consisted of enzyme preparations heated in a boiling-water bath for 25 min. Suspensions were incubated at 37°C for 1 h and washed twice in ice-cold KCl. Whole-mounted preparations were shadowcast.

Indirect labeling of β_2m sites. Bacterial suspension (400 μl) was pelleted at 12,000 × *g* for 2 min and suspended in 100 μl of human β_2m (Sigma; M-4890, lot 113F-04202) 5 μg/ml, or in chewing-stimulated whole saliva, diluted 1/2 in KCl. For controls, bacteria were suspended in β_2m-free buffer. After mixing and incubation at room temperature for 1 h, bacteria were washed in 900 μl of KCl, suspended in 200 μl of KCl, mounted onto Formvar- and carbon-coated Ni grids, and kept in a moist chamber. The grids were then covered with rabbit anti-human β_2m (Nordic Tilburg, The Netherlands; lot 23-582) diluted 1/200 in PBST (0.01 M phosphate buffer, pH 7.2, containing 0.14 M NaCl and 0.05% [vol/vol] Tween 20) for 1 h. The anti-β_2m antiserum and a normal rabbit serum used as a control had been absorbed for 1 h by suspending a pellet from 1.5 ml of a bacterial suspension (OD_{550} = 1.0) in 400 μl of serum. After centrifugation three times at 12,000 × *g*, the supernatant serum was used. After three washes for 10 min each with PBST, the grids were covered for 30 min. with goat anti-rabbit IgG-gold conjugate with a mean diameter of 5 nm (Janssen Pharmaceutica, Bersee, Belgium; lot 50307) diluted 1/20 in PBST. The grids were then washed three times with PBST and once with distilled water.

Direct β_2m-gold labeling method. Gold probes of 9 and 23 nm (G_9 and G_{23}) were prepared by the method of Frens (11) and stabilized with either human β_2m, pH 6.0, human fibronectin, pH 6.9 (Sigma; F-2006, lot 54F-9320), or polyethylene glycol 20,000 (PEG) at both pH 6.0 and 6.9 by the method of Bendayan (1). The gold probes were diluted in KCl with 0.02% PEG. Briefly, gold colloid was prepared by adding different volumes of 1% (wt/vol) citric acid to boiling 0.01% (wt/vol) tetrachloroauric acid. After adjusting the pH to 6.0 or 6.9, the lowest concentrations of protein needed to stabilize the colloid in 1.4% (wt/vol) NaCl were determined. The proteins were added to the colloids in 10% excess, and after 5 min PEG was added to a final concentration of 0.1%. The gold probe was centrifuged for 1 h at 100,000 × *g* in an IEC model B-60 ultracentrifuge (Damon Co., Needham, Mass.), and the pellet was suspended in buffer. Controls consisted of PEG only. The gold concentration was equalized between the test and control vials by measuring the OD at 520 nm. The size of the gold probe was measured by EM, and the presence of protein on the surface of the colloid was determined by using 14.6% (wt/vol) UA. Before use, gold probes were diluted in KCl with 0.02% (wt/vol) PEG and centrifuged at 12,000 × *g* for 3 min to remove aggregates. Two direct labeling methods were used. For the first, bacteria were mounted on grids, exposed to different concentrations of gold probes for 1 h, and then washed. For the second, they were first reacted with the gold probes in suspension, washed, and then mounted onto grids. The whole-cell specimens were then shadowcast with Pt-Pd at a 45° angle.

Surface structure and β_2m-gold labeling of embedded and sectioned bacteria. Washed suspensions of strains OMZ65 and 10449 grown in chemically defined medium were used. The direct labeling method with G_9 and G_{23} was used to detect β_2m-binding sites in both thick (0.5 to 1.0 μm) and ultrathin (60 nm) sections, cut in a Sorval MT5000 ultramicrotome (Dupont, Newtown, Conn.) with a diamond knife (Diatome; Reichart-Jung, Scarborough, Ontario). Bacteria were first reacted with the gold probe, washed twice in KCl with 0.05% Tween 20 and then in KCl with 10% (vol/vol) glycerol, mixed with 2% Noble agar, and mounted on filter paper. Freeze-substitution was carried out as described by Beveridge, Harris, and Humphrey (Proc. Microsc. Soc. Canada, Fredericton, New Brunswick, 1985, vol. 12, p. 22). Briefly, pieces of the filter paper were plunged into liquid propane, transferred to tubes containing 2% OsO_4 and 2% uranyl acetate in acetone, and cooled with liquid nitrogen. Freeze-substitution was carried out at −80°C for 48 h, and the specimens were then transferred to acetone at room temperature, washed in acetone, and embedded in Epon (CanEM, Guelph, Ontario). The thick sections were poststained with UA. The thin sections were stained with UA and then with Reynolds lead citrate.

EM examination. All samples were examined in a Philips

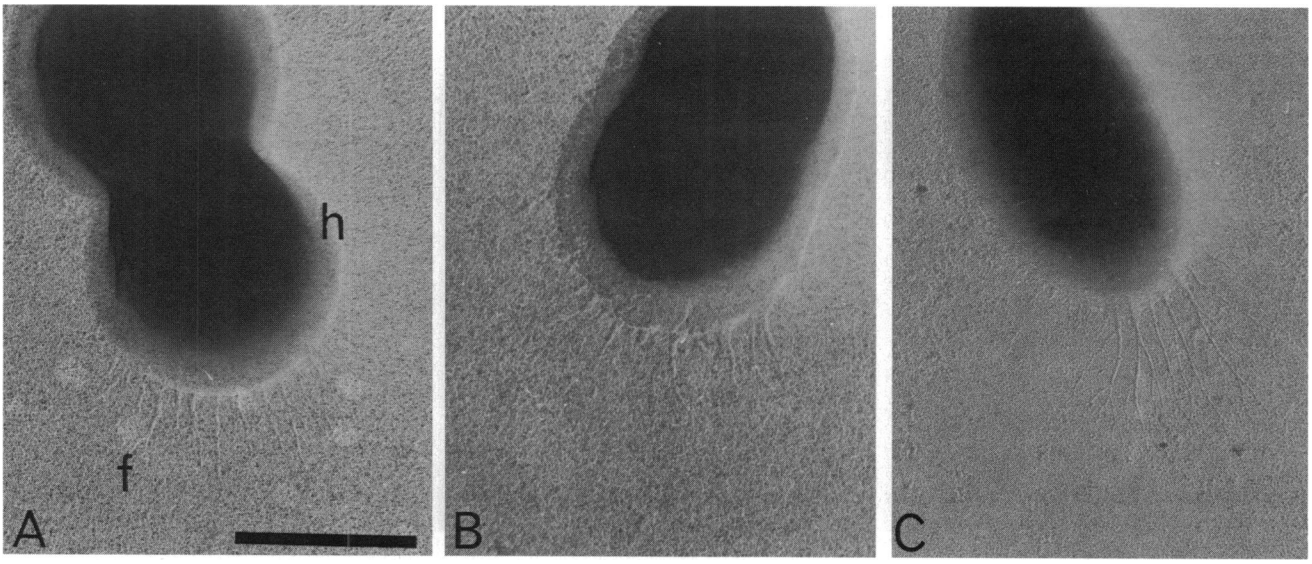

FIG. 1. Shadowcast preparations of whole cells of mutans streptococci. (Bar represents 0.5 μm in this and following electron micrographs.) (A) Strain 3720; (B) strain 10449; (C) strain OMZ65. All strains displayed a halo (h) and fibrillar appendages (f). Proximal ends of appendages were often detected within the halo area (C).

400 EM at 60 to 110 keV, depending on the thickness of the sections. For stereo images, photomicrographs were taken with a 10° to 20° difference in the tilt of the specimen.

RESULTS

Surface characteristics of whole-mounted cells. In shadowcast preparations, the bacteria appeared as central electron-dense bodies surrounded by a less dense halo, 50 to 100 nm wide (Fig. 1). Fibrillar protrusions radiating through this halo were evident most often at the poles of the cells and were occasionally detected at both poles of a single cell. The fibrillar appendages were approximately 5 nm wide and 400 to 700 nm long. Many of the appendages were branched. In stereo images, shadowcast cells appeared slightly dome-shaped and were surrounded by a flatter brim, or halo.

The fibrillar appendages could be detected on all strains tested, including the fresh isolates. They were present on OMZ65, ME1, and LM7 grown in TSB, THB, and chemically defined medium. The use of the last for OMZ65 yielded slightly more fibrils, but differences among these media were not obvious. Growth in BHI with 5% sucrose, however, yielded a thick interbacterial extracellular substance, and

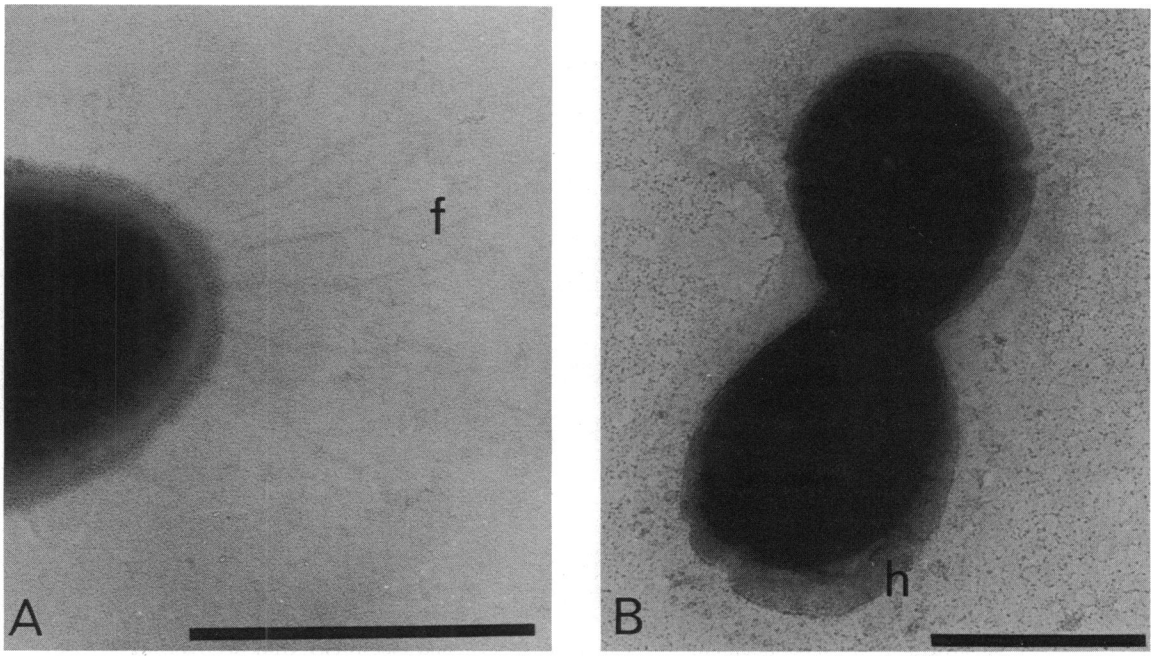

FIG. 2. Whole cells of OMZ65 stained with Reynolds lead citrate (A) or UA (B). Fibrils (f), of similar appearance as in shadowcast preparations, were seen only in lead citrate-stained specimens. h, Halo.

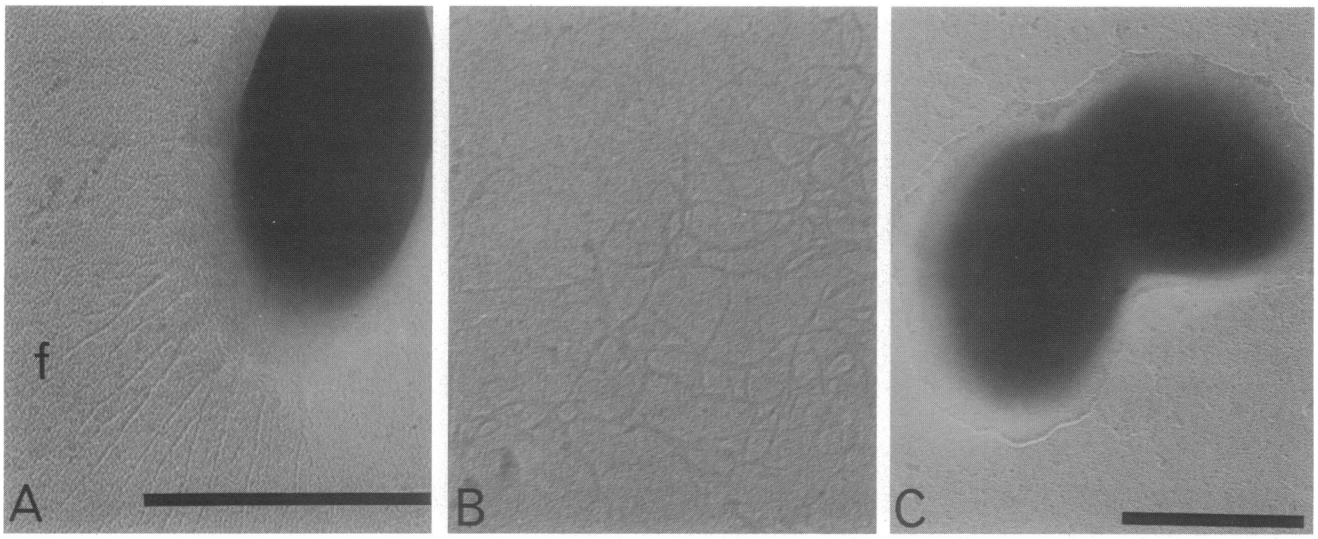

FIG. 3. Shadowcast specimens of physically and chemically treated whole cells of OMZ65. Fibrils (f) remained intact on cells which had been heated to 100°C for 10 min (A). Sonication removed some of the surface fibrils, releasing them into the supernatant (B). Fibrils were not detected on cells after trypsin digestion for 1 h (C).

fibrils were not found so easily. Of all the strains, OMZ65 had the highest density of these structures and was therefore chosen for further investigation.

Staining of whole-mounted OMZ65 with Reynolds lead citrate at pH 11.0 showed surface fibrils with a shape and location identical to that seen by shadowcasting (Fig. 2A). Staining with 2% PTA at pH 1.2 showed similar structures. Staining with 0.1% ruthenium red or 2% PTA at pH 0.3 did not reveal the fibrils, nor did negative staining with 2% PTA at pH 7.0 or staining with 1% ammonium molybdate at pH 7.0. UA also failed to stain the surface fibrils, but the halo could be visualized (Fig. 2B).

Heat, sonication, and protease treatment. Heating OMZ65 to 100°C for 10 min did not affect the surface fibrils (Fig. 3A). Sonication for 30 s seemed to remove or distort some of the surface structures, as seen on cells remaining in the centrifuged pellet, and fibrillar structures forming a meshwork could be found after shadowcasting the sonic supernatant (Fig. 3B). The thickness and appearance of these structures were similar to those of the fibrils on whole bacteria (Fig. 1).

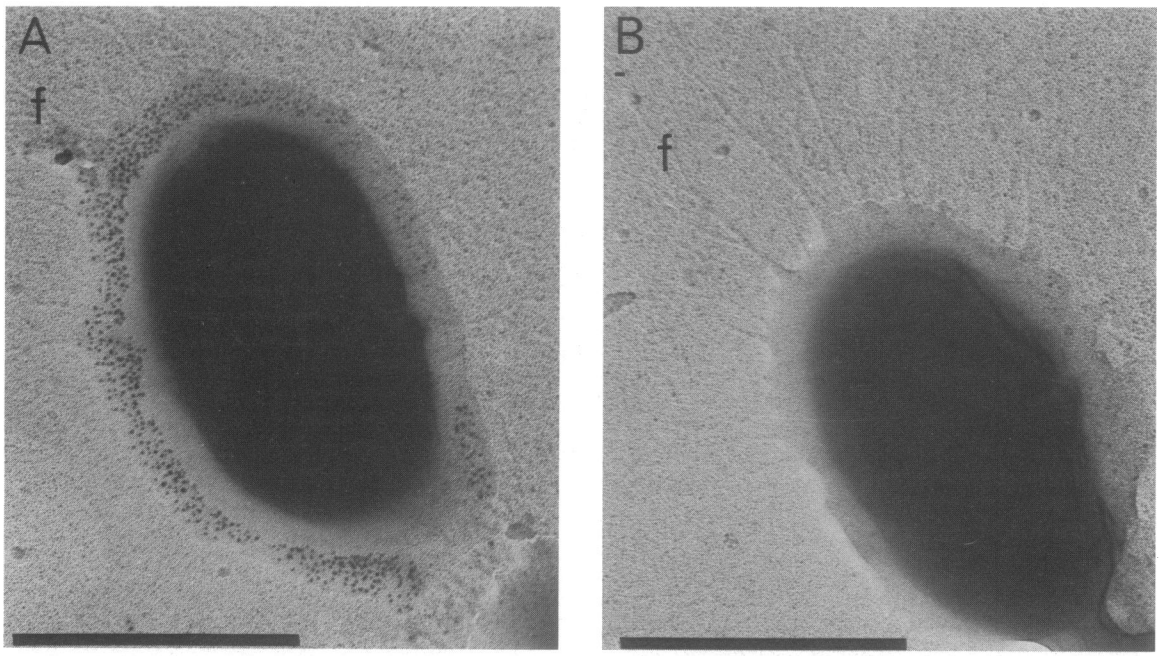

FIG. 4. Electron photomicrographs of whole-mounted, indirect immunostained, and shadowcast OMZ65. (A) OMZ65 reacted with human β_2m and then in sequence with rabbit anti-β_2m and goat anti-rabbit IgG conjugated to 5-nm gold particles. The gold is bound to the cell periphery, not to fibrils (f). (B) OMZ65 control, treated as in panel A but without β_2m.

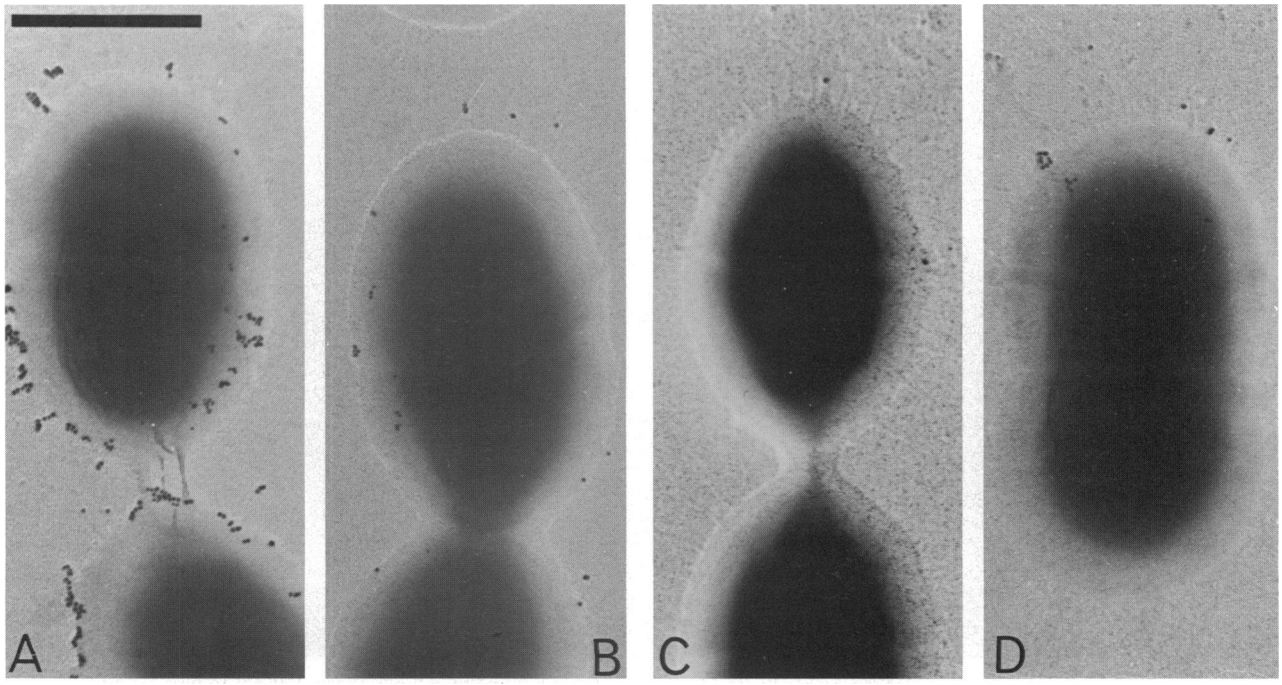

FIG. 5. Electron photomicrographs of whole-mounted OMZ65 reacted with (A) G_9-β_2m (note binding to periphery), (B) G_9-PEG, and (C) G_9-fibronectin. (D) Strain BHT, a known non-β_2m-binding strain, reacted with G_9-β_2m.

Trypsin removed all surface appendages from OMZ65 when enzyme treatment lasted 1 h (Fig. 3C). In the 15-min sample, there was little evidence that fibrils had been removed or digested. Protease K and both heat-inactivated enzyme preparations had no effect.

Indirect immunogold labeling. The three gold probes demonstrated slightly different binding patterns when whole-mounted bacteria were studied. With the indirect technique, rather dense gold label was visible mostly along the halo or the periphery of the cells (Fig. 4). The controls showed no or only very little label. Gold label was not found on the fibrillar appendages. Not all bacteria in a given sample showed reactivity with β_2m, but there was a clear difference in the proportion of β_2m-binding cells and the degree of labeling per cell between established binding (ME1 and OMZ65) and nonbinding (BHT) strains. Saliva-treated cells, used to test

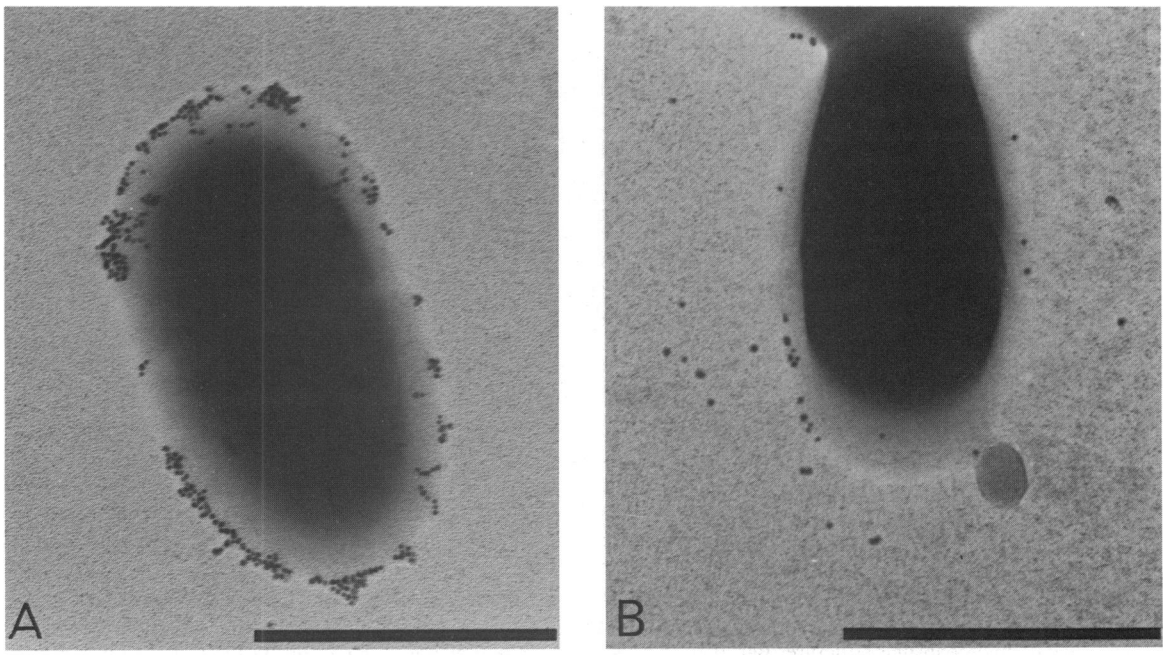

FIG. 6. Electron photomicrograph of strain 10449 reacted with (A) G_9-β_2m (the gold probe clusterd on the cell surface) and (B) G_9-PEG.

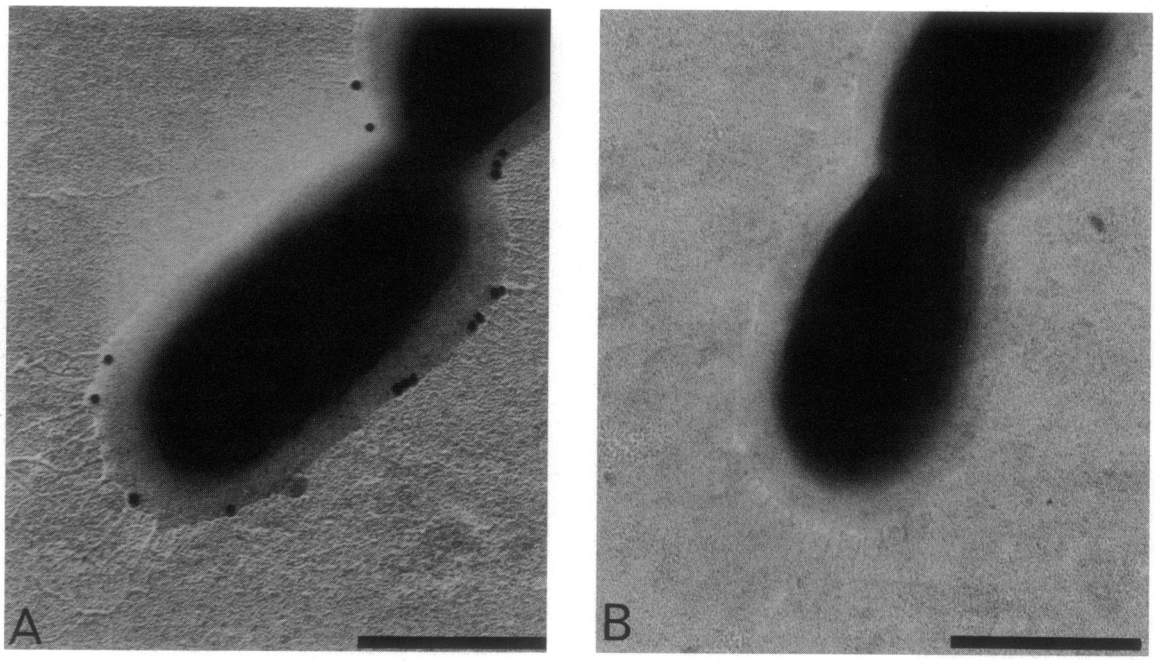

FIG. 7. Electron photomicrograph of OMZ65 reacted with (A) G_{23}-β_2m (note binding to cell periphery, but not to fibrils) and (B) G_{23}-β_2m after preheating the bacteria to 60°C (note presence of fibrils but no binding of the gold probe).

the absorption of salivary β_2m, showed the same reaction pattern, but the label was not as dense. Gold label was seen on bacteria grown in all three media.

Direct protein-gold labeling. The direct method, in which larger gold particles (G_9 and G_{23}) were used, revealed fewer binding sites per cell than did the indirect method, but a greater proportion of bacteria in each sample exhibited binding (<30% of cells by the indirect versus almost all cells by the direct method). Figures 5A, B, and C illustrate the results of an experiment with OMZ65 first mounted on grids and then reacted with either G_9-β_2m, G_9-PEG, or G_9-fibronectin. For this strain, binding occurred only with G_9-β_2m. Strain BHT, the negative control, bound neither probe (Fig. 5D). Strains KPSK2 and 10449 (Fig. 6) bound more of the G_9-β_2m and also more of the control G_9-PEG probes than OMZ65 (Fig. 5) and ME1. Addition of Tween 20

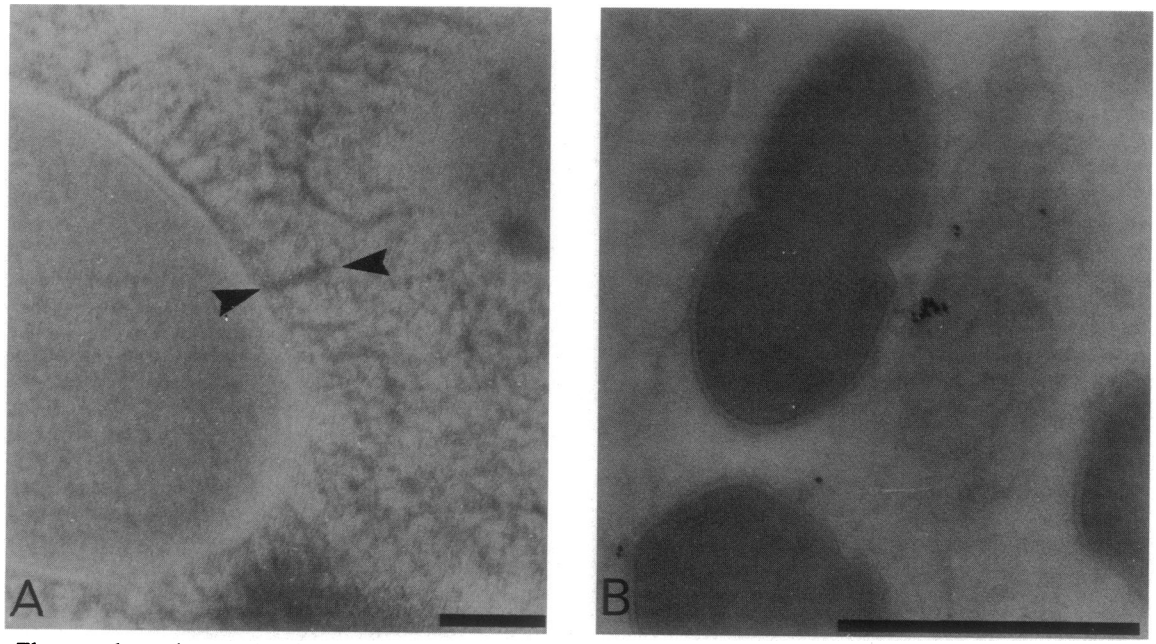

FIG. 8. Electron photomicrographs of ultrathin sections. (A) OMZ65 preserved by freeze substitution and poststained with Reynolds lead citrate. Arrows indicate microcapsular structure. Bar, 0.1 μm (this photomicrograph only). (B) Strain 10449 reacted with G_9-β_2m and stained with UA. The gold probe appeared at a distance from the cell.

almost abolished G_9-PEG binding, but did not affect binding of G_9-β_2m. The surface appendages showed very sparse, if any, label, and the label was mostly associated with the periphery of the cell, even though binding also occurred on the central part of the cell. The binding of G_{23} followed the same pattern as G_9, but even fewer particles bound to the bacteria. No label was seen on the fibrillar appendages. Figure 7 shows OMZ65 reacted with G_{23}-β_2m. In Fig. 7B, the bacteria were heated to 60°C for 10 min prior to the binding experiment, which is known to abolish β_2m binding (7). No or little label could be detected compared with the control. As in previous experiments, heating to 60°C did not affect the fibrillar appendages.

Exposure of the probes to bacteria in suspension resulted in a different labeling pattern than that observed for whole, grid-mounted cells. The gold probe was distributed over the bacterial surface rather than limited to its periphery. Bacteria were mostly clumped in these samples.

Embedded and sectioned specimens. Ultrathin sections of bacteria grown in chemically defined medium and post-stained, following freeze substitution, with lead citrate exhibited thin strands of stainable material extending up to 1/5 of the cell diameter from the cell wall, possibly indicating collapsed capsular material (Fig. 8A). Similar structures were stained with ruthenium red. Ultrathin sections reacted with G_9-β_2m revealed sparse gold labeling which was not always associated with the cell wall, but rather present at a distance from it (Fig. 8B). The gold probe was always present in close association with the cells and not randomly distributed as background staining in the embedding material.

To verify that the gold bound to cellular structures, thick sections were cut. The stereophotograph (Fig. 9) illustrates a three-dimensional representation of strain 10449 reacted with G_9-β_2m prior to embedding and thick sectioning. Very little gold label was associated with the immediate cell wall structures; most was bound in clusters to the UA-stainable fuzz extending up to one cell diameter from the cell surface. These fuzzy structures sometimes formed strands bridging between cells or extending into cell-free areas, but they did not resemble the fibrils seen on grid-mounted cells because they were stained by UA. The wispy fuzz was observed on bacteria grown in both chemically defined medium and THB; OMZ65 had somewhat more fuzz than 10449.

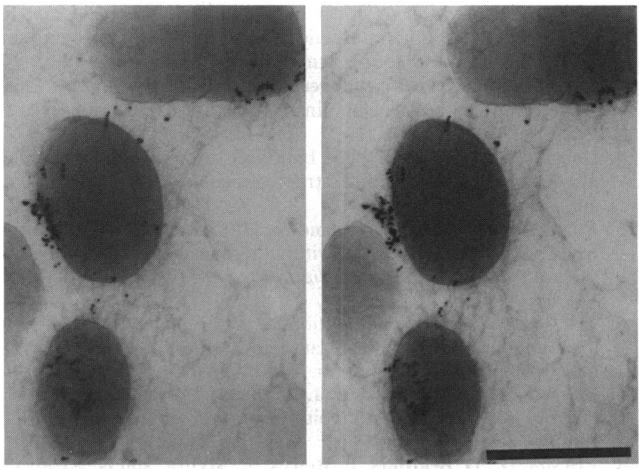

FIG. 9. Stereo electron photomicrographs of a UA-stained thick section of strain 10449; should be viewed through stereoglasses. Note the extension of the delicate bacterial fuzz and the binding of the gold probe to this rather than to the cell wall.

DISCUSSION

In this report, binding sites for human β_2m on the surface of mutans streptococci were located by both indirect immunogold and direct ligand-gold EM. By using appropriate controls, specific binding was demonstrated for purified monomeric human β_2m as well as for the β_2m naturally present in human saliva. The selective distribution of the bound gold probes depended on the EM method used. The probe concentrated close to the body of the cell in grid-mounted whole-cell preparations. In contrast, the probe clustered in fuzzy structures distant from the cell wall in embedded preparations which were used in an attempt to maintain spatial orientation of fragile surface structures. Although this report provides evidence that the elaboration of long appendages, 4 to 6 nm in diameter, is a common trait among the several species which now constitute the mutans group of streptococci, neither EM approach yielded label on the long fibrillar appendages which we have described.

Binding kinetics for β_2m, in an aggregated form, to oral streptococci and group A, C, and G streptococci have been reported previously (7, 8, 21). Wagner et al. also demonstrated the binding of aggregated β_2m to a short cell surface fuzz by using an indirect immunoelectron microscopy method based on a ferritin-antibody conjugate and limited to thin-sectioned specimens of these nonoral streptococci (30). Group A, C, and G streptococci have a higher binding capacity than oral streptococci for aggregated β_2m. Mutans streptococci are known to have from 70 to 1,700 β_2m receptors per cell (7); thus, the degree of labeling in the present study was consistent with previous observations. Some other differences in their binding characteristics include heat sensitivity, salt concentration dependence, and susceptibility to proteolytic enzymes (7, 8). The binding of monomeric β_2m to oral streptococci is calcium dependent and detergent sensitive (6), whereas binding of β_2m aggregates can be demonstrated in a variety of buffers, even those containing detergents (7, 8).

The various approaches to labeling used in the present study underlined the differences between the interaction of monomeric and aggregated β_2m with mutans streptococci. With the indirect method, in which monomeric β_2m was reacted with the bacteria, a lower percentage of the cells in each sample was labeled compared with the direct method. The reasons for this are not clear. The conjugation of β_2m to the gold colloid probably displayed β_2m in a manner mimicking an aggregated form and perhaps could have even exposed sequences of β_2m amino acids with a higher affinity for bacteria due to altered folding of the molecule. Similarly, the higher avidity of aggregated β_2m, as seen with glutaraldehyde- or liposome-induced aggregation (2, 21), has been ascribed to multiple-point attachment. The insensitivity of G_9-β_2m binding to the presence of Tween 20 also suggests that it may represent an aggregated form. The heat sensitivity of β_2m binding was consistent with previous results with radiolabeled aggregates (7). It is significant that adsorption of β_2m onto a colloid surface does not abolish its affinity for bacteria but enhances it. This phenomenon has also been discussed recently by Hay and Gibbons (Int. Assoc. Dent. Res. 1986, abstr. no. S-43, p. 719) in relation to proline-rich salivary proteins, which bind to bacteria feebly in their native state but act as bacterial attachment receptors when adsorbed to calcium phosphate beads that mimic teeth.

The literature on surface appendages of the mutans group of streptococci is suprisingly scant. Fibrillar appendages have been mentioned as a finding only a few times by investigators using various EM techniques (19, 24, 26), with Holt and Leadbetter's report (19) the only one based on more than one serotype. In this communication, we have demonstrated the consistent presence of mostly polar surface fibrillar appendages on both shadowcast and chemically stained whole-mount preparations of streptococci of the mutans group. The appendages were detected on all strains which had been grown in three media. *S. sobrinus* OMZ65 displayed the most densely arranged fibrils, which were sensitive to trypsin but not to heat or to protease K. The staining characteristics of the fibrils distinguish them from the strands of condensed fuzzy structures and much shorter, perhaps microcapsular material which we observed in freeze-substituted embedded specimens.

It could be expedient to interpret the fibrillar appendages as dried capsular material, as has been done for other streptococci (22). A brief look at other shadowcast preparations (22) might reveal a similar structure, but the difference between those findings and what was described here is clear. *S. mutans* does not produce significant amounts of extracellular polysaccharide when grown in sucrose-free medium. Even though the ultrathin sections might suggest the presence of lead citrate- and ruthenium-staining capsular material, it did not extend nearly as far as the fibrils. The strands of capsular material on group B streptococci were demonstrated exclusively on antibody-stabilized cells, but our bacteria were not treated with antiserum prior to shadowing. Furthermore, in contrast to the polar fibrils, capsular strands were found all around the periphery of group B streptococci. Our findings were not an effect of the direction of shadowing, as the lead citrate-stained appendages demonstrated the same polarity. Partial removal of the fibrillar structures by sonication and their recovery in the supernatant as a network of fibrils of comparable size to those on the intact cells suggest that they are specific structures. Boiling the cells would most likely disrupt a delicate capsule, if present, but the fibrils were still found after such treatment. It is thus unlikely that the fibrils represent a dried capsule.

On whole cells, the β_2m-gold probe bound primarily to limited segments of the periphery or the halo of the cell, and on cells with greater binding capacity, label bound over the whole cell surface. It is conceivable that the peripheral binding pattern resulted from a concentration of binding sites due to drying-induced distortion of the cell. The binding of the gold probes to bacteria in suspension yielded a more uniform distribution, all around the cell. However, clustering of binding sites was still observed (Fig. 9). The fibrillar appendages were consistently and remarkably free of β_2m label.

The visualization of surface structures and β_2m-binding site distribution by stereo imaging in thick sections adds a new dimension to studies of bacteria-ligand interactions. A cloudlike, wispy fuzz, extending substantially beyond the cell wall than either previously described or previously appreciated, contained most of the gold label, which was apparently at a considerable distance from the cell wall compared with what we could interpret from photographs of whole-mounted cells. In ultrathin sections it was not as easy to appreciate association between the gold probe and the cells, as the gold was sparse and present at a distance from the cell wall. The chemical nature of the wispy, fuzzy structures bearing the β_2m receptors is as yet unknown. They are distinct from the more organized fibrils seen by shadowcasting. The amount and distribution of label were unaffected by the growth medium. Positive binding after growth in a chemically defined medium suggests that the exposure of β_2m-binding sites at the streptococcal surface is not sucrose dependent. Moreover, a difference was observed between strains of *S. mutans* and *S. sobrinus*, which are known to differ markedly in their salivary pellicle- and glucan-binding functions (13). The two *S. mutans* strains bound somewhat more G_9-β_2m than did the two *S. sobrinus* strains, which is opposite to their glucan-binding capacities.

To our knowledge, the three-dimensional structure of surface components such as the fuzzy coat or capsules surrounding the cell has not been considered in previous studies of bacteria-ligand interactions. Yet the way in which potential binding sites are displayed on the bacterial surface and the possible flexion of surface structures to form zones or pockets of reactive sites probably has a great effect on the degree of binding. Therefore, application of our approach to localization of other adhesin-related antigens, such as the glucosyl transferases and antigen B (I/II) of mutans streptococci, seems warranted. Likewise, appreciation of three-dimensional structure might also raise questions about the pattern in which surface antigens are exposed to the host and explain differences in mucosal immunogenicity. Thus, the ability of bacteria to bind host molecules such as β_2m, a human histocompatibility component, and expose them on delicate structures at a distance from the cell wall may hinder immune system recognition and targeting by the host. The way in which ligand-binding sites are displayed might be a clever adaptation which indigenous microorganisms have evolved to maintain a low immunogenic profile.

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