

Serology of *Streptococcus sanguis*: Localization of Antigens with Unlabeled Antisera

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A new technique for the electron microscope localization of bacterial surface antigens, using unlabeled antisera, has been developed. This technique, called the "immuno-coating reaction" was applied to serological studies of *Streptococcus sanguis*, using antisera which had been shown to be specific for this species by gel diffusion analysis. The results of the immuno-coating reaction confirmed the specificity of the antisera for *S. sanguis*. In addition, the data indicate that some of the antigens responsible for the reaction were proteins which formed filamentous processes on the surface of the cell.

The development of fluorescent antibody labeling for use with the light microscope was followed by the introduction of ferritin labeled antibody conjugates for use with the electron microscope (14). The ferritin provided the electron-dense label which could be easily observed in the electron microscope; these labeled antibodies have been used to localize surface antigens in both gram-positive and gram-negative bacteria (10, 15, 16). In the process of studying ferritin labeled globulins against strains of *Streptococcus sanguis*, we noted that control sections of the bacteria treated with unlabeled antisera and stained with uranyl acetate and lead citrate had electron-dense coatings on the surface of the cells; such coatings were not present on sections treated with normal sera. Antigen-antibody complexes have been observed on the surfaces of viruses (9) and bacterial appendages by negative staining techniques and were referred to as a "coating reaction." A nonspecific coating reaction was described for *Staphylococcus aureus* cells treated with human immunoglobulin G (18). In keeping with this nomenclature, we have called the reaction reported in this paper an "immuno-coating reaction" to indicate that immunological specificity is implied. The simplicity of the immuno-coating reaction relative to the preparation of ferritin conjugates suggested it might be useful not only for localizing bacterial antigens, but as a routine serological tool. The purpose of this investigation was to determine: (i) whether the immuno-coating reaction was immunologically specific and (ii) if the reaction could be used for serological investigations.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains studied included the following: *S. sanguis* strains 6223, M5, and B4 which were isolated from dental plaque (13); Challis and Wickey (supplied by J. Ranhand, National Institute of Allergy and Infectious Diseases); *S. sanguis* strains ATCC 10556, 10557, and 10558; and *S. mitis* strains ATCC 903, 9811, and 6249. The last three strains, which had been previously designated as *S. mitis*, have been shown to have antigenic and physiological properties characteristic of *S. sanguis* (2). *S. mutans* strains FA-1, Pk-1, LM-7, HS-6, and GS-5 (supplied by R. J. Gibbons, Forsyth Dental Center, Boston, Mass.) and *S. faecalis*, strain S161, were also used. The cultures of *S. sanguis* and *S. faecalis* were grown in brain heart infusion broth (Difco) at 37 C for 18 h; cultures of *S. mutans* were grown anaerobically by using gas generating packs (H₂ + CO₂; Difco). The medium and growth conditions were the same as used for *S. sanguis*.

Immunization. M-5 cells from 18-h broth cultures were washed three times in 0.15 M NaCl and suspended at a concentration of 0.4 mg/ml (dry weight) in 0.15 M NaCl containing 0.2% formalin. The vaccine was injected intravenously in female New Zealand rabbits twice weekly, for 4 weeks. The dose for the first four injections was 0.2 ml, and for the last four it was 1.0 ml. The rabbits were bled by cardiac puncture 5 days after the last injection. Preimmune sera were obtained from all rabbits and used for the controls.

Gel diffusion techniques. Immuno-electrophoresis and Ouchterlony analyses were performed in borate buffered agar, pH 8.4, as described previously (13).

Absorption of serum. Strain ATCC 10557 has been shown to have only two antigenic components (precipitins) in common with the M-5 strain. These antigens, designated c and d, have been identified by immuno-electrophoresis, are found in autoclaved ex-

tracts of whole cells (11), and can be separated from the extract of M-5 by precipitation with 5% trichloroacetic acid (Rosan, unpublished observation). A mixture of c and d antigens (2 mg, dry wt) was dissolved in 0.5 ml of saline; 2 ml of M-5 antisera was added, and the mixture was incubated at 4 C for 24 h. The precipitate was removed by centrifugation, and the absorbed serum was tested against an extract containing all of the antigens. Absorption was repeated until the c and d antigens could no longer be detected in the extracts of strains M-5 and 10557 by gel diffusion analyses.

Procedure for the immuno-coating reaction.

Cells from 18-h cultures were washed three times with 0.15 M NaCl. Pellets obtained after centrifugation were resuspended in 0.5 ml of undiluted M5 antisera and incubated at room temperature for 30 min; the cells were washed three times in saline, and the pellets were fixed and embedded for electron microscopy. Controls for this experiment included the following: (i) *S. sanguis* cells exposed to preimmune sera (normal sera), (ii) *S. mutans* and *S. faecalis* cells incubated with M-5 antisera, (iii) M-5 cells treated with diluted M-5 antisera, (iv) absorption with specific antigen preparations, and (v) detection of M-5 antibody bound to cells with ferritin labeled sheep anti-rabbit globulin.

Immunoferritin controls. Ferritin-conjugated anti-rabbit globulins were prepared as follows: Ferritin which had been crystallized six times (Research Division, Miles Laboratories, Inc., Illinois) was conjugated to sheep anti-rabbit globulin (Research Division, Miles Laboratories, Inc., Kankakee, Ill.), utilizing xylylene m-discocyanate (Polysciences, Inc., Warrington, Pa.) as a coupling reagent according to the method of Singer (14) as modified by Hsu (5). M-5 cells were first incubated with rabbit anti-M-5-sera as described above under procedure for the immuno-coating reaction. The cells were washed, and the pellets were incubated with 0.25 ml of ferritin-conjugated anti-rabbit globulins at room temperature for 30 min. This was followed by three washes in 0.15 M saline. Controls included the following: (i) M-5 cells plus normal sera, followed by treatment with ferritin labeled anti-rabbit globulin, (ii) M-5 cells plus ferritin labeled anti-rabbit globulin.

Preparation of cells for electron microscopy.

Pellets of treated and washed cells were prefixed in a solution containing 2.5% glutaraldehyde and 2% paraformaldehyde in sodium cacodylate buffer, pH 7.3 (6). The pelleted cells were postfixed in 2% osmic acid in s-collidine buffer at pH 7.3, washed, and stained in block with a 0.5% solution of uranyl acetate in Veronal-acetate buffer, pH 5.2. The specimens were dehydrated in graded ethanol solutions and embedded in Epon (9). Sections cut at 0.1 μ m were stained with uranyl acetate and lead citrate (14). The specimens were examined in Philips EM-300 electron microscope.

Treatment with proteolytic enzymes. Strain M-5 and 10557 cells were treated with pepsin and trypsin as follows. For pepsin treatment, 45 mg of cells (wet wt) were suspended in 20 ml of a 0.4% pepsin solution (Worthington Biochemical Corp., Freehold, N.J.) in

0.02 N HCl. For trypsinization, 50 mg of cells (wet wt) were suspended in 25 ml of a 0.1% trypsin solution (Worthington Biochemical Corp., Freehold, N.J.) in 0.05 M phosphate buffer at a pH of 7.5; controls were incubated in the HCl or buffer solutions without the enzymes. All incubations took place at 37 C for 5 h. After incubation, a sample of each cell suspension was washed, incubated with M-5 antisera (immuno-coating reaction), washed again, and processed for electron microscopy. A sample of untreated cells was also processed for electron microscopy.

RESULTS

The typical morphology of untreated M-5 cells is shown in Fig. 1. The cell wall consists of an electron-dense inner layer and a less dense outer layer which ranged in thickness from 12 to 15 nm. Hair-like, filamentous structures extending beyond the cell wall for a distance of up to 55 nm can be observed along the periphery of the cell.

M-5 cells incubated with M-5 antisera have a distinct layer of intermediate electron density (60-70 nm thick) surrounding the cell wall (Fig. 2). This layer could be readily distinguished from the cell wall in properly oriented sections because of differences between their fine structure. The cell wall appeared more homogeneous than the electron-dense coating.

The intensity of the reaction was reduced with dilution of the M-5 antisera and was completely absent at a 1:50 dilution. The morphology of M-5 cells treated with normal rabbit sera and then washed (Fig. 3) remained almost indistinguishable from that of untreated cells (Fig. 1); no electron-dense coating was visible. However, the fine filamentous projections extending from the periphery of the cell walls of untreated cells were not as prominent on cells treated with normal sera, possibly because the hair-like extensions were removed partially during the repeated washings and centrifugations.

None of the other species of microorganisms incubated with M-5 antisera demonstrated any trace of an electron-dense coating. A typical result with *S. mutans*, strain LM-7, is shown in Fig. 4.

Incubation of strain M-5 with rabbit M-5 antisera followed by ferritin-conjugated anti-rabbit globulin resulted in ferritin tagging of the coating layer (Fig. 5). Although the ferritin marker penetrated the dense coating layer to varying degrees, most of the ferritin remained on the surface of the dense coat.

M-5 cells incubated with normal rabbit sera followed by ferritin-conjugated anti-rabbit globulin had neither an electron-dense coat, nor

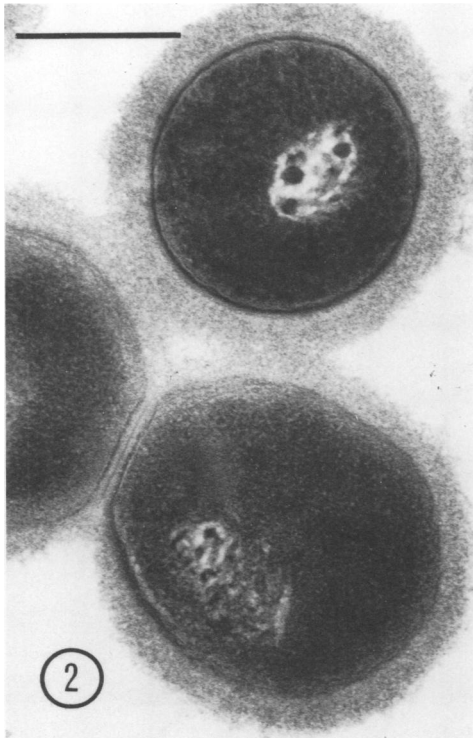
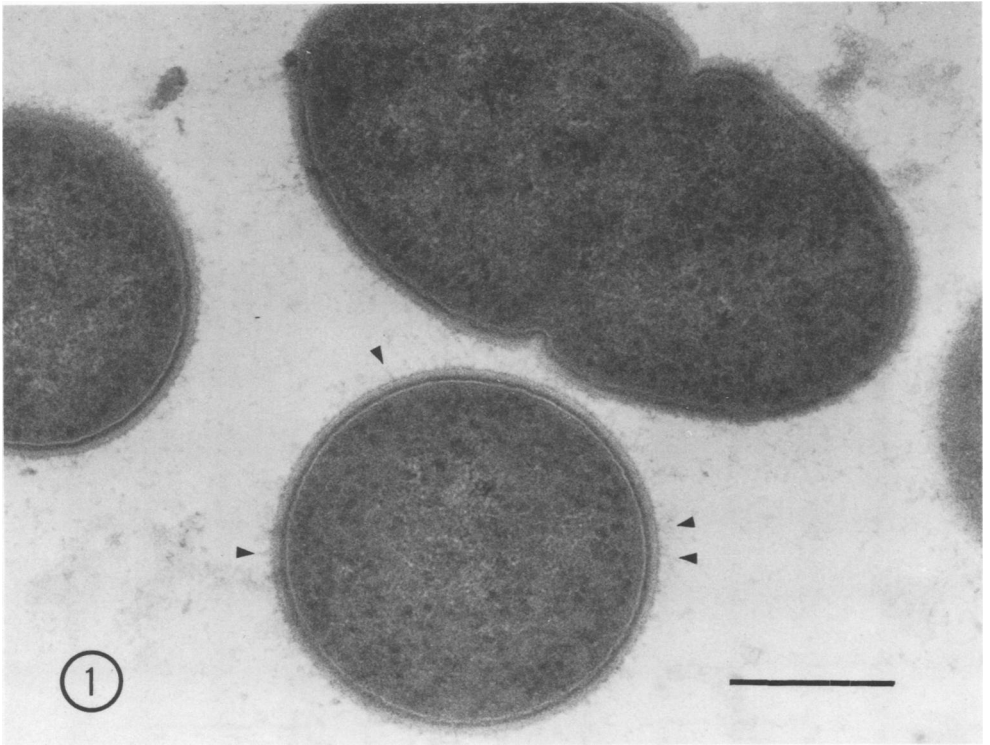


FIG. 1. Electron micrograph of thin section of *S. sanguis*, strain M-5. Magnification in micrographs i. $\times 80,000$, and the bar is $0.25 \mu\text{m}$. The arrow indicates filamentous processes.

FIG. 2. Electron micrograph of thin section of strain M-5 incubated with M-5 antisera.

FIG. 3. Electron micrograph of thin section of strain M-5 treated with normal sera.

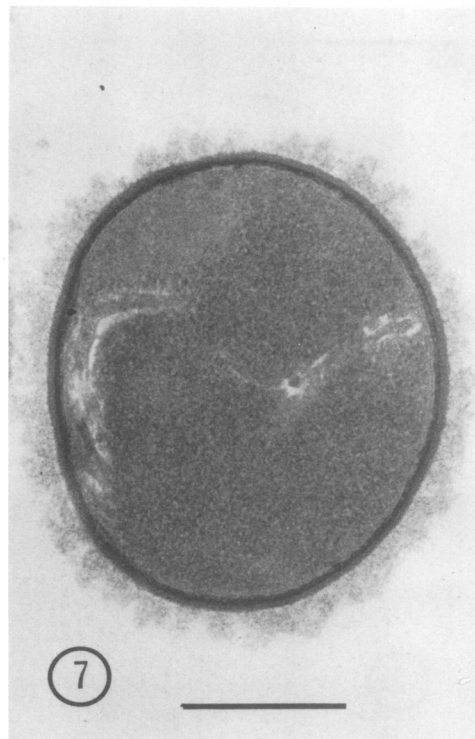
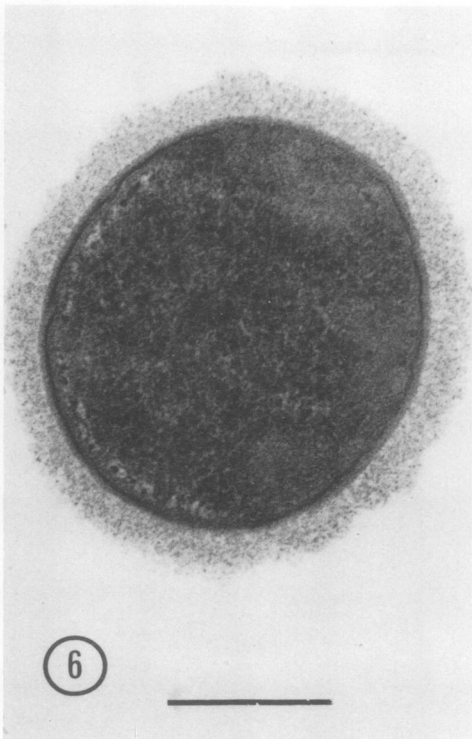
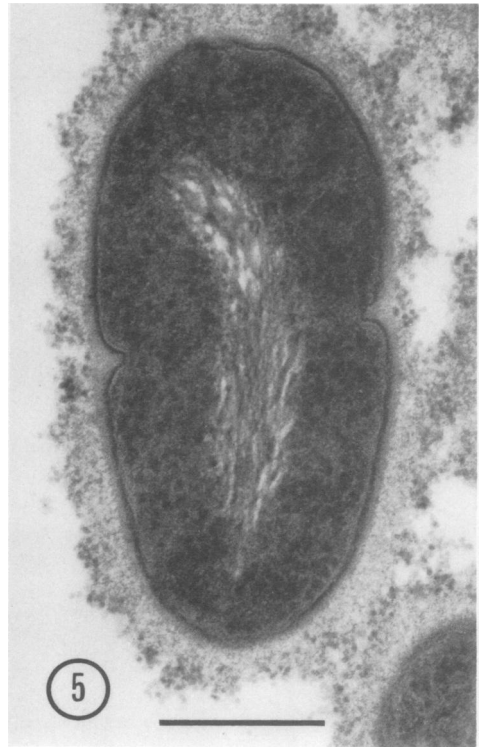
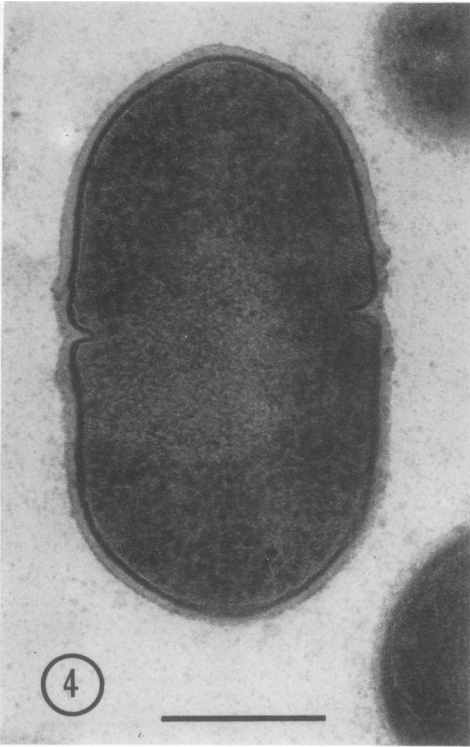


FIG. 4. Electron micrograph of thin section *S. mutans* strain LM-7 treated with M-5 antisera.

FIG. 5. Electron micrograph of thin section of strain M-5 treated with M-5 antisera followed by ferritin conjugated anti-rabbit globulin.

FIG. 6. Electron micrograph of thin section of *S. sanguis*, strain Wicky, treated with M-5 antisera.

FIG. 7. Electron micrograph of thin section of *S. sanguis*, strain 10557 treated with M-5 antisera.

was any ferritin marker observed on the cell surface. Ferritin labeled anti-rabbit globulin also did not react with untreated M-5 cells. The results of these studies indicated that M-5 sera reacted specifically with homologous cells to give an immuno-coating reaction. Thus far, the results confirmed previous studies employing gel diffusion analysis, which also indicated that M-5 antisera did not react with *S. mutans*, *S. faecalis*, and other heterologous bacteria (13). However, these previous studies did show that M-5 antisera cross-reacted broadly with strains of *S. sanguis*. Could these cross reactions also be observed in the immuno-coating reaction?

Reactions with other strains of *S. sanguis*.

The immuno-coating reaction formed a moderately electron-dense coating on the periphery of all strains of *S. sanguis* treated with anti M-5 serum. Three of these strains, Wicky, 10558, and 6223, demonstrated a relatively compact and uniform coating similar to strain Wicky (Fig. 6). The other strains had a coating which was less compact or uniform and had a somewhat lacy appearance similar to that shown for strains 10557 (Fig. 7). The control cells treated with normal sera did not have a coating reaction.

M-5 antisera absorbed with the c and d antigens no longer demonstrated a coating reaction with 10557 cells (Fig. 8). However, M-5 cells did react with the absorbed serum, but the intensity of the reaction was reduced markedly (Fig. 9).

Treatment with proteolytic enzymes. The normal morphology of *S. sanguis* includes very thin filamentous processes radiating from the periphery of the cell wall (Fig. 1). Trypsin treatment partially removes some of these processes and is associated with a reduction in intensity of the immuno-coating reaction. Pepsin treatment results in complete removal of the filamentous processes; in strain 10557 which has only the c and d antigens in common with M-5, removal of the filamentous processes result in elimination of the coating reactions (compare Fig. 7 with Fig. 10). In strain M-5, pepsin treatment results in marked reduction of the intensity of the coating reaction (compare Fig. 2 with Fig. 11).

DISCUSSION

Two types of immuno-coating reaction were observed among the strains studied. In the first type, the coating appeared as a compact, well defined, electron-dense layer of relatively uniform width. The other type appeared less well defined or uniform and had a somewhat lacy structure. These differences could be simply

due to differences in the number or types of antigens found in different strains, even though these antigens may not have been detected in the heated extracts used in gel diffusion analysis. It is also possible that at least part of the immuno-coating reaction is caused by antigens or cross reactions which were either undetected or too weak to be detected in immuno-diffusion analyses.

Evidence is available, however, that part of the immuno-coating reaction is associated with the c and d antigens. Strain 10557 contains only the c and d antigens in common with M-5; M-5 antisera absorbed with the c and d antigens no longer gives a coating reaction with 10557 cells, and the coating reaction with M-5 cells was reduced. The isolated c and d antigens are hydrolyzed by trypsin and pepsin (B. Rosan, International Association for Dental Research Abstr., p. 265, 1972). The former enzyme reduces the immuno-coating reaction with 10557 cells, whereas pepsin eliminated entirely the immuno-coating reactions with these cells. Treatment of M-5 cells with these proteases markedly reduced the immuno-coating reaction but did not eliminate it entirely; the remaining immuno-coating reactions are presumably due to other antigens in the M-5 cell wall. The simultaneous disappearance or reduction of the coating reaction and the filamentous processes strongly suggests that some of the antigenic determinants responsible for the reaction are located in these processes. The close relationship between these filaments and the c and d antigens is also suggested by the disappearance or reduction of the coating reaction after absorption of the sera with these protein antigens.

Although the c and d antigens could not be detected in gel diffusion analysis of strains 903 and 10556, these strains did possess filamentous processes on their surfaces. The less well defined coating reaction observed in these strains might result because of a weaker antigen-antibody interaction, or because the coating reaction is more sensitive than the precipitin reaction and can detect cross-reactions which are not visible in gel diffusion. In any event, the similarity in cross reactions obtained by immunodiffusion and the immuno-coating reaction not only confirms the specificity of the M-5 antisera but suggests that the immuno-coating reaction is a valid technique for detecting serological relationships among bacteria.

The morphological appearance of the hair-like, filamentous processes on the surface of *S. sanguis* cells resembles the "fuzzy coat" described by W. F. Liljemark and R. J. Gibbons (International Association for Dental Research Abstr, p. 107, 1972) in *S. miteor* and *S. salivarius*

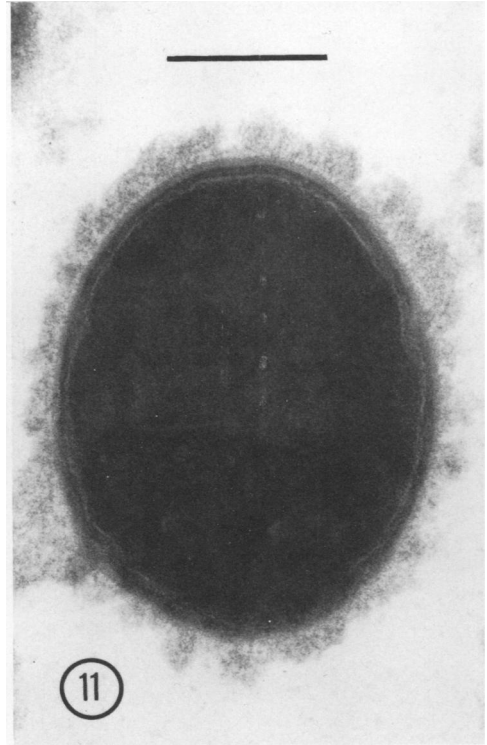
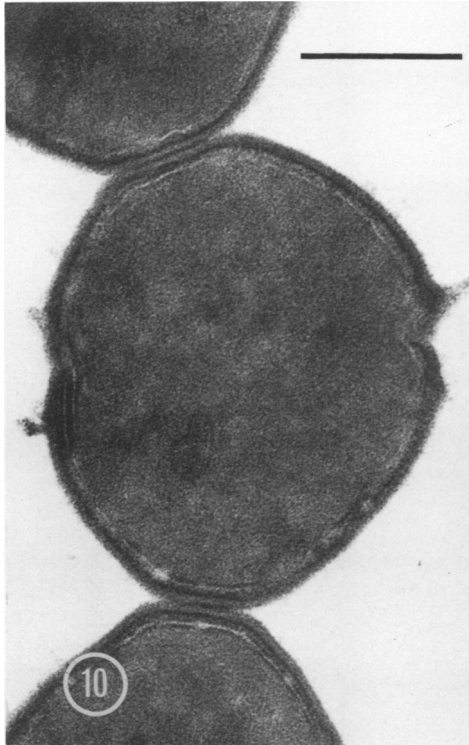
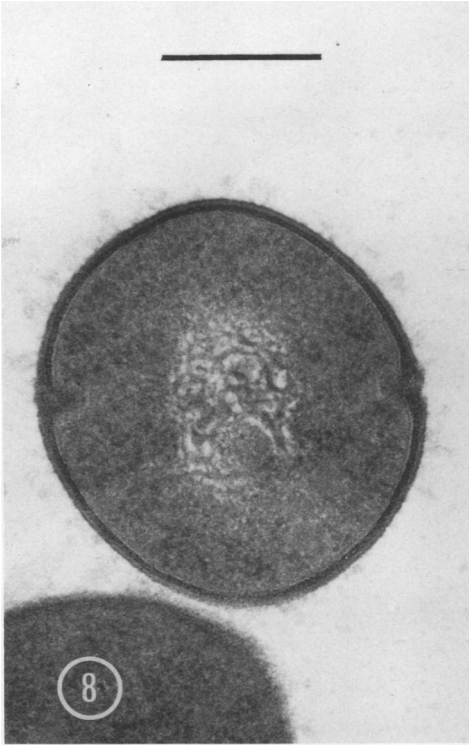


FIG. 8. Electron micrograph of *S. sanguis*, strain 10557, treated with M-5 antisera absorbed with the c and d antigens.

FIG. 9. Electron micrograph of thin section of strain M-5 treated with M-5 antisera absorbed with the c and d antigens.

FIG. 10. Electron micrograph of thin section of strain 10557 after treatment with pepsin followed by incubation with M-5 antisera.

FIG. 11. Electron micrograph of thin section of M-5 after treatment with pepsin followed by incubation with M-5 antisera.

(4). The susceptibility of these processes to digestion by trypsin is similar to that reported for *S. pyogenes* (16) and for *S. salivarius* (4).

Ellen and Gibbons (3) have postulated that these filaments are necessary for the adherence of *S. pyogenes* to epithelial cells; similar mechanisms have been postulated for the adherence of *S. salivarius* and other indigenous bacteria to epithelial cells (3). It is possible that analogous mechanisms might be involved in the preference that *S. sanguis* cells shows for adhering to tooth surfaces (1, 17). Thus, the immuno-coating reactions combined with other immuno-chemical approaches might provide a useful tool for furthering our understanding of the mechanisms governing the selective distribution of bacteria on various surfaces in the oral cavity.

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