INFECTION AND IMMUNITY, May 1981, p. 830-839
0019-9567/81/050830-10$02.00/0

Vol. 32, No. 2

Localization of *Streptococcus mutans* GS-5 Glucosyltransferases and Intracellular Invertase

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Antibodies directed against *Streptococcus mutans* GS-5 intracellular invertase and glucosyltransferase fractions capable of synthesizing primarily water-soluble or insoluble glucans were used to ultrastructurally localize the enzymes by means of the unlabeled antibody peroxidase-antiperoxidase method. This immunocytochemical procedure revealed that the intracellular invertase was associated primarily with the cytoplasmic membrane of the cariogenic organism. The glucosyltransferase complex responsible for insoluble glucan synthesis was localized as aggregates attached to the cell surface or extracellular polysaccharides of strain GS-5. In contrast, the glucosyltransferase activity synthesizing primarily water-soluble glucans was distributed uniformly over the cell surface or in association with extracellular polysaccharides. These results are discussed relative to the sucrose-metabolizing ability of *Streptococcus mutans*.

The central role of sucrose metabolism in the development of human dental caries initiated by *Streptococcus mutans* has been supported by numerous investigations (9). The dietary disaccharide serves as a substrate for the synthesis of glucan molecules by *S. mutans* and stimulates colonization of tooth surfaces by the organism. In addition, recent results (21) indicate that the organism metabolizes sucrose to lactic acid more rapidly than most other oral microorganisms. These two properties of *S. mutans* appear to contribute significantly to the cariogenic potential of this oral bacterium.

At least five different enzyme systems capable of metabolizing sucrose have been demonstrated in *S. mutans*: glucosyltransferase (GTF; EC 2.4.1.5) (10), fructosyltransferase (EC 2.4.1.10) (3), intracellular (8) and extracellular (6) invertases (EC 3.2.1.26), and the sucrose phosphotransferase system (25, 27). Most investigations concerning these activities have focused on the purification and characterization of the individual enzymes, and relatively little information is currently available regarding the cellular location of each activity.

Recently, the cytochemical and immunocytochemical localization of the sucrose-metabolizing enzymes of *S. mutans* GS-5 was reported (1). Utilizing antibodies directly against partially purified GTF fractions in conjunction with the indirect fertitin labeling technique, it was demonstrated that GTF activity is closely associated with the cell surface and extracellular polysaccharide material. However, due to limitations of the cytochemical procedure used, it was not possible to differentiate between the location of the fructosyltransferase and invertase activities since both are capable of releasing glucose from sucrose.

The present investigation involves the utilization of the peroxidase-antiperoxidase (PAP) indirect immunocytochemical procedure (26) to clarify the location of the invertase and GTF enzymes. With the use of antisera directed against highly purified intracellular invertase (19) and GTF responsible for synthesizing soluble glucan, this technique permitted a more precise differentiation and localization of the saccharolytic enzymes, both intracellularly and extracellularly. The results are discussed in terms of the potential roles of the sucrose-metabolizing enzymes in the cariogenic properties of the organism.

MATERIALS AND METHODS

Culture and growth conditions. The *S. mutans* GS-5 culture used in this study was maintained by monthly transfer between brain heart infusion agar slants and by storage in Todd-Hewitt (TH) broth at −65°C. For electron microscopy an overnight culture grown in TH broth was inoculated into 15 ml of either the chemically defined medium of Terleckyj et al. (29) containing 2% glucose or TH broth containing 2% sucrose, and grown for 18 h at 37°C. The two control cultures, *Staphylococcus aureus* and *Escherichia coli*, were propagated in TH broth for 18 h at 37°C.

Preparation of antisera. Antisera against highly purified invertase and GTF-B, a fraction responsible for synthesizing soluble glucans, were produced as previously reported (15, 19). The GTF-B fraction displays a single band on sodium dodecyl sulfate-poly-

acrylamide gel electrophoresis and contains no detectable lipoteichoic acid, but does contain trace amounts of dextranase activity (14). The insoluble glucan-synthesizing fraction, GTF-A, was heterogeneous (13) and aggregated, and it contained multiple bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and demonstrable dextranase and fructosyltransferase activities. Gamma globulin fractions (2) were lyophilized and stored at 10°C until immediately before use. The PAP complex was prepared according to the procedure described by Sternberger (26). Additionally, our initial investigations utilized PAP kindly supplied by L. A. Sternberger (Rochester, N.Y.).

Immunocytochemical localization of enzymes. Cells were obtained from the media by centrifugation at 4,000 × *g* for 20 min. After a rinse in 0.05 M phosphate buffer (pH 7.2) containing 0.9% NaCl (PBS), the bacteria were fixed for 1 h at 4°C in 1% glutaraldehyde in PBS. After two washes in PBS at 4°C, the cells were stored at 10°C overnight in PBS. The next day, the specimen was washed two more times in PBS and enrobed in a small volume (1 to 2 drops) of 1% agarose. After solidification, the agarose cell button was cut into 1-mm cubes with a razor blade, and the specimens were dehydrated and embedded in Epon (18). Silver-to-gold sections were cut with a diamond knife and retrieved on 300-mesh nickel grids. For immunocytochemical localization, the PAP procedure described by Childs et al. (5) was slightly modified as follows: (i) etch sections in 10% H_2O_2 for 3 min; (ii) rinse in distilled water and blot on filter paper; (iii) float on drop of 1:30 dilution of normal goat serum in PBS for 5 min; (iv) float on drops of rabbit anti-GTF-A or -B globulins diluted in PBS to 140 μg of protein (8) per ml, or on drops of anti-invertase at 1.0 μg/ml of PBS, and react at 10°C for 48 h; (v) jet rinse grids in PBS, float on drop of 1:30 normal goat serum for 5 min, and jet rinse in PBS; (vi) float on drop of 1:30 goat anti-rabbit immunoglobulin G for 5 min and jet rinse in PBS; (vii) float on 1:30 normal goat serum for 5 min and then float on a drop of PAP (1:35 dilution in PBS containing 1% normal goat serum) for 5 min; (viii) jet rinse in 0.05 M tris(hydroxymethyl)aminomethane buffer (pH 7.6), followed by diaminobenzidine and H_2O_2 reaction mixture for peroxidase (23); and (ix) jet rinse in distilled water and float on drop 4% OsO_4 for 8 min, followed by rinse in distilled water.

Controls were treated in exactly the same manner except that (i) normal rabbit globulins were reacted with the sections of *S. mutans* at step iv, and (ii) sections of a mixed culture of *S. aureus* and *E. coli* were reacted with the anti-GTF and invertase sera. Electron micrographs were prepared as previously described (1).

RESULTS

Localization of GTF-A. The partially purified enzyme complex capable of synthesizing adherent glucans, GTF-A (14), was localized by the PAP technique with anti-GTF-A gamma globulin. In cells grown in the defined medium with no contaminating sucrose, the results indicate that the enzyme complex exists as aggregates associated with the cell surface (Fig. 1 to 3). The cytochemical label, which can be found some distance from the cell cross sections (Fig. 1), probably represents specific staining of GTF on cell surfaces which were exposed by grazing cuts. In some instances, the electron-light area of the cell wall is evident in such sections (Fig. 1, arrowheads). It is also likely that some of the intercellular label is localizing enzyme complexes that were shed from the cells and trapped in the agarose matrix used to enrobe the cells before embedding. An examination of the labeling pattern at higher magnification (Fig. 2) demonstrated the random localization of the GTF-A complex along the cell surface, usually in direct contact with the cell wall. In addition, the GTF-A complexes appear to be involved in "bridges" (Fig. 3, arrowheads) between adjacent cells which may represent glucan-GTF complexes postulated previously as mediators of cell-cell agglutination (7). Such complexes could be formed in the TH broth preinocula even though the cells were cultured in glucose-chemically defined medium. Cells which had been grown in TH medium containing sucrose synthesized copious amounts of extracellular polysaccharides which appeared as electron-light fibrils and tufts against a gray background (Fig. 4). The GTF-A enzyme aggregates were usually localized at the edges of polysaccharide clumps or in spaces inside of these clumps (Fig. 4).

The control experiments utilizing normal rabbit globulin at a 1:100 dilution (140 μg of protein per ml) demonstrated little or no labeling of the *S. mutans* cells (Fig. 5). When specific antisera against invertase or GTF were reacted with sections of the mixed *S. aureus* and *E. coli* culture, no labeling was seen (Fig. 6). Membranes of the *S. aureus* cells would often appear darkened, probably due to the reaction of catalases with the H_2O_2-diaminobenzidine reaction mixture; moreover, the absence of PAP complexes over these areas indicated that antibodies were not involved in the reaction.

Localization of GTF-B. In the absence of sucrose, the labeling pattern of GTF-B, the lower-molecular-weight enzyme responsible for soluble glucan formation, shows that the enzyme is nonaggregated and uniformly distributed throughout the culture as evidenced by the presence of PAP complexes over all of the section except for the cell wall (Fig. 7). This is in contrast to GTF-A, which, even in the absence of sucrose, is aggregated and tightly associated with the cell surfaces (Fig. 1 to 4).

When cells were grown in the presence of sucrose, GTF-B was localized in association with the cells and extracellular polysaccharides (Fig. 8), where it could be seen to attach to the fibrillar

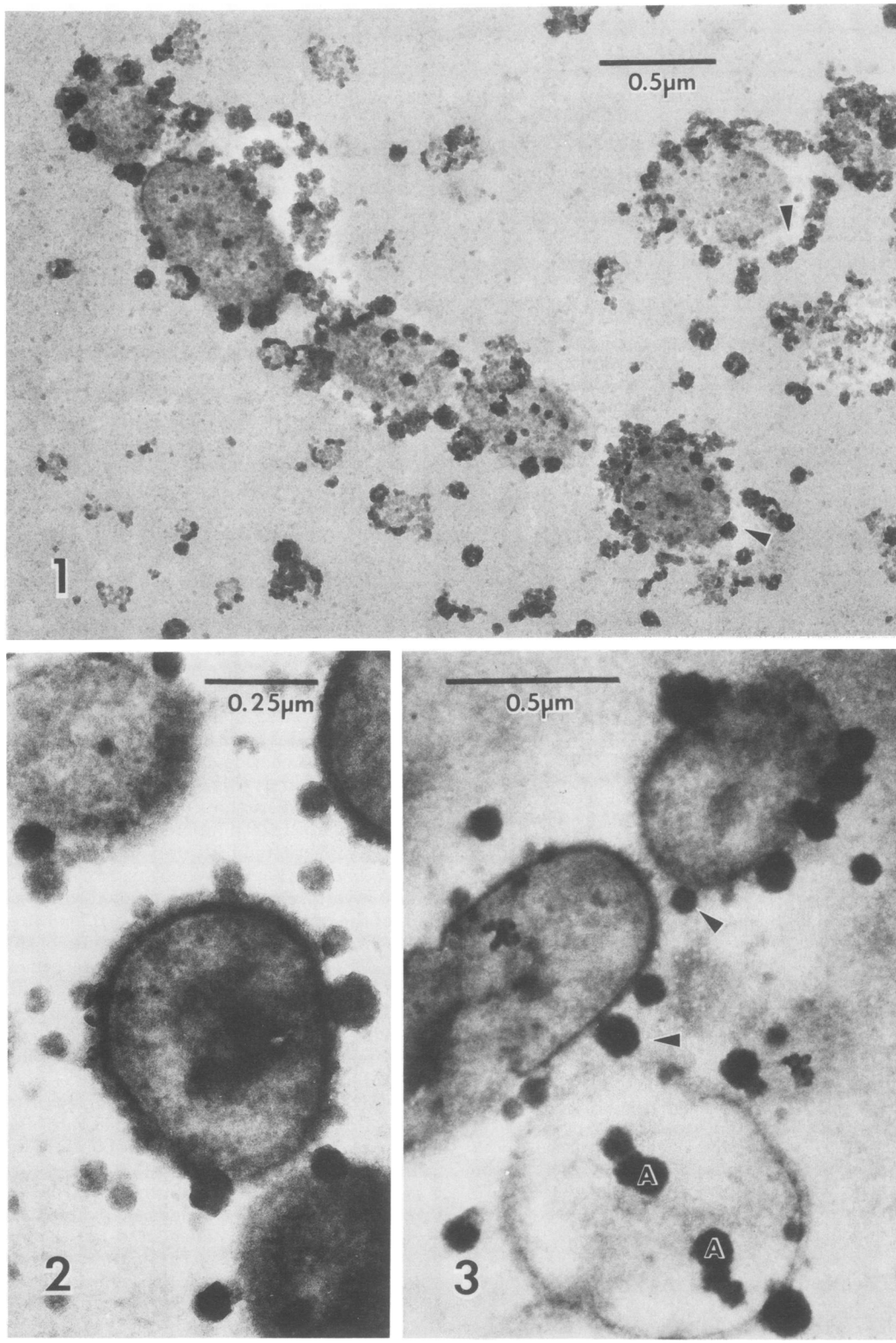


FIG. 1. *Immunocytochemical localization of GTF-A in S. mutans GS-5 grown in defined medium containing glucose and reacted with rabbit anti-GTF-A globulin fraction containing 140 μg of protein per ml of diluent. GTF-A enzyme is localized as 55- to 100-nm aggregates usually associated with the cell walls (arrowheads).*

FIG. 2. *High magnification of GS-5 cells reacted with anti-GTF-A as described for Fig. 1.*

FIG. 3. *Cells reacted with anti-GTF-A as described for Fig. 1 show association of enzymes with cell walls and cell-to-cell bridges (arrowheads). Dead cell at bottom of micrograph shows intracellular aggregates of GTF-A (A).*

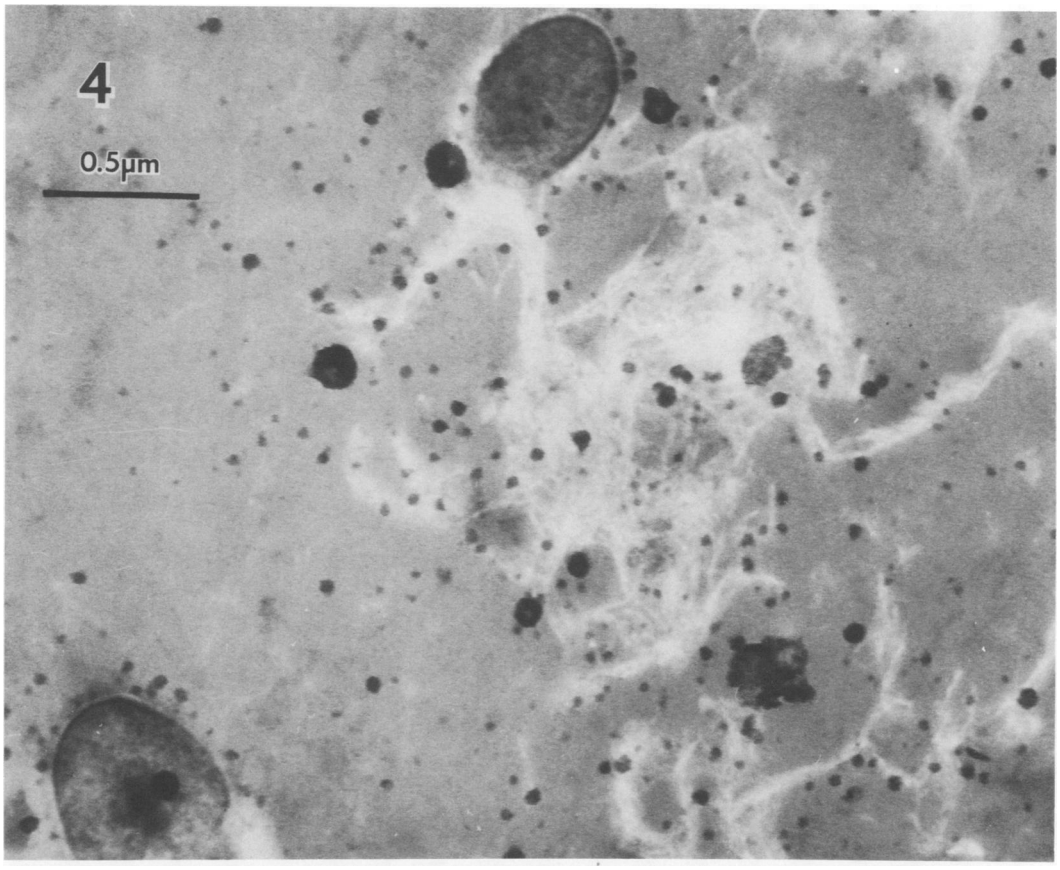
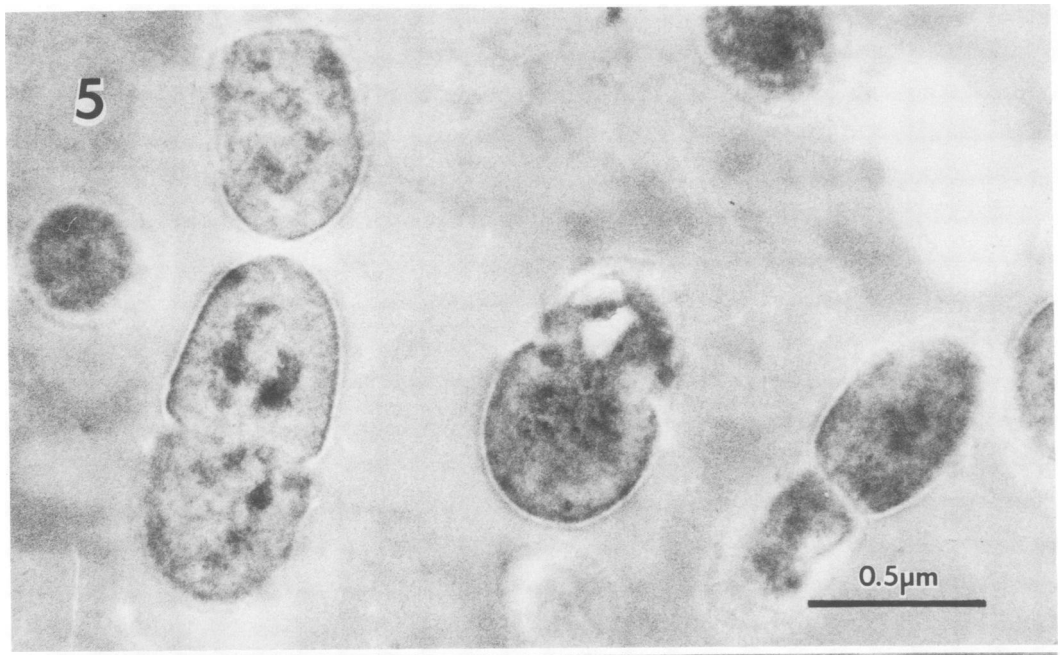


FIG. 4. *S. mutans cells grown in sucrose-containing TH medium synthesized large amounts of extracellular polysaccharides. The immunocytochemical localization of GTF-A was accomplished as described for Fig. 1. Note association of enzyme with polysaccharides and cell walls.*

and globular polysaccharides described in a previous study (12). This finding is in contrast to GTF-A, which localizes as only large aggregates associated with cell surfaces and extracellular polysaccharides (Fig. 4). Control specimens using nonspecific rabbit globulins gave identical results to the previous controls for the GTF-A experiments (Fig. 5). When anti-GTF-A and -B were mixed together and reacted with GS-5 cells, we noted the presence of both large and small aggregates associated with cell surfaces and extracellular space (figure not shown).

Localization of intracellular invertase. The availability of highly purified preparations of intracellular invertase made it possible to prepare specific anti-invertase gamma globulins (19). The application of this antibody onto sections of strain GS-5 cells in conjunction with the PAP procedure demonstrated that this enzyme was primarily associated with the cellular membrane (Fig. 9). Extensive labeling of the area near the cytoplasmic membrane was clearly demonstrated by the presence of PAP complexes, which could be seen at high magnification to trace the membrane along the forming cross wall and possibly even into the central mesosome (Fig. 9, arrow). PAP complexes were also found in the cytoplasm of the cells, indicating that invertase is not strictly located at the membrane; however, little or no label was found extracellularly (Fig. 9).

Unfortunately the PAP technique did not allow any discrimination between location of the enzyme at the inner or outer faces of the cytoplasmic membrane. Clear spaces in the cells represent artifactual holes induced by H_2O_2 during the etching procedures. No labeling of the *S. aureus* or *E. coli* cells could be found when the anti-invertase serum was applied to sections of the mixed culture (figure not shown). When normal rabbit globulin was reacted with *S. mutans* cells, no label was found as previously described (Fig. 5). The results of all labeling experiments are summarized in Table 1.



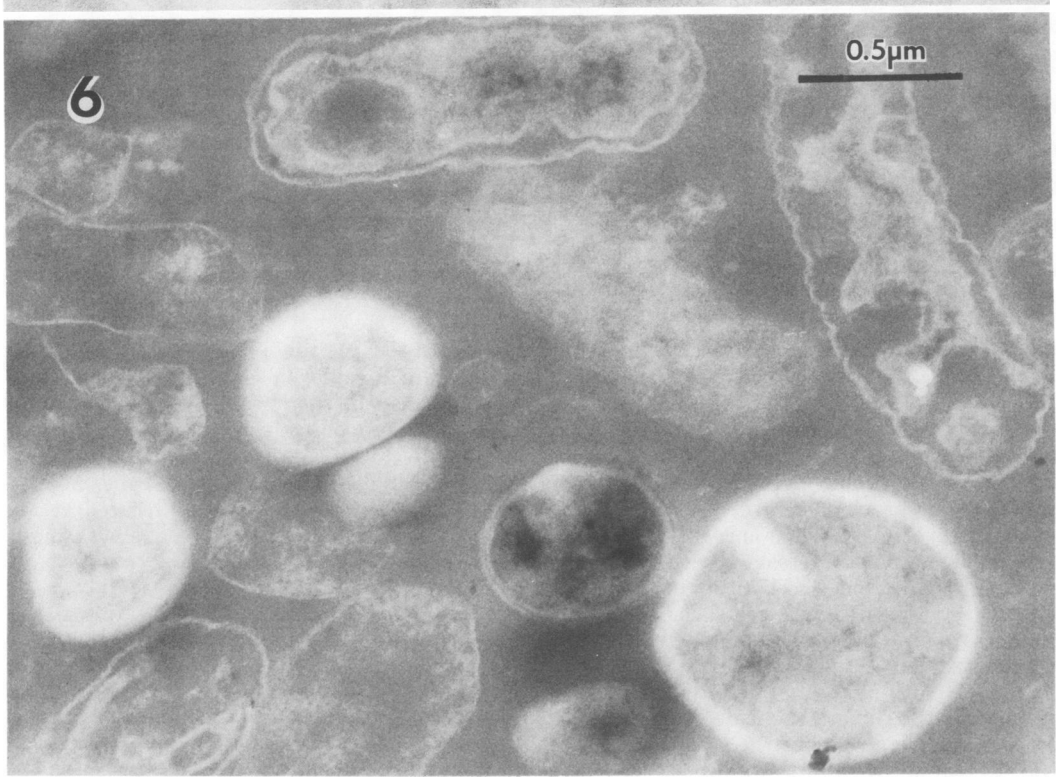


FIG. 5. *Immunocytochemical control. S. mutans cells were grown in TH broth containing sucrose. Normal rabbit globulin was used in the staining procedure at a 1:100 dilution (150 μg of protein per ml).*

FIG. 6. *Immunocytochemical control. S. aureus and E. coli cells grown in TH broth containing sucrose were reacted with anti-GTF-A; no staining is seen.*

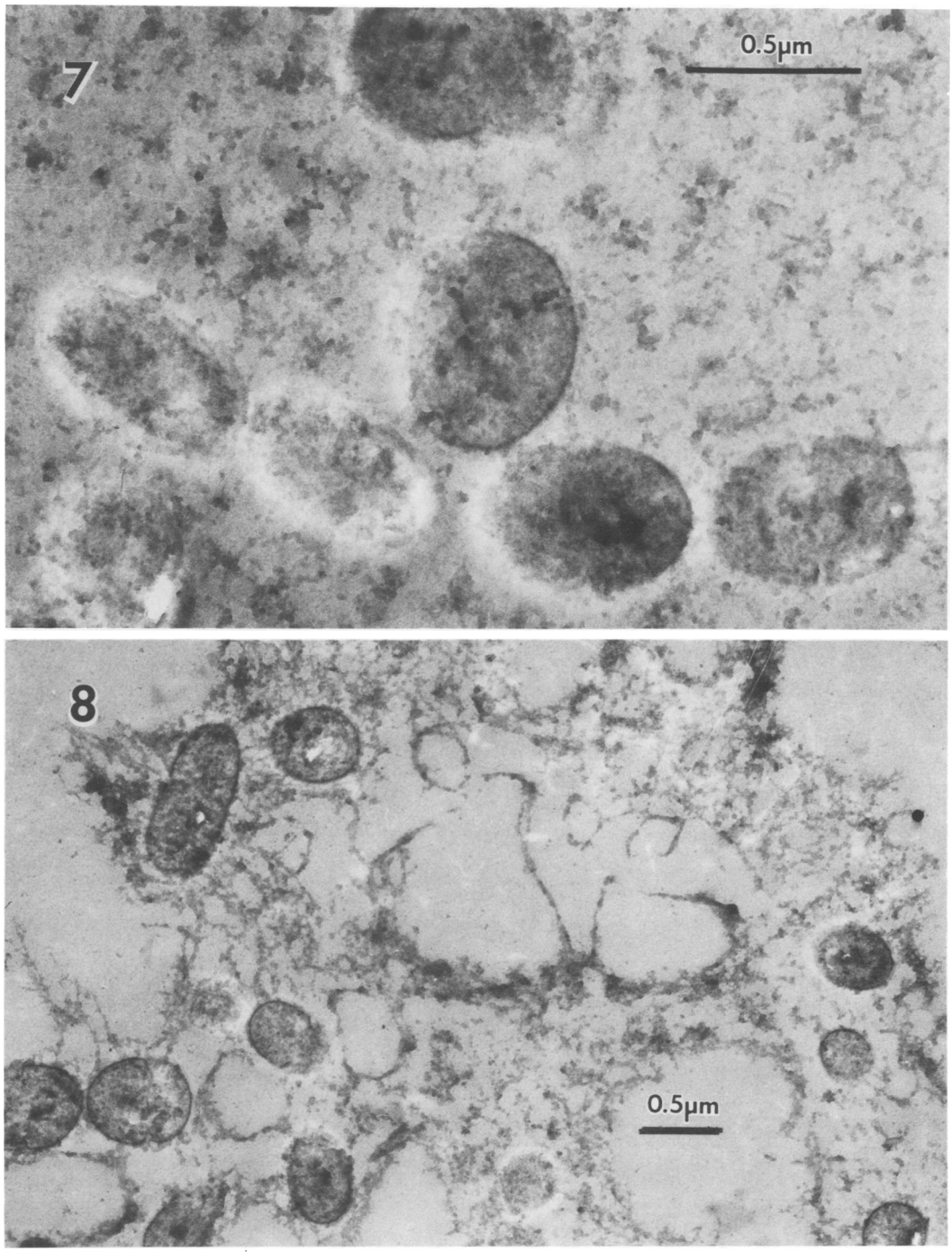


FIG. 7. *Localization of GTF-B in S. mutans GS-5 grown in glucose defined medium and reacted with rabbit anti-GTF-B gamma globulin fraction containing 140 μg of progein per ml.*

FIG. 8. *Localization of GTF-B along polysaccharide fibrils synthesized by cells grown in medium containing 2% sucrose. Sections were stained as described for Fig. 7.*

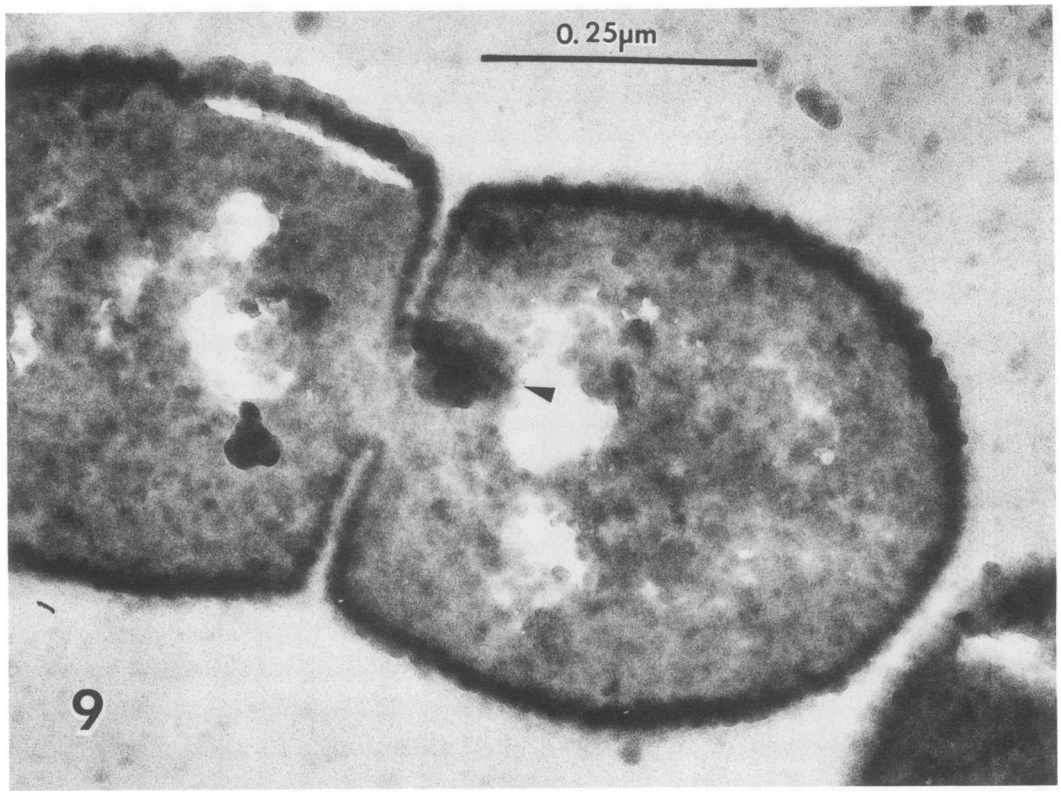


FIG. 9. *Immunocytochemical localization of intracellular invertase with specific antiserum (1.0 μg of protein globulin per ml) applied to GS-5 cells grown in medium containing sucrose.*

TABLE 1. *Location of GTF and invertase enzymes in S. mutans GS-5*

Enzyme	Location in:	
	Absence of sucrose	Presence of sucrose
GTF-A	Large aggregates in association with cell surfaces	Large aggregates in association with cell surfaces and globular polysaccharides
GTF-B	Small aggregates uniformly distributed intracellularly and extracellularly	Small aggregates associated primarily with fibrillar and globular extracellular polysaccharides
Invertase	Cytoplasmic membranes, mesosomes, cytoplasm	Cytoplasmic membranes, mesosomes, cytoplasm

DISCUSSION

The present investigation used antibody directed against highly purified GTF-B preparations, which synthesize primarily water-soluble glucans, to demonstrate that this nonaggregated enzyme is rather uniformly distributed over the cell surface of strain GS-5. These results confirm previous observations involving antibody directed against purified GTF-B preparations and the indirect ferritin labeling technique (16). In contrast to the location of the GTF-A fraction, relatively little of the GTF-B fraction could be demonstrated specifically associated with the cell surface of strain GS-5. This may indicate that the purified GTF-B fraction does not have great affinity for the cell surface of the organism grown in the absence of sucrose as previously suggested (11). Since soluble glucan-synthesizing activity has been demonstrated in the GTF-A fraction (14), the inability to demonstrate aggregates with anti-GTF-B might also indicate that the GTF-B enzyme may not be as firmly associated with the water-insoluble polysaccharides.

The PAP labeling technique demonstrated

that GTF-A complex occurs as aggregates associated with both the cell surface and extracellular polysaccharides. It is not possible, however, to determine whether the complex is directly associated with the cell wall or with wall-bound polysaccharide. Measurement of the size of the GTF aggregates indicated a range of 55- to 100-nm diameter which is similar to that calculated by an indirect ferritin labeling technique (1). However, it is not possible to directly compare the aggregate sizes since the electron-dense product of the PAP procedure is the result of an enzymatic reaction on the section surface and is therefore variable.

Since both anti-GTF-A and anti-GTF-B cross-react with both antigens (16), it might be expected that both antibody preparations would display similar PAP-labeling patterns. However, both antibody preparations affected cell-associated GTF activity to different degrees (15, 16), with anti-GTF-A showing much more reactivity than anti-GTF-B. These studies also demonstrated that anti-GTF-A readily aggregated GS-5 cells, whereas anti-GTF-B did so only weakly. These previous results as well as the present results may indicate that the GTF-B antigen in the cell surface GTF aggregates is not readily accessible to antibodies, or that the cell surface aggregates contain more than one GTF antigenic species.

The GTF-A complex utilized to prepare antibodies for the present study contained other components in addition to the enzyme(s) involved in insoluble glucan synthesis (14). However, recent results (16) suggest that a major antibody fraction against this complex is directed against adherent glucan-synthesizing GTF activity. Furthermore, the labeling pattern with anti-GTF-A is similar to that observed in the previous investigation utilizing an anti-GTF antibody preparation preabsorbed with cells of a mutant strain of GS-5 which synthesized little insoluble glucan (1). Nevertheless, the unavailability of significant quantities of homogeneous GTF preparations capable of synthesizing insoluble glucans exclusively indicates that the labeling experiments with anti-GTF-A are not entirely unequivocal. Further purification of the GTF-A fractions has yielded an enzyme fraction with properties identical to GTF-B but no resolvable fraction capable of synthesizing only water-insoluble glucans (Kuramitsu, unpublished data). Furthermore, it is still not clear whether a single GTF species capable of producing both soluble and insoluble glucans is produced by *S. mutans* or whether multiple enzymes are involved. Both antibody preparations used in the present investigation will cross-react with GTF-A and GTF-B (15).

The present results involving the anti-GTF-A and anti-GTF-B preparations with the PAP labeling technique are similar to those using the same antibody preparations in conjunction with the indirect ferritin labeling technique (16). Both procedures demonstrate that the GTF-A complex exists as aggregates associated with the cell surface while the GTF-B enzyme is associated with the surface in a more diffuse and less aggregated form. Although the present results involving the PAP labeling technique are also in general agreement with another recent observation utilizing the indirect ferritin labeling procedure (1), a direct comparison between these two studies cannot be made. The GTF preparations used as antigens in the present investigation (14) were purified by a procedure distinct from the earlier investigations (1) and exhibit different properties from the antigens of the latter study (Bozzola, unpublished data). In this regard, neither antigen preparation of the latter investigation synthesized primarily water-insoluble glucans, as the GTF-A antigen of the present study did. A major advantage of the PAP procedure over the ferritin experiments (1, 16) is that the reaction takes place on the surface of the cut sections. Consequently, the PAP procedure makes it possible to localize antigens which are normally inaccessible to antibodies, e.g., at intracellular or deeply buried antigenic sites. Additionally, the technique is considerably more sensitive than the ferritin procedure and approaches the level of antigen detection of the radioimmunoassays (22). For these reasons the present results demonstrate more clearly the aggregated nature of cell surface GTF-A activity and the more diffuse GTF-B distribution, which were only suggested by the ferritin labeling procedure (16). One disadvantage of the PAP labeling technique relative to other electron microscopic labeling techniques is the lack of fine-structure detail in the former procedure. This results from embedding cells fixed only with glutaraldehyde and subsequent sectioning and reaction of antibodies, PAP, and peroxidase substrates on the section surface. In this procedure osmium tetroxide is utilized in the last step of sample preparation and does not add to the preservation of ultrastructural detail. However, to our knowledge the present micrographs represent the clearest resolution of enzyme localization with the PAP technique and bacterial cells.

The demonstration that significant GTF-A activity is present on the cell surface of strain GS-5 cells indicates that this colonization-dependent activity (14) can play a role in the attachment of the organism to tooth surfaces. Thus the aggregated adherent glucan-synthesiz-

ing GTF-A activity isolated from culture supernatant fluids of GS-5 also exists as high-molecular-weight complexes on the cell surface. Such complexes, rather than the soluble unaggregated GTF activity, may be required to synthesize highly branched α-1,3- and α-1,6-linked adherent glucans. The presence of the soluble disaggregated GTF-B activity on the cell surface of *S. mutans* may play a role in glucan-mediated aggregation since GTF-B–glucan interactions have been previously demonstrated (7, 14). Thus, both cell surface GTF activities could play significant roles in plaque formation in vivo.

Recent results involving a cytochemical procedure for localizing free glucose released by sucrose-hydrolyzing activities demonstrated that such activities are associated with the cell wall, in the cytoplasm, and in the membrane fraction of strain GS-5 (1). However, since intracellular and extracellular invertases, fructosyltransferase, and GTF in the absence of primer dextran (6) are all capable of releasing glucose from sucrose, it was not possible to specifically identify the enzymes responsible for the localized reactions. The present investigation used specific antibodies in conjunction with the PAP procedure to demonstrate that the invertase is associated primarily with the membrane fraction. Therefore, at least a portion of the sucrase activity demonstrated in the membrane fraction of the earlier investigation (1) can now be ascribed to the intracellular invertase activity. Recent results have confirmed this observation by the immunological demonstration of invertase activity in isolated membrane fractions of strain GS-5 (Wondrack and Kuramitsu, unpublished data). Since this enzyme can be isolated from broken-cell preparations of strain GS-5 in large quantities (19), the enzyme is probably loosely associated with the membranes and may exist as an extrinsic rather than an integral membrane protein (24).

The recently described membrane-associated sucrose phosphotransferase system of *S. mutans* (25, 27) has been demonstrated to convert sucrose to sucrose 6-phosphate, which may be the true substrate for the membrane-associated invertase. In this regard, reports from several laboratories indicate that the K_m's for sucrose of the intracellular invertases from different strains of *S. mutans* are relatively high (13, 20, 28). Lending further support to this hypothesis, a recent investigation has demonstrated that the intracellular invertase activity from *S. mutans* 6715 copurifies with the sucrose 6-phosphate hydrolase activity (4). Therefore, the present demonstration that this activity is associated with the membrane fraction of *S. mutans* is compatible with the possible role of the enzyme in sucrose transport. The presence of invertase (sucrose 6-phosphate hydrolase) activity in the membrane could facilitate the entry of sucrose hydrolytic products into the cell cytoplasm for subsequent fermentation.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Research grants DE05039 (J.J.B.) and DE03258 (H.K.K.) from the National Institute of Dental Research.

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