JOURNAL OF BACTERIOLOGY, Apr. 1974, p. 304-311

Vol. 118, No. 1
Printed in U.S.A.

Morphological Study of *Streptococcus mutans* and Two Extracellular Polysaccharide Mutants

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Received for publication 12 November 1973

Two extracellular polysaccharide mutants of *Streptococcus mutans* GS-5 were obtained and examined. The mutants were distinguished by colonial morphology and by growth on and adherence to hard surfaces. A technique was devised which allowed these bacteria to be studied as they appeared when grown on a hard surface in liquid medium which contained sucrose. Negative stains, replicas, and scanning electron micrography clearly revealed differences in cellular aggregation due to the various extracellular polysaccharides produced. Comparison of sections of the adherent parent strain (GS-5) with those of the nonadherent mutant (GS-511) allowed the extracellular polysaccharide(s) responsible for adhesion to be visually localized.

The interrelation of *Streptococcus mutans* and sucrose with dental caries has been studied and reviewed extensively (6, 7, 9). It has been found to involve the production of extracellular glucans. These glucans, which are synthesized specifically from sucrose, are responsible for attachment of the bacteria to dental surfaces. Several enzymes (glucosyltransferases) are responsible for synthesis of these glucans (11). The relative proportions of the various linkages, the frequency of branching, and the molecular weight of the glucans are probably controlled by the kinds and amounts of glucosyltransferases present. It has been demonstrated that certain types of extracellular glucans are of major importance in adherence of *S. mutans* to teeth (10). Mutants of *S. mutans* which differ in the type of extracellular glucans produced would be useful in elucidation of the specific polysaccharide(s) required for adherence to teeth. The present report describes two such mutants derived from *S. mutans* GS-5.

Previous morphological studies of *S. mutans* and its extracellular polysaccharides were limited to specimens of plaque (20, 21), sucrose-grown cultures (12), or purified polysaccharide obtained from various oral streptococci (19). A technique was developed for study of the above-mentioned mutants which allowed examination of the bacteria by a variety of electron microscopy procedures, as they appeared when grown on a hard surface covered by a liquid medium. A comparative study of these mutants has facilitated visualization and identification of the polysaccharides responsible for adherence of *S. mutans* GS-5 to dental surfaces.

MATERIALS AND METHODS

Cultures and culture conditions. A culture of *S. mutans* GS-5 was obtained from R. J. Gibbons, Harvard School of Dental Medicine, Cambridge, Mass. Mutant GS-511 was obtained from GS-5 by ultraviolet (UV) irradiation (3, 14). Strain GS-514 was derived from GS-511 by treatment with nitrous acid (14). The bacteria were maintained by weekly transfer onto Trypticase soy agar (BBL) or Mitis-Salivarius agar (Difco) plates.

Identification of mutants was done on sucrose agar with the following ingredients per liter: Trypticase sucrose agar (BBL), 33.5 g; yeast extract, 3.0 g; sucrose, 40 g; agar, 10 g; and crystal violet, 0.8 mg. The cultures to be used for electron microscopy were grown in sucrose broth which consisted of the following ingredients per liter: Trypticase (BBL), 20 g; sucrose, 40 g, NaCl, 8 g; KCl, 0.5 g; Na_2HPO_4, 1.15 g; KH_2PO_4, 0.5 g; and K_2CO_3, 1.0 g. When 2 g of glucose was substituted for sucrose, this medium was designated glucose broth. All cultures in this study were incubated at 35 C in an atmosphere of 90 to 95% N_2 and 5 to 10% CO_2.

Production of mutants. The parent strain, GS-5, was cultured for 10 h in 10 ml of glucose broth, washed twice, and resuspended in 20 ml of sterile phosphate-buffered saline. The suspension was transferred to a petri dish, placed on a magnetic stirrer, and exposed to UV-radiation for 60 s (399 ergs per cm^2 per s at 7 cm above stirrer plate). A 4.0-ml sample was then serially diluted to 10^{-3}, and 0.1-ml portions were spread onto sucrose agar.

A selected isolate, GS-511, obtained from the UV-treatment, was grown in 10 ml of glucose broth for 8 h, washed twice and resuspended in 10 ml of nitrous acid (sterilized by membrane filtration) at pH 4.7, and incubated at 26 C for 5 min. A 1.0-ml portion was diluted to 10^{-4} in phosphate-buffered saline, and 0.1 ml-samples were spread onto sucrose agar.

Physiological characteristics. The sugar fermentations were done as suggested by Edwardsson (Table 1) (5). For determination of arginine hydrolysis, the medium and method described in Methods in Microbiology were used (13). Polysaccharides were prepared by the methods of Gibbons (7) or Newbrun (18). Carbohydrate content of the material was estimated by use of anthrone reagent (22). Amount of protein present in the polysaccharides was determined with the Folin phenol reagent (15). Hydrolysis of the polysaccharides was accomplished with 4 N HCl in sealed vials at 100 C for 1 h. The HCl was evaporated under vacuum at 45 C, and the resulting monosaccharides were separated by a chloroform-acetic acid-distilled water (30:35:5, vol/vol/vol) solvent on Whatman no. 1 filter paper (1). The locations of the monosaccharides were detected with ammoniacal $AgNO_3$.

Electron microscopy. A technique was developed which allowed electron micrographs to be taken of bacteria as they appeared when grown on a hard surface in a liquid medium containing sucrose. In this procedure, a sterilized microscope slide, a cover slip, and a cover slip with Formvar-carbon-coated grids were placed in a sterile plastic petri dish. Sucrose broth was added, inoculated with the desired organism, and incubated at 35 C for a predetermined time. The above components were then treated as follows. The slide and plain cover slip were removed and gently washed two to three times in double-distilled water; the slide was air-dried for three to four days, and the cover slip was fixed overnight in 3% gluteraldehyde and buffered to pH 6.0 with Veronal acetate. The dried slide was processed for replicas (17), and the cover slip was rinsed thoroughly in double-distilled water, air- or freeze-dried, coated with 40 to 50 nm of palladium-gold (60 to 40 alloy), and examined in a Kent Cambridge Mark II Stereoscan operating at an accelerating voltage of 30 kV and at a 45° angle tilt. The cover slip with attached grids was dipped three times in double-distilled water, and the grids were removed to a filter paper and stained with 2% phosphotungstic acid at pH 5.5. The bacteria on the bottom of the petri dish were then embedded in situ as follows: after a gentle wash with Veronal acetate, the sample was fixed for 24 h in Veronal acetate-buffered 1% OsO_4 (16), dehydrated, and gradually infiltrated with Epon at 0.5 h-intervals by three changes of 3:1, 1:1, 1:3 (vol/vol) mixtures of absolute ethanol-Epon. After three more changes of pure Epon, a thin layer of resin was poured onto the petri dish and polymerized at 60 C for 48 h. The hardened plastic was allowed to cool for 24 h, after which the Epon layer was stripped off, sectioned, and examined with a Hitachi HU-11A transmission electron microscope at an accelerating voltage of 50 kV.

RESULTS

Isolation of mutants. After exposure of *S. mutans* GS-5 to UV light, several isolates were obtained at a frequency of one per thousand parental colonies. These colonies, which differed from the heaped, adherent morphology of GS-5, were smooth, glossy, and mucoid. These isolates were cloned and examined for polysaccharide production in sucrose broth and for fermentation of sugars (Table 1). All of these isolates were found to be identical in all characteristics examined. One of the isolates, GS-511, was then chosen for further study. Following treatment of GS-511 with nitrous acid, one new isolate was found at a colonial frequency of approximately 10^{-4}. This isolate, designated GS-514, produced colonies which were smooth, dull, shrunken, and adherent. For both mutants, all characteristics examined have remained stable for over two years of weekly passages.

Physiological characteristics. An important characteristic of the two mutants was the striking physical differences in the extracellular polysaccharides produced when grown in sucrose broth. The cultures appeared as follows: GS-5 produced an extracellular polysaccharide adherent to the culture vessel walls; GS-511 made a polysaccharide that was dispersed and nonadherent; and GS-514 formed a soft gel throughout the medium. The purified polysaccharides from all three strains proved to be 97 to 103% carbohydrate, as determined by anthrone reagent (22). Protein content, as estimated by the method of Lowry (15), was less than 1% in GS-5 polysaccharide and undetectable in the material obtained from the two mutants. By paper chromatography, glucose was found to be the major component of the hydrolyzed polysaccharide. A minor component of fructose was also found in all samples.

Other physiological tests provided identical results for GS-5 and GS-511 (Table 1). GS-514 was differentiated by the production of a higher terminal pH from sugar fermentations. The acidity was found to be approximately 0.5 pH unit higher with all sugars except mannitol, which was approximately 1.1 pH units higher. Also, GS-514 failed to grow on Mitis-Salivarius agar which contained 40% sucrose.

Electron microscopy. Negative stains revealed clear differences in the three strains. Strain GS-5 showed a greater tendency to form clumps and cell aggregates (Fig. 1a). A large amount of extracellular material was found, both adherent to the cells and in the background (Fig. 1a, arrow). This material stained heavily with the phosphotungstic acid and largely obscured the cellular details. Mutant GS-511, in contrast, grew uniformly over the grid surface, and the soluble nature of the polysaccharide left the cellular details clearly visible (Fig. 1b). The obvious presence of mesosomes and cell septa demonstrated how little polysaccharide adhered to the cell surfaces (Fig. 2). Strain GS-514 neither formed clumps, as did

TABLE 1. *Physiological characteristics of S. mutans GS-5 and the two mutants derived from it, GS-511 and GS-514*

Physiological tests	GS-5	GS-511	GS-514
Fermentation of[a]:			
arabinose	−	−	−
starch	−	−	−
xylose	−	−	−
fructose	+	+	+
galactose	+	+	+
glucose	+	+	+
inulin	+	+	+
lactose	+	+	+
maltose	+	+	+
mannitol	+	+	±[b]
raffinose	+	+	+
sorbitol	+	+	+
sucrose	+	+	+
trehalose	+	+	+
Reaction on blood agar	γ	γ	γ
Hydrolysis of arginine	−	−	−
Growth on M-S agar[c]	+	+	−

[a] Sugar fermentation was considered positive if terminal pH was less then pH 5.5.

[b] Terminal pH was 5.7.

[c] Mitis-Salivarius agar containing 40% sucrose.

GS-5, nor did it grow as uniformly over the surface as GS-511. The gel-like polysaccharide produced largely obscured cellular detail, but it was not as electron opaque as the polysaccharide of GS-5 (Fig. 1c). Electron-dense spots, approximately 23 nm in diameter, consistently appeared on the cell surface of GS-514 (Fig. 1c, arrow).

When sectioned specimens of a 9-h culture of GS-5 were examined, cell aggregates and microcolonies which had adhered tenaciously to the petri dish were found. The cells were always embedded in extracellular material. At the periphery of a microcolony, both globular and fibrillar polysaccharides were observed, whereas only fibrillar structures were present toward the center (Fig. 3). The fibrillar structures were composed of two parallel protofibrils, each 2.0 nm wide, to which an amorphous electron-dense material was often adherent (Fig. 3, insert). Thus, three structural types of polysaccharides, similar to those described by Guggenheim (12), were observed in the 9-h cultures. When 24-h cultures were studied, the entire sample appeared to be the same as the center of the 9-h microcolonies (Fig. 4a).

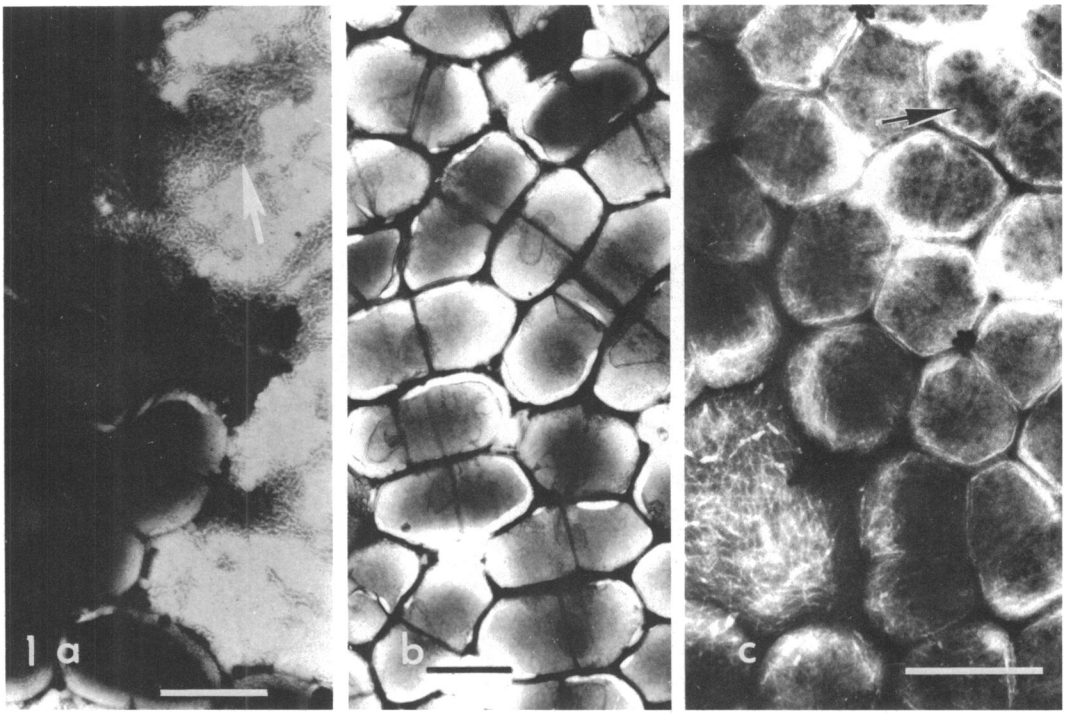
Electron micrographs of sections of GS-511 revealed chains of streptococci distributed in a random fashion over the surface of the petri dish. No aggregates or microcolonies, as was the case with GS-5, were formed by GS-511. The only extracellular polysaccharide present in the GS-511 sections adhered to the cells and was similar in nature to the globular, cell-associated material of GS-5 (Fig. 4b). In contrast to GS-5, the sections of 9-h and 24-h cultures of GS-511 were essentially identical. This mutant did not adhere firmly to the culture vessel, and the samples had to be handled carefully in order to prevent the loss of much material. When micrographs of 24-h cultures of GS-514 were examined, the cells were found to be enmeshed in long fibrils of polysaccharide (Fig. 4c). The fibrils were composed of two protofibrils, each approximately 5.0 nm wide, with a large amount of amorphous material adherent to the fibrils. Examination of 9-h cultures of this mutant revealed only a globular, cell-associated material and complete absence of long fibrils (Fig. 5). The fibrils appeared only as the medium gelled.

Scanning electron micrography confirmed the characteristics of the three strains. The parent strain, GS-5, formed aggregates and microcolonies (Fig. 6a), whereas mutant strain GS-511 grew uniformly over the surface (Fig. 6b). The streptococcal chains of GS-514 appeared to form piles and clumps (Fig. 6c). It was suspected that these clumps occurred as the sample dried and the gel structure of the polysaccharide collapsed. Freeze-dried preparations confirmed this suspicion. The three-dimensional structure of the gel was preserved by freeze-drying, and chains of streptococci were supported in a network of extracellular material (Fig. 6d). Freeze-dried samples of the other two strains did not differ significantly from the air-dried specimens.

Replicas of the three strains were consistent with the observations indicated above. The tendency of GS-5 to form mounds made it difficult to prepare. Replicas of these revealed bacteria deeply embedded in extracellular material (Fig. 7a). GS-511 cell outlines were cleanly reproduced (Fig. 7b). Ridges at sites of future cell division were easily observable. Globular protrusions, which measured about 47 nm across, were found on cell surfaces (Fig. 7b, arrow). These were probably the remains of the globular extracellular polysaccharide seen in sections. The GS-514 cells appeared to have been covered, along with much of the background, with extracellular material (Fig. 7c).

DISCUSSION

These mutants demonstrate the major role which extracellular polysaccharides play in de-



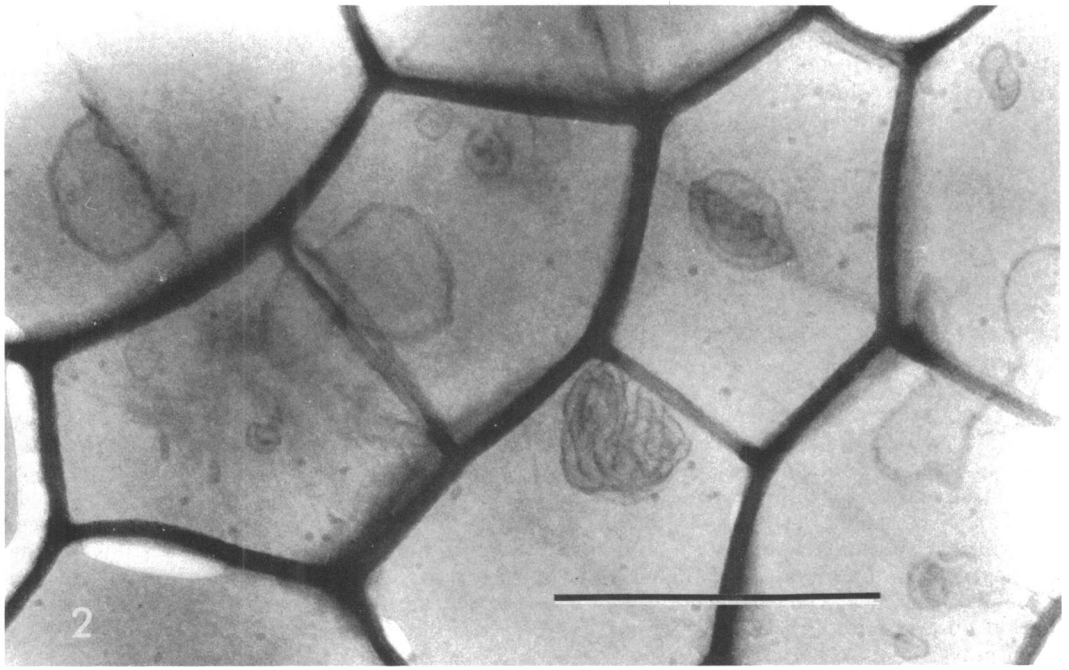


FIG. 1. *Negatively stained electron micrographs of (a) S. mutans GS-5 and extracellular polysaccharide mutants (b) GS-511 and (c) GS-514. GS-5 cells were heavily coated with phosphotungstic acid-positive material. Note fibrillar extracellular material in background of GS-5 (a, arrow). Arrow indicates 0.22-μm spots consistently present in GS-514 (c, arrow). Marker, 0.5 μm.*

FIG. 2. *Negative stains of S. mutans GS-511 demonstrating the soluble nature of the polysaccharide via the clearly visible cellular details.*

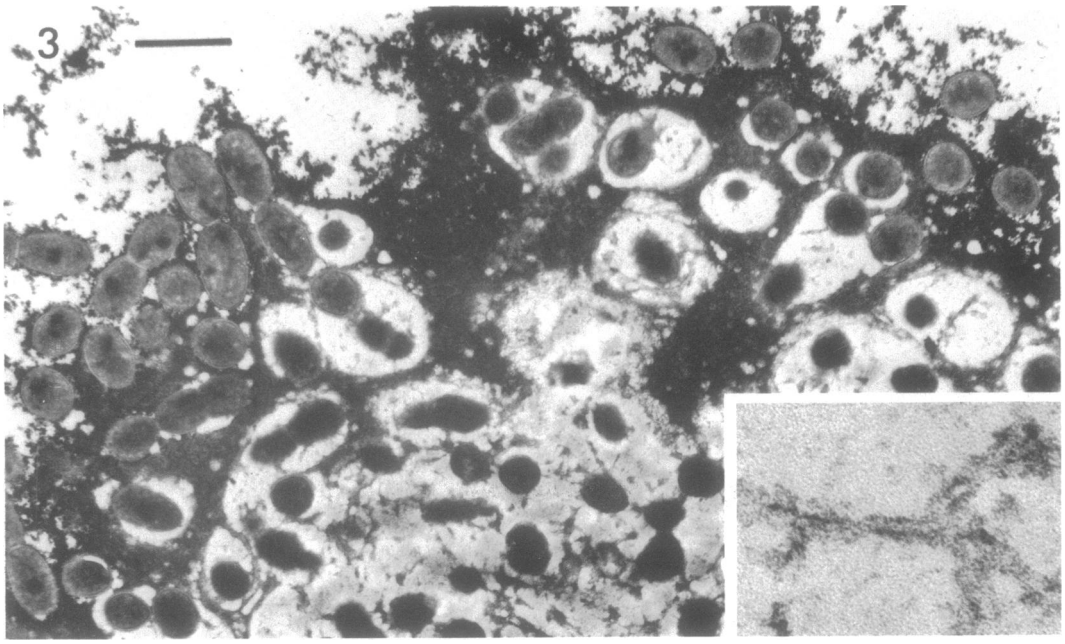


FIG. 3. *Section taken through a microcolony (9-h culture) of S. mutans GS-5. Bar, 1 μm. Insert demonstrates fibrillar structures from center of colony and composed of two parallel protofibrils, each 2.0 nm wide.*

termination of colonial characteristics. The parent strain, *S. mutans* GS-5, possessed a characteristic system of glucosyltransferases and synthesized both soluble glucans and the insoluble glucans responsible for the adherence of *S. mutans* to hard surfaces (9). Guggenheim suggested that the fibrillar polysaccharide observed in sections of *S. mutans* OMZ176 were α(1–3)-linked and were responsible for attachment of the bacteria (12). He recently confirmed the presence of this linkage and suggested that the insoluble glucans of this fraction glued the bacteria to surfaces (10).

Micrographs of GS-5 showed two distinct kinds of material, which appeared to be consistent with the polysaccharides described by Guggenheim (12): (i) a globular material in close association with the cell surface, which was found at the periphery of the microcolonies, and (ii) a fibrillar substance found in the intercellular spaces, both at the periphery and the center of the microcolonies. The extracellular polysaccharide observed in the GS-511 sections consisted only of globular material, closely associated with the cells.

In the present study, the difference between parent strain GS-5 and mutant GS-511 was the loss of the ability by the latter to adhere to surfaces. Therefore, it can be assumed that the globular extracellular polysaccharide of GS-511 is not responsible for the adherence of *S. mutans* to hard surfaces.

From these studies, it appears that the adherence of *S. mutans* is due to certain fibrillar extracellular polysaccharides. De Stoppelar described a polysaccharide mutant of *S. mutans*, similar to GS-511, which did not produce caries in test animals (3). Thus, the loss of pathogenicity was probably related to loss of the ability to produce the fibrillar polysaccharides. It will be necessary to check the mutants described in this study for cariogenicity before a definite correlation can be made.

The polysaccharide and the colonies produced by GS-514 were similar to those of *Streptococcus sanguis* (4). The gel-like polysaccharide neither adhered to hard surface nor caused the cells to aggregate and, to this degree, was similar to GS-511. Only a study of the glucosyltransferases and the linkages of the polysaccharides will reveal the relation of the mutants to GS-5 and specifically define which polysaccharide is required for adherence.

ACKNOWLEDGMENTS

We thank Judy Murphy for her assistance with the scanning electron microscope preparations and the Carbondale Southern Illinois University Electron Microscope Center for use of the instruments, including a Cambridge Mark IIA

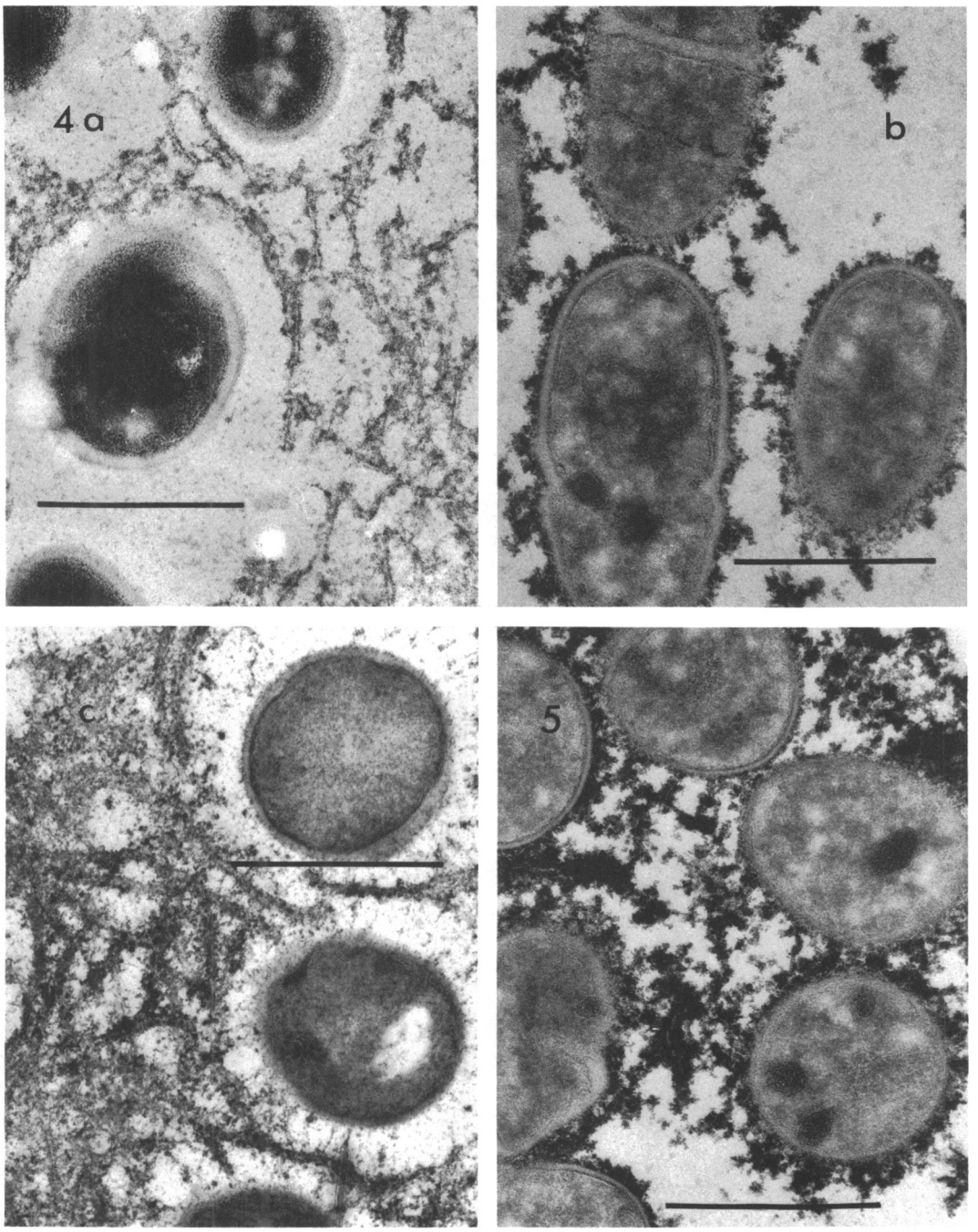


FIG. 4. *Sectioned samples of 24-h cultures of S. mutans (a) GS-5, (b) GS-511, and (c) GS-514. Marker, 0.5 μm.*

FIG. 5. *A 9-h culture of GS-514 mutants. Note globular nature of the extracellular material found in young cultures compared with the fibrillar material present in 24-h cultures. Marker, 0.5 μm.*

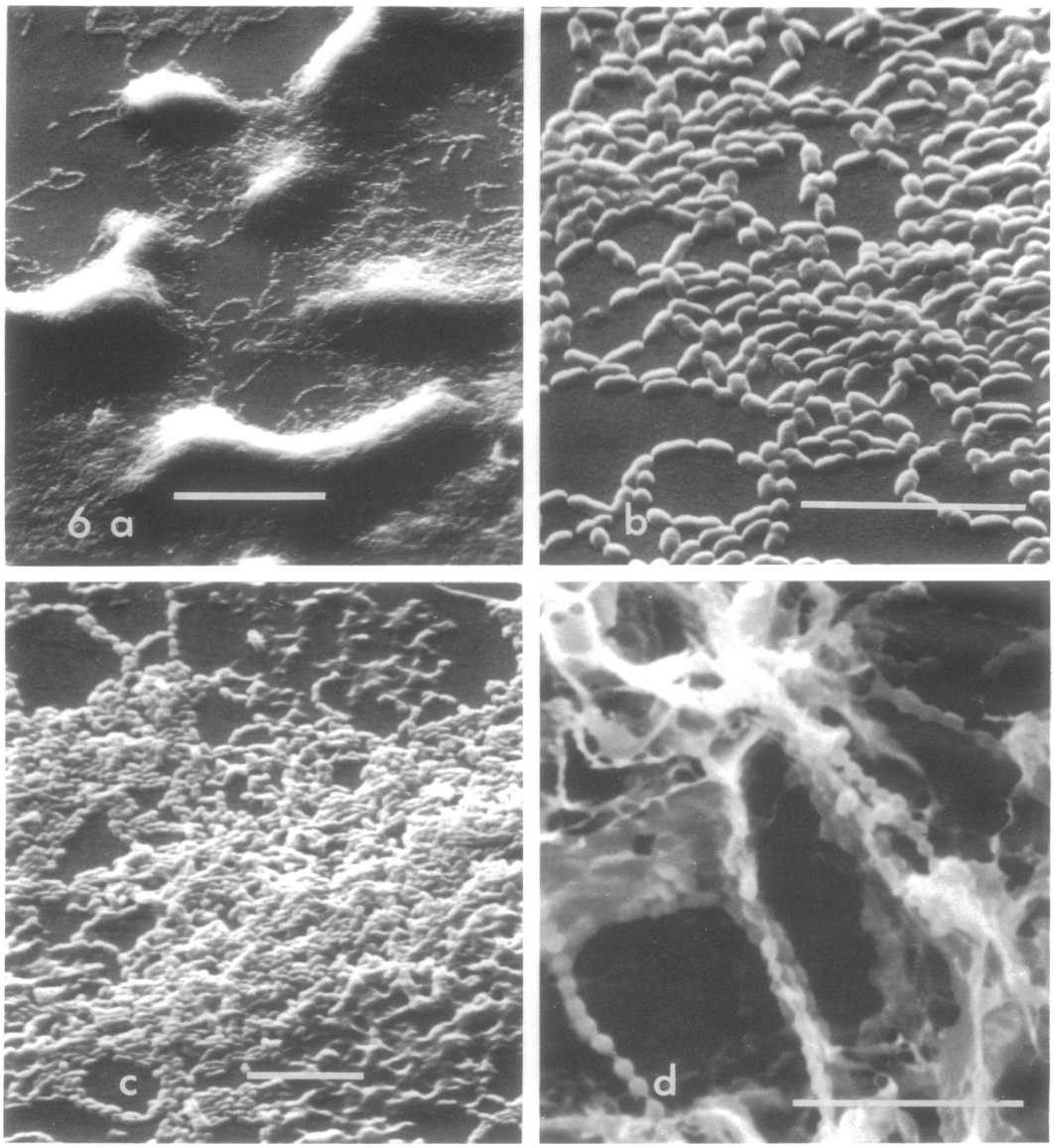


FIG. 6. *Scanning electron micrographs of S. mutans (a) GS-5, (b) GS-511, and (c) GS-514 revealing differences in colonial characteristics due to differences in extracellular polysaccharides. (d) Scanning electron micrograph of freeze-dried preparation of mutant GS-514. Three-dimensional structure of the gel is apparent. (a) Marker, 20 μm; (b), (c), (d) markers, 0.5 μm.*

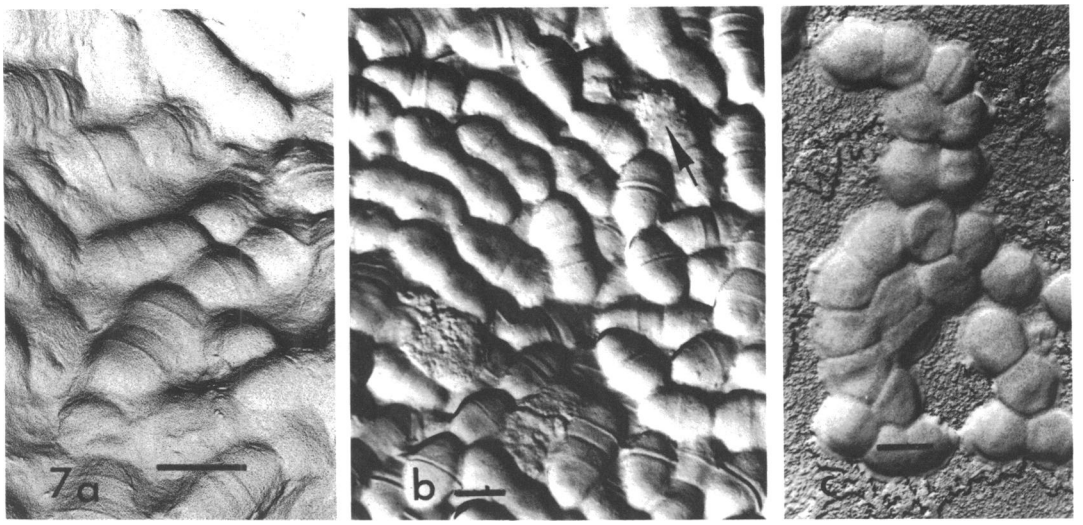


FIG. 7. *Electron micrographs of replicas of (a) S. mutans GS-5, (b) mutant GS-511, and (c) GS-514 grown on the surface of a microscope slide. Globular protrusions, approximately 45 nm in diameter, found on cell surfaces of GS-511 are indicated by arrow (b). Marker, 5 μm.*

Stereoscan scanning electron microscope purchased with Biomedical Sciences support grant no. FR-1 SO5 FR07118-01. The original strain of *S. mutans* GS-5 was obtained from R. J. Gibbons, Harvard School of Dental Medicine, Cambridge, Mass.

This research was supported by training grant no. DE-00144-09 from the National Institute of Dental Research.

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