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COMPARISON OF CELL SEDIMENT AND SURFACE GROWN "TEST PLAQUE" USING SCANNING ELECTRON MICROSCOPY

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Abstract

Scanning electron microscopy was used to compare the morphology, integrity and distribution of bacterial cells in a test plaque grown on the surface of enamel with that of the cell sediment plaque routinely used in a short-term intraoral caries model. Cultures of *S. mutans* IB-1600 or *S. sobrinus* 6715-13 were grown in complex media supplemented with either 2.0% sucrose (glucan plaque) or 0.2% glucose (non-glucan plaque). Cell sediment (CS) plaque was prepared by centrifuging the cultures after incubation, recovering the cell sediment, and spreading it on Metricel membrane filter paper. Surface grown (SG) plaque was prepared by suspending saliva-coated bovine enamel in the culture medium, incubating, and recovering the enamel assembly with bacterial accumulations. Cell morphology and integrity, as well as the appearance of glucan-like material produced by the cells, was similar in both CS and SG test plaques. The cell distribution however, varied in the SG plaque from extremes of all cells to all glucan, whereas the cell sediment plaque was more uniform in cell distribution. A highly standardized test plaque minimizes variability in the intraoral caries model. These findings support the contention that the bacterial cells in a cell sediment plaque are similar in morphology, integrity and glucan production to surface grown plaque, and have the added advantage of uniform distribution, which makes the cell sediment plaque more appropriate for intraoral caries model studies.

Key Words: Scanning electron microscopy, mutans streptococci, morphology, glucan.

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Introduction

Organisms belonging to the mutans streptococci group have been implicated as important etiological agents in dental caries (Hamada and Slade, 1980; van Houte, 1980; Loesche, 1986). *Streptococcus mutans* and *Streptococcus sobrinus* strains have been singled out for their virulence traits with respect to their rate of acid production, and growth rate and survival in an acid environment. An additional virulence trait is the synthesis of glucan from sucrose which is known to promote the accumulation of mutans streptococci in dental plaque (Gibbons and van Houte, 1975) and has been shown to enhance the cariogenic potential of plaque (Zero *et al.*, 1986a, 1992).

To specifically evaluate the virulence characteristics of a particular bacterial strain, studies must be conducted in isolation of other plaque microorganisms. Plaque formed "naturally" in the mouth is comprised of various types of bacteria, making it difficult to evaluate a particular microorganism. Various *in vitro* models have been used to study the properties of oral bacteria, including the chemostat (Ellwood, 1976) and the Bristol mouth (Curzon *et al.*, 1984). These models, respectively, rely on either bacterial growth in a culture suspension or growth on an enamel specimen.

More recently, the intraoral enamel demineralization test (IEDT) (Brudevold *et al.*, 1984) has been applied to the study of microbial virulence factors (Zero *et al.*, 1986a, 1986b, 1992). The IEDT model permits the study of microbial virulence factors in the human mouth. This model uses "test plaque" prepared from cell sediments (CS) of pure bacterial cultures which are loaded onto plastic holders, and held in the mouth on a palatal appliance.

The purpose of this study was to compare the morphology, integrity, and distribution of bacterial cells in a test plaque grown on the surface of enamel, with those of cell sediment plaque routinely used in the IEDT caries model.

Table 1. A comparison of cell attributes and growth conditions for *S. mutans* and *S. sobrinus* test plaques.

Cell attributes	*S. mutans* test plaques				*S. sobrinus* test plaques			
	"non-glucan"		"glucan"		"non-glucan"		"glucan"	
	CS	SG	CS	SG	CS	SG	CS	SG
cells are of normal morphology	+	+	+	+	+	-	+	?
cells are intact	+	+	+	+	+	-	+	?
cells are uniformly distributed on surface	+	+	+	-	+	-	+	-
cells produce a glucan-like material	not applicable		+	+	not applicable		+	+

A combination of "+ and +" for a particular cell attribute implies similarity between the growth conditions.

A combination of "+ and -" for a particular cell attribute implies a difference between the growth conditions.

A "?" indicates that this particular cell attribute could not be determined in this growth condition.

Materials and Methods

Preparation of enamel assembly

Permanent bovine incisors were obtained from a local slaughter house. Enamel blocks measuring 5 mm x 5 mm x 2 mm were sectioned from the labial surface, polished to a flat surface, then stored in saline solution. A groove was prepared with a #169 high-speed tungsten carbide burr on the dentine side of the enamel sections to orient a 0.914 mm resilient orthodontic wire. The wire was mechanically attached to the dentine with a light cured composite (Silux plus, 3M, St. Paul, MN). The enamel sections were suspended by the wire in a 250 ml bottle (Wheaton, Millville, NJ) containing double-deionized water. The assembly was sterilized by exposure to ethylene oxide at 38°C for 5 hours, followed by a 12 hour period of aeration.

Whole saliva, stimulated by chewing parafilm, was collected from one subject and clarified by centrifugation at 12,000 *g* for 30 minutes. The supernatant was decanted and the pellet discarded. The supernatant was heated to 60°C for 45 minutes, recentrifuged and stored at -4°C until use (see below).

Surface grown (SG) test plaque

The sterile enamel blocks were immersed in the clarified saliva for 2 hours in order to permit pellicle formation. Two enamel blocks were used for each set of conditions. All procedures were carried out in a positive pressure lamina flow hood. A sterile culture medium was prepared, consisting of trypticase tryptose yeast (TTY) broth known to be essentially free of sucrose (Hamada and Torri, 1978) [trypticase (BBL®, Becton, Dickinson and Co., Cockeysville, MD), 15 g/l; tryptose (Difco Labs., Detroit, MI) 4 g/l; yeast extract, 4 g/l; K_2HPO_4, 2 g/l; KH_2PO_4, 5 g/l; Na_2CO_3, 2 g/l; NaCl, 2 g/l]. The basal TTY medium was supplemented with either 0.2% glucose or 2.0% sucrose, followed immediately by inoculation with either *S. mutans* Ingbritt IB-1600 (serotype c) or *S. sobrinus* 6715-13 (serotype d). The saliva treated enamel assembly was promptly suspended into the inoculation medium. Incubation was carried out anaerobically in gas pack jars at 37°C for 18 hours. After incubation, the enamel assemblies were removed from the culture medium under the lamina flow hood, and prepared for examination of the bacterial accumulations by scanning electron microscopy (SEM) (see below).

Cell sediment (CS) test plaque

The cell sediment plaque samples were prepared using the same culture conditions as described above; however, no enamel blocks were suspended into the medium. The cultures were harvested by centrifugation (10,000 *g*, 10 minutes, 4°C) and the cell sediment washed twice in cold buffer (100 mmol/l KCl; 20 mmol/l $KHCO_3$). During each wash, the cell sediment was homogenized in a Wheaton glass mortar. After final centrifugation, the cell sediment was spread on filter paper (grade 613, VWR Scientific, Inc., San Francisco, CA) and spatulated to remove excess moisture from the sediment. The cell sediment was spread as a smear layer on Metricel membrane filter paper (GA-6, pore size 0.45 μm, Millipore Corp., Bedford, MA) and prepared

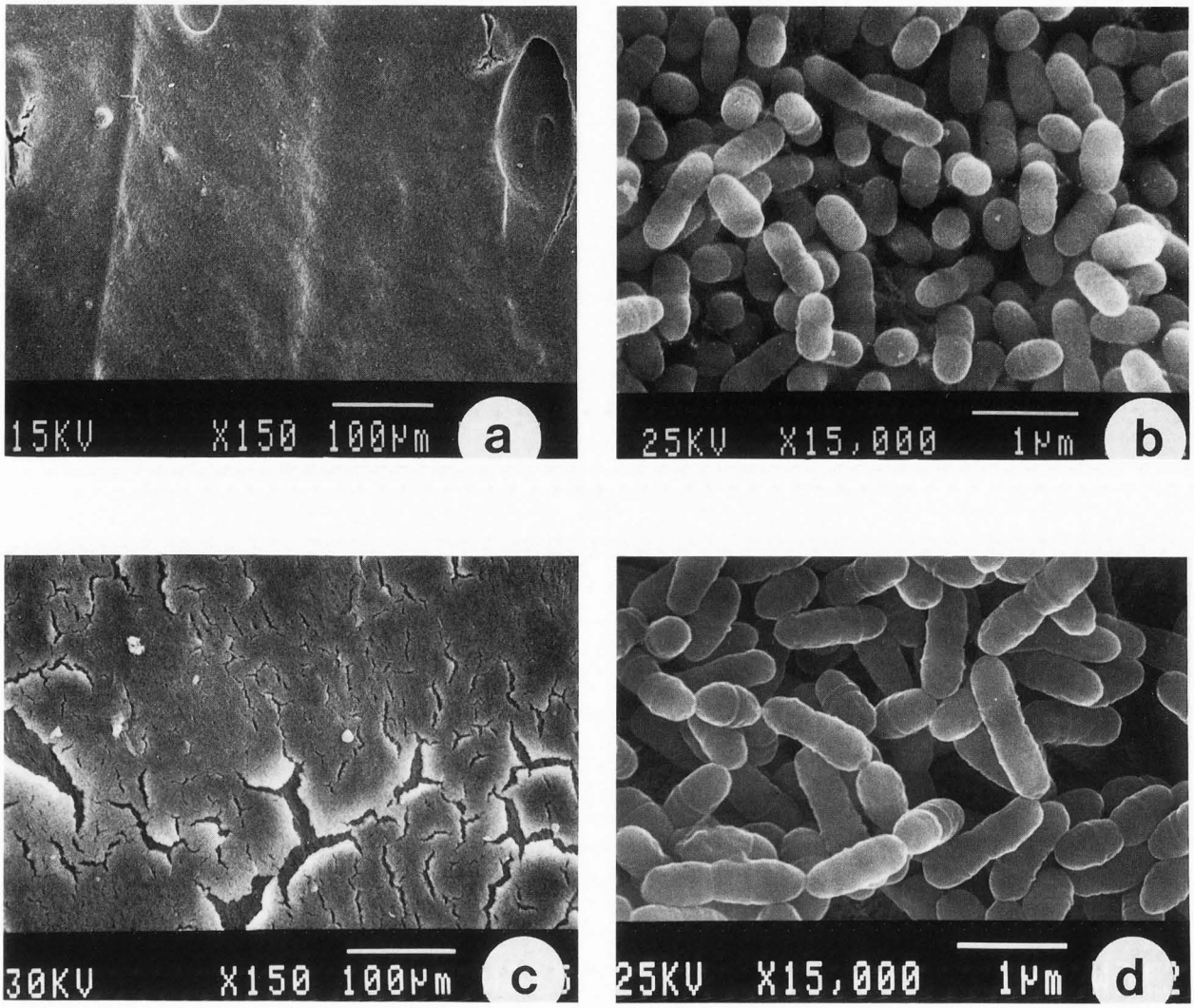

Figure 1. Scanning electron micrographs of "test" plaques of *S. mutans* Ingbritt-1600 (serotype c) cultured in TTY media supplemented with 0.2% glucose ("non-glucan"): (**a** and **b**) cell sediment; (**c** and **d**) surface grown. Bars = 100 μm (a and c) and 1 μm (b and d).

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for SEM examination (described below).

Scanning electron microscopy sample preparation

Surface grown and cell sediment samples were each immersed for 1 hour in 1.0% glutaraldehyde in 0.05 mol/l PO_4 buffer, pH 6.8, followed by two rinses for 10 minutes each in 0.05 mol/l PO_4 buffer. The samples were post-fixed in 1.0% osmium tetroxide (OsO_4) in 0.1 mol/l PO_4 buffer, pH 7.2, for 1 hour followed by two rinses for 10 minutes each in 0.1 mol/l PO_4 buffer.

The test plaques were dehydrated in a graded series of ethanol and air dried. Samples were mounted on aluminum SEM stubs using a conductive silver adhesive, then sputter coated (Hummer VI, Anatech Ltd., Alexandria, VA) with a thin (10 nm) layer of Au/Pd alloy to enhance conductivity. The sample surfaces were examined extensively in a JEOL-820 SEM (JEOL USA, Peabody, MA) at magnifications up to 30,000x.

Results

Table 1 is a graphic comparison of the various cell attributes and growth conditions for each of the bacterial strains studied.

S. mutans plaques

Non-glucan plaque The cells in the cell sediment (CS) (Figs. 1a and 1b) and surface grown (SG) (Figs. 1c

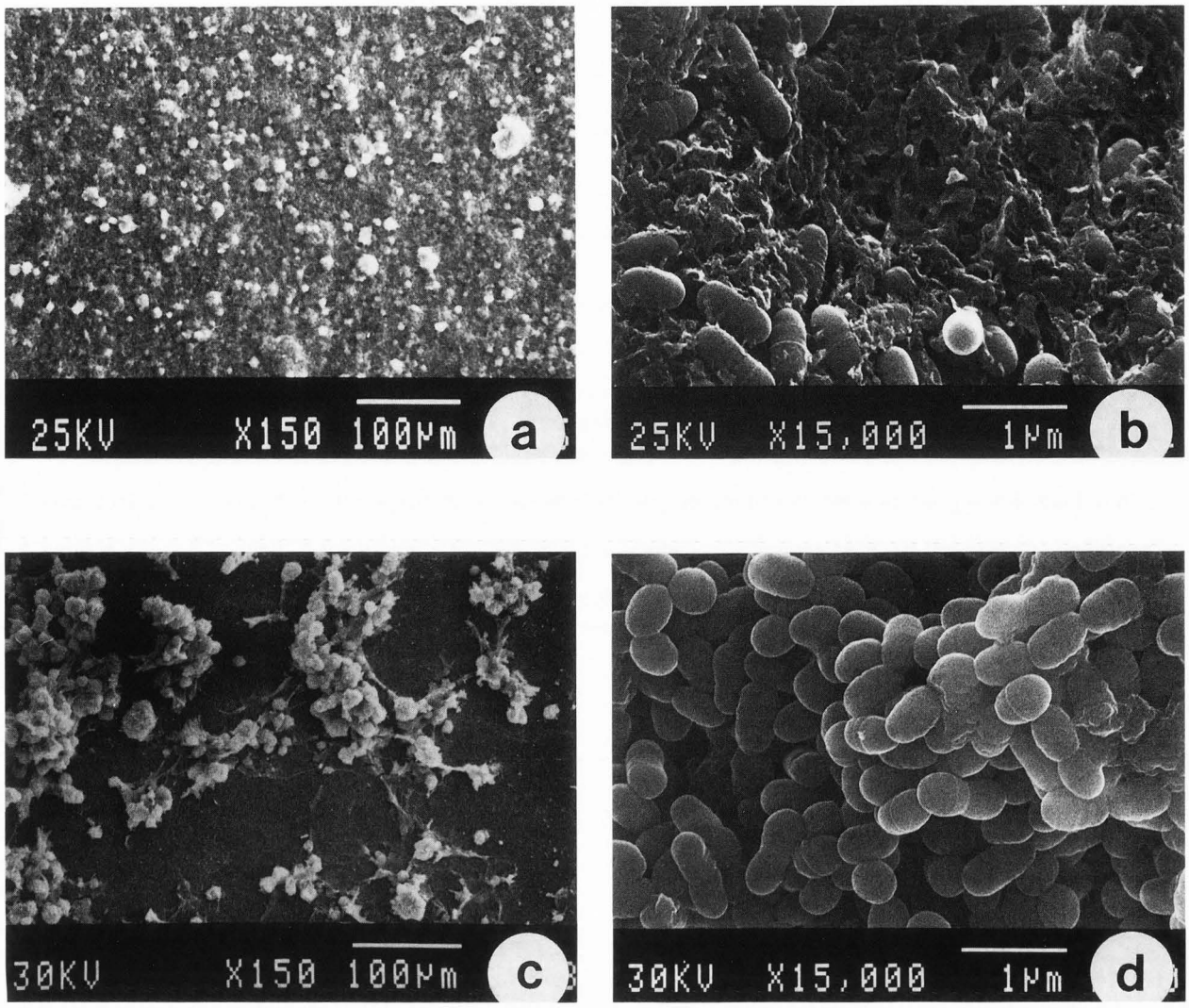

Figure 2. Scanning electron micrographs of "test" plaques of *S. mutans* Ingbritt-1600 (serotype c) cultured in TTY media supplemented with 2.0% sucrose ("glucan"): (**a** and **b**) cell sediment; (**c** and **d**) surface grown. Bars = 100 μm (a and c) and 1 μm (b and d).

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and 1d) *S. mutans* test plaques grown in the absence of sucrose (non-glucan) were similar in morphology. Both the CS and SG test plaques consisted of intact closely packed cells with single and multiple cleavage planes. Cell chains, characteristic of mutans streptococci, were shorter in the CS plaque, most likely due to the vigorous preparation procedures. At low magnification (Fig. 1c), the SG test plaque appeared to be a uniform, dense mat of cells covering the enamel surface, similar in distribution to the CS plaque (Fig. 1a) which had been mechanically spread across the filter paper.

Glucan plaque The morphology and integrity of the *S. mutans* cells grown in the presence of sucrose (glucan plaque) was similar in both the CS (Figs. 2a and 2b) and SG (Figs. 2c and 2d) test plaques. The cells in both growth conditions (CS and SG) produced an extracellular material consistent with the appearance of glucan. The uniformity of distribution varied between the two growth conditions, most likely due to the homogenization and spatulation of the CS plaque. The CS test plaque contained a homogenous mixture of cells and extracellular material, whereas surface grown *S. mutans* test plaque contained clumps of cells surrounding an inner core of glucan material. At low magnification, the variation in the nature of the SG test plaque was apparent (Fig. 2c), with areas consisting of cells alone, glucan

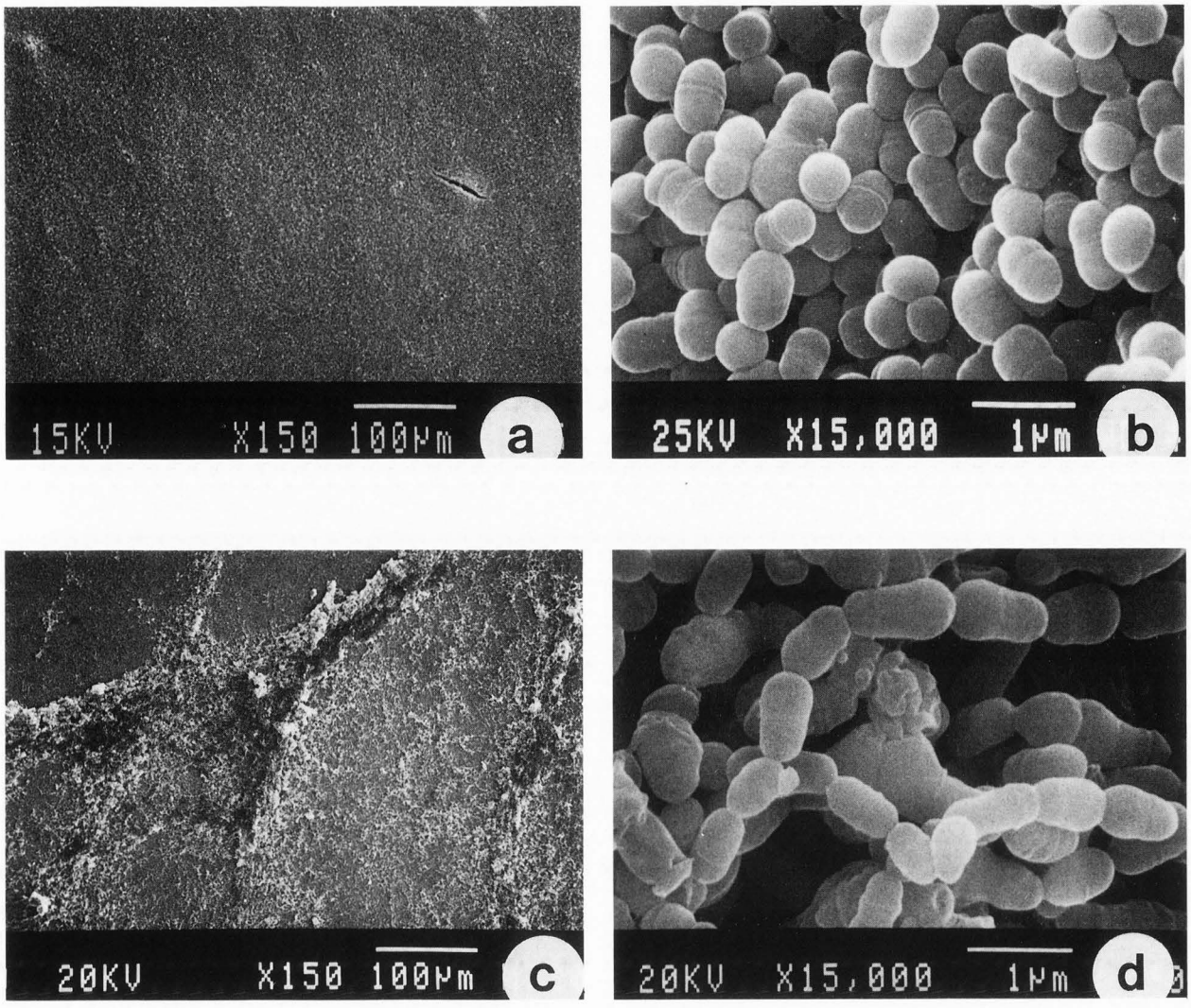

Figure 3. Scanning electron micrographs of "test" plaques of *S. sobrinus* 6715-13 (serotype d) cultured in TTY media supplemented with 0.2% glucose ("non-glucan"): (**a** and **b**) cell sediment; (**c** and **d**) surface grown. Bars = 100 μm (a and c) and 1 μm (b and d).

material alone and combinations of cells and glucan material. This was in contrast to the uniformity of the CS plaque seen at low magnification in Figure 2a.

S. sobrinus plaques

Non-glucan plaque The cells in the CS, *S. sobrinus*, non-glucan test plaque (Figs. 3a and 3b) were intact closely packed cells of normal morphology spread in a uniform layer. The SG, *S. sobrinus*, non-glucan test plaque (Figs. 3c and 3d), however, contained cells of irregular morphology, with swelling or "ballooning" occurring either at one end of the cell or in the center at the cell division site. There were also disrupted cells at various stages of lysis mixed with intact normal appearing cocci. At low magnification (Fig. 3c), the non-uniformity of surface coverage by the SG plaque was apparent.

Glucan plaque The CS *S. sobrinus* glucan test plaque (Figs. 4a and 4b) was a uniform layer of plaque consisting mainly of intact normal appearing cells interspersed in an extracellular (glucan) matrix, with a few irregular balloon-shaped cells present. The SG *S. sobrinus* glucan test plaque (Figs. 4c and 4d) consisted of clumps of cells coated in glucan material with voids between the clumps. Cell morphology was obscured by the glucan material coating.

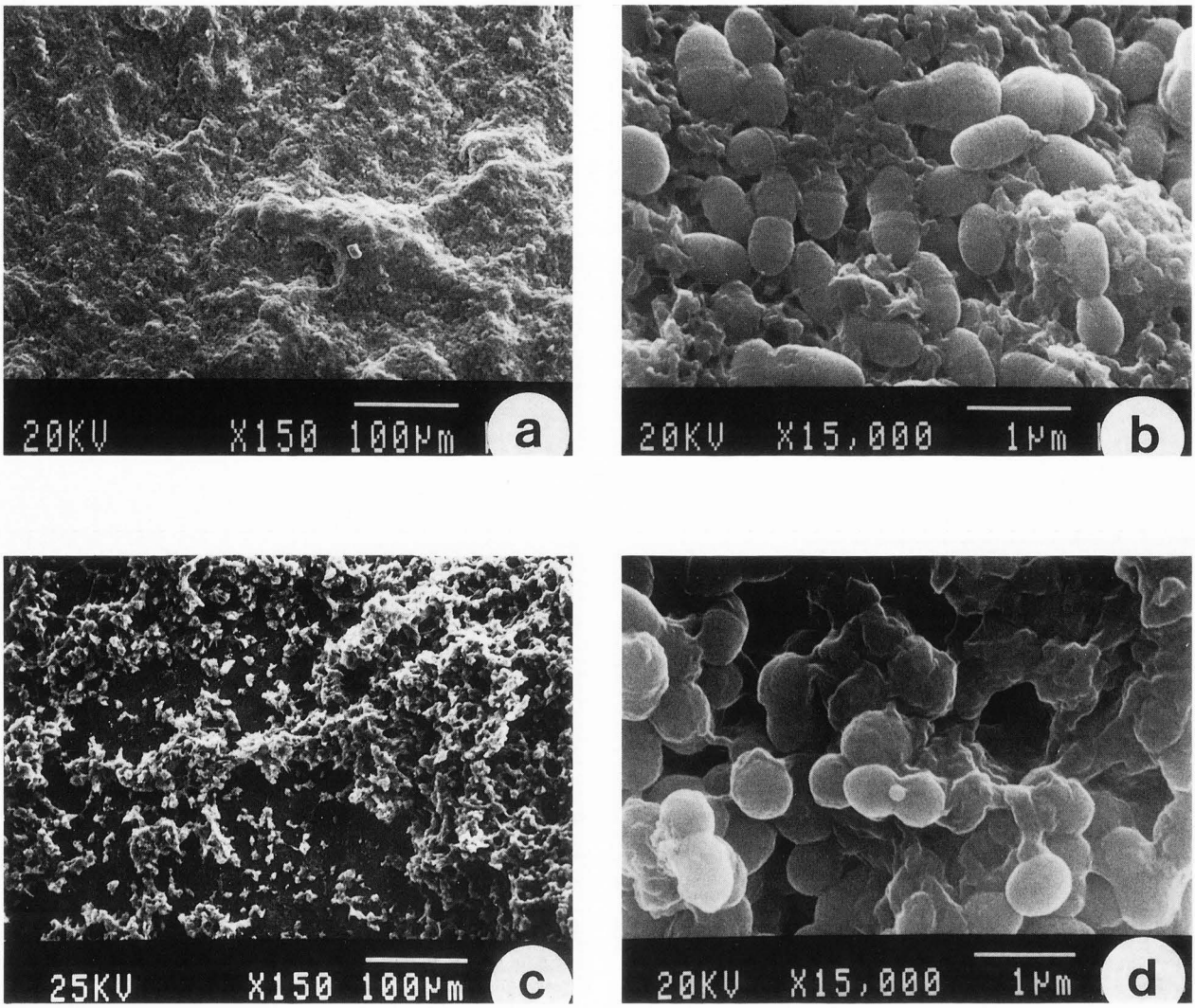

Figure 4. Scanning electron micrographs of "test" plaques of *S. sobrinus* 6715-13 (serotype d) cultured in TTY media supplemented with 2.0% sucrose ("glucan"): (**a** and **b**) cell sediment; (**c** and **d**) surface grown. Bars = 100 μm (a and c) and 1 μm (b and d).

Discussion

Pure strains of *S. mutans* (serotype c) grown on the surface of agar plates with and without sucrose have previously been examined with SEM (Moro *et al.*, 1986). Cells grown in the absence of sucrose were clearly seen as discrete cells, while cells grown in the presence of sucrose were embedded in an extracellular polysaccharide matrix. The extracellular matrix formed by the test plaques used in our study was similar in appearance to that of previous studies. When the growth media in this study contained added sucrose, both cell sediment and surface grown test plaques produced an extracellular material that was generally fibrillar in nature and closely resembled the appearance of water-insoluble glucan described by Toda *et al.* (1987).

Koga *et al.* (1988) examined the cellular adherence properties of *S. mutans* to saliva-coated human enamel. When incubated in the presence of sucrose, cell-to-cell attachments via amorphous substances (consistent with the appearance of glucan) were observed in the SEM. However, in the absence of sucrose, no intercellular attachments were observed although the cells adhered to the enamel surface. We also observed adherence of our

test strains to saliva-coated enamel for both culture conditions (with or without sucrose).

Cell morphology within the *S. mutans* strain was not affected by growth conditions (CS or SG). No apparent cell destruction was observed in the cell sediment plaques, despite the vigorous preparation procedures of homogenization and spatulation.

The *S. sobrinus* strain appeared to be more sensitive to growth conditions, resulting in altered cell morphology in the SG plaque. Although this effect was also seen in the CS glucan plaque (Fig. 4b), it was most prevalent in the SG non-glucan plaque (Fig. 3d). The reason for this finding is not certain. It is possible that growth of *S. sobrinus* may have been influenced by local conditions present at the enamel surface. The competition for substrate among closely packed cells may have resulted in changes in cell morphology. Another possibility is that localized changes in salt concentration may have resulted in alterations in cell morphology, since we observed that salt crystals formed on the enamel surface of some of the samples during incubation in the grown medium. This contention is supported by two previous studies in which ultrastructural changes were induced in *S. sobrinus* by varying the concentration of zinc ions (Scheie *et al.*, 1989), and changing the ratio of bicarbonate to potassium in the growth medium (Tao *et al.*, 1987).

Differences in the uniformity of cell distribution were observed between the CS and SG plaques. SG plaque cells grew in clumps, creating variations in cell densities and glucan material across the enamel surface. Repeated homogenization and spatulation of the CS plaque produced a uniform distribution of cells, or cells and glucan, without causing disruption of the cells. The homogeneity of the CS test plaque can be considered an advantage in the IEDT model. A highly standardized test plaque minimizes variability due to differences in the acidogenic response of the plaque between one test and another. In contrast, the SG test plaque was highly variable, especially for cells grown in the presence of sucrose. The distribution of cells and glucan material varied from extremes of all cells to all glucan. Differences in the distribution of cells in plaque can result in marked variation in the demineralizing potential of plaque (Zero *et al.*, 1986b, 1992), thus complicating the evaluation of the virulence characteristics of a specific bacterial strain. Previous studies involving the IEDT model have been carried out utilizing CS plaque samples prepared in the same manner to that used in this investigation (Zero *et al.*, 1986a, 1986b, 1992). The results in this current investigation support the use of a cell sediment plaque, rather than a surface grown plaque, for IEDT model studies.

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Discussion With Reviewers

T. Sabet: What is the basic chemical composition of the clarified saliva? Did the authors test for the presence of antibodies against each of the 2 bacteria (in this study) in the saliva?

Authors: The clarification of saliva is a standardization procedure to remove cellular debris and bacteria from whole saliva, while leaving proteins and glycoproteins which are involved in pellicle formation. Antibodies can be associated with the salivary pellicle; however, in this study, their role would be to enhance bacterial adhesion to the enamel surface, rather than to initiate an immunologic response.

P.A. Adriaens: In cell sediment samples, the interface between the enamel and the plaque mass may not be comparable to the interface in a surface grown sample. How will this difference affect the test results related to demineralization of the sample?

Authors: There may be a difference. However, to grow bacteria on a surface requires metabolic activity, which leads to the production of acid and results in demineralization of the enamel surface. Thus, a direct comparison, in a controlled manner, of the surface grown and cell sediment plaque is not possible.

P.A. Adriaens: Which kind of methods were used to quantitatively or qualitatively evaluate the subjective observations of the specimens? How do these support the statements made concerning the "uniform" distribution of bacterial cells? Which statistical analyses were performed?

Authors: Sample size was $n = 2$, which is not appropriate for statistical analysis. A thorough examination of the samples was carried out in the SEM, and the micrographs shown are representative.