Sucrose-Promoted Accumulation of Growing Glucosyltransferase Variants of *Streptococcus gordonii* on Hydroxyapatite Surfaces

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Streptococcus gordonii exhibits a phase variation involving expression of high (Spp⁺) or low (Spp⁻) glucosyltransferase activity. The related bacterial accumulation on hydroxyapatite (HA) and saliva-coated HA surfaces was examined and found to be significant. Spp⁺ cells growing anaerobically in a defined medium utilize about 30% of the glucose available from sucrose to make insoluble glucans. These glucans formed cohesive masses on HA beads, which contained 80 to 90% of the total bacteria. The bacterial polymer mass had a volume of about 40 μm^3 and contained more than 5×10^{10} viable cells per cm^3 . In the absence of sucrose, the beads were saturated by 1×10^8 to 2×10^8 Spp⁺ cells. Spp⁻ bacteria, which make 30-fold less glucan than do Spp⁺ bacteria, did not accumulate on surfaces in numbers significantly above the saturation level of 1×10^8 to 2×10^8 cells in the presence or absence of sucrose. Insoluble glucan synthesized by Spp⁺ cells from sucrose also enabled these bacteria to accumulate on saliva-coated HA seven times more effectively than the Spp⁻ cells and 10 times more effectively than the Spp⁺ cells grown in medium without sucrose.

Streptococcus gordonii, which was originally classified among the taxonomically diverse species *Streptococcus sanguis*, is found principally on the tooth surface and is an early colonizer of this site (4, 18). The many aspects of initial adhesion of these organisms has been studied in detail (8, 24). However, successful colonization also requires that the attached organisms subsequently grow and accumulate on the tooth surface. This phase, which results in plaque formation, is less well understood. The early plaque formed by these streptococci may serve as a substratum for attachment by additional oral bacteria and therefore be of importance to the maturation of dental plaque. These later colonizers may attach to the plaque directly by binding to the streptococci via lectinlike interactions (20) or indirectly by binding to other organisms which have directly attached to the streptococci (17). Secondary bacteria may also bind to streptococcal cell products such as glucans (2). Therefore, the accretion of bacteria in the developing plaque can involve homotypic or heterotypic bacterial interactions or interactions with cell products.

Recently, a phase variation involving glucosyltransferase (GTF) activity in *S. gordonii* has been described (27). In broth cultures, *S. gordonii* switches reversibly from high to low levels of GTF activity at frequencies of 10^{-4} to 10^{-3} in both directions. Normally, *S. gordonii* produces glucan polymers which make the colonies hard and cohesive and cause them to stick tenaciously to the surface of sucrose agar plates. This sucrose-promoted phenotype is designated Spp⁺. The lower-level GTF variants are soft on sucrose agar plates, and this phenotype is designated Spp⁻. The glucans produced from sucrose by the constitutive, extracellular GTF of *S. gordonii* can be both water-soluble polymers containing principally α -1,6 glucosidic linkages and water-insoluble polymers containing higher proportions of α -1,3 glucosidic linkages, with some α -1,4 linkages (11, 19). The

type of polymer produced is affected by environmental conditions (3, 21) and by the availability of acceptor molecules (19). The latter is best described for the GTF of *S. gordonii* 10558, which tends to make more water-soluble glucans in the presence of acceptor molecules such as dextran, whereas the enzyme synthesizes greater proportions of insoluble polymers in the absence of acceptor molecules (19).

The high frequency of reversible switching between high and low levels of GTF activity may indicate that this enzyme and/or the polymers it synthesizes from sucrose are ecologically relevant to this organism. In classical, washed-cell adhesion tests (1, 5), which measure initial reversible and subsequent irreversible adhesion of nongrowing cells (8), neither the GTF nor the glucans it produces from sucrose enhanced initial adhesion of *S. gordonii* to hydroxyapatite (HA) and saliva-coated HA (S-HA) surfaces (31). A system was developed, therefore, to quantitatively study the role that GTF variation may play in the accumulation of growing cells on HA and S-HA surfaces. This system has the advantage of allowing extracellular products as well as cell surface molecules to play a role in cell attachment and accumulation.

Strain Challis Spp⁻ variants and Spp⁺ revertants were used to study the role of the level of GTF activity in the accumulation of growing cells on glass, HA, and S-HA bead surfaces in the presence and absence of sucrose or dextran. Strain Challis was used because GTF variation in this strain is most thoroughly described (27). In addition, strain Challis has little specific adhesiveness to HA or S-HA, and thus any effects related to GTF activity may be more apparent. The Spp⁺ strain G9B was also used to study the role of GTF in the accumulation of a bacterial strain with more specific affinity for S-HA (24) and to confirm that our findings were not unique to strain Challis. These studies suggest a role for GTF and its glucans in the sucrose-promoted accumulation of *S. gordonii* cells on both HA and S-HA surfaces.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains were stored at -70°C in 50% glycerol. Strain CH1C1 is an Spp^{-} variant of the Spp^{+} parental strain Challis, and strain CH1D2 is its Spp^{+} revertant (27). Strain G9B was obtained from B. Rosan and is Spp^{+} . Strains Challis and G9B, previously classified as *S. sanguis*, were classified as *S. gordonii* based upon biochemical reactions in the API-ZYM and LRA osidase systems (Analytab Products, Plainview, N.Y.) using the criteria of Kilian et al. (15).

Cells were grown in chemically defined FMC broth medium (28), which contains no complex proteins or carbohydrates to influence adhesion. Cultures were incubated anaerobically (≤ 10 ppm of oxygen) at 36°C in a Coy chamber (Coy Manufacturing Co., Ann Arbor, Mich.) containing a gas mixture of 85% N_2 , 10% H_2 , and 5% CO_2 . Under these conditions the medium had an oxidation-reduction potential of -100 to -200 mV as determined by the E_h indicator dyes methylene blue, Nile blue, and phenosafranin (13). Late-log-phase cultures were diluted to 2×10^5 cells per ml in fresh medium to determine growth curves and for accumulation tests. In some cases the medium was supplemented with additional sucrose and/or dextran (Sigma). Cells were then grown in 1-ml aliquots in optically clear, 1-dram (ca. 3.7-ml) glass vials (Fisher Scientific Co.) capped with aluminum foil to allow adequate gas exchange. Vials were mixed at 10 rpm on a rotating drum (New Brunswick Scientific Co., Edison, N.J.).

Growth curves were established over a period of 1 to 27 h for all the strains studied. Growth was measured spectrophotometrically by A_{520} , and the pH of the cultures was measured with a Sensorex microelectrode (Thomas Scientific Co.) at each time point; pH correlated with A_{520} readings and was used to monitor the phase of growth (see Results). The number of viable bacteria was measured following sonication of the cultures for 10 s with a microprobe sonicator (Heat Systems Ultrasonics, Inc.) to break up streptococcal chains. Dilutions were plated on brain heart infusion (BHI) (Difco) agar plates, and CFU were counted after 24 h of anaerobic incubation at 36°C . Total bacterial counts were determined with a Petroff-Hausser counting chamber (Thomas).

Preparation of substrata. HA beads (BDH, Poole, England) in 10-mg amounts were placed into optically clear glass vials containing buffered KCl (2 mM KH_2PO_4 , 2 mM K_2HPO_4 , 1 mM CaCl_2 , 50 mM KCl [pH 6.8] [1]), autoclaved, and allowed to equilibrate overnight at 4°C . Seventeen-milligram amounts of acid-washed glass beads of 0.2-mm average diameter (Thomas), which gave a surface area equivalent to that of 10 mg of HA beads, were placed into vials and autoclaved in deionized water.

To prepare S-HA beads, whole, unstimulated saliva was collected from three healthy, male donors with no evidence of oral disease. The saliva was collected on ice and centrifuged at $12,000 \times g$ for 15 min. The supernatant was heat treated at 60°C for 30 min and recentrifuged, and the resulting supernatant was stored at -20°C (1, 5). Absence of viable bacteria in the prepared saliva was confirmed on BHI plates incubated anaerobically at 37°C . No GTF activity was evident in undiluted saliva on activity gels (25). Saliva was thawed quickly and diluted 1:4 in buffered KCl immediately before adsorption onto HA beads. Ten milligrams of sterilized, buffer-equilibrated HA beads were pretreated with diluted saliva on a rotating drum (10 rpm) at ambient temperature for 90 min. All bead preparations were washed

twice with 1 ml of sterile, buffered KCl and used immediately as substrata for nongrowing, washed bacteria in the adhesion tests or for growing bacteria in the accumulation tests.

Accumulation tests. Bacteria from late-log static cultures were diluted to ca. 2×10^5 cells per ml in fresh FMC medium. Sucrose and/or dextran was added as required, and in some cases 2 μCi of [^3H]thymidine (Amersham) per ml was added to radiolabel the bacteria. One-milliliter aliquots were dispensed immediately onto substrata of glass or HA beads. To study accumulation on S-HA beads, 1-ml aliquots were incubated in rotating glass vials without beads until cultures had reached early log phase, when ca. 1×10^7 to 2×10^7 cells per ml were present. These cultures were then transferred into vials containing S-HA beads.

The vials containing the bacteria and beads were placed on a rotator (10 rpm) in an anaerobic chamber, and the bacteria were allowed to grow at 36°C for various time periods from 1 to 27 h. At intervals, cultures were removed, the pH was measured, and the numbers of cells present on the beads and unattached in the fluid phase of the culture (supernatant) were determined.

Viable counts were determined for the unattached cells in the supernatant as described above. Beads were rinsed twice with 2 ml of sterile, buffered KCl (pH 6.8) to remove unattached bacteria and treated with 5 U of *Penicillium* sp. dextranase (EC 3.2.1.11; grade III; Sigma) in 100 mM potassium phosphate buffer (pH 6.0) for 30 min at 37°C . The beads were then sonicated to break up bacterial chains and polymers and to release bacteria from the beads. Bacteria were then diluted and plated as described above. Neither sonication nor dextranase treatment significantly reduced cell viability. Total counts of both unattached cells in the supernatant and the cells associated with the beads were determined with a Petroff-Hausser counting chamber.

In radiolabeled cultures, the unattached cells were centrifuged at $1,000 \times g$ and washed twice with buffered KCl to remove unincorporated label. The beads were washed twice with 2 ml of buffered KCl to remove loosely attached bacteria. This procedure also removed any potential unincorporated label from the bacterial insoluble polymer mass (BPM) which formed around the beads in some cultures grown with sucrose. Digestion of the BPMs with dextranase showed that the amount of free label released from the solubilized, washed BPMs was not above background. After the addition of 4 ml of Biosafe II scintillation cocktail (Research Products International Co., Mount Prospect, Ill.), the radioactivity of the supernatants and beads was counted in a Beckman scintillation counter. The recovered radiolabel counts in these tests were in agreement with total and viable cell counts. The percent accumulation on the beads was determined as the percentage of the total radiolabel counts associated with the beads.

Washed-cell adhesion tests. Modified washed-cell adhesion tests (1, 5) were performed. Bacteria were grown anaerobically to late log phase in the presence of 2 μCi of [^3H]thymidine per ml. The radiolabeled cells were washed twice by centrifugation at $1,000 \times g$ and resuspended to a concentration of 10^8 cells per ml in buffered KCl, and 1-ml volumes were placed into polystyrene tubes (Falcon; Becton Dickinson Labware, Lincoln Park, N.J.) containing HA or S-HA beads. Cells were rotated with the beads (10 rpm) at 37°C for 3.5 h, when maximum adhesion occurred. The supernatants containing the unattached cells were removed, and the beads were rinsed twice with 1 ml of buffered KCl. The radioac-

tivity of the supernatants and washed beads was counted as described above.

Viscosity tests. Viscous water-soluble polymer production was measured with a Canon-Manning microviscometer (Canon Instrument Co., State College, Pa.) with a calibrated range of 0.8 to 4 centistokes.

Measurement of insoluble polymer. BPMs were washed extensively in sterile buffered KCl at 4°C for 24 h to remove diffusible sucrose and water-soluble polymers. The final wash had no detectable hexoses as determined by anthrone reaction (32). The BPMs were then treated with dextranase and sonicated as described above. The mixture was then centrifuged at $1,000 \times g$ to remove the bacteria, and the resulting cell-free supernatants, containing the digested polymer, were analyzed by an anthrone reaction against a glucose standard curve. Results were expressed as milligrams of glucose equivalents per milliliter.

Scanning electron microscopy. Glass and HA beads on which growing bacteria had accumulated were fixed in 2.5% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.3) for 90 min, rinsed in buffer, postfixed in 1% osmium tetroxide, and serially dehydrated in ethanol. Samples in 100% ethanol were air dried to better maintain structural integrity. After sputter coating with 12-nm gold particles, samples were examined on a scanning electron microscope (model 100B; Amray, Bedford, Mass.).

Statistical analyses. Differences between pairs of strains and treatments were analyzed by the one-tailed Student *t* test for small samples. All samples were taken in duplicate, and tests were repeated at least twice. Least squares regression analysis was used to calculate coefficients of determination (r^2) to predict the stage of growth from pH measurements.

RESULTS

Establishment of growth parameters in FMC medium. Growth curves were established for rotating cultures in FMC medium under anaerobic conditions and in the absence of beads. Growth curves were similar for Challis and its derivatives; generation times averaged 60 min. A_{520} measurements correlated with viable counts for cultures grown without sucrose and were linear and inversely proportional to the pH of the culture throughout early to late log phases. A typical plot of pH versus A_{520} had a coefficient of determination of 0.9972 by least squares regression analysis.

The total and viable counts of cultures grown in the presence of 1% (wt/vol) sucrose were essentially the same as those of the cultures grown without sucrose at each time point. The pH values of the cultures grown with 1% sucrose were consistently within 0.2 pH unit of the cultures grown without sucrose for all strains tested. Because the relationship of A_{520} and pH held for all strains and for both growth conditions, pH was used to monitor the phase of growth. This was particularly important when the medium was supplemented with 1% sucrose because bacterial polymer aggregates formed in the *Spp*⁺ cultures in mid- to late log phase, which precluded the use of absorbance readings to monitor growth.

Influence of sucrose on accumulation of strain Challis on HA and glass beads. The distribution of the cells attached to the beads or unattached in the fluid phase (supernatant) was monitored throughout the growth cycle in FMC medium (Fig. 1A). The total number of bacteria in the system increased from an initial count of about 2×10^5 cells to more than 2.5×10^9 cells at late log phase. From about 1 to 7 h, the

FIG. 1. Distribution of strain Challis cells between HA beads and supernatant. (A) Cells growing in FMC without sucrose; (B) cells growing in FMC supplemented with 1% sucrose. The numbers of viable bacteria attached to the beads (●) and unattached in the supernatant (□) and the percentage of the total cells in the system that were attached to the beads (△) are shown as a function of culture age.

cells were essentially equally distributed between the beads and the supernatant. The number of bacteria on the beads then increased faster than the number in the supernatant so that by 10 h essentially 90% of the total of 3.5×10^7 cells in the system were attached to the bead surfaces. Bacterial cell numbers continued to increase on the beads until the available sites on the beads became saturated with about 1×10^8 to 2×10^8 cells. Thereafter, the number of cells continued to increase only in the supernatant. Consequently, because the total number of cells in the system continued to increase while the number of cells on the beads remained essentially the same, the percentage of the total number of cells in the system that were attached to the beads decreased.

FIG. 2. Scanning electron micrograph of BPM of strain Challis on HA beads. Bacteria grown to late log stage in FMC medium supplemented with 1% sucrose formed glucans which cover cell aggregates and obscure the bead surface. Bacteria on the glucan surface are seen at the lower left. Similar patterns were seen with glass beads. Magnification, $\times 4000$.

The distribution of cells grown in FMC medium supplemented with 1% sucrose (Fig. 1B) was similar for the first 10 h to that seen in FMC cultures (Fig. 1A). Thereafter, however, in the sucrose-supplemented cultures the number of cells associated with the beads continued to increase. Therefore, throughout the remaining test period, as the total number of cells increased to more than 2.5×10^9 , over 90% of the cells remained associated with the beads. Comparisons of viable and total counts showed that essentially all the bacteria were viable.

The greatly enhanced accumulation on the beads by strain Challis in the presence of sucrose was associated with the formation of both water-soluble and water-insoluble glucans (see below). Under these test conditions, copious amounts of water-insoluble glucans enmeshed the bacteria and beads to form a large, cohesive BPM, which by the late log stage of growth had a volume of greater than $40 \mu\text{m}^3$ and contained viable bacteria at concentrations greater than 5×10^{10} cells per cm^3 . Scanning electron microscopy specimens showed that the majority of bacteria were enmeshed within and covered by the polymer mass, but in occasional areas bacteria could be seen on the surface of the polymer matrix (Fig. 2). The glucan nature of the polymers associated with the supernatant and beads was confirmed by the finding that the viscosity of the supernatant was substantially reduced and the BPM was readily dispersed following dextranase digestion.

The patterns of accumulation of growing cells on surfaces were essentially identical when either glass or HA beads were used as substrata (data not shown). When the unattached cells in the supernatant were used as inocula for new tests, either with or without sucrose, the typical patterns of accumulation during growth (Fig. 1) were seen.

Effects of exogenous dextrans on accumulation of strain Challis on HA beads. Dextrans are present in plaque in vivo and are known to affect GTF activity and products of other oral streptococci (9, 19). Exogenous dextrans were added to the medium, and the percentage of strain Challis cells accumulated on the beads was determined at the mid- to late log stage of growth when more than 90% of the cells were

FIG. 3. Inhibition of sucrose-promoted accumulation of strain Challis by exogenous dextran. Mean percentage ($\pm$ the standard deviation) of strain Challis cells attached to HA beads at late log phase is shown for FMC cultures grown in the presence (▨) and absence (■) of 1% sucrose and in the presence of various concentrations of 72-kDa dextran. The relative amounts of insoluble glucan present on the beads in each sucrose culture are shown at the top and ranged from ca. 1.5 mg of glucose equivalents (++) to $<30 \mu\text{g}$ of glucose equivalents (-).

viable and maximum differences in accumulation could be seen (Fig. 1). The addition of dextran alone to the medium had little effect on the accumulation of cells on the beads (Fig. 3). However, in the presence of 1% sucrose, the addition of exogenous dextrans to the growth medium decreased the amount of insoluble polymer formed in a concentration-dependent manner. The number of bacteria associated with the beads at dextran (72 kDa) concentrations from 0.1 to 0.0003% decreased accordingly ($P \leq 0.001$, Fig. 3). This effect was similar when dextrans of 9 to 2,000 kDa were used (data not shown).

Accumulation of strain Challis Spp⁺ and Spp⁻ variants on HA beads. The Spp⁻ variants of strain Challis have only about 20% of the GTF activity of the parent and Spp⁺ revertant strains (27). This difference was not particularly evident in the synthesis of soluble polymer, although the supernatants of the Spp⁺ cultures were consistently more viscous than those of the Spp⁻ cultures (Fig. 4). More importantly, the Spp⁻ cells made considerably less insoluble polymer than did the Spp⁺ cells (Fig. 5). By the late log phase of growth, strain CH1C1 made $55 \mu\text{g}$ glucose equivalents per ml of insoluble glucan compared with $1,500 \mu\text{g}$ of glucose equivalents per ml made by strains Challis and CH1D2.

The decreased ability of the Spp⁻ strain CH1C1 to make soluble and insoluble polymers was reflected in the decreased ability of these cells to accumulate on HA beads in numbers greater than those afforded by the available adhesion/adsorption sites. Moreover, essentially no grossly visible BPM was formed by CH1C1 cells grown in FMC medium supplemented with sucrose. Therefore, the pattern of accu-

FIG. 4. Water-soluble polymer formation by *Spp*⁺ and *Spp*⁻ strains as a function of culture age. Viscosity of the supernatants of the *Spp*⁺ strains Challis (□) and CH1D2 (■) and the *Spp*⁻ strain CH1C1 (▲) in an accumulation test in which FMC supplemented with 1% sucrose was used. The contribution to the viscosity of equivalent numbers of cells in FMC (●) was minimal.

mulation of *Spp*⁻ cells grown either with or without sucrose was similar to that of Challis cells grown without sucrose (Fig. 6). The addition of higher concentrations (up to 3% [wt/vol]) of sucrose to the medium did not enhance the

FIG. 5. Water-insoluble polymer formation by *Spp*⁺ and *Spp*⁻ strains as a function of culture age. Beads from the accumulation tests for which results are shown in Fig. 4 were washed extensively and treated with dextranase, and the cell-free digests were analyzed for equivalent glucose units by anthrone reaction. Glucose equivalents per milliliter recovered from the *Spp*⁺ strains Challis (□) and CH1D2 (■) and from the *Spp*⁻ strain CH1C1 (▲) are plotted on a log scale.

FIG. 6. Percentage of growing cells of strains Challis and CH1C1 attached to HA beads from early log phase through early stationary phase of growth. Shown are strain Challis grown without sucrose (■), strain Challis grown with 1% sucrose (□), strain CH1C1 grown without sucrose (▲), and strain CH1C1 grown with sucrose (△). Cells were labeled with [³H]thymidine, and the radioactive counts associated with the beads as a percentage of total cell-incorporated label was calculated for each time point. The total numbers of cells present at each time point were equivalent for both strains under both growth conditions.

accumulation of *Spp*⁻ cells. The total number of cells in the test system was the same for both strains with or without sucrose.

As was seen with strain Challis (Fig. 3), the addition of exogenous dextran proportionally decreased the accumulation of the *Spp*⁺ revertant CH1D2 on bead surfaces in the presence of 1% sucrose ($P \leq 0.002$, Table 1). However, dextran had little effect on the accumulation of the *Spp*⁻ strain CH1C1 in the presence or absence of sucrose. Similar results were seen with other independently derived *Spp*⁻ variants and *Spp*⁺ revertants of strain Challis (data not shown).

Accumulation of *Spp*⁺ and *Spp*⁻ strains on S-HA. In vivo, the tooth surface is covered by a salivary pellicle, which markedly influences the ability of bacteria to attach (7). To study the accumulation of growing cells on S-HA beads, the accumulation test was modified in order to reduce exposure of the salivary pellicle to culture products and to minimize elution of saliva from the beads. Bacteria were grown with and without 1% sucrose to early log phase, when ca. 1×10^7 to 2×10^7 cells were present. No grossly visible BPM or bacterial aggregation was present at this time. Each 1-ml culture was then transferred to a vial containing either sterile S-HA or HA beads, and incubation was continued for 4 h to allow the bacteria to grow to mid-log phase, when over 5×10^8 cells were present. At various times a vial was chosen for enumeration of the bacteria and the percentage of cells associated with the beads was determined. The total number of cells present at each time point was similar for each strain in both media.

As can be seen in Fig. 7A, modification of the accumulation test by transfer of the growing cells to the substrata

TABLE 1. Effects of sucrose and dextran on accumulation of growing *Spp*⁺ and *Spp*⁻ strains on HA

Concn (%) ^a		% Accumulation ^b of strain:		
Sucrose	Dextran	Challis <i>Spp</i> ⁺	CH1C1 <i>Spp</i> ⁻	CH1D2 <i>Spp</i> ⁺
0	0	21.95 ± 3.15	18.55 ± 1.55	22.40 ± 1.20
	0.003	26.50 ± 2.35	21.25 ± 1.15	20.20 ± 1.20
	0.03	21.05 ± 4.15	22.50 ± 0.30	19.55 ± 3.45
1	0	76.15 ± 5.05	22.45 ± 1.35	83.70 ± 3.40
	0.003	52.75 ± 4.15	23.70 ± 2.60	66.70 ± 7.70
	0.03	25.80 ± 5.20	29.15 ± 4.05	38.45 ± 1.75

^a Concentrations (grams percent) of sucrose and/or dextran (72 kDa) present in FMC medium throughout growth.

^b Percentage of total cells present that were on bead surfaces at mid- to late log phase of growth.

reduced the percentage of sucrose-promoted attachment of strain Challis on HA compared with that in the original test (Fig. 1B). However, 4×10^8 to 5×10^8 cells were attached to the beads by 4 h, which represented a five- to sixfold net increase in the number of bacteria accumulated on the beads. In the absence of sucrose (Fig. 7A), only about 10^8 cells, or 25% of the total bacteria, had accumulated on the beads by 4 h. This was similar to the maximum number of cells found in the original accumulation assay (Fig. 1A) and represented a near saturation state. Strain CH1C1 cells accumulated on HA in the presence or absence of sucrose in a pattern similar to that of strain Challis without sucrose (data not shown).

Strain Challis accumulated less well on S-HA than on HA (Fig. 7B). At 1.5 h in medium without sucrose, there were ca. 2×10^7 cells on the S-HA beads, but there was no significant increase in the number of accumulated bacteria by 4 h. The presence of sucrose, however, significantly enhanced the accumulation of strain Challis, allowing more than 3×10^8 cells to accumulate on the S-HA beads, which was more than 10-fold higher than in FMC alone.

The relevance of the *Spp*⁺ state to the accumulation of strain Challis on S-HA surfaces was apparent from comparisons with the *Spp*⁻ strain CH1C1 (Fig. 7C). The accumulation patterns of the *Spp*⁻ strain CH1C1 on S-HA were similar in the presence or absence of sucrose and paralleled that of strain Challis on S-HA grown in the absence of sucrose (Fig. 7B). Again, there was no significant increase in

TABLE 2. Comparative adhesion and accumulation of *S. gordonii* cells on HA and S-HA in the presence and absence of sucrose

Strain	<i>Spp</i>	Sucrose	Adhesion ^a		Accumulation ^b	
			HA	S-HA	HA	S-HA
Challis	+	-	6.8×10^6	9.0×10^5	1.2×10^8	2.4×10^7
		+	8.7×10^6	8.0×10^5	3.3×10^8	3.1×10^8
CH1C1	-	-	7.5×10^6	4.2×10^6	1.3×10^8	3.0×10^7
		+	1.0×10^7	3.8×10^6	1.2×10^8	4.2×10^7
G9B	+	-	1.7×10^7	1.5×10^7	2.5×10^8	1.6×10^8
		+	2.1×10^7	1.3×10^7	4.6×10^8	4.5×10^8

^a Number of [³H]thymidine-labeled, washed, nongrowing bacterial cells attached to 10 mg of HA or S-HA beads. A subsaturating concentration of cells (10^8) was incubated with beads for 3.5 h at 37°C in the washed-cell adhesion test in the presence (+) or absence (-) of 1% sucrose. Errors between tests were less than 5%.

^b Number of [³H]thymidine-labeled growing cells attached to 10 mg of HA or S-HA beads at mid- to late log phase. Bacteria were grown in FMC medium with (+) or without (-) 1% sucrose to early log phase and then transferred onto HA or S-HA beads to continue growth for 4 h. Errors between tests were less than 5%.

the number of bacteria attached to the S-HA beads during the incubation period. Final cell numbers were more than sevenfold less than the number of strain Challis cells accumulated on S-HA when both strains were grown in the presence of sucrose. Similar patterns were also seen with other *Spp*⁻ strains (data not shown). The difference between the S-HA percent accumulation curves for strains Challis and CH1C1 may reflect the increased ability of all *Spp*⁻ strains examined to attach to S-HA in the washed-cell adhesion tests (31). Adhesion, which is elevated two- to fivefold over that of *Spp*⁺ cells, was not sucrose dependent (Table 2; 31).

Accumulation of strain G9B on HA and S-HA. The sucrose-promoted accumulation of G9B cells was studied to confirm that this process occurs in other *Spp*⁺ *S. gordonii* strains. In the original accumulation test strain G9B showed a time- and growth phase-associated pattern of cell distribution on HA beads similar to that of strain Challis. Generation time was ca. 60 min, and cultures grew to a final density of more than 2.5×10^9 cells per ml by late log phase (data not shown). In the modified accumulation test, 85 to 95% of the cells were attached to the HA beads in early log phase in the absence of sucrose. By mid- to late log phase ca. 50% of the total cells

FIG. 7. Percentage of growing cells of strains Challis and CH1C1 attached to HA or S-HA beads. Early-log-phase cultures were transferred to HA or S-HA beads and allowed to continue to grow. (A) Percentage of strain Challis cells attached to HA when grown in FMC alone (■) or FMC with sucrose (□). (B) Percentage of strain Challis cells attached to S-HA when grown in FMC (■) or FMC with sucrose (□). (C) Percentage of strain CH1C1 cells attached to S-HA in FMC (▲) or FMC with sucrose (△).

present were associated with the beads (Table 2). The higher number of strain G9B cells associated with the HA beads at late log phase compared with the number of cells of strain Challis associated with the beads is consistent with the greater number of potential initial attachment sites available for strain G9B, as suggested by results in the washed-cell adhesion assays ($P \leq 0.007$, Table 2).

The presence of 1% sucrose significantly increased the accumulation of strain G9B on HA beads so that ca. 90% of the cells remained associated with the HA beads from early through late log phase. This increased accumulation on the beads was associated with the production of a water-insoluble polymer which formed a typical, cohesive BPM around the beads. By late log phase the strain G9B BPM contained $>2 \times 10^9$ cells and 1.2 mg of glucose equivalents per ml of insoluble glucan. More importantly, in the presence of sucrose the accumulation of strain G9B on S-HA was significantly enhanced ($P \leq 0.001$, Table 2) and cell numbers more than doubled.

DISCUSSION

In contrast to classical, washed-cell adhesion tests, the growing-cell accumulation tests described here allow the cell surface components and extracellular products to play a role in attachment and growth of the organisms on surfaces. The accumulation test demonstrates distinct and significant differences in the accumulation of Spp⁺ and Spp⁻ strains on both HA and S-HA surfaces. These differences are associated with different amounts of cohesive, insoluble glucan synthesized by strains of the two phenotypes. After an initial period in which the cells are evenly distributed between the supernatant and beads, cell numbers increase more rapidly on the bead surfaces. This increased number of cells on the beads occurs in FMC medium both with and without sucrose and therefore is probably unrelated to glucan synthesis. A possible explanation for this finding may be the promotion of increased growth by nutrients concentrated on the bead surfaces by adsorption, possibly coupled to changes in the metabolism of the attached bacteria (29).

In sucrose-free medium, once the surface sites were saturated, bacterial cell numbers continued to increase only in the supernatant. Transfer of the unattached cells to a new accumulation test resulted in a typical distribution pattern, suggesting that these cells were not, as a whole, an attachment-deficient subpopulation. However, this does not rule out the possibility that these cells were in a transitory nonadhesive state during a critical period of their growth cycle when they initially contacted the beads.

The same pattern of cell distribution was shown by both Spp⁺ and Spp⁻ cells in the absence of sucrose. In contrast, in the presence of sucrose the number of Spp⁺ cells on the beads increased above the level of initial saturation. This increase in cell number was associated with both soluble and insoluble polymer formation. The soluble polymer makes the fluid phase more viscous and could possibly serve to initially drive more cells onto the beads and/or promote adhesion by increasing contact time between the attaching cells and the substratum (23). This is unlikely to contribute significantly to the accumulation of Spp⁺ bacteria, however, since noticeable soluble polymer synthesis lagged behind insoluble polymer synthesis and accumulation. It appears, therefore, that insoluble polymer production is critical for cell accumulation in numbers beyond the level of initial saturation. The high concentration of cells within the BPM ($>5 \times 10^{10}$ cells per cm³), with a viability of 90 to 100%, is somewhat surprising.

This population appears to grow at essentially the same rate as the cells in FMC medium without sucrose. It is possible that the BPM concentrates nutrients which allow the cells to maintain normal growth rates and reach these high cell numbers.

The insoluble glucans formed in this system are associated with the beads and the attached bacteria. Little insoluble glucan was found free in the supernatant. This does not appear to be due to the inability of the unattached supernatant cells to produce insoluble polymer. Upon transfer to new medium, these cells attached to beads and formed BPMs in the presence of sucrose, as had the parental culture. A possible explanation for the association of insoluble polymer synthesis with attached bacteria is that GTF production or activity may change when cells are bound to a solid surface. Studies suggest that the *S. mutans* *gtf* gene is more actively transcribed when cells are attached to HA beads in the presence of sucrose (14). The possibility that a similar phenomenon occurs in *S. gordonii* is suggested by the observation that cells grown in rotating polystyrene tubes, to which the cells do not readily attach, showed greatly delayed and decreased insoluble polymer synthesis ($<30 \mu\text{g}$ of glucose equivalents per ml by late log phase). When beads were added to these cultures, however, insoluble glucans were made (>1 mg of glucose equivalents per ml) and surrounded the beads, forming a typical BPM (data not shown).

An alternative explanation is that molecules adsorbed to the surface or sequestered by the attached bacterial populations cause conformational changes in the GTF molecule which favor insoluble glucan production. This has been demonstrated for *S. mutans* GTF, the conformation of which is altered by high salt concentrations or salivary phospholipids to favor insoluble polymer production (26). It is not known if the GTF activity of *S. gordonii* is modified in surface-associated populations, although it is known that a 156-kDa form of GTF predominates within the BPM, whereas a 170-kDa form is associated with the unattached cells in the presence of sucrose (30).

Sucrose-promoted accumulation of Spp⁺ cells on S-HA surfaces is more relevant to the in vivo environment and suggests that this mechanism of colonization and accumulation may play a role in the oral cavity. Factors in saliva, such as free dextrans or factors that solubilize bacterial glucans, may promote or inhibit insoluble polymer production (6, 7, 9, 12). Such factors are host specific and reflect the composition of the resident oral flora and salivary biochemistry (7, 12). The GTF of strain Challis is biochemically similar to the better-characterized GTF of *S. gordonii* 10558 (19) in that soluble dextran acceptors reduce insoluble glucan synthesis. This inhibition severely reduced sucrose-promoted accumulation in vitro and presumably could have a significant impact in vivo. The soluble and insoluble glucans produced by *S. gordonii* theoretically may displace salivary proteins, glycoproteins, or polymers (23) from the salivary pellicle or mask these components, thus affecting colonization. Some salivary components may serve a protective function in blocking cell attachment (10), and their displacement or masking could enhance attachment of cells to the tooth surface.

The in vivo relevance of sucrose-promoted accumulation of *S. gordonii* remains to be determined, although there is some evidence that sucrose promotes colonization by some streptococci now classified as *S. gordonii* and *S. sanguis* (16). Recent evidence suggests that *S. sanguis* sensu stricto is more important as an initial colonizer of the tooth surface

(22). If these preliminary in vivo findings are confirmed and *S. sanguis* is established as the primary colonizer, then the accumulation of *S. gordonii* on early and established plaque, perhaps involving insoluble glucans that enmesh the bacterial cells and their products, may be more relevant to maintaining these organisms in the oral environment. In addition to maintaining *S. gordonii* in the plaque, the glucans made by these organisms can be bound by other plaque bacteria (2) and may serve as a substratum for additional organisms, thereby determining the nature of the mature plaque.

The function of the Spp⁻ variants is an intriguing question. These variants have no enhanced ability to accumulate on either HA or S-HA surfaces when sucrose is provided as a substrate for glucan synthesis but do show increased adhesion to S-HA (31). The latter suggests that changes relevant to initial adhesion occur in addition to changes in GTF activity during phase variation (31). It has been postulated (10) that on a nonshedding surface such as a tooth, it would be ecologically advantageous for an organism to be able to switch between adhesive and nonadhesive states depending upon the environmental conditions. This could potentially give the cells the opportunity to be released from a surface in order to colonize new sites. Changes in adhesive properties would be particularly relevant to survival once a site is saturated and/or the nutrients in the microenvironment are depleted. It is conceivable that the Spp⁻ variants, which arise from Spp⁺ cells, may be released from plaque developing under the influence of sucrose. Studies in support of this role for Spp⁻ cells will be presented in a future paper.

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REFERENCES

- Appelbaum, B., E. Golub, S. C. Holt, and B. Rosan. 1979. In vitro studies of dental plaque formation: adsorption of oral streptococci to hydroxyapatite. *Infect. Immun.* 25:717-728.
- Bourgeois, G., and B. C. McBride. 1976. Dextran-mediated interbacterial aggregation between dextran-synthesizing streptococci and *Actinomyces viscosus*. *Infect. Immun.* 13:1228-1234.
- Buchan, R. A., and H. F. Jenkinson. 1990. Glucosyltransferase production by *Streptococcus sanguis* Challis and comparison with other oral streptococci. *Oral Microbiol. Immunol.* 5:63-71.
- Carlsson, J., H. Grahnen, G. Jonsson, and S. Wikner. 1970. Establishment of *Streptococcus sanguis* in the mouths of infants. *Arch. Oral Biol.* 15:1143-1148.
- Clark, W., L. Bammann, and R. Gibbons. 1978. Comparative estimates of bacterial affinities and adsorption sites on hydroxyapatite surfaces. *Infect. Immun.* 19:846-853.
- Cowan, M. M., K. Parrish, R. E. Kessler, C. Pyle, Jr., K. G. Taylor, J. E. Ciardi, and R. J. Doyle. 1988. Glucan-binding factor in saliva. *Infect. Immun.* 56:2912-2917.
- Gibbons, R. J. 1989. Bacterial adhesion to oral tissues: a model for infectious diseases. *J. Dent. Res.* 68:750-760.
- Gibbons, R. J., I. Etherden, and W. Peros. 1985. Aspects of the attachment of oral streptococci to experimental pellicles, p. 77-84. *In* S. E. Mergenhagen and B. Rosan (ed.), *Molecular basis of oral microbial adhesion*. American Society for Microbiology, Washington, D.C.
- Gibbons, R. J., and P. H. Keyes. 1969. Inhibition of insoluble dextran synthesis, plaque formation and dental caries in hamsters by low molecular weight dextran. *Arch. Oral Biol.* 14:721-724.
- Gibbons, R. J., and J. van Houte. 1975. Bacterial adherence in oral microbial ecology. *Annu. Rev. Microbiol.* 29:19-44.
- Grahame, D. A., and R. M. Mayer. 1984. The origin and composition of multiple forms of dextranucrase from *Streptococcus sanguis*. *Biochim. Biophys. Acta* 786:42-48.
- Hamada, S., and H. D. Slade. 1980. Biology, immunology and cariogenicity of *Streptococcus mutans*. *Microbiol. Rev.* 44:331-384.
- Hewitt, L. F. 1950. Oxidation-reduction potentials in bacteriology and biochemistry, 6th ed. E. and S. Livingstone, Edinburgh.
- Hudson, M. C., and R. Curtiss. 1990. Regulation of expression of *Streptococcus mutans* genes important to virulence. *Infect. Immun.* 58:464-470.
- Kilian, M., L. Mikkelsen, and J. Henrichsen. 1989. Taxonomic study of viridans streptococci: description of *Streptococcus gordonii* sp. nov. and emended descriptions of *Streptococcus sanguis* (White and Niven 1946), *Streptococcus oralis* (Bridge and Sneath 1982), and *Streptococcus mitis* (Andrews and Horder 1906). *Int. J. Syst. Bacteriol.* 39:471-484.
- Kilian, M., B. Nyvad, and L. Mikkelsen. 1986. Taxonomic and ecological aspects of some oral streptococci, p. 391-400. *In* S. Hamada, S. M. Michalek, H. Kiyono, L. Menator, and J. R. McGhee (ed.), *Molecular microbiology and immunology of Streptococcus mutans*. Elsevier Science Publications, Amsterdam.
- Kolenbrander, P. E., and R. A. Andersen. 1986. Multigenic aggregations among oral bacteria: a network of independent cell-to-cell interactions. *J. Bacteriol.* 168:851-859.
- Loe, H., E. Theilade, and S. B. Jensen. 1965. Experimental gingivitis in man. *J. Periodontol.* 36:177-187.
- Mayer, R. M. 1987. Dextranucrase: a glucosyltransferase from *Streptococcus sanguis*. *Methods Enzymol.* 138:649-661.
- McIntire, F. C., A. E. Vatter, J. Baros, and J. Arnold. 1978. Mechanism of coaggregation between *Actinomyces viscosus* T14 and *Streptococcus sanguis* 34. *Infect. Immun.* 21:978-988.
- Niven, C. F., Z. Kiziuta, and J. C. White. 1946. Synthesis of a polysaccharide from sucrose by streptococcus s.b.e. *J. Bacteriol.* 51:711-716.
- Nyvad, B., and M. Kilian. 1990. Comparison of the initial streptococcal microflora on dental enamel in caries-active and caries-inactive individuals. *Caries Res.* 24:267-272.
- Robb, I. D. 1984. Stereobiochemistry and function of polymers, p. 39-49. *In* K. C. Marshall (ed.), *Microbial adhesion and aggregation*. Dahlem Konferenzen. Springer-Verlag KG, Berlin.
- Rosan, B., R. Eifert, and E. Golub. 1985. Bacterial surfaces, salivary pellicles, and plaque formation, p. 69-76. *In* S. Mergenhagen and B. Rosan (ed.), *Molecular basis of oral microbial adhesion*. American Society for Microbiology, Washington, D.C.
- Russell, R. R. B. 1979. Use of Triton X-100 to overcome the inhibition of fructosyltransferase by SDS. *Anal. Biochem.* 97:173-175.
- Schachtele, C. F., S. K. Harlander, J. W. Bracke, L. C. Ostrum, J. B. Mattais, and R. J. Billings. 1978. *Streptococcus mutans* dextranucrase: stimulation by phospholipids from human sera and oral fluids. *Infect. Immun.* 22:714-720.
- Tardif, G., M. Sulavik, G. W. Jones, and D. B. Clewell. 1989. Spontaneous switching of the sucrose-promoted colony phenotype in *Streptococcus sanguis*. *Infect. Immun.* 57:3945-3948.
- Terleckyj, B., N. P. Willett, and G. D. Shockman. 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. *Infect. Immun.* 11:649-655.
- van Loosdrecht, M. C. M., J. Lyklema, W. Norde, and A. J. B. Zehnder. 1990. Influences of interfaces on microbial activity. *Microbiol. Rev.* 54:75-87.
- Vickerman, M. M. 1991. Ph.D. thesis. University of Michigan, Ann Arbor.
- Vickerman, M. M., D. B. Clewell, and G. W. Jones. Oral Microbiol. Immunol., in press.
- Yemm, E. W., and A. J. Willis. 1954. The estimation of carbohydrates in plant extracts by anthrone. *Biochem. J.* 57:508-514.