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Sucrose-dependent accumulation of oral streptococci and their adhesion-defective mutants on saliva-coated hydroxyapatite

M. M. Vickerman[1], G. W. Jones[2]
[1]Department of Biologic and Materials Sciences, School of Dentistry and [2]Department of Microbiology and Immunology, School of Medicine, University of Michigan, Ann Arbor, USA

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The adhesion and accumulation of oral streptococci on saliva-coated hydroxyapatite was examined in strains representing species that appear in initial plaque (*Streptococcus sanguise* FC1 and *Streptococcus oralis* C5) and in more mature plaque (*Streptococcus gordonii* G9B). Washed cells of strains FC1 and C5 did not attach better to saliva-coated hydroxyapatite than did strain G9B, suggesting that the degree of initial adhesiveness does not alone account for the temporal appearance of these bacteria in dental plaque. Growing cells of each strain were also examined for their ability to accumulate on saliva-coated hydroxyapatite. The addition of sucrose to the medium promoted the accumulation of strain G9B more than it promoted the accumulation of strains FC1 and C5. Sucrose also enhanced the accumulation of adhesion-defective mutants of each strain to levels similar to those of the respective parent strains. These results suggest that sucrose-dependent accumulation may facilitate the colonization of the tooth surface by these species of oral streptococci when adhesion is limited by reduced bacterial adhesiveness or limited pellicle-binding sites.

Key words: streptococci; hydroxyapatite; saliva; adhesion; dental plaque

M. M. Vickerman, Department of Biologic and Materials Sciences, 300 North Ingalls Building, University of Michigan, Ann Arbor, MI 48109-0402, USA

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The heterogeneous group of oral streptococci previously classified as *Streptococcus sanguis* and now classified as *Streptococcus sanguis*, *Streptococcus gordonii* and *Streptococcus oralis* (18) appears to occupy significantly different niches in the oral cavity (9, 19). All 3 species are considered early colonizers of the tooth surface (21), but ecological studies that use these new taxonomic designations suggest that temporal differences exist in the appearance of these species in dental plaque. *S. oralis* and *S. sanguis* are more often isolated from early plaque and therefore appear to be initial or primary colonizers of the salivary pellicle (9, 27). Of these species, only *S. oralis* appears before tooth eruption (34). *S. gordonii*, in contrast to *S. sanguis* and *S. oralis*, appears more commonly in more mature plaque and on oral epithelial surfaces (9). These differences may reflect differences in the colonization abilities of the newly defined species.

Initial colonization of the tooth surface requires bacterial adhesion to the adsorbed salivary pellicle. This may involve interactions between specific adhesins on the bacterial cell surface (10, 30) and specific pellicle receptors (13, 26). *In vitro* kinetic studies indicate that the number of bacteria that can colonize the surface in this way must be limited by the number of available receptor sites (1) and is significantly less than the number of bacteria found in mature plaque. The large numbers of bacteria in dental plaque, therefore, probably arise through additional mechanisms of surface colonization that are independent of the number of initial binding sites present on the salivary pellicle-coated tooth surface.

Colonizing bacteria that occupy initial binding sites in the salivary pellicle form a substratum consisting of bacterial cells and their products. This modified surface provides additional sites of attachment for secondary colonizers that may include the progeny of the initial colonizing species. Attachment of secondary colonizers may involve bacterial aggregation through homotypic and heterotypic cell interactions (21) or bacterial aggregation mediated by host factors such as salivary components (22, 23). Bacterial products may also mediate secondary colonization and/or accumulation of plaque bacteria. Glucan polymers that have been synthesized from dietary sucrose via the bacterial extracellular enzyme glucosyltransferase may facilitate bacterial accumulation. Bacteria may bind directly to the enzyme and its glucan

products (8, 14, 33) or may bind to amphipathic molecules such as lipoteichoic acids that are complexed to glucans (29, 42). Although sucrose-associated accumulation mechanisms are best defined for mutans streptococci (24), recent evidence suggests that sucrose and resulting glucan synthesis may be important in the accumulation of *S. gordonii* strain Challis (40). These *in vitro* studies show that accumulation may be facilitated by passive occlusion of multiplying bacterial cells, as glucans are synthesized in the developing dental plaque (41).

An effective accumulation mechanism does not preclude the existence of an effective mechanism of initial attachment, although these two mechanisms may be independent. The present studies were therefore designed to investigate the relative importance of initial adhesion and sucrose-promoted accumulation in the establishment of growing streptococci on saliva-coated hydroxyapatite. Initial colonizers of the tooth surface may heavily depend on attachment to the salivary pellicle, whereas later colonizers may rely more on accumulation mechanisms as the initial sites of adhesion in the salivary pellicle become fully occupied. To test this hypothesis, 3 well-characterized oral streptococcal species representing initial colonizers (*S. sanguis* strain FC1 and *S. oralis* strain C5) and later colonizers (*S. gordonii* strain G9B) were examined for their ability to attach to and accumulate on hydroxyapatite and saliva-coated hydroxyapatite beads. Although adhesive mechanisms have been studied in all these strains (1, 4, 11, 30), there is little or no information on their accumulation. Our second objective was to examine the role of sucrose-dependent accumulation in these strains. To determine the relative importance of this mechanism when initial attachment is impaired, nonadhesive mutants of each strain with significantly reduced adhesion to saliva-coated hydroxyapatite were generated. Our results suggest that sucrose-dependent accumulation may play a significant role in later colonization of the tooth surface and help cells to overcome reduced initial adhesion.

Material and methods

Bacteria and mutant strain isolation

All bacterial strains were stored in 50% glycerol at −70°C. Mutant strains with reduced abilities to attach to saliva-coated hydroxyapatite were obtained from laboratory strains of *S. gordonii* strain G9B (obtained from B. Rosan, University of Pennsylvania, Philadelphia), *S. sanguis* strain FC1 and *S. oralis* strain C5 (obtained from R. Gibbons, Forsyth Dental Center, Boston, MA), by the following method. Cultures were mutagenized with nitrosoguanidine (28), cultured overnight in brain heart infusion broth (Difco Laboratories, Detroit, MI) and then washed and suspended in buffered KCl (1) to a concentration of 1×10^9 bacteria/ml. Bacterial suspensions were then mixed (Labquake, Labindustries, Berkeley, CA) with equal volumes of 2.5% (wt/vol) hydroxyapatite flakes (BDH, Poole, UK) that had been coated with heat-sterilized (60°C), whole, unstimulated saliva (40). After 60 min of mixing, the flakes were allowed to settle out by gravity, and the bacteria that remained unattached in the supernatants were cultured and subjected to additional rounds of selection with saliva-coated hydroxyapatite flakes. Supernatants containing unattached cells then were diluted and plated onto brain heart infusion agar and incubated anaerobically (Coy Chamber, Coy Manufacturing Co., Jackson, MI) at 37°C. Plates with isolated colonies were flooded with saliva-coated hydroxyapatite flakes, rinsed in buffered KCl and examined microscopically for colonies to which flakes did not attach. These colonies were picked and purified as presumptive adhesion-defective mutant strains and their nonadhesive nature confirmed in a standard adhesion test (see below). Identities were confirmed by comparison of carbohydrate utilization patterns (7) and chromosomal DNA restriction digest patterns (2) of mutant and respective parent strains.

Adhesion to hydroxyapatite and saliva-coated hydroxyapatite beads

Adhesion tests with washed, nongrowing, ^{3}H-thymidine (Amersham, about 50 Ci/mM)-labeled cells from mid- to late-log phase anaerobic cultures have been previously described (39, 40). Briefly, bacterial suspensions in 1 ml of buffered KCl were mixed with 10 mg of hydroxyapatite or saliva-coated hydroxyapatite beads in polystyrene tubes on a rotating drum (New Brunswick Scientific Co., Edison, NJ) for 90 min at room temperature. Supernatants containing unattached cells and washed beads, with attached cells, were counted separately in a scintillation counter. The percentage adhesion was calculated as the number of attached cells/total number of cells ×100 (39, 40). Affinity constants (K_a values) were calculated (1) from linear regression plots of unattached cells versus the ratio of unattached to attached cells over concentration ranges of 1×10^7 to 6×10^9 bacteria/ml. Bacterial concentrations were determined by total counts and were related to radioactive counts. Assays were done in triplicate and repeated at least 3 times.

Accumulation assays

To determine the amount of sucrose-independent and sucrose-dependent accumulation, the standard accumulation test was done with hydroxyapatite beads as previously described (40). Briefly, bacteria were grown in one ml volumes of FMC (38) medium and FMC medium containing 1% (wt/vol) sucrose (FMCS) (41) with 10 mg of hydroxyapatite beads (BDH) in 4 dram glass vials (Fisher Scientific Co., Pittsburgh, PA). Vials were rotated (10 rpm) at 36°C under anaerobic conditions (Coy Chamber). The pH and absorbance readings at 520 nm (A_{520}) of cultures were monitored to estimate the growth phase (40). At mid- to late-log stage, supernatants containing the unattached bacteria and beads with attached bacteria, were treated separately with dextranase (EC 3.2.1.11; Sigma Chemical Co., St. Louis, MO) and sonicated to disperse bacterial chains and insoluble glucan polymer (40). The numbers of unattached and attached ^{3}H-thymidine-labeled bacteria were determined by scintillation counts. The correlation of ^{3}H-thymidine counts to viable bacterial counts was confirmed in representative tests with each strain. The percentage accumulation was calculated as the percentage of the total number of bacteria present that was attached to the beads (40).

A modified accumulation test was used to measure accumulation on saliva-coated hydroxyapatite beads because of the lability of saliva in the standard test system (40). One-ml cultures grown anaerobically in FMC and in FMCS were transferred at the early- to mid-log phase of growth to new vials containing 10-mg quantities of saliva-coated hydroxyapatite beads. Incuba-

tion was then continued as above for 4 to 6 h until the cultures reached the mid- to late-log phase of growth. Tests with hydroxyapatite beads were included as controls. The percentages of bacteria that accumulated on the beads at these times were calculated.

Buccal cell adhesion assays

Overnight cultures of bacteria were washed twice and resuspended in buffered KCl to 1×10^8 cells/ml. Buccal cells were scraped from the buccal surface of healthy male donors with a wooden applicator, washed twice in buffered KCl and resuspended to 1×10^5 cells/ml. Equal volumes of bacteria and epithelial cells were mixed together in tubes on a rotating drum at 36°C. Cell smears were prepared on glass slides, heat-fixed and stained with crystal violet. Bacteria on each of 100 cells were counted microscopically (12). Controls of buccal cells incubated without bacteria were also counted to determine the numbers of indigenous bacteria per cell. Tests were done in triplicate and repeated 3 times.

Hydrophobicity measurements

Phase partitioning of bacteria between hexadecane (Sigma Chemical Co.) and PUM buffer, pH 7.1 (31) as well as adsorption to phenyl-Sepharose (Pharmacia, Piscataway, NJ) beads (35) were used as two measurements of relative hydrophobicity. In the first method, bacteria (1×10^9/ml) were mixed on a vortex mixer with 0.25 volumes of hexadecane (Sigma Chemical Co.), and the percentage of bacteria that entered the organic phase was calculated from a comparison of the A_{400} reading of the aqueous phase before and after mixing. In the second method, bacteria (1×10^9/ml) were mixed with an equal volume of 25% phenyl-Sepharose beads in buffer for 30 min at room temperature and the percentage of the bacteria adsorbed to the beads calculated from A_{400} readings taken before and after treatment.

Lipoteichoic acid assays

Lipoteichoic acid was measured by enzyme-linked immunosorbent assay (ELISA) as previously described (42). Parent and mutant strains were grown anaerobically in FMC medium until cultures reached the same A_{520} at the mid- to late-log phase of growth. Bacteria recovered from 1 ml of culture by centrifugation were resuspended in water and extracted with hot phenol-water at 68°C (16); the remaining bacteria-free supernatant was similarly extracted to determine the amount of released lipoteichoic acid. The dialyzed extracts were adsorbed to polyvinyl plates (Dynatech Laboratories, Alexandria, VA) and the lipoteichoic acid detected with polyclonal rabbit antibody specific for the glycerolphosphate moiety of lipoteichoic acid (17), (a gift from R. E. Kessler), horseradish conjugated goat anit-rabbit immunoglobulin (Bio-Rad Laboratories, Richmond, CA) and hydrogen peroxide/TMB (3,3′,5,5′-tetramethylbenzidine) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Column-purified lipoteichoic acid from strain Challis (a gift from R. E. Kessler) was used to construct standard curves over the concentration range of 50 ng/ml to 0.8 ng/ml (42). Concentrations of lipoteichoic acid recovered from the bacterial cells and from supernatants of 1-ml cultures were calculated from least squares regression analyses.

Insoluble glucan assays and glucosyltransferase activity gels

In cultures containing sucrose, macroscopic masses of attached bacteria and their products, including water-soluble and water-insoluble glucans, formed on hydroxyapatite and saliva-coated hydroxyapatite beads by the mid- to late-log phase of growth. Sucrose-dependent accumulation has previously been shown to be associated with the formation of water-insoluble glucan polymers on hydroxyapatite beads by *S. gordonii* strain Challis (40). To see whether this association held true for the strains in the present study, the insoluble glucan present on the beads in mid- to late-log phase cultures of each parent and mutant strain was measured as previously described (40). The masses on the beads were washed to remove water-soluble glucans and sucrose. The remaining water-insoluble glucan was digested with dextranase and sonicated. The beads and bacteria were removed from the digests by centrifugation and the solubilized glucan in the supernatant assayed by the anthrone reaction (43) against a glucose standard (40); the results were expressed in glucose equivalents per ml.

Glucosyltransferase activities were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) activity gels (32) as previously described (37). Parent and mutant strains were grown to late-log phase when culture turbidities at A_{520} were similar. Culture supernatants were boiled in SDS and electrophoresed in 8.75% polyacrylamide. Gels were incubated with Triton X-100 and 3% sucrose and then stained with pararosaniline (Sigma Chemical Co.) to visualize the resulting glucan bands.

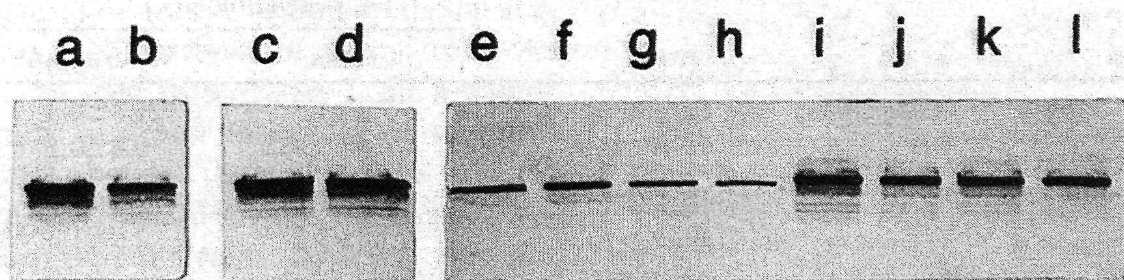

Fig. 1. SDS-PAGE gel showing glucosyltransferase activities of parent and mutant strains grown in the absence and presence of sucrose. **a.** Strain VJ5. **b.** Strain VJ5 with sucrose. **c.** Strain C5. **d.** Strain C5 with sucrose. **e.** Strain FC1. **f.** Strain FC1 with sucrose. **g.** Strain VJ1. **h.** Strain VJ1 with sucrose. **i.** Strain G9B. **j.** Strain G9B with sucrose. **k.** Strain VJ9. **l.** Strain VJ9 with sucrose.

Results

Comparison of mutant and parent strains

Mutant strains with reduced abilities to attach to saliva-coated hydroxyapatite surfaces (see below) were isolated following counterselection of adhesive bacteria with saliva-coated hydroxyapatite flakes. These strains were recognized initially by the inability of colonies to bind saliva-coated hydroxyapatite flakes. Strain VJ9, derived from the *S. gordonii* strain G9B, strain VJ1, de-

rived from the *S. sanguis* strain FC1 and strain VJ5 derived from the *S. oralis* strain C5, were chosen for further study. The 3 mutant strains had the same carbohydrate utilization and the same DNA restriction digest patterns as their respective parent strains (data not shown).

Stained glucan bands on activity gels (Fig. 1), which reflect the amount of glucosyltransferase activity (37), showed that the glucosyltransferase enzyme of each mutant strain was comparable to that of its parent. Only one major glucosyltransferase band was detected in each of the 6 strains, and these all appeared to be of the same molecular mass (about 175 kDa); lower-molecular-weight bands with glucosyltransferase activities were probably due to cleavage of the native glucosyltransferases by endogenous proteases (25). The glucosyltransferase enzymes of all 3 parent strains and their mutants appeard to be constitutive with respect to the substrate sucrose, since no consistent differences in the intensities of the glucosyltransferase bands of cultures grown with and without sucrose were observed (Fig. 1).

Hydrophobicity, which has been associated with adhesion in oral streptococci (3, 6), was measured by two methods to characterize the cell surface properties of mutant strains. All 3 parent strains were hydrophobic in both test systems, with more than 80% of the bacteria binding to either phenyl-Sepharose beads (Fig. 2A) or to the hexadecane droplets thereby entering the alkane phase in the phase partition test (Fig. 2B). In contrast, the 3 mutant strains (Fig. 2) were markedly more hydrophilic ($P<0.001$ to <0.0001) than their respective parent strains.

Lipoteichoic acid may contribute to the hydrophobicity of oral streptococci and to their adhesive properties (6, 29) and has been implicated in *S. gordonii* accumulation (42). Since the 3 mutant strains were all relatively hydrophilic, cellular lipoteichoic acid concentrations were measured to determine whether this cell surface property was related to the adhesive changes in our mutants. Accordingly, extracts of whole cells and of bacteria-free culture supernatants were assayed for lipoteichoic acid by ELISA. As can be seen from Table 1, *S. gordonii* strain VJ9 cells contained significantly less lipoteichoic acid than the parent G9B cells ($P\leq0.001$). The lipoteichoic acid content of the supernatant fluid,

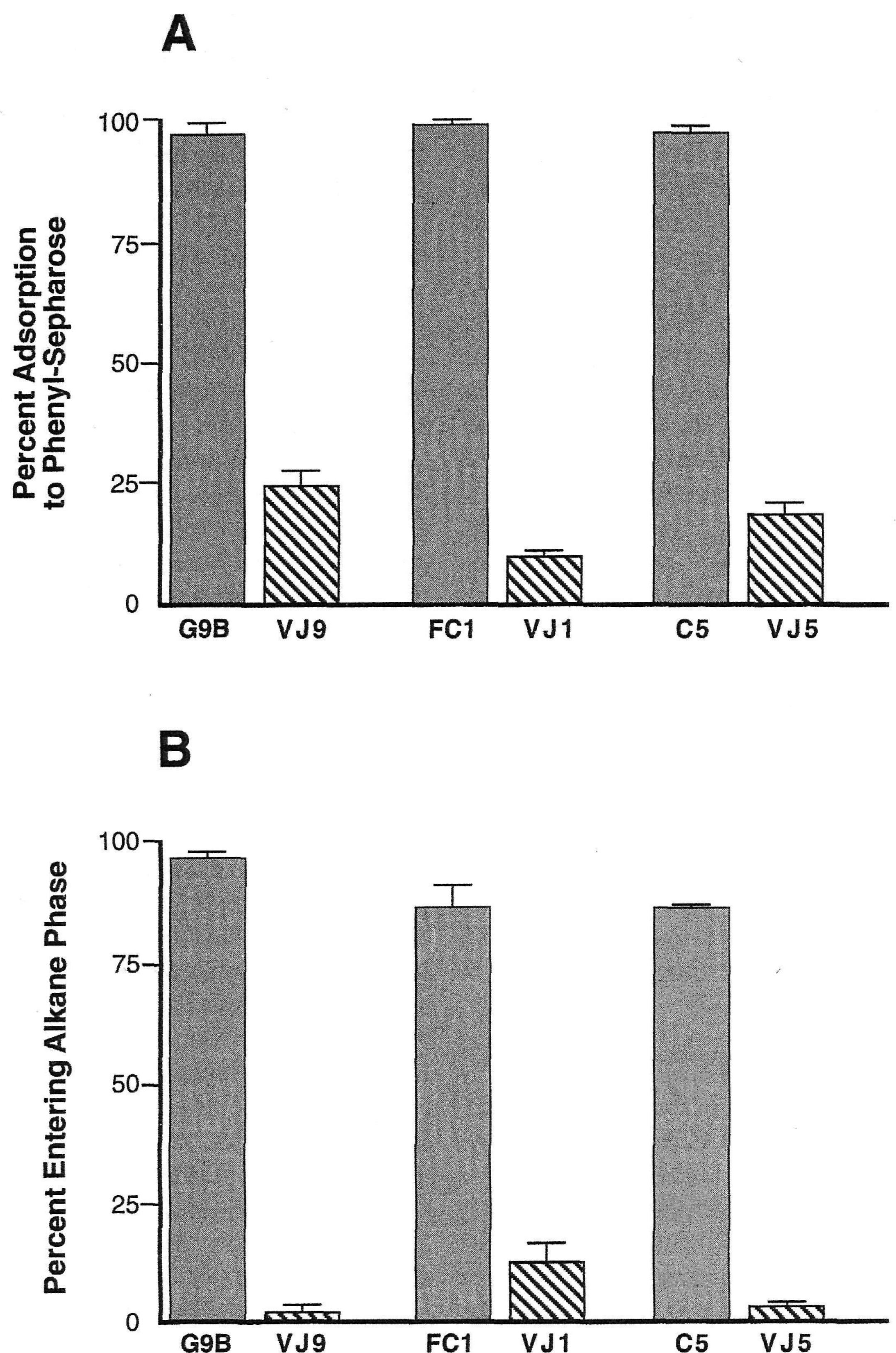

Fig. 2. Hydrophobic properties of parent (stippled) and mutant (hatched) strains. **A.** Percentage of 1×10^9 cells adsorbed to phenyl-Sepharose after 90 min of incubation at room temperature. **B.** Percentage of 1×10^9 cells/ml that bind to hexadecane droplets and enter the alkane phase after vortexing for 2 min. Error bars represent standard deviations.

Table 1. Lipoteichoic acid concentrations of streptococcal cells and culture supernatants

			Lipoteichoic acid (μg)[a]	
Species	Strain	Origin	Cells	Supernatant
S. gordonii	G9B	Parent	9.2±1.3	1.3±0.3
	VJ9	G9B	1.4±0.7*[b]	0.6±0.1**
S. sanguis	FC1	Parent	4.2±1.5	1.2±0.5
	VJ1	FC1	13.7±3.3**	2.8±1.3***
S. oralis	C5	Parent	13.6±1.0	≤0.3[c]
	VJ5	C5	≤0.3	≤0.3

[a] Mean lipoteichoic acid content of bacterial cells recovered from 1 ml of culture and of 1 ml of bacteria-free culture supernatant was assayed by ELISA with rabbit anti-polyglycerol-phosphate antiserum. [b] Lipoteichoic acid concentrations of mutant cells and culture supernatants were compared with those of the respective parent strains in a one-tailed Student's *t*-test: * $P<0.001$; ** $P<0.01$; *** $P<0.06$. [c] None detected by ELISA.

Table 2. Adhesion of parent and mutant strains to human buccal cells

Species	Strain	Adhesion[a]
S. gordonii	G9B	107.1±7.2
	VJ9	6.9±2.2*[b]
S. sanguis	FC1	84.9±25.3
	VJ1	9.0±3.6*
S. oralis	C5	73.3±20.2
	VJ5	3.7±2.2*

[a] Mean number of streptococci per buccal cell (±SEM) as determined microscopically.
[b] * $P \leq 0.005$ for adhesion compared to the respective parent strain by one-tailed Student's *t*-test.

however, showed that the lower concentration of cell-associated lipoteichoic acid of strain VJ9 was probably not caused by the greater release of lipoteichoic acid from the cells into the culture medium. In contrast, the cells of *S. sanguis* strain VJ1 contained significantly more lipoteichoic acid than the cells of its parent strain FC1 ($P \leq 0.01$) and more lipoteichoic acid tended to be released by strain VJ1 into the growth medium. No lipoteichoic acid was detected by this method in the *S. oralis* mutant strain VJ5 nor in spent culture supernatant fluids of the parent strain C5; *S. oralis* cell walls contain ribitol teichoic acids and choline (20), which may have been detected by the anit-lipoteichoic acid antiserum in extracts of the parental strain C5 cells. It was apparent, therefore, that for these strains, the detectable cellular concentrations of lipoteichoic acid were unrelated to the hydrophobic properties of the cell surfaces and that the cellular lipoteichoic acid of the 3 mutant strains were divergent.

Since it has been shown that some oral streptococcal species can occupy more than one niche in the oral cavity and that *S. gordonii* is recovered more often from oral mucosa than the other 2 species (9), a relevant adhesive characteristic to measure was the ability of the bacteria to adhere to oral epithelial surfaces such as those of buccal cells. As can be seen from Table 2, all 3 parent strains attached to buccal cells significantly better than did the mutant strains ($P \leq 0.004$). The differences between parent strains, however, were not significant.

Adhesion to saliva-coated hydroxyapatite and hydroxyapatite beads

Streptococcal cell chain lengths were similar for parent and mutant pairs of strains under the conditions of the adhesion test. All 3 parent strains attached to saliva-coated hydroxyapatite beads. *S. gordonii* strain G9B, however, attached statistically better than *S. sanguis* strain FC1, and strain FC1 attached better than *S. oralis* strain C5 at the non-saturating concentrations of 1×10^8 bacteria/ml ($P \leq 0.001$, Table 3). Affinity constants for the parent strains were between 0.7 and 1.6. The 3 mutant strains were significantly impaired in their ability to attach to saliva-coated hydroxyapatite beads ($P \leq 0.0003$ compared with their respective parent strains, Table 3). Of these, only strain VJ5 had a measurable K_a for saliva-coated hydroxyapatite beads, but this was significantly reduced compared with the K_a of its parent strain C5 (Table 3).

No significant differences in either adhesion to or affinity for hydroxyapatite compared to saliva-coated hydroxyapatite were apparent for strain G9B (Table 3). In contrast, strains FC1 and C5 attached better to hydroxyapatite beads than they did to saliva-coated hydroxyapatite beads ($P \leq 0.001$) and had increased affinities for this surface (Table 3). Selection of mutant strains with reduced ability to attach to saliva-coated hydroxyapatite surfaces also resulted in significant reductions in the adhesion of the 3 mutant strains to hydroxyapatite surfaces ($P \leq 0.0003$ compared with their respective parent strains, Table 3). Attachment of the mutant strains to hydroxyapatite beads, however, was significantly better ($P \leq 0.00003$) than attachment to saliva-coated hydroxyapatite beads, and this was reflected by increased affinity constants for the hydroxyapatite surface (Table 3). Thus, it appeared that the mutant strains suffered defects in specific adhesive mechanisms involved in the attachment to saliva-coated hydroxyapatite. The nature of these defects was not investigated further in the present studies.

Accumulation of growing cultures on hydroxyapatite and saliva-coated hydroxyapatite surfaces

Accumulation was measured and expressed as the percentage accumulation (i.e., the percentage of the total bacteria (unattached and attached) that were attached to the beads) and as the accumulation index (i.e., the percentage of the bacteria attached to the beads in the presence of sucrose divided by the percentage of the bacteria attached to the beads in the absence of sucrose). Since the total cell growth of the parent and mutant strains was similar for medium with or without sucrose (data not shown) and since results were determined for the same phase of growth for all strains, the percentage accumulation reflects the relative numbers of bacterial cells accumulated on the beads. Because the strains differed in their growth rates (data not shown) and in their initial adhesiveness to hydroxyapatite and saliva-

Table 3. Adhesion of nongrowing parent and mutant streptococci to saliva-coated hydroxyapatite and hydroxyapatite beads

		Substratum			
		Saliva-coated hydroxyapatite		Hydroxyapatite	
Species	Strain	Percentage adhesion[a]	K_a[b]	Percentage adhesion	K_a
S. gordonii	G9B	63.7±4.3	1.4±0.4	65.6±8.3	1.3±0.3
	VJ9	0.9±0.1*[c]	-----	14.2±1.3*	0.5±0.2
S. sanguis	FC1	28.8±2.3**	0.7±0.3	54.2±1.2***	4.4±0.4
	VJ1	1.5±0.2*	-----	19.8±2.3*	0.4±0.2
S. oralis	C5	17.7±0.5**	1.6±1.1	30.5±2.8***	4.3±1.5
	VJ5	2.2±0.1*	0.2±0.1	14.7±0.5*	1.7±0.8

[a] Percentage of 1×10^8 washed, ^{3}H-thymidine-labeled bacteria attached to 10 mg of saliva-coated hydroxyapatite or hydroxyapatite beads after 90 min of incubation. [b] Affinity constants (K_a) calculated (1) by regression analyses (R values ≥ 0.95) of plots of number of unattached cells versus number of unattached/number of attached cells present after 90 min of incubation of various concentrations of bacteria (1×10^7 to 6×10^9 bacteria/ml) with 10 mg of saliva-coated hydroxyapatite or hydroxyapatite beads. The regression curves for the attachment of strains VJ9 and VJ1 to saliva-coated hydroxyapatite were non-Langmurian. [c] Comparison by one-tailed Student's *t*-test of the adhesion of: * mutant and parent strain pairs to hydroxyapatite and saliva-coated hydroxyapatite ($P \leq 0.0003$); ** strain FC1 and strain C5 with strain G9B to saliva-coated hydroxyapatite ($P<0.0001$); and *** strain FC1 ($P<0.04$) and strain C5 ($P<0.001$) with strain G9B to hydroxyapatite.

Table 4. Accumulation of growing streptococcal cultures in standard assay on hydroxyapatite beads in the absence and presence of sucrose

Strain	Percentage accumulation[a]		Accumulation index[b]	Bead-associated glucan[c]
	FMC	FMCS		
G9B	43.9	97.8	2.2	1015
VJ9	8.0	95.8	12.0	951
FC1	21.8	49.1	2.3	77
VJ1	5.5	46.0	8.4	97
C5	5.8	11.2	1.9	983
VJ5	1.1	8.7	7.9	195

[a] Percentage accumulation was calculated as the (number of bacteria attached to the beads/total number of bacteria present) ×100 at mid- to late-log growth phase when accumulation approached maximum. Cultures were grown in defined medium (FMC) or FMC supplemented with 1% (wt/vol) sucrose (FMCS). [b] The accumulation index was calculated as the percent accumulation of cultures grown in the presence of sucrose (FMCS)/percentage accumulation of cultures grown in the absence of sucrose (FMC); an index >1.0 indicates the degree to which sucrose promoted accumulation. [c] Water-insoluble bacterial glucan polymer (expressed as μg of glucose equivalents/ml) associated with the beads at mid- to late-log phase of growth, as determined by anthrone reactions.

Table 5. Accumulation on saliva-coated hydroxyapatite and hydroxyapatite beads in the absence and presence of sucrose in the modified accumulation assay

Strain	Percentage accumulation on hydroxyapatite[a]		Accumulation index[b]	Percentage accumulation on saliva-coated hydroxyapatite		Accumulation index
	FMC	FMCS		FMC	FMCS	
G9B	36.4	79.6	2.2	21.3	92.4	4.3
VJ9	23.6	87.0	3.7	8.7	52.2	6.0
FC1	14.3	28.0	2.0	2.3	3.9	1.7
VJ1	4.2	39.5	9.5	2.8	7.3	2.6
C5	15.6	50.0	3.2	15.1	33.0	2.2
VJ5	3.9	24.9	6.4	3.9	18.5	4.7

[a] One-ml cultures in FMC or FMCS medium were transferred during the early to mid log phase of growth to vials containing hydroxyapatite or saliva-coated hydroxyapatite beads and incubation continued for 4 to 6 h. The percentage accumulation was calculated as the percentage of the total number of bacteria present that were attached to the beads at the end of the test. [b] Accumulation index was calculated as the percent accumulation in FMCS/percent accumulation in FMC; an index >1.0 indicates the degree to which sucrose promoted accumulation.

coated hydroxyapatite beads (Table 3), the accumulation index was used to evaluate the relative effects of sucrose on accumulation.

Of the 3 parent strains, sucrose promoted the accumulation of strain G9B the greatest (Table 4). The accumulation indices for hydroxyapatite beads were very similar for all 3 parent strains (Table 4). As expected from the adhesion assay results, all 3 mutant strains attached significantly less than did their parent strains when sucrose was not present. Accordingly, the sucrose-independent accumulation of the adhesion-defective mutant strains was also less than that of the parent strains. In the presence of sucrose, however, the percentage accumulation values of parent and mutant pairs on hydroxyapatite were similar (Table 4). The compensatory effect of sucrose for the lower initial adhesion of the mutant strains was reflected by their markedly increased accumulation indices (Table 4). All 6 strains produced water-insoluble glucan polymer that accumulated on the hydroxyapatite beads (Table 4); however, no correlation existed between the amount of glucan on the beads and the percentage accumulation.

A modified method was employed for measuring accumulation on saliva-coated hydroxyapatite beads (40). Experiments with hydroxyapatite beads were included as controls and for comparisons with the standard assay. As can be seen (Table 5), the percentage accumulation values of the parent and mutant strains and their accumulation indices on hydroxyapatite beads in this modified method were consistent with the results of the standard method (Table 4). Sucrose promoted the accumulation of all 3 parent strains on saliva-coated hydroxyapatite beads, but the accumulation of strain G9B was about 3-fold greater than the accumulation of strain C5 and approximately 20-fold greater than that of strain FC1. Sucrose also promoted the accumulation of the 3 adhesion-defective mutant strains on saliva-coated hydroxyapatite beads. The accumulation of all 3 adhesion-defective mutants was greatly enhanced in the presence of sucrose so that accumulation approached or exceeded that of the parent strain. This was most clearly demonstrated by strain VJ9.

Discussion

Once the initial sites in the salivary pellicle on the tooth surface are occupied, additional bacterial accumulation results from the addition of bacteria derived from the progeny of the surface-attached population and/or bacteria originating from other oral sites. The present studies investigated the hypothesis that one basis for the sequential appearance of oral streptococcal species in dental plaque (9, 19, 27) is that primary colonizing species such as *S. oralis* and *S. sanguis* are more effective at adhesion to salivary pellicle sites, whereas later, secondary colonizers such as *S. gordonii* are more effective at accumulation. Accordingly, the relative abilities of representative strains of each of these species to attach to and accumulate on saliva-coated hydroxyapatite was examined. Unexpectedly, the initial adhesion of nongrowing cells of the two primary colonizing species, *S. sanguis* strain FC1 and *S. oralis* strain C5, to the salivary pellicle was significantly less than the attachment of the later colonizing species *S. gordonii* strain G9B, although the affinity constants of the 3 strains were similar. The accumulation tests used here allow cell surface components and extracellular cell products to play a role in the attachment and growth of bacteria on the bead surfaces (40). Strain G9B also showed the greatest accumulation of growing cells on saliva-coated hydroxyapatite and hydroxyapatite in the absence of sucrose, suggesting that the cell surface properties that influence initial adhesion may also influence sucrose-

independent accumulation in these strains. Moreover, strain G9B was more than 3-fold more effective than strain C5 and 20-fold more effective than strain FC1 at sucrose-dependent accumulation on saliva-coated hydroxyapatite beads. The high degree of sucrose-dependent accumulation for strain G9B is consistent with our previous finding of an effective sucrose-dependent accumulation mechanism in another *S. gordonii* strain, Challis (40), and may indicate that well developed accumulation mechanisms are important for establishment of this species in more mature plaque when the number of pellicle-binding sites becomes limited.

Although sucrose-dependent accumulation was most effective in strain G9B, sucrose also promoted the accumulation of strains FC1 and C5 significantly. However, the mechanisms of accumulation by these 3 species may differ. In *S. gordonii* strains G9B and Challis, sucrose-dependent accumulation has been correlated with the production of water-insoluble glucan polymers (40). The present studies do not indicate that such a straightforward relationship exists in strains FC1 and C5. This indicates that other sucrose-dependent factors may be involved in the accumulation of these strains. These may include interactions of several types; for example, bacterial cell surface components such as glucosyltransferase may interact with water-soluble polymers (14, 33). Sucrose is also known to affect complexes between glucans and the lipoteichoic acid or other cell surface amphipathic molecules (5) and to influence accumulation (42). Finally, carbohydrate-inducible genes such as those described in mutans streptococci that are related to *gtf* expression (15) and carbohydrate metabolism (36) may influence properties involved in accumulation. The nature of the sucrose-dependent accumulation for the oral streptococci in the present studies is intriguing and requires further investigation.

The second hypothesis pursued in this study was that effective accumulation may be more important for oral streptococci under conditions of reduced bacterial adhesiveness or when fewer binding sites are recognized (and hence available) in the salivary pellicle on the tooth surface. To investigate this possibility, mutant strains with decreased abilities to attach to saliva-coated hydroxyapatite flakes were generated. Characterization of the mutant strains demonstrated that all also were reduced in their abilities to attach to buccal cells and to hydroxyapatite beads and that they were more hydrophilic than the parent strains; cell wall lipoteichoic acid concentrations were altered, but such changes could not be directly related to reduced cell surface hydrophobicity or adhesion to saliva-coated hydroxyapatite. However, all mutant strains had glucosyltransferase activities comparable to those of the respective parent strains; thus, the mutants were appropriate tools for studying the possible contributions of glucan polymers and sucrose-dependent accumulation in strains with reduced adhesive abilities. In the absence of sucrose, the mutants of strains G9B and C5 were less effective at accumulation on hydroxyapatite and saliva-coated hydroxyapatite than the parents. Surprisingly, the accumulation of the adhesion-defective mutant of strain FC1 was comparable to that of the parent on the saliva-coated hydroxyapatite substratum, indicating that this strain's adhesive and sucrose-independent accumulation mechanisms on this substratum may be independent. In the presence of sucrose, all 3 mutant strains showed significant sucrose-dependent accumulation, which was reflected by the accumulation indices. This resulted in numbers of mutant attached cells reaching more than 50% of the numbers of parent cells that accumulated on these surfaces. As with the parent strains discussed above, this accumulation was not directly related to the amount of water-insoluble glucan polymers. We have demonstrated previously that 2 strains of *S. gordonii* accumulate in water-insoluble glucan polymers on hydroxyapatite and saliva-coated hydroxyapatite surfaces to concentrations equivalent to those found in dental plaque (40). These results suggest that the physical and chemical properties of the polymers produced from sucrose and their interactions with cell surface components, as well as with the substratum, may be more important in determining the numbers of polymer-associated bacteria attached to a surface than the total concentrations of the polymers.

The results of these studies do not support our hypothesis that the temporal appearance of these streptococcal species is reflected by their ability to attach initially to saliva-coated hydroxyapatite. However, the results do support the hypothesis that reduced initial adhesion to saliva-coated hydroxyapatite may be overcome by sucrose-dependent accumulation, which may provide a colonization advantage for these bacteria. Although the nature of the sucrose-dependent accumulation was not the focus of the present study, these results clearly indicate that sucrose-dependent accumulation mechanisms exist in oral streptococci and that these mechanisms may be independent of those involved in initial bacterial attachment to saliva-coated hydroxyapatite surfaces. Furthermore, the studies with adhesion-defective mutants indicate that sucrose-dependent accumulation may be advantageous at least for these representative strains of oral streptococci when the binding sites in the salivary pellicle are no longer available. Although sucrose-dependent accumulation is classically thought to confer advantages primarily to the mutans streptococci (24), these *in vitro* results suggest that *in vivo*, sucrose-dependent accumulation mechanisms may play a role in plaque development and promote the colonization of the tooth surface by other strains of oral streptococci.

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