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Ecological Implications of Glucosyltransferase Phase Variation in *Streptococcus gordonii*

M. M. VICKERMAN,[1,2] D. B. CLEWELL,[2,3] AND G. W. JONES[2]*

Departments of Cariology and General Dentistry[1] and of Biologic and Materials Sciences,[3] School of Dentistry, and Department of Microbiology and Immunology, School of Medicine,[2] University of Michigan, Ann Arbor, Michigan 48109-0620

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When sucrose is provided as a substrate for glucosyltransferase (GTF), Spp$^+$ cells of the oral bacteria *Streptococcus gordonii* grow embedded in an insoluble glucan mass associated with surfaces. Spp$^-$ phase variants with lower GTF activity, which either arise from or are grown with Spp$^+$ cells, segregate preferentially as unattached cells in the culture supernatants. Conversely, Spp$^+$ revertants preferentially accumulate on surfaces. GTF phase variation, therefore, may facilitate the dispersion of *S. gordonii* cells throughout the oral cavity.

Streptococcus gordonii is found principally on the tooth surface in dental plaque (5, 7) and grows on sucrose agar plates as hard, cohesive colonies (Spp$^+$) (18) as a result of production of glucans (3, 6, 13) via extracellular glucosyltransferase (GTF) (12). Reversible phase variation results in Spp$^-$ variants, which have about 20% of the GTF activity of Spp$^+$ cells and form soft colonies on sucrose agar plates (18). When the cells are grown in sucrose-containing medium, the cells with these two phenotypes show different patterns of accumulation on hydroxyapatite and glass beads (20). Additional accumulation of Spp$^+$ cells is promoted by the production of cohesive, insoluble glucan polymers from sucrose which enmesh the beads and over 90% of the growing bacteria to form a single bacterial polymer mass (BPM) (20). Spp$^-$ variants make significantly less insoluble glucan and show no significant sucrose-promoted accumulation (20). Consequently, the number of Spp$^-$ cells that accumulate on these surfaces is essentially the same in the presence and absence of sucrose and equivalent to that of Spp$^+$ cells growing in the absence of sucrose (20). We have speculated (20) that GTF phase variation may function to conserve bacteria in the body, as has been proposed for phase variation in the antigens of some pathogens (16). The different accumulation patterns of Spp$^+$ and Spp$^-$ cells in the presence of sucrose could cause differential partitioning of Spp$^+$ and Spp$^-$ bacteria between the BPM and the fluid phase in culture and, hence by implication, segregation of the two phenotypes in the oral cavity between the dental plaque and the saliva.

If Spp$^-$ variants arising from Spp$^+$ cells are not physically entrapped in the insoluble polymer formed by Spp$^+$ cells or if Spp$^-$ cells are less able to bind to this polymer, then they should be recovered as unattached cells at frequencies that increase with the number of generations. To test this hypothesis, the parental strain, Challis (Spp$^+$), was grown anaerobically with glass beads (Thomas) in 1-ml volumes of chemically defined FMC (19) and FMC supplemented with 1% (wt/vol) sucrose (FMCS) media as previously described (20). Cells were grown on a rotating drum to mid to late log phase when accumulation approached maximum, ca. 10^9 total bacteria were present and BPMs formed in FMCS cultures (20). The unattached bacteria (ca. 10^7 cells) were collected from the supernatants of FMCS cultures (3,000 × *g* for 5 min), resuspended in 1 ml of fresh medium, and reincubated in new vials containing glass beads until the same final growth phase. This procedure was repeated for three or four passages. An equivalent number of cells from the supernatants of FMC cultures were similarly transferred. At each passage, one FMCS culture and one FMC culture were randomly selected for quantitation. After recovery of the bacteria from the beads by dextranase treatment and sonication (20), the Spp$^+$ and Spp$^-$ cells present in the supernatants and on the beads were enumerated on sucrose agar plates (18). All recovered Spp$^-$ isolates were confirmed by biochemical tests (4) and by colony phenotype on 5% sheep blood Trypticase soy (BBL Microbiology Systems, Cockeysville, Md.) and mitis salivarius (Difco) agar plates as strain Challis. Growth curves, acid production and sensitivity to the recovery procedures of representative Spp$^-$ isolates from each passage were indistinguishable from those of strain Challis, indicating that increased growth rates, aciduricity, or acidogenicity did not favor recovery of either phenotype. GTF activity gel (15) patterns were consistent with those of Spp$^-$ variants described previously (18).

In FMC cultures, the occurrence of Spp$^-$ variants on beads and in supernatants was similar for all passages (ca. 5×10^{-5} [Table 1]). In contrast, in FMCS cultures, greater numbers of Spp$^-$ variants were present in supernatants than on beads and the number of Spp$^-$ variants in supernatants increased with each passage ($P = 0.001$). This suggested that Spp$^-$ variants arising from Spp$^+$ populations were less efficiently sequestered in the BPMs synthesized by Spp$^+$ cells, resulting in their increased recovery as unattached cells.

To test the hypothesis that Spp$^+$ revertants arising from Spp$^-$ cells in FMCS cultures preferentially attach to surfaces, the Spp$^-$ strain CH97 (18) was grown as described above. In this case, however, the beads, to which ca. 10^7 bacteria initially attached, were rinsed and fresh medium was added. The cultures were grown until ca. 10^9 total cells were present, and the beads with attached cells were transferred for three or four passages. Spp$^+$ revertants on the beads and in the supernatants of randomly selected vials were quantitated at each passage, as described above.

In FMCS medium, the number of Spp$^+$ revertants on the beads was higher than in the supernatants and increased with each passage ($P \leq 0.001$ [Table 2]). In contrast, in FMC

* Corresponding author.

TABLE 1. Enrichment of unattached Spp$^-$ variants in the supernatants of Spp$^+$ cultures

Passage[a]	Location[b]	FMCS		FMC	
		Spp$^-$/total[c]	Frequency[d]	Spp$^-$/total	Frequency
1	SN	1/10,599	$\sim 9.4 \times 10^{-5}$	0/28,656	$< 3.0 \times 10^{-5}$
	B	0/48,594	$< 2.1 \times 10^{-5}$	1/57,804	$\sim 1.7 \times 10^{-5}$
2	SN	1/17,744	$\sim 5.6 \times 10^{-5}$	0/67,558	$< 1.5 \times 10^{-5}$
	B	1/47,642	$\sim 2.1 \times 10^{-5}$	0/27,540	$< 3.6 \times 10^{-5}$
3	SN	14/7,474	1.8×10^{-3}	1/21,052	$\sim 4.7 \times 10^{-5}$
	B	8/18,427	4.3×10^{-4}	1/14,459	$\sim 6.9 \times 10^{-5}$

[a] Number of passages of unattached cells in supernatants in a representative experiment (see text).
[b] SN, unattached bacteria in supernatant; B, bacteria attached to beads.
[c] Number of Spp$^-$ colonies/total number of Spp$^+$ and Spp$^-$ colonies examined. Recovery of both phenotypes was ≥95%, as determined by comparison of viable and total microscopic counts (20).
[d] Ratio of number of recovered Spp$^-$ variants to total number of colonies examined on sucrose agar plates. Replicate experiments gave similar results; coefficients of determination (R^2) values calculated by least-squares regression for supernatants were 0.73 to 0.88.

cultures the numbers of Spp$^+$ revertants were similar in supernatants and on beads and occurred at expected frequencies of about 5×10^{-5} for all passages. Spp$^+$ revertants had the same biochemical profiles, growth curves, acid production, and sensitivity to the recovery procedures as strain CH97 did. These experiments suggested that Spp$^+$ revertants preferentially accumulated on surfaces when sucrose was available.

The distribution of phase variants was examined in mixed FMC and FMCS cultures inoculated with both Spp$^+$ and Spp$^-$ strains. If Spp$^-$ cells do not become included in the BPMs formed by Spp$^+$ cells in FMCS, then their distribution between beads and supernatants in FMCS mixed cultures should be similar to their distribution in FMC mixed cultures in which Spp$^+$ cells cannot form BPMs. Approximately 10^5 cells of strain Challis and an equivalent number of Spp$^-$ cells of strain CH97 or CH1C1 were inoculated into FMC and FMCS media with glass beads. Similarly inoculated mixed cultures of strain Challis with the GTF-negative strain CHA1 and pure cultures of all four strains were used as controls. To reduce variations due to potential differences in inoculum sizes, the accumulation index for each strain was calculated as the number of bacteria attached to the beads at mid to late log stage of growth divided by the total number of bacteria of that strain in the culture. Accumulation indices for pure cultures grown in FMCS are usually ≥0.8 and ≤0.2 for Spp$^+$ and Spp$^-$ strains, respectively, whereas in FMC accumulation indices are ≤0.2 for both phenotypes (20). The larger accumulation index of the Spp$^+$ cells in FMCS reflects their sucrose-promoted accumulation. The ratio of the accumulation index of a strain in FMCS to its accumulation index in FMC reflects its sucrose-promoted accumulation. Thus, in mixed cultures containing both Spp$^+$ and Spp$^-$ strains, the ratio of the accumulation index of the Spp$^-$ strain in FMCS to its accumulation index in FMC reflects any effects the Spp$^+$ strain has on the accumulation of the Spp$^-$ strain.

Sucrose-promoted accumulation of strain Challis occurred in the presence or absence of Spp$^-$ cells in FMCS and typical BPMs formed. In mixed cultures, the ratio of the accumulation index of strain Challis in FMCS to the accumulation index in FMC (14.1 [Table 3]) indicates that BPM production by strain Challis significantly increased its own accumulation, consistent with previous results (20). In FMCS, the accumulation index of strain Challis in mixed cultures was the same as that in pure cultures (ratio of 1.0), showing that the presence of Spp$^-$ cells did not modify the accumulation of strain Challis. The accumulation indices of the Spp$^-$ strains CH1C1 and CH97 in FMCS and FMC

TABLE 2. Enrichment of attached Spp$^+$ revertants arising from Spp$^-$ populations

Passage[a]	Location[b]	FMCS		FMC	
		Spp$^+$/total[c]	Frequency[d]	Spp$^+$/total	Frequency
1	SN	2/77,878	2.5×10^{-5}	1/15,709	$\sim 6.4 \times 10^{-5}$
	B	23/73,504	3.1×10^{-4}	2/21,264	9.4×10^{-5}
2	SN	5/18,018	2.7×10^{-4}	1/15,888	$\sim 6.3 \times 10^{-5}$
	B	33/53,952	6.1×10^{-4}	3/38,536	7.8×10^{-5}
3	SN	3/15,203	2.0×10^{-4}	2/58,608	3.4×10^{-5}
	B	20/21,059	9.5×10^{-4}	2/32,536	6.1×10^{-5}
4	SN	7/26,023	2.7×10^{-4}	1/41,232	$\sim 2.4 \times 10^{-5}$
	B	25/25,344	1.0×10^{-3}	1/18,712	$\sim 5.3 \times 10^{-5}$

[a] Number of passages of cells attached to washed beads in a representative experiment (see text).
[b] SN, unattached bacteria in supernatant; B, bacteria attached to beads.
[c] Number of Spp$^+$ colonies/total number of Spp$^+$ and Spp$^-$ colonies examined. Recovery of both phenotypes was ≥95%, as determined by comparison of total microscopic and viable counts.
[d] Ratio of number of Spp$^+$ revertants to total number of colonies examined on sucrose agar plates. Replicate experiments gave similar results; the coefficient of determination (R^2) value calculated by least-square regression was 0.93 for beads.

TABLE 3. Exclusion of Spp$^-$ and GTF-negative bacteria from the insoluble glucan matrix produced by Spp$^+$ cells in mixed cultures

Strain[a]	Phenotype	Accumulation index[b]		Ratio[c]
		FMCS	FMC	
Challis[d]	Spp$^+$	0.99	0.07	14.1
CH1C1	Spp$^-$	0.10	0.20	0.5
CH97	Spp$^-$	0.30	0.28	1.1
CHA1	GTF$^-$	0.10	0.09	1.1

[a] Strain CH1C1 and CH97 are independently derived Spp$^-$ derivatives of the parental strain Challis (18). Strain CHA1 is a spontaneous GTF-negative derivative of strain Challis and does not revert to Spp$^+$ at detectable frequencies; genetic analysis suggests that strain CHA1 is defective in the *gtf* structural gene (17).

[b] Number of bacteria attached to the beads/total number (attached and unattached) of bacteria of the strain in mixed cultures grown in FMCS or FMC medium; mixed cultures were composed of strain CH1C1, CH97, or CHA1 with strain Challis.

[c] Ratio of accumulation index of the strain in FMCS mixed culture to accumulation index of same strain in FMC mixed culture. A ratio approaching 1.0 indicates that the BPMs formed by Spp$^+$ cells in FMCS have no influence on the accumulation of Spp$^-$ or GTF$^-$ cells on the bead surfaces.

[d] Results of mixed cultures of strain Challis with strain CH1C1; similar results (±10%) were obtained with strains CH97 and CHA1.

TABLE 4. Influence of precolonization of surfaces by Spp$^-$ strains on accumulation in the presence of BPMs produced by Spp$^+$ bacteria

Primary strain[a]	Phenotype	Accumulation index[b]		Ratio[c]
		FMCS	FMC	
Challis[d]	Spp$^+$	0.98	0.06	16.3
CH1C1	Spp$^-$	0.11	0.18	0.6
CHA1	GTF$^-$	0.24	0.13	1.8

[a] Strain allowed to colonize beads initially at ca. 10^7 cells; primary strain supernatants were removed, secondary strain bacteria were added, and the cultures then grown to mid to late log phase when ca. 10^9 total (attached and unattached) cells were present. Strain CH1C1 or CHA1 was added to beads precolonized by strain Challis. Strain Challis was added to beads precolonized by strain CH1C1 or CHA1.

[b] Number of cells of the primary strain attached to the beads/total number (attached and unattached) of the primary strain present at mid to late log phase in FMCS or FMC medium.

[c] Ratio of the accumulation index of cultures grown in FMCS to the accumulation index of cultures grown in FMC. A ratio approaching 1.0 indicates no sucrose-promoted accumulation or inclusion in the BPM attached to the beads.

[d] Values shown are for strain Challis as the primary strain with strain CH1C1 as the secondary strain; similar results (±10%) were found with strain CHA1 as the secondary strain.

indicate that the BPM formed by the Spp$^+$ strain Challis did not increase the accumulation of the Spp$^-$ strains (ratios of 0.5 and 1.1 [Table 3]). Total numbers of the Spp$^-$ bacteria recovered from the mixed and pure FMC and FMCS cultures were similar, suggesting that noninclusion of Spp$^-$ cells in the BPM made by strain Challis was not due to interstrain antagonism. The responses of the GTF-negative strain CHA1 in these tests were similar to those of the Spp$^-$ strains (Table 3).

Although Spp$^+$ and Spp$^-$ cells initially attach to glass beads equally well in FMC and FMCS (20), in mixed cultures competition between strains for initial sites of attachment may occur. To reduce the potential influence of this competition, strain CH1C1 or the control strain CHA1 was allowed to precolonize the beads before strain Challis cells were added. Strains CH1C1 and CHA1 (primary strains) were grown in FMC and FMCS to early log phase when the bacteria were approximately equally distributed between the supernatant and bead phases of the cultures and the number of bacteria on the beads was at a subsaturating level of ca. 10^7 cells (20). The supernatants were removed and replaced with strain Challis cultures of the same age which had been grown in the same medium without glass beads (secondary strain). Strain Challis cultures of this age produced no visible polymer in FMCS. In the converse experiment, strains CH1C1 and CHA1 (now secondary strains) were added to beads precolonized by strain Challis (now the primary strain). The cultures were then grown to mid to late log stage. The accumulation index for each strain was calculated as described above.

The ratios of the accumulation indices in FMCS to FMC demonstrated that sucrose-promoted accumulation of the Spp$^+$ strain Challis occurred as expected when it was the primary strain (ratio of 16.3 [Table 4]). Even when strain Challis was added as the secondary strain to beads precolonized with strain CH1C1 or CHA1, an almost eightfold increase in the accumulation index was observed. The accumulation indices of strain CH1C1 (ratio of 0.6 [Table 4]) indicate that there was no significant increase in accumulation of the Spp$^-$ strain when it was the primary strain and strain Challis was added to produce glucans. Again, the responses of the control strain CHA1 were similar to those of the Spp$^-$ strain CH1C1.

The results of the selection and mixed culture experiments are consistent and support the hypothesis that in the presence of sucrose, Spp$^-$ strains are released from the enveloping insoluble glucan mass being actively synthesized by neighboring Spp$^+$ cells. Conversely, Spp$^+$ variants arising from Spp$^-$ cell populations utilize available sucrose and accumulate as bacterial glucan masses attached to the surfaces. An ecological scenario consistent with these findings is based on the suggestion (7) that for bacteria like *S. gordonii*, which colonize the nonshedding surface of the tooth, the production of less-adhesive forms would be ecologically advantageous if the site becomes overpopulated or otherwise unfavorable for growth. These less-adhesive forms could be more readily released to colonize new sites. Dense populations of *S. gordonii* occur within the BPM ($>5 \times 10^{10}$ viable bacteria cm^{-3} [20] equivalent to 10^{11} cells per g [dry weight]), which approach bacterial concentrations in dental plaque (7). If these in vitro findings can be extrapolated to in vivo situations, then it is feasible that a similar release of Spp$^-$ cells from densely populated plaque occurs. The present studies did not address the question of environmentally induced effects on switching. However, this possibility is supported by the finding that the frequency of switching for both the Spp$^+$ strain Challis and the Spp$^-$ strain CH97 grown anaerobically in defined FMC medium was on the order of 10^{-5}, whereas higher frequencies of 10^{-4} to 10^{-3} were found in nutritionally complex medium cultures grown in 5% CO_2 (18). The differential production of insoluble glucans by attached Spp$^+$ and Spp$^-$ cells may reflect changes in gene expression of bacteria associated with surfaces as has been proposed for *Streptococcus mutans* (8) and marine vibrios (1, 2). There is only limited evidence that sucrose promotes colonization in vivo by *S. gordonii* (10). The complex oral environment contains more acidogenic and aciduric organisms, such as mutans streptococci, which have a selective advantage and tend to increase in the presence of sucrose (11). In this potentially adverse environment, the ability of *S. gordonii* cells to utilize the mechanism described here to detach from plaque matrices could be

advantageous and raises the question of whether the plaque environment may influence the frequency of GTF phase variation.

Once Spp^- cells are released from the plaque surface, reattachment is essential to avoid clearance by saliva. The greater ability of Spp^- cells to attach to saliva-coated hydroxyapatite beads (21), compared with the ability of Spp^+ cells, suggests that Spp^- cells, in addition to having lower GTF activity and a greater chance of being released from polymer matrices, might have additional phenotypic changes that confer advantages in colonization of the unsaturated salivary pellicle or equivalent sites. Direct studies of the nature described here on saliva-coated beads are precluded by the lability of adsorbed saliva (20). However, with shorter incubation periods, Spp^+ cells show sucrose-promoted accumulation on saliva-coated hydroxyapatite beads (20), suggesting that similar partitioning of Spp^+ and Spp^- cells could occur in the oral environment. The release of cells with low GTF activity may not be specific for Spp^- variants, because the GTF-negative strain CHA1 behaves similarly.

Although *S. gordonii* was previously classified among the taxonomically diverse *Streptococcus sanguis* (9) and both species appear to be components of early plaque, some recent evidence suggests that *S. gordonii* is more important in mature plaque (5, 14). If the preliminary in vivo findings are confirmed, then accumulation of *S. gordonii* on early and established plaque, which consists of bacterial cells and their products enmeshed in polymers similar to BPMs, may be relevant to the maintenance of these organisms in the oral environment. Specific interactions of *S. gordonii* phase variants with the BPM substratum are being investigated and will be presented in a future paper.

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