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***Streptococcus gordonii*'s Sequenced Strain CH1 Glucosyltransferase Determines Persistent But Not Initial Colonization of Teeth of Rats**

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Abstract

Objective—Extracellular glucan synthesis from sucrose by *Streptococcus gordonii*, a major dental plaque biofilm bacterium, is assumed important for colonization of teeth; but this hypothesis is untested *in vivo*.

Methods—To do so, we studied an isogenic glucosyltransferase (Gtf)-negative mutant (strain AMS12, *gtfG*[−]) of *S. gordonii* sequenced wild type (WT, strain Challis CH1, *gtfG*⁺), comparing their *in vitro* abilities to grow in the presence of glucose and sucrose and, *in vivo*, to colonize and persist on teeth and induce caries in rats. Weanling rats of two breeding colonies, TAN:SPFOM(OM)BR and TAN:SPFOM(OMASF)BR, eating high sucrose diet, were inoculated with either the WT (*gtfG*⁺), its isogenic *gtfG*[−] mutant, or reference strains of *Streptococcus mutans*. Control animals were not inoculated.

Results—*In vitro*, the *gtfG*[−] strain grew at least as rapidly in the presence of sucrose as its WT *gtfG*⁺ progenitor, but formed soft colonies on sucrose agar, consistent with its lack of insoluble glucan synthesis. It also had a higher growth yield due apparently to its inability to channel carbon flow into extracellular glucan. *In vivo*, the *gtfG*[−] mutant initially colonized as did the WT as but, unlike the WT, failed to persist on the teeth as shown over time. By comparison to three *S. mutans* strains, *S. gordonii* WT, despite its comparable ecological success on the teeth, was associated with only modest caries induction. Failure of the *gtfG*[−] mutant to persistently colonize was associated with slight diminution of caries scores by comparison with its *gtfG*⁺ WT.

Conclusions—Initial *S. gordonii* colonization does not depend on Gtf-G synthesis; rather, Gtf-G production determines *S. gordonii*'s ability to persist on the teeth of sucrose-fed rats. *S. gordonii* appears weakly cariogenic by comparison with *S. mutans* reference strains.

Keywords

S. gordonii; glucosyltransferase; colonization persistence; biofilm; plaque; dental caries

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INTRODUCTION

Streptococcus gordonii, a species now officially distinguished taxonomically from *Streptococcus sanguis*,^{1,2} binds α -amylase and is one of the “pioneer” species numerically abundant on teeth of salivary α -amylase-secreting hosts only, including humans and rats.^{3–5} Extracellular glucan synthesis from sucrose by glucosyltransferase (Gtf) has long been associated with the ability of the formerly-termed sanguis streptococci to colonize the teeth.^{6–8} This belief probably grew from findings that had previously established an analogous relationship with regard to the extracellular glucans synthesized by mutans streptococci.^{9–11}

The single *S. gordonii* Gtf enzyme, whose gene is designated *gtfG*¹², makes both α -1,6- and α -1,3-linked extracellular glucans from sucrose,^{13–16} analogous to the glucans produced by the mutans streptococci. Also, the mutans streptococci and *S. gordonii* Gtf enzymes share chemical structure characteristics of sucrose hydrolysis and glucan binding domains. The glucans produced by the *S. gordonii* Gtf and the glucan binding capabilities of the enzyme itself have been postulated to play roles in oral biofilm formation.¹⁷ Although a role of extracellular glucans in surface colonization has been demonstrated *in vitro* based on the ability of glucan-synthesizing *S. gordonii* to accumulate on saliva-coated hydroxyapatite beads in the presence of sucrose,^{18–20} this has never been directly assessed *in vivo*.

Other factors have been demonstrated to influence tooth surface colonization by *S. gordonii*: viz. its colonization of rats is complexly influenced by two cell-surface α -amylase binding proteins in a sucrose-independent but sucrose-augmented fashion.²¹ Still other cell-surface proteins have been implicated in sucrose-independent colonization, based mostly on data from binding to saliva-coated hydroxyapatite,^{22–24} and a very short-term *in vivo* study that could not have discriminated between oral mucosal and tooth surface colonization in mice eating a sucrose-free as-well-as a possibly sucrose-contaminated (molasses) diet, using mutants with defects of cell-surface-associated polypeptides CshA and CshB.²⁵

The present study addresses the role of Gtf-G in colonization of the teeth of rats by use of a well-characterized, sequenced *S. gordonii* strain²⁶ and its isogenic mutant that is deficient in the enzyme Gtf-G that, thus, cannot synthesize extracellular glucan.²⁷ It therefore tests the hypothesis that Gtf-G is seminal to sucrose-associated colonization and persistence in the biofilm on the teeth. It simultaneously seeks to assess whether the production of glucans by *S. gordonii* influences their cariogenicity.

MATERIALS AND METHODS

Bacterial strains and growth studies

S. gordonii strains Challis CH1 and its isogenic mutant AMS12²⁷ were studied. Strain AMS12 was constructed by allelic exchange in which an internal 1.7-kb *HindIII* fragment of the structural gene, *gtfG*, was replaced with a *lacZ*/erythromycin resistance determinant. The resulting gene encodes a truncated ~560-amino acid protein with no Gtf activity.¹²

Strain phenotypes were confirmed qualitatively [API 20 Strep, bioMérieux, Marcy l'Etoile, France] and growth rates were measured in chemically-defined (protein-free) FMC^{21,28} medium to seek any undetected phenotypic changes as well as expected behaviors. Because of the tendency of extracellular glucan-synthesizing bacteria to clump in the presence of sucrose, thus making growth analysis by turbidimetric methods problematic, growth rates were assessed by analysis of bacterial protein.^{29,30} For some *in vivo* studies, comparisons with *Streptococcus mutans* strains BM71,³¹ NCTC-10449S³² and LT11³³ were also made. All cultures were maintained at -70°C before study. After recovery from rats, colonies resembling

inoculants by their phenotypes on appropriate agar media were re-identified by API 20 strips and, furthermore, chromosomal DNA isolated from the recovered strains using standard molecular biology techniques gave similar restriction fragment length profiles; DNA probes designed to anneal to the chromosomal regions flanking *gtfG* hybridized to the expected sized fragments in Southern blot analyses.³⁴

Experimental design, animals, diet, inoculation, recovery of bacteria

Three *in vivo* experiments were done. Their designs are depicted in Figs. 1A, 1B, and 1C. They were approved by the Institutional Animal Care and Use Committee of the fully accredited Center for Laboratory Animal Care, University of Connecticut Health Center. Procedures were slight modifications of those previously detailed.^{32,35} Specifically, weanling rats of two different specific pathogen free Osborne-Mendel (SPFOM) rat colonies, designated TAN:SPFOM(OM)BR and TAN:SPFOM(OMASF)BR, were studied. Importantly, both colonies have an indigenous flora free of α -amylase-binding bacteria, as tested by ¹²⁵I-amylase binding and fluorescent antibody to amylase-binding protein assays, and also free of mutans streptococci, as demonstrated by repeated culturing of the dentition of progeny after provision of a sucrose-rich diet.^{21,36} The TAN:SPFOM(OMASF)BR colony was derived by Caesarean delivery of pups of TAN:SPFOM(OM)BR females; these pups were foster-fed by germ-free rats and subsequently inoculated by the so-called altered Schaedler flora (ASF)³⁷ typical of rodent gut.³⁸ The resultant gnotobiotic breeding colony was expanded in the gnotobiotic facility, then housed in our strict barrier facility and further expanded to enable experiments.³⁷ For those experiments, weanling 21 day old rats of the TAN:SPFOM(OM)BR colony were randomly distributed to groups of equal size, as stipulated for each experiment, and fed diet 2000, containing 56% sucrose^{21,34} and, one day later, inoculated with either CH1 or AMS12. As a positive control *S. mutans* BM71 was used to inoculate an additional group and as a negative control one group was not inoculated (Fig. 1A.). Inocula were of equal doses (~10⁹ CFU), as confirmed by counting of CFU after spiral plating. To be assured that the more complex oral flora of rat colony TAN:SPFOM(OM)BR, by comparison with the TAN:SPFOM(OMASF)BR colony [Tanzer et al., Abstract 2895, 82nd General Session, International Association for Dental Research, Honolulu, 2004] was not interfering with the colonization of the inoculants or the development of carious lesions, two experiments using the latter colony of rats were also done. Again, one group remained un-inoculated. The design of one of them also included simultaneous study of three strains of known virulent *S. mutans*, BM71, 10449S and LT11, as internal controls for colonization and characterization of the comparative intensity of caries expression (Fig. 1B.). In another experiment with the latter rat colony, only CH1 and AMS12 were studied, but the time course of their colonization of the teeth and either emergence or subsidence was more closely characterized (Fig. 1C.). An un-inoculated group served to check for cross contamination.

Recoveries of inoculants in two (Fig. 1A and 1B) of the three experiments were done both at mid-experiment and at the time of euthanasia. At mid experiment, rats' teeth were swabbed for relative quantification of inoculants among the total flora. Immediately after animals were euthanized by anesthetic overdose, the molar teeth of one hemi-mandible were excised *en bloc*, sonified to dislodge and disperse their floras, and cultured as previously detailed.^{35,36} In the short-term, time-course-of-colonization experiment (Fig. 1C), rats were euthanized at 1, 7, 14, or 21 days after inoculation and their molar teeth harvested (as above) and colonization characterized.

Samples were spiral plated onto: Trypticase soy sheep blood agar (BA, for total recoverable flora); mitis salivarius agar (MS, for total streptococci, including possible cross/extraneous contaminants and revertants, and for antibiotic-sensitive *S. gordonii* Challis CH1 and *S. mutans* strains which were identified by their typical colonial morphologies); MS

supplemented with 10 µg/mL erythromycin (MSE, for *S. gordonii* AMS12); MS supplemented with 200 µg/mL streptomycin sulfate (MSS, for *S. mutans* 10449S). Because the colonial morphology on sucrose-supplemented agar of AMS12 is smooth and soft, and not unlike that of *Enterococcus faecalis* on MS agar, samples were also plated on Enterococcal agar [Difco-Becton Dickinson, Sparks, MD, USA] to be certain that misidentifications did not occur. This was especially important because enterococci are commonly resistant to a variety of antibiotics and the best quantification plate for AMS12 was MSE. Additionally, colony forming units (CFUs) that appeared similar to either *E. faecalis* or AMS12 were picked to confirm identities by use of API 20 strips. Recoveries were expressed in both absolute CFU values and as relative values, the percentage of total recoverable CFU on BA that was the inoculant. Data could be expressed only in relative terms from the swab samples of the teeth, due to the variable size of swab samples.

Caries scoring

As previously detailed,³⁶ heads were defleshed by dermestid beetles and assigned random number codes which were entered into the laboratory computer, thereby blinding the caries scorer to the experimental history of specimens. Lesions were scored according to the method of Keyes,³⁹ as modified by Larson.⁴⁰ Only after scoring had been completed and all scores entered into the computer, did the computer decode the random numbers and constitute the experimental groups³⁶, and data were plotted as previously recommended.⁴¹

Statistics

As previously detailed,³⁶ one way ANOVA was used for evaluation of both microbiological and caries data. Differences among groups were isolated by Fisher's LSD method.^{42,43} Microbiological data expressed in percentage terms were arcsine transformed to improve the normalcy of distribution before ANOVA. Caries data as well as absolute microbiological CFU data were statistically managed without transformation.

RESULTS

No phenotypic distinctions were detected between strains CH1 (*gtfG*⁺) and AMS12 (*gtfG*⁻) except as related to extracellular glucan production, as revealed by shiny, adherent, hard, erythromycin-sensitive colonies on MS of the WT by comparison with the shiny, non-adherent, soft and erythromycin-resistant colonies of the mutant on MSE, as previously described.¹² Growth rates (Fig. 2), monitored in vigorously vortexed culture tubes, were the same for strains CH1 and AMS12 in FMC supplemented with either glucose or sucrose. However, when the chemically defined medium was supplemented with sucrose, thus enabling Gtf-G synthesis, growth yield for the *gtfG*⁺ WT cell was lower than for its *gtfG*⁻ mutant AMS12, as previously observed for mutans streptococci.⁴⁴

Un-inoculated animals remained free of both mutans streptococci and *S. gordonii* at the date of euthanasia in all experiments, and there was no evidence of cross-contamination among groups or reversion of mutants to WT pheno- or genotype. Additionally, pheno- and genotypes of *S. gordonii* recovered from rats were confirmed to be identical, as far as could be determined, to the inoculants.

During the *in vivo* studies with TAN:SPFOM(OM)BR animals, CH1 on day 13 was 16.2 ± 4.5 % of the swab-detected total recoverable flora while AMS12 was 0.039 ± 0.02 %; BM71 was 30.5 ± 11.5 % of the swab-detected total recoverable flora. The difference between the CH1 and BM71 recoveries was not significant. During the first experiment with the TAN:SPFOM(OMASF)BR colony, CH1 on day 21 was 16.8 ± 6.70 % of the swab-detected total recoverable flora while AMS12 was 0.19 ± 0.17 % (data not shown). Recoveries for the three mutans

streptococci ranged from 19.7 to 34.7 % for this parameter. The differences for *S. gordonii* CH1 and AMS12 swab recoveries, thus, in mid-experiment for the two experiments were highly significant ($p < 0.001$); but the differences in recoveries for the *S. gordonii* CH1 were not statistically different from that of any of the *S. mutans* strains for the two experiments.

At the date of euthanasia, no detectable AMS12 colonies were recovered from the extracted and sonified three mandibular molars of TAN:SPFOM(OM)BR animals, whereas the recovery of CH1 was $35.4 \pm 8.92 \times 10^7$ CFU, representing 76.8 ± 8.25 % of the total recoverable flora (Fig 3, panels A and B, respectively). Similarly, from the TAN:SPFOM(OMASF)BR rats recoveries of CH1 were $22.4 \pm 4.40 \times 10^7$ CFU, representing 59.1 ± 10.6 % of the total recoverable flora and recoveries of AMS12 were $0.03 \pm 0.02 \times 10^7$ CFU, representing 0.10 ± 0.07 % of the total recoverable flora (Fig 3, panels C and D, respectively). These differences, for both experiments, were highly significant ($p < 0.001$). Thus, the WT colonized the rats' teeth extremely well, but the *gtfG*⁻ mutant did not persistently colonize the rats.

In the latter experiment, three *S. mutans* strains included as internal controls (BM71, 10449S and LT11) colonized the TAN:SPFOM(OMASF)BR rats and were recovered by tooth swabbing at 21.7 ± 7.7 % (BM71), 19.7 ± 9.5 % (10449S) and 34.7 ± 11.6 % (LT11) at day 21 (data not shown). At the time of euthanasia, on day 42, BM71 was $100.7 \pm 9.52 \times 10^7$ CFU/3 mandibular molars, representing 78.2 ± 3.7 % of their flora for BM71-inoculated rats, 10449S was $91.6 \pm 13.4 \times 10^7$ CFU/3 mandibular molars, representing 80.9 ± 8.63 % of their flora, for 10449S-inoculated rats, and LT11 was $138 \pm 9.9 \times 10^7$ CFU/3 mandibular molars, representing 88.7 ± 4.4 % of their flora, for LT11-inoculated rats, respectively (Fig 3, panels C and D). These values for the *S. mutans* strains on both absolute count and percentage recoverable flora bases were statistically about 66.6 to 75.8% higher than those for the *S. gordonii* *gtfG*⁺ WT ($p = 0.019$). Nonetheless, it is clear both the *S. gordonii* WT stain and all of the *S. mutans* strains dominated the tooth surface biofilm flora.

A time-course experiment was done to better characterize when the *gtfG*⁻ mutant declined in the ecology of the teeth. Groups of six rats, either un-inoculated, or inoculated with CH1 or AMS12 were euthanized at 1, 7, 14, and 21 days post-inoculation. Recoveries from sonified erupted molars were not distinguishable on either absolute count (Fig 4A) or relative count bases (Fig. 4B) between the WT *S. gordonii* *gtfG*⁺ (CH1) and its *gtfG*⁻ deficient mutant (AMS12) up to the 7th post-inoculation day. At the 14th day post-inoculation, while the absolute counts of this pair on the teeth were not different, the percentage recovery of the *gtfG*-deficient mutant declined in the microbial community of the tooth ($p < 0.001$). By the 21st day after inoculation, while the *gtfG*⁺ WT was a high percentage of the recoverable flora and in high absolute numbers, the *gtfG*⁻ isogenic mutant essentially no longer persisted in the flora of the biofilm ($p < 0.001$). There were no statistically significant differences in the recovery of the WT among the values on the 7th, 14th and 21st day on either absolute count or relative count bases ($p = 0.814$ and $p = 0.124$, respectively).

Enamel (Keyes E) caries scores for two rat colonies are presented in Fig. 5. In the TAN:SPFOM(OM)BR experiment (Fig 5A and B) total caries (the sum of fissure and smooth surface) scores associated with inoculation by CH1, by comparison with the un-inoculated group, were modestly increased ($p = 0.039$), but not significantly so in association with inoculation by AMS12 ($p = 0.160$). Analysis of the data by tooth surface type revealed increases for fissure surfaces (Fig 5A) were significantly increased, albeit modestly, both by 13 %, by comparison with those of the un-inoculated group ($p < 0.020$). There was no fissure score difference between CH1 and AMS12-inoculated rats ($p = 0.968$). No statistically significant increase of smooth surface caries (Fig 5B) was associated with inoculation by either CH1 or AMS12. Total, fissure, and smooth surface caries scores for the BM71-inoculated group were significantly elevated by comparison with the un-inoculated group by 59.4, 75, and 33%, respectively (all $p < 0.02$).

They were significantly increased by comparison with those for the *S. gordonii* inoculated animal groups only on the smooth surfaces of the teeth ($p < 0.016$). Dentinal penetration scores (Keyes Dm) were very low (data not shown) and not different among the *S. gordonii*-inoculated groups, for any tooth surface category; there was significant carious lesion penetration into dentin in all categories of surfaces for the BM71-inoculated group ($p < 0.001$; data not shown).

In the experiment with the lower background flora colony (TAN:SPFOM(OMASF)BR) (Fig. 5C and D), analysis by tooth surface type showed that both the CH1- and AMS12-inoculated groups had modestly higher scores than the un-inoculated group with regard to fissures Fig 5C ($p = 0.013$ and $p = 0.003$, respectively), smooth surfaces Fig 5D ($p = 0.010$ and $p = 0.030$, respectively), and total enamel caries scores (both, $p < 0.001$ and $p < 0.001$). Differences in caries scores between groups inoculated by CH1 or AMS12 were not significant for total enamel caries scores ($p = 0.650$) or fissure caries scores ($p = 0.546$), but were so for smooth surface scores ($p = 0.031$), with the *gtfG*⁺ strain CH1 having slightly more cariogenicity than its *gtfG*⁻ mutant AMS12.

By additional contrast, caries scores (total, fissure, and sum of smooth surfaces) associated with inoculation by any of the *S. mutans* strains were greatly increased for all categories of enamel surfaces by comparison with either those of the un-inoculated rats or those inoculated by either of the *S. gordonii* strains (all, $p < 0.001$) (Fig 5C and D). Again, dentinal penetration of lesions in animals inoculated by the *S. gordonii* isogenic pair was nil, while dentinal penetration of lesions by all of the *S. mutans* strains was substantial (data not shown) and significantly different (all, $p < 0.001$).

DISCUSSION

S. gordonii is an excellent colonizer of the teeth, consistent with earlier observations in both humans and rats.^{3-5,21,36} While initiation of colonization is not dependent on Gtf-G production, as shown here, persistence of colonization is. Thus, the *gtfG*⁺ genotype of the WT sequenced strain Challis CH1 colonizes strongly and persists as a major component of the bacterial community on the tooth surface. However, results with strain AMS12 indicate that inactivation of *gtfG*, while not compromising initial colonization, profoundly compromises persistence in the plaque biofilm. These phenomena were established with two colonies of rats, both free of competing indigenous mutans streptococci and free of all α -amylase binding bacteria,^{21,36} such as *S. gordonii*.

The equal growth rates of the *gtfG*⁺ and *gtfG*-deficient cells, as monitored by bacterial protein synthesis in glucose- and in sucrose-supplemented protein-free defined medium, with lower growth yield for the *gtfG*⁺ WT, is consistent both with the lack of undetected mutations which could have affected growth rate of the *gtfG*⁻ mutant and its expected failure to divert carbon flow from sucrose to extracellular glucans, by contrast with its progenitor. As previously demonstrated for the mutans streptococci, diversion of sucrose, but not glucose, carbon flow to extracellular glucan product causes lower molar growth yields.⁴⁴ We have no data on *in vivo* growth rates and yields of the WT/isogenic mutant pair, and do not know how to assess them in steady state, but it is highly unlikely that the failure of the mutant to persist in the plaque ecology can be attributed to its inability to grow at a normal rate, based upon the current *in vitro* observations.

Cariogenicity (virulence) of sequenced *S. gordonii* WT CH1 is modest despite its excellent colonization of teeth in sucrose-fed rats, consistent with previous data.^{21,36} In the face of the modest cariogenicity of the WT strain, it is not surprising that only minor loss of cariogenicity can be detected for the *gtfG*⁻ mutant. The relatively minor cariogenicity of *S. gordonii* is dramatically evidenced by simultaneous expression of high virulence of three *S. mutans* strains

in these sucrose-fed rats. It is remarkable, nonetheless, that even transient colonization by *S. gordonii* *gtfG*⁻ AMS12 has demonstrable effects on smooth surface caries increments in these experiments in which that strain had effectively disappeared from the oral flora by 21 days after inoculation, as documented in these 3 separate experiments.

Others have reported that non-mutans streptococci, frequently described with the old term *S. sanguis*, can induce caries in mono-infected gnotobiotic rats which, of course, have no competing flora.^{45–48} Due in part to the appropriate taxonomic reclassification of the oral streptococci,^{1,2} it is rather unclear what species of non-mutans streptococci resembling the *sanguis* streptococci were studied in the past in experimental animals (and humans), especially in reports before 1990. The literature is further difficult to interpret as to the reported failure of caries induction associated with *S. sanguis*-inoculation of conventional test animals, because these animals were not documented to be free of indigenous *S. sanguis*, or even free of indigenous mutans streptococci, which could potentially compete with the establishment of new inocula or express their own virulence. It is additionally of potential confusion to readers that the new taxonomy now uses the term *S. sanguinis*,^{1,2} rather than *S. sanguis*, for one of the formerly-termed *sanguis* streptococci.

No caries induction occurs when rats are colonized by *S. gordonii* compared with un-inoculated control animals fed the same diet if they are fed a powdered starch diet, even though this sucrose-free, powdered high starch diet supports good colonization of the teeth.^{21,36} Clearly, *S. gordonii* can colonize the teeth in the absence of sucrose. This is likely a phenomenon related, at least in part, to multiple cell surface adhesins, including its α -amylase-binding surface proteins, AbpA and AbpB^{49–51} which function *in vivo* in a sucrose-independent but sucrose-augmented fashion.²¹ Other sucrose-independent, major cell wall surface adhesions, such as CshA, CshB, SspA, SspB and Hsa^{22–25} that are hypothesized to play a role in colonization, although not yet characterized *in vivo*, may also be involved. Initial colonization is thus potentially quite complicated. However, from the presently reported experiments with Challis-CH1 and its *gtfG*-deficient mutant, one can conclude that even in the setting of a high sucrose diet, initial colonization is glucan-independent, but persistent colonization is glucan-dependent.

It may be speculated that the deletion of a functional Gtf enzyme in *S. gordonii* can have multiple effects that might alter the potential of this organism to persist in the rat oral cavity. The presence of the 174 kDa surface-associated Gtf may sterically mask or unmask other cell surface adhesins that may affect attachment or detachment of *S. gordonii* or other species on the tooth surface. The glucans synthesized by the enzyme may contribute directly or indirectly to biofilm structure. In addition to its role as a glucan synthesizing and glucan binding protein Gtf also affects sugar metabolism which may in turn lead to pleiotrophic changes in cell properties that affect colonization.

In conclusion, our data on Gtf in colonization of *S. gordonii* do not support its role in initial colonization of teeth but rather do support its role in persistence in the tooth surface biofilm. Studies to further examine the role of Gtf of *S. gordonii* and the quality and quantity of glucans it produces, are in progress.

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Fig. 1.

In vivo experimental designs. Three experiments are depicted. For each, the Calendar of Events is shown on the X-axis. For each, after initial pooling of weaned 21 day old rats, provision of diet and demineralized sterile water, animals were distributed randomly to the groups and inoculated as identified within the bars representing the experimental groups. Swab and euthanasia dates are stipulated. After euthanasia, molars from one hemi-mandible are sonified. Fig. 1A represents the study done with rat colony TAN:SPFOM(OM)BR. There were 8 rats per group. Fig. 1B represents the study done with rat colony TAN:SPFOM(OMASF)BR. There were 10 animals per group. Fig. 1C represents a second study done with rat colony TAN:SPFOM(OMASF)BR designed to delineate the time course of disappearance of the

GtfG⁻ mutant. There were initially 24 animals in each of the three experimental groups. Six rats of each of these groups were euthanized on the days indicated. Other details of the *in vivo* experimental procedures are given in Materials and Methods.

Fig. 2.

Typical semi-logarithmic growth curves of *S. gordonii* CH1 and AMS12. The data for each strain in the presence of 0.06 M carbohydrate are identified by specific symbols. Growth at 37 °C was measured by bacterial protein analysis in the protein free chemically defined medium²⁸. To do the experiment, log phase cultures were inoculated from the same growth media into pre-warmed large flasks, vigorously stirred, and 10 ml aliquots transferred to culture tubes that were incubated and sequestered at the times indicated. After vortex mixing, cultures were filtered through 47 mm, 0.22 µm pore size nitrocellulose acetate discs (GSWP, Millipore, Bedford, MA), quick washed twice with 10 ml ice-cold water and protein in the bacteria trapped on the filters subsequently extracted and analyzed.²⁹

Fig. 3. Colonization of weanling rats' teeth by *S. gordonii* strains CH1 and AMS12, and by *S. mutans* strains BM71, 10449S and LT11, at date of experiment termination. Group inoculation status at the outset of the experiment is indicated on the X-axis. Two experiments are represented: Data for the experiment with the TAN:SPFOM(OM)BR rat colony (n=8/group), which was terminated 34 days post-inoculation, are presented in Fig 3A and 3B. Data for the experiment with the TAN:SPFOM(OMASF)BR rat colony (n=10/group), which was terminated 42 days post-inoculation, are presented in Fig 3C and 3D. Panels A and C present the Mean $\pm$ SEM of absolute count recoveries of the indicated inoculants from 3 mandibular molars at termination. Panels B and D present the Mean $\pm$ SEM for relative count recoveries

of the indicated inoculants as a percentage of total recoverable flora from 3 mandibular molars at termination.

Fig 4. Time course of recovery of *S. gordonii* strains CH1 and AMS12 from sonified erupted molar teeth of TAN:SPFOM(OMASF)BR rats at stipulated dates (X-axis) after inoculation. Panel A gives mean $\pm$ SEM absolute count recoveries of the indicated inoculants, while Panel B gives Mean $\pm$ SEM relative count recoveries of the indicated inoculants as a percentage of total recoverable flora. No *S. gordonii* were recovered from any un-inoculated animal.

Fig. 5.

Bar diagrams of hemi-mandibular enamel caries scores as a function of tooth surface category type, plotted according to the recommendations of Rinehimer and Tanzer.⁴¹ Data for the experiment with the TAN:SPFOM(OM)BR rat colony (n=8/group), which was terminated 34 days post-inoculation, are presented in Panels A and B. Data for the experiment with the TAN:SPFOM(OMASF)BR rat colony (n=10/group), which was terminated 42 days post-inoculation, are presented in Panels C and D. To the left of the midlines, Panels A and C, are plotted fissure (sulcal) scores. To the right of the midlines, Panels B and D, are plotted smooth surface scores, the components of which are bucco-lingual, approximal, and morsal surfaces.^{39,40} Means $\pm$ SEM values are represented for each tooth surface category identified by bar shades. Inoculation status is indicated to the left of the horizontally stacked bars.