Oral Implantation in Humans of *Streptococcus mutans* Strains with Different Degrees of Hydrophobicity

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The more hydrophobic, rough-colony-forming, streptomycin-resistant *Streptococcus mutans* parent strains GW Sm' and LK Sm' and the less hydrophobic, smooth-colony-forming, streptomycin-resistant variant strains GW36 Sm' and LK36 Sm' were implanted in oral cavities. Strains GW Sm' and LK Sm' implanted significantly better than strains GW36 Sm' and LK36 Sm'. The hydrophobicity of and the colony morphology formed by the different *S. mutans* strains did not seem to be affected throughout the experiment.

Previous studies on initial adherence of oral streptococci to hydroxyapatite and tooth surfaces have shown that the selective manner by which surfaces in the oral cavity are colonized depends, at least in part, on the number of binding sites and the binding force between colonizing bacteria and the surfaces involved (7). The nature of these interactions has not been satisfactorily revealed to date. It has been suggested that electrostatic forces are primarily responsible in plaque formation (14). Others (1, 13) have confirmed that such forces are likely to be involved but that other forces are probably important as well (J. Olsson, Ph.D. thesis, University of Göteborg, Sweden, 1978). Recently, a hypothesis on the adherence of *Streptococcus sanguis* to hydroxyapatite was published that took into consideration both electrostatic and hydrophobic interactions as equally important factors in initial bacterial adherence (4, 12).

Recent studies on fresh clinical isolates of plaque bacteria show that oral streptococci are markedly hydrophobic (17, 19). Westergren and Olsson (19) have presented evidence that the adherence of *Streptococcus mutans* to saliva-coated hydroxyapatite is dependent on hydrophobic interactions. Similarly, hydrophobic binding has been shown to participate in initial adherence of *S. sanguis* to saliva-coated hydroxyapatite (8, 11).

The aim of the present study was to compare oral implantation in humans of freshly isolated *S. mutans* strains and variants of these strains, which, after repeated subculture, showed decreased hydrophobicity and ability to adhere in vitro.

**MATERIALS AND METHODS**

*Test subjects.* Twenty-nine adults volunteered for the implantation experiments. They gave fully informed consent to participate in the study, which was approved by the local ethics committee. The subjects were told to maintain their ordinary dietary and oral hygiene habits throughout the study.

*Microorganisms.* The *S. mutans* parent strains GW and LK and the variant strains GW36 and LK36 have been previously described (19). Briefly, strains GW and LK were originally isolated from human teeth and only subcultured twice. Strains GW36 and LK36 are variants of strains GW and LK, respectively, obtained after 36 transfers on blood agar medium. Strains GW36 and LK36 have decreased hydrophobicity and ability to adhere in vitro compared with their parent strains GW and LK. Strains GW and LK form rough colonies on blood agar and mitis salivarius agar (Difco Laboratories, Detroit, Mich.), whereas strains GW36 and LK36 form smooth colonies. Before implantation, strains GW, LK, GW36, and LK36 were made resistant in one step to 200 μg of streptomycin.

*Experimental design.* Streptomycin-resistant strains GW Sm' and GW36 Sm' were each implanted twice in 10 subjects (experiment 1) and once in another 10 subjects (experiment 2). Strains LK Sm' and LK36 Sm' were each implanted once in nine subjects (experiment 3). The implantations were performed on the following days: in experiment 1, days 3 (strain GW Sm'), 21 (strain GW36 Sm'), 39 (strain GW36 Sm'), and 46 (strain GW Sm'); in experiment 2, days 3 (strain GW36 Sm') and 37 (strain GW Sm'); and in experiment 3, days 3 (strain LK36 Sm') and 10 (strain LK Sm'). Before each of the implantations, chlorhexidine treatment was given. The experimental design is shown in Fig. 1. Bacterial samples were collected immediately before the implantations and at various times after the implantations. The samples were collected in experiment 1 on days 1, 2, 5, 9, and 16 after the first implantations of strains GW Sm' and GW36 Sm' and on days 1, 2, and 5 after the second implantations of these strains. Samples were collected on days 1, 2, 5, 9, 16, 22, 25, 29, and 32 after the implantations in experiment 2 and on days 1 and 5 after the implantations in experiment 3.

*Inoculum.* Lyophilized stock cultures of strains GW Sm', LK Sm', GW36 Sm', and LK36 Sm' were grown overnight in streptococcal broth (9). A total of 25 (experiments 1 and 3) and 55 (experiment 2) tubes containing 9 ml of the streptococcal broth were inoculated with 0.5 ml of the overnight culture and incubated at 37°C for 6.5 h (i.e., late log phase). The cells were collected by centrifugation and washed once in 5 ml of saline. The cells were then pooled and suspended in 35 ml of saline. From this bacterial suspension, 1 ml was used for enumeration of the inoculum. The remaining suspension was used for implantation.

*Implantation.* The test subjects rinsed their mouths with 3 ml of the bacterial suspension for 2 min. While the rinse was kept in the mouth, approximal tooth surfaces were flossed with unwaxed dental floss. All implantations were made at the same time of day.

*Chlorhexidine treatments.* Because chlorhexidine has been shown to reduce oral levels of *S. mutans* (6), this agent was used in the present study for suppression of previously

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implanted bacteria to minimize the possibilities for interference of such persistent cells with just-introduced test cells. In an effort to ensure standardized experimental conditions, each implantation was preceded by chlorhexidine treatments. At 48 and 24 h before implantation, the test subjects rinsed their mouths with 10 ml of a 0.2% chlorhexidine solution (Hibigene; ICI, Macclesfield, England) for 2 min and cleaned approximal tooth surfaces with unwaxed dental floss, which was moistened with chlorhexidine.

Bacterial samples. Unstimulated saliva was used for enumeration of streptomycin-resistant *S. mutans* cells. Saliva (1 ml) was serially diluted in 0.05 M phosphate buffer containing 0.4% KCl (pH 7.1) and cultured on mitis salivarius agar with streptomycin (200 μg) by a micropipette method (18). The agar plates were incubated in 5% CO2–95% N2 at 37°C for 48 h. The saliva samples were collected at the same time of day.

Hydrophobicity test. The hydrophobicity of the inocula and of isolates of implanted *S. mutans* was tested by the method described by Rosenberg and co-workers (15). Briefly, this method measures the adherence of bacteria to hexadecane. Bacteria are grown overnight in brain heart infusion broth, washed twice, and suspended in 10 mM potassium phosphate buffer (pH 7.2) to an absorbance of 0.5 at 436 nm. Then, 3 ml of this suspension was agitated on a Whirlimixer together with 100 μl of hexadecane for three successive 1-min periods with 15-min intervals. During agitation, the hexadecane forms small drops which transfer adhering bacteria to the top phase. The absorbance in the suspension 15 min after the third agitation was used as a measure of hydrophobicity. In the present study, bacteria in suspensions having a final absorbance of less than 0.1 were regarded as highly hydrophobic. Bacteria which gave rise to a final absorbance of greater than 0.4 were considered to have low hydrophobicity.

Statistical analyses. The results were analyzed by the Wilcoxon signed-rank test (3), a nonparametric method for comparison of two groups in paired samples. Thus, a comparison was made of implantations of strains GW Sm' and LK Sm' versus strains GW36 Sm' and LK36 Sm', respectively, in paired bacterial samples, i.e., in samples from the same individual and from corresponding sampling occasions after two implantations. The differences in recovered implanted bacteria for each pair were examined, ranked, and signed. The sum of positive or negative signed ranks for all pairs was tested by referring to an appropriate table of critical levels.

RESULTS

At the start of the three experiments, none of the test subjects harbored streptomycin-resistant *S. mutans* strains. Streptomycin-resistant strains GW Sm' and LK Sm' had the same high hydrophobicity (absorbance of less than 0.1) and formed similar rough colonies as streptomycin-sensitive strains GW and LK. Similarly, streptomycin-resistant strains GW36 Sm' and LK36 Sm' had the same low hydrophobicity (absorbance of greater than 0.4) and formed similar smooth colonies as the streptomycin-sensitive strains GW36 and LK36. The inocula of the different *S. mutans* strains contained the following numbers of CFU: in experiment 1, 7.2 x 10^8 (GW Sm'), 2.1 x 10^8 (GW Sm'), 0.9 x 10^8 (GW36 Sm'), and 0.9 x 10^8 (GW Sm'); in experiment 2, 0.7 x 10^8 (GW36 Sm') and 1.1 x 10^10 (GW Sm'); and in experiment 3, 1.2 x 10^5 (LK36 Sm') and 0.2 x 10^5 (LK Sm').

The more hydrophobic parent strain GW Sm' implanted better than its less hydrophobic variant strain GW36 Sm'. In experiment 1, the median value of streptomycin-resistant *S. mutans* in the test subjects was, on almost all sampling occasions, about 10-fold greater after implantation of strain GW Sm' than after implantation of strain GW36 Sm' (Fig. 2). When the Wilcoxon signed-rank test was used for comparison of each of the implantations of strain GW Sm' versus those of strain GW36 Sm', it was found that the recovery of implanted bacteria was significantly greater (*P < 0.01*) on all five sampling occasions after the first implantation of strain GW Sm' and on two of three sampling occasions after the second implantation of this strain than on corresponding sampling occasions after the implantations of strain GW36 Sm'. In experiment 2, a similar pattern was found. The median value of streptomycin-resistant *S. mutans* in the test
subjects was greater after implantation of strain GW Sm' than after implantation of strain GW36 Sm' on all sampling occasions (Fig. 3). The difference in recovered implanted bacteria between these implantations was statistically significant on all but the first sampling occasion. The more hydrophobic parent strain also implanted better than its less hydrophobic variant in experiment 3. In this experiment, the median values of recovered streptomycin-resistant S. mutans in the test subjects were, on the two sampling occasions, 3- and 45-fold greater after the implantation of strain LK Sm' than after the implantation of strain LK36 Sm' (Table 1). When the Wilcoxon signed-rank test was used, it was found that, on the second sampling occasion, the difference in recovered implanted bacteria between the implantations of strains LK Sm' and LK36 Sm' was statistically significant (P < 0.01).

Immediately before introduction of the test strains, low levels of streptomycin-resistant S. mutans (<100 CFU/ml of saliva) persisted in three subjects before the second implantation and in five subjects before the fourth implantation in experiment 1. No such bacteria were found before any of the other implantations in any of the experiments. After the second implantation in experiment 1, when the smooth-colony-forming strain GW36 Sm' had been implanted, streptomycin-resistant S. mutans which formed rough colonies was also detected. On the last sampling occasion after this implantation, the streptomycin-resistant S. mutans strain that formed rough colonies even predominated over those that formed smooth colonies. After all other implantations, only streptomycin-resistant S. mutans which formed colonies with a morphology corresponding to that of the most recently introduced test strain was recovered. Thus, in experiment 2, in which GW36 Sm' was the first strain to be implanted and implantations were followed for up to 32 days, all recovered streptomycin-resistant S. mutans isolates formed smooth colonies after the implantation of strain GW36 Sm' and rough colonies after the implantation of strain GW Sm'. When the hydrophobicity of representative isolates of these colonies was tested on each sampling occasion, it was found that the low hydrophobicity of strain GW36 Sm' and the high hydrophobicity of strain GW Sm' remained the same after implantation as before implantation. In all experiments, when testing the hydrophobicity of inocula and recovered implanted bacteria, the values of the absorbancies were in most cases less than 0.05 or greater than 0.45 and were never between 0.1 and 0.4.

**DISCUSSION**

The more hydrophobic, rough-colony-forming, streptomycin-resistant S. mutans parent strains GW Sm' and LK Sm' implanted significantly better than the less hydrophobic, smooth-colony-forming variant strains GW36 Sm' and LK36 Sm', respectively. This seemed to be the case regardless of the order in which the strains were implanted in the oral cavity. The hydrophobicity of and the colony morphology formed by the different S. mutans strains were not affected throughout the experiment.

![Graph](image)

**TABLE 1. Streptomycin-resistant S. mutans in saliva in nine subjects 1 and 5 days after implantations of strains LK36 Sm' and LK Sm' (experiment 3)**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Implanted S. mutans (CFU x 10^2 per ml of saliva)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
</tr>
<tr>
<td>Day 1</td>
<td></td>
</tr>
<tr>
<td>LK36 Sm'</td>
<td>0.5</td>
</tr>
<tr>
<td>LK Sm'</td>
<td>1.8</td>
</tr>
<tr>
<td>Day 5</td>
<td></td>
</tr>
<tr>
<td>LK36 Sm'</td>
<td>0.04</td>
</tr>
<tr>
<td>LK Sm'</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*ND, Not detected, i.e., <20 CFU/ml of saliva.

Values differ significantly (P < 0.01 according to Wilcoxon signed-rank test) from corresponding values obtained with strain LK36 Sm'.
lished in the mouth. However, it seems unlikely that persisting streptomycin-resistant S. mutans could have been of any significance concerning competition for the same binding sites, as it were found in only low numbers and only before two of eight implantations, one with strain GW36 Sm³ and one with strain GW Sm³.

It is worth noting that the less hydrophobic, smooth-colony-forming strain GW36 Sm³ could persist in several of the test subjects for as long as 32 days. The parental strain of GW36 Sm³, strain GW36, is probably a mutant of the original strain GW, which has been subsequently favored by selective nutritional pressure on blood agar (19). Accordingly, one could expect that a back mutation would occur, with a subsequent selection of such mutants by nutritional pressure in the mouth after implantation of strain GW36 Sm³. However, as was previously mentioned, no revertants were found after implantation of strain GW36 Sm³. Given ideal conditions, S. mutans has a mean generation time of 40 min (5). For microorganisms colonizing smooth tooth surfaces in vivo, a mean generation time of 3 to 4 h has been calculated (16). In vivo growth of S. mutans in fissures has been estimated to have a mean generation time of 6 to 14 h (10). The slower growth rate in vivo of S. mutans is likely to result in a slower selection for mutants. Furthermore, our use of streptomycin-resistant strains could have been of significance for the selection of mutants. Studies in rats by Bammann and co-workers (2) indicate that streptomycin-resistant S. mutans strains colonize teeth less efficiently than the wild type. These investigators assumed that the streptomycin-resistant strains of S. mutans, since they adhered as well as the wild type, had impaired colonization properties because of altered growth rate. Thus, a slower growth rate in vivo and a slower growth rate due to the streptomycin-resistant labeling of strain GW36 might be one reason that no revertants appeared during the 32 days after implantation of strain GW36 Sm³.

It has previously been shown that strains GW36 and LK36 have impaired ability to adhere to saliva-coated hydroxyapatite compared with strains GW and LK (19). The streptomycin-resistant strains tested in the present study had the same hydrophobicity as their streptomycin-sensitive parent strains GW, LK, GW36, and LK36. Therefore, it is reasonable to assume that the relatively low level of implantation in the human oral cavity of the variant strains GW36 Sm³ and LK36 Sm³ could be due to an impaired ability of these strains to adhere to the tooth surfaces.

Little is known about the molecular basis for hydrophobic interactions between oral bacteria and salivary pellicles. Recent studies by Nesbitt et al. (11) and Gibbons et al. (8) indicate that adherence of S. sanguis to saliva-coated hydroxyapatite is strongly related to the bacterial surface hydrophobicity and that surface fibrils are primarily responsible for both surface hydrophobicity and adherence specificity (8). The basis for the differences in implantation observed in our study, using strains of S. mutans with different hydrophobicity, is not well understood. The postulated hydrophobic interactions could be either specific, e.g., lectin-like, or nonspecific, e.g., involving lipoteichoic acid. Preliminary results from our laboratory reveal that the hydrophobic component of strains GW and LK is protein in nature and is not lipoteichoic acid. Furthermore, both parent and variant strains are devoid of surface fibrils and have the same appearance under scanning electron microscopy. It might be argued that the impaired implantation of strains GW36 Sm³ and LK36 Sm³ could be due to a loss of surface components other than those associated with surface hydrophobicity or to a change of properties other than the ability to adhere, e.g., a decreased growth rate in vivo. However, the fact that both pairs of strains behaved similarly, having a different surface hydrophobicity as the common denominator, tends to minimize these possibilities.

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LITERATURE CITED


