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Role of sucrose in the fitness of *Streptococcus mutans*

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Introduction: Dental caries has been closely linked to fermentable carbohydrates as key environmental factors. Sucrose has been identified as the most cariogenic carbohydrate. *Streptococcus mutans*, considered to be the primary pathogen causing dental caries, is able to utilize sucrose as a nutrient source, partially for the production of intracellular storage components and for the production of extracellular glucans via the glucosyltransferases GtfB, GtfC, and GtfD. The following study explores the competitiveness and fitness of *S. mutans* when grown with different concentrations of sucrose.
Methods: Growth competition with oral streptococci and antimicrobial susceptibility in static biofilm models grown without sucrose or with 0.1% or 0.5% sucrose were investigated using confocal laser scanning microscopy. The numbers of surviving *S. mutans* of both wild-type and an isogenic Gtf-negative mutant after antimicrobial treatment were determined as colony-forming units.
Results: *S. mutans* was able to establish microcolonies with increasing sucrose concentration in the presence of other streptococcal competitors during biofilm development. The antimicrobial susceptibility decreased when sucrose was available as substrate and was dependent on the presence of the Gtfs.
Conclusion: The increased resistance against antimicrobial treatment was associated with the availability of sucrose, but was not influenced much by the concentration used during this study. The resistance was strongly associated with the Gtf activity, excluding any intracellular metabolic effect of sucrose in the resistance mechanism.

Key words: antibiotic resistance; biofilm; *Streptococcus mutans*; sucrose

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Dental plaque is one of the best characterized microbial biofilms and its ecological balance (bacterial homeostasis) is crucial to maintaining a healthy, caries-free status and may protect the host from invasion by potentially disease-causing bacteria (17). *Streptococcus mutans* is a common member of the healthy dental biofilm but under certain circumstances it can increase in numbers, initiating demineralization, which eventually leads to tooth decay. The ability of *S. mutans* to increase in proportion and suppress the growth of or displace other members of the dental plaque is attributed to the expression of cell-density-dependent virulence properties, e.g. natural competence and bacteriocin production, as well as to its acidogenic and aciduric abilities (14, 16). However, most of the identified virulence factors have not been linked directly to the cariogenic potential of *S. mutans* under *in vivo* conditions. A well-characterized, clinically relevant factor in caries development is the ability of *S. mutans* to metabolize sucrose. Sucrose is considered the most cariogenic dietary carbohydrate because it can function as fermentable disaccharide and serve as a substrate for intracellular polysaccharide synthesis. More importantly, sucrose is the substrate for glucosyltransferase (Gtf)-mediated, sucrose-dependent glucan production, which promotes the adhesion of *S. mutans* to the tooth surface. These glucans are synthesized from sucrose by the enzymatic action of three types of Gtf: GtfB and GtfC synthesize mainly water-insoluble glucans (>85%) with α(1–3) glucosidic bonds (mutan); GtfD forms water-soluble glucans (>70%) with α(1–6) glucosidic bonds (dextran) (20). The inactivation of the *gtf* genes in *S. mutans* leads to diminished virulence in rodent models, proving the *in vivo* importance of this virulence property (33). Gtfs are common among the oral streptococci that are able to produce glucan polymers from sucrose (2), but only the mutans streptococci, like *S. mutans* or *S. downei*, seem to be able to synthesize glucans that promote strong adhesion (4,

13). However, there are limited reports available regarding how different sucrose concentrations may influence the fitness and ability of *S. mutans* to become dominant in the dental plaque. Since biofilm formation has been attributed to an increase in resistance towards antimicrobials and a more protective mode of growth (6), we were interested in further investigating the sucrose-concentration-dependent biofilm formation of *S. mutans*. In this study, we present data on the ability of *S. mutans* to compete against other members of the oral streptococci and cope with antimicrobial stress when cultured in the presence of different concentrations of sucrose.

Materials and methods

Bacterial strains and media

The bacterial strains used during the study are listed in Table 1. All strains were routinely grown in brain–heart infusion (BHI) broth (Difco, Sparks, MD) or on BHI agar plates at 37°C anaerobically (90% N_2/5% CO_2/5% H_2). Filter-sterilized sucrose was supplemented when indicated.

Biofilm growth and confocal laser scanning microscopy (CLSM)

For single-species biofilm growth, *S. mutans* was grown overnight in BHI, and diluted 1 : 100 into BHI (final cell density approximately 10^6 colony-forming units). For mixed biofilms, *S. mutans*, *S. oralis*, *S. mitis*, *S. sobrinus*, and *S. gordonii* were grown separately in BHI overnight, subsequently adjusted to an absorbance at 600 nm (A_{600}) of 1.0 and mixed in a 1 : 100 dilution in BHI. Sucrose was added as indicated, compensated with different concentrations of glucose to equalize the overall content of carbon source. The cell suspensions were inoculated into the Lab-Tek®II Chamber Slide™ System (Nalge Nunc International, Naperville, IL), where the objective slide was replaced with a thin cover slide for proper CLSM. Biofilms were typically grown as a static culture for 16 h at 37°C anaerobically. The biofilm cells were stained with CellTracker™ Orange CMTMR [5-(and-6)-(((4-chloromethyl)-benzoyl) amino)tetramethylrhodamine] or the LIVE/DEAD Bacterial Viability kit, which visualizes the integrity of bacterial plasma membranes, according to the manufacturer's instructions (Molecular Probes, Eugene, OR). CLSM was performed using LSM 5 PASCAL with LSM 5 PASCAL software (Carl Zeiss, Jena, Germany). The microscope was equipped with detectors and filter sets for monitoring red fluorescence [excitation wavelength: 540–580 nm (560 CWL), dichroic mirror wavelength: 595 nm (LP), barrier wavelength: 600–660 nm (630 CWL)] and green fluorescence [excitation wavelength: 465–495 nm (480 CWL), dichroic mirror wavelength: 505 nm (LP), barrier wavelength: 515–550 nm (535 CWL)]. Images were obtained with a 40 × 1.4 Plan-Neofluar oil objective (Carl Zeiss, Jena, Germany).

Antimicrobial treatment of *S. mutans* biofilms grown with different concentrations of sucrose

An overnight culture of either *S. mutans* strain UA140 (wild-type) or strain UA140 *BCD* in BHI was diluted 1 : 100 in BHI 0.5% glucose, BHI 0.4% glucose plus 0.1% sucrose, or BHI 0.5% sucrose. Aliquots of 1 ml were transferred to 1.5-ml incubation tubes (in triplicate) and incubated overnight at 37°C anaerobically. The next day the cells were centrifuged at 16,000 ***g*** for 1.5 min in a tabletop centrifuge and the medium was completely removed with a pipette. Subsequently, 500 µl of either Listerine® Cool Mint (Pfizer, Morris Plains, NJ), containing the essential oils thymol 0.064%, eucalyptol 0.092%, methyl salicylate 0.060%, and menthol 0.042% as active ingredients, or PerioGard® (Colgate, Piscataway, NJ), containing 0.12% chlorhexidine gluconate, was added to two of the 1.5-ml incubation tubes. The cells were vortexed to resuspend planktonic cells and were further incubated for 30 s. As a control, 500 µl phosphate-buffered saline (PBS) was added to one tube. After 30 s the cells were centrifuged for 16,000 ***g*** for 1.5 min and washed twice with BHI to remove any residual antimicrobial agent. All cells were sonicated to break up the biofilms as well as cell-chains (this was controlled by visual inspection under the microscope), serially diluted, and plated on BHI agar plates. For CLSM imaging, the biofilms were grown as described above; the medium was replaced with 500 µl Listerine for 30 s, followed by a wash step with BHI and subsequent fluorescent staining before CLSM microscopy.

Table 1. Bacterial strains

Strain	Relevant characteristics	Reference
UA140	*S. mutans*; wild-type	(24)
JK2gfp	UA140::Φ(*ldh$_p$-gfp*)	(19)
UA140 BCD	UA140, GtfBCD$^-$	(7)
S. oralis	Wild-type	ATCC 10557
S. mitis	Wild-type	ATCC 33399
S. sobrinus	OMZ 176; wild-type	B. Guggenheim and (21)
S. gordonii	DL1; wild-type	(22)

Bacterial enumeration and statistics

Bacteria were counted after serial dilution in PBS (from 10^{-3} to 10^{-7}). Three dilutions were plated in duplicate on BHI agar plates. Bacterial counts were normalized to the PBS control to calculate the percentage of survivors. The results were expressed as the percentage of survivors of the antimicrobial-agent-treated cells relative to the PBS-treated control cells. All experiments were repeated three times. Descriptive statistics, including the mean and standard deviation, were calculated. Statistical analysis of data was performed using QUICKCALCS online calculators (http://www.graphpad.com/quickcalcs/index.cfm) using the t-TEST software to compare the means of two groups. Data were considered significantly different if the two-tailed *P* value was ≤0.05.

Results

Sucrose-dependent establishment of *S. mutans* microcolonies in multispecies biofilms

Sucrose-dependent biofilm formation of *S. mutans* involves the synthesis of glucan-polymers by coordinated action of the glucosyltransferases GtfB, GtfC, and GtfD, thus enabling *S. mutans* to adhere firmly to surfaces. The concentration of sucrose in the growth medium has an influence on the thickness and stability of single-species *S. mutans* biofilms (13). We were interested in determining if the concentration of sucrose can influence the composition of a multispecies biofilm containing *S. mutans* as well as other oral streptococci. We analyzed the growth of *S. mutans* in submerged static biofilms in the presence of mitis, mutans, and sanguinis group members, comprising (see Table 1) in part the so-called early colonizers of dental plaque. Their similar nutritional requirements mean that they are considered as direct competitors of *S. mutans*. The inoculation took place at a 1 : 1 ratio at the same time because this had been demonstrated earlier to provide a situation in which all strains could grow and no inhibition by bacteriocins and H_2O_2 production would occur (15).

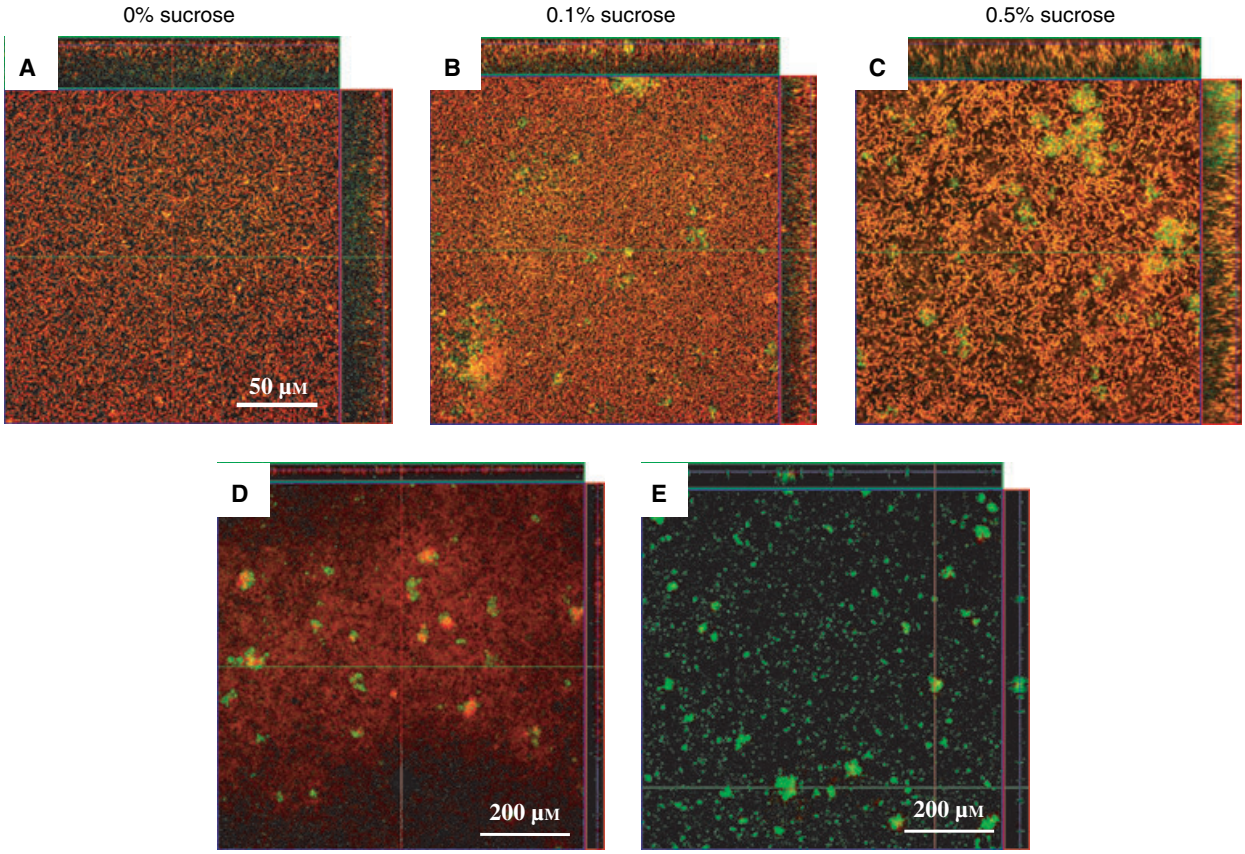

Fig. 1. CLSM images of mixed streptococcal biofilms (*Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus sobrinus*, *Streptococcus gordonii* shown by red fluorescence and *S. mutans* strain JK2gfp by green fluorescence; 1 : 1 ratio) grown in the presence of different sucrose concentrations: (A) 0% sucrose/0.5% glucose, (B) 0.1% sucrose/0.4% glucose, (C) 0.5% sucrose/0% glucose. Pictures were taken at an overall magnification of ×400. (C) and (D) show a mixed biofilm grown with 0.5% sucrose before and after washing the biofilms on a rotary shaker for 2 min with PBS. Pictures were taken with an overall magnification of ×100. Cells were stained with CellTracker™ Orange CMTMR and grown anerobically overnight at 37°C.

We used strain JK2gfp, a green fluorescent UA140 strain expressing the fluorescent marker *gfp*, to distinguish *S. mutans* and stained all the streptococci with CellTracker™ Orange. As shown in Fig. 1A–C, the mixed streptococcal community was able to build a biofilm on the glass surface. Figure 1B,C demonstrate that the presence of sucrose in the growth medium enables *S. mutans* to form microcolonies at both sucrose concentrations (0.1% and 0.5%). These microcolonies were not visible in biofilms grown without sucrose, where only single cell-chains of *S. mutans* were detectable by careful inspection. These single cells might well be the original inoculum and not from growth. Interestingly, washing the biofilms on a rotary shaker for 2 min with PBS before microscopic re-examination revealed decreased numbers of oral streptococci, but *S. mutans* microcolonies were still attached (see Fig. 1C,D) when grown with 0.1% and 0.5% sucrose. These observations indicate that *S. mutans* has a better potential to establish itself in a competitive biofilm if sucrose is available.

Antimicrobial susceptibility of single-species *S. mutans* biofilms grown with different concentrations of sucrose

Our previous work demonstrated that single-species biofilms of *S. mutans* grown with sucrose withstand shear-stress better with increasing concentrations of the carbohydrate (13). Combined with the results from the multispecies biofilms, these data suggest that *S. mutans* has an advantage when sucrose is available for glucan synthesis compared to growth situations were no sucrose is present. Since the growth of bacteria in biofilms generally leads to a more resistant phenotype against antimicrobial agents (6), we tested the susceptibility to Listerine and chlorhexidine of *S. mutans* biofilms grown with different concentrations of sucrose compared to planktonic cells grown without sucrose. Both mouth-rinses are widely used to reduce the amount of bacteria in the dental plaque and therefore are of clinical relevance (1, 18). The percentage of survivors of strain UA140 after Listerine treatment is presented in Fig. 2A. Cells grown as planktonic cultures without sucrose were most susceptible; the percentage of survivors was only 0.0029%. When grown in 0.1% sucrose the percentage increased in a statistically significant manner to 0.029% (a 10-fold increase) ($P \leq 0.05$). However, with 0.5% sucrose the percentage of survivors was increased further to 0.08% (2.7-fold); this further increase was not statistically significant. In addition, we tested the susceptibility of UA140 against chlorhexidine (Fig. 2B). Compared to the Listerine treatment, the cells were 7×10^3 times more resistant when tested under growth conditions without sucrose, with a percentage of survivors of 20%. Addition of sucrose at both concentrations (0.1% and 0.5%) increased the resistance against chlorhexidine about three-fold, to 87% and 75%, respectively. The difference between no sucrose and sucrose was statistically significant ($P \leq 0.05$). However,

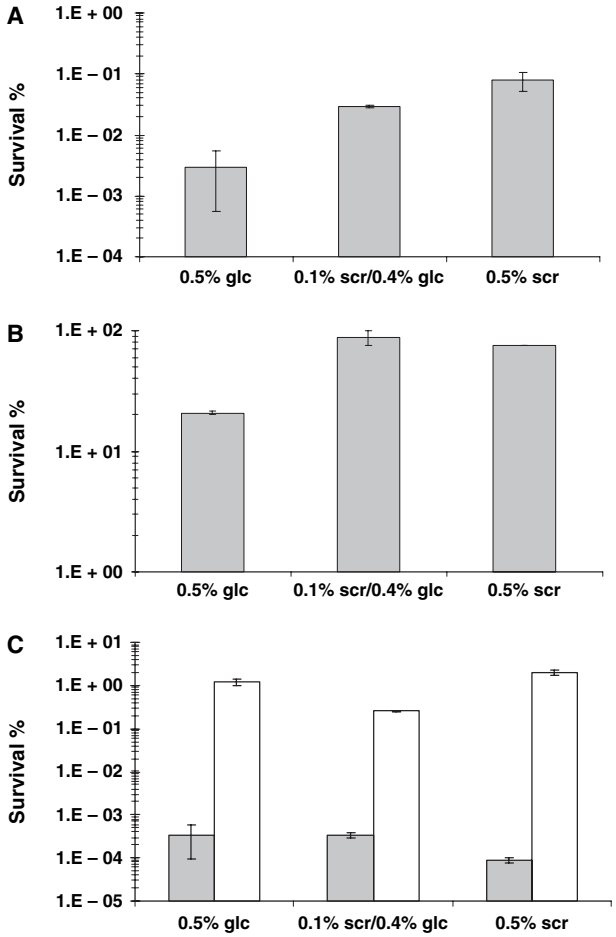

Fig. 2. Susceptibility of *Streptococcus mutans* wild-type and Gtf⁻ mutant grown as planktonic cells or static biofilm with different sucrose concentrations and treated with Listerine and chlorhexidine. (A) Percentage of survivors of UA140 after Listerine treatment, grown as planktonic culture without sucrose (compensated for carbohydrate content with 0.5% glucose) and biofilm cultures with sucrose (0.1% sucrose/0.4% glucose and 0.5% sucrose). (B) Percentage of survivors of UA140 after chlorhexidine treatment, grown as planktonic culture without sucrose (compensated for carbohydrate content with 0.5% glucose) and biofilm cultures with sucrose (0.1% sucrose/0.4% glucose and 0.5% sucrose). (C) Susceptibility of a UA140 Gtf⁻ mutant grown as static cultures with different sucrose concentrations treated with Listerine (gray bars) and chlorhexidine (white bars). The percentage of survivors as mean and standard deviation (see Materials and methods for details) are presented.

consistent with the data obtained with Listerine, there was no statistically significant difference between 0.1% and 0.5% sucrose.

The susceptibility of UA140 to Listerine was also tested with biofilms grown on glass surfaces with 0.1% and 0.5% sucrose, because growth without sucrose did not give a stable biofilm for staining with the LIVE/DEAD Bacterial Viability staining under these conditions (Fig. 3). The amount of red fluorescent cells before treatment with Listerine was comparably low in the biofilms grown with both concentrations of sucrose. After staining with the LIVE/DEAD Bacterial Viability fluorescent dyes, the number of red fluorescent cells increased dramatically, indicating a compromised cell membrane (Fig. 3). In both biofilms, areas were visible that still had bacteria showing a strong green fluorescence, and it seemed that 0.5% sucrose led to better protection against Listerine. Since the LIVE/DEAD Bacterial Viability fluorescent dye propidium iodide is only able to cross compromised membranes, it was not clear if the red bacteria were dead or whether their membrane was weakened, and the cells might have been able to recover from the treatment. We therefore consider the plating data for enumeration as more reliable (see Fig. 2).

Taken together, these results display the protective advantage to *S. mutans* cells grown with sucrose, enabling the formation of a biofilm structure.

Antimicrobial susceptibility of *S. mutans* Gtf mutants

The glucosyltransferases GtfB, GtfC, and GtfD synthesize the water-soluble and water-insoluble glucans that promote attachment and adhesion of *S. mutans* to the tooth surface (28, 31). We were interested in determining if the antimicrobial susceptibility towards Listerine and chlorhexidine was influenced mainly through the presence of glucans and the formation of a biofilm structure, or if the metabolic availability of sucrose could render the cells resistant. We conducted the same susceptibility test with strain UA140 GtfBCD⁻, which is negative for GtfB, GtfC, and GtfD, growing with no sucrose, 0.1% sucrose, and 0.5% sucrose. As presented in Fig. 2C, the mutant strain was equally susceptible to Listerine and chlorhexidine when grown with and without sucrose. However, UA140 GtfBCD⁻ seemed to be more resistant to chlorhexidine, confirming the results obtained with the wild-type UA140. Of particular interest was the difference observed between wild-type cells and the GtfBCD⁻ mutant cells treated with Listerine or chlorhexidine when grown as planktonic cultures without sucrose. In both cases the wild-type strain showed an eight-fold (Listerine; 0.0029% vs. 0.00033%) and a 16-fold (chlorhexidine, 20% vs. 1.19%) higher resistance against the antimicrobial agent than the mutant strain. Although only statistically significant for the chlorhexidine-treated cells ($P \leq 0.05$), this suggests a protective role of the Gtf enzymes against the tested antimicrobials.

Discussion

The objective of this study was to determine how the availability of sucrose influences the competitiveness and fitness of *S. mutans* in a biofilm. Although the role of sucrose in the cariogenic process has been well established (25, 26), the influence of different sucrose concentrations on the biofilm formation abilities of *S. mutans* has only recently been investigated using a more quantitative and qualitative approach (13). Sucrose clearly provides an advantage to *S. mutans* in a competitive situation with other selected oral streptococci (Fig. 1) compared to growth without sucrose. It seems that *S. mutans* failed to grow in the competitive situation tested when no sucrose was present, because we cannot exclude the possibility that the sparse green fluorescent bacteria were simply the cells used for

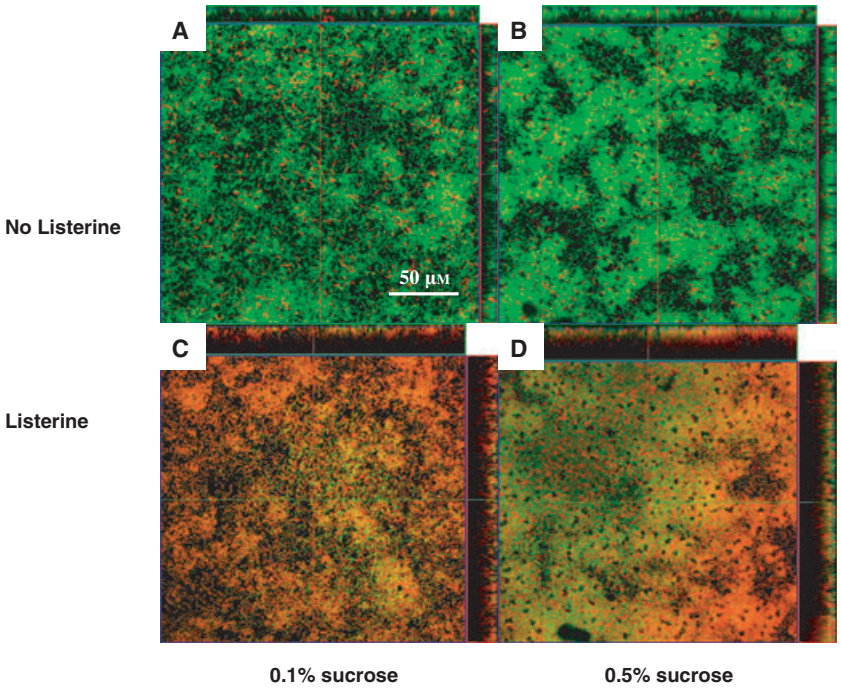

Fig. 3. CLSM images of *Streptococcus mutans* biofilms before and after Listerine treatment. The cells were stained using the LIVE/DEAD Bacterial Viability kit: green fluorescence indicates viable cells; red fluorescence indicates cells with compromised membranes. Pictures were taken with an overall magnification of ×400. (A) No Listerine, 0.1% sucrose/0.4% glucose; (B) no Listerine, 0.5% sucrose/0% glucose; (C) plus Listerine, 0.1% sucrose/0.4% glucose; (D) plus Listerine, 0.5% sucrose/0% glucose.

inoculation. During the biofilm formation process, *S. mutans* was able to grow in clusters, building up microcolonies when sucrose was available. These microcolonies were not composed of *S. mutans* alone, but were mingled with other streptococci, when grown with both concentrations of sucrose (0.1% and 0.5%). Pratten and Wilson reported that the composition of dental plaque had a greater proportion of streptococci when grown with sucrose in a constant-depth biofilm fermenter (23). In addition, high-frequency carbohydrate consumption increases the proportions of mutans streptococci and lactobacilli while decreasing the number of competing streptococci like *S. sanguinis* in dental plaque (29). Our results support that sucrose increases the competitiveness of *S. mutans* in a mixed species environment. During the process of sucrose-dependent biofilm formation in this mixed species model, glucans could be produced by all streptococci. It was shown that the Gtf enzymes from commensal streptococci contribute to the overall production of glucans in the dental plaque (30). This could lead to co-adhesion to glucans produced by other species, which might explain the microcolonies observed during biofilm development, which consisted not only of *S. mutans* but also of a few other streptococci. However, it is very obvious that the microcolony development was restricted to *S. mutans* because no other dense cluster-like structures were observed.

The ability to develop a biofilm has been attributed to an increase in resistance against antimicrobial agents. This is an important aspect regarding the occurrence of multidrug-resistance bacteria. Although oral bacteria are not causing any life-threatening diseases, the oral biofilm could be a reservoir for bacterial species acquiring, developing, and transferring new resistance mechanisms when challenged with a sub-lethal concentration of antimicrobials. It has been shown that *S. mutans* grown in a biofilm is more competent to take up DNA (16). In addition, under a high-density cell environment, as is commonly found in biofilms, the production of the bacteriocin mutacin IV can lead to lysis of competing streptococcal species, thus releasing heterologous DNA for consecutive uptake by *S. mutans* or possibly other oral streptococci (14). Genetic exchange has also been reported between *S. gordonii* and *Treponema denticola* (32). Hence, the interspecies genome–genome interactions (12) might have a deeper impact on resistance mechanisms, even if just one species develops a specific resistance. Therefore, it is important to understand the role of sucrose-dependent biofilm development by *S. mutans* and the susceptibility to antimicrobial treatment. The ability to withstand shear-forces was markedly increased when *S. mutans* grew in the presence of sucrose as a static or dynamic (flow cell) biofilm culture. Interestingly, the relationship of increased sucrose concentration and biofilm adhesion strength was not linear, indicating a threshold around 0.5% sucrose (13). The results from the present study demonstrated a similar outcome regarding the antimicrobial susceptibility. There was a significant increase in the number of surviving bacteria when *S. mutans* was grown with sucrose compared to growth without sucrose, but there was no significant difference between the two sucrose concentrations used. A possible explanation could be that the biofilm formation reaches a steady state after a certain amount of glucan polymer is built up. *S. mutans* encodes *dexA* for the glucan-degrading enzyme DexA (dextranase) (5). This enzyme seems to have a function in the glucan-turnover process because a mutant was more adherent, indicating an increased presence of glucans, but further studies are not available. In addition, individual cells of *S. mutans* grown with 1% sucrose as probed with atomic force microscopy after 6 h and 12 h of growth showed a similar stickiness (8).

The difference in susceptibility towards the tested antimicrobials, Listerine and chlorhexidine, was also described earlier with *S. gordonii* in a saliva-conditioned, flow-cell system (9). Chlorhexidine was described as being bacteriostatic *in vivo* (3) and one of the characteristics of chlorhexidine is that it can bind to soft and hard tissues in the mouth and might prevent colonization by bacteria (1), but it seems to be not as effective against already existing streptococcal and oral biofilms (9, 23). The mechanism of antimicrobial action of Listerine or essential oils, as the main ingredient in Listerine, is not well characterized. However, Listerine was most effective in decreasing the number of bacteria after 30 s of treatment. We also tested another strain of *S. mutans*, GS5 (10), with a similar result, excluding strain-specific susceptibility towards Listerine (data not shown). The LIVE/DEAD Bacterial Viability fluorescent kit was used to confirm the plating data. Planktonic cells of *S. mutans* appeared all fluorescent red, indicating a compromised membrane. However,

growth as a biofilm with either 0.1% sucrose or 0.5% sucrose increased the proportion of green cells, which indicates viability. Although the experiments were performed with monospecies biofilms and with planktonic cells as control, they might still be applicable to the situation in dental plaque, because the antimicrobial potential of Listerine on dental plaque has been demonstrated in several studies – reviewed in ref. (27). Recently it has been described that a dual-species biofilm of *S. mutans* and *Veillonella parvula* has a percentage of survivors after antimicrobial treatment that is several log values higher compared to the single-species biofilms (11). This clearly demonstrates that the composition of the biofilm has an impact on the percentage of survivors and that mutual strategies have evolved to cope with stress situations. The ability to survive antimicrobial treatment in a biofilm has been attributed to several potential mechanisms like limited diffusion, adaptive stress response, physiological heterogeneity, and the presence of phenotypic variants. We could see a much higher susceptibility towards both antimicrobials in the Gtf$^-$ mutants. The resistance seemed to be linked to the glucan production, since there was no difference in percentage of survivors when the mutants were grown with or without sucrose. Therefore, the availability of sucrose for metabolic purposes did not confer more resistance. The colony morphology of the surviving bacteria did not show a significant difference in the appearance, which excluded obvious phenotypic variances. Further studies need to address if the survivors are more resistant towards a re-treatment and if there is a limited diffusion of Listerine or chlorhexidine in biofilms. An interesting finding is that the Gtf$^-$ mutants were more susceptible when grown without sucrose. There might be some protection given by the presence of the protein. Thus the role of the Gtf enzymes in the cariogenicity of *S. mutans* might exceed just the simple role of glucans synthesis.

In conclusion, we demonstrated that sucrose-dependent biofilm formation of *S. mutans* gave this oral streptococcus an advantage in resistance towards antimicrobials and promoted enhanced competitiveness in a mixed species biofilm. Both attributes help to better understand the mechanisms by which *S. mutans* can become predominant in the dental plaque, and eventually lead to the development of carious lesions of the enamel tooth surface.

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