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# Influence of Growth Mode and Sucrose on Susceptibility of *Streptococcus sanguis* to Amine Fluorides and Amine Fluoride-Inorganic Fluoride Combinations

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**This study evaluated the susceptibility to amine fluorides (AmFs) of planktonic and biofilm cultures of *Streptococcus sanguis* grown with and without sucrose. Cultures were incubated with AmFs (250 mg of fluoride liter$^{-1}$) for 1 min. The susceptibility of biofilms was less than that of the planktonic form and was further decreased by growth in the presence of sucrose.**

Caries, one of the most prevalent infectious diseases of humans, is preceded by accumulation of bacterial plaque, an oral biofilm on the tooth surface. Bacteria in biofilms have been demonstrated to be less susceptible to antimicrobial agents than their planktonic counterparts (5, 19). In addition, sugars are known to alter the surface properties of bacteria (11) and can also lead to the production of environment-modifying secondary metabolites. All of these factors may alter the susceptibility of the organism to antimicrobial agents. The purpose of this study was to determine the susceptibility of *Streptococcus sanguis*, a primary colonizer of dental enamel, to amine fluorides and inorganic fluorides, which are anticaries agents (3, 9, 10, 13, 16, 17). The effects of sucrose and growth mode on susceptibility were investigated independently and together to determine the relative importance of each factor. Combinations of amine fluoride with inorganic fluorides were investigated for possible additive antimicrobial activity (12). *S. sanguis* NCTC 10904 was used in all experiments. In most experiments, the nutrient supply consisted of a mucin-containing medium (MCM) containing the following (per liter): proteose peptone, 5.0 g (Oxoid, Basingstoke, England); hog gastric mucin, 2.5 g (Sigma, Poole, England); yeast extract, 2.0 g (Oxoid); Lab Lemco powder, 1.0 g (Oxoid); sodium chloride, 0.35 g (BDH, Poole, England); calcium chloride, 0.2 g (BDH); potassium chloride, 0.2 g (BDH); and urea, 0.5 g (Sigma).

Planktonic cultures were grown aerobically by continuous culture at 37°C. Bacteria were grown either in MCM or in MCM supplemented with 36 g of sucrose liter$^{-1}$ at a flow rate of 0.03 liter h$^{-1}$ (dilution rate = 0.06 h$^{-1}$). The achievement of the steady state was determined by plate counts on tryptone soy agar (TSA) (Oxoid).

Biofilm-derived suspended cells were obtained from the effluent of a glass-packed tube (GPT) device. The physiological state of such cells would be more like that of bacteria in saliva, which are mostly derived from biofilms on oral surfaces, than that of cells grown by traditional planktonic culture methods. The device consisted of a 280-cm length of silicone rubber tubing filled with 710- to 1,180-μm-diameter glass beads (Sigma) inoculated with an overnight nutrient broth (Oxoid) culture of *S. sanguis*. Following a 4-h static attachment phase, MCM was pumped through the device at a flow rate of 0.03 liter h$^{-1}$ (dilution rate = 2.31 h$^{-1}$). Effluent steady-state CFU concentration was determined as for chemostat cultures.

Biofilms, growths formed by microorganisms existing on the tooth surface, were grown in a constant-depth film fermentor (CDFF) as described by Wilson et al. (18). Hydroxyapatite discs (5-mm diameter) (U.S. Bio-interfaces Inc., San Diego, Calif.), recessed to a depth of 300 μm, were used as the substratum for biofilm growth; bacteria grown on these were designated CDFF biofilms. The CDFF was operated aerobically at 37°C, the turntable speed was 3 rpm, and MCM was delivered to the CDFF at a flow rate of 0.03 liter h$^{-1}$. For biofilms grown in MCM plus sucrose, a 10% solution of sucrose was pulsed into the CDFF three times a day for 33-min periods at a flow rate of 0.6 liter h$^{-1}$. To determine steady state, CDFF biofilms were suspended in 1 ml of phosphate-buffered saline, pH 7.3 (PBS) (Oxoid), and plate counts of the resulting suspensions were performed on TSA.

Three amine fluorides obtained from GABA International, Therwil, Switzerland, were used in the study: Olaflur, $C_{27}H_{60}F_2N_2O_3$; Oleaflur, $C_{22}H_{45}FNO_2$; and Steraflur, $C_{22}H_{47}FNO_2$. Other than the amine fluoride, preparations contained (per liter) 2 g of polyethylene glycol-hydrogenated castor oil (GABA International), 50 g of ethanol (BDH), 0.25 g of potassium acesulfame (Hoechst, Frankfurt, Germany), and 25 g of xylitol (Sigma). This solution and deionized water were used as negative controls. Table 1 specifies the concentrations of amine and inorganic fluorides used in the experiments.

For GPT effluent and chemostat cultures, 3.0 ml of agent was incubated with $2.4 \times 10^7$ CFU for 1 min at room temperature. This corresponds to a mean fluoride/CFU ratio of $1.6 \times 10^{-5}$ mol of fluoride per $1 \times 10^7$ CFU of *S. sanguis*. The agents and cells were vortexed to mix in the last second of the incubation, and then 100 μl was immediately transferred to 900 μl of neutralizing solution (Difco, Detroit, Mich.). The suspension was serially diluted in PBS and plated on TSA. A pan containing five CDFF biofilms grown in MCM (mean total viable count = $2.4 \times 10^7 \pm 0.9 \times 10^7$ CFU) was aseptically removed from the CDFF and placed in a sterile tube. Agent (3.0 ml) was then carefully added to the tube. After 1 min at room temperature, the pan was removed from the agent and each separate biofilm was suspended in 1.0 ml of sterile PBS

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TABLE 1. Amine fluoride and inorganic fluoride concentrations in solutions used for antimicrobial activity testing

| Active ingredients of solutions[a] | Concn of amine (mg liter$^{-1}$) | Amt of amine (mol) | Concn of inorganic cation (mg liter$^{-1}$) | Amt of inorganic cation (mol) |
|---|---|---|---|---|
| Olaflur ($C_{27}H_{60}F_2N_2O_3$) | 3,250 | 0.0071 | | |
| Oleaflur ($C_{22}H_{45}FNO_2$) | 4,671 | 0.013 | | |
| Steraflur ($C_{22}H_{47}FNO_2$) | 4,697 | 0.013 | | |
| Olaflur-tin(II) fluoride | 1,625 | 0.0035 | 390 | 0.0033 |
| Olaflur-zinc fluoride | 1,625 | 0.0035 | 215 | 0.0033 |
| Zinc fluoride | | | 430 | 0.0066 |

[a] All solutions had a fluoride concentration of 250 mg liter$^{-1}$.

and vortexed for 1 min. The suspension was serially diluted in PBS and plated on TSA. For biofilms grown in MCM plus sucrose, a pan containing five biofilms (mean total CFU count = $4.6 \times 10^7 \pm 3.0 \times 10^7$) was aseptically removed from the CDFF and placed in a sterile tube. Test solution (5.7 ml) was carefully added to the tube (to achieve the same CFU-to-fluoride ratio used with MCM-grown CDFF biofilms). After 1 min at room temperature, the pan was removed from the agent and each separate biofilm was suspended in 1.0 ml of sterile PBS containing 0.1 g of 710- to 1,180-μm-diameter glass beads (Sigma) and vortexed for 5 min. The resultant suspension was serially diluted in PBS and plated on TSA.

pH was determined prior to antimicrobial testing by using Whatman type CS pH strips (pH 3.8 to 5.5 and 5.2 to 6.8) and a Shindengen (Camlab) pH boy-p2 pH meter for the chemostat and GPT cultures. Only the pH meter was used for determining biofilm pH. All pH measurements were made at room temperature.

In order to determine whether the amine fluoride with the greatest charge-to-mass ratio, Olaflur, could bind to the exopolymer (EPS) produced by the organism, cell-free preparations of the exopolymer were used. A stationary-phase *S. sanguis* culture (5 ml) in MCM was filter sterilized into 15 ml of 10% sucrose in MCM salts (0.83 g of NaCl, 0.2 g of $CaCl_2$, and 0.2 g of KCl per liter) and incubated at 37°C for 5 days. The resulting suspension was centrifuged at 3,500 rpm (15 min in a Centaur 2 centrifuge [Fisons]), and the pellet was resuspended in 25 ml of MCM salts; this step was repeated five times. The last pellet was resuspended in 10 ml of MCM salts. Olaflur (1 ml) (250 mg of fluoride liter$^{-1}$) or the negative control solution (as described above) was added either to 1 ml of MCM salts or to EPS made up to a 1-ml volume with MCM salts. After vortexing, the suspensions were filter sterilized to remove EPS and the absorbance was measured at 270 nm (the amine absorbance peak, determined by scanning spectrophotometry) by using the negative control plus MCM salts (50:50) to zero the spectrophotometer.

The specific growth rate (CFU formed per CFU per hour) for chemostat cultures grown in MCM was 0.06 h$^{-1}$ ($n = 4$). The mean specific growth rate for the GPT culture was 0.012 h$^{-1}$ ($n = 4$), which was fivefold lower than that of the chemostat culture. Biofilms of *S. sanguis* attached to the glass beads in the GPT were observed by microscopy. Bacteria in the fluid phase (pH 5.9 ± 0.2) existed as dense clumps, short chains, and single cells rather than the long chains associated with planktonic cultures. Chemostat cultures grown in MCM plus sucrose (pH 5.4 ± 0.2) contained aggregates of cells and exopolymeric material that were not observed in chemostat cultures grown solely in MCM (pH 6.0 ± 0.2). CDFF biofilms of *S. sanguis* grown in MCM consisted of distinct microcolonies of various sizes. The mean viable count per MCM-grown biofilm at steady state was $4.8 \times 10^6 \pm 1.9 \times 10^6$ CFU. CDFF biofilms of *S. sanguis* grown in MCM plus sucrose (mean viable count per biofilm at steady state: $9.2 \times 10^6 \pm 15.4 \times 10^6$ CFU, pH 5.6 ± 0.2) covered a far greater proportion of the substratum and consisted of stacks of microcolonies surrounded by extensive extracellular material.

Zinc fluoride solution and basal medium did not significantly reduce (with respect to the deionized-water control group) the viability of *S. sanguis* cultures, regardless of growth mode or the addition of sucrose to the growth media (Table 2). Cultures treated with the Olaflur-tin fluoride combination were significantly killed in chemostat and GPT cultures grown in MCM but not in chemostat cultures grown in MCM plus sucrose or CDFF biofilms grown in MCM. *S. sanguis* was significantly susceptible to Olaflur, Oleaflur, and Steraflur (250 mg of fluoride liter$^{-1}$) under all culturing conditions except as CDFF biofilms grown in MCM plus sucrose. CDFF biofilms grown in MCM were less susceptible than planktonic cultures grown in MCM, as were planktonic cultures grown in MCM plus sucrose. The order of activity of the amine fluorides against MCM-grown CDFF biofilms was Olaflur > Oleaflur > Steraflur.

The addition of cell-free preparations of *S. sanguis* exopolymer to Olaflur had no effect on the concentration of amine ions remaining in solution. Hence, there was no reduction in the concentration of amine left in solution following the addition of up to 7 mg (dry weight) of exopolymer to the solution of Olaflur (3.25 mg of amine).

The aim of this investigation was to determine the importance of growth mode and the presence of sucrose in growth media, independently and together, on the susceptibility of *S. sanguis* to amine fluorides and amine fluorides and inorganic fluoride combinations. CDFF biofilms were less susceptible than planktonic cultures grown in the same medium. Cultures grown in MCM plus sucrose were less susceptible to amine fluorides than cultures grown in MCM in the same growth mode. The decreases in susceptibility caused by biofilm growth and growth in sucrose-containing media were additive. The double-charged amine cation from the Olaflur preparation was more effective than the singly charged amine cations, as has been previously demonstrated with other cationic species (1). Inorganic fluoride compounds did not increase the sensitivity of *S. sanguis* to amine fluoride solutions.

Several explanations have been proposed for the differences in susceptibility of biofilms and planktonic cultures. Bacterial growth rate has previously been demonstrated to affect the susceptibility of bacteria to antimicrobial compounds (6, 20). GPT cultures grown in MCM had a mean growth rate approximately fivefold lower than that of chemostat cultures grown in the same medium. Both cultures were killed by a 1-min exposure, suggesting that the amine fluoride susceptibility of the organism is not greatly affected by phenotypic changes due either to a reduced growth rate or to being derived from biofilms.

Another factor to consider is pH. Cationic antimicrobial agents are thought to exhibit greater activity at alkaline pH (1). This agrees with the observation that cultures grown in MCM were more susceptible than cultures grown in MCM plus sucrose, which had a lower pH. It is notable, however, that CDFF biofilms grown in MCM plus sucrose were less susceptible than their planktonic counterparts, indicating that factors other than pH are involved in decreased susceptibility of the CDFF biofilms.

CDFF biofilms and planktonic cultures grown in MCM plus sucrose contained high concentrations of exopolymer compared to the MCM-grown cultures and had reduced susceptibility to amine fluorides. Ionic binding of cationic antimicrobial agents to polyanionic exopolymers (4, 8) and chemical bonding

between agent and exopolymer (2, 14) have been previously reported to protect cells within biofilms and aggregates. However, cell-free preparations of the exopolymer did not bind Olaflur, the double-charged amine fluoride, suggesting that ionic and chemical interactions between the exopolymer and amine fluoride are unlikely to account for the reduced susceptibility of *S. sanguis* biofilms to amine fluorides.

Diffusion limitation (4) has been linked with reduced susceptibility of biofilm bacteria, particularly when short treatment times are used (7, 15), due to the extensive matrix surrounding the cells. CDFF biofilms of *S. sanguis* grown in MCM plus sucrose had a greater amount of matrix per cell than CDFF biofilms grown in MCM and were also less susceptible to amine fluorides. It is debatable whether diffusion limitation alone is sufficient to account for this reduced susceptibility, considering the low molecular weight of the amine ions; however, the matrix may exclude the amine fluorides by other mechanisms—e.g., electrostatic repulsion or hydrophobic interactions. In conclusion, the results of this study have shown that biofilms similar in physiological state to those which exist on the tooth surface in vivo were less susceptible to amine fluorides than planktonic cultures and suspended cells originating from biofilms. Growth in sucrose decreased the susceptibility of biofilms to amine fluorides further. The decreased susceptibility of biofilms to these agents could not be accounted for by altered growth rate, pH, or binding of the agent to exopolymer and so remains to be determined.

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TABLE 2. Susceptibility of *S. sanguis* to amine fluorides and amine fluoride-inorganic fluoride combinations

| Organism group and form[a] | Presence of sucrose in medium | Mean CFU recovered after incubation with[b]: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Deionized water | Olaflur-tin(II) fluoride | Olaflur-zinc fluoride | Zinc fluoride | Olaflur | Oleaflur | Steraflur | Basal medium |
| A, planktonic | No | $(4.8 \pm 0.1) \times 10^6$ | 0 | 0 | $(4.6 \pm 1.3) \times 10^6$ | 0 | 0 | 0 | $(5.0 \pm 0.3) \times 10^6$ |
| B, biofilm derived | No | $(1.4 \pm 0.1) \times 10^7$ | 0 | 0 | $(8.7 \pm 1.8) \times 10^6$ | 0 | 0 | 0 | $(7.3 \pm 1.5) \times 10^6$ |
| C, intact biofilm | No | $(3.5 \pm 2.1) \times 10^7$ | $(2.7 \pm 1.3) \times 10^6$ | $(1.9 \pm 1.1) \times 10^6$ | $(2.9 \pm 0.8) \times 10^7$ | $(5.3 \pm 2.8) \times 10^3$ | $(2.2 \pm 0.2) \times 10^4$ | $(8.4 \pm 6.8) \times 10^5$ | $(9.8 \pm 4.5) \times 10^6$ |
| D, planktonic | Yes | $(5.4 \pm 1.5) \times 10^6$ | $(7.8 \pm 2.3) \times 10^5$ | $(7.4 \pm 5.8) \times 10^4$ | $(4.4 \pm 1.6) \times 10^6$ | $(4.8 \pm 8.1) \times 10^4$ | $(6.0 \pm 8.4) \times 10^3$ | $(5.3 \pm 6.5) \times 10^4$ | $(4.0 \pm 1.5) \times 10^6$ |
| E, intact biofilm | Yes | $(5.3 \pm 4.2) \times 10^7$ | | | | $(2.8 \pm 0.4) \times 10^6$ | $(1.5 \pm 0.9) \times 10^7$ | $(4.0 \pm 3.2) \times 10^7$ | |

[a] Planktonic cultures were grown by continuous culture. Biofilm-derived culture was grown in the GPT device. Biofilms were grown in the CDFF.
[b] Fluoride-containing solutions (i.e., all media except deionized water and basal medium) were adjusted to 250 mg of fluoride $liter^{-1}$. Values are means of viable counts from triplicate runs of each device.

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