

## Transformation of Fluoride Resistance Genes in *Streptococcus mutans*

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To study the nature of fluoride resistance in *Streptococcus mutans*, we transformed DNA extracted from fluoride-resistant mutants of *S. mutans* GS-5 into fluoride-sensitive cells of the same strain. Transformation with DNA from first-step mutants produced transformants with resistance to either 600 or 1,000 µg of sodium fluoride per ml, both of which are within the first-step resistance range (400 to 1,000 µg/ml). In five of six of these transformation experiments, however, the transformant resistance levels were greater than those of their respective DNA donors. Transformation with DNA from a second-step mutant resistant to 1,600 µg/ml resulted in transformants resistant to 600 µg/ml, similar to some transformants receiving DNA from first-step mutants. When a second-step mutant resistant to 3,000 µg/ml was used as a DNA donor, four different levels of resistance were seen in the transformants (600, 1,000, 1,500, and 2,000 µg/ml). In many cases, the growth rates of the transformants (first and second step) were faster than those of the DNA donors. Additionally, many of the transformants demonstrated abrupt shifts in growth rates at relatively low culture densities.

Fluoride is currently the most often and widely used agent to control dental caries. Fluoridation of drinking water has dramatically reduced the incidence of tooth decay. Caries have been found to be reduced by 50% by administering a concentration of one part per million of fluoride (1 µg/ml) in drinking water (3).

One consequence of the widespread use of fluoride to control dental caries is the possible selection and maintenance of a population of oral microorganisms demonstrating resistance to fluoride. Bunick and Kashket (2) demonstrated fluoride resistance in both *Streptococcus salivarius* and *Streptococcus mutans*.

Brussock and Kral (1) isolated spontaneous first- and second-step fluoride-resistant mutants of *S. mutans* GS-5 using a stepwise selection procedure. First-step mutants exhibited six different levels of maximal resistance ranging from 400 to 1,000 µg of sodium fluoride per ml and were isolated at a frequency of  $6.4 \times 10^{-10}$ . Second-step mutants exhibited two levels of maximal resistance, 1,600 and 3,000 µg of sodium fluoride per ml, and were isolated at a frequency of  $1.4 \times 10^{-8}$ . Brussock and Kral (1) reported that in all cases, resistant strains retained higher levels of resistance than the parental strain; second-step mutants exhibited higher levels of resistance than first-step mutants. The characteristics of the fluoride-resistant mutants were similar to those of the parental strain. There were no distinguishable differences between strain GS-5 and the mutant strains with respect to colony and cellular morphology. The parental and mutant strains were also similar biochemically.

The work of Brussock and Kral (1) led to an interest in the genes involving fluoride resistance.

It was not until 1981 that Perry and Kuramitsu (4) demonstrated gene transfer in *S. mutans* by the process of transformation. Their study involved the transformation of three strains of *S. mutans* comprising three different serotypes with DNA derived from streptomycin-resistant strains. Their results represented the first demonstration of genetic transformation of *S. mutans*.

To study the nature of sodium fluoride resistance in *S.*

*mutans*, we transformed DNA extracted from fluoride-resistant mutants of *S. mutans* into fluoride-sensitive cells of the same strain. The transformants were characterized by routine Gram stains, appearance of typical colony morphology, typical appearance of growth in Todd-Hewitt glucose broth, and generation times.

### MATERIALS AND METHODS

**Organism.** *S. mutans* GS-5 (serotype c) was obtained from the laboratory of F. L. Macrina, Department of Microbiology, Medical College of Virginia, Virginia Commonwealth University, Richmond. The fluoride-resistant mutants were isolated by Brussock and Kral (1) as previously described.

**Isolation of crude DNA.** Fluoride-resistant mutants of *S. mutans* were inoculated from frozen-glycerol culture (1) into 100 ml of Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, Md.) containing glucose (1%). Cells were lysed by the glycine-enhanced cell lysis method described by Reider and Macrina (5). After lysis of the cells, 10 ml of ice-cold 100% ethanol was pipetted down the side of the tube. This formed a layer on top of the aqueous suspension. The DNA precipitated at the interface. The mixture was left overnight at  $-25^{\circ}\text{C}$ . The next morning, a clean glass rod was inserted into the suspension and rotated. The DNA fibers wound around the rod. In succession, the DNA-bearing rod was dipped into 80, 70 and 50% ethanol, respectively. This removed the sodium lauryl sulfate. The rod was then transferred to a tube containing 5 ml of  $0.1 \times \text{SSC}$  buffer ( $1 \times \text{SSC}$  is 0.015 M sodium citrate and 0.15 M sodium chloride). The DNA was removed by scraping the rod against the side of the tube. The DNA was resuspended in the SSC buffer by incubation overnight at  $4^{\circ}\text{C}$ . The next morning, 0.5 ml of  $10 \times \text{SSC}$  buffer (pH 8.2) was added to the DNA suspension. DNA can resuspend more easily in  $0.1 \times \text{SSC}$  buffer but is more stable stored in  $1 \times \text{SSC}$  buffer. The suspension was then microcentrifuged (Fisher Scientific Co., Fair Lawn, N.J.) and membrane filtered into a sterile test tube. The tube was sealed tightly and stored at  $4^{\circ}\text{C}$ .

**Transformation procedure.** The procedure used for transformation was a modification of the procedure used by Perry and Kuramitsu (4). *S. mutans* GS-5 was grown in Todd-

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TABLE 1. Comparison of resistance levels and growth characteristics in fluoride-resistant transformants and fluoride-resistant DNA donors

Nature of DNA donors	Strain no. of DNA donor	Generation time of DNA donor $\pm$ SD (min)	Resistance level of DNA donor ( $\mu\text{g/ml}$ ) <sup>a</sup>	No. of transformants tested	Avg generation time of transformants $\pm$ SD (min)	Resistance level of transformants ( $\mu\text{g/ml}$ ) <sup>a</sup>
First-step fluoride-resistant mutants	A38	43 $\pm$ 5	400	10	47 $\pm$ 2	600
	A35	46 $\pm$ 4	500	10	37 $\pm$ 4	1,000
	A52	43 $\pm$ 3	700	10	37 $\pm$ 4	1,000
	A40	44 $\pm$ 4	800	10	36 $\pm$ 5	1,000
	A51	44 $\pm$ 3	900	10	39 $\pm$ 8	1,000
	A33	50 $\pm$ 4	1,000	10	47 $\pm$ 4	1,000
Second-step fluoride-resistant mutants	A64	60 $\pm$ 3	1,600	10	47 $\pm$ 3	600
	A69	56 $\pm$ 4	3,000	4	39 $\pm$ 3	600
				2	44 $\pm$ 6	1,000
				2	38 $\pm$ 4	1,500
				2	36 $\pm$ 1	2,000
Transformants with A69 DNA	A100	46 $\pm$ 4	1,000	10	48 $\pm$ 3	600
	A102	45 $\pm$ 3	1,500	10	41 $\pm$ 6	1,000
	A103	43 $\pm$ 7	2,000	10	49 $\pm$ 2	600

<sup>a</sup> Resistance to sodium fluoride.

Hewitt glucose broth supplemented with heat-inactivated horse serum (10%) for 72 h (daily transfers) at 37°C. From the overnight culture, a 1:10,000 dilution was made into the same medium and incubated for 5 h at 37°C. In a sterile tube, 0.1 ml of the DNA solution (approximately 0.5  $\mu\text{g/ml}$ ) and 0.33 ml of the 5-h culture were gently mixed. This mixture was then incubated for 4 h at 37°C. Samples of 100- $\mu\text{l}$  of the culture (approximately  $3 \times 10^5$  CFU/ml) were spread onto Todd-Hewitt glucose agar plates containing sodium fluoride (500  $\mu\text{g/ml}$ ). Three control experiments were also performed. In one control experiment, 100  $\mu\text{l}$  of the competent *S. mutans* GS-5 cells (without prior incubation with DNA) were spread onto the selective medium. This served as a negative control. In a second control experiment, competent *S. mutans* GS-5 cells were mixed with DNase-treated (DNase I; Sigma Chemical Co., St. Louis, Mo.) A33 DNA (first-step mutant) and DNase-treated A69 DNA (second-step mutant) and then spread onto the selective medium. In a final control experiment, competent *S. mutans* GS-5 cells were incubated with *S. mutans* GS-5 DNA followed by plating on the selective medium. Dilutions of the transformation mixture were also spread onto fluoride-free agar to determine viable counts. After incubation at 37°C in candle jars for 48 to 96 h, the plates were examined for fluoride-resistant transformants.

**Determination of maximal level of resistance.** All fluoride-resistant transformants and the control strains were streaked with an inoculating needle from fluoride-free Todd-Hewitt glucose broth onto Todd-Hewitt glucose agar plates containing increasing concentrations (in 100- $\mu\text{g/ml}$  increments) of sodium fluoride. Plates were incubated in candle jars at 37°C for 48 to 96 h and examined for growth. These experiments were performed in triplicate.

**Characteristics of resistant transformants.** Routine Gram strains were performed throughout the experiments. Growth characteristics in Todd-Hewitt glucose broth and on Todd-Hewitt glucose agar were noted. Generation times were determined in fluoride-free Todd-Hewitt glucose broth at 37°C. (Generation time experiments were performed in triplicate.) Spectrophotometric measurements (675 nm) were recorded at the time of inoculation and at time intervals

thereafter until maximal growth was achieved. A 24-h reading was also recorded.

## RESULTS

Brussock and Kral (1) isolated spontaneous, first-, and second-step fluoride-resistant mutants of *S. mutans* GS-5. Attempts to transform fluoride-sensitive cells with DNA from these strains were successful. Between 100 and 300 transformant colonies were observed on any one plate. The frequency of isolation for all transformants was between  $10^{-3}$  and  $10^{-4}$  transformants per CFU. No colonies were observed when just competent cells, competent cells with DNase-treated DNA, or competent cells with parental strain DNA were spread onto selective media.

Of those transformants isolated, 10 from each transformation experiment were retained for further study. Transformations with DNA from first-step DNA donors A38, A35, A52, A40, and A51 all resulted in transformants whose resistance levels were greater than those of the DNA donors (Table 1). Only transformants containing first-step A33 DNA demonstrated resistance levels identical to that of the donor (Table 1).

Transformation experiments with DNA from the second-step mutants are also presented in Table 1. Again, of those strains isolated, 10 from each transformation experiment were retained for further study. With both donors (A64 and A69), the transformants demonstrated fluoride resistance levels lower than those of the DNA donors. With strain A64 as the DNA donor, all the transformants demonstrated a single level of resistance (600  $\mu\text{g}$  of sodium fluoride per ml), much less than that of the donor. When strain A69 was used as the DNA donor, transformants showing four different levels of resistance (600, 1,000, 1,500, and 2,000  $\mu\text{g}$  of sodium fluoride per ml) were isolated. Again, these resistance levels were less than that of the donor.

In another set of experiments, cells that were previously transformed with DNA from strain A69 (A100, A102, and A103) were used as DNA donors (Table 1). In all three cases, the levels of resistance in these secondary transformants were less than those of the DNA donors (A100, A102, and A103).

The range of average generation times for each group of transformants was between 36 and 49 min (Table 1), a relatively narrow range and one that includes most of the generation times of the DNA donors. However, there were two obvious differences between the growth profiles of the transformants and the DNA donors. First, many of the transformants (33 of 110) demonstrated generation times that were less than those of the DNA donors. Second, 43 of the 110 transformants demonstrated rapid, exponential growth rates only up to optical densities between 0.1 and 0.2 and then very abruptly switched to very slow growth. These cultures achieved maximal optical densities of only 0.35 to 0.69. The other 57 transformants demonstrated growth curves similar to those of the DNA donors, all achieving maximal optical densities greater than 1.0.

Growth of the transformants in both Todd-Hewitt glucose broth and on Todd-Hewitt glucose agar resembled that of the parental strain, GS-5. All transformants occurred as gram-positive cocci in chains. There were no discernible differences in cell size or chain length between the resistant transformants and strain GS-5. When grown in the absence of fluoride for at least 48 h, all transformants isolated retained their original maximal levels of resistance when reintroduced to fluoride-containing media.

#### DISCUSSION

Spontaneous, first-, and second-step fluoride-resistant mutants of *S. mutans* GS-5 were isolated by Brussock and Kral (1). The procedure used enabled the isolation of mutant colonies which could be studied on an individual basis. Brussock and Kral (1) demonstrated retention of the original maximal levels of resistance when transformants were passaged through fluoride-free media for at least 50 generations. The transformation experiments reported here demonstrated that resistance in each of the isolates was indeed a stable characteristic and probably genotypic. The frequency of isolation of transformants ( $10^{-3}$  to  $10^{-4}$ ) was approximately  $10^6$  times greater than the frequency of isolation of spontaneous mutants and strongly suggests that the resistant colonies did not arise spontaneously. Successful transformation was also supported by lack of colony growth on any of the control plates.

Many of the resistance levels reported here are only two to three times that of the parental strain. That being the case, reproducibility of resistance levels is crucial if the transformants are to be considered truly different from each other and substantially different from the parental strain. In all experiments (greater than 200) in which the resistance level of the parental strain was 300  $\mu\text{g}$  of sodium fluoride per ml, the resistance levels of the transformants (as well as those of the first- and second-step mutants) were identical to those reported here. In rare cases (less than 2% of the experiments), a resistance level other than 300  $\mu\text{g}/\text{ml}$  for the parental strain was observed. Owing to the rarity of these observations, these discrepancies were attributed to technical error. Apparently, a 100- $\mu\text{g}/\text{ml}$  increment in sodium fluoride concentration is large enough to allow for consistent reproducibility.

Transformation with DNA from five of the six first-step mutants resulted in transformants each of which demonstrated a resistance level greater than its respective DNA donor. Four of those five resulted in transformants resistant to 1,000  $\mu\text{g}$  of sodium fluoride per ml, which appears to be the upper limit of resistance for first-step mutants. The other set of transformants in that group of five demonstrated resistance to 600  $\mu\text{g}$  of sodium fluoride per ml, which, coincidentally, was also seen when the donor was second-step mutant A64 or transformants A100 and A103. This suggests that 600 and 1,000  $\mu\text{g}$  of sodium fluoride per ml represent critical first-step resistance levels.

Transformation with DNA from two second-step mutants gave two different sets of results. As mentioned previously, when A64 acted as the DNA donor, the transformants all demonstrated a single level of resistance (600  $\mu\text{g}/\text{ml}$ ) within the first-step resistance level range. Transformation with DNA from strain A69, the mutant resistant to the highest level (3,000  $\mu\text{g}/\text{ml}$ ), resulted in transformants with four different levels of resistance. Two of those levels were within the first-step range (600 and 1,000  $\mu\text{g}/\text{ml}$ ), and two were within the second-step range (1,500 and 2,000  $\mu\text{g}/\text{ml}$ ). When transformants A100, A102, and A103 were used as DNA donors, the secondary transformants all demonstrated resistance levels within the first-step range, either 600 or 1,000  $\mu\text{g}$  of sodium fluoride per ml. Again, these two levels appear critical. No transformants with the level of resistance of the donor were observed.

Coincidentally, in some cases the acquisition of fluoride resistance was accompanied by faster growth. A number of transformants grew with generation times in the 30- to 40-min range, while most of the DNA donor strains and strain GS-5 grew with generation times near 45 min, and some grew even slower. There was no statistical correlation between generation time and level of fluoride resistance. Nor was there a correlation between generation time and high or low maximal optical density. A reasonable explanation for the fact that approximately 40% of the transformants cease rapid growth at relatively low optical densities (0.1 to 0.2) is not at hand.

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