In Vitro Fluoride Resistance in a Cariogenic *Streptococcus* [1]

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ABSTRACT. Stable fluoride-resistant mutants of *Streptococcus mutans* GS-5 were isolated with a stepwise selection procedure. First-step mutants were isolated at a frequency of 6.4×10^{-10} and demonstrated six maximal levels of resistance ranging from 400-1000 ug/ml sodium fluoride. Second-step mutants with higher levels of resistance were isolated at a frequency of 1.4×10^{-8}. Second-step mutants demonstrated two maximal levels of resistance, 1600 and 3000 ug/ml sodium fluoride. Other than fluoride resistance, the characteristics of both first- and second-step mutants were similar to those of the parental strain. Growth rates did differ, however. First-step mutants exhibited slightly longer mass doubling times than the parental strain (average of 50 *vs* 45 min, respectively). Second-step mutants exhibited substantially longer mass doubling times (average of 71.5 min). The results suggest that fluoride resistance may be regulated by more than one gene, and that high levels of resistance may be due to a cumulative effect of at least two genes.

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INTRODUCTION

The most effective practical measure for the control of dental caries is the administration of fluoride in drinking water and dentifrices. As a consequence of the widespread use of fluoride, there is a demonstrated accumulation of fluoride by plaque material in the oral cavity. Recent studies have indicated that fluoride can be concentrated within bacterial cells, and that the bacterial concentration may account for the high levels of fluoride present in plaque relative to the lower levels in saliva and serum (Kashket and Rodriguez 1976, Whitford et al. 1977).

Another consequence of the use of fluoride is the selection and maintenance of a fluoride-resistant population of oral bacteria. Naturally-occurring, fluoride-resistant cells of *Streptococcus mutans* were isolated from the plaque of xerostomia patients using daily fluoride gel (1%) therapy for 2.3 to 4.5 years (Streckfuss et al. 1980). Colonies resistant to 600 ppm fluoride were isolated; however, the fluoride tolerance dropped to 165-330 ppm after seven transfers in the absence of fluoride. This transient resistance to fluoride has been seen in several oral microorganisms (Green and Dodd 1957, Williams 1964).

Studies have demonstrated both phenotypic adaptation and genotypic resistance of several strains of oral bacteria to fluoride. Williams (1967) showed adaptation of *Streptococcus zymogenes* and *Streptococcus faecalis* to 100 mM fluoride by serial passage through increasingly higher concentrations of fluoride. This resistance was lost when the organisms were grown for two to three days in fluoride-free media.

Stable resistance to fluoride has been demonstrated in a variety of oral bacteria. Fluoride-resistant isolates of *S. mutans* were obtained by inoculating a series of BHI broth tubes with 0 to 900 ppm fluoride in 50-ppm intervals (Streckfuss et al. 1980). Cells growing at 400-600 ppm were considered to be fluoride resistant. The stability of this resistance was verified by alternate culturing in BHI broth with and without fluoride. Bunick and Kashket (1981) also demonstrated fluoride resistance in *Streptococcus salivarius* by plating cells on mitis salivarius agar containing 5.3 mM sodium fluoride. After plating 1.2×10^{10} CFU, seven colonies arose after aerobic growth for 24 hours at 37°C. They also determined the frequency of mutation to be 5.8×10^{-10} which is consistent with a single point mutation.

Fluoride resistance has also been induced with radiation and chemical mutagens. Hamilton (1969) irradiated *S. salivarius* cells for 10-15 seconds with 2437 Å of ultraviolet light on spread plates containing sodium fluoride. He found resistant isolates on 1.2 mM and ′.4 mM, but not 4.8 mM sodium fluoride. He also noted that the resistant isolates grew more slowly on fluoride than did adapted cells. Rosen et al. (1978) induced fluoride resistance in *S. mutans* cells with both ultraviolet radiation and acriflavin. They obtained mutants resistant to 600 ppm fluoride.

The aim of our investigation was to isolate and study individual, stable, spontaneous, fluoride-resistant mutants of *S. mutans* GS-5 in order to better understand the nature of fluoride resistance. We describe a procedure used to isolate both first- and second-step resistant mutants, and report the finding of various levels of resistance within each step.

METHODS AND MATERIALS

ORGANISM. *Streptococcus mutans* GS-5 was obtained from M. J. Pucci, Department of Microbiology, Virginia Commonwealth University, Richmond, Virginia.

ISOLATION OF MUTANTS. *Streptococcus mutans* GS-5 cells were inoculated from a frozen glycerol culture (Kral and Callaway 1984) into 20 ml of Todd Hewitt broth (BBL, Becton Dickinson and Co., Cockeysville, MD) containing glucose (1%). The culture was incubated overnight at 37°C and allowed to grow to an optical density of approximately 1.35 (approximately 10^{10} CFU/ml), as measured by a Spectronic 20 (Bausch and Lomb, Rochester, NY) at 675 nm. The cells were harvested by centrifugation at 5000 × g for 10 min (Sorvall GLC-2B, DuPont Instruments, Newtown, CT), washed once with sterile PKMN buffer (10 mM potassium phosphate, 75 mM potassium chloride, 75 mM sodium chloride, 1 mM magnesium chloride), maintained at a pH of 7.0 (Eisenberg et al. 1981), resuspended in PKMN buffer, and incubated at 37°C for 1-2 h (this incubation allows the cells to use up reserve energy sources, reducing residual background growth). Following incubation, the cells were centrifuged again at 5000 × g for 10 min and resuspended in PKMN buffer at a cell density of approximately 10^{11} CFU/ml. Aliquots of 100 ul of the cell suspension were spread onto duplicate Todd Hewitt glucose agar plates (pH 7.2) containing increasing concentrations (0-1000 ug/ml at 50-ug/ml increments) of sodium fluoride. The sodium fluoride was dissolved in distilled water, autoclaved, and aseptically added to sterile agar (50°C) prior to pouring the plates. Membrane filtration was not employed due to loss of a significant quantity of the sodium fluoride on the filter.

Spread plates were placed in candle jars and incubated at 37°C for 48 h. Following incubation, each plate was examined for the appearance of fluoride-resistant colonies. *Streptococcus mutans* GS-5 is naturally resistant to 300 ug/ml sodium fluoride. Resistant colonies were transferred with sterile toothpicks to fresh Todd Hewitt glucose agar plates containing the same concentration of sodium fluoride as the plates from which they were isolated. Following incubation and

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growth, these isolates were transferred to Todd Hewitt glucose broth containing 300 ug/ml sodium fluoride in order to prepare frozen glycerol cultures and for further study.

To isolate second-step resistant mutants, cells of the first-step isolate, A26, were inoculated into 60 ml of Todd Hewitt glucose broth containing 300 ug/ml sodium fluoride, and incubated at 37°C. Following overnight growth to approximately 0.5 optical density units, the cells were spread on Todd Hewitt glucose agar plates containing 800, 1200, 1600, 2000, and 2400 ug/ml sodium fluoride. The resistant isolates were transferred to broth (containing 300 ug/ml sodium fluoride) for frozen storage.

DETERMINATION OF MAXIMAL LEVEL OF RESISTANCE. All fluoride-resistant isolates and the parental strain, GS-5, were streaked with an inoculating needle from fluoride-free Todd Hewitt glucose broth onto Todd Hewitt glucose agar plates containing increasing concentrations of sodium fluoride. Plates were incubated in candle jars at 37°C for 48 h and examined for growth.

CHARACTERIZATION OF RESISTANT MUTANTS. All fluoride-resistant isolates, as well as strain GS-5, were characterized biochemically, physiologically, and morphologically. Gram stains were performed to test for culture purity, cell arrangement, and Gram reaction. The presence of catalase was assayed with hydrogen peroxide. To test for the ability to ferment mannitol and sorbitol to acidic end products, cells were inoculated into M. R. S. "Complete" Medium (DeMan et al. 1960) without beef extract and incubated at 37°C overnight. Two loopsful of each culture were transferred into 5 ml of M. R. S. "Identification" Medium (DeMan et al. 1960) containing mannitol or sorbitol as the sole energy source plus phenol red (0.5 g/l) as a pH indicator. Cultures were incubated at 37°C for 24-48 h and examined for color change indicative of acid production. Colony morphology on mitis salivarius agar (Difco Laboratories, Detroit, MI) was examined following incubation at 37°C for 48 h. To determine if extracellular polysaccharide was produced in the presence of sucrose, cells were streaked onto Todd Hewitt sucrose (1%) agar plates, incubated at 37°C for 48 h, and screened for a whitish cap over each colony indicative of extracellular polysaccharide synthesis. To determine if resistance to fluoride was a stable characteristic, isolates were grown in fluoride-free Todd Hewitt glucose broth for at least 50 mass doublings and then challenged with sodium fluoride. Mass doubling times were determined in fluoride-free medium at 37°C. Growing cells were transferred to fresh Todd Hewitt glucose broth (prewarmed to 37°C) and incubated. Spectrophotometric measurements (675 mm) were recorded at the time of transfer and every hour thereafter for 8 h. An overnight measurement was also recorded. Mass doubling times of selected isolates growing in broth containing 300 ug/ml sodium fluoride were also determined with the procedure already described.

RESULTS

Spontaneous, first-step, fluoride-resistant mutants of *S. mutans* GS-5 were isolated as individual colonies on a thin background of residual growth on Todd Hewitt glucose agar plates containing 150, 250, and 300 ug/ml sodium fluoride. The frequency of isolation was 6.4×10^{-10}. Twenty-three isolates were retained for further study. Although some of the mutants were isolated on concentrations of sodium fluoride to which the parental strain is insensitive (approximately 300 ug/ml), all were found to possess greater resistance than the parental strain. These isolates as well as their maximal levels of resistance are listed in Table 1. Several maximal levels of resistance ranging from 400 to 1000 ug/ml sodium fluoride were found within the first-step isolates.

Second-step fluoride-resistant mutants were isolated from a culture of the first-step isolate, A26, on Todd Hewitt glucose agar plates containing 1200 ug/ml sodium fluoride. The frequency of isolation was 1.4×10^{-8}. As seen in Table 2, second-step isolates possessed higher levels of fluoride resistance than first-step isolates. Again different levels of resistance were seen. Eight of 10 strains retained for further study demonstrated maximal resistance to 1600 ug/ml sodium fluoride, whereas two strains showed maximal resistance to 3000 ug/ml.

TABLE 1

Maximal levels of resistance and mass doubling times of first-step sodium fluoride (NaF)-resistant mutants of. S. mutans.
*Parental strain-*S. mutans *GS-5*.

Strain number	Maximal level of NaF-resistance at pH 7.2, (ug/ml)	Mass doubling time (min)
GS-5	300	45
A25	1000	47
A26	1000	58
A27	1000	52
A32	1000	58
A33	1000	50
A34	1000	50
A35	500	47
A36	900	47
A37	1000	50
A38	400	48
A40	800	47
A41	800	48
A42	800	48
A43	800	48
A44	800	51
A45	1000	47
A46	800	47
A47	800	48
A48	800	51
A49	800	47
A50	800	48
A51	900	48
A52	700	56

All of the fluoride-resistant isolates and the parental strain were catalase-negative and fermented glucose, mannitol, and sorbitol to acidic end products. All strains were gram-positive cocci occurring in chains. There were no discernible differences in cell size or chain length between the resistant isolates and strain GS-5.

On mitis salivarius agar, resistant colonies appeared small, opaque, round, and volcano-shaped as did colonies of the parental strain. All isolates grew within 48 h.

All resistant and parental colonies produced extracellular polysaccharide material when grown on Todd Hewitt sucrose agar. The polysaccharide appeared as a whitish cap over each colony. The capsular material was not produced by cells grown on glucose media.

TABLE 2

Maximal levels of resistance and mass doubling times of second-step sodium fluoride (NaF)-resistant mutants of S. mutans. *Parental strain-A26.*

Strain number	Maximal level of NaF-resistance at pH 7.2, (ug/ml)	Mass doubling time (min)
A26	1000	58
A64	1600	63
A65	1600	65
A66	1600	75
A67	1600	68
A68	1600	62
A69	3000	61
A70	3000	75
A71	1600	62
A72	1600	94
A73	1600	90

When grown in the absence of fluoride for at least 50 mass doublings, all of the resistant isolates retained their original maximal levels of resistance when reintroduced to media containing fluoride.

The mass doubling times for first- and second-step mutants, grown in fluoride-free medium are given in Tables 1 and 2, respectively. Mass doubling times for first-step mutants ranged from 47-58 min with an average of 50 min (the parental strain has a mass doubling time of approximately 45 min). Second-step mutants exhibited slower mass doubling times, ranging from 61 to 94 min with an average of 71.5 min. There was no statistical correlation between mass doubling time and maximal level of resistance for either first- or second-step mutants.

Mass doubling times of selected mutants, representing each level of maximal resistance, growing in broth containing 300 ug/ml sodium fluoride, are given in Table 3. The mass doubling times of the first-step isolates and the parental strain were considerably slower, whereas mass doubling times of second-step isolates remained unchanged compared to growth in fluoride-free media.

DISCUSSION

We have isolated both first- and second-step spontaneous, fluoride-resistant mutants of *S. mutans* GS-5, a bacterium originally described by Gibbons (1966), and later shown to be strongly adherent and highly virulent with respect to cariogenic disease (Bozzola et al. 1977, Johnson et al. 1977). The described procedure allowed for the isolation of mutant colonies that could be studied individually. Retention of the original level of fluoride resistance, when passaged through fluoride-free media for at least 50 mass doublings, indicated that each of the isolates possessed a genotypic mutation. Preliminary transformation studies have confirmed this (unpublished data). The frequency of isolation for first-step mutants was 6.4×10^{-10}, consistent with a single point mutation. Bunick and Kashket (1981) have reported a similar frequency of mutation (5.8×10^{-10}) for fluoride resistance in *S. salivarius*. Second-step mutants exhibiting higher levels of resistance were isolated from a first-step mutant at a frequency of 1.4×10^{-8}. The frequency of isolation for second-step mutants was 22 times higher than the frequency of isolation for first-step mutants. This may be due to the finding that the doubling times of first-step mutants growing in broth containing 300 ug/ml sodium fluoride were markedly increased, whereas the second-step doubling times remained unchanged. Since the procedure for isolation of second-step mutants included growing first-step mutants in broth plus 300 ug/ml sodium fluoride, those cells which had mutated spontaneously to withstand greater concentrations of fluoride were probably growing at faster rates than the first-step parental cells, thus enhancing their frequency of isolation. The discovery of first- and second-step mutants suggests that two mutational events occurred, most likely in separate genes. These mutations apparently have a cumulative effect. Attempts are being made to isolate third-step fluoride-resistant mutants.

Various levels of resistance were found within each step. First-step isolates exhibited six different maximal levels of fluoride resistance at pH 7.2, ranging from 400 to 1000 ug/ml sodium fluoride. Second-step isolates exhibited two maximal levels of resistance, 1600 and 3000 ug/ml sodium fluoride. The presence of these various levels of resistance within each step again suggests that more than one gene is responsible for fluoride resistance.

The characteristics of our 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. Like strain GS-5, the isolates were catalase-negative and fermented mannitol and sorbitol to acidic end products. An identifying characteristic of *S. mutans* is its ability to produce extracellular polysaccharide in the presence of sucrose (Guggenheim 1968). This polysaccharide is important in the adherence of *S. mutans* to the tooth surface and, therefore, to the entire cariogenic process (Marsh 1980). Each of the isolates produced extracellular polysaccharide in the presence of sucrose. The mass doubling times of the isolates did differ from that of strain GS-5. First-step mutants exhibited slightly longer doubling times than the parental strain in fluoride-free media. Second-step mutants had longer mass doubling times than either strain GS-5 or any of the first-step mutants. There was no correlation between mass doubling time and level of fluoride resistance.

Bunick and Kashket (1981) reported that stable resistance to fluoride appeared to be genotypic and possibly regulated by a single gene product. Our data indicate that more than one gene and, therefore, more than one gene product are involved in fluoride resistance, and that high levels of resistance may be due to a cumulative effect of at least two genes.

TABLE 3

Mass doubling times of selected fluoride-resistant mutants of S. mutans *in Todd Hewitt glucose broth plus 300 ug/ml sodium fluoride (NaF).*

Strain number	Mass doubling time (min)	Maximal level of NaF-resistance (ug/ml)
GS-5	98	300
A26	90	1000
A35	80	500
A36	60	900
A38	70	400
A40	65	800
A52	70	700
A65	60	1600
A69	60	3000

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