Comparison of Cariogenic Characteristics between Fluoride-sensitive and Fluoride-resistant *Streptococcus mutans*

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Abstract

The aim of this study is to compare cariogenic characteristics of fluoride-sensitive *Streptococcus mutans* [fluoride-sensitive (FS) *S. mutans*] and fluoride-resistant *Streptococcus mutans* [fluoride-resistant (FR) S. mutans] in the presence of sucrose, and to evaluate its effect on cariogenic biofilm formation. *S. mutans* ATCC 25175 was continuously cultured in trypticase soy broth (TSB) containing NaF (70 ppm) for 40 days to generate FR *S. mutans*. FS and FR *S. mutans* were inoculated in TSB with or without 2% sucrose, and optical density and pH were measured every hour. An oral biofilm was formed using saliva bacteria and analyzed through confocal laser scanning microscopy and CFU count. Finally, the expression of glucosyltransferases genes of both *S. mutans* was investigated through RT-PCR. FR *S. mutans* exhibited slower growth and lower acidogenicity in the presence of sucrose compared to FS *S. mutans*. Both cariogenic and single species biofilm formation was lower in the presence of FR *S. mutans*, along with reduced number of bacteria. FR *S. mutans* showed significantly low levels of gtfB, gtfC, and gtfD expression compared to FS *S. mutans*. On the basis of results, FR *S. mutans* may be less virulent in the induction of dental caries.

Key words : *Streptococcus mutans*, Fluoride, Fluoride-resistance, Biofilm, Dental caries

Ⅰ. Introduction

Dental caries is caused by the breakdown of the demineralization/remineralization balance as a consequence of frequent sugar intake and changes in the oral environment due to low pH conditions[1]. When sugar is supplied frequently or salivary secretion is too scarce to neutralize the acid produced, pH decrease in the biofilm may enhance the acidogenicity and acidurance of the non-mutans bacteria, resulting in establishment of a more acidic environment. As these changes disrupt microbial homeostasis, an ecological shift occurs in the oral biofilm toward more proportion of cariogenic bacteria such as mutans streptococci and lactobacilli, along with gradual decrease of non-mutans and further accelerating caries lesion progress[1]. This caries etiology is known as 'ecological plaque hypothesis', emphasizing the dynamic stability of the biofilms[2]. Among cariogenic bacteria, *Streptococcus mutans* is considered to be closely related with the formation of cariogenic biofilms, as it possesses caries-related virulence factors, such as acidogenicity, aciduricity, and adhesin[3].

S. mutans produces abundant lactic acid by metabolizing carbohydrates using lactate dehydrogenase (LDH) and, thereby, decreasing the environmental pH value in biofilms[4]. Further, as *S. mutans* presents aciduricity (acid tolerance) mediated by a membrane bound F-ATPase (F-type adenosine triphosphatase) proton pump[5], it is competent at metabolizing carbohydrates into lactic acid even though the external pH of its environment is decreased[5,6]. Furthermore, this bacterium contributes to bacterial adhesion and biofilm formation by synthesizing glucan from sucrose using its glucosyltransferases (Gtfs)[3].

The sucrose-dependent mechanism of *S. mutans* to biofilm formation is by producing extracellular polysaccharide (EPS) with three types of glucosyltransferases (GtfB, -C, and -D) encoded by *gtf* genes[3,7]. GtfB and GtfC mainly synthesize water-insoluble glucan, also called mutan, comprising α(1-3) glucosidic bonds, whereas GtfD synthesizes water-soluble glucan, also called dextran, comprising α(1-6) glucosidic bonds[8]. GtfB is absorbed to enamel and binds other oral microorganisms, GtfC is absorbed to pellicle, and GtfD forms readily metabolizable polysaccharides while also acting as a primer for GtfB. Overall, the Gtfs contribute to cariogenic biofilm formation and accumulation, resulting in dental caries[9].

Fluoride is commonly used to prevent dental caries due to its remineralization effects and antimicrobial activity against bacteria and biofilms[10]. Fluoride has been shown to inhibit the growth and metabolism of cariogenic bacteria even at sub-minimum inhibitory concentrations (1 mmol/L)[11,12]. At sub-millimolar levels, fluoride can directly inhibit bacterial metabolism by binding to a variety of enzymes in the form of F-, Hydrogen fluoride(HF), or metal-F complexes. In addition, at micromolar levels, HF acts as a transmembrane proton carrier to enhance the proton permeability of the cell membrane, resulting in cytoplasm acidification, reduction of acid tolerance, and inhibition of glycolysis[13,14].

However, prolonged use of fluoride prophylaxis has resulted in the emergence of fluoride resistance in bacteria[15]. Accordingly, studies on fluoride-resistant *S. mutans* strains have been conducted to identify its cariogenic characteristics, but the results have been inconsistent[16], requiring more studies on characteristics of fluoride-sensitive (FS) and -resistant (FR) *S. mutans* regarding the effects of changes in *gtf* expression and formation of cariogenic biofilms.

This study aimed to compare the cariogenic characteristic of FR *S. mutans* with that of FS *S. mutans* in the presence of sucrose and to determine its effect on cariogenic biofilm formation using salivary bacteria.

Ⅱ. Materials and Methods

1. Bacterial strain and culture conditions

S. mutans ATCC 25175 was used in the following experiments. The bacteria were cultured in trypticase soy broth (TSB; BD Biosciences, Franklin Lakes, NJ, USA) in the presence and absence of fluoride at 37℃ in anaerobic conditions. For cariogenic biofilm formation, salivary bacteria were cultured in the presence of FS and FR *S. mutans* in brain-heart infusion (BHI; BD Biosciences, Franklin Lakes, NJ, USA) medium containing 2% sucrose.

2. Preparation of fluoride resistant *S. mutans*

To decide fluoride concentration of the culture media to make fluoride resistance, the susceptibility of *S. mutans* for fluoride had been investigated by serial dilution method from 1000 ppm of fluoride. The 50% inhibitory concentration of fluoride against *S. mutans* growth was found to be between 62.5 and 125 ppm, and 70 ppm of fluoride concentration was decided for the study. 10,000 ppm of NaF solution was filtered with 0.22 μm polyvinylidene fluoride (PVDF) membrane and added to TSB media while adjusting the concentration to 70 ppm. Isogenic fluoride-resistant *S. mutans* from parental *S. mutans* strains was generated by sequential culture in TSB containing 70 ppm of NaF for 40 days. In order to isolate FR *S. mutans*, sequentially cultured-*S. mutans* was streaked on trypticase soy agar (BD Biosciences) containing 200 ppm of NaF, and a single colony was picked and inoculated into TSB containing 70 ppm of NaF. The identity of *S. mutans* was determined by gram staining and polymerase chain reaction (PCR) using specific primers follows as 5′-CTC AAC CAA CCG CCA CTG TT-3′ and 5′-GTT TAA CGT CAA AAT TAG CTG TAT TAG C-3′. All isogenic strains were kept frozen at -85℃.

3. Comparison of cariogenic related factors

In order to investigate growth and acidogenicity, the concentration of FS and FR *S. mutans* was adjusted to 0.4 optical density at 660 nm wavelength using fresh medium, and 5 mL of FS and FR *S. mutans* media were each inoculated into 200

mL of BHI broth with or without 2% sucrose. The bacterial growth and pH were measured every hour. The growth of *S. mutans* was evaluated by optical density (OD) measurement at 660 nm using a spectrophotometer (Biotek instrument, Winooski, VT, USA) after transferring 200 μL of bacterial suspension into a 96-well plate (SPL bioscience, Gyeonggi, Korea). The pH of the culture media was measured using a pH meter (Orion A211; Thermo Fisher Scientific, Waltham, MA USA) after transferring 7 mL of culture media into a fresh 50 mL conical tube (SPL bioscience).

4. Preparation of conditioning plate for biofilm formation

Pooled saliva of 10 healthy donors were centrifuged at 8,000 × g for 10 min at 4℃. The supernatant was filtered with a 0.22 μm polyvinylidene fluoride (PVDF) membrane and diluted to twofold with phosphate-buffered saline (PBS, pH 7.2). The prepared saliva was added to 8-well chamber glass slip and polystyrene 24-well plate. The chamber and the plate were dried at 40℃ in a drying oven and sterilized in an UV sterilizer, and these procedures were repeated five times.

5. Biofilm formation and observation

Salivary biofilm was formed according to the method described by Lee[17]. Unstimulated saliva was collected from 10 healthy donors and pooled. The pooled saliva was mixed with BHI broth containing 2% sucrose and centrifuged at 1,200 × g for 10 min at 4℃ to remove debris. The supernatant was transferred into two fresh tubes, and then FS or FR *S. mutans* (1 × 10^6 cells) was added respectively.To analyze biofilm formation, the mixture was vortexed for 15 s, and 1 mL and 400 μL of the mixtures were inoculated into a conditioned polystyrene 24-well plate and a 8-well glass chamber. The plates were incubated in an anaerobic chamber at 37℃ for 36 hours to form biofilm. The biofilm formed polystyrene 24-well plate was washed three times with PBS to remove planktonic bacteria, and the biofilm was physically detached with a scraper (Corning Co., Corning, NY, USA). The bacterial suspensions were serially diluted from 10 to 10^7 with BHI and inoculated on BHI agar plates to count whole bacteria or mitis-salivarius bacitracin (MSB) agar plates to count *S. mutans*, respectively. For the observation of the biofilm formation, 8-well glass chamber was washed three times with PBS and stained with SYTO 9 (Invitrogen, Eugene, OR, USA) according to the manufacturer's

instructions. The cariogenic biofilm including FS and FR *S. mutans* was observed with a confocal laser scanning microscope LSM 700 (CLSM; Zeiss, Carl-Zeiss, Oberkochen, Germany). A z-stack of three randomly chosen locations from 0 to 30 μm was taken per sample and the 3D biofilm image was obtained using ZEN software (Carl-Zeiss).

6. Investigation of glucosyltransferase expression

RT-PCR was performed to investigate the expression of *gtf*B, *gtf*C, and *gtf*D genes from FS and FR *S. mutans* cultured in BHI including 1% sucrose. Total RNA from sequentially cultured-FS and FR *S. mutans* was isolated with a TRIzol® Max bacterial RNA isolation kit (Invitrogen Life Tech, Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized by mixing total RNA (1 μg) and Maxime™ RT premix with random primers (iNtRON, Gyeonggi, Korea) in a 20 μL reaction volume and incubating the mixture at 45 ℃ for 1 h. cDNA were mixed with 25 μL of SYBR Premix Ex Taq and 0.4 mM of each primer pair in a 50 μL final volume and subjected to 40 PCR cycles (95℃ for 15 s, 60℃ for 15 s, and 72℃ for 33 s) on an ABI PRISM 7500 Fast RT-PCR system (Applied Biosystems, Foster City, CA, USA). The PCR products were investigated for non-specific amplification using a dissociation curve of amplification.

The primer sequences used for RT-PCR were as follows: 5' -AGC AAT GCA GCC AAT CTA CAA AT-3' and 5'-ACG AAC TTT GCC GTT ATT GTC A-3' for *gtf*B gene; 5'-CTC AAC CAA CCG CCA CTG TT-3' and 5'-GTT TAA CGT CAA AAT TAG CTG TAT TAG C-3' for *gtf*C gene; 5'-CAC AGG CAA AAG CTG AAT TAC A-3' and 5'-AAT GGC CGC TAA GTC AAC AG-3' for *gtf*D gene; and 5'-CTA ATT CAC CTG TAC GAG-3' and 5'-CCG AAT CTT CTG TAA G-3' for the 16s rRNA gene. 16s rRNA of *S. mutans*, which is a house keeping gene, was used as a reference to normalize the expression levels of *gtf* genes. The process was performed four times.

7. Statistical analysis

All statistical analyses were performed by Mann-Whitney test using SPSS 21 (SPSS Inc., Chicago, IL, USA). The data are presented as mean ± standard deviation or as median and interquartile range. *p* values less than 0.05 were considered statistically significant.

Ⅲ. Results

1. Growth of FS and FR *S. mutans*

Growth of FR *S. mutans* was found to be significantly lower than that of FS *S. mutans* after 3 hours to the end point in the presence of sucrose ($p < 0.05$). At the end point, the growth of FR *S. mutans* was about the half of FS *S. mutans* (Fig. 1A). In sucrose-free condition, no significant difference was found between FS and FR *S. mutans* at the end point of growth (Fig.

1B). However, there was a significant difference from 3 to 7 hours after inoculation. The growth of FR *S. mutans* from 3 to 7 hours was lower than that of FS *S. mutans*, indicating that FR *S. mutans* required longer time to reach the stationary phase.
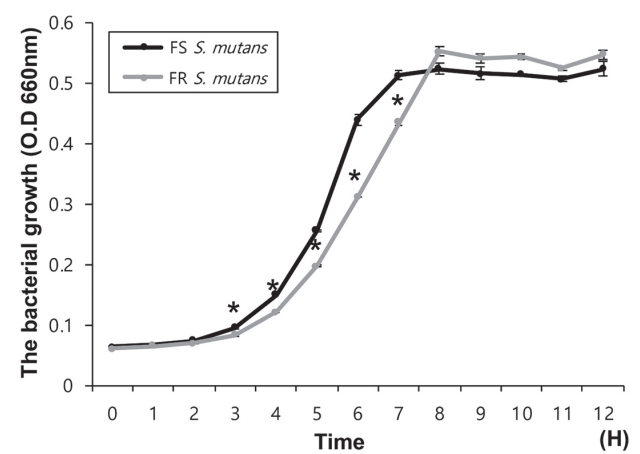
2. Acidogenicity of FS and FR *S. mutans*

Acid production by *S. mutans* was evaluated through pH changes in spent culture media in the presence or absence of 2% sucrose (Fig. 2). As shown in Fig. 2A, the pH value of spent
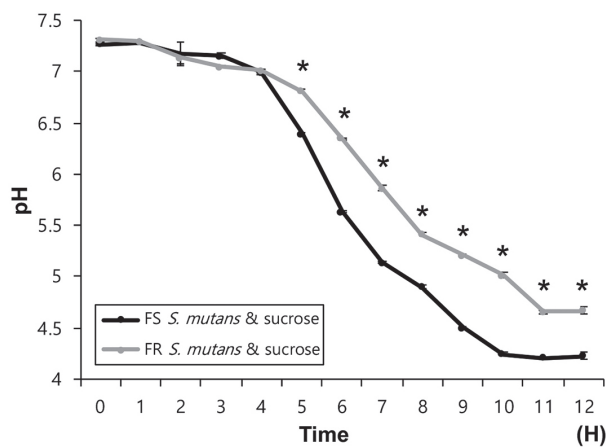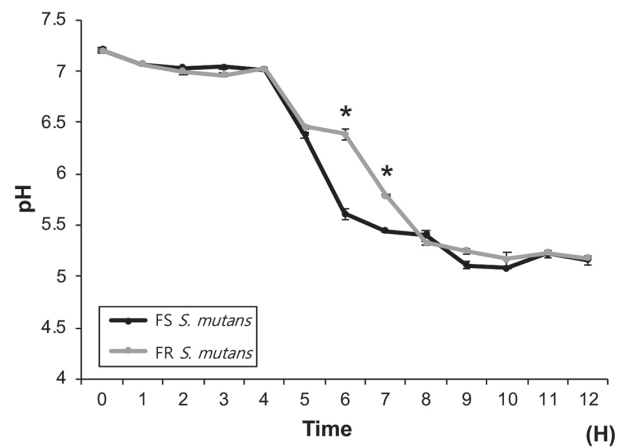
A. Sucrose
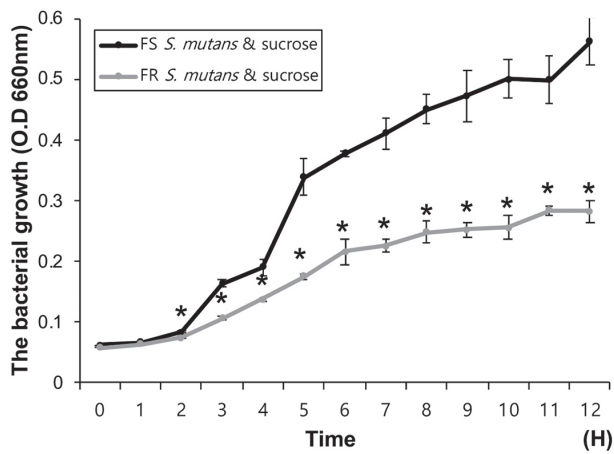
B. Sucrose free

Fig. 1. Bacterial growth of FS and FR *S. mutans*. Optical density values of FS and FR *S. mutans* cultured in BHI media (A) with or (B) without 2% sucrose.
p value from Mann-Whitney U test.

A. Sucrose


B. Sucrose free

Fig. 2. Acid production of FS and FR *S. mutans*. PH values of BHI media inoculated with FS or FR *S. mutans* (A) with or (B) without 2% sucrose.
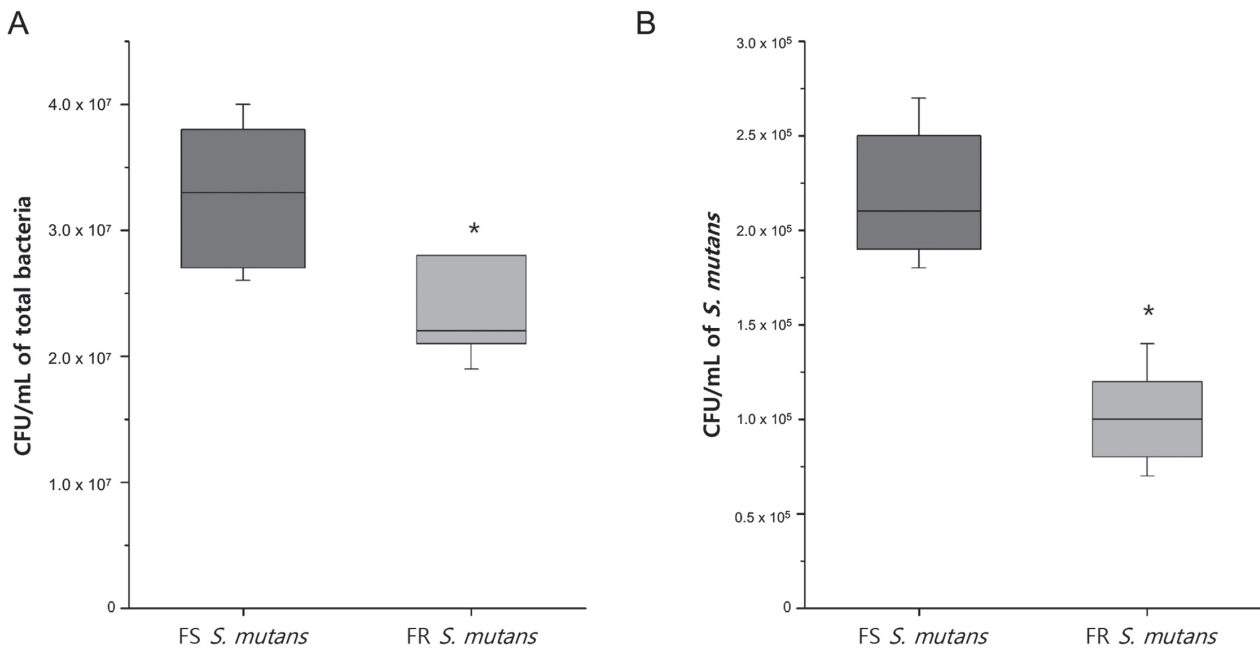*p* value from Mann-Whitney U test.

media of FS *S. mutans* was significantly lower than that of FR *S. mutans* from 5 hours in the presence of sucrose ($p < 0.05$). No significant difference was found in the end pH value between FS and FR *S. mutans* in the sucrose-free condition (Fig. 2B). However, a significant difference in pH value after 6 and 7 hours showed longer time for FR *S. mutans* to reach a pH below 5.5.

3. Comparison of salivary and single species biofilm formed with FS or FR *S. mutans*

CLSM results showed that FR *S. mutans* formed less biofilm in both salivary biofilm, and single species biofilm compared to FS *S. mutans* (Fig. 3). Bacterial levels in salivary biofilm and single *S. mutans* biofilm were determined and the results are shown in Fig. 4. The number of total bacteria and *S. mutans* was significantly lower in the salivary biofilm with FR *S. mutans* ($p < 0.05$). BHI and MSB agar were used to determine the total bacteria and *S. mutans* counts in the salivary biofilm, respectively.
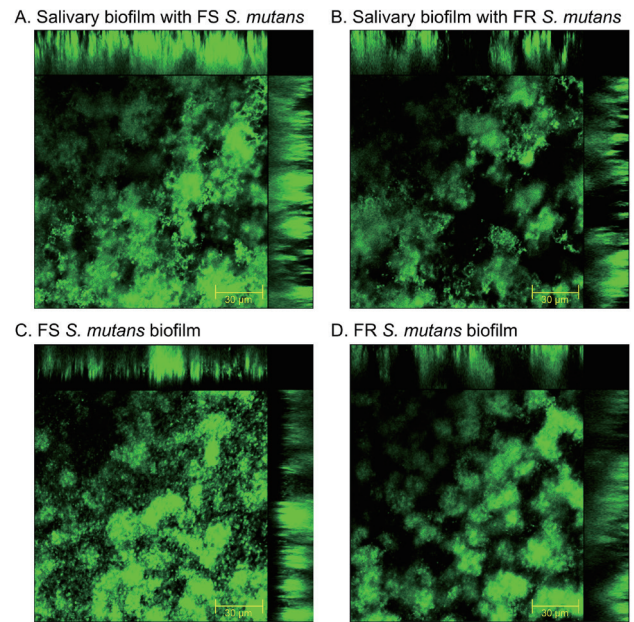
Fig. 3. Confocal laser scanning microscopic images biofilm formed with FS and FR *S. mutans*. (A) Salivary biofilm formed with FS *S. mutans*, (B) salivary biofilm formed with FR *S. mutans*, (C) FS S. mutans biofilm, (D) FR *S. mutans* biofilm. CLSM was performed with a Zeiss LSM 700 microscope using a 40x/1.3 oil immersion lens. Bar 30 μm.

Fig. 4. Bacterial count in salivary biofilms formed with FS or FR *S. mutans*. (A) CFU of total bacteria in BHI agar plates, (B) CFU of *S. mutans* in MSB agar plates.
*p* value from Mann-Whitney U test.

4. Comparison of *gtf* expression between FS and FR *S. mutans*

As biofilm formation exhibited a difference between FS and FR *S. mutans*, Gtfs production as a biofilm formation related factor in FS and FR *S. mutans* was compared (Fig. 5). The expression of *gtf*B, *gtf*C, and *gtf*D was significantly reduced to about 30%, in FR *S. mutans* compared to FS *S. mutans* ($p < 0.05$).

IV. Discussion

Fluoride has been recognized as a major factor for decreasing caries due to its antimicrobial and remineralization effects[18]. However, prolonged use of fluoride has been reported to cause mutation of *S. mutans* and adaptation to fluoride[19]. Studies on FR *S. mutans* have reported different characteristics of FR *S. mutans* compared to the FS parental strain[16], and phenotypic and genotypic changes in FR *S. mutans* have been evaluated[20-22]. In this study, the cariogenic properties of FR *S. mutans* were compared with those of FS *S.*
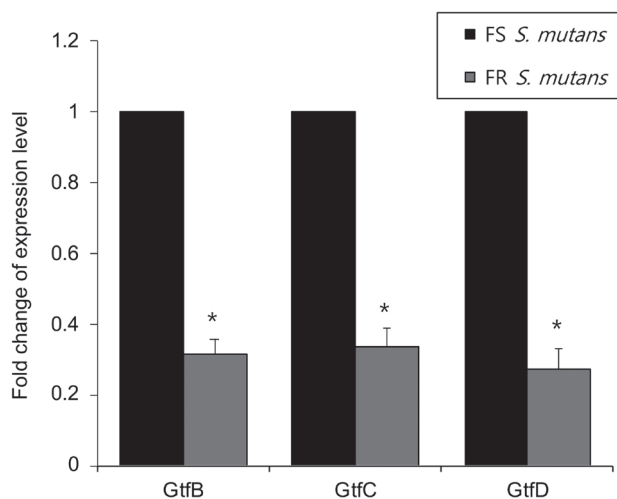
Fig. 5. Comparison of expression of gtf genes in FS and FR *S. mutans*. After isolation of total RNA, gtfB, gtfC, and gtfD expression in FS and FR *S. mutans* was analyzed by RT-PCR. The experiment was performed four times, and the gene expression data were shown as the average fold change with means and their standard deviations.
p value from Mann-Whitney U test.

mutans in the presence of sucrose, and the effect of FR *S. mutans* on salivary biofilm formation was assessed.

In previous study, FR *S. mutans* was mostly generated through a stepwise procedure to select colonies that grew in the presence of 400 - 600 ppm of fluoride under laboratory conditions, and its characteristics were compared with those of FS *S. mutans*[22]. However, due to different experimental designs and different strains used, no conclusive result was obtained[16]. Our study generated FR *S. mutans* by long term exposure of FS *S. mutans* to a 50% inhibitory concentration of fluoride, and fluoride resistance was verified by streaking on 200 ppm trypticase soy agar.

Sucrose is considered the most cariogenic dietary carbohydrate as its fermentation induces low pH in biofilms and it serves as a substrate for the formation of extracellular and intracellular polysaccharides[23]. Acidogenicity and growth of FS and FR *S. mutans* was compared with and without 2% sucrose in planktonic conditions. The acidogenicity of *S. mutans* was compared by evaluating pH changes. When FR *S. mutans* was cultured without sucrose, the end pH and growth value of spent media was not significantly different from that of FS *S. mutans*. However, media containing FR *S. mutans* took longer time to reach a pH below 5.5, which is the critical pH for the demineralization to occur[24]. A significant difference between FS and FR *S. mutans* was found in the sucrose added condition. In FR *S. mutans*, end growth was decreased to half, and the end pH value was high compared to that of FS *S. mutans*. From the results, it seems that sucrose acts as an inhibitor for growth and acid production in FR *S. mutans*. Under stress conditions, *S. mutans* show stress mechanisms, such as alteration in F-ATPase activity, catabolic pathways, and gene expressions[25]. The results of the present study suggest the modification of sucrose metabolic activity in FR *S. mutans*, resulting in a shift to decreased cariogenicity. This is in line with a previous study that lower cariogenicity of FR *S. mutans* was found in rats[26].

As prevention and treatment of dental caries focus on the microbial environment as well as on the cariogenic bacteria[1], cariogenic and single species biofilm formation with FS or FR *S. mutans* was observed. FR *S. mutans* formed reduced mass of salivary biofilm and single species biofilm compared to FS *S. mutans*, and the number of total bacteria and *S. mutans* was also significantly reduced. Oral biofilms are complex polymicrobial communities embedded within an extracellular matrix, present as a highly organized 3D structure adhered to

the tooth[27,28]. It is consists of various microbial constituents including bacterial strains and EPS, which is one of the major components of biofilms and virulent factor in cariogenic biofilm formation[23]. Water soluble and water insoluble glucans are synthesized from sucrose by Gtfs in *S. mutans* and contribute to sucrose-dependent adhesion and biofilm formation[9].

When *gtf* expression of FS and FR *S. mutans* was compared through RT-PCR, the expression of *gtf*B, *gtf*C, and *gtf*D of FR *S. mutans* was significantly reduced compared to that of FS *S. mutans*. Reduction of *gtf*B, *gtf*C, and *gtf*D expression in FR *S. mutans* indicates that both water insoluble and soluble glucan were produced at lower levels by FR *S. mutans* than FS *S. mutans*, resulting in reduced adhesion to the surface, cell adhesion, and biofilm formation[9]. Expression of *gtf* genes in *S. mutans* can be changed in the presence of other oral bacteria[27], and fluoride did not seem to have an inhibitory effect on the Gtf activity of *Streptococcus* species in previous studies[29,30]. However, our results showed that FR *S. mutans* expressed significantly lower levels of *gtf* gene expression and formed a reduced mass of cariogenic biofilm compared to FS *S. mutans* with the salivary bacteria.

The concentration of fluoride in plaques can be elevated by the use of fluoride-enriched dentifrice[13,31] and topical NaF[32]. Although fluoride seems to decrease after initial exposure[33], it persists for a long time and even sub-millimolar levels of fluoride can affect the physiology of microbial cells[34]. Generally, daily mouthwash of 0.05% NaF (approximately 230 ppm F) for 1 min is recommended to prevent dental caries[35]. However, lower concentration of NaF (10, 50, and 125 ppm F) has also been suggested to show inhibitory effects on virulence factors and composition of *S. mutans* biofilm[29], and fluoride concentration in biofilm is known to increase along with duration of fluoride exposure[32]. This suggests that frequent use of low concentrations of fluoride would be effective in preventing dental caries by direct antimicrobial effects of fluoride and generation of FR *S. mutans*.

However, the limitation of this study is that as stresses from the oral environment are periodic rather than continuous and natural variations occur in the pH of plaques, generation of FR *S. mutans* in the oral environment is not easy[10]. Also, investigation of the stability of the resistance acquired form long term exposure of fluoride, and the affects of FR *S. mutans* to other microbial species should be considered in the future studies.

Ⅴ. Conclusion

In this study, long term exposure of *S. mutans* to 50% inhibitory level of fluoride generated FR *S. mutans*. Sucrose metabolic activity and acid production were reduced in FR *S. mutans*. Moreover, decreased salivary biofilm formation was observed along with decreased *gtf* gene expression. These findings suggest that FR *S. mutans* may be less virulent than FS *S. mutans* in inducing dental caries and may be effective in maintaining oral microbial homeostasis and reducing dental caries.

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국문초록

불소 민감성 *Streptococcus mutans*와 불소 저항성 *Streptococcus mutans*의 우식원성 특성 비교

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이 연구의 목적은 불소 민감성(fluoride-sensitive) *Streptococcus mutans* (FS *S. mutans*) 와 불소 저항성(fluoride-resistant) *Strepto-coccus mutans* (FR *S. mutans*)의 우식원성 특성을 비교하는 것이다. *S. mutans* ATCC 25175 균주를 NaF (70 ppm)를 포함한 trypticase soy broth에서 40일 동안 배양하여 FR *S. mutans*를 획득하였다. 2% 자당 유무에 따른 FS와 FR *S. mutans*의 성장과 산 생성 변화를 비교하였고, 타액 세균을 이용하여 FS와 FR *S. mutans* 바이오필름을 형성하여 공초점 레이저 현미경으로 관찰하고 세균 수를 측정하였다. 또한 RT-PCR을 통해 FS와 FR *S. mutans*의 gtf 유전자 발현 정도를 비교하였다. FR *S. mutans*는 FS *S. mutans* 보다 자당을 이용한 성장과 산 생성이 모두 낮았다. 타액 세균과 단일 균주 바이오필름의 형성 또한 FR *S. mutans*가 FS *S. mutans* 보다 적었고, 더 낮은 *gtf*B, *gtf*C 및 *gtf*D 발현을 보였다. 이 연구를 통해 FR *S. mutans*는 FS *S. mutans* 보다 감소된 우식원성 특성을 가지고 있음을 관찰하였고, 불소의 주기적 사용은 *S. mutans* 세균의 활성을 낮추어 우식 발생을 줄이는데 도움이 될 수 있을 것이다.