



UvA-DARE (Digital Academic Repository)

Fluoride resistance in *Streptococcus mutans*

Liao, Ying

Publication date

2017

Document Version

Other version

License

Other

[Link to publication](#)

Citation for published version (APA):

Liao, Y. (2017). *Fluoride resistance in Streptococcus mutans*. [Thesis, fully internal, Universiteit van Amsterdam].

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

Chapter 5

The fitness cost of fluoride resistance for *Streptococcus mutans* in biofilms

A modified version of this chapter has been accepted as:
Cai Y, Liao Y, Brandt BW, Wei X, Liu H, Crielaard W, van Loveren C, Deng DM. 2017. The fitness cost of fluoride resistance for different *Streptococcus mutans* strains in biofilms. *Front Microbiol* 8:1630. doi: 10.3389/fmicb.2017.01630



Abstract

The cariogenic bacterium *Streptococcus mutans* can develop stable resistance to fluoride through chromosomal mutations *in vitro*. Clinically fluoride-resistant *S. mutans* has, however, seldom been isolated, despite the wide application of fluoride in oral-care products. This is possibly due to a decreased fitness in the fluoride-resistant strains. The aim of this study was to compare the fitness of 48-h biofilms of two fluoride-resistant strains *S. mutans* UF35 and UA159FR (UAFR) with the wild-type fluoride-sensitive strain UA159 in absence of fluoride. The fitness of biofilms was examined by exposure to chlorhexidine or to low pH. The engineered UF35 strain contains one point chromosomal mutation, while UAFR, selected from NaF-containing agar plates, has multiple chromosomal mutations. All biofilms were grown for 48 h under a constantly neutral pH or a pH-cycling (8 h of neutral pH and 16 h of pH 5.5) condition. The formed biofilms were first treated with chlorhexidine or solutions of pH 3.0, after which their lactic acid production was quantified. The biomass of all biofilms was also quantified. UAFR biofilms behaved differently from UF35 and UA159 biofilms, while the latter two behaved similar. The biomass of UAFR biofilms was 2-4 fold higher and the UAFR biofilms were more resistant to CHX and low pH. The fluoride-resistance of UAFR and UF35 strains in biofilms was further confirmed by treating the biofilms with NaF solutions. The level of NaF resistance of three biofilms is generally ranked as the following: UAFR > UF35 > UA159. In conclusion, indeed there is a fitness consequence in UAFR, but surprisingly this fluoride-resistant strain, under the described conditions, performs better. Also UF35 does not display fitness loss: it performs equally well as the wild-type fluoride-sensitive strain.

Introduction

The intensive use of antimicrobials has dramatically increased the incidence of antimicrobial resistance among human microbial isolates (Holmes et al. 2016). Generally, a bacterial species acquires resistance to antimicrobials through two routes: *de novo* mutation or horizontal gene transfer of resistance determinants (Andersson and Hughes 2010). Irrespective of how the resistance was acquired, the antimicrobial-resistant microbes could suffer a decrease in biological fitness (Andersson and Hughes 2010). These fitness costs made antimicrobial-resistant microbes less competitive than susceptible strains when the selective pressure from antimicrobials was removed, which might eventually result in the loss of the antimicrobial resistance (Andersson and Hughes 2010). However, some studies have shown that the fitness costs could be modulated by multiple factors including environmental factors and the occurrence of compensatory mutations (Andersson and Hughes 2010; Marcusson et al. 2009; Melnyk et al. 2015) and might not be able to reverse the resistance at the community level.

As the most effective caries-preventive agent, fluoride has been added to water supplies and to daily oral care products, such as toothpaste, mouthwash and dental floss, for almost 70 years (Birkeland and Torell 1978). It not only protects dental hard tissues by inhibiting demineralization and enhancing remineralization, but also functions as an antimicrobial agent (Ten Cate 2004; Van Loveren 2001). Fluoride is able to suppress bacterial growth and metabolism (Brown et al. 1980; Van Loveren et al. 1991c). To counteract this suppression, several bacterial species were found to acquire stable fluoride resistance, although most of the resistant strains were obtained in a laboratory setting (Bunick and Kashket 1981; Hamilton 1969a; Sheng and Liu 2000; Streckfuss et al. 1980). We previously identified multiple single nucleotide mutations in the genome of a fluoride-resistant *Streptococcus mutans* strain (Liao et al. 2015), indicating that the stable resistance to fluoride was the result of gene mutations.

Despite the wide application of fluoride-containing products in our daily life, fluoride-resistant bacteria have seldom been isolated from clinical samples. It is possible that some bacterial cells might have become fluoride resistant in dental biofilms, but might suffer from a decreased fitness and hence be unable to survive *in vivo*. In order to understand the impact of fluoride resistance on bacterial fitness, several laboratory-derived fluoride-resistant

S. mutans strains were compared to their isogenic wild-type strains on growth, acidogenicity and surface adherence *in vitro* or in animal models (Lau and Kral 1987; Liao et al. 2015; Rosen et al. 1978; Van Loveren et al. 1991a). However, the results from these studies were inconsistent. For example, the fluoride-resistant *S. mutans* strain C180-2FR, was reported less acidogenic than its parent strain in one study (Van Loveren et al. 1991c), but found equally acidogenic to the same parent strain in another study (Liao et al. 2015). Van Loveren *et al.* (Van Loveren et al. 1991a) studied the competition between C180-2FR and its parent strain C180-2 in a rat model. The fluoride-resistant C180-FR strain colonized less and was eventually outgrown by its parent strain. However, Hoelscher and Hudson (Hoelscher and Hudson 1996) characterized another fluoride-resistant *S. mutans* isolate (NCH105) and reported that this strain bound to the tooth surface to the same extent as the parent strain UA130. Differences in experimental design or different strains used in these studies may be the reasons for these discrepancies. As a result, it is hard to draw definitive conclusions about the fitness costs of fluoride resistance.

The aim of this study was to compare the fitness of two fluoride-resistant *S. mutans* strains (UAFR and UF35) with their isogenic wild-type strain UA159 (UA) in biofilms. The fluoride-resistant strain UAFR was created by culturing UA on agar plates containing increasing concentration of fluoride (Zhu et al. 2012), while strain UF35 was engineered by changing one single nucleotide (-44A→C) in the promoter region *mutp* of UA159 (Liao et al. 2016). Both strains displayed slower growth rate than the wild-type strain in suspensions. *S. mutans* biofilms rather than planktonic cultures were examined, since biofilms better mimic the bacterial life-style in dental plaque. Moreover, various bacterial growth environments in an oral cavity were simulated by growing the biofilms under either constantly neutral pH or pH-cycling conditions. The pH-cycling consists of a period of 8 h at neutral pH and a period of 16 h at pH 5.5.

Materials and Methods

Bacterial strains and growth conditions

The strains used in this study were the fluoride-sensitive *S. mutans* strain UA159 and two fluoride-resistant strains (UF35 and UAFR). The construction of strain UF35 was described in Liao *et al.* (Liao et al. 2016). The strain UAFR

(accession: NZ_CP007016.1) was kindly provided by Professor Zhimin Zhang (Jilin University, Changchun, China). All strains were routinely maintained anaerobically (90% N₂, 5% CO₂, 5% H₂) on Brain-Heart Infusion (BHI) agar at 37°C. Biofilms were grown in a semi-defined biofilm medium (BM) (Deng et al. 2009b). The pH of BM was adjusted to either 7.0 by adding 76 mM K₂HPO₄ and 15 mM KH₂PO₄, or to 5.5 by adding 100 mM acetic acid. BM with 0.4% of glucose (BMG) or 0.2% of sucrose (BMS) was used as medium for pre-cultures and biofilm growth, respectively.

Biofilm growth

Biofilms were grown in an active attachment model (Nunc, Roskilde, Denmark) to avoid bacterial sedimentation (Li et al. 2014). This model consists of a standard 96-well microtiter plate and a lid with 96 polystyrene pegs that fit into the wells (Nunc™, Roskilde, Denmark). Overnight (16 h) cultures of *S. mutans* UA159, UF35 and UAFR in BMG were adjusted to a final OD₆₀₀ of 0.035 in fresh BMS (pH 7.0). Two hundred microliter of each culture was dispensed into each well of the microtiter plates. The lid (with the pegs) was then placed in the wells and the samples were incubated anaerobically. After 8 h, half of the biofilms (grown on the pegs) were transferred to wells containing BMS of pH 7.0, while the other half was transferred to BMS of pH 5.5. The biofilms were further incubated for 16 h. Then the biofilms were transferred to wells containing BMS of pH 7.0 for another 8 h. Part of these 32-h biofilms were exposed to NaF treatments. The other part was transferred to either BMS of pH 7.0 or BMS of pH 5.5 for an additional 16 h. These 48-h biofilms (not exposed to NaF treatments) were challenged by either chlorhexidine (CHX) or low pH. The schema of biofilm growth and biofilm processing is illustrated in Figure 1. All experiments were repeated three times. For each test condition, four replicates were used in every experiment.

Sodium fluoride (NaF) treatments

A pilot study had shown that the biofilms displayed detectable phenotypic changes only after incubation with NaF for 16 h. Therefore, NaF was added to the growth medium at 32 h and its effect on biofilm growth and lactic acid production was subsequently examined after 16-h incubation. In detail, the 32-h biofilms, grown under either constantly neutral pH or under the pH-cycling conditions, were incubated in BMS (pH 7.0 or pH 5.5) containing various con-

centrations of NaF (0-12.5 mM) for 16 h. The growth of the biofilms in NaF-medium during 16 h was quantified by a crystal violet assay. The capabilities of the 48-h biofilms to produce lactic acid were also tested.

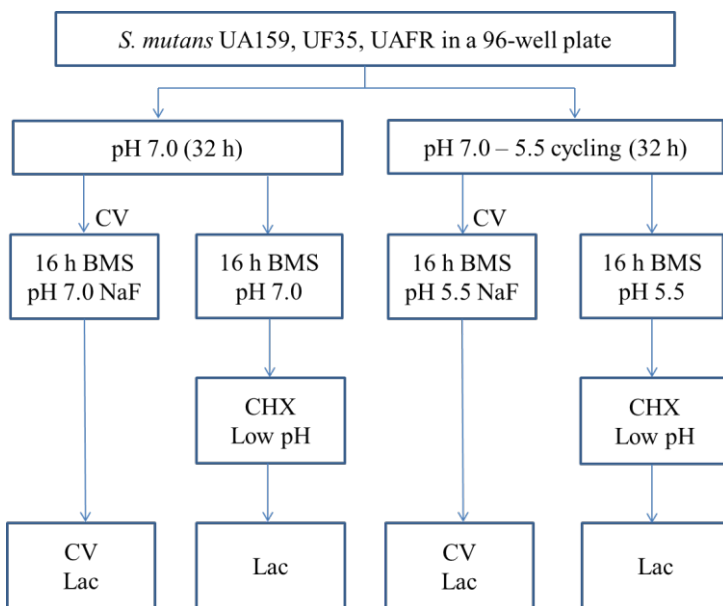


Figure 1. The experimental design of the biofilms growth and treatments. CV: crystal violet assay. Lac: lactic acid quantification.

Chlorhexidine (CHX) treatments

The 48-h biofilms (not exposed to NaF treatments; see Figure 1) were inserted into wells containing 200 μ L CHX solutions at concentrations of 0, 0.01%, 0.02% and 0.04% for 5 min. The CHX solutions were prepared from a 20% chlorhexidine digluconate solution (Sigma-Aldrich, St. Louis, MO, USA). The treatments were stopped by inserting the biofilms into a neutralizing solution (6% Tween 80, 0.6% lecithin and 0.068% potassium phosphate, pH 7.0) for 10 min (Kara et al. 2006). Thereafter, the capabilities of the biofilms to produce lactic acid were evaluated.

Low-pH challenge

The 48-h biofilms (not exposed to NaF treatments; see Figure 1) were rinsed with sterile distilled water and then inserted into wells containing acid solution of pH 3.0. The acid solution was prepared from 40 mM potassium phos-

phate/citrate buffer (Svensäter et al. 1997). The duration of the treatment varied depending on the growth conditions of the biofilms. The biofilms grown under constantly neutral pH condition were treated for 1, 3 and 5 min, while the biofilms grown under pH-cycling were treated for 5, 10 and 30 min. Treatments were terminated by inserting the biofilms into 300 mM HEPES [4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid] buffer solution (pH 7.0) for 10 min. The lactic acid production of the biofilm was evaluated directly after the treatment.

Crystal violet staining assay

A crystal violet staining assay was used to quantify the biomass of the biofilms with or without NaF treatments. The pegs with biofilms were first inserted into a 0.01% crystal violet solution (200 μ L/well) for 5 min, washed twice with distilled water and then inserted into 2% sodium deoxycholate to destain for 5 min. The absorbance of the destaining solution was measured at 608 nm using a spectrophotometer (Spectramax Plus, Molecular Device, Sunnyvale, California, USA). The change of biomass during the 16-h overnight incubation was calculated as: Biofilm growth is equal to the OD₆₀₈ of 48-h biofilms minus the OD₆₀₈ of 32-h biofilms. The data are presented as the percentage of biofilm growth in a NaF group relative to that in the corresponding non-NaF treated control group.

Lactic acid quantification

In order to quantify the capability of the biofilms to produce lactic acid, the pegs with biofilms were incubated in an assay buffer containing 1% glucose (200 μ L/well) for 1 h at 37°C. The assay buffer was prepared from BM without yeast extract to avoid biofilm growth during 1-h incubation (pH 7.0). The lactic acid concentration in the buffer solution was quantified by an enzymatic-spectrophotometric method (Belli et al. 1995; Gutmann and Wahlefeld 1974). This method is based on an enzymatic conversion of L-lactate to pyruvate with concomitant reduction of NAD to NADH. The increase in absorbance at 340 nm is proportional to NADH formation. The data is presented as the percentage of lactic acid production of each treatment group relative to the corresponding non-treatment control groups.

Viable cell counts

Each individual peg with biofilms was cut off with a sterile scalpel without disturbing the biofilms and placed in 1ml CPW (5 g yeast extract, 1 g peptone, 8.5 g NaCl, 0.5 g L-cysteine hydrochloride per liter, adjusted to pH 7.3). Biofilms were dispersed by sonication on ice for 1 min with 1s pulse at an amplitude of 40 W (Vibra cell™, Sonics & Materials Inc., USA). Serially diluted samples were plated onto BHI agar plates. The plates were incubated anaerobically for 3 days and colony-forming units (CFU) were counted.

Statistical analysis

The data were analyzed with the Statistical Package for Social Science (SPSS, Version 20.0). One-way analysis of variance (ANOVA) was used to compare the biofilm formation of three *S. mutans* strains at 32 h and 48 h, followed by a Bonferroni's post-hoc test. An independent Student's *t*-test was further applied to compare the 32-h and 48-h biomass formation of each strain. The difference was considered significant if $p < 0.05$. The responses (percentage of biomass increase and lactic acid production relative to the control group) of these three *S. mutans* biofilms to NaF, CHX and low-pH treatments were analyzed by two-way ANOVA analysis, using strains and treatment conditions as the independent variables. When the interaction between strains and treatment conditions was significant, new two-way ANOVA analyses were carried out for each of the 3 pairs of strains. The difference was considered as significant if $p < 0.016$ after Bonferroni correction. All tests were done for the biofilms grown under constantly neutral pH condition and the biofilms grown under pH-cycling condition separately.

Results

In this study, three aspects of fitness were examined for all tested strains in absence of NaF: biofilm formation, response to CHX treatments and response to low-pH challenges. In addition, the inhibition of NaF on biofilm growth and lactic acid production was examined for these three strains to confirm the fluoride-resistance of UF35 and UAFR in biofilms.

Biofilm formation

The capabilities of biofilm formation of three strains, measured with the crystal violet staining assay, are presented in Figure 2. The biomass of these strains was measured at 32 h and 48 h. Irrespective of the pH regime during biofilm growth and irrespective of the biofilm age, UAFR produced significantly more biomass than UF35 and UA159, while the latter two strains did not differ in biofilm formation. The viable-cell plate counts of the 48-h biofilms supported the results from the crystal violet staining assay. The logCFU per biofilms were: 8.1 ± 0.5 (neutral pH), 7.8 ± 0.3 (pH-cycling) for UAFR; 7.1 ± 0.4 (neutral pH), 7.3 ± 0.1 (pH-cycling) for UF35 and 7.4 ± 0.3 (neutral pH), 7.2 ± 0.3 (pH-cycling) for UA159. In accordance with the differences in biomass and CFUs, the lactic acid production of 48-h UAFR biofilms was also significantly higher than the other two biofilms. No difference in lactic acid production was observed between UF35 and UA159 biofilms irrespective of the growth conditions (data not shown).

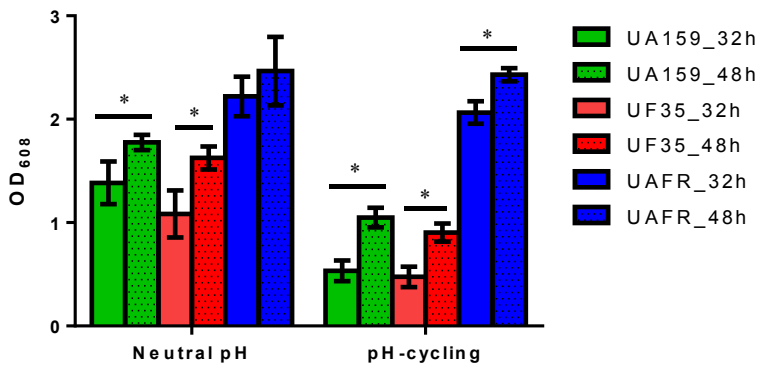


Figure 2. Biomass of 32-h and 48-h biofilms. The biofilms of *S. mutans* UA159, UF35 and UAFR were grown under either a constantly neutral pH or a pH-cycling condition for 32 h and 48 h. The biomass was quantified by the crystal violet staining assay and presented as the OD value at 608 nm. * indicates the significant difference between the 32-h and 48-h biofilms of each strain, $p < 0.05$.

Response of biofilms to CHX and low-pH challenge

As stated above, *S. mutans* UAFR biofilms produced significantly more lactic acid than UF35 and UA159 biofilms. Therefore, the treatment efficacies of CHX and low pH are presented as the percentage reduction in lactic acid pro-

Chapter 5

duction of the treated samples relative to the non-treated samples (Figure 3 and 4).

Figure 3 shows that the reduction in lactic acid production in biofilms was enhanced with increasing concentrations of CHX. Under constantly neutral pH, the CHX concentration-dependent reduction in UAFR biofilms seemed to be the strongest among three strains, but the differences did not reach statistical significance (Figure 3A). However, under pH-cycling conditions, this reduction in UAFR biofilms was significantly less than the reduction in the other two biofilms (Figure 3B). UF35 biofilms showed similar responses to the CHX treatments as the wild-type UA159 biofilms.

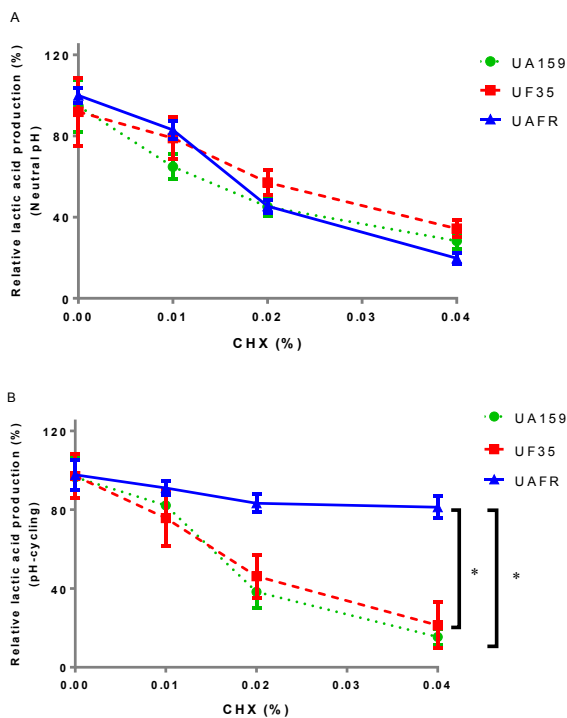


Figure 3. Response of 48-h biofilms to CHX treatments. The 48-h biofilms of *S. mutans* UA159, UF35 and UAFR were treated by CHX for 5 min. The lactic acid production of the biofilms is shown. The data are presented as the percentage of lactic acid production in the CHX treatment groups relative to the corresponding non-treatment control group. * indicates the significant difference between treatment response of each strain, $p < 0.016$.

With increasing durations of the low-pH challenge, the reduction in lactic acid production in biofilms became more obvious (Figure 4). Again, *S. mutans* UAFR biofilms behaved differently from UF35 and UA159 biofilms,

but the difference was only observed for the biofilms grown under constantly neutral pH conditions (Figure 4A). Under this condition, UAFR biofilms displayed a delayed response (longer than 1min) to low-pH challenge, while the other two biofilms already showed reductions in lactic acid production after being treated for 1-min. Moreover, the effect of the duration of the low-pH challenge varied upon the biofilm growth conditions: when the biofilms were grown under the constantly neutral pH conditions, the low-pH challenge reduced the lactic acid production of all biofilms to undetectable levels within 5 min; while this took 30 min when the biofilms were grown under pH-cycling conditions.

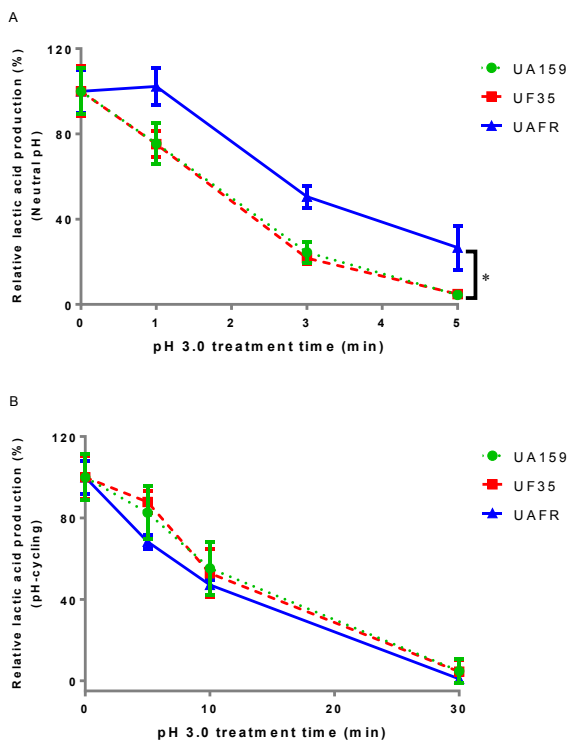


Figure 4. Response of 48-h biofilms to low-pH challenges. The 48-h biofilms of *S. mutans* UA159, UF35 and UAFR strains were treated with pH 3.0 buffer solutions for various periods of time. The lactic acid production of the biofilms is shown. The data are presented as the percentage of lactic acid production in low-pH treatment groups relative to the corresponding non-treatment control group. * indicates the significant difference between treatment response of each strain, $p < 0.016$.

Response of biofilms to NaF treatments

To confirm that the two *S. mutans* UF35 and UAFR biofilms are indeed less sensitive to fluoride than the wild-type UA159 biofilms, we examined the response of these three biofilms to the growth medium containing NaF (Figure 5). The NaF treatments did not affect the biofilm growth of UAFR, but reduced that of UF35 and to a larger extent that of UA159, in a dose-dependent manner (Figure 5A and 5B). A similar result for these three biofilms was obtained for the NaF concentration-dependent reduction in lactic acid production (Figure 5C and 5D). The level of NaF resistance of three strains is generally ranked as the following: UAFR > UF35 > UA159.

Since the treatment efficacy of NaF is known to be pH-dependent, the tested concentration range of NaF differed in the biofilms grown under two pH conditions, being 3-12 mM for constantly neutral pH and 0.25-1 mM for pH-cycling condition.

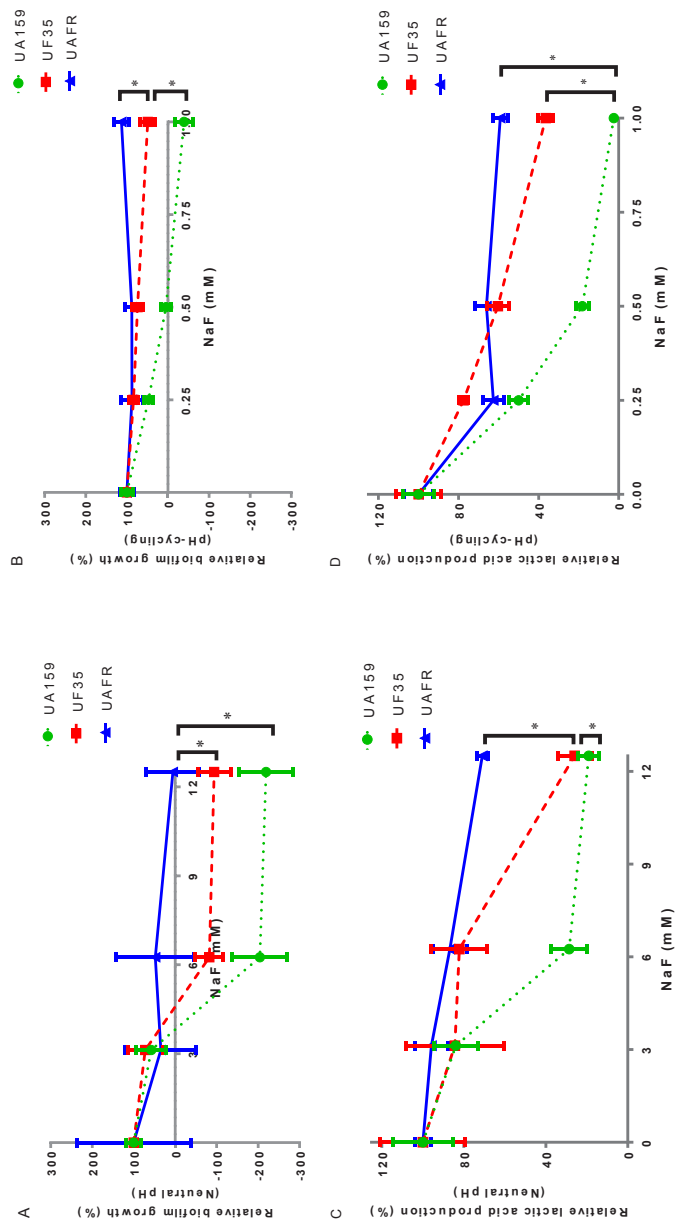


Figure 5. Response of various biofilms to 16-h NaF incubations. The 32-h biofilms of *S. mutans* UA159, UF35 and UAFR were grown with various concentrations of NaF for 16 h under either constantly neutral pH or pH-cycling conditions. The biomass and lactic acid production of the biofilms are shown. The data are presented as the percentage of biofilm growth or lactic acid production relative to the corresponding non-treatment control group. * indicates the significant difference between treatment response of each strain, $p < 0.016$.

Discussion

In this study, we compared the fitness of two *S. mutans* fluoride-resistant strains with that of their parent fluoride-sensitive wild-type strain. Our data showed that independent of pH conditions (during biofilm growth), neither of the fluoride-resistant strains has a reduced fitness as compared to the fluoride-sensitive strain. In contrast, one of the strains UAFR displayed stronger fitness in biofilm formation and resistance to CHX and low-pH challenges than the wild-type strain and the other fluoride-resistant strain UF35, in absence of fluoride. Moreover, strain UAFR coped with NaF treatments better than UF35. As stated above, these two fluoride-resistant strains differ in the number of chromosomal mutations. Strain UF35 contains one mutation in the promoter region of the fluoride antiporter-coding genes and resists fluoride challenge through up-regulation of fluoride antiporters (Liao et al. 2016). Strain UAFR was obtained by stepwise selection on agar plates containing increasing concentration of fluoride (Zhu et al. 2012). The genetic mechanism of fluoride resistance in UAFR is still unknown. We have identified up to 24 single nucleotide polymorphisms (SNPs) in the UAFR genome by comparing its genome sequence with that of the wild-type strain UA159. One of these SNPs locates in the (same) promoter region of fluoride antiporter genes as in UF35 (data not shown). The differences in fitness and in fluoride-resistance between UF35 and UAFR may be related to the number of chromosomal mutations in their genomes. In a previous study, a series of isogenic *Escherichia coli* strains carrying up to 5 fluoroquinolone resistance mutations were constructed and examined for the fitness cost resulting from fluoroquinolone resistance (Marcusson et al. 2009). The strains containing more than three mutations possessed a better fitness in growth rate in absence of fluoroquinolone than those containing less than three mutations. In our case, the additional mutations in UAFR may lead to the improved fitness, including enhanced biofilm formation and resistance to CHX.

The fitness of a bacterium can be influenced by its growth conditions (Bjorkman et al. 2000). In this study, we observed that strain UF35, when grown in biofilms, responded similarly to the low-pH challenge as the wild-type strain, while it was previously found to be more susceptible to the same treatment than the wild-type strain when grown in suspension (Liao et al. 2016). Thus, the bacterial lifestyle can lead to differences in fitness. Similarly,

we observed that the resistance of UAFR biofilms to CHX and low-pH challenge also depended on the biofilm growth conditions. The higher resistance of UAFR biofilms to a 1-min low-pH challenge could be due to the higher biomass of UAFR as compared to the wild-type strain. The treatment time (1 min) may have been too short to allow effective penetration of acid solution into the dense biofilm layer. However, the resistance of UAFR to CHX treatments when the biofilms were grown under pH-cycling condition cannot be simply explained by its high biomass, since the same concentrations of CHX was applied to the biofilms grown under either pH condition. Further studies are necessary to investigate the role of different gene mutations in UAFR strain in its resistance to CHX treatments.

As shown in the treatment design of this study (Figure 1), the duration of low-pH challenge (up to 30 min) was much longer for the biofilms grown under pH-cycling conditions than for the biofilms grown under constantly neutral pH conditions (up to 5 min). This increased resistance to the low-pH challenge might be related to the acid tolerance level of the biofilm grown under pH cycling. It is known that *S. mutans* is able to alter its physiology under acidic conditions in order to survive: this adaptive response is referred as the acid tolerance response (ATR) (Welin-Neilands and Svensäter 2007). Several studies have demonstrated that planktonic cells and cells in biofilms survived acid killing better after a prior exposure to low but nonlethal pH values (McNeill and Hamilton 2003; Welin-Neilands and Svensäter 2007). In our case, the biofilms grown under pH-cycling conditions have been exposed to pH 5.5 for 16 h before the acid killing treatment, which might trigger the adaptive response and result in stronger resistance to acid killing.

This study showed that duration of 16 h was required to measure an inhibitory effect of NaF on the biofilms. It also determined the inhibitory concentrations of NaF on biofilms, being 3-12 mM when the pH of NaF solutions was neutral and 0.25-1 mM when the pH of NaF solutions was 5.5. Although these are *in vitro* findings, they are helpful in estimating the selection pressure that a daily usage of NaF can impose on the oral microbes. *In vivo*, the concentration of fluoride remained in dental biofilms after application of fluoride-containing products was reported to be between 0.06 and 0.3 mM F (Duckworth et al. 1987; Naumova et al. 2012). In a healthy oral ecosystem, where the pH of dental biofilms is mostly neutral, the daily application of fluoride would not impose any selection pressure, since our data showed the effective treatment concentrations of NaF should be above 3 mM (57 ppm F) when the pH of NaF so-

lution was neutral. However, this might not be the case for the dental biofilms in caries-susceptible subjects or at approximal sites, where the pH of these biofilms often remains low for an extensive period of time (Jensen and Schachtele 1983). Our data showed that the fluoride-resistant biofilms (UF35) produced significantly more lactic acid (1.05 ± 0.07 mM) than the fluoride-sensitive UA159 biofilms under pH cycling after being treated with 0.25 mM NaF. In the caries-susceptible biofilms *in vivo*, the daily application of fluoride would likely promote fluoride-resistant strains. Therefore, fluoride-resistant strains might only present in a specific niche in the oral cavity.

In summary, this study compared the fitness of two fluoride-resistant strains with its wild-type fluoride-sensitive strain. In the absence of fluoride, neither fluoride-resistant strain showed compromised fitness as compared to the wild-type strain. In addition, the fluoride-resistant strain containing multiple chromosomal mutations exhibited better fitness than the fluoride-resistant strain containing a single chromosomal mutation and the wild-type strain. Additional studies are needed to understand the role of multiple chromosomal mutations in the fitness and fluoride resistance.

Funding

The authors declare no conflicts of interest. This work was supported by the National Natural Science Foundation of China (81670983), and Guangdong Science and Technology Department (2015A020212004, 2013B021800060).

Acknowledgements

The authors thank Prof. Zhimin Zhang for providing the strain UA159-FR.