Fluoride resistance in Streptococcus mutans
Liao, Ying

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Chapter 3

A single nucleotide change in the promoter mutp enhances fluoride resistance of Streptococcus mutans

A shortened version of this chapter has been published as:
Abstract
Microorganisms can utilize fluoride antiporters to escape toxicity of fluoride. Recently, we identified a single nucleotide mutation in the promoter of the fluoride antiporter-coding genes in a naturally selected fluoride-resistant *Streptococcus mutans* strain. In this study we explored the role of this mutation in fluoride resistance and in the virulence of *S. mutans* in a defined genetic background.

An *S. mutans* UA159 mutant with the single nucleotide mutation -44A→C in the promoter *mutp* was constructed using PCR-based site-directed mutagenesis. We examined the growth and lactic acid production of the wild-type and mutant strains in the presence of fluoride. The response of the two strains to acidic stress was compared using an acid-killing assay. The expression of the genes downstream of *mutp* in both strains was quantified with real-time PCR. The activities of the wild-type and mutated promoters were evaluated using green fluorescence protein (GFP) reporter strains.

At physiological level, fluoride resistance in growth and lactic acid production was stronger in the mutant strain than in the wild-type strain. However, the mutant strain was less tolerant to acidic stress than the wild-type strain. The single nucleotide mutation in *mutp* significantly upregulated the expression of the downstream genes and increased the activity of the promoter independently of the presence of fluoride.

These results suggest that the single mutation in the promoter *mutp* confers fluoride resistance on *S. mutans* by constitutively increasing the promoter activity and up-regulating the downstream fluoride antiporter-coding genes.
Mutation in mutp enhances fluoride resistance

Introduction

Fluoride is highly abundant in the earth’s crust (Mason and Moore 1982). It is widespread in air, water and soil. Its concentrations vary depending on location, ranging from $<10 \mu\text{M}$ to $>100$ mM in water and $<0.5$ mmol/kg to $>100$ mmol/kg in soil (IPCS 2002). Especially in ground water, its concentration is higher than most other anions (Jagtap et al. 2012). Since fluoride was discovered as a therapeutic agent for caries prevention, it has been used in various oral hygiene products, in concentrations ranging from 50 mM to 140 mM in toothpaste, to 647 mM in fluoride gels (Lagerweij et al. 2006; Walsh et al. 2010).

Because fluoride is toxic for bacteria, fungi, plants and animals at high concentrations, it has been used as an antimicrobial agent for a long time (Weinstein and Davidson 2004). The mechanisms of fluoride toxicity for various species are complex (Marquis et al. 2003). It is thought that fluoride can efficiently enter bacterial cells at acidic pH values as hydrogen fluoride (HF) and dissociates when exposed to the more neutral intracellular pH (Marquis et al. 2003). These intracellular fluoride ions inhibit the activities of essential enzymes, such as enolases, ureases and phosphatases (Marquis et al. 2003). There are also reports that fluoride affected the activity of phosphoryl transfer enzymes through the formation of the inorganic complexes with metallic ions, such as aluminium or beryllium (Barbier et al. 2010).

Due to the extensive use and toxic effects of fluoride, microorganisms are able to develop resistance to fluoride. For example, more than 100 mM sodium fluoride (NaF) is required to inhibit the growth of Candida albicans or Escherichia coli. A fluoride-resistant Streptococcus mutans strain was isolated from an xerostomia patient who had received topical fluoride treatment (263 mM NaF) daily (Streckfuss et al. 1980). Recent studies have revealed that a fluoride antiporter protein (CrcB) helps microbes to combat fluoride toxicity. This protein is a membrane-associated transporter with specificity for fluoride, and can export fluoride ions (F-) to maintain a low F- level in the cells (Baker et al. 2012; Li et al. 2013; Ren et al. 2012). It has been shown that the expression of this protein is regulated by riboswitches, a class of regulatory RNA molecules (Baker et al. 2012). These riboswitches are able to sense and bind to fluoride, and thus to regulate the expression of the fluoride antiporter. Although homologues of crcB can be identified in the three domains of life (bacteria,
archaea and eukaryota), the fluoride-sensing riboswitches are much less prevalent. They are absent in eukaryotic organisms such as fungi and plants, and prokaryotic organisms such as \textit{S. mutans}, \textit{Staphylococcus} and \textit{Salmonella} (Baker et al. 2012). The role of the fluoride antiporter has been shown in some of these organisms (Li et al. 2013), but little is known about the regulation of this system.

In a previous study, we compared the genome sequence of a naturally selected fluoride-resistant \textit{S. mutans} strain with that of a fluoride-sensitive strain using whole genome shotgun Illumina sequencing, and discovered one single nucleotide mutation (-44A→C) located in the promoter of two tandem genes coding for the fluoride antiporters (Liao et al. 2015). The expression of these genes was much higher in the fluoride-resistant strain than in the fluoride-sensitive strain. Although this evidence indicated that the mutation is involved in regulating the fluoride antiporters, it is uncertain whether the fluoride resistance is due to the mutation. This is because more differences were found in the genome comparison than the mutation mentioned above.

The aim of the present study was to investigate the role of this single nucleotide mutation in the fluoride resistance of \textit{S. mutans} in a defined genome environment. To this end, we constructed a mutant strain from fluoride-sensitive \textit{S. mutans} strain UA159 with the same mutation (-44A→C) in the promoter of the fluoride antiporter-coding genes referred to above. The promoter region will be referred to as mutp.

\textbf{Materials & methods}

\textit{Bacterial strains, plasmids and media}

Bacterial strains and plasmids used in this study are listed in Table 1. \textit{E. coli} was grown in Luria-Bertani medium or on Luria-Bertani agar aerobically at 37°C. \textit{S. mutans} strains were routinely cultured anaerobically (90% N₂, 5% CO₂, 5% H₂) at 37°C using either Brain Heart Infusion (BHI) broth or agar. Where indicated, ampicillin (Amp) (100 µg / ml) or erythromycin (Em) (300 µg / ml) was used for \textit{E. coli} and Em (10 µg / ml) for \textit{S. mutans}. 
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain / plasmid</th>
<th>Relevant characteristics</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F-, endA1deoR, recA1, hsdR17 (rK, mK), supE44, thi-1, gyrA96, relA</td>
<td>Thermo Scientific, MA, USA</td>
</tr>
<tr>
<td>EC1000</td>
<td>RepA+ MC1000, Km+, carrying a single copy of the pWV01 repA gene in the glgB gene</td>
<td>(Leenhouts et al. 1996)</td>
</tr>
<tr>
<td><strong>S. mutans strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UA159</td>
<td>Wild-type strain</td>
<td>(Ajdic et al. 2002)</td>
</tr>
<tr>
<td>UF35</td>
<td>UA159 mutant, mutation in mutp (-44 A→C)</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pJET1.2 / blunt</td>
<td>Ampr</td>
<td>Thermo Scientific, MA, USA</td>
</tr>
<tr>
<td>pORI280</td>
<td>Emr, lacZ+, ori+ of pWV01, replicates only in strains providing repA in trans</td>
<td>(Leenhouts et al. 1996)</td>
</tr>
<tr>
<td>pLY35</td>
<td>pJET1.2 with mutp from UA159; Ampr</td>
<td>This study</td>
</tr>
<tr>
<td>pLY35M</td>
<td>pJET1.2 with mutp (-44 A→C); Ampr</td>
<td>This study</td>
</tr>
<tr>
<td>pORI280-35M</td>
<td>pORI280 with mutp (-44 A→C); Emr; lacZ+</td>
<td>This study</td>
</tr>
<tr>
<td>pDM45</td>
<td>E. coli-streptococcal shuttle vector; Emr; harboring gfpmut2</td>
<td>This study</td>
</tr>
<tr>
<td>pLY26</td>
<td>mutpUA159:gfpmut2 in pDM45</td>
<td>This study</td>
</tr>
<tr>
<td>pLY27</td>
<td>mutpUF35:gfpmut2 in pDM45</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Construction of the mutp -44A→C mutant**

The promoter mutp mutant of S. mutans UA159 was constructed in two steps: 1) Creation of the point mutation. A 697 bp region at mutp, containing the adjacent up-stream and down-stream sequences, was PCR amplified from genomic DNA of strain UA159 and inserted into a commercial cloning vector pJET1.2 (Thermo Scientific, MA, USA). A standard site-directed mutagenesis (mutp -44A→C) was carried out on the constructed plasmid. 2) Construction of the clean mutp mutant. The 697 bp of mutp with the mutation was inserted into a suicide vector pORI280 and the resulting plasmid was transformed into S. mutans UA159 (Leenhouts et al. 1996). The transformants in which a plasmid had integrated in the chromosome via a single crossover event were grown overnight in Todd-Hewitt broth with 10 µg / ml Em. Cultures were then diluted in the same medium without antibiotics to density of 10 cells/ml, followed by growth for approximately 30-40 generations. Dilutions of the cultures were spread on BHI agar plates supplemented with 40 µg / ml 5-bromo-4-chloro-3-indolylgalactopyranoside (X-gal). After an anaerobic incubation for 48 h, white colonies were selected from the plates. Either wild-type or mutant strains ap-
peared white due to a second crossover event. Sanger sequencing was performed on the white colonies to confirm the presence of the mutation (Deng et al. 2007b). The mutant was designated UF35 and examined further at physiological level and genetic level.

**Construction of Green fluorescent protein reporter strains**

Green fluorescent protein (GFP) reporter fusions were constructed and transformed in *S. mutans* and *E. coli* following a reported procedure (Deng et al. 2007a). Briefly, the mutp regions from *S. mutans* UA159 and UF35 were PCR-amplified with primers mutp_gfp_F and mutp_gfp_R (Table 2) and ligated into a shuttle vector pDM45, which contains a promoterless green fluorescent protein coding gene (gfpmut2) (Cormack et al. 1996), resulting in pLY26 (with the wild-type mutp) and pLY27 (with mutp -44A→C). Both constructs were transformed into either *E. coli* DH5α or *S. mutans* wild-type strain UA159 after the sequences of the insertions were confirmed with Sanger sequencing. The reporter strains were used to examine the activity of promoter mutp.

**Table 2. Primers used in this study**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide sequence (5’ to 3’)*</th>
<th>Product sizeb</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>mutp_F</td>
<td>GAATTCCTTAATCCCCATCTAACCTCACTCT</td>
<td>697</td>
<td>Amplification of fragment containing mutp (-44A) from UA159 genomic DNA for mutagenesis</td>
</tr>
<tr>
<td>mutp_R</td>
<td>GCAATGCAATACCATAGGAATACCTCACT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mutp_M_F</td>
<td>CAAAAAAGGCTTGGTCTAGCTTTCTTTTTT</td>
<td>697</td>
<td>PCR-based site-directed mutagenesis in mutp (-44A→C)</td>
</tr>
<tr>
<td>mutp_M_R</td>
<td>GAAGAAAGAAAGCTAGACGCAAGGCTTTTTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mutp_con_F</td>
<td>CAACGATGCCAACACAA</td>
<td>875</td>
<td>Confirmation for mutation in mutp (-44A→C)</td>
</tr>
<tr>
<td>mutp_con_R</td>
<td>TTAACAGCGCCACTCTCTCTCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mutrt_F</td>
<td>ATGGTGAGCGATATGTA</td>
<td>146</td>
<td>Real time PCR; expression of mut</td>
</tr>
<tr>
<td>mutrt_R</td>
<td>TGTCTAGAAAGACGAATGCT</td>
<td>131</td>
<td>Real time PCR; expression of perA</td>
</tr>
<tr>
<td>perA rt_F</td>
<td>TTACTGCCTGCTGGTATGG</td>
<td>140</td>
<td>Real time PCR; expression of perB</td>
</tr>
<tr>
<td>perA rt_R</td>
<td>TGCTGATAAGGTATATCATCTGTAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>perB rt_F</td>
<td>AGATGCTAATCCTTGGTA</td>
<td>106</td>
<td>Amplification of mutp region from UA159 and UF35 for GFP fusions</td>
</tr>
<tr>
<td>perB rt_R</td>
<td>TATGGTCTTCTCTTTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mutp_gfp_F</td>
<td>CATATGAGGCCCTCTCTTCTTTACCTAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mutp_gfp_R</td>
<td>GCATGCAGCTCATATTACTGGGCTATTTA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*restriction sites are in boldface: EcoRI, GAATTC; SphI, GCATGC; NdeI, CATATG

b in bp
Mutation in *mutp* enhances fluoride resistance

**Growth assays**

The growth of *S. mutans* wild-type (UA159) and the mutant (UF35) strains in the presence of fluoride were examined both on solid agar plates and in broth. For growth on solid agar, *S. mutans* was first grown in BHI broth overnight. Five microliter of 10^4 to 10^5-fold serially diluted overnight cultures were spotted on BHI agar plates supplemented with 0, 2, 4, 8, 12 mM of NaF. All plates were incubated anaerobically at 37°C for 3 d before recording images. For growth in broth, the overnight cultures were first diluted at the ratio of 1:20 in fresh BHI broth and grown until early exponential phase (OD_{600} ~ 0.3). NaF was then added to reach the final concentrations of 0, 5, 10, 20 mM. The OD_{600} of all groups was recorded every 30 min for a total duration of 20 h at 37°C in SpectraMax Plus 384 (Molecular Devices, CA, USA). Data was plotted with GraphPad Prism (version 4.00 GraphPad Software, San Diego, California, USA). The experiments were repeated twice.

**Lactic acid production**

Lactic acid production of *S. mutans* UA159 and UF35 strains was examined in the presence of glucose and NaF at pH 7.0 or pH 5.5. The *S. mutans* strains were grown in BHI broth until late exponential phase. Cells (approximately 10^9 CFU/ml) were harvested and resuspended in either pH 7.0 or pH 5.5 buffered medium. The designated amount of glucose (0.4%) and NaF were then added to the suspensions. The medium was either buffered with 90 mM potassium phosphate to maintain pH 7.0 or with 30 mM 2-(N-morpholino)ethanesulfonic acid (MES) to maintain pH 5.5. The final concentration of NaF was 10mM for pH 7.0 condition and 1.5 mM for pH 5.5 condition. In the control groups, Milli-Q was used to replace NaF. All mixtures were incubated at 37°C for 1 h. At the end of the incubation, the mixtures were incubated at 80°C for 5 min to release the intracellular acid. The supernatants were collected for lactic acid determination after 2 min centrifugation at 16,100 x g at room temperature. The lactic acid concentration was determined enzymatically, by measuring the amount of NADH formed during the conversion from lactate to pyruvate in the presence of L-lactate dehydrogenase and NAD (Gutmann and Wahlefeld 1974). The experiment was performed in triplicate.
Acid tolerance response (ATR)

*S. mutans* UA159 and UF35 strains were also examined for their abilities to survive at a killing pH (pH 3.0). Induction of acid tolerance response (ATR) was performed as previously described with minor modifications (Svensäter et al. 1997). *S. mutans* was grown until mid-exponential phase (OD<sub>600</sub> = 0.5) in BHI broth, harvested with centrifugation (4,528 × g for 10 min at 4°C) and resuspended in BHI broth supplement with 40 mM potassium phosphate/citrate buffer (pH 5.5) at a density of approximately 1<sup>9</sup> colony forming units (CFU) / ml. After 2 h incubation at 37°C, suspensions were washed with 50 mM KCl and 1 mM MgCl<sub>2</sub>, resuspended in 40 mM potassium phosphate / citrate buffer (pH 3.0) and followed by 45 min incubation at 37°C. Samples were taken before the incubation at pH 3.0 and at 15, 30 and 45 min during the incubation, serially diluted and plated on BHI agar. CFUs were counted after 48 h anaerobic incubation. The survival rates were calculated as \( \frac{N}{N_0} \), where \( N_0 \) was the initial population of cells and \( N \) was the population after exposure to killing pH for 15, 30 and 45 min. The ATR experiment was performed in triplicate.

Gene expression

The expression of the genes downstream of *mutp*, namely *mut*, *perA* and *perB*, was examined in *S. mutans* wild-type and mutant strains by real-time PCR. *S. mutans* strains were grown in BHI broth without fluoride until early exponential, late exponential and stationary phases. Samples were taken at three phases and processed as previously described (Liao et al. 2015). Briefly, total RNA was isolated using the Genejet RNA kit (Thermo Scientific, MA, USA), followed by DNase treatment with the TURBO DNA-free kit (Life Technologies, Carlsbad, USA) and cDNA synthesis with the RevertAid first strand cDNA synthesis kit (Thermo Scientific, MA, USA). Primers used for real-time PCR are listed in Table 2. *gyrA* and *recA* were used as reference genes (Brenot et al. 2005; Smoot et al. 2001). Specificity of PCR reactions was confirmed with melting curve analysis. A \( 2^{\Delta\Delta CT} \) method was used to calculate relative gene expression (Schmittgen and Livak 2008). The experiment was performed in triplicate.
Mutation in mutp enhances fluoride resistance

**Green fluorescence assay**
The activity of wild-type mutp and mutated mutp was examined in either *E. coli* DH5α or *S. mutans* UA159 reporter strains using a green fluorescence assay. Overnight cultures were diluted in PBS or PBS containing 10mM NaF till approximately $10^7$ CFU/ml. After 1 h incubation at 37°C, the fluorescence signals of the dilutions were measured with BD Accuri C6 Cytometer (BD Biosciences, CA, USA) using 488 nm as excitation wavelength and 530 nm as emission wavelength. Ten thousand events were collected in each sample. Data were acquired and analyzed with CFlow software (BD Biosciences, CA, USA). Mean fluorescence intensities (FI) were calculated for events gated from forward-scatter and side-scatter analysis. The corresponding strain containing pDM45 (the construct with promoterless GFP fusion) was used as a negative control. Fluorescence intensities of the reporter strains were calculated by subtracting the mean FI emitted by the negative control from the mean FI of the reporter strains. The experiment was performed in triplicate.

**Statistical analysis**
Data was analyzed with IBM SPSS Statistics version 23.0 (IBM Corporation, NY, USA). The unpaired Student’s *t* test was performed for comparisons between survival rates of the two strains in ATR, gene expression of two strains in different growth phases, and fluorescence intensities of two GFP constructs in different strains. Three-way ANOVA was performed to determine the effect of the independent variables (pH, fluoride and strain) and their interactions on lactic acid production. Subsequently, unpaired Student’s *t* test was performed to compare the lactic acid production of two strains under different pH and fluoride conditions. Differences were considered statistically significant at $p < 0.05$ for comparison of survival rates in ATR and fluorescence intensities; at $p < 0.0167$ for comparisons of gene expression and $p < 0.0125$ for comparisons of lactic acid production (after Bonferroni correction).

**Results**

**Differences between wild-type and mutant strains at physiological level**
The wild-type *S. mutans* UA159 and the single-nucleotide-mutant UF35 strains were examined for their physiological characteristics, including their abilities
to grow and produce lactic acid in the presence of fluoride, and also to respond to acidic stress.

Irrespective of the test methods, the mutant strain clearly grew better in the presence of NaF than the wild-type strain. On solid agar, the mutant strain grew until the concentration of NaF reached 12 mM, while the wild-type strain stopped growing when NaF was 4 mM (Figure 1). In broth, the growth of the

Figure 1. Representative Growth of \textit{S. mutans} UA159 and UF35 on BHI agar plates. NaF is supplemented at increasing concentrations from (A) to (D): (A) without NaF; (B) 2 mM NaF; (C) 4 mM NaF; (D) 8 mM NaF. Overnight grown cultures were serially diluted $10^i$-fold (D1) to $10^j$-fold (D5).
Mutation in *mutp* enhances fluoride resistance

mutant strain was inhibited by 20 mM NaF, while that of the wild-type strain was completely inhibited at half that concentration (Figure 2). When fluoride was absent, the doubling time for both the wild-type and mutant strains was similar, at 48.0 ± 1.4 min and 51.0 ± 0.8 min, respectively.

**Figure 2.** Growth of *S. mutans* (A) UA159, and (B) UF35 in BHI broth without NaF (open circles) or with 5 mM (filled circles), 10 mM (open squares), 20 mM (filled squares) NaF. Data are expressed as means ± standard deviation of two independent experiments.

Since the efficacy of fluoride is different at neutral and acidic pH (Marquis et al. 2003), we wished to examine the effect of fluoride on lactic acid production in different pH conditions. To do so, we conducted a pilot study in
which we selected the minimum inhibitory concentrations of NaF for lactic acid production of the wild-type strain, which were 10 mM for pH 7.0 and 1.5 mM for pH 5.5. Figure 3 shows that the selected concentrations of NaF significantly inhibited the lactic acid production of both wild-type and mutant strains at the designated pH ($p < 0.005$). However, in the presence of NaF, the mutant strain produced significantly more lactic acid than the wild-type strain. The interaction of pH, fluoride and strain did not significantly affect lactic acid production ($p = 0.138$).

![Figure 3](image)

**Figure 3.** Lactic acid production within 1 h by *S. mutans* UA159 and UF35 at (A) pH 7.0 and (B) pH 5.5 with and without NaF. Means ± standard deviation of three independent experiments are shown. *** indicates $p < 0.001$.  

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Mutation in \textit{mutp} enhances fluoride resistance

In contrast, comparison of the acid tolerance showed different fitness patterns between these two strains. The 2 h incubation at pH 5.5 had no effect on the viable cell counts (10^9 CFU/ml) of either the wild-type strain or the mutant strain. But exposure to pH 3.0 reduced the survival rate of the mutant strain significantly more than that of the wild-type strain (Figure 4).

![Figure 4](image)

**Figure 4.** Survival of \textit{S. mutans} UA159 and UF35 during a 45-min exposure to the killing pH (3.0). The survival rates were calculated as $N / N_0$, where $N_0$ was the initial population of cells and $N$ was the population after exposure to killing pH for 15, 30 and 45 min. Means ± standard deviation of three independent experiments are shown. * indicates $p < 0.05$. ** indicates $p < 0.01$.

**Differences between wild-type and mutant strains at genetic level**

To understand the molecular mechanism underlying this fluoride resistance and loss of acid tolerance, we further examined the expression of the genes downstream of \textit{mutp}, and also the activities of the wild-type and the mutated \textit{mutp}. Figure 5 displays the organization of genes downstream of \textit{mutp} in the genome of \textit{S. mutans} UA159 (Figure 5A) and the relative expression of the three downstream genes (Figure 5B ~ D). The genes \textit{mut} and \textit{perA} form an operon. The genes \textit{perA} and \textit{perB} encode homologues of the fluoride antiporter and share 58% sequence similarity. Throughout the growth phases (early exponential, late exponential and stationary), the expression of all three downstream genes in the mutant strain was 6-fold higher than that in the wild-type strain, even when fluoride was not present.
Figure 5. (A) Arrangement of the promoter mutp and the downstream genes. The location of the mutation -44A→C is shown with the vertical red line. The sequences of mutp from UA159 and UF35 are shown in the box below. The mutation is shown in red. The predicted -10 element is in the purple box; the predicted -35 element is in the red box. The start codon of the first downstream gene is shown in blue. mut, encodes chorismate mutase; perA, encodes a homologue of fluoride antiporter; perB, encodes another homologue of fluoride antiporter and shares 58% identity with perA. The bar graphs (B to D) show the relative expression of the downstream genes of mutp in S. mutans UA159 and UF35 at early exponential phase (B), late exponential phase (C) and stationary phase (D). Overall expression of each selected gene in UF35 relative to that in UA159 is presented as average fold-change ± standard deviation. This experiment was repeated three times. The significance level (α) was set at 0.0167 (after Bonferroni correction for three genes). ** indicates \( p < 0.005 \). *** indicates \( p < 0.0001 \).

Since the wild-type mutp and the mutated mutp has been separately
fused with GFP, the activities of these two promoters could be followed by the fluorescence intensities (FI) of the reporter strains. Figure 6 shows the FIs of the constructs containing the wild-type mutp or the mutated mutp. In both S. mutans UA159 and E. coli DH5α, the strains containing the mutated mutp exhibited significantly higher FI than those with the wild-type mutp \((p < 0.0001)\). The FIs of the reporter S. mutans strains were also examined after addition of 10 mM NaF for 1 h. The presence of fluoride did not have any influence on the FIs of the strains (data not shown).

**Figure 6.** Quantification of fluorescence of the GFP reporter strains: S. mutans UA159 containing the wild-type mutp-GFP construct or the mutated mutp-GFP construct; and E. coli DH5α containing the wild-type mutp-GFP construct or the mutated mutp-GFP construct. Fluorescence values emitted by the negative control (strains containing pDM45) were subtracted from the mean fluorescence intensities of the reporter strains. Means ± standard deviation of triplicate experiments are shown. AU, arbitrary units. *** indicates \(p < 0.0001\).

**Discussion**

This study demonstrates that a single mutation in the mutp promoter region in S. mutans, namely -44A→C, results in a clear resistance to fluoride in terms of growth and lactic acid production. The fluoride resistance may be due to up-regulation of the downstream fluoride antiporter homologues through the enhanced mutp activity. As stated previously, some microorganisms, such as E. coli and Bacillus anthracis, were able to up-regulate the fluoride antiporter through fluoride-sensing riboswitch RNAs and thereby to alleviate the deleterious effects of fluoride (Baker et al. 2012). But not all prokaryotes possess fluo-
ride-sensing RNAs (Baker et al. 2012). Our data might indicate an alternative mechanism whereby they can relieve the toxic effects of fluoride.

Unlike previous results that showed fluoride-related regulation of fluoride antiporters, the high activity of mutated mutp we observed and the resulting high expression of fluoride antiporters were not related to fluoride. In other words, the single mutation leads to constitutively high activity of the promoter. Moreover, as high activity of the mutated mutp could be seen not only in *S. mutans*, the bacterial species where mutp was derived, but also in *E. coli*, an unrelated bacterial species, the activity of mutated mutp may be regulated not by *S. mutans*-specific transcriptional factors, but by a more universal mechanism.

The antimicrobial resistance caused by mutations in promoters has been reported for various microorganisms, including *E. coli*, *Staphylococcus aureus* and *Campylobacter jejuni* (Caroff et al. 2000; Ng et al. 1994; Zeng et al. 2014). These mutations are often located at the -35 element (Normark and Normark 2002; Sherman et al. 1996; Toprak et al. 2012), an important RNA polymerase binding site (Murakami et al. 2002). It is thought that specific nucleotide-sequence patterns within the -35 element could lead to an arrangement of hydrogen bonds that facilitates the activity of RNA polymerase and hence overexpression of downstream genes (Castagnoli 1987). *In silico* examination of mutp shows that the -44A→C mutation locates in the putative -35 element (Liao et al. 2015). A comparison between the canonical sequence of the -35 element (TTGACA) and the sequence of the -35 element from wild-type mutp (TAGAAG) or mutated mutp (TAGACG) shows that the mutated sequence is more similar to the canonical sequence (Figure 5A) (Liao et al. 2015; Murakami et al. 2002). Therefore, the -44A→C mutation likely leads to better binding between RNA polymerase and mutp, resulting in an increased transcription rate (Jaurin et al. 1982) and thus up-regulation of the fluoride antiporter.

The ability of *S. mutans* to endure acidic stress is an important virulence factor that allows this species to be dominant in the oral microbiome in an acidic environment and to cause dental caries (Matsui and Cvitkovitch 2010). A previous *in vitro* study showed that a fluoride-resistant *S. mutans* strain could tolerate acid stress better than the fluoride-sensitive strain (Zhu et al. 2012). Using a similar experimental setup, we evaluated the acid tolerance of the constructed mutant strain, but found lower acid tolerance than in the
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wild-type strain. The increased sensitivity of the mutant strain to low pH may have been due to an energy-spilling “futile proton cycle” (Russell 2007). The fluoride antiporter, which was identified as an F\(^-\)/H\(^+\) antiporter, also helps with proton influx (Stockbridge et al. 2012). The protons extruded by proton pumps can reenter the cells through the antiporter, causing a futile cycle. Meanwhile, as the overproduction of fluoride antiporters requires energy, it also sensitizes the mutant strain to low pH. It is generally believed that acquisition of drug resistance, particularly the resistance mediated by chromosomal mutations, may incur a fitness cost in bacteria. However, compensatory mutations may partly or fully ameliorate this cost (Andersson 2003). Our constructed mutant strain contains one mutation in the chromosome, while the fluoride-resistant strain used in our previous study was naturally selected from fluoride containing agar plates and has multiple mutations in the chromosome (Liao et al. 2015). Although it is therefore likely that the resistance to fluoride impairs the acid tolerance of S. mutans, an alternative mutation or mutations may compensate for this fitness cost, or even enhance the fitness. We are carrying out further studies to investigate the potential alternative mutations.

In conclusion, our study shows that the novel -44A→C mutation in the promoter of fluoride antiporter-coding genes leads to fluoride resistance in S. mutans through increased promoter activity and increased expression of downstream genes. This mechanism for gaining fluoride resistance has the potential for use by a wide range of microorganisms. The sensitivity to acidic stress of the fluoride-resistant mutant is increased as a biological cost of fluoride resistance, which may thus affect the competitiveness of the resistant strain in the environment.

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