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A Single Nucleotide Change in the Promoter mutp Enhances Fluoride Resistance of Streptococcus mutans

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Previously, we identified a single nucleotide mutation in the promoter (mutp) of the fluoride antiporter-coding genes in a naturally fluoride-resistant Streptococcus mutans strain. Here, we studied the role of this mutation in a defined genetic background. The results confirmed that this mutation alone confers fluoride resistance on S. mutans, as shown by growth and lactic acid production assays. This resistance was explained by constitutively higher mutp promoter activity and upregulation of the fluoride antiporter-coding genes.

Fluoride is highly abundant in nature and is extensively used in oral hygiene products (1, 2). At high concentrations, it can be bactericidal. Due to the extensive use of fluoride, many microorganisms have developed resistance to fluoride (3). Recent studies revealed that a conserved fluoride antiporter protein (CrcB) mediates microbial fluoride resistance by exporting fluoride ions (F−) to maintain a low F− level in the cell (4, 5). The expression of the fluoride antiporter genes can be regulated by fluoride-sensing riboswitches (4). However, these riboswitches are absent in microorganisms such as Streptococcus mutans and staphylococci (4). Little is known about the regulation of the fluoride antiporter genes in these microorganisms.

In a previous study, we compared the genome sequence of a naturally selected fluoride-resistant S. mutans strain to that of a fluoride-sensitive strain using whole-genome shotgun sequencing and discovered a single nucleotide mutation (−44A→C) located in the promoter of two tandem genes coding for the fluoride antiporters (6). The expression of these genes was 10-fold higher in the fluoride-resistant strain than in the fluoride-sensitive strain. Although this evidence indicated that this mutation is involved in the expression regulation of the fluoride antiporters, it is uncertain whether this mutation alone causes fluoride resistance, since an additional seven mutations were found in the genome comparison. Thus, we aimed to investigate the role of this single nucleotide mutation in the fluoride resistance of S. mutans in a defined genomic environment. The promoter containing this mutation is referred to as mutp.

We constructed a clean mutp mutant from S. mutans strain UA159 using site-directed mutagenesis and an unlabeled gene replacement system (7). The successful introduction of the base change, −44A→C, in mutp was confirmed by Sanger sequencing. The corresponding mutant strain was designated UF35 and char-

FIG 1 Representative growth of S. mutans strains UA159 and UF35 on BHI agar plates supplemented with increasing concentrations of NaF: 0 mM NaF (A), 2 mM NaF (B), 4 mM NaF (C), and 8 mM NaF (D). Overnight-grown cultures were serially diluted 10-fold (D1) to 105-fold (D5). This experiment was done in triplicate.
characterized further at the physiological and genetic levels. The primers used in the construction of this strain are listed in Table S1 in the supplemental material.

First, we examined the growth and lactic acid production of the *S. mutans* UA159 and UF35 strains in the presence of fluoride. To evaluate the growth of *S. mutans*, we spotted 5 μL of 10⁻¹ to 10⁻⁵-fold serially diluted overnight cultures onto brain heart infusion (BHI) agar plates supplemented with 0, 2, 4, and 8 mM NaF and incubated the plates anaerobically at 37°C for 3 days. UF35 was still able to grow at 8 mM NaF, while UA159 stopped growing at 4 mM NaF (Fig. 1). The growth rate of UA159 was compared to that of UF35 in BHI broth without fluoride. The doubling time of both strains was similar (48.0 ± 1.4 min for UA159 and 51.0 ± 0.8 min for UF35) (data not shown).

Since the antimicrobial efficacy of fluoride depends on the pH (8), we harvested late exponential cultures and resuspended the cells in pH 7.0 or pH 5.5 buffered medium containing 0.4% glucose and 10 or 1.5 mM NaF, respectively. The medium was 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. In the control groups, Milli-Q water replaced NaF. The lactic acid concentration in the suspension was determined after 1 h incubation (9). The lactate production was similar for both strains in the absence of fluoride. However, in the presence of fluoride, the mutant strain produced about 4-fold more lactate than the wild-type strain (P = 0.001). The difference was even more obvious at pH 5.5, with the mutant strain producing about 20-fold more lactate than the wild-type strain (P < 0.001). Collectively, these data indicated that a single base change in *mutp* indeed enables *S. mutans* to resist higher concentrations of fluoride.

Next, we examined the expression of three genes downstream of *mutp* to understand the mechanism of this fluoride resistance. The arrangement of *mutp* and the downstream genes *mut*, *perA*, and *perB* is shown in Fig. 2A. The *mut* and *perA* genes form an operon. The *perA* and *perB* genes encode homologues of the fluoride antiporter CrcB. The bar graphs (B to D) show the relative expression of the downstream genes of *mutp* in *S. mutans* UA159 and UF35 at the 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 ± SD. This experiment was repeated three times. The significance level (α) was set at 0.0167 (after Bonferroni correction for three genes). **, P < 0.005; ***, P < 0.0001.

FIG 2 (A) Arrangement of the promoter *mutp* and the downstream genes. The location of the −44A→C mutation is shown with the vertical red line. The sequences of *mutp* from strains UA159 and UF35 are shown in the box below. The mutation is shown in red. 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* and *perB* encode homologues of the fluoride antiporter CrcB. The bar graphs (B to D) show the relative expression of the downstream genes of *mutp* in *S. mutans* UA159 and UF35 at the 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 ± SD. This experiment was repeated three times. The significance level (α) was set at 0.0167 (after Bonferroni correction for three genes). **, P < 0.005; ***, P < 0.0001.
fluoride antiporter and share 58% identity. The expression of these three genes was quantified as described in our previous study (6). In brief, the S. mutans cells at the early exponential, late exponential, and stationary phases were collected and subjected to total RNA extraction, DNase treatment, cDNA synthesis, and real-time PCR. The 2−ΔΔCT method was used to calculate relative gene expression (10). As shown in Fig. 2, the expression of all three downstream genes in UF35 was 6-fold higher than that in UA159 for the three different growth phases, although fluoride was absent. Based on these results, we confirmed that UF35 acquired fluoride resistance through higher expression of the fluoride antiporters (perA and perB), driven by a constitutively higher activity of the mutated mutP.

We further found that the −44A→C mutation is located in the putative −35 element, an RNA polymerase binding site (11). Indeed, antimicrobial resistance caused by mutations in the −35 element has often been reported for various microorganisms (12–14). Specific nucleotide-sequence patterns within the −35 element reportedly can lead to an arrangement of hydrogen bonds that facilitates the activity of RNA polymerase, leading to the higher expression of downstream genes (15). The sequence of the −35 element from mutated mutP (TAGAGG) was more similar to the canonical sequence (TTGACA) than that of wild-type mutP (TAGAGG). It is likely that the −44A→C mutation results in better binding of RNA polymerase to mutP and hence an increased transcription rate (16).

The ability of S. mutans to endure acidic stress is an important virulence factor that allows this species to dominate the oral microbiome in an acidic environment and to cause dental caries (17). A naturally selected fluoride-resistant S. mutans strain was reported to tolerate acid stress better than the fluoride-sensitive strain (18). Here, we characterized the acid tolerance strain UF35, the clean mutant, following a similar protocol (18). In brief, the cells of strains UA159 and UF35 were harvested at the mid-exponential phase and preincubated in BHI broth supplemented with 40 mM potassium phosphate/citrate buffer (pH 5.5) for 2 h at 37°C. The cells were then exposed to pH 3.0 for 45 min. Before exposure and after 15, 30, and 45 min of exposure, CFU counts were determined. Exposure to pH 3.0 reduced the survival rates of both strains. However, the survival rate was significantly lower in UF35 than in UA159 (data not shown). After 45 min, UA159 exhibited a >10-fold higher survival rate than UF35 (P < 0.05). This result was opposite to that of the previous study (18). The reason for this discrepancy is not clear. Since the naturally selected fluoride-resistant S. mutans strain, which was studied by Zhu et al. (18), contains several mutations in its genome compared to the wild-type strain (data not shown), we cannot exclude the possibility that these additional mutations are related to this discrepancy. We hypothesize that the increased sensitivity of UF35 to low pH is due to an energy-spilling “futile proton cycle” (19). The fluoride antiporter not only acts as an F+/H+ antiporter but also facilitates proton influx (20). The protons extruded by proton pumps can reenter the cells through the antiporter, causing a futile cycle.

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 the downstream genes. The sensitivity to acidic stress of the fluoride-resistant mutant 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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