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### Fluoride resistance in *Streptococcus mutans*

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Chapter 7  
**Summary and future perspectives**



### Summary

Fluoride has been used as the most effective anti-caries agent for over five decades. Prolonged exposure of oral bacteria to high concentrations of fluoride can lead to the development of fluoride resistance. This thesis describes a number of experiments focusing on phenotypic and genotypic characteristics of fluoride-resistant strains of *Streptococcus mutans*, which is the most studied cariogenic bacterial species.

In **Chapter 2** we examined these characteristics of the fluoride-resistant strain *S. mutans* C180-2FR and its parental strain C180-2. *S. mutans* C180-2FR had a significantly stronger ability to grow in the presence of fluoride than C180-2. The two strains also exhibited obviously distinct colony morphologies. Next, in the hope of locating genes involved in fluoride resistance, we applied whole-genome shotgun (WGS) sequencing for the two strains. The whole genome sequences of these two strains were then compared. Single nucleotide polymorphisms (SNPs) in the genome of *S. mutans* C180-2FR were identified and confirmed by traditional Sanger sequencing. The functions of these genomic changes were further studied by quantifying the expression of the genes which contained SNPs. The genome comparison revealed eight SNPs in C180-2FR, which were located in five genes and two intergenic regions. Gene expression data showed that three genes downstream of a promoter containing a SNP were constitutively up-regulated in C180-2FR compared to C180-2. Interestingly, two out of the three genes, *perA* and *perB*, are homologs of *eriC<sup>F</sup>*, which is a confirmed fluoride antiporter-coding gene. This study proved that WGS sequencing is a useful method to uncover genomic changes in fluoride-resistant *S. mutans* strains.

The relationship between the mutation in the promoter (*mutp*) of fluoride antiporter-coding genes and fluoride resistance was further studied in **Chapter 3**. A mutant strain harbouring the mutation in *mutp* was constructed from the fluoride-sensitive strain *S. mutans* UA159. This mutant strain, named UF35, was characterized for its ability to grow and produce lactic acid in the presence of fluoride. We showed that this mutant strain, UF35, was able to grow with higher concentrations of fluoride than *S. mutans* UA159. In the presence of fluoride, UF35 produced significantly more lactic acid than UA159. However, the acid tolerance response (ATR) assay showed that the mutant strain was less tolerant to acidic stress than the wild-type strain. This increased

sensitivity of UF35 to low pH may be due to an energy-spilling “futile proton cycle” mediated by the fluoride antiporters. The effect of the mutation on *mutp* activity was evaluated by quantifying not only the expression of downstream genes, but also the fluorescence of reporter strains. Results from both measurements confirmed that the mutated *mutp* was constitutively more active than the wild-type *mutp*. We therefore concluded that the single nucleotide mutation in *mutp* increases the promoter activity and up-regulates the expression of the fluoride antiporters, leading to enhanced fluoride resistance in *S. mutans*.

Data from Chapter 3 confirmed the role of one of the eight mutations found in Chapter 2. While the role of fluoride export as a mechanism of fluoride resistance is easy to understand, it may not be the only way bacteria use to acquire fluoride resistance. Previous studies have shown that the same parental strain can develop different levels of fluoride resistance, indicating multiple genes may be involved in the process. To identify other factors related to fluoride resistance, in **Chapter 4**, the available genome sequences of two naturally selected fluoride-resistant strains (UA159FR and C180-2FR) and those of their parental strains (UA159 and C180-2) were analyzed. Common chromosomal regions with mutations in both fluoride-resistant strains were identified. The gene expression and enzyme activity of affected genes were evaluated. Mutations were found in three common loci, related to two promoters of functional genes and one pathway. Consistent with our previous study (Chapter 2), we found mutations in *mutp* in the fluoride-resistant strains and up-regulation of the fluoride antiporters in the two strains compared to their parental strain. This further emphasized the role of the fluoride antiporters in fluoride resistance. Mutations were also located in the promoter *glpFp*, whose downstream gene (*glpF*) encodes a glycerol uptake facilitator protein. A significantly lower expression of *glpF* was observed in the two fluoride-resistant strains than in the wild-type strains, which may change the membrane permeability and thus the influx of fluoride. The genes encoding enolase (*eno*) and pyruvate kinase (*pyk*), two key glycolytic enzymes, also contained mutations in the fluoride-resistant strains. *S. mutans* C180-2FR has two mutations in *pyk*, while UA159FR has one mutation in *eno* and *pyk*, respectively. We evaluated the effect of these mutations on the enzyme activities. Pyruvate kinase in *S. mutans* C180-2FR was completely deactivated because of the amino-acid substitution Y419D. Enolase in *S. mutans* UA159FR was less inhibited by fluoride than enolase in the wild-type strain UA159. The results described in Chapter 4 provided novel candidate genes for mechanisms of fluoride resistance related to fluoride transport and

glycolysis.

After characterizing the genotype of fluoride-resistant *S. mutans* strains, we investigated an important trait of fluoride-resistant strains, namely the ability to tolerate challenges, or the fitness of the strains. In **Chapter 5**, two fluoride-resistant strains, *S. mutans* UF35 as described in Chapter 3 and UA159FR in Chapter 4, as well as their wild-type strain UA159, were characterized for their fitness. Biofilms instead of planktonic cultures were used, as the former better mimic the bacterial lifestyle in dental plaque. The fitness of the biofilms of the two fluoride-resistant strains and their wild-type strain was examined by challenging them with fluoride, chlorhexidine and lethal pH (pH 3.0). Both *S. mutans* UF35 and UA159FR showed stronger resistance to fluoride than the wild-type strain UA159. The biofilms of *S. mutans* UA159FR were more resistant to chlorhexidine and killing pH than the biofilms of UF35 and UA159. In addition, the biomass of UA159FR was significantly higher compared to the other two strains. We thus concluded that the fitness of the fluoride-resistant *S. mutans* strains was better (UA159FR) than or equal (UF35) to the wild-type strain.

In addition to the fitness, the acidogenesis, or the ability to produce lactic acid, which contributes to the development of dental caries, is an important phenotype of *S. mutans*. In many studies, fluoride-resistant *S. mutans* strains are reported to produce more lactic acid than fluoride-sensitive strains in the presence of fluoride, indicating a higher glycolytic activity in fluoride-resistant strains. This may be due to the changes in the regulation of glucose uptake, as suggested by our data from Chapter 4. The mutation identified in *pyk* and the changed activity of pyruvate kinase in *S. mutans* C180-2FR may lead to changes in intracellular phosphoenolpyruvate concentration and thus alterations in glucose uptake. In **Chapter 6**, we compared glucose-uptake activity of a fluoride-sensitive and fluoride-resistant strain to better understand their difference in acid production in the presence of fluoride. The overall glucose-uptake activity and the expression of genes related to the glucose uptake of *S. mutans* C180-2 and C180-2FR in the absence and presence of fluoride was quantified. In addition, the activity of the PEP-dependent phosphotransferase system (PTS) in these two strains was evaluated. We observed significantly less inhibition of the overall glucose-uptake activity by fluoride in *S. mutans* C180-2FR compared to C180-2. The presence of fluoride triggered a clear up-regulation of glucose uptake-related genes in C180-2, while no differential gene regulation was observed in C180-2FR. In the absence of fluoride, C180-2 showed a sig-

nificantly higher PEP-PTS activity than C180-2FR. In the presence of fluoride, the two strains showed similar PEP-PTS activities. Data from this chapter reveals that glucose uptake is differently regulated in fluoride-sensitive and fluoride-resistant strains. The difference is visible both in the absence and in the presence of fluoride.

Overall, this thesis shows that fluoride-resistant *S. mutans* strains exhibit both phenotypic and genotypic changes. Multiple genomic mutations related to fluoride resistance have been identified. The regulation of the fluoride antiporters may be an efficient and essential way for *S. mutans* to acquire fluoride resistance. Other factors, including changes in glycolysis, may also play a role in the mechanism of fluoride resistance. Upon acquiring fluoride resistance, the bacteria experience changes in the fitness and glucose uptake. These phenotypical alterations can influence their ability to survive in the oral cavity under different challenges.

### **Future perspective**

The experiments described in this thesis are limited to lab-derived fluoride-resistant *S. mutans* strains. One major concern is whether fluoride resistance has widely occurred in the population during the last five decades, as fluoride has been globally used. To date, we can only find two reports of clinical fluoride-resistant *S. mutans* isolates, which are from 30 years ago. There has been no report on the prevalence of fluoride-resistant *S. mutans* among populations. To understand the clinical relevance of fluoride resistance, a prevalence study is needed. Specifically, it is worth mentioning that we should carefully define fluoride resistance. Fluoride resistance may develop gradually. *S. mutans* could have evolved to be more resistant to fluoride than 50 years ago. An ideal study would compare the ability of *S. mutans* isolates to resist fluoride between a group of people exposed to fluoride for a long time and another group without fluoride exposure. As it is difficult to find a population which is not exposed to fluoride, the reference for fluoride resistance can also be found in previous studies. For example, most studies find that the majority of fluoride-sensitive wild-type *S. mutans* strains are able to resist 100-300 ppm (5.3-15.8 mM) fluoride (Brussock and Kral 1987; Van Loveren et al. 1991b). Therefore, strains which can grow with higher than 300 ppm (15.8 mM) fluoride should be regarded as fluoride-resistant.

As stated previously, *S. mutans* may apply different methods to acquire

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fluoride resistance. Instead of using one mechanism, bacterial cells may combine several mechanisms to develop a higher level of fluoride resistance. We did find multiple chromosomal regions and proteins that are potentially related to fluoride resistance. However, our current data cannot confirm whether these factors contribute synergistically to bacterial fluoride resistance. A possible target for future studies would be to introduce candidate mutations in the fluoride-sensitive strains separately and construct mutant strains with different combinations of mutations. A comparison of the fluoride resistance of these mutant strains can determine the additive effect of the mutations.