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Fluoride resistance in *Streptococcus mutans*

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Chapter 1

General introduction

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Chapter 1

Fluoride is the most popular caries-preventive agent. It is widely used in many oral care products, such as toothpastes, mouthwashes, and gels (Anusavice et al. 2005). In addition, fluoride intake through drinking water is also very common: in 2012, about 435 million people from 25 countries received water fluoridated at the recommended concentration, while people from another 28 countries have naturally fluoride-containing water with fluoride concentrations sometimes above the recommended level (Lennon 2006). It is believed that the anti-caries effects of fluoride are due to its ability to protect dental hard tissues and inhibit bacterial growth and metabolism. On the one hand, fluoride in saliva can be absorbed to the surface of apatite crystals in acidic environments, which arrests demineralization. When the pH rises, fluorhydroxyapatite becomes highly supersaturated, which enhances the remineralization process (Buzalaf et al. 2011). This protection of enamel can be observed at fluoride concentrations as low as 0.02 ppm (0.001 mM) (Jacobson et al. 1991). On the other hand, fluoride is toxic to bacterial cells and can function as an antimicrobial, specifically at concentrations during and shortly after the use of oral hygiene products. Consecutive application of fluoride at 250–12,300 ppm (13–647 mM) has been shown to significantly reduce the numbers of *Streptococcus mutans* in dental plaque (Beighton and McDougall 1977; Loesche et al. 1975; Van Loveren 2001). The antimicrobial action of fluoride is enacted in three main aspects of the metabolism of oral bacteria: the acidogenicity, acidurance and adherence to the tooth surface (Hamilton and Ellwood 1978; Meurman 1988; Van Loveren 2001).

Bacteria have evolved different abilities to withstand certain levels of fluoride (Maltz and Emilson 1982). Fluoride-resistant strains of several oral bacterial species, including *S. mutans*, *Streptococcus salivarius* and *Streptococcus sanguinis*, have been created in laboratories (Brussock and Kral 1987; Bunick and Kashket 1981; Hamilton 1969a). Generally, a fluoride-resistant strain is able to grow in an environment containing 400-1000 ppm (21.1-52.6 mM) fluoride, depending on the strain. This level of fluoride is at least 3 times higher than that fluoride-sensitive strains could withstand (Brussock and Kral 1987; Van Loveren et al. 1993). The acquired fluoride resistance can be transient or permanent. Transient resistance is quickly lost by bacterial cells, already after one to seven passages, in a fluoride-free medium (Streckfuss et al. 1980). It is possibly acquired through phenotypic adaptation. Several transient fluoride-resistant *S. mutans* strains have been isolated from xerostomia patients

(Brown et al. 1983; Streckfuss et al. 1980). Stable, or permanent, fluoride resistance persists for at least 50 generations after the strain is cultivated without fluoride (Brussock and Kral 1987). The laboratory-derived fluoride-resistant strains mostly show stable fluoride resistance.

The stable fluoride-resistant strains allow researchers to examine cariogenicity and fitness of the strains and to investigate the mechanism of the acquired resistance. This knowledge might help us to better understand the potential impact of fluoride on oral bacteria after 50 years of daily fluoride application at high concentrations. Since *S. mutans* has been widely recognized for its major role in cariogenesis, fluoride resistance of this species has been most often studied (Loesche 1986).

Mode of antimicrobial action of fluoride

To understand the characteristics of fluoride-resistant strains and the mechanism of acquired fluoride resistance, the mode of antimicrobial action of fluoride against *S. mutans* must be known. A brief summary of the antimicrobial mechanisms of fluoride in *S. mutans* is shown in the Figure. The inhibitory effect of fluoride on intracellular metabolism depends on the influx of hydrogen fluoride (HF), which diffuses into bacterial cells, and dissociates to the proton (H^+) and fluoride ion (F^-) in the cytoplasm (Hamilton 1990). This process speeds up when the pH of the extracellular environment decreases, as this facilitates the association of H^+ and F^- to HF (Marquis et al. 2003). Therefore, when the extracellular pH lowers, F^- and H^+ accumulate faster in the cytoplasm and the antimicrobial effect is stronger. The strong pH-dependence of the fluoride effect is well recognized: the inhibitory levels of fluoride for the glycolysis are as high as 10 mM at neutral pH, but are only in the micro-molar range at pH 4.0 (Marquis et al. 2003).

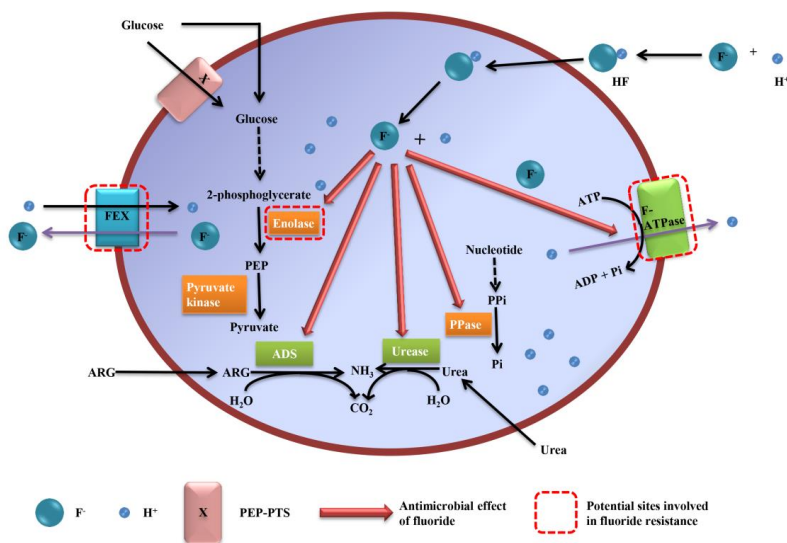


Figure. Mechanisms for the antimicrobial effects of fluoride and potential sites involved in the mechanisms of fluoride resistance. FEX, fluoride exporters; ARG, arginine; ADS, arginine dehydratase system; PPi, inorganic pyrophosphate; PPase, pyrophosphatase; Pi, inorganic phosphate; PEP, phosphoenolpyruvate. Red arrows show the inhibitory effect of fluoride on the enzymes. Red dashed boxes indicate sites which are potentially involved in fluoride resistance. F-ATPase and enolase are involved in both the antimicrobial action of fluoride and the potential mechanisms of fluoride resistance.

The intracellular F^- and H^+ can directly or indirectly affect enzymatic activities and physiological processes in the cell, leading to lower acid production, acid tolerance and adherence of *S. mutans* to tooth surfaces (Belli et al. 1995; Bender et al. 1985; Hamilton and Ellwood 1978; Marquis 1990; Meurman 1988; Rolla and Melsen 1975; Van Loveren 2001). It has been found that enolase, which is involved in glycolysis, can be competitively inhibited by F^- (Guha-Chowdhury et al. 1997). This inhibition is observed for purified enolase as well as enolase from permeabilized cells (Curran et al. 1994; Guha-Chowdhury et al. 1997; Van Loveren et al. 2008). In addition, enolase is also indirectly inhibited by the acidification of the cytoplasm caused by the accumulation of H^+ (Belli et al. 1995; Marquis et al. 2003). Moreover, enolase does not only play a role in the glycolytic process, but also catalyzes the production of phosphoenolpyruvate (PEP) for glucose uptake through the PEP-dependent phosphotransferase system (PTS). Thus, the inhibition of the enolase activity

by F^- also has a negative effect on glucose uptake (Germaine and Tellefson 1986; Hamilton and Ellwood 1978).

S. mutans experiences rapid and dynamic pH fluctuations from pH 7.0 to below pH 3.0 in the oral cavity after dietary carbohydrate intake of the host (Jensen and Wefel 1989; Matsui and Cvitkovitch 2010). The ability of *S. mutans* to withstand these repetitive cycles of acid shocks is defined as acidurance, or acid tolerance (Matsui and Cvitkovitch 2010). It is one of the major virulence factors of *S. mutans*. In the presence of fluoride, this ability has largely diminished (Marquis 1990; Marquis et al. 2003; Welin-Neilands and Svensäter 2007). The glycolysis of *S. mutans* arrests at pH 6.0 in the presence of 10 mM F^- , while in the absence of F^- it is only inhibited at a pH lower than 5.0 (Marquis 1990). The survival rate of *S. mutans* after exposure to a lethal pH (3.5) decreases 77% in presence of fluoride (500 mM) (Welin-Neilands and Svensäter 2007). The acidification of the cytoplasm via the influx of HF as well as the inhibition of the proton-extruding F-ATPase account for the reduction in acidurance of *S. mutans* (Bender et al. 1986; Marquis 1990; Van Loveren 2001). F-ATPase, also known as ATP synthase, is a membrane-bound protein consisting of two domains, F_0 and F_1 . ATP hydrolysis by F-ATPase is obligatorily coupled to proton extrusion through the F_0 pore in the membrane (Bender et al. 1986). Therefore, F-ATPase is closely related to acid tolerance of bacteria (Bender et al. 1986). F^- can bind to F-ATPase in the presence of Al^{3+} (Marquis et al. 2003), and the activity of the enzyme is reduced by 50% with less than 100 ppm (5.26 mM) fluoride (Pandit et al. 2013; Sutton et al. 1987). Previous studies on fluoride inhibition of F-ATPase were done with either the purified enzyme or in permeabilized cells. The inhibition of F-ATPase in intact cells remains unknown (Marquis 1995; Pandit et al. 2013; Sutton et al. 1987). It is worth mentioning that the development of modern biotechnology makes this type of study possible. The combination of single-molecule fluorescence resonance energy transfer (smFRET) and confocal microscopy allows the observation of regulatory conformational changes of specific proteins (Duncan et al. 2014). Such an approach has been taken to study the inhibition of *E. coli* membrane F-ATPase by aurovertin (Johnson et al. 2009) and is thus promising to confirm the fluoride inhibition of F-ATPase in *S. mutans* cells.

Fluoride can affect the adherence of *S. mutans* to enamel, which is a cariogenic trait of *S. mutans*. Yet, there is no consensus on how fluoride influences the ability to adhere (Meurman 1988; Rolla and Melsen 1975; Streckfuss

Chapter 1

et al. 1980). While an obvious decrease in adherence of *S. mutans* to hydroxyapatite was found with 500 ppm (26.3 mM) F⁻ or even less in some *in vitro* studies (Meurman 1988; Shani et al. 2000), others reported hardly any change in adherence with more than 5,000 ppm (263 mM) F⁻ (Rolla and Melsen 1975; Streckfuss et al. 1980). As glucosyltransferases (GTF) play an important role in bacterial adhesion (Schilling et al. 1989), the effect of fluoride on GTF activities have been studied. Yet, no inhibition of the GTF activity by fluoride was reported (Guo et al. 2014; Pandit et al. 2011; Shani et al. 2000). Whether the fluoride inhibition of the adherence of *S. mutans* contributes to caries prevention requires further studies.

In addition to the abovementioned actions, other mechanisms may also play roles in the antimicrobial activity of fluoride. Recently, fluoride is found to inhibit alkali production (Burne and Marquis 2000; Marquis et al. 2003). This is due to the inhibition of either urease or the arginine deiminase system (ADS) by fluoride. The former is very sensitive to fluoride, with 50% inhibition by 0.3 mM F⁻ (Burne and Marquis 2000; Clancy and Burne 1997). The ADS, however, is less sensitive than the urease system and its inhibition by fluoride requires low pH values (Burne and Marquis 2000; Curran et al. 1998). Fluoride can also affect metabolism by binding to pyrophosphatase in the presence of Mn²⁺ (Marquis et al. 2003). Pyrophosphatase is responsible for the release of pyrophosphate (PPi) from nucleotide triphosphates and is therefore involved in a variety of physiological processes, including biosynthesis and regulation of metabolism (Marquis et al. 2003; Parfenyev et al. 2001).

Occurrence of fluoride resistance

Laboratory-derived fluoride-resistant *S. mutans* strains have been isolated through either one-step or step-wise procedures (Brussock and Kral 1987; Hoelscher and Hudson 1996; Lau and Kral 1987; Rosen et al. 1978; Van Loveren et al. 1989; Zhu et al. 2012). In the one-step procedures, the wild-type cells were directly spread on agar plates containing high concentrations of NaF (highest at 26.3 mM), and fluoride-resistant colonies were selected from these plates (Brussock and Kral 1987; Hoelscher and Hudson 1996; Lau and Kral 1987). In the step-wise procedures, fluoride-resistant strains were obtained by culturing the fluoride-sensitive parent strains on agar plates containing increasing concentrations of NaF to a maximum of 52.6 mM (Van Loveren et al.

1989; Zhu et al. 2012). In the 1970s, *S. mutans* strains were made resistant to fluoride by exposure to ultraviolet light or acriflavin (Rosen et al. 1978). However, this method became less popular because of the non-specific nature of these mutagens and the latent chromosome lesions (Auerbach 2013).

Reports on clinically isolated fluoride-resistant *S. mutans* strains are scarce. Streckfuss and co-workers (1979) reported seven fluoride-resistant *S. mutans* isolates from radiation-induced xerostomia patients receiving daily topical application of 1% (238 mM) NaF gel (Streckfuss et al. 1980). These isolates were obtained with the one-step selection method. The isolates which were able to grow in culture media containing 400-600 ppm (21.1-31.6 mM) fluoride were considered “fluoride resistant” (Streckfuss et al. 1980). The same research group later found that sustained fluoride treatment increased the ratio of fluoride-resistant to fluoride-sensitive strains (Brown et al. 1983). These two studies are the only reports on the isolation of fluoride-resistant strains from clinical samples. So far, there have been no reports on the prevalence of naturally occurring fluoride-resistant bacteria in the oral cavity. The reasons for the lack of this type of studies are unknown. It might be related to the little attention that the antimicrobial function of fluoride has received, as fluoride has mainly been studied for its role in protecting dental hard tissue.

Characteristics of fluoride-resistant strains

One of the foremost concerns related to fluoride resistance in oral bacteria is whether fluoride-resistant strains impose risks on oral health. Studies have been focused on several characteristics of fluoride-resistant strains: stability of the resistance, the acidogenicity, the fitness and the *in vivo* cariogenicity. A summary of the fluoride-resistant *S. mutans* strains and their characteristics is shown in the Table.

The stability, or persistence, of fluoride resistance is studied as an indication of whether the fluoride-resistant strains can prosper in the oral cavity in the long run. Resistance to fluoride induced *in vitro* is usually stable, and remains at similar resistance levels after as many as 500 transfers in the absence of fluoride (Hamilton 1969a; Streckfuss et al. 1980). However, the *S. mutans* isolates from xerostomia patients with long-term fluoride application seem to have a transient resistance, as the resistance was lost after seven transfers in fluoride-free medium (Streckfuss et al. 1980). Very little has been done to

Chapter 1

characterize these transient fluoride-resistant isolates. The characterizations discussed below are all based on the stable fluoride-resistant strains.

The acidogenicity of fluoride-resistant *S. mutans* strains has been studied in comparison with the corresponding wild-type fluoride-sensitive strain. The outcome of the comparison depended on the presence/absence of fluoride. When fluoride was absent, some fluoride-resistant strains produced acid at a lower rate than the wild-type strains at the environmental pH between 4.0 and 7.0 (Eisenberg et al. 1985). One fluoride-resistant strain was reported to produce acid at a higher rate than the wild-type strain when the environmental pH was below 6.0 (Van Loveren et al. 1991c). Another fluoride-resistant strain was found to produce acid at the same rate as the wild-type strain (Hoelscher and Hudson 1996). However, when fluoride was present, all fluoride-resistant strains were more acidogenic than the fluoride-sensitive strains (Hoelscher and Hudson 1996; Sheng and Liu 2000; Van Loveren et al. 1991c).

Table. Fluoride-resistant strains and their characterizations.

Fluoride-resistant <i>S. mutans</i> strain	Lab / clinical isolate	Level of resistance	Stable / transient resistance	Characteristic	Potential mechanism	Reference
1144/600FR	Lab isolate; step-wise derived from <i>S. mutans</i> #1144	600 ppm (31.6 mM)	Stable	N/A	Potential involvement of genotypic mutation	(Streckfuss et al. 1980)
6715/600FR	Lab isolate; step-wise derived from <i>S. mutans</i> 6715	600 ppm (31.6 mM)	Stable	Decreased ability to form biofilms on tooth compared to the wild-type in the absence of fluoride; Higher adherence percentage than the wild-type with 600 ppm (31.6 mM) fluoride.	Potential involvement of genotypic mutation	(Streckfuss et al. 1980)
6715-derived	Lab isolate; induced by exposure of <i>S. mutans</i> 6715 to ultraviolet light	600 ppm (31.6 mM)	N/A	Lower cariogenicity than the wild type <i>in vivo</i> (rats).	N/A	(Rosen et al. 1978)
C180-2FR	Lab isolate; one-step derived from <i>S. mutans</i> C180-2	500 ppm (26.3 mM)	Stable	Faster acid production than the wild-type when pH below 6.0 (in the absence of fluoride); Unable to bind to enamel when the wild-type was present; Similar cariogenicity compared to the wild-type <i>in vivo</i> (rats).	Similar activity of enolase or F-ATPase compared to the wild-type; No mutation in the enolase gene.	(Van Loveren et al. 1991a; Van Loveren et al. 2008; Van Loveren et al. 1989)
FA-1-derived	Lab isolate; induced by exposure of <i>S. mutans</i> FA-1 to acriflavin	600 ppm (31.6 mM)	N/A	Higher cariogenicity than the wild type <i>in vivo</i> (rats).	N/A	(Rosen et al. 1978)
GS-5 A25 – A73	Lab isolates; step-wise derived from <i>S. mutans</i> GS-5	400-3000 ppm (21.1-157.9 mM)	Stable	N/A	Potential involvement of multiple gene mutations	(Brussock and Kral 1987)

Table. (continued)

NCH105	Lab isolate; one-step derived from <i>S. mutans</i> UA130	1000 ppm (52.6 mM)	Stable	Higher acid production and glucose uptake than the wild-type with fluoride present at pH 6.5 and pH 6.0. Similar ability with the wild type to bind to artificial tooth pellicles.	Higher F-ATPase ability than the wild type in the presence of fluoride; stronger enolase activity than the wild type in the presence of fluoride; Two mutations found in enolase gene.	(Hoelscher and Hudson 1996; Mitsuhashi et al. 2014)
S-126-derived	Lab isolate; induced by exposure of <i>S. mutans</i> S-126 to ultraviolet light	600 ppm (31.6 mM)	N/A	Lower cariogenicity than the wild type <i>in vivo</i> (rats).	N/A	(Rosen et al. 1978)
U41, U48, U53, U60, U64, U80, U85	Clinical isolates	600 ppm (31.6 mM)	Transient	Similar to adherence patterns of lab-derived fluoride-resistant strains.	Potential involvement of horizontal plasmid transfer	(Streckfuss et al. 1980)
UA159FR	Lab isolate; step-wise derived from <i>S. mutans</i> UA159	1000 ppm (52.6 mM)	N/A	Higher ability to resist acid stress than the wild type.	N/A	(Zhu et al. 2012)

The fitness of fluoride-resistant strains was assessed in two ways: the ability to survive acidic pH and to compete with the wild-type strains to adhere to tooth surfaces. There is only one report on the acid tolerance of fluoride-resistant *S. mutans*, which found stronger acid tolerance of a fluoride-resistant strain than its parental strain (Zhu et al. 2012). Controversial results have been reported for the competition test, which examined the competition between fluoride-resistant and fluoride-sensitive strains to bind to enamel. A fluoride-resistant strain was unable to bind to teeth when its wild-type strain was present (Van Loveren et al. 1991a; Van Loveren et al. 1990). This result was challenged by Hoelscher, who found another fluoride-resistant strain to be capable of binding to the same extent as the wild-type strain when they had been mixed in equal proportions (Hoelscher and Hudson 1996). The mechanism of this competition is not very clear. Likely the growth rate of each strain determines the outcome of the competition. In the former study (Van Loveren et al. 1991a; Van Loveren et al. 1990), the growth rate of the fluoride-resistant strain was reported to be lower than its wild-type strain, while the strains in the latter study exhibited similar generation time (Hoelscher and Hudson 1996).

A few *in vivo* studies examined the cariogenicity of fluoride-resistant *S. mutans* strains in rats. The results vary depending on the strains and the parameters used in the experiment. Van Loveren (1989) reported less severe dental lesions in rats superinfected with a fluoride-resistant strain than in rats superinfected with the wild-type strain (Van Loveren et al. 1989). This result was in line with the results of the competition test, in which the same strains were examined (Van Loveren et al. 1991a; Van Loveren et al. 1990). However, when the number of all visible lesions, including enamel and dentine lesions, were taken into account, the two strains showed similar cariogenic potentials, whether fluoride was included in the diet or not (Van Loveren et al. 1989). Rosen (1978) also reported lower cariogenicity of three fluoride-resistant strains when compared to their wild-type strains (Rosen et al. 1978). Another strain, made resistant to fluoride by exposure to ultraviolet light, exhibited at least as much ability as its wild type to cause caries (Rosen et al. 1978). As no conclusions can be drawn from these *in vivo* studies, due to their different experimental designs as well as their limited sample sizes, the cariogenicity of fluoride-resistant strains *in vivo* remains an important issue for further studies.

Mechanisms of fluoride resistance

As previously stated, *S. mutans* can acquire either transient or stable fluoride resistance. The former was noticed in xerostomia patients with daily topical application of fluoride (Brown et al. 1983; Streckfuss et al. 1980). It was proposed that this transient resistance was related to the horizontal transfer of plasmids (Brown et al. 1983; Streckfuss et al. 1980). Fluoride-resistant *S. mutans* strains lost these plasmids when fluoride was absent and rapidly reversed back to fluoride-sensitive state (Brown et al. 1983; Streckfuss et al. 1980). Till now, there is no evidence to support this hypothesis.

In contrast to transient fluoride resistance, stable fluoride resistance is believed to be due to chromosomal mutations. Different approaches have been applied to identify genes that are related to the stable fluoride resistance. Until 2008, the research focus has been on enolase and F-ATPase, which are known to be essential in the antimicrobial action of fluoride. In 2012, a fluoride exporter, or fluoride antiporter, and its regulation were unexpectedly discovered in bacteria during a study on the binding of various metabolites to bacterial RNA (Baker et al. 2012; Breaker 2012). These recent studies brought new concepts or candidates for the mechanism of fluoride resistance. The Figure shows not only the antimicrobial target sites of fluoride, but also the potential sites which are involved in fluoride resistance.

Enolase and F-ATPase

Enolase and F-ATPase are two important enzymes that are sensitive to fluoride. Hence, these two enzymes were originally considered as the most possible sites involved in fluoride resistance. Meanwhile, enolase and F-ATPase were also thought to be involved in the mechanisms of fluoride resistance. It was hypothesized that they were insensitive to fluoride in fluoride-resistant *S. mutans* strains. In the presence of fluoride, the cellular level of PEP could be maintained, which provided sufficient substrates for the PEP-dependent PTS, as well as for glycolysis (Hoelscher and Hudson 1996).

However, the published evidence is not sufficient to prove the above hypothesis. The activities of purified enolase or enolase in permeabilized cells were compared *in vitro* between fluoride-sensitive strain and the derived fluoride-resistant strains. No difference was observed between the enolase activities for the two strains, regardless the presence of fluoride (Bunick and Kashket

1981; Van Loveren et al. 2008). Recently, a study reported that enolase from a fluoride-resistant strain was less sensitive to fluoride. However, the difference was not large enough to explain the resistance in metabolism (Mitsuhata et al. 2014).

The results of studies on the F-ATPase activity in fluoride-resistant strain were also inconsistent. One study showed that F-ATPase in a fluoride-resistant strain was insensitive to fluoride at pH 5.0, while the F-ATPase in the corresponding fluoride-sensitive wild-type strain was sensitive to fluoride under the same pH condition (Hoelscher and Hudson 1996). However, another study did not find any difference between two strains, both at pH 4.0 and pH 7.0 (Van Loveren et al. 2008).

In efforts to identify mutations responsible for fluoride resistance, researchers have sequenced genes coding for enolase and F-ATPase (Mitsuhata et al. 2014; Sheng et al. 2005; Van Loveren et al. 2008). No mutation was found in the F-ATPase-coding gene in the fluoride-resistant strain (Sheng et al. 2005). In the enolase-coding gene (*eno*) of a fluoride-resistant strain NCH105, one mutation was located (Mitsuhata et al. 2014), while in another strain no mutation was identified (Van Loveren et al. 2008). The *eno* mutation in the fluoride-resistant strain NCH105 led to an amino acid alteration from proline to leucine (P173L) (Mitsuhata et al. 2014). However, in the three-dimensional conformation models the mutation is not located nearby any known F⁻ binding site (Mitsuhata et al. 2014).

The reason for the inconsistent findings with enolase and F-ATPase in fluoride resistance is unclear. Since different fluoride-resistant strains were tested in different studies, it is possible that the approach (proteins or regulation pathways) employed by each strain to resist fluoride is strain-dependent.

Fluoride exporters

In 2012, Breaker et al. discovered that two gene families, which were previously predicted to code for proteins involved in camphor resistance (*crcB*) and ClC-type ion channel protein (*eriC^F*), have identical biochemical roles (Baker et al. 2012; Breaker 2012). Both gene families encoded fluoride exporters (Baker et al. 2012; Rapp et al. 2006) and are directly related to the fluoride resistance of microorganisms. The deletion of *crcB* in *Escherichia coli* or *Candida albicans* leads to a 200-350 fold higher sensitivity of the mutant to fluo-

ride compared to the wild-type strain. The resistance to fluoride can be restored by supplementing *eriC^F* from another species, *Bacillus cereus* (Baker et al. 2012; Li et al. 2013). The genes *crcB* and *eriC^F* are conserved in the bacterial kingdom. Most bacterial species harbour only *crcB* in their genome, while a few of them have only *eriC^F* (Baker et al. 2012). *S. mutans* has two *eriC^F* genes in tandem with the same orientation (Men et al. 2016). The involvement of these two genes in fluoride resistance has been confirmed by two gene knockout studies (Men et al. 2016; Murata and Hanada 2016). Both gene knockout studies found that *S. mutans* became 100-fold more sensitive to fluoride after knocking out both *eriC^F* copies (Men et al. 2016; Murata and Hanada 2016). Differently, one of the two studies reported increased fluoride sensitivity by knocking out either of the two *eriC^F* copies (Murata and Hanada 2016), while the other discovered that only the second *eriC^F* copy was required for fluoride resistance (Men et al. 2016). Up till now, it is not clear why *S. mutans* possesses two copies of fluoride exporter-coding genes. Breaker (2012) proposes that this might be a more recent adaptation the species made to the bursts of extremely high fluoride concentrations delivered with oral health products (Breaker 2012).

Currently, the fluoride exporter, or fluoride antiporter, is identified as a subclass of the bacterial CLC anion-transporting proteins based on its protein structure. In contrast to canonical CLCs, which are weakly selective for Cl⁻ and other monovalent anions, this fluoride exporter greatly prefers F⁻ over Cl⁻, even though F⁻ is usually strongly hydrated and difficult to develop host-guest compounds (Stockbridge et al. 2012). The protein has a “double-barrelled” channel architecture in which two F⁻ tunnels span the membrane (Stockbridge et al. 2015). The narrow pores and unusual anion coordination which exploits the quadrupolar edges of conserved phenylalanine rings indicate its preference for F⁻ (Stockbridge et al. 2015). Unlike all other CLC transporters which employ 2-to-1 stoichiometry, the fluoride exporter exchanges F⁻ with H⁺ with 1-to-1 stoichiometry (Picollo et al. 2012; Stockbridge et al. 2012).

The regulation of the fluoride exporter varies among different bacterial species. Many bacterial species, including those from the orders Lactobacillales and Bacillales, regulate the fluoride exporters using fluoride riboswitches (Baker et al. 2012). Fluoride riboswitches are fluoride-binding RNA molecules, which are stabilized once bound by fluoride (Ames et al. 2010). The fluoride-bound riboswitches can then activate expression of genes coding for the fluo-

ride exporters (Baker et al. 2012). However, riboswitches are not identified in *S. mutans* cells (Baker et al. 2012; Breaker 2012). How fluoride exporters are regulated in *S. mutans* and whether the regulation of fluoride exporters is related to fluoride resistance requires further investigations.

Involvement of multiple factors

Back in 1987, Brussock and Kashket isolated fluoride-resistant *S. mutans* strains in two steps. The second-step isolates showed higher levels of fluoride resistance than the first-step isolates. They proposed that different mutations occurred in each step contribute synergistically to fluoride resistance (Brussock and Kral 1987). However, there is a limited amount of studies trying to identify candidate genes/proteins which are potentially involved in fluoride resistance. No study has been performed to explore the additive effect of multiple factors.

Outline of this thesis

This thesis aims to: (1) investigate the genotypic characteristics of fluoride-resistant *S. mutans* and identify candidate genes/proteins involved in fluoride resistance; (2) evaluate the virulence-related phenotypic characteristics, including fitness and glucose uptake activity, of fluoride-resistant *S. mutans*.

In **Chapter 2**, the genotypic changes in a fluoride-resistant *S. mutans* strain were identified using whole-genome shotgun (WGS) sequencing and bioinformatics analyses. The expression of genes related to identified single nucleotide polymorphisms (SNPs) was compared between the fluoride-resistant strain and its parental wild-type strain.

In **Chapter 3**, the relationship between a mutation in the promoter of the fluoride antiporter-coding genes and fluoride resistance was investigated. A mutant with a defined genetic background was constructed and the growth and acid production was examined in the presence and absence of fluoride. A potential mechanism used by *S. mutans* to acquire fluoride resistance is discussed.

In **Chapter 4**, the available whole genome data from two fluoride-resistant *S. mutans* strains and those from their parental strains were analyzed. Common chromosomal regions with mutations present in both fluoride-resistant strains were identified. The function of these loci was evaluated with related gene expression and corresponding enzyme activities.

In **Chapter 5**, the fitness of the biofilms of two fluoride-resistant

Chapter 1

strains was compared with that of a wild-type fluoride-sensitive strain in the absence of fluoride. These biofilms were examined for their responses to chlorhexidine or acidic challenges.

In **Chapter 6**, the differences in glucose transport systems between a fluoride-sensitive and a fluoride-resistant *S. mutans* strain were explored. The total glucose uptake activity and PEP-PTS activity from the two strains were assayed in the absence and presence of fluoride. Expression of several glucose uptake-related genes was quantified and compared between the two strains.

In **Chapter 7**, the results from previous chapters were summarized and an outlook for future studies is presented.