Fluoride resistance in Streptococcus mutans

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Chapter 6

The regulation of glucose uptake in fluoride-sensitive and fluoride-resistant *Streptococcus mutans* strains

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Abstract

Fluoride-resistant *Streptococcus mutans* strains are isolated after exposure of the wild-type strains to high dose of fluoride for a long time. These strains usually exhibit higher acid production than the wild-type strains in the presence of fluoride. Previous studies did not find difference in the enolase activity between fluoride-sensitive and fluoride-resistant strains to explain the different acid production. Such difference in glycolytic activity may be due to changes in other parts of sugar metabolism. This study aimed to compare the effect of fluoride on glucose uptake by a fluoride-sensitive and fluoride-resistant *S. mutans* strain, as well as to identify differences in the regulation of PEP-PTS activity between these two strains. We observed less severe inhibition of glucose-uptake activity by fluoride in the fluoride-resistant strain *S. mutans* C180-2FR. Genes related to glucose uptake were up-regulated upon fluoride application in the fluoride-sensitive strain *S. mutans* C180-2. No such regulation was seen in C180-2FR. In addition, the PTS activity in C180-2 was significantly higher than that in C180-2FR in the absence of fluoride. Our data suggested that glucose uptake is differently regulated in fluoride-sensitive and fluoride-resistant *S. mutans* strains. These differences may be related to the transport of fluoride ions and deactivation of pyruvate kinase.

Introduction

Due to its well-established anti-caries effect, fluoride has been widely used in a variety of oral health products for over 50 years (Birkeland and Torell 1978; Lennon 2006). The long-term exposure of oral bacteria to high concentrations of fluoride, which inhibits both bacterial growth and metabolism, leads to development of fluoride resistance (Van Loveren 2001). Such fluoride-resistant *Streptococcus mutans* have been isolated in clinics and laboratories (Brown et al. 1980; Streckfuss et al. 1980; Van Loveren et al. 1991c). Studies on of fluoride-resistant *S. mutans* strains showed that they not only survive environments with fluoride (with as high as 600 ppm F), but also produce more acids than the fluoride-sensitive strains in the presence of fluoride (Hoelscher and Hudson 1996; Van Loveren et al. 1991c).

The higher glycolytic activity of fluoride-resistant *S. mutans* in presence of fluoride, illustrated by higher acid production, was first thought to be related to the insensitivity of enolase to fluoride inhibition (Bunick and Kashket 1981). Fluoride ion (F^-) binds to enolase and inhibits the enzymatic activity in wild-type *S. mutans* cells, which results in the inhibition of glycolysis (Marquis et al. 2003). It was hypothesized that this enzyme was less sensitive to F^- in fluoride-resistant bacteria (Bunick and Kashket 1981). However, previous studies found that enolase from fluoride-sensitive and fluoride-resistant strains was similarly inhibited by F^- (Bunick and Kashket 1981; Van Loveren et al. 2008). Thus, the increased ability to metabolize sugar in the presence of fluoride by fluoride-resistant strains seems not to be due to changed glycolytic activity, but changes in other pathways.

Several researchers reported a significant decrease in levels of early glycolytic intermediates, especially glucose-6-phosphate, in fluoride-sensitive cells in the presence of fluoride (Bender et al. 1985; Hamilton 1969b). The intracellular glucose-6-phosphate concentration can be affected by different physiological processes. Most importantly, as the final product of both the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) and non-PTS system, it is used to indirectly evaluate the glucose-uptake activity (Bender et al. 1985; Meza et al. 2012). It was thus proposed that, in addition to enolase, the glucose uptake pathway in fluoride-sensitive bacterial cells is sensitive to fluoride inhibition (Bender et al. 1985). In fluoride-resistant strains, this process was thought to be less inhibited by fluoride (Hamilton 1969b).

While no direct evidence for this has been found, we previously found

that pyruvate kinase in a fluoride-resistant *S. mutans* strain C180-2FR was completely deactivated (Chapter 4). Pyruvate kinase, a key enzyme in the glycolysis, catalyzes the transformation from PEP to pyruvate. PEP is the phosphoryl-group donor in PEP-PTS, which is the major glucose-uptake system in *S. mutans* (Vadeboncoeur and Pelletier 1997). The deactivation of pyruvate kinase will largely result in changes in the intracellular PEP concentration and thus differences in the PTS regulation. Alterations in PTS can lead to changes in the glucose-uptake activity.

The current study aimed to compare the effect of fluoride on glucose uptake by a fluoride-sensitive and fluoride-resistant *S. mutans* strain, as well as to identify the difference in the regulation of PEP-PTS activity between these two strains. To this end, glucose-uptake activity and related gene expression in a fluoride-sensitive *S. mutans* strain C180-2 and its lab-derived fluoride-resistant strain C180-2FR was compared with and without fluoride. Specifically, the activity of PEP-PTS was also examined and compared between these two strains.

Materials and Methods

Bacterial strains and growth conditions

The strains used in this study were *S. mutans* C180-2 and its lab-derived fluoride-resistant mutant strain C180-2FR(Van Loveren et al. 1991b). Both strains were grown in Brain Heart Infusion (BHI) broth or on BHI agar anaerobically (90% N2, 5% CO2, 5% H2) at 37°C.

Determination of extracellular glucose uptake

Both strains were grown in BHI broth until mid-exponential phase ($OD_{600} = 0.6$). Cells were pelleted by centrifuging at 4, 528 x g for 10 min at 4°C and resuspended with 1/7 original volume with 300 mM 4-(2-hydroxythyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.0 buffer to make a cell density of ~ 7 x 10^9 colony forming unit (CFU) / ml. After resuspension, the cells were washed twice with the same buffer and then split into three aliquots. One of the following conditions was added to each aliquot: potassium fluoride (final concentration at 10 mM), chlorhexidine di-gluconate (final concentration at 0.06 mM), or Milli-Q (control). Based on the results of a pilot study (data not shown), we selected 10 mM fluoride to use in this study. Potas-

sium fluoride was used instead of sodium fluoride to avoid adding sodium ions, which are known to inhibit the glucose transport process (Marsh et al. 1984). After adding glucose to a final concentration of 3.3 mM, all aliquots were incubated at 37°C and samples were taken at 0, 5, 10, 15, and 20 min during the incubation, pelleted and the supernatants were used immediately for the glucose assay.

Glucose was quantified enzymatically using the Glucose (HK) Assay Kit (Sigma-Aldrich, Missouri, USA). Briefly, glucose is phosphorylated to glucose-6-phosphate by hexokinase. Glucose-6-phosphate is then oxidized by NAD^+ and glucose-6-phosphate dehydrogenase to 6-phosphogluconate and NADH. The amount of glucose is thus determined by the change of absorbance at 340 nm as NAD^+ reduces to NADH. The activity of exogenous glucose uptake was calculated as the speed of extracellular glucose depletion. The residual glucose-uptake activity was determined by comparing the activity of each group with that of the control group, which was set as 100%.

PEP-PTS assay

The activity of PEP-PTS was examined with permeabilized cells. Permeabilization of the cells was performed as previously described with minor modifications (Kornberg and Reeves 1972). Briefly, cultures of both *S. mutans* strains were grown in BHI broth until mid-exponential phase ($OD_{600} = 0.6$) and harvested by centrifuging at 4, 528 x g for 10 min at 4°C. Cells were resuspended in 1/10 original volume with permeabilization buffer containing 20 mM potassium phosphate pH 7.0 and 5 mM $MgCl_2$. Toluene was added to a final concentration of 10% (v/v) after which the mixture was vigorously agitated and then incubated for 15 min at 37°C. Next, the cells were frozen twice in liquid nitrogen with thawing at 37°C after each freezing. They were harvested by centrifuging again at 4, 528 x g for 10 min at 4°C and resuspened in permeabilization buffer. The permeabilized cells were stored at -80°C until used.

The internalization of glucose through the PEP-PTS can be summarized as the following reaction: PEP + glucose → glucose-6-phosphate + pyruvate. The translocation of the phosphoryl group is completed via several proteins including Enzyme I (EI), histidine protein (HPr), and enzyme II (EII) (Vadeboncoeur and Pelletier 1997). The ability of cells to transport glucose through the PEP-PTS was evaluated with the permeabilized cells by quantifying the reduction of PEP. The reaction included permeabilized

cells, 5 mM glucose, and different concentrations of NaF (0-10 mM) in 20 mM potassium phosphate buffer pH 7.0. The reaction was performed at 37°C and initiated by the addition of 1 mM PEP. The reduction of PEP was kinetically monitored for 40 min using a SPECTRAmax PLUS^{384} (Molecular Devices Corp., Sunnyvale, Calif. USA) at 240 nm. The activity of the PEP-PTS was calculated as the reduction of PEP per min per mg dry weight. The experiment was performed in triplicate.

Gene expression in fluoride-challenged glycolysis

To measure gene expression, both *S. mutans* C180-2 and C180-2FR were grown in BHI broth until late exponential phase (OD_{600} = 0.8) and pelleted with centrifugation at 4,528 x g for 10 min at 4°C. The pellets were resuspended with a pH 7.0 phosphate-buffered semi-defined medium containing vitamins and amino acids (Deng et al. 2009a). The suspensions were split into two portions. Glucose was added to all samples with a final concentration of 22.2 mM. Either Milli-Q or a final concentration of 10 mM NaF was added to each sample. All samples were incubated at 37°C for 1 h before harvesting with centrifugation at 16,100 x g for 2 min at room temperature. The supernatants were used for lactic acid determination as previously described (Gutmann and Wahlefeld 1974) as a way to confirm the effect of fluoride in the two strains. The pellets were used for downstream RNA extraction and real-time PCR.

Total RNA of the samples was extracted using Genejet RNA kit (Thermo scientific, MA, USA), following the instructions from the manufacturer. Genomic DNA contamination was removed with the TURBO-DNA-*free* Kit (Life Technologies, Carlsbad, USA). Next, cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, MA, USA) with random hexamer primers. Expression of four genes (Table 1) was examined with real-time PCR. Two of them, encoding the glucose-specific transporter subunit (*ptsG*) and sucrose-specific transporter subunit (*scrA*), are involved in the PEP-PTS (Ajdic et al. 2002). The other two genes encode a sucrose phosphorylase (*gtfA*) and a membrane protein (*msmG*) that are involved in a previously reported non-PTS system (Tao et al. 1993). Primers used in this study are listed in Table 1. The genes *gyrA* and *recA* were used as reference. The relative expression of the four genes was calculated based on either the strain or the presence of NaF. For the former analysis, the relative gene expression was calculated by dividing the gene expression of C180-2FR by that of

C180-2. For the latter analysis, the relative expression was calculated by dividing the gene expression in the presence of fluoride by that in the absence of fluoride.

Table 1. Primers used in this study

Gene	Product	Sequence(5'-3')
gtfA	Sucrose phosphorylase	GTATTGGTGTGGTTGATGT
		AGTTATTATATTCGGCAGTTGA
msmG	Multiple sugar-binding ABC transporter permease	GTTGCCATTGCTGATTCTT
		GCTCCATTGCTCATACCA
ptsG	PTS system glucose-specific transporter subunit IIABC	CACCTTAGCAGAGAATGGA
		TAACGGATGATGGACTTGAA
scrA	PTS system sucrose-specific transporter subunit IIABC	ACTATTGGTGCTTATACAGGAT
		GCCATACAGCCACAAGTA

Statistics

Data was analyzed with GraphPad Prism (version 5.00, GraphPad Software, San Diego, California, USA). Two-way ANOVA was performed to determine the effect of different treatments on glucose-uptake activity in the two strains. The PEP-PTS activity was also examined with two-way ANOVA to decide the effect of different NaF levels on PTS activity in two strains. Student's *t* test was performed for comparisons between gene expressions of two strains. *P* values were corrected for multiple testing using the false discovery rate (FDR). Differences were considered statistically significant at $p < 0.05$.

Results

Lactic acid production

We observed significantly different lactic acid production by the fluoride-resistant strain *S. mutans* C180-2FR and fluoride-sensitive *S. mutans* C180-2 (Figure 1). When NaF was absent, *S. mutans* C180-2 and C180-2FR produced similar amount of lactic acid. The use of 10 mM NaF completely abolished the lactic acid production of C180-2, while in C180-2FR there was no inhibition of acid production (Figure 1).

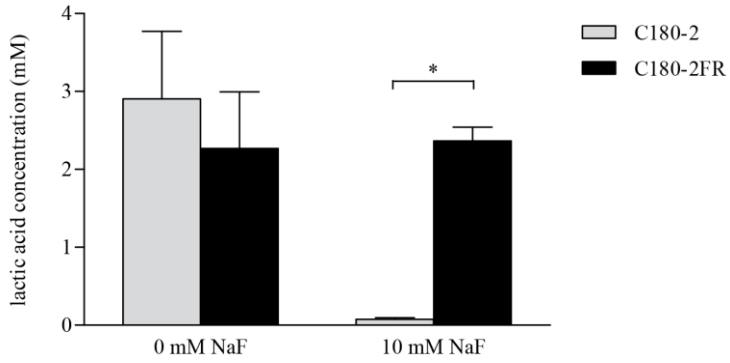


Figure 1. Lactic acid production of *S. mutans* C180-2 and C180-2FR with and without NaF. * $p < 0.05$.

Inhibition of glucose uptake

Figure 2 shows the residual glucose-uptake activity in the presence of 0.06 mM chlorhexidine di-gluconate or 10 mM potassium fluoride in *S. mutans* C180-2 and C180-2FR. In control groups, *S. mutans* C180-2 took in glucose significantly faster than C180-2FR, at 12.6 nmol / (min. mg dry weight) and 9.0 nmol / (min. mg dry weight) respectively ($p < 0.01$). In the presence of 0.06 mM chlorhexidine di-gluconate, glucose-uptake activity in both strains was significantly reduced (Figure 2). *S. mutans* C180-2 remained approximately 21% residual activity, while C180-2FR remained 43% of the activity of the control group. The involvement of 10 mM potassium fluoride led to significant inhibition in C180-2, with 60% remained activity (Figure 2). The glucose uptake in C180-2FR was not obviously affected by potassium fluoride (Figure 2).

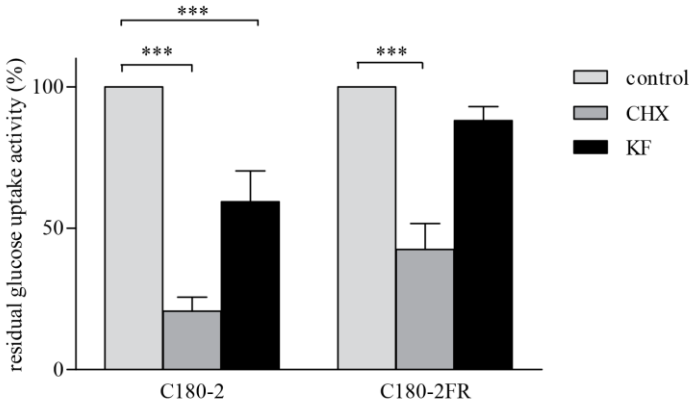


Figure 2. Residual glucose-uptake activity with chlorhexidine or potassium fluoride treatment in *S. mutans* C180-2 and C180-2FR. The activity of the control groups was set as 100%. *** $p < 0.001$.

PEP-PTS activity

The effect of the fluoride concentration on PEP-PTS activity in two *S. mutans* strains is shown in Figure 3. When fluoride was absent, *S. mutans* C180-2 showed a significantly higher PEP-PTS activity than C180-2FR (66.2 nmol PEP reduced / min. mg dry weight and 48.3 nmol PEP reduced / min. mg dry weight, respectively; Figure 3). An clear inhibition of PEP-PTS was observed in both *S. mutans* C180-2 and C180-2FR once fluoride was added to the system. With 1 mM NaF, *S. mutans* C180-2 and C180-2FR showed about 40% and 30% of the original activity, respectively. There was no significant difference between the absolute values of PEP-PTS activity from the two strains (Figure 3). The inhibitory effect of 5 mM NaF was almost the same as that of 1 mM NaF in both strains (Figure 3).

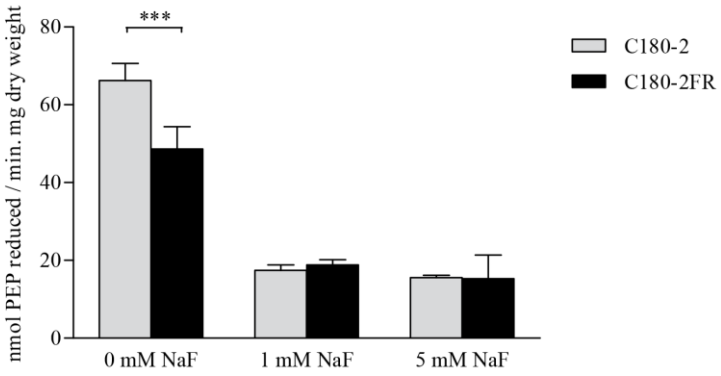


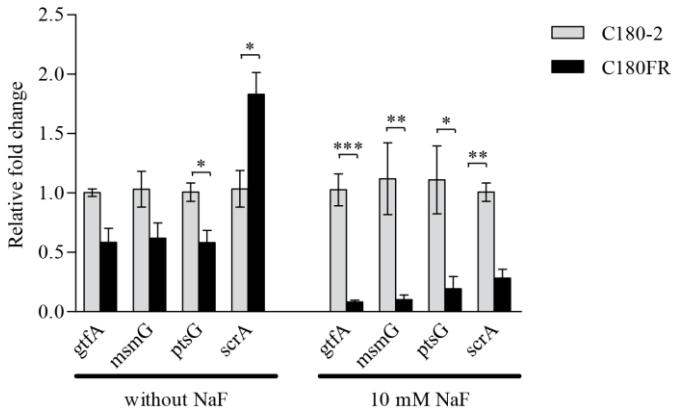
Figure 3. PEP-PTS activity of permeabilized cells of *S. mutans* C180-2 and C180-2FR exposed to concentrations of NaF. The activity of PEP-PTS is shown as the amount of PEP reduced per min per mg dry weight. *** $p < 0.001$.

Expression of PTS and non-PTS genes

The relative expression of the selected genes is shown in Figure 4. When there was no fluoride in the system, no obvious difference of the expression of *gtfA* and *msmG* was seen between the two strains (Figure 4A). *S. mutans* C180-2FR expressed about 0.5-fold *ptsG* and 1.7-fold *scrA* compared to C180-2 ($p < 0.05$, Figure 4A). When fluoride was added in the system, all tested genes showed significantly lower expression in C180-2FR than in C180-2 (Figure 4A): The expression of *gtfA* and *msmG* in C180-2FR was about 10-fold lower than that in C180-2 and both *ptsG* and *scrA* expressed about 5-fold less in C180-2FR (Figure 4A).

The influence of fluoride on the gene expression in the two strains is shown in Figure 4B. *S. mutans* C180-2, *gtfA* and *msmG* expressed 6- to 7-fold more in the presence of NaF than in the absence of NaF ($p < 0.01$ for *gtfA*; $p < 0.05$ for *msmG*). The other two genes, *ptsG* and *scrA,* also were up-regulated in the presence of NaF, however this was not statistically significant (Figure 4B). For *S. mutans* C180-2FR, all four genes showed similar levels of expression, irrespective of the presence of NaF (Figure 4B).

A.



B.

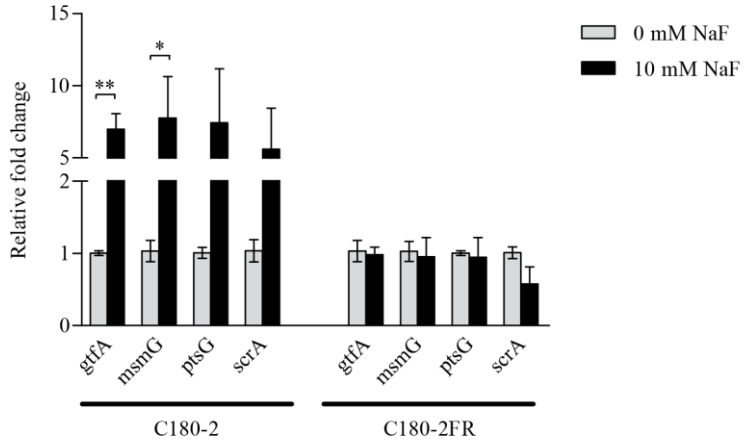


Figure 4. Expression of the two PTS (*ptsG* and *scrA*) and two non-PTS (*gtfA* and *msmG*) related genes (A) in *S. mutans* C180-2FR relative to C180-2 in the absence and presence of NaF; (B) in the presence of NaF relative to that in the absence of NaF in the two *S. mutans* strains. All *p* values were corrected for multiple testing using the false discovery rate (FDR). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Discussion

The different glycolytic activities between fluoride-sensitive and fluoride-resistant *S. mutans* strains have been previously reported. So far this phenomenon is not explained. The current study revealed that the uptake of glucose was differently regulated in fluoride-sensitive and fluoride-resistant *S. mutans* strains, both in the absence and presence of fluoride. The difference of regulation can be seen in the activity of glucose uptake and PEP-PTS, as well as the expression of glucose uptake related genes.

Similar to previous findings, we noticed a significantly stronger ability to produce lactic acid by the fluoride-resistant *S. mutans* C180-2FR than its wild-type strain *S. mutans* C180-2 (Figure 1) (Van Loveren et al. 1991c). In line with this difference in acid production, we found that the inhibition of the glucose-uptake activity by fluoride was more severe in *S. mutans* C180-2 than in C180-2FR (Figure 2). In the wild-type strain C180-2, the inhibition of glucose uptake by fluoride is thought to be due to the inhibition of enolase (Hoelscher and Hudson 1996). This enzyme catalyzes the transformation from 2-phosphoglycerate to PEP, thus producing substrate for the PEP-PTS (Guha-Chowdhury et al. 1997). Previous studies have confirmed that enolase can be bound and competitively inhibited by micromolar levels of fluoride (Marquis et al. 2003). The reduced production of PEP due to the inhibition of enolase is proposed to affect the efficiency of PEP-PTS (Hoelscher and Hudson 1996; Van Loveren 2001). However, the reason for the less severe inhibition of glucose uptake by fluoride in *S. mutans* C180-2FR is not clear yet. Previous studies have shown that the enolase activities of *S. mutans* C180-2 and C180-2FR were inhibited at the same level by fluoride (Van Loveren et al. 2008). This indicates that the less inhibition of glucose uptake by fluoride in *S. mutans* C180-2FR is not related to enolase activity. Further investigations are required to explain the insensitivity of glucose uptake in *S. mutans* C180-2FR to fluoride.

We noticed a significantly higher PEP-PTS activity in C180-2 than in C180-2FR in the absence of fluoride (Figure 3). The application of 0.06 mM chlorhexidine di-gluconate, which is known to specifically inhibit PEP-PTS (Marsh et al. 1983), led to stronger inhibition of glucose-uptake activity in C180-2 than in C180-2FR (Figure 2). The direct and indirect evidence above indicate that the glucose uptake in the two strains is constitutively differently regulated. PEP-PTS is regulated by a variety of factors, including phosphoryla-

tion of proteins and protein-protein interaction (Deutscher et al. 2014). The phosphorylation state of the PTS proteins is mainly controlled by the ratio of PEP to pyruvate, which are the substrate and product of the phosphorylation of EI and EII (Deutscher et al. 2014; Hogema et al. 1998). Interestingly, we have shown in our previous study that pyruvate kinase, the enzyme catalyzing the transformation from PEP to pyruvate, was completely deactivated in C180-2FR (Chapter 4). The deactivation of this enzyme indicated that, on the one hand, other pathways are activated in these cells to produce pyruvate. One such potential bypass would be via the aminotransferases (Yoneyama et al. 2011). On the other hand, the balance between PEP and pyruvate may be broken. If the PEP-to-pyruvate ratio becomes lower due to the deactivation of pyruvate kinase and action of bypasses, PTS proteins tend to be dephosphorylated, suggesting a weaker PEP-PTS activity. In order to confirm the regulation mechanism, the intracellular PEP-to-pyruvate ratio in S. mutans C180-2FR should be determined and compared to that in C180-2.

In our current study, the expression of genes related to glucose uptake was significantly lower in C180-2FR than in C180-2 when fluoride was present (Figure 4A), which was in contrary to the result from glucose-uptake activity. Further examination of the gene expression profiles revealed that the two strains responded differently to fluoride in gene expression. C180-2 up-regulated (gtfA and msmG) or tended to up-regulate (ptsG and scrA) genes upon the application of fluoride (Figure 4B). No obvious regulation of these genes by fluoride was seen in C180-2FR (Figure 4B). The insensitivity of gene expression in C180-2FR to fluoride may relate to the over-expression and hyper-function of fluoride antiporters. It has been proved in our previous study that C180-2FR expresses significantly more fluoride antiporters than C180-2, which are responsible for the efflux of fluoride ions (F-) (Baker et al. 2012; Liao et al. 2015). The more efficient efflux of F- can result in lower intracellular F- levels, which leave the glucose uptake genes undisturbed.

The discrepancy between the low glucose-uptake activity and high glucose uptake gene expression in S. mutans C180-2 in the presence of fluoride could be explained as a compensatory event and response to stressful challenges. Similar up-regulation of genes was observed in cells grown with limited glucose (Moye et al. 2014). It is known that genes could be regulated in a either negative or positive feedback loop (Maamar et al. 2007). The function of the enzymes involved in glucose uptake can be inhibited by different challenges in the environment, either the limited access to glucose or the presence of fluoride

(Moye et al. 2014). A compensatory feedback may emerge to cope with the perturbations raised due to the stresses (Kitano 2004). The up-regulation of glucose uptake-related genes may be an action taken by the bacteria to compensate the decreased ability to internalize sugars (Moye et al. 2014). The outcome of the compensatory up-regulation was limited, as the function of the enzymes requires further post-transcriptional regulations (Bobrovskyy and Vanderpool 2016).

In summary, we confirmed in this study that the glucose uptake is differently regulated in fluoride-sensitive and fluoride-resistant S. mutans strains. The differences are seen in total glucose-uptake activity, PTS activity and glucose uptake gene expressions. The transport of fluoride ions and deactivation of pyruvate kinase could be involved in the regulation mechanisms.