Quantitative comparison of mRNA expression of glucosyltransferase(GTF) between xylitol-resistant(X^R) and xylitol-sensitive(X^S) mutans streptococci

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Abstract

Since the long-term exposure of mutans streptococci to xylitol is known to select for xylitol-resistant (X^R) natural mutants, the occurrence and survival of such X^R strains were performed in batch culture methods.

The aim of the study was to compare the differentiation and quantification of mRNA expression of the *gtf* genes of X^R and X^S mutans streptococci.

Using a real-time reverse-transcription polymerase chain reaction, the expression of each *gtf* was determined. In X^R strains, the relative levels of transcription of *gtfB* and *gtfC* were decreased while that of *gtfD* was increased, suggesting the presence of independent promoters.

It also suggested that mutation related to production of glucosyltransferase occurred under the exposure of xylitol could explain the caries-preventive mechanisms of xylitol.

Key words : Xylitol resistant(X^R), *gtf*, Glucosyltransferase

Ⅰ. INTRODUCTION

Xylitol has been used as a reliable substitute for sugar to inhibit the dental caries. It has been shown that a total or partial replacement of sucrose by xylitol in the diet decreased the formation of dental plaque and dental caries[1-5]. Various mechanisms have been put forward as explanations for the caries-prevention[6-8]. It has been suggested that not only is xylitol not fermented by most dental plaque microorganism but also it interfere with the *in vitro* growth of the organism including mutans streptococci[9-13]. However, there are a lot of reports with controversy about the inhibiting growth of cariogenic bacteria[14-16]. Though several hypotheses have been introduced to explain the growth inhibitory effect of xylitol, no clear evidence has yet shown the nature of the effect. Besides, Wennerholm and Emilson[17] found that the mean levels of salivary mutans streptococci returned to their pre-xylitol consumption levels within three months of habitual xylitol consumption. Birkhed *et al.*[18] found no difference in the relative number of

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Streptococcus mutans (S. mutans) in plaque before and after a three-month period of frequent xylitol consumption.

Investigation of the xylitol-mediated growth inhibition of mutans streptococci showed that xylitol was transported and phosphorylated by a constitutive fructose-PTS in mutans streptococci and that non-metabolizable xylitol-phosphate accumulated intracellularly[19,20]. Trahan *et al.*[20] have shown that the accumulation is followed by a xylitol-phosphate dephosphorylation and by efflux of xylitol. A so called 'futile cycle' is thus established which results in a futile expenditure of energy and a partial inhibition of growth of mutans streptococci on glucose. However, consecutive periods of growth of mutans streptococci on glucose in the presence of xylitol result in a progressive loss of sensitivity to xylitol by the bacterial population as a consequence of a selective enrichment of naturally-occurring xylitol-insensitive or xylitol-tolerant mutant lacking the constitutive fructose-PTS responsible for xylitol uptake. These xylitol-insensitive or xylitol-tolerant mutants were also termed "xylitol-resistant(X^R)" when they were first partially characterized and have retained this designation[19].

Such a selection process also occurs *in vivo* in humans xylitol consumers[21]. Like the laboratory X^R strains, they had a low constitutive fructose-PTS specific activity and a very low xylitol-phosphorylating capacity as compared with xylitol-sensitive(X^S) isolates.

Mutans streptococci produces three glucosyltransferase, coded by *gtfB*, *gtfC*, and *gtfD*, whose action is essential for sucrose-dependent cellular adhesion which plays an important role in the formation of dental biofilm inducing dental caries[22].

The purpose of the present study was to compare the differentiation and quantification of mRNA expression of the *gtf* genes of X^R and X^S mutans streptococci.

Ⅱ. MATERIALS AND METHODS

X^R and X^S strains of S. mutans culture conditions

Three *S. mutans* strains (*S. mutans* Ingbritt, *S. mutans* LM7, and *S. mutans* GS5) were used in the experiments. The isogenic X^R mutants of the parental *S. mutans* strains were selected after sequential cultivations in the presence of 1% xylitol and 1% glucose according to the method by Trahan *et al.*[19] All strains were kept frozen at -80 ℃. As needed, a culture was started from a frozen liquid culture. The identity of the strains and the absence of contamination were controlled for each experiment by Gram staining and by PCR method. *S. mutans* strains was cultured in brain-heart infusion broth (BHI, Difco, Detroit, Mich. USA)

Extraction of total RNA

S. mutans strains used were grown at 37 ℃ to an optical density of 1.0 at 550 nm. The cell suspension (1 ml) was inoculated into 100 ml of fresh broth and incubated at 37 ℃. When the culture reached optical densities of 0.6 (mid-exponential phase), the cells were collected by centrifugation at 3000 × g, 10 min. RNA samples were extracted by a hot-phenol method[23]. Briefly, after the cells were washed with phosphate buffered saline(pH 7.4), an equivalent number of cells (determined at an optical density of 1.0 at 550 nm) was collected by centrifugation at 3000 × g, 10 min. Total RNA was isolated by using TRIzol reagent (Invitrogen Life Technology Carlsbad, Calif, USA) according to the manufacturer's instructions. The RNA was precipitated by ethanol, and suspended in diethylpyrocarbonate (DEPC; Sigma, St. Louis, MO, USA) treated water. Crude RNA was further purified by means of an RNeasy RNA isolation column with digestion of RNase-free DNaseI, as recommended by the manufacturer. Purified RNA was extracted from the column with 100 μl of DEPC-treated water and stored at -80 ℃.

Quantification of RNA

DNA and RNA amounts were determined with the use of PicoGreen double-stranded DNA quantitation kit and a RiboGreen RNA quantitation kit with a Fluorometer, respectively.

Primer Design

The outline of real-time quantitative RT-PCR and

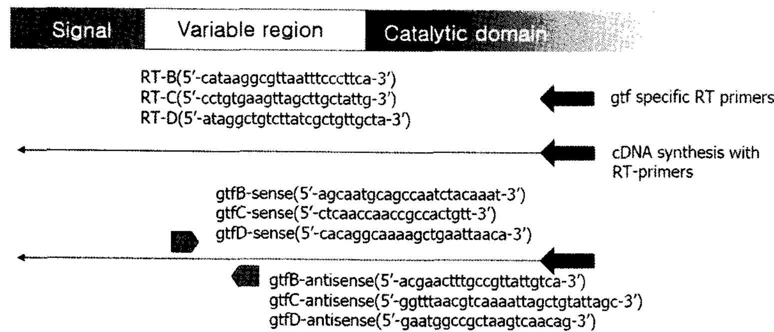

Fig. 1. Outline of and primers for real-time quantitative RT-PCR. The target for PCR was the 5' one-third region of the *gtf* genes shown at the top.

primers used in this study is shown in Fig. 1. The first-strands synthesis of cDNA was primed by means of gene-specific primers. The primers RT-B1117 (5'-cataaggcgttaatttcccttca-3'), RT-C1195 (5'-cctgtgaagttagcttgctattg-3'), and RT-D1164 (5'-ataggctgtcttatcgctgttgcta-3') were designed corresponding to the 5' region of the genes encoding the catalytic domain from the *gtfB, gtfC, gtfD* genes of *S. mutans*. The primer sets B442f (5'-agcaatgcagccaatctacaaat-3') and B537r (5'-acgaactttgccgttattgtca-3') and D514r (5'-gaatggccgctaagtcaacag-3') were designed for PCR amplification, corresponding to the hyper-variable region 580-870 bp upstream of the RT primers. The expected sizes for each PCR product from *gtfB, gtfC, gtfD* were 98 bp, 93 bp, and 83 bp, respectively. The primer set RecA/F1 (5'-ccggaatcttctggtaag-3') and RecA/R1 (5'-ctaattcacctgtacgag-3'), corresponding to the recA gene of *S. mutans*, was designed to compare with the expression of the housekeeping gene.

Reverse transcription and Real-time PCR

The reverse transcription (RT) reaction mixture (20 μl) containing 2.5 μM of each RT-primer, 1 μg of total RNA sample, and 200 U of Super Script II reverse transcriptase (GIBCO, USA) was incubated at 42 ℃ for 50 min, after which the cDNA sample was stored at -20 ℃. Real-time quantitative PCR was performed by GeneAmp 7500 Sequence Detection System (PE Applied Biosystems) with Premix RT-PCR kit (Takara, Kyoto, Japan). The reaction mixture (25 μl) contained 0.5 μl of the cDNA sample and 0.025 μM of the appropriate PCR primer. The cycle profile was as follows: 1 cycle at 50 ℃ for 2 min, 1 cycle at 95 ℃ for 1 min, 40 cycles at 95 ℃ for 15 sec, and 60 ℃ for 20 sec, and a slow ramp (20 min) from 60 to 95 ℃. The dissociation curve was captured during this slow ramp. Experiments were performed in triplicate. The critical threshold cycle (Ct) was defined as the cycle at which fluoroscence became detectable above the background and was inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer set with Ct values obtained from amplification of known quantities of the genomic DNA isolated from *S. mutans*. The standard curves were used for transformation of the Ct values to the relative number of DNA molecules. Data was expressed as means and standard deviations of triplicate experiments. The contamination of genomic DNA was determined with control reactions that contained no reserve transcriptase.

Protein analysis

The bacterial cells were collected by centrifugation at 6,500 × g for 10 min, washed twice in PBS and resuspended in PBS. The cells were lysed by incubating them with 100 μg of lysozyme per ml on ice for 30 min and subsequently sonicated with an ultrasonic processor (six 10-s bursts at 18 W with a 10-s cooling period between bursts; Sonics & Materials, Inc. Newtown, Conn, USA). The protein were separated

on an sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis. The gels were stained with Coomassie blue. Prestained markers were from Bio-rad (Boston, MA, USA).

Statistical analysis

Inter-group differences of various factors were estimated by a statistical analysis of variance (ANOVA) for factorial models. Student's t-test was used to compare individual groups.

Ⅲ. RESULTS

PCR by specific primer of *S. mutans* X^R and X^S was performed to investigate the possible contamination in the course of sequential cultivation(Fig. 2).

Isolated total RNA samples contained non-detectable amounts of double-stranded DNA by PicoGreen double-stranded DNA kit. The amounts of total RNA of each group are shown in Fig. 2. An equal amount of total RNA (1 μg) from each group was used for quantification of the transcript levels of the *recA* genes and the *gtf* genes. Using RT-PCR, we observed no significant difference in the expression of the *recA* gene from each sample, as well as interference by dimer of primer (data not shown). Though the level of *gtf* mRNA was different according to each strains, it has the same tendency. The level of *gtfB* and *gtfC* were significantly different between the XS and X^R groups and that of *gtfD* was limitedly different in both strains. The expression of *gtfB* and *gtfC* was decreased in X^R group though the expression pattern of *gtfD* was reverse in both strains(Fig. 3).

Surface proteins expressed by SDS-PAGE has characteristic of 100 kDa band in X^R of *S. mutans* Ingbritt(Fig. 4). In the other strains, no difference was detected (data not shown).

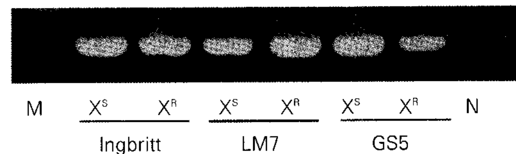

Fig. 2. Identification of *S. mutans* gene by PCR. PCR amplification with spiecies-specific primer.
Lane M, Marker; Lane 2 and 3, *S. mutans* Ingbritt; Lane 4 and 5, *S. mutans* LM7; Lane 6 and 7, *S. matans* GS5; Lane 8, Negative control.

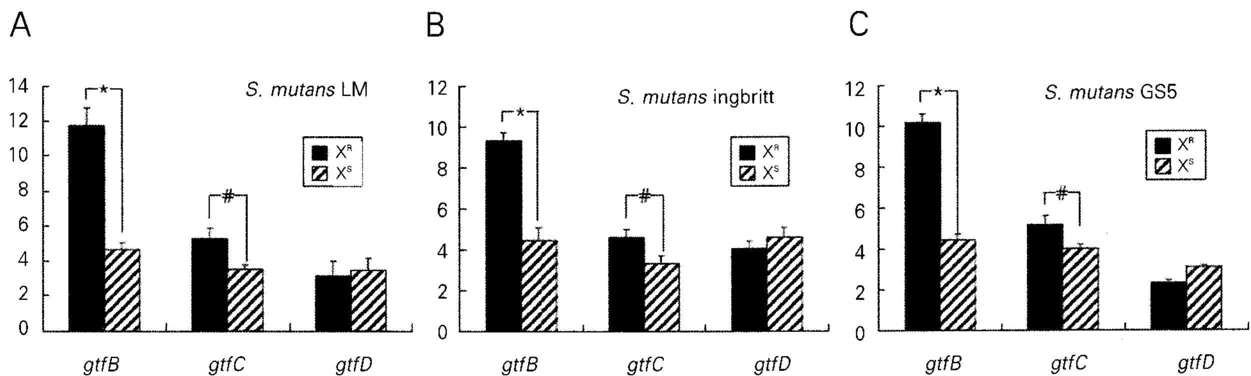

Fig. 3. Total RNA and relative quantities of *gtfB, gtfC,* and *gtfD* mRNA in the mid-exponential growth phase of *S. mutans* LM7, GS, Ingbritt. Amounts of extracted total RNA were assessed by means of a RiboGreen RNA quantification kit with a fluorometer. Following reverse transcription from 1 ug of total RNA, the amount of each *gtf* cDNA was determined by real-time PCR. Data were expressed as means and standard deviation of triplicate experiments. There was a statistical difference ($p<0.05$, ANOVA) according to the genes.
* statistically different # statistically different

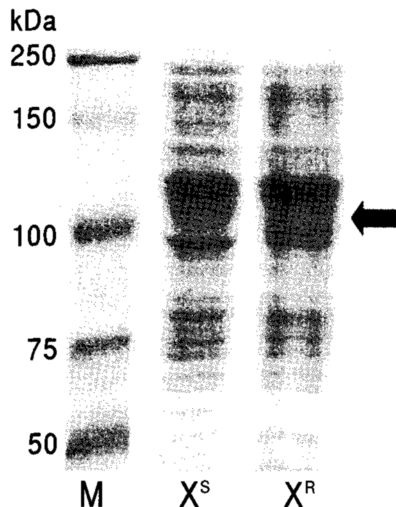

Fig. 4. Comparison of the protein profiles of cellular extracts from *S. mutans* Ingbritt xylitol sensitive (X^S) and xylitol-resistant (X^R) strain. Cells were grown at 37 ℃, extracted, and the protein extracts were analyzed by SDS-PAGE as described in Materials and Methods.

Ⅳ. DISCUSSION

The use of xylitol as an anticariogenic agent is being widely considered as it does not induce dental caries, and partial substitution of sucrose with xylitol in the human diet resulted in a reduction in the incidence of caries[24]. It was suggested that xylitol can stimulate many a existing defense mechanisms of pathogenesis of dental caries such as salivary flow and the maintenance of a high pH in oral fluid and dental plaque[24]. Some effects of xylitol on the ecosystem of dental plaque, especially cariogenic bacteria have also been reported such as lessening of glucosyltransferase, invertase and sugar permease activities, and modification of the proportion of soluble polysaccharides in plaque[14,25]. However, they are measured by traditional methods though the exact mechanism are not clearly understood. Real-time-PCR is advantageous for the measurement of levels of specific RNAs of the growing bacteria in respects of sensitivity and specificity. The conventional method like Northern blotting generally require a lot of RNA. In addition, since the coding region of the gtf genes is approximately 5000bp, it is difficult to isolate whole size mRNA thoroughly. However, in the present study, each gtf transcription could be detected from a 1200-bp 5' fragment, which is one of the advantages compared with the conventional methods. Trahan and Mouton[21], who first reported the emergence of resistance against xylitol suggested that the loss of sensitivity might be explained by several mechanisms such as depression of preexisting enzymes which allow the cells to utilize xylitol and repression of enzymatic systems accountable for the inhibition phenomenon and also loss of a plasmid coding for such an enzymatic system as well as mutational events. However, insensitive cells could not grow at the expense of xylitol and no return to a sensitive state was observed when xylitol was removed from the growth medium by Gauthier *et al.*[26]. Even though no plasmid was detected in sensitive cells, they concluded that resistance resulted from a mutational event.

A shift from xylitol-sensitive to a xylitol resistant population under the selective pressure of xylitol in the oral environment is probably the most dramatic effect of xylitol on the oral microorganism that has been reported to date[27,28]. At first, it could seem to be controversy, however, evidence is accumulating to suggest that the natural mutants selected by xylitol are less virulent and may have a clinical significance by contributing to the establishment of a less cariogenic plaque in xylitol consumers.

Cariogenic bacteria have several virulence factors such as acid production, hydrophobicity, adhesivity, adaptation to the external environment. Glucosyltransferase whose cooperative action is essential for cellular adhesion to the tooth surface. Adhesive glucan mediate attachment of bacteria to the tooth surface as well as to other bacteria so that they also have an effect on the physiological status of plaque biofilm, and contribute to the cariogenicity of *S. mutans*. This is the first report of distinctive transcriptional analysis of the gtf genes in xylitol sensitive and xylitol resistant *S. mutans* growing cells and the result from the dynamic process of gtf expression in sucrose-dependent cellular adhesion. GTFB, which mainly synthesize water-insoluble glucans, are encoded by the *gtfB* genes, and is one of the main virulence factors of *S. mutans*. In the present study, *gtfB* expression was decreased in all the X^R strains, which could support the hypothesis; 'X^R is less virulent than X^S', though it is still controversial.

PCR technique is widely accepted to the dental research fields, especially real-time PCR might be useful experimental tool for examining the differentiation and quantitation of a certain protein expression inducing the characteristics of virulences. In the present study, X^R *S. mutans* Ingbritt but not the other strains revealed about 100 kDa band in SDS-PAGE . Although the total amount of mRNA of *gtfB* and *gtfC* was decreased in all the X^R strain, the degree was different according to the original strains. It is suggested that the independent promoters present related to the expression of GTFB, GTFC, and GTFD.

Further examination using the sensitive and analytical methods like 2-D electrophoresis would be necessary to define the accurate anticariogenic effects of xylitol.

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Abstract

Mutans streptococci의 자일리톨 내성균주와 감성균주의 glucosyltransferase mRNA의 정량적 비교 연구

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자일리톨에 장기간 노출된 mutans streptococci는 자일리톨에 내성이 발현되어 자일리톨 내성균주가 생성된다고 알려져 있다.

본 연구의 목적은 mutans streptococci에서 자일리톨 내성균주와 감성균주의 *gtf* 유전자 발현량을 각각의 유전자별로 정량적으로 분석하고 비교하는 것이다.

실시간 역전사 중합효소연쇄반응법을 이용하여 각각의 *gtf* 발현을 조사한 결과 *gtfD*는 증가한 반면, *gtfB*와 *gtfC*는 감소하였는데 이는 각 유전자의 독립된 조절기전이 존재함을 보여주는 것이다. 또한 자일리톨에 노출된 mutans streptococci에서의 glucosyltransferase와 연관된 유전자변형이 자일리톨의 치아우식증 예방효과의 작용기전 중 하나임을 알 수 있었다.

주요어 : 자일리톨 내성, *gtf*, Glucosyltransferase