

## Cloning and expression of *Streptococcus mutans* GS-5 glucosyltransferase

Su-Kyeong Kim, Jae-Gon Kim, Byeong-Ju Baik, Yeon-Mi Yang,  
Kyung-Yeol Lee, Jeong-Yeol Park

*Department of Pediatric Dentistry and Institute of Oral Bioscience, School of Dentistry, Chonbuk National University*

### Abstract

Dental caries is an infectious disease caused by mutans streptococci, and is a primary etiologic agent of dental caries in humans. The molecular pathogenesis of mutans streptococcal-associated dental caries occurs in three phases. Firstly, *S. mutans* attaches to tooth surface via a cell surface adhesion termed antigen I/II. In the second phase, the glucosyltransferase(GTFs) synthesize polymers like glucans in the presence of sucrose. In the third phase, the multivalent glucans interacts with glucan binding proteins (GBPs) and they make dental plaque and accumulation of microorganisms.

Many studies and clinical trials have indicated that a mucosal immune response to these antigens(Ag I/II, GTFs, GBPs) of *S. mutans* can influence the pathogenesis of dental caries. So these antigens can be important vaccine candidates for immunologic intervention against dental caries.

In this study, we cloned the genes for GTFb, GTFc, GTFd from *S. mutans* GS-5 and did the nucleotide sequence analysis. And the recombinant proteins of GTFd and N-terminus of GTFd were expressed. Intact GTF which we get from this experiment can be used for antibody production specific for any GTF activity domain through animal experiment.

**Key words** : *Streptococcus mutans*, Glucosyltransferase, Glucan

### I. INTRODUCTION

Among oral bacteria, mutans streptococci have been known as causative agents of dental caries. Based on DNA homology, mutans streptococci are divided into several species—*S. mutans*, *S. rattus*, *S. sobrinus* etc. And these species can be subdivided in-

to eight serotypes by their surface glycoproteins. *S. sobrinus* and *S. mutans* are most often isolated from humans and associated with the dental caries<sup>1-3</sup>.

The molecular pathogenesis of mutans streptococci appears to involve several phases, each of which may offer targets for immunological intervention<sup>4,5</sup>. The first phase involves the initial attachment of the mutans streptococci to the glycoprotein or the dental pellicle. This is mediated by an adhesion from mutans streptococci that is known as antigen I/II (also known as P1 and Pac)<sup>6</sup>. The second phase, which is also called as accumulation phase, depends on the presence of sucrose, as well as glucosyltransferas-

교신저자 : 김 재 곤

전북 전주시 덕진구 덕진동

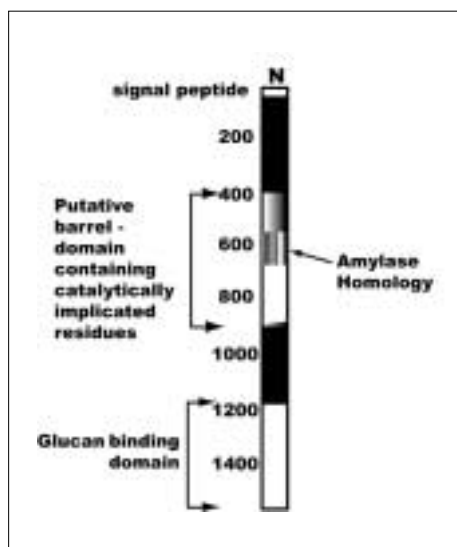
전북대학교 치과대학 소아치과학교실

Tel: 063-250-2121, 2223 Fax: 063-250-2131

E-mail: pedodent@chonbuk.ac.kr

es(GTFs) and glucan-binding proteins(GBPs) from mutans streptococci. After cleaving sucrose into its component saccharides(glucose and fructose), mutans streptococci GTFs synthesize glucans. In the third phase, the multivalent glucans that have been produced interact with GBPs and with the glucan-binding domain of GTFs. The aggregation and the multiplication of the bacteria result in the accumulation of biofilms<sup>2,5,7,8</sup>. Like this, cell association and accumulation through glucan binding is critical for the cariogenic plaque formation and therefore prevention of this process by sIgA antibody will be one of ideal approaches to interfere dental caries process<sup>14,18,22</sup>.

Antibody could block the receptors necessary for colonization(e.g. adhesins) or accumulation(e.g., glucan-binding domains of GBPs and GTF), or inactivate GTF enzymes responsible for glucan formation<sup>5,9-13</sup>. The activity of GTF is mediated through both catalytic and glucan-binding functions. The catalytic activity of GTF appears to be associated with several residues in the N-terminal one-third of the molecule. The C-terminal region of GTF molecule contains a pattern of repeating sequences which have been identified in all GTFs from mutans streptococci (Fig. 1)<sup>7,8</sup>.



**Fig. 1.** Schematic representation of glucosyltransferase as a major candidate caries immunogen. The catalytic or sucrose-binding and splitting region is located at the N-terminal one-third of glucosyltransferase, while the glucan-binding region at the C-terminal one-third of the molecule.

Previously, induction of sIgA antibody in humans by oral or topical GTF administration resulted in the reduction of indigenous mutans streptococci<sup>5</sup>. Passive administration of antibody to GTF in the diet also can protect rats from experimental dental caries<sup>5</sup>. Thus, the presence of antibody to glucosyltransferase in the oral cavity prior to infection can significantly influence the disease outcome, presumably by interference with one or more of the functional activities of the enzyme.

The ultimate aim of this experiments is to develop anticaries vaccine by recombinant GTF protein production. And in this point of view, it is different from other studies, which used GTF protein directly purified from bacteria. For the first step, I cloned 3 main GTFs from *S. mutans* GS-5 and subcloned the full length GTFd and amino-terminal half fragment of GTFd protein. And until now, the expression of GTFd is in progress. Continuously, GTFb and GTFc will be expressed for antibody production or inactivation of their catalytic or glucan-binding functions.

## II . MATERIALS AND METHODS

### 1. Materials

All chemicals and plastic wares were purchased from Sigma (St. Louis, MO, U.S.A.) and Falcon Labware (Frankline Lakes, NJ, U.S.A), respectively. Oligonucleotides were ordered from Genetech (Yusung, Korea) and T vector was supplied from Takara (Shiga, Japan). Restriction enzymes were obtained from KOSCHEM (Seongnam, Korea).

### 2. Bacterial culture

*S. mutans* GS-5 was grown in BHI medium for 16h at 37 in an anaerobic chamber and 200 µl of overnight culture was transferred into 5ml of a fresh medium, leaving for further incubation.

### 3. Preparation of *S. mutans* GS-5 genomic DNA

The genomic DNA from *S. mutans* GS-5 was prepared as described previously<sup>5,15</sup>. Briefly, 5 ml of *S. mutans* GS-5 was collected and resuspended in 4 ml of TE buffer containing 1 ml of 25% glucose. After 30

min incubation at 37°C, 1 mg of lysozyme was added into the bacterial suspension and further incubated at 37°C for 1h. RNAs in the lysates were removed by adding 0.5 mg of RNase A and then proteins in the lysates were digested by 0.5 mg of proteinase K. The cleared lysates were treated with SDS for 12 h at 4°C and then genomic DNA was precipitated by ethanol. Finally, the genomic DNA was resuspended in 1 ml of TE buffer.

4. PCR amplification of GTFb, GTFc or GTFd genes

The genomic DNA was used as templates for amplifying GTFb, GTFc or GTFd genes in 50 µl of PCR reaction containing Taq polymerase, dNTP, and oligonucleotides. The primers specific for GTFb, GTFc or GTFd genes were designed and synthesized as described in Table 1. GTFd genomic DNA was fragmented into smaller pieces by digestion with BamHI and SmaI enzymes. And GTFb or GTFc genomic DNA were digested with BglII and KpnI restriction enzymes. The genomic DNA was denatured by 5 min incubation at 95°C and then target genes were amplified for 30 cycles of PCR reaction (1 min 95°C, 1 min 55°C, 1 min 72°C).The PCR product was then treated for 5 min at 72°C.

5. Cloning of GTFb, GTFc or GTFd genes

About 2 µl of PCR products were ligated into T vector following manufacture 's protocol and the ligation mixtures were transformed into competent Top 10 cells. The resulting antibiotics resistant bacteria were selected from agar plates and screened by checking the plasmid from each bacterial colony.

First, plasmids were compared in size with that of control vector in agarose gels to reveal the presence of any insert DNA. Second, digestions of plasmids with restriction enzymes were performed to further prove the presence of target genes. The plasmids containing expected insert size were then sequenced to confirm the presence of target gene. The DNA sequencing was performed by BMRC (Daejeon, Korea).

6. Sequence analysis of GTF genes from *S.mutans* GS-5

The DNA sequence of cloned genes was analyzed by comparing with the previously known sequences. For GTF sequence analysis, the known sequence from *S. mutans* GS-5 (GenBank # D78181) was used as standard. The programs, Multalin<sup>14)</sup> and ESP-ript<sup>15)</sup>, were used to generate sequence alignment between two genes.

7. Expression of recombinant GTFd protein in bacterial expression system

In order to express cloned genes in *E. coli* system, gene fragments of GTFd and GTFd N-terminal were subcloned to pQE expression vector. BamHI and Sall were used to transfer N terminus of GTFd gene(GTFd<sub>mr</sub>), BamHI and SmaI for full length GTFd. The ligation mixtures were transformed in Top10 and M15 bacterial strains. Transformed M15 bacterial colonies were inoculated and grown overnight at 37°C in Luria Broth containing 100 µl/ml of ampicillin and 25 µg/µl of kanamycin. The cells will be further grown in the presence of IPTG to induce expression of the fusion protein.

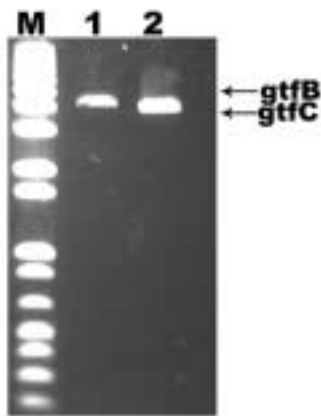
**Table 1.** List of oligonucleotides used to amplify gtfB and gtfC or gtfD genes. The small case shows the extra nucleotides to help gene cloning. The underlined sequences are the locations of restriction enzyme sites

	Sequence
gtfB5	ggaAGATCTAATGAATCGAAATCCCAA
gtfB3	cggGGTACCTTAGTTAATCCGAACTCG
gtfC5	ggaAGATCTACTGACGACCGTCAACAG
gtfC3	cggGGTACCTCACATAACGATTACCAG
gtfD5	gatcGGATCCGAAACAGAACAGCAGACCTCAG
gtfD 3	tccCCCGGGTTAATAATATCTAGCGATACCCCAACG
gtfD M5	GATAAAAGT <u>CATATGGATTGGGAT</u>
gtfD M3	ATCCCAATCC <u>CATATGACTTTTATC</u>

### III. RESULTS

#### 1. PCR product of GTFb, GTFc or GTFd gene

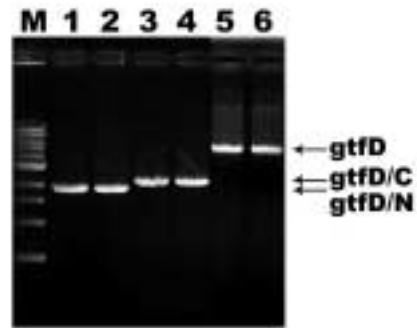
The genomic DNA from *S. mutans* GS-5 was isolated and used as templates for GTF gene amplification. The primers specific for GTFb, -c, -d genes were separately designed based on the published sequences. PCR was performed yielding 4.3 kb GTFb, and 4 kb GTFc (Fig. 2). In a similar way, 4.6 kb full length GTFd and N-terminal or C-terminal half size of GTFd genes were also amplified (Fig. 3).



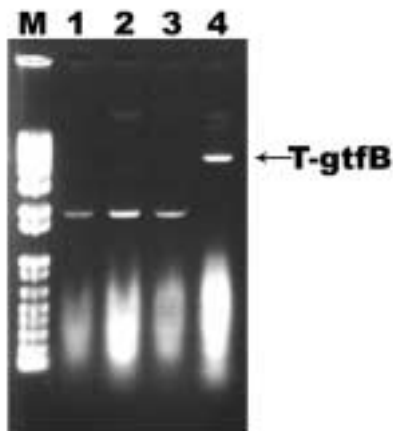
**Fig. 2.** PCR amplification of *S. mutans* GS-5 genes encoding GTFb and GTFc genes. The genes for GTFb and GTFc were amplified by PCR using the genomic DNA linearized by restriction enzyme digestion. M indicates size marker.

#### 2. Cloning of into T-vector of GTFb, GTFc or GTFd gene

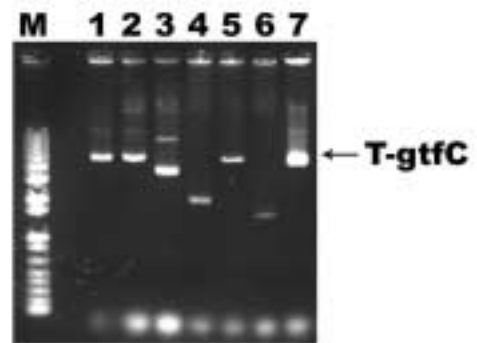
PCR products were ligated into T vector, yielding T vector cloned with GTF genes. The cloned T vectors were transformed into an *E. coli* strain, Top 10. Plasmids were prepared from antibiotics-resistant colonies and separated in agarose gel. In the agarose gel, the fourth lane band is higher than the others because it includes insert gtfB DNA (Fig. 4). There are several bands including expected size of gtfC inserts (Fig. 5). Similarly, the second lane band of the



**Fig. 3.** PCR amplification of *S. mutans* GS-5 genes encoding GTFd genes. In addition to full-length genes (GTFd), truncated genes encoding 5' or 3' segments were also amplified.



**Fig. 4.** Screening of plasmids cloned with PCR-amplified GTFb genes. The genes encoding GTFb were cloned into T vector and bacterial transformants were screened to figure out the presence of insert DNA. Arrows indicates the positions of plasmids with insert DNA.

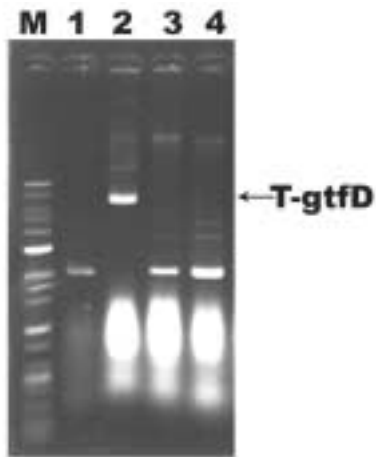


**Fig. 5.** Screening of plasmids cloned with PCR-amplified GTFc genes. The genes encoding GTFc were cloned into T vector and bacterial transformants were screened to figure out the presence of insert DNA. Arrows indicates the positions of plasmids with insert DNA.

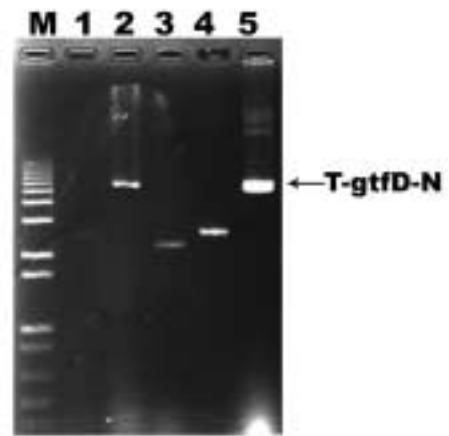
gtfD agarose gel shows 7.6 kb band size corresponding total size of T-vector and GTFd gene(Fig. 6). GTFd N-terminus as well as GTFd full length was ligated into T-vector because sometimes cloning and expression of smaller fragment are helpful for easy protein expression or development of specific antigen epitope(Fig. 7).

### 3. Sequence analysis of cloned genes

After checking the presence of any inserts in the ligated plasmids, we confirmed the integrity of cloned GTFb, GTFc, GTFd or GTFd N-terminal sequences by nucleotide sequencing. For GTFd analysis, the corresponding sequence from *S. mutans* GS-5 was compared with that of the previously-cloned gene. The representative sequence alignment was shown in Fig. 8. There was no miss match at all.



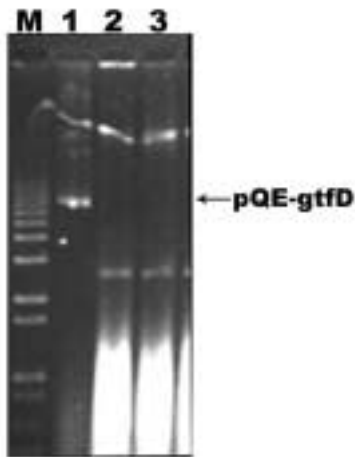
**Fig. 6.** Screening of plasmids cloned with PCR-amplified GTFd genes. The genes encoding GTFd were cloned into T vector and bacterial transformants were screened to figure out the presence of insert DNA. Arrows indicates the positions of plasmids with insert DNA.



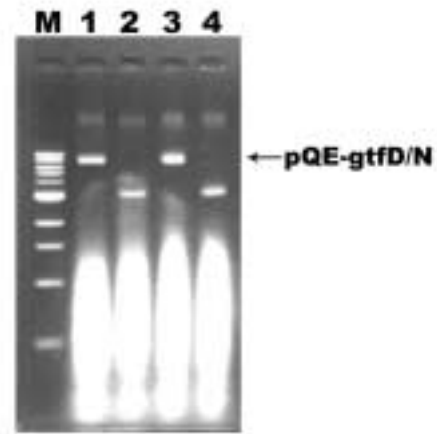
**Fig. 7.** Screening of plasmids cloned with PCR-amplified GTFd-N terminus genes. The genes encoding GTFd-N terminus were cloned into T vector and bacterial transformants were screened to figure out the presence of insert DNA. Arrows indicates the positions of plasmids with insert DNA.



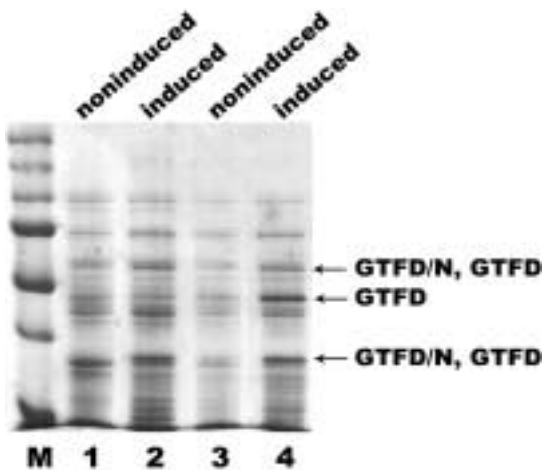
**Fig. 8.** Sequence alignment between cloned GTFd and the corresponding region in reported GTFd genes. The sequence of the cloned GTFd genes was compared to that of known GTFd genes.



**Fig. 9.** Screening of plasmids of GTFd cloned in pQE-30 vector. The pQE- GTFd plasmids were transformed in M15 bacterial strains, and their transformants were screened for the presence of recombinant expression vector.



**Fig. 10.** Screening of plasmids of GTFd-N terminus cloned in pQE-30 vector. The pQE-GTFd-N terminus plasmids were transformed in M15 bacterial strains, and their transformants were screened for the presence of recombinant expression vector.



**Fig. 11.** Protein expression pattern with crude cell lysates of GTFd and GTFd-N terminus. The first and second lanes are the crude cell lysates of GTFd. The third and fourth lanes are the crude cell lysates of GTFd-N terminus. M indicates marker and there are some changes of band patterns between before and after of induction.

#### 4. Subcloning GTFd or GTFd N fragment into expression vector

GTFd full length and N terminus were subcloned into pQE-30 expression vector according to the procedure suggested by the manufacturer(Qiagen). And the pQE-GTFd and pQE-GTFd/N transformed into competent M15 host cells. GTFd and GTFd/N plas-

mids were isolated and purified for the induction of recombinant protein. Plasmids with expected GTFd and GTFd/N genes were prepared from antibiotics-resistant colonies and separated in agarose gel (Fig. 9, 10). One colony containing pQE-GTFd plasmid and two colonies containing pQE-GTFd-N terminus were obtained.

#### 5. GTFd, GTFd-N terminus expression

Before the purification using Ni-NTA resin, we compared that there are some changes of protein expression between before the induction and after the induction. For induction, 1ml of M 15 host cell including pQE-GTFd or pQE-GTFd-N terminus were harvested. And 30  $\mu$ l of 1X SDS sample buffer was added to each sample. After that, all samples were heated at 100 with 30  $\mu$ l of 1X SDS sample buffer and microcentrifuged. 5  $\mu$ l of each sample were gel loaded.

GTFd/N shows the increased band at above 50 kDa and below 37 kDa. GTFd shows the increased band at above and below 50 kDa, and below 37 kDa. It may imply the expression of GTFd and GTFd/N proteins because these expression patterns can not be detected from other protein expressions (Fig. 11).

Currently, the conditions to maximize the yield of target proteins are being tested. The purified protein

will be used to make antibodies for the future experiment of vaccine development. Hopefully, segmented fragments will be also useful to determine an efficient epitope for the induction of protective immunity against dental caries.

#### IV. DISCUSSION

The extracellular polysaccharides synthesized from sucrose via the glucosyltransferases(GTFs) of *S. mutans* have been recognized to be important virulence factors for the induction of human dental caries (Loesche,1986)<sup>15)</sup>. *S. mutans* synthesizes  $\alpha$ -(1-3) and  $\alpha$ -(1-6)-linked glucan polymers through the concerted action of three secreted GTFs that are encoded by the genes GTFb, GTFc and GTFd<sup>18,19)</sup>.

The tandemly arranged GTFb and GTFc genes encode the enzymes(GTF-I and GTF-SI, respectively) responsible for the synthesis of water-insoluble,  $\alpha$ -(1-3)-rich glucan. GTFd encodes GTF-S, which is responsible for the synthesis of  $\alpha$ -(1-6)-linked, water-soluble glucan<sup>2,5)</sup>. The principal roles of the extracellular glucans of *S. mutans* are to facilitate the adhesion of the organism and its accumulation on teeth, and to establish an extracellular polysaccharide matrix for the organism that may provide it with increased protection against mechanical host-clearance forces and which may afford it some protection against host immune defences<sup>20,21)</sup>. Like this, GTF takes a key role in the formation of a biofilm and bacterial colonization so it can be a target for the caries vaccine development<sup>22-26)</sup>.

There are many routes for caries vaccine delivery such as gut-associated lymphoid tissue(GALT), nasal-associated lymphoid tissue(NALT), tonsils, minor salivary gland, rectal etc. All routes have some advantages or disadvantages. For example, oral route includes the detrimental effects of stomach acidity on antigen, or relatively distant inductive sites. Therefore, the recent mucosal administration of antigen delivery often includes adjuvants which can potentiate the immune response<sup>27-35)</sup>.

In 2003, Vincent et al.<sup>36)</sup> made *S. mutans* mutants, which absent of a 155-kda glucosyltransferase S(Gtf-S) by knocking-out special regulator. And they confirmed these mutants are defective in sucrose-dependent adherence and significantly less cariogenic. So

they concluded that regulator can also control *S. mutans*-induced cariogenesis.

Several lines of studies indicate that the presence of specific antibody might modify the course of infection and disease with cariogenic mutans streptococci. For example, antibody specific GTF, when incubated *in vitro* with sucrose and growing cultures of mutans streptococci, markedly reduced the amount of plaque formed on hard surface<sup>2)</sup>.

Like this, antibody specific for any GTF activity domain can inactivate GTFs and interfere with synthesis of glucans, inhibiting accumulation of mutans streptococci. So we focused on production of recombinant GTF protein and the following antibody production in order to develop anticaries vaccine in the future. As a first step, we cloned three types of GTFs from *S. mutans* GS-5, and then subcloned GTFd and its N-terminal into pQE expression vector. At the current moment, the expression of GTFd and GTFd N-terminal is confirmed. In the crude cell lysate extracts of GTFd and GTFd N-terminal(Fig. 11), there were many fragmentations although its significance is not clear at this moment. But some efforts will be put to improve the expression conditions. And it is also needed that some changes such as host strains, vectors, growth conditions etc to increase the protein expression level.

Also, there may exist some difference and close interaction among all three types of GTFs. For example, glucan binding protein C plays an important role in sucrose-dependent adhesion by binding to the soluble glucan synthesized by GTFd especially<sup>19)</sup>. So it is necessary to continue the study about the purification each active domain of GTFb, GTFc, GTFd and their interactions. Furthermore, the effective antibody delivery system to human is another problem have to be solved in the future.

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국문초록

## *Streptococcus mutans* GS-5 Glucosyltransferase의 클로닝과 발현

김수경 · 김재곤 · 백병주 · 양연미 · 이경열 · 박정렬

전북대학교 치과대학 소아치과학교실

치아우식은 주로 *mutans streptococci*에 의해 야기되는 감염성 질환으로서 주 원인균에는 *streptococcus mutans*가 있다. *S. mutans*가 치아우식을 유발하는 분자 생물학적 기전은 몇 가지 단계를 포함한다. 먼저 *S. mutans*는 AgI/II와 같은 세포 표면의 섬유성 단백질을 매개로 치면의 타액성 피막에 일차적으로 부착한다. 두번째 단계에서 자당의 존재하에 glucosyltransferase(GTF)는 glucan과 같은 다당체를 합성하게 된다. 마지막으로 이렇게 합성된 glucan은 glucan binding proteins와 상호작용하여 치면세균막을 형성해서 세균의 군집화를 가능하게 한다.

많은 실험과 임상연구에서 *S. mutans*의 주요 항원(Ag I/II, GTFs, GBPs)들이 치아우식 병리기전에 영향을 준다고 알려져왔고, 따라서 이런 항원들이 면역계에 작용하여 치아우식을 막는 백신으로 이용가능하다.

본 실험은 *streptococcus mutans* GS-5로부터, GTFb, GTFc, GTFd 유전자를 복제하고 염기서열분석을 하였으며, 이중 GTFd가 먼저 재조합 단백질을 생산을 위해 발현 벡터에 클로닝되었으며, 이로부터 단백질이 발현됨을 확인하였다. 이번 실험에서 얻은 순수 GTF 항원은 동물실험을 통해 특정 GTF 활성부위에 대한 항체 생산에 이용될 수 있을 것이다.

**주요어** : 연쇄상구균, Glucosyltransferase, Glucan