

## PRODUCTION OF GLUCOSYLTRANSFERASE B AND GLUCANS BY *STREPTOCOCCUS MUTANS* STRAINS ISOLATED FROM CARIES-FREE INDIVIDUALS

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### ABSTRACT

Glucosyltransferase B is an enzyme produced by *Streptococcus mutans*, which catalyzes synthesis from sucrose of insoluble glucans that provide support to the biofilm. It is one of the main virulence factors in the generation of dental caries. However, its role is unclear in caries-free individuals who carry the bacteria. The aim of this study was to determine the production of glucosyltransferase B and the production of glucans by *Streptococcus mutans* strains isolated from biofilms of 30 caries-free individuals. Strains were grown in Todd Hewitt broth. Extracellular proteins were obtained by ammonium sulfate precipitation and associated membrane proteins by extraction with urea. The presence of GtfB was determined by molecular weight by SDS-PAGE and confirmed by Western blotting using a specific monoclonal antibody and polysaccharide production by elec-

trophoretic separation, incubation with sucrose and Schiff staining. The results show that 96.7% of strains of *Streptococcus mutans* produce a band at the position of the molecular weight corresponding to the glucosyltransferases, of which 63.4% are positive by Western blot. Polysaccharides are produced by 93.3% of the strains. Conclusions: *Streptococcus mutans* strains isolated from biofilm of healthy individuals produce virulence factors associated with dental caries, such as glucans and glucosyltransferase B. This indicates that there are conditions in the oral cavity different from these factors that keep the individual free from dental caries, which should be investigated in the search for strategies to control the disease.

Key words: glucosyltransferase B, dental caries, glucans, virulence factors, *Streptococcus mutans*.

## PRODUCCIÓN DE GLUCOSILTRANSFERASA B Y GLUCANOS POR CEPAS DE *STREPTOCOCCUS MUTANS* AISLADAS DE INDIVIDUOS LIBRES DE CARIES

### RESUMEN

La glucosiltransferasa B es una enzima producida por *Streptococcus mutans*, que a partir de la sacarosa, cataliza la síntesis de glucanos insolubles los cuales dan soporte a la biopelícula, siendo uno de los principales factores de virulencia para la generación de la caries dental. Sin embargo, no se ha esclarecido su papel en los individuos libre de caries, portadores del microorganismo. El objetivo de este estudio fue determinar la producción de glucosiltransferasa B y la producción de glucanos por cepas de *Streptococcus mutans* aisladas de biopelícula de 30 individuos libres de caries. Las cepas fueron cultivadas en caldo Todd Hewitt y las proteínas extracelulares fueron obtenidas por precipitación con sulfato de amonio las proteínas asociadas a membrana por extracción con urea. La presencia de GtfB fue determinada por peso molecular por SDS-PAGE, confirmada por Western Blot utilizando un anticuerpo específico y la produc-

ción de polisacáridos por separación electroforética, incubación con sacarosa y coloración de Schiff. Los resultados muestran que el 96.7% de las cepas de *Streptococcus mutans* producen una banda a la altura del peso molecular correspondiente a las Gtf, de las cuales son reactivas por western blot el 63.4%. El 93.3% de las cepas producen polisacáridos. Conclusiones: las cepas de *Streptococcus mutans* aisladas de biopelícula de individuos sanos producen factores de virulencia asociados a la caries dental como glucosiltransferasa B y glucanos lo que indica que hay condiciones en la cavidad oral diferentes a estos factores que mantienen al individuo libre de caries dental, los cuales deben ser investigados en la búsqueda de estrategias para controlar la enfermedad.

Palabras clave: Glucosiltransferasa B, Caries dental, Glucanos, Factores de virulencia, *Streptococcus mutans*.

### INTRODUCTION

Dental caries is one of the most prevalent infectious pathologies in the world<sup>1</sup>. In Colombia, 82% of the population is estimated to have had caries

experience<sup>2</sup> and despite knowledge of its origin, no 100 % effective method to prevent it has been found yet. The role of *Streptococcus mutans* in cariogenic activity is its ability to adhere to the

initially acquired film, produce acids and synthesize insoluble and soluble glucans that help maintain plaque<sup>3</sup> by producing glucosyltransferases (Gtfs) which metabolize sucrose into free glucans and fructose<sup>4</sup>.

There are three types of Gtf: GtfD, which synthesizes soluble glucans, and GtfB and GtfC, which synthesize insoluble glucans. The two main properties of these proteins are metabolizing sucrose and binding to glucan, acting as another factor in the adherence of this molecule called GBP<sup>5</sup>. It has been shown that GtfB and GtfC are essential in establishing the extracellular polysaccharide matrix in dental biofilm, and GtfB is proposed as being responsible for *S. mutans* forming microcolonies<sup>6</sup>. The presence of GtfB has been proposed as a marker for dental caries activity<sup>7</sup> and its importance in the physiopathogenesis of the disease has led to the design of prevention strategies such as vaccines<sup>8-10</sup> and blockage by means of natural substances such as apigenin, which modulates the expression of the genes GtfB and GtfC<sup>11</sup>, chocolate<sup>12</sup> and *Epigallocatechin gallate* tea<sup>13</sup>, among others<sup>14</sup>.

The probability of the existence of bacterial strains without the GtfB virulence factor or not expressing it arises insofar as the environmental conditions and host susceptibility can alter bacterial physiology. Thus, the same pathogen may cause disease in one individual yet not in another. Studies by Perrone and Fontana show the existence of fibrillose structures that have a defined role in adhesion processes and which have already been characterized in previous studies. A study of caries-resistant and caries-active subjects shows two bands recognized by anti P1 and anti Gtf antibodies, and these bands were found in more subjects with than without caries, suggesting that there are differences in the composition of the fimbriae of *S. mutans* in patients with and without caries, attributing to it a major role among the virulence factors of *S. mutans* in the physiopathogenesis of the disease<sup>15, 16</sup>.

As the role of the insoluble glucane-producing Gtfs has been shown to be important in aiding caries production, the aim of this study is to determine whether *S. mutans* strains isolated from caries-free patients have modified GtfB and polysaccharide production, and whether this makes them non-pathogens and compatible with a healthy state.

## MATERIALS AND METHODS

### Population and Sample

A descriptive study was performed on a total of 30 *S. mutans* strains isolated from bacterial plaque from 30 patients (one strain from each patient). All individuals were clinically healthy for dental caries (ICDAS 0), with permanent dentition, without systemic compromise and who had not received prior fluorine or sealant treatment or dental care. Before the sample was taken, the patients signed an informed consent form according to the guidelines of Resolution 008430 of 1993, which rules research on human beings in Colombia. Controls were a strain of *S. mutans* isolated from a patient with a high caries index (ICDAS 4) and a strain of *S. mutans* ATCC #31989 serotype c. (American Type Culture Collection Manassas, VA USA).

### Percentage of bacterial plaque

Once a patient was classified as healthy, O'Leary's<sup>17</sup> bacterial plaque index was taken using a plaque stain (Quirident, Bogotá, Colombia). After rinsing with water, surfaces with positive plaque were counted. For each patient the percentage was calculated by dividing number of stained surfaces by the total number of surfaces and multiplied by 100.

### Isolation and identification of *S. mutans*

Samples of bacterial plaque were taken from the vestibular surfaces of all the teeth with a sterile swab, and placed in Todd Hewitt medium (Oxiod Ltd, Cambridge, UK) for transportation, to be used for isolating *S. mutans* grown initially on mitis salivarius agar (Difco- Becton Dickinson, NJ, USA), supplemented with potassium tellurite (Oxiod) and incubated at 37°C for 24 hours under microaerophilic conditions. Biochemical identification tests were performed for which *S. mutans* is positive (VP, esculin, LAP, manose, sorbitol, lactose, threonine, inositol) using an API 20 Strep kit (BioMerieux S.A., Paris, Francia). After isolation and identification, each strain was preserved in trypticase soy medium (Difco) with 3% glycerol (Merck KGaA, Darmstadt, Germany) and frozen at -85°C (Nuair Laboratory Equipment Supply, Plymouth, MN, USA).

### Extraction of GtfB

Each strain was de-frozen at room temperature and subject to Gram staining, and reconstituted in 10 ml of Todd Hewitt (Oxiod) at 37° C for 24 hours, in a

microaerophilic atmosphere. Then it was transferred to 10 ml of the same culture medium and left to incubate under the same conditions for 18 hours. For precipitation of extracellular glucosyltransferases, 60% saturated 10 ml ammonium sulfate solution (Merck) was added to the 10 ml culture, and left at room temperature for 30 minutes. The sample was centrifuged at 10000 rpm for 5 minutes (Eppendorf Centrifuge 5403, Eppendorf Hamburg, Germany) and the precipitate was saved. To extract the glucosyltransferases associated to the membrane, the precipitate was suspended in 500 ml of 8M urea (Sigma Chemical Co, St Louis, MO, USA) in 10 mM potassium phosphate buffer at pH 7.2, incubated for 1 hour at room temperature with vigorous vortexing (Vortex Maxi Mix II 37600, Thermo Fisher Scientific Inc. Waltham, MA, USA).

After centrifuging at 10000 rpm for 5 minutes, the supernatant was removed and the precipitate extracted again under the same conditions. The two supernatants were put together and frozen at  $-20^{\circ}\text{C}$ . (Nuair Laboratory Equipment Supply)<sup>18</sup>.

## Identification of GtfB

### Electrophoretic separation

SDS-PAGE electrophoresis of the GtfF extracts was done on 10% polyacrylamide gel, with the buffer system described by Laemli in 1978, at constant 150v for 1 hour, in a double cell Mini protean II chamber (BIO-RAD Laboratories, Hercules, CA, USA)<sup>19</sup>. A pattern of high range molecular weight for SDS-PAGE (BIO-RAD, cat. No. 161-0309) was used. After the electrophoretic run, the gels were stained with Coomassie brilliant blue R-250 (BIO-RAD) to determine the molecular weights of the proteins obtained. Strains showing a band in the position of the molecular weight corresponding to GtfB were defined as positive. The gels that were used for determining polysaccharide production and transfer were not stained.

### Transfer

The proteins separated by electrophoresis were transferred to nitrocellulose paper using the method described by Towbin et al.<sup>20</sup>. The gels from the SDS-PAGE were placed in contact with 0.45 mm nitrocellulose (NC) membranes (BIO-RAD), between several layers of filter paper. The transfer was done at constant 250 mA for 3 hours in buffer

Tris 25 mM; glycine 129 mM; methanol 20%, in a mini transfer cell (BIO-RAD). Transfer efficiency was checked by evidence of the pre-stained molecular weight band pattern on the NC paper. The NC membranes were blocked with 10% skimmed milk in TTBS buffer at pH 7.5 (tris 10 mM- NaCl 150 mM-Tween 20 0.1%) for two hours.

### Western Blot

The nitrocellulose membranes were cut into strips 0.5 cm wide, incubated with monoclonal anti-GtfB antibody (P 136, provided by Dr. Kazuo Fukushima of the Department of Biochemistry and Molecular Biology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba, Japan), diluted in TTBS 1:100, for 1 hour at room temperature while shaken. After incubation, they were rinsed 3 times with TTBS and incubated with alkaline phosphatase-conjugated anti-mouse anti-serum (Sigma Chemical CO. 1418), in TTBS diluted 1:5000 for 1 hour. They were rinsed in the same way as the first incubation and stained by adding BCI-p 0.015%-NBT 0.03% in a tris saline buffer solution (tris 100 mM-NaCl 100 mM-MgCl<sub>2</sub> 5 mM) as a substrate until the bands appeared. The reaction was stopped with distilled water. The approximate molecular weight of the bands observed was calculated using a calibration curve, plotting log molecular weight against relative mobility of each protein.

### Determination of polysaccharide production

The gels obtained after electrophoresis were incubated in a 50mM imidazole (Sigma Chemical Co) for 24 hours at room temperature to allow renaturation of the proteins in the gel. Then they were submerged in 5% sucrose buffer solution (Merck) for a further 24 hours<sup>21</sup>, the gels were rinsed with distilled water and following the instructions in the glycoprotein detection kit (Sigma Chemical Co), incubated for 2 hours in the fixing agent, and rinsed plentifully before placing them in oxidant solution, rinsing again with distilled water and placing them in Schiff stain to color the bands where polysaccharides were produced. Then they were left in fixing solution and dried in a gel dryer (BIO RAD gel dryer model 583). The strains with a magenta colored band in the GtfB molecular weight position were considered positive for polysaccharide production.

## RESULTS

Table 1 summarizes the results of the 30 Gtf-enriched extracts obtained from the *S. mutans* strains isolated and percentage of bacterial plaque from caries-free subjects.

Electrophoresis on polyacrylamide gel of the 30 extracts showed bands corresponding to the molecular weight of the enzyme studied in 96.7% of the cases, as well as a series of bands of other molecular weights of *S. mutans* proteins (Fig. 1A).

Considering that proteins such as PAc and the other Gtfs are within the GtfB molecular weight range, they were identified by using the specific monoclonal anti- GtfB antibody P136 (Fig. 1B). The enzyme was not detected in 36.6% of the *S. mutans* strains analyzed.

The Schiff stain for detecting polysaccharides confirms that 93.3% of the strains have the ability to synthesize glucans, which appear in the position corresponding to Gtf molecular weight. The different strengths of the magenta color show that polysaccharide production is highly variable under the same conditions of culture and extraction, with the color ranges varying from 11% to 97% (Fig. 1C).

Of the 19 (63.3%) GtfB-positive strains, 17 (89.5%) showed ability to synthesize glucans and 36.6% produce polysaccharides in absence of GtfB, which may be attributed to the presence of the other two sacrolytic enzymes GtfC or GtfD. Bacterial plaque was present in all patients it the study in varying percentages. The two lowest values for bacterial plaque (9.1 and 10.87%) coincide with a Gtf-negative strain and a non-polysaccharide producing strain, respectively. Nevertheless, strains with similar characteristics were isolated from other caries-free individuals with high levels of bacterial

**Table 1: Summary of results of tests carried out in this study.**

Strain Nº	Electrophoresis	GtfB	Polysaccharides	% of polysaccharides	% of bacterial plaque*
01	+	+	+	60	38
07	+	+	+	77	26
08	+	+	+	50	40
12	+	+	+	41	19.2
13	+	+	+	13	23
19	+	+	-	0	10.87
23	+	+	+	11	40
25	+	+	+	37	52
31	+	+	+	27	50.6
32	+	+	+	62	40
33	-	+	-	0	32
34	+	-	+	97	38
37	+	-	+	75	12
38	+	-	+	23	9.1
39	+	-	+	15	53
40	+	-	+	47	24.2
41	+	-	+	41	32
42	+	-	+	30	35
43	+	-	+	55	17
44	+	-	+	47	18.7
45	+	-	+	72	42
46	+	-	+	89	24
47	+	+	+	91	57
48	+	+	+	24	23
49	+	+	+	33	18
50	+	+	+	48	22
51	+	+	+	41	14
52	+	+	+	21	23
53	+	+	+	46	30
54	+	+	+	46	24
Positive control	+	+	+	72	ND
ATCC	+	+	ND	ND	

ND: not determined; \*: caries-free subjects

plaque, therefore it cannot be stated that there is a relationship between plaque index and presence or absence of GtfB.

It proves that the production of polysaccharides and GtfB has no incidence of the development of bacterial plaque or on the absence of caries, because an individual may be caries-free in spite of the presence of these two variables.



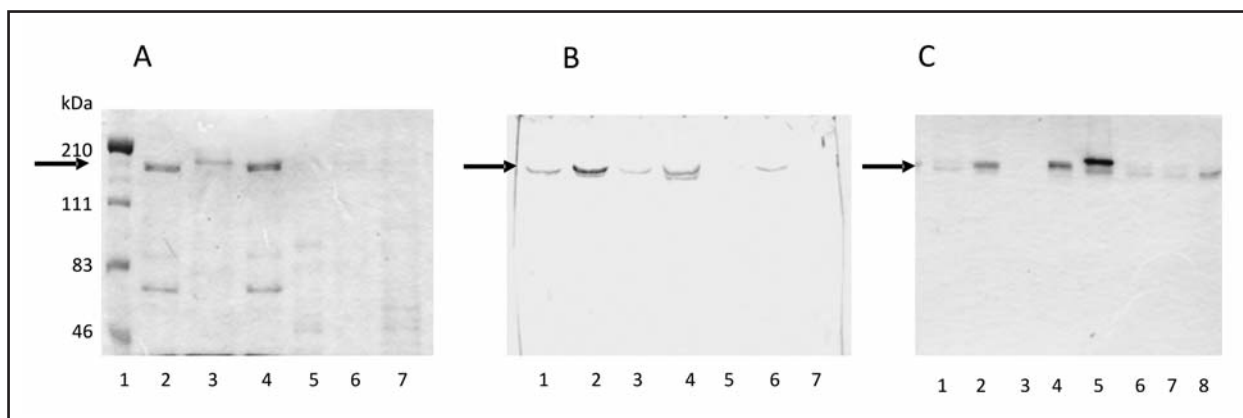


Fig. 1: Representative pictures of the experiments performed. 1A: 10% polyacrylamide gel electrophoresis stained with Coomassie blue. Lane 1: high molecular weight range pattern. Lanes 2, 3, 4, 6 and 7: positive samples for a band in the position of GtfB molecular weight. Lane 5: absence of the band corresponding to GtfB. 1B: Detection of GtfB with a specific monoclonal antibody P136. Lanes 1, 2, 3, 4 and 6 positive samples for GtfB; lanes 5 and 7 non-GtfB-producing strains. 1C: polysaccharide production in the position corresponding to the molecular weight of Gtfs. There is variability in the intensity of the magenta coloring of the producer strains (lanes 1, 2, 4, 6, 7 and 8). Lane 3: absence of polysaccharide synthesis.

## DISCUSSION

Various factors play a part in the physiopathogenesis of caries, including the host, the environment and the microorganism. It has been shown that when a microorganism or its virulence factors – in this case GtfB – intervene, there might be a marked reduction in caries indices in experimental animals<sup>22</sup>.

The first evident outcome of the study is that most strains in the oral cavity of caries-free patients are able to produce the enzyme GtfB and to synthesize polysaccharides. This confirms that there are other factors, either in the microorganism or in the host, which may alter either the enzyme or its products, and may be explained as follows:

1. The enzyme GtfB has undergone changes in shape or structure in post-transcription or post-translation events, which may alter its catalytic region or glucan-binding domain<sup>23</sup>. This happens, for example, in Asp 451 in GtfB, which causes enzymatic ability to be lost when substituted, or mutagenesis of Asp 413, Trip 491, His 561<sup>24</sup>. The biochemical role of these amino acids is unknown, but it is presumed that they either bind to sucrose at specific sites or act as proton donors and determine the shape of the active form of the protein<sup>25</sup>.

2. The bacteria's exaggerated expression of dextranases which enable the breakdown of the glucans that have been synthesized by Gtf activity<sup>26</sup>. These dextranases with specificity for alpha 1,6 bonds have already been described as 66 kDa proteins with optimal activity at pH 6.0<sup>27</sup>. Research by Per-

rone et al. describes an unidentified 66 kDa protein which seems to be important because of its differences between healthy and caries-susceptible individuals. In addition, there is evidence of dextranase production in Gram-negative bacteria from the oral cavity, associated to periodontitis<sup>16, 28</sup>.

3. Moreover, and as a negative control, the expression of the self-inhibitory peptide described by Eto et al. may be assumed. Based on the fact that several enzymes have self-inhibitory sequences, Eto et al. built peptides from the GtfB sequences and examined the capacity of each, finding that the peptide corresponding to the sequence 1176-1194 inhibited enzyme activity<sup>29</sup>.

It is important to highlight that in this case polysaccharide production does not show the enzymatic activity of GtfB alone, but also the activity of the other two Gtfs, as they appear to have a similar location in the gel due to their molecular weight and therefore must be found and activated in the same way to produce glucans.

Regarding host factors that may affect GtfB after it has been expressed and is found in one of its two forms – extracellular or associated to the membrane – it is assumed that its activity may be altered by the presence of immunoglobulin A. This theory would be validated by the studies by Fontana et al., which show that healthy individuals have greater activity of this humor factor for defense in the oral cavity<sup>15</sup>. Gtf-specific IgA inhibits glucan production by binding to a catalytic site on the enzyme or by binding

to other epitopes and causing changes in shape that prevent it from working<sup>30</sup>.

In addition to the control by IgA, these strains might have a serotype making them more susceptible to phagocytosis by polymorphonuclears in the oral cavity, suggesting that serotype-specific studies should be conducted on them<sup>31</sup>.

Although there are few studies on the topic, it is equally important to consider each individual's genetic component, which may modify the individual immune response according to the major histocompatibility complex and other substances in the saliva such as the non-immunoglobulin factor (GIF), an alpha-amylase that can inhibit GtfB from *Streptococcus mutans*. Moreover, these salivary alpha-amylases can cover the sites in the salivary film, thus producing competitive inhibition with Gtfs.

Another important factor in controlling Gtfs is each individual's environmental pH, sucrose availability and ions that may interfere with enzyme activity, which are difficult to study in persons who may have very different diets<sup>23</sup>. In addition, there is the likelihood of the subjects acquiring fluorine by another means such as iodized salt. However, although this would explain the healthy state, how would it explain caries in subjects of the same age and similar local conditions?

The second important result indicates that there are non-GtfB-producing *S. mutans* strains able to synthesize polysaccharides in healthy subjects. Other studies have shown the existence of a minority of strains that do not produce this enzyme. This may be due to genetic damage to the base sequence by mutations or alteration in the promoter regions, if it is accepted that GtfB and GtfC have separate promoters, as suggested by Goodman<sup>32</sup>.

This result shows that GtfB is not essential to polysaccharide production, due to the existence of GtfC and GtfD, which are also involved in the mechanisms of adhesion and coaggregation. An article by Fujiwara et al. shows that the expression of GtfB is greater than that of GtfC and GtfD, but reports that

although GtfB synthesizes more insoluble glucans, it is not as important as GtfC for microorganism adhesion. GtfB seems to be more useful in aggregation processes during the exponential growth phases<sup>33</sup>. In fact, GtfC has greater affinity for hydroxyapatite in presence of saliva, whereas GtfB has greater affinity for bacterial surfaces<sup>34</sup>. Fujiwara et al. also report that, in contrast to previous knowledge, these two genes do not operate under the action of a single promoter, but independently, although maximum adhesion of *S. mutans* occurs in presence of the three enzymes.<sup>33</sup> This study agrees with others that report variability in the production of these enzymes, which is attributed to genetics. It has even been mentioned that the difference in the production of GtfC is more marked than that of the other two enzymes, depending on the serotype of *Streptococcus mutans*<sup>4, 15, 18</sup>.

The reason suggested for the non-expression of an enzyme is the genetic polymorphism that has been found in clinical specimens in addition to the natural genetic transformation that may occur in the bacterial plaque where the bacterium acquires genes that alter its virulence factors<sup>35</sup>. The new genetic material might come from dead microorganisms or plasmids<sup>36</sup>.

As all subjects had bacterial plaque, it should be studied qualitatively rather than quantitatively, i.e. it should be determined whether it has a greater content of insoluble polysaccharides or presence of other cariogenic microorganisms.

It may be concluded from the results of this study that GtfB is a virulence factor that is produced but controlled in caries-free individuals, although it is not known how it is controlled. This research has shown that the factors that enable caries control are diverse. The multiple factors of the etiology make it difficult to find a method for controlling the disease that is efficient and affordable to everyone. GtfB and the protein PAc have been the target of most research, and their composition, action mechanisms and genetics are being explored more and more often.

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