Since 1983
EPITHEORESE KLINIKES FARMAKOLOGIAS KAI FARMAKOKINETIKES | 42(Sup1): 23-29 (2024)

Received: 15 July 2024 | **Accepted**: 11 September 2024 | **Published**: 27 December 2024

Open Access | **RESEARCH**

Relationship between glycosyltransferase enzyme and biofilm formation by *Streptococcus mutans* isolated from dental childhood caries

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ABSTRACT

Background: Dental caries, known as tooth decay, is mainly caused through *Streptococcus mutans*. The *S. mutans* produce acids and leading to cavity formation through demineralization of enamel layer. The biofilm formed by *S. mutans*, composed of glucan polysaccharides, extracellular DNA, and proteins, plays an essential role in caries expansion. *Aim*: The correlation between glycosyltransferase (Gtf) activity, responsible for glucan matrix synthesis, and biofilm formation in *S. mutans* was investigated. *Methodology*: The samples, conducted in Al-Shaab Dental Specialist Center in Baghdad, included 120 child patients diagnosed with caries. Dental plaque samples were collected and analyzed for bacterial isolation, identification, and biochemical tests. Gtf activity was estimated by spectrophotometer using Wu and co-workers method. *Results*: A significant positive correlation between Gtf activity and biofilm production was found by statistical analysis, with a robust correlation coefficient (r=0.967) at 48 hours of biofilm formation. *Conclusion*: These results underline the crucial role of Gtf in *S. mutans* biofilm formation, offering visions into the interaction between Gtf activity and caries development. Understanding this relationship may contribute to the development of strategies targeting biofilm disruption for improved oral health.

KEYWORDS

glycosyltransferase, biofilm, *Streptococcus mutans*, malachite green, dental caries

How to cite this article: Almijbilee M. M. A., Mahdi M. A., Authman S. H.: Relationship between glycosyltransferase enzyme and biofilm formation by *Streptococcus mutans* isolated from dental childhood caries. *Epitheorese Klin. Farmakol. Farmakokinet.* 42(Sup1): 23-29 (2024). DOI: 10.61873/RGJG6383

Publisher note: PHARMAKON-Press stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. INTRODUCTION

Dental caries, normally mentioned as tooth decay or cavities, is defined as the demineralization and resulting deterioration of the enamel on teeth, leading to the creation of cavities. *Streptococcus mutans* is identified as the primary etiological agent due to the creation of acids from the breakdown of different carbohydrates, causing dental enamel erosion, persist in low pH environments, and attached on the teeth surfaces in a robust acidic biofilm community [1]. Furthermore, the bio-

film traps acids on the teeth surfaces and help progress of carious ulcers while shielding the bacteria that live inside it. Extracellular DNA (eDNA), glucan polysaccharides, and proteins make up the extracellular substance of *S. mutans* [2]. Organisms from all domains of life have a diverse range of biomolecules with covalently bonded sugar moieties connected to lipids and proteins (glycoconjugates). The cell glycocalyx is made up of glycans on the cell surface and serves a variety of functions in cell recognition and signaling [3-5]. The biofilm's most important and most researched component is the glucan matrix, which provides *S. mutans* with an acidic microenvironment, mechanical stability, and a surface on which to adhere [6]. *GtfB*, *GtfC*, and *GtfD* are the extracellular glucosyltransferases that create and organize the glucan matrix. GTFs hydrolyze sucrose and polymerize the glucose moiety into sticky glucose polymers in environments with high sucrose content [7]. *S. mutans* generates three Gtfs enzymes (*GtfB*, *GtfC*, and *GtfD*), for each with a different but comparable expression pattern. These three proteins are encoded by the *GtfB*, *GtfC*, and *GtfD* genes, in that order. While *GtfB* and *GtfC* are in an operon arrangement sectioned by 198 bp and manifest to have coordinately organized promoters, indicating that they can be co-transcribed and the same regulatory mechanism is submissive, *GtfD* is existed upstream of the *GtfB*/C locus and has a separate promoter [8].

GTFs are involved in the process of glycoconjugate synthesis. GTFs facilitate the creation of glycosidic bonds by facilitating the transfer of sugar moieties from donor molecules that have been activated to target acceptor molecules. These compounds include sugar phosphates nucleotide, nucleotide monophosphate sugars and diphosphate sugars. The reaction strategy of *O*-GTs using nucleotide diphosphate sugars as donors is as follows [9]:

$$[\mathrm{NDP-sugar} + \mathrm{acceptor-OH} \rightarrow \mathrm{NDP} + \mathrm{acceptor-O-sugar}]$$

A biochemical GT test was recently disclosed in which GT activity is linked to the phosphatase-catalyzed hydrolysis of the nucleoside diphosphate (e.g., UDP) produced as a subsequent product of the GT process [10]. A well-established phosphate detection technique using the malachite green reagent is used to quantify the free phosphate produced by the phosphatase [11].

Herein, the glycosyltransferase (Gtf) enzyme was produced by *S. mutans*, which isolated from 120 patients from Al-Shaab Dental Specialist Center in Baghdad, Iraq. The Gtf enzyme was evaluated by colorimetric method using malachite green to detect the free phosphate produced by the enzyme. the biofilm produced by *S. mutans* was estimated using microplate method and the relation the biofilm of *S. mutans* and the Gtf enzyme were determined using SPSS software.

2. METHODOLOGY

2.1. Ethical approval

This study was conducted in accordance with the ethical standards outlined by the Research Ethics Committee of Mustansiriyah University, College of Science. Prior to the initiation of the research, ethical approval was obtained from the Biomedical Research Ethics Committee, adhering to the guidelines stipulated for biomedical research involving human participants.

The study was classified as a minimal-risk investigation, with all procedures reviewed to ensure compliance with ethical norms, participant safety, and data confidentiality. Informed consent was secured from all participants or, in cases involving minors or individuals unable to provide consent, from their legal guardians. The consent process was designed to be clear and non-coercive, ensuring participants were fully informed about the study's objectives, procedures, risks, and benefits. Confidentiality of personal and health information was maintained by anonymizing participant data through coding and removing identifiers as early as possible.

2.2. Study groups

The study was place between 2023 and 2024 and involved 120 children from the Al-Shaab Dental Specialist Center in Baghdad, Iraq. The study examined bacterial strains from dental plaque of 120 individuals (average age 6.5 ± 0.75) diagnosed with deciduous tooth caries (ECC). The World Health Organization's epidemiological study guidelines were used to perform the examinations [12].

2.3. Dental plaque collection

Following patient categorization for the study, supragingival plaque samples were obtained with sterile dental explorers. Prior to plaque collection, each individual washed their mouth with distilled water for 1 minute. Material was obtained from fasting individuals in the morning (between 8 and 12 a.m.), before to toothbrushing and clinical evaluation. The recovered plaque was placed in sterile tubes containing 0.5 mL of phosphate buffered saline (PBS), which inhibited O_2, and transported to

the lab on ice within 1 hour. Plaque samples were sonicated for 30 seconds, vortexed to form a homogenous suspension, then centrifuged at 2000 × g for 10 minutes at 4°C. A total of 50 µl aliquots of cleared supernatants were employed for standard plate culture procedures on Mitis salivarius bacitracin agar (MSBA) comprising (90 g/L) mitis salivaris agar supplied with (150 g/L) sucrose and 200 IU/L bacitracin. All plates were incubated for 48 hours at 37°C in an atmosphere of 85% N_2, 10% CO_2, and 5% O_2. Colony counts more than 130 (CFU/plate) were judged positive [13].

2.4. Morphological identification

Following collecting pure colony samples on Mitis Salivaris agar, single colonies were injected onto the surface of TSA plates with 5% human blood and incubated under the previously described conditions. The features of each colony were then assessed, including shape, color and colony size of the bacterial isolates were evaluated after incubation at 37°C for 24 hours in anaerobic environment by using microbiological a candle jar. The kind of hemolysis, Gram-staining, form, clump, and cells arrangement were tested under a microscope [13].

2.5. Bacterial identification

In the Catalase test, a clump of growth from a pure bacterial culture is transferred to a slide, and 3% hydrogen peroxide is added. The produce of bubbles indicates a positive result, confirming the production of catalase [14].

The Carbohydrate fermentation test evaluates bacterial isolates capacity to use several sources (raffinose, sorbitol, and mannitol) as a single source of energy and carbon. Bacterial isolates are injected into sterile tubes containing brain heart infusion broth with one of each Carbohydrate source and cultured anaerobically at 37°C for 24 hours. A change in hue from red to yellow in the indication indicates a favorable outcome [13].

2.6. Detection of biofilm production isolates

Brain heart infusion broth was used to adjust the previously generated culture to 0.5 McFarland. It was then diluted 100 times in brain heart infusion broth supplied by 2% sucrose. In 96 flat bottom microtiter plate wells, 200 µL of the diluted cultures of each test isolate and the standard reference strain were distributed (3 wells per isolate). The microtiter plate was incubated at 37 °C for 48 hours, after which they were treated as follows: The bacterial culture was decanted by inverting the microtiter plate, which was then washed (3-4) times with sterile normal saline (0.9%). 200 µL of methanol is added to each well and allowing the plates stay at room temperature for 20 minutes, the settled methanol was decanted and the plates were allowed to dry. A 200 µL of 0.1% crystal violet was added to each well to stain the plates and waiting for 15 minutes. After that, they were cleaned triple with distilled water and let to dry at room temperature while inverted. Then resolving the adhering cells and any biofilms they had generated, for dissolving the dye a 200 µL of 33% glacial acetic acid was added to each well. A microplate reader was then used to determine the OD at 600 nm. All medical isolate was examined three times; the average of the result was calculated beside the standard deviation. The average OD values of each test isolate were subtracted from the mean OD value found for control wells, which contained sterile medium only [15]. Every negative test and control strain had an OD value, which was then computed, and the OD cutoff values (ODc) were assessed as previously mentioned [16].

2.7. Preparation of malachite green reagent

To prepare the malachite green reagent 17.6 g of ammonium heptamolybdate tetrahydrate were dissolved in 400 mL of water, and 240 mL of 70% $HClO_4$ were added to create a solution. After being dissolved in 5 mL of ethanol, 1.9 g of malachite green carbinol hydrochloride was gradually combined with the molybdate and perchloric acid mixture. After dissolving 10 g of Pluronic F68 in 30 mL of distilled water with careful stirring, the final volume was then brought to 1L using distilled water [17]. Whatman filter paper was used to filter the mixture of reagents (sedimentation is another method that may be used to separate the insoluble residue) kept at room temperature in a brown plastic bottle [18].

The components of the Malachite B reagent are 0.38 mM malachite green oxalate and 0.35% w/v polyvinyl alcohol (low molecular weight; 98–99% hydrolyzed). Glassware was acid-washed to guarantee that any phosphate was eliminated. The PVA was dissolved in microwave heat after being added to distilled water to create the B reagent. After cooling to room temperature, malachite green oxalate was added and the volume was adjusted as needed [19].

2.8. Determination of glucosyltransferase Gtf activity

A glycosyltransferase reaction was conducted in a 96-well plate with 50 µL of reaction buffer (25 mM

Tris, 150 mM NaCl, 5 mM $MgCl_2$, and 5 mM $MnCl_2$, pH 7.5) and 2 µL 0.1 M GlcNAcβ-benzyl acceptor at room temperature for a duration of 20 minutes. The reaction initiated by adding 5 µL 10 mM UDP-Gal. To assess glycosyltransferase activity, a well containing all component except for the bacterial suspension was included as a blank control. Substrates were added to each well, followed by 15 µL of bacterial suspension, 30 µL of malachite reagent A, and 100 µL of water to stop the reactions. Each well received 30 µL of malachite reagent B, which was added, gently mixed, and allowed to incubate for 20 minutes at room temperature before color development took place. A multiwell plate reader was then used to read the plate at 620 nm [19-20].

2.9. Data analysis

SPSS was carried out to calculate the statistical analysis. To look into the connection between glycosyltransferase activity and biofilm formation, the Pearson correlation coefficient (*r*) was calculated. A *p*-value of 0.05 after Bonferroni adjustment was deemed significant. Software from Origin Lab was used to prepare the graphs.

3. RESULTS

3.1. Bacterial isolation

The study was conducted in Al-Shaab Dental Specialist Center in Baghdad involving 120 patients. After culture of Dental Plaque revealed a 45% positivity rate, with *S. mutans* being the predominant isolate 26.7% and 18.3% other bacteria. While other sample without growth 55%.

3.2. Bacterial identification

The initial identifications were made using morphological and cultural characteristics of bacterial isolates cultured on Mitis Salivaris Bacitracin agar medium. The isolates' spherical, Gram-positive cells and appearance in medium chains were revealed by the results. The colonies of these isolates have a smooth surface, frosted glass appearance, rising, and extreme convexity.

Results from an analysis of the biochemical properties of bacterial isolates thought to be from the genus Streptococcus revealed that these isolates were Gram positive, catalase-negative, and capable of fermenting sugars such as mannitol, sorbitol, and raffinose. The VITEK-2 technology was also used to verify that the 32 bacterial isolates were *S. mutans*.

3.3. Analysis of biofilm formation by microplate method

The investigation into *Streptococcus mutans* biofilm formation revealed distinct adherence levels among the tested cultures. Figure 1 indicates, out of 32 cultures, 3 were classified as non-adherent, 4 as weakly adherent, 10 as moderately adherent, and 15 as strongly adherent.

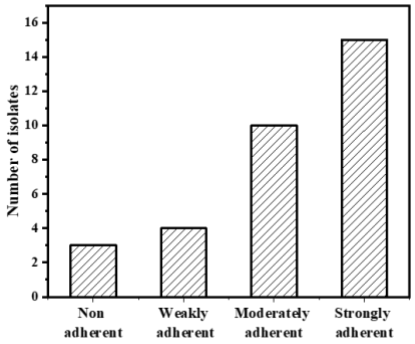

Figure 1. The Biofilm formation ability among *S. mutans* isolated from Dental childhood caries patients.

3.4. Determination of Glycosyltransferase activity and their relation with biofilm formation by *S. mutans*

The Gtf enzyme activity was estimated by determine the free phosphate produced by the enzyme with malachite green dye for the 32 isolates, the Gtf activity was 0.40 – 0.95 for the non-adherent. The Gtf activity increased to double about 1.50 – 2.10 for the weakly adherent. The highest Gtf was 11.80 (for the strongly adherent isolate) while the lowest value was 8.65. A substantial correlation coefficient (r=0.967) was discovered at the 48-hour point of biofilm formation, indicating the strongest strength of this correlation (Figure 2).

4. DISCUSSION

The biofilm formation results categorization provides valuable insights into the varying capacities of *S. mutans* to adhere to surfaces, potentially representing different stages of biofilm development. The distribution of adherence levels underscores the complexity of biofilm formation and highlights the need for further research to comprehend the dynamics of bacterial colonization. These findings lay the foundation for potential strategies aimed at disrupting or preventing biofilm formation, particularly in the context of oral health. The results obtained

are consistent with the findings of another local study by Al-Kazirragy [21], which reported that 53% of the tested isolates were strong producers, and 46% were good or mild producers. Very few *S. mutans* isolates with substantial biofilm were discovered by Gibbons and Etherden. These variations can be attributed to variations in growing conditions, including pH, ionic pressures, and the quantity of subcultures [22] Microorganisms are more likely to cling to biomedical surfaces when biofilm is present, shielding them from the host immune response and antimicrobial therapy [23].

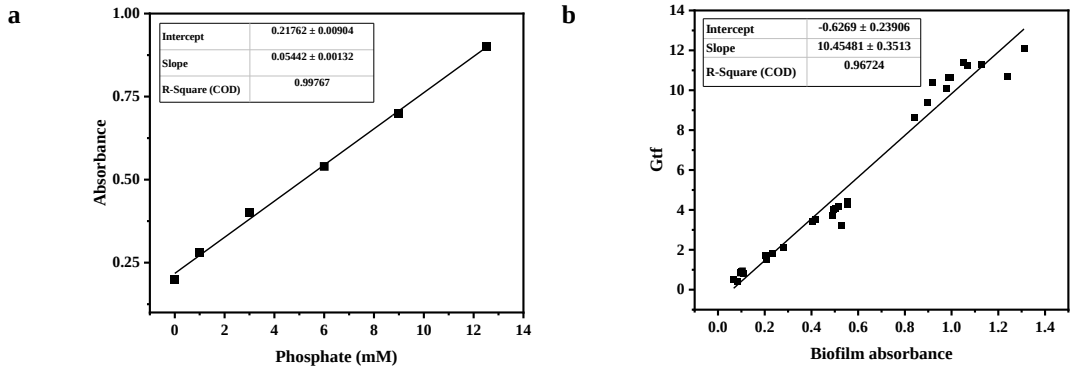

Figure 2. **a**: The calibration curve of phosphate with malachite green. **b**: the correlation between the Gtf and biofilm absorbance.

Determination of Glycosyltransferase activity and their relation with biofilm formation by *S. mutans* reveal a strong correlation between the activity of glycosyltransferases and the biofilm production capability of *S. mutans*. Glycosyltransferases are enzymes that play a crucial role in the synthesis and transfer of glycosidic bonds within the cell, and their involvement in biofilm formation has long been an area of scientific interest. The result obtained from the study suggest a positive correlation, which indicating that as the activity of glycosyltransferases increases, the biofilm production capability of *S. mutans* increases.

A substantial correlation coefficient (r=0.967) was discovered at the 48-hour point of biofilm formation, indicating the strongest strength of this correlation. A correlation coefficient approaching suggests a highly positive linear relationship between the two variables, in this case, glycosyltransferase activity and biofilm production. The statistical significance of this correlation is underscored by a low p-value (p<0.0001).

S. mutans is renowned for its ability to form robust biofilms on tooth surfaces, contributing significantly to the etiology of dental caries. Biofilms provide a protected microenvironment, enhancing bacterial resistance to mechanical removal, host defenses, and antimicrobial agents. The cooperative community structure within these biofilms facilitates the exchange of genetic material, contributing to the adaptation and persistence of *S. mutans* within the oral microbiota [24-25].

Glycosyltransferases, particularly glucosyltransferases (Gtf), are key enzymes in the synthesis of extracellular polysaccharides that constitute the biofilm matrix. *S. mutans* produces three Gtfs (*GtfB*, *GtfC*, and *GtfD*) that convert dietary sucrose into glucans, providing the structural backbone of the biofilm matrix. The enzymatic activity of Gtfs directly influences the architecture and adhesive properties of *S. mutans* biofilms, impacting their overall virulence [26].

Glycosyltransferases play a crucial role in the initial adhesion of *S. mutans* to tooth surfaces. Gtf-mediated synthesis of adhesive glucans promotes bacterial attachment, facilitating the establishment of biofilm communities. Furthermore, these enzymes contribute to the intercellular adhesion within the biofilm, enhancing its structural integrity and resistance to mechanical forces [27-28].

Biofilms formed by *S. mutans* create a microenvironment characterized by low pH due to the fermentation of dietary carbohydrates. Glycosyltransferases play a dual role not just only by contributing to the biofilm matrix but also by promoting acid production. This acidic milieu favors demineralization of tooth enamel, which leading to the initiation and progression of dental caries [8,29].

5. CONCLUSION

The high correlation between glycosyltransferase (Gtf) and *S. mutans* biofilm (r=0.967) carry significant implications for our understanding of *S. mutans* biofilm formation, a process integral to the pathogenicity of this bacterium in the context of dental caries. By analytic the temporal dynamics of the glycosyltransferase-biofilm production relationship, the study not only provides valued insights into the underlying molecular mechanisms but also suggests potential targets for involvement strategies pointed at disrupting biofilm formation.

ACKNOWLEDGMENTS
The authors would like to express their deepest appreciation to national health factory for kindly providing the use of critical equipment and instruments required for the effective completion of this study on the determine the biofilm formation isolate.

CONFLICT OF INTEREST STATEMENT
The authors declare no conflicts of interest.

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