Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 339 (2004) 2883–2888
Carbohydrate RESEARCH

Effect of carbohydrates on fructosyltransferase expression and distribution in *Streptococcus mutans* GS-5 biofilms

Ramona Rozen, Gilad Bachrach and Doron Steinberg*

Institute of Dental Sciences, Faculty of Dentistry, Hebrew University-Hadassah, PO Box 12272, Jerusalem 91120, Israel

Received 7 January 2004; accepted 1 July 2004
Available online 28 October 2004

Abstract—*Streptococcus mutans* produces a fructosyltransferase (FTF) enzyme, which synthesizes fructan polymers from sucrose. Fructans contribute to the virulence of the biofilm by acting as binding sites for *S. mutans* adhesion and as extracellular nutrition reservoir for the oral bacteria. Antibodies raised against a recombinant *S. mutans* FTF were used to test the effect of glucose, fructose, and sucrose on FTF expression in *S. mutans* GS-5 biofilms. Biofilms formed in the presence of fructose and glucose showed a higher ratio of FTF compared to biofilms formed in the presence of sucrose. Confocal laser scanning microscopy images of *S. mutans* biofilms indicated a carbohydrate-dependent FTF distribution. The layer adjacent to the surface and those at the liquid interface displayed high amounts cell-free FTF with limited amount of bacteria while the in-between layers demonstrated both cell-free FTF and cells expressing cell-surface FTF. Biofilm of *S. mutans* grown on hydroxyapatite surfaces expressed several FTF bands with mole- cular masses of 160, 125, 120, 100, and 50 kDa, as detected by using FTF specific antibodies. The results show that FTF expression and distribution in *S. mutans* GS-5 biofilms is carbohydrate regulated.

Keywords: *Streptococcus mutans*; Fructosyltransferase; Protein expression; Biofilm; Bacterial amount; Immunofluorescence; Confocal microscopy

1. Introduction

Streptococcus mutans has been implicated as the primary etiological agent in dental caries formation.[1,2] One of the cardinal virulence properties of *S. mutans* is attributed to its ability to produce extracellular polysaccharides from sucrose, which facilitate their adhesion and act as nutrition reservoir in periods of limited nutrition.[2,3] Fructosyltransferase (FTF) enzyme is produced from a single *S. mutans ftf* gene,[4] and synthesizes large amounts of inulin-type fructan polymers from sucrose, while *Streptococcus salivarius* produces copious amounts of levan-type fructans.[5] Enzymatically active *S. salivarius* FTF or *S. mutans* FTF can be found either as cell-surface associated or cell-free (secreted),[6,7] in saliva, or immobilized on the acquired pellicle or in the dental plaque biofilm.[8] Infection of rats with *S. mutans* in which the *ftf* gene has been inactivated resulted in reduced caries lesions.[9] Fructans may influence the pathogenesis of dental caries by serving as short-term storage for polysaccharides in the dental plaque[4] and by serving as binding sites for adhesion of specific oral bacteria to hard surfaces.[10]

Bacteria may exhibit a distinct mode of growth and differential gene expression in various growth environments. Indeed, transcription of *ftf* is affected by several environmental conditions including growth rate[11] and sucrose exposure.[12–14]

In this study FTF expression, in the enzymatic/protein level, was tested in *S. mutans* biofilm in the presence of glucose, fructose, and sucrose.

2. Experimental

2.1. Antiserum FTF preparation

In order to obtain a purified antigen for FTF antibody, we have cloned *ftf* in *E. coli* as described by Rozen

* Corresponding author. Tel.: +972 2 6757633; fax: +972 2 6758561; e-mail: dorons@cc.huji.ac.il

0008-6215/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2004.07.028

et al.[15] In brief: the *S. mutans* GS-5 *ftf* gene, excluding the signal peptide, was amplified using PCR with Expand High Fidelity PCR System (Roche Diagnostics GmbH, Mannheim, Germany) and primers (FTF 1314, 5′-GGGGGATCCCAGGCAGATGAAGCCAATTC-3′ and FTF 1315, 5′-CCAGGTACCTTTAAAACCAATGCTTACACAGAAAGC-3′) into which *Bam* HI and *Kpn* I sites (underlined) were incorporated. The 2.2 kb PCR product, was gel purified (JETSORB gel extraction kit, GENOMED, GmbH, Oeynhausen, Germany), digested with *Bam* HI and *Kpn* I simultaneously (Boehringer Mannheim, Mannheim, Germany) and cloned into the pQE30 expression vector (Qiagen). The resulting construct (pQEFTF) introduces an N-terminal His_6 tag, which aids in protein purification. Recombinant FTF was expressed in *E. coli* SG13009 [pREP4] and purified using a nickel–nitrilotriacetic [Ni–NTA] column, under denaturating conditions as recommended by the manufacturer (Qiagen).

The Ni–NTA purified recombinant FTF was subjected to 10% SDS-PAGE according to the method of Laemmli.[16] The gel bands containing the recombinant FTF were homogenized and injected to rabbits to elicit polyclonal rabbit sera as described by Rozen et al.[15]

The immunization protocol was approved by the animal care and use committee of Sigma Israel.

2.2. Quantification of biofilm formation and FTF expression

Quantification of cell-free FTF and *S. mutans* expressing FTF in biofilms was examined as described before.[17] *S. mutans* biofilms were formed in 24-well polystyrene plates (Nunc, Roskilde, Denmark). Biofilm growth was initiated by inoculating 50 μL of overnight-grown *S. mutans* into 2 mL of tryptone–vitamin (TV) medium [3.5% tryptone (Difco), thiamine–HCl (0.2 μg/mL), nicotinamide (1 μg/mL), riboflavin (0.2 μg/mL)] (Sigma) supplemented with 1% of the tested carbohydrates. After incubation at 37 °C under 5% CO_2 for 24 h, the wells were rinsed twice with 0.5 mL PBS and then fixed with 3.7% formaldehyde for 20 min. Wells were rinsed twice with 0.5 mL distilled water, and incubated with blocking solution [PBS containing 1.5% bovine serum albumin (PBS–BSA)] for 10 min. Wells were incubated with rabbit anti-FTF serum (prepared as described before, diluted 1:100 in PBS–BSA) for 60 min, and rinsed with PBS. Wells were incubated with fluorescent Cy^2-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., PA, USA) (diluted 1:50 in PBS–BSA) for 45 min in the dark, and rinsed with PBS. After incubation with antibodies, wells were stained with 4′,6-diamidino-2-phenylindole (DAPI) solution (1 μg/mL) (Sigma) for 30 min and rinsed three times with 0.5 mL distilled water. Wells without biofilms were incubated with either DAPI or with antibodies, and used as blank controls. Each assay was performed in triplicate. The fluorescence intensity for Cy^2 (cell-associated and cell-free FTF expression in the biofilm) and DAPI staining (total bacterial staining) in biofilms was detected using a fluorescence micro-plate reader (FLUOstar$^+$ Galaxy, BMG Laboratories, Offenburg, Germany). The ratio between FTF expression (fluorescence detected with Cy^2 dye) per bacteria (fluorescence detected with DAPI) was calculated.

2.3. FTF expression at different biofilm layers

Biofilms were constructed using a modified method of Hazlett et al.[18] *S. mutans* GS-5 biofilms were grown in 4-well glass bottom chamber slides which the walls of the wells can be disconnected from the bottom slide (Lab-Tek II, Nunc, IL, USA) using a modified method of Hazlett et al.[18] Biofilms growth was initiated in individual wells of chamber slides (Lab-Tek) by inoculating 25 μL of overnight-grown *S. mutans* into 1 mL of TV medium supplemented with the tested carbohydrates. After incubation at 37 °C in 5% CO_2 for 24 h, the wells were rinsed twice with 0.5 mL distilled water and the biofilm was fixed with 3.7% formaldehyde for 5 min. Rabbit anti-FTF serum was used at a 1:100 dilution and fluorescent Cy^2-conjugated goat anti-rabbit IgG (Jackson) was used at a 1:50 dilution. After incubation with antibodies, wells were stained with propidium iodide (P.I.) (0.5 μg/mL) (Sigma) for 15 min and rinsed with distilled water. Then, the walls of the 4-well chamber slides were gently removed, 5 μL mounting solution composed of: 3% 1,4-diazabicyclo[2.2.2]octane (DABCO), 77% glycerol, 20% PBS, and 0.1% NaN_3 was added on each biofilm, and biofilms were covered with cover slips and sealed with nail varnish.

Immunofluorescent cross section images of *S. mutans* biofilms were acquired by means of confocal laser scanning microscopy (CLSM), using a Zeiss Axiovert 135M microscope and the Carl Zeiss Confocal system, run with Ver. 3.95 software (Zeiss, Germany). CLSM images demonstrated bacteria not expressing cell-surface FTF stained with only PI (red), secreted cell-free FTF stained with only Cy^2 (green), and bacteria expressing cell-surface FTF stained with both PI and Cy^2 (yellow). A series of optical sections at 2.5–5 μm increments were taken throughout the full depth of biofilms. Data analysis of optical sections was calculated using Image-Pro PLUS Ver. 4.1 software (Media Cybernetics, MD, USA). The 3-D confocal images of biofilms were reconstituted and processed for display using Adobe PhotoShop Ver 5.0 software.

2.4. Molecular weight of cell-associated FTF in biofilms

Overnight *S. mutans* GS-5 cultures were sedimented at 3500×*g* for 10 min, and re-suspended in saline to an optical density 1.0 at 600 nm. Bacterial suspension (2 mL)

was added to 200 mg HA-beads (ISS Priss Ceramic, size 80 μm; Bio-Rad, CA, USA) (pre-washed with saline) and incubated at 37 °C in 5% CO_2 for 2 h, while rotating on a shaker. At the end of incubation period, the beads were washed twice with saline to remove unbound bacteria. Bacteria bound to HA-beads were grown for additional 24 h in 20 mL TV medium supplemented with tested carbohydrates. Bacteria were harvested as already described. Bacterial pellets (biofilm bacteria bound to HA) were washed twice with saline to remove unbound bacteria and re-suspended in 1.2 mL lysis buffer (2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetra-acetic acid, 0.5 mM EDTA, 1% Triton X-100, 25 mM Tris–phosphate, 25 mM K_2HPO_4 in 10% glycerol, pH 7.8) supplemented with 1 mM of phenymethylsulfonyl fluoride (PMSF) (Sigma). Cell suspensions and HA-beads were placed in 2 mL microfuge tubes containing 600 μL of glass beads (106 μm, Sigma). Cells were disrupted four times using the FastPrep cell disruptor (Bio 101, Savant Instruments, Inc., NY, USA) at a speed of 6 m/s for 45 s, with ice-cooling between disruptions. The lysates were centrifuged at 10,000×*g* for 5 min and the supernatant fluids were used as the cell-associated FTF preparations. Protein concentration of the cell-associated preparations was determined by the Bio-Rad protein assay.

The size of the FTFs obtained was determined by Western immunodetection. Briefly, 15 μg of cell-associated samples were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Rabbit anti-FTF was used at a 1:2000 dilution and peroxidase conjugated goat anti-rabbit IgG (Jackson) was used at a 1:20,000 dilution. The SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) was used for FTF detection. Blots were exposed to radiographic films, and films were scanned using a Flour-S MultiImager apparatus (Bio-Rad).

2.5. Statistical analysis

Each experiment was conducted three times. Data obtained was analyzed using *t-tests*. The degree of significance was determined at $p < 0.05$.

3. Results

3.1. Construction and purification of a recombinant FTF

A histidine tagged FTF, which lacks the signal peptide, was expressed in *E. coli*. Following IPTG induction, a recombinant protein of approximately 100 kDa was detected on Coomassie-stained SDS-PAGE. The recombinant FTF was purified using Ni–NTA resin and was found to have fructosyltransferase activity as tested by zymography.

Purified recombinant FTF was used to immunize rabbits (Sigma). The anti-FTF serum obtained reacted with the recombinant FTF and with cell-associated FTF samples from *S. mutans* GS-5 (data not shown).

3.2. Effect of carbohydrates on biofilm formation and FTF expression

FTF expression and total bacterial amount in *S. mutans* biofilms was influenced by the presence of the tested carbohydrates (Fig. 1). The amount of total bacteria in the presence of sucrose was significantly higher (5.7–8.6 times, $p < 0.05$) compared to biofilms formed in the presence of glucose or fructose (Fig. 1). FTF expression in biofilms formed with sucrose was only slightly higher (about 15%, $p < 0.05$) compared to biofilms formed with the other carbohydrates (Fig. 1). High ratio of FTF per bacteria was detected in biofilms formed in the presence of fructose (8.4 ± 0.8) and glucose (5.8 ± 1.2), while biofilms formed in the presence of sucrose exhibited the lowest FTF per cell ratio (1.2 ± 0.3) (Fig. 1).

3.3. FTF expression at different biofilm layers

Carbohydrate-dependent FTF expression in *S. mutans* biofilms was analyzed in correlation to biofilm depth (Fig. 2). CLSM images of biofilms, formed after 24 h growth demonstrated bacterial cells, FTF, and interstitial spaces between bacterial cells aggregates. The thickness of *S. mutans* biofilms formed was also found to be carbohydrate depended. Biofilms formed in the presence of sucrose were of average depth of 70 μm (Fig. 2B), while biofilms formed in the presence of glucose or fructose had an average depth of only 21–22 μm (Table 1). Cross sections of the biofilm formed with sucrose, indicated high amounts of cell-free FTF at sections between

	Glucose	Fructose	Sucrose
Bacterial amount	2,061 ± 306	1,367 ± 220	11,835 ± 263
FTF amount	11,956 ± 230	11,842 ± 310	14,218 ± 497
Ratio FTF/ bacteria	5.8 ± 1.2	8.4 ± 0.8	1.2 ± 0.3

Figure 1. *S. mutans* GS-5 biofilms formed on 24-well polystyrene plates after 24 h growth in tryptone vitamin base medium supplemented with different carbohydrates. *S. mutans* biofilms stained with DAPI (total bacterial amount) or Cy^2-fluorescent antibody (FTF expression). The ratio between FTF expression per total bacteria is expressed as fluorescence arbitrary numbers ± standard deviation of triplicate samples.

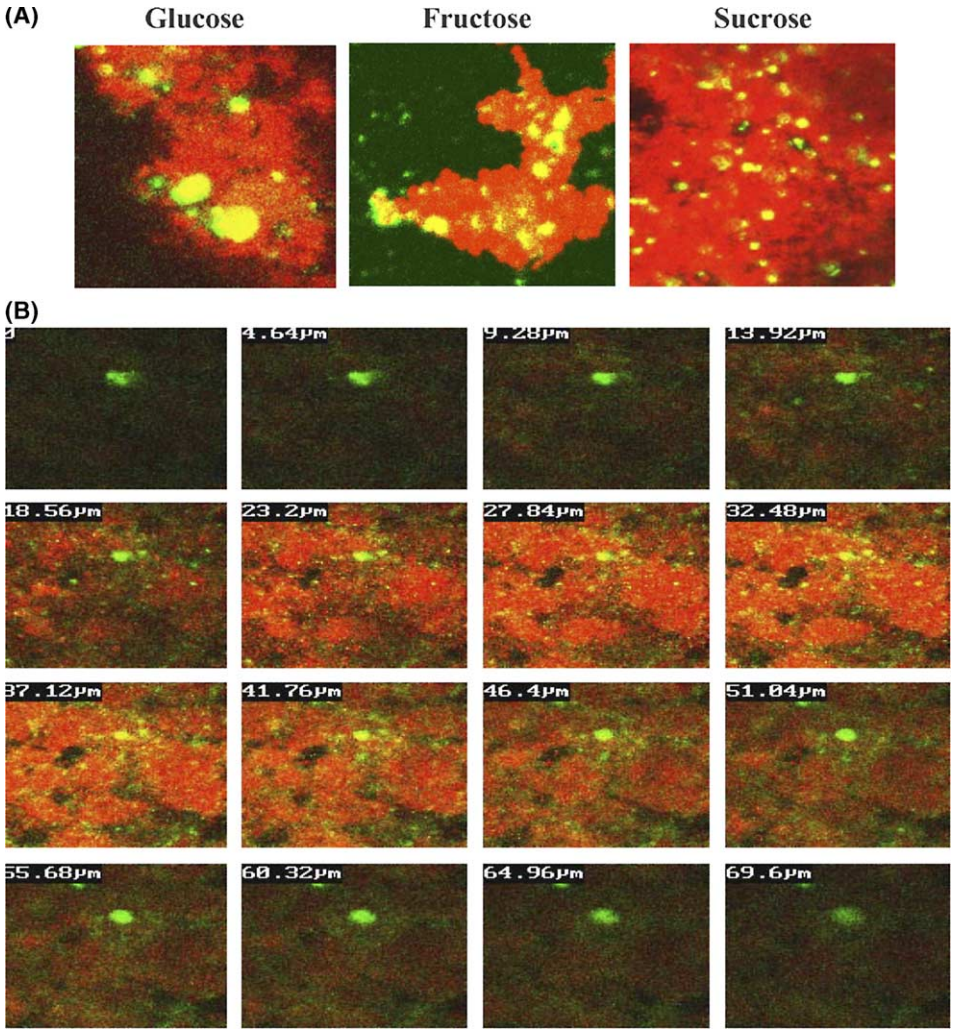

Figure 2. Confocal laser scanning microscope (CLSM) images of *S. mutans* GS-5 biofilms formed in glass chamber slides. (A) Biofilms formed in the presence of different carbohydrates. (B) Cross section of biofilms formed in the presence of sucrose. Bacteria not expressing FTF on the surface were stained with propidium iodide (P.I.) (red). Cell-free FTF was detected with Cy^2-fluorescent antibody (green). Bacteria expressing cell-surface FTF were stained for both P.I. and Cy^2 (yellow).

Table 1. Confocal laser scanning microscopy analysis of *S. mutans* GS-5 biofilms

	Biofilm depth (μm)		
	Growth medium supplemented with[a]		
	Glucose	Fructose	Sucrose
Thickness (mean)	22	21	70
Layers with limited amount of bacteria[b]	0–4; 20–22	0–2; 19–21	0–8; 62–70
Layers with bacteria and cell-free FTF[c]	4–5; 16–20	15–19	8–15; 48–62
Layers with bacteria, cell-surface, and cell-free FTF[d]	5–16	2–15	15–48

Data analysis of optical sections taken throughout biofilm depth.
[a] Bacteria were grown for 24 h on glass chamber slides in a tryptone vitamin (TV) medium supplemented with different carbohydrates.
[b] Biofilm layers containing cell-free FTF and limited amount of bacteria.
[c] Biofilm layers containing bacterial cells not expressing FTF on the surface and cell-free FTF.
[d] Biofilm layers containing bacteria expressing cell-surface FTF and cell-free FTF.

0–8 μm, adjacent to the surface, and 62–70 μm (green), adjacent to the liquid interface, with limited amounts of bacteria (red). The layer bordering the bacteria free layers demonstrated a mixture of cell-free FTF and bacteria, while the in-between layer demonstrated a mixture of cell-free FTF and cells expressing FTF (Fig. 2B). High amounts of cell-free FTF and low amounts of bacteria were founds at 0–4 μm and at 20–22 μm, in biofilms

exposed to glucose, while in biofilms formed with fructose, the cell-free FTF layers were between 0–2 and 19–21 μm. The in-between layers of biofilms formed with glucose and fructose demonstrated a similar distribution of bacterial cells expressing FTF and cell-free FTF as in the presence of sucrose (Table 1).

3.4. MW characterization of biofilm *S. mutans* cell-associated FTF

Cell-associated samples from *S. mutans* biofilms, grown for 24 h on HA surfaces with different carbohydrates, revealed several FTF bands with molecular weights of 160, 125, 120, 100, and 50 kDa as detected by western immunodetection.

4. Discussion

S. mutans grown in biofilm were found to exhibit a distinct mode of growth and differential gene expression compared to planktonic bacteria. Expression of enzymes such FTF may differ in biofilm environment compared to planktonic conditions. In the present study, we examined the expression of FTF in the protein level using anti-FTF serum. Antibodies were used because they enable direct detection and quantification of both cell-free and cell-associated FTF, as well as characterization of changes in their MWs distribution which cannot be done by monitoring FTF expression on a nucleic acid level. Moreover the anti-FTF serum enabled us the detection of FTF in both cell lysates and in intact biofilm cells and the distribution of FTF in different layers of the biofilm both as cell free and cell associated.

Since the carbon source availability is an important environmental component in dental biofilm formation, we tested the effect of abundant simple sugars (glucose, fructose, and sucrose) on FTF expression in *S. mutans* biofilms. Our results show that FTF expression in *S. mutans* biofilms is carbohydrate-regulated. In biofilm grown with sucrose, a slightly higher amount of FTF was detected compared to biofilms grown with glucose or fructose. The thick and dense biofilm formed in the presence of sucrose allows a relative higher amount of FTF expression compared to the other tested carbohydrates, however the low ratio of FTF expression per bacteria in biofilm formed at the presence of sucrose results from the higher amounts of cells found in biofilm exposed to sucrose compared to biofilms formed with glucose or fructose. CLSM analysis has shown a relative higher amount of cell-free FTF (secreted) in biofilms formed with sucrose, thus resulting in a low cell-associated FTF per bacteria ratio. These results are in agreement with other reports showing a rapid release of cell-associated FTF from *S. salivarius* in the presence of sucrose.[6,7]

High ratios of FTF expression per bacterial cell were detected in *S. mutans* biofilms formed in the presence of fructose or glucose, indicating that relatively more bacteria are expressing FTF, although their absolute number is low. The high ratio of FTF per cell was detected in biofilms formed in the presence of fructose, suggesting that fructose could induce cell-associated FTF expression in *S. mutans*. Other studies have also shown that fructose may influence bacterial properties. For example: Kiska and Macrina[13] have shown that addition of fructose to glucose grown bacterial cells, resulted in a temporary drop in *ftf*-CAT expression which lasted for 2 h, and was followed by an increase in *ftf*-CAT expression to levels higher than initial levels. Fructose was also found to induce lipoteichoic acid expression and secretion[19] as well as fructanase production in *S. mutans*.[20]

Our CLSM findings show that not all biofilm bacteria express surface FTF. The distribution of cell-free FTF and cells expressing FTF had a similar profile among the three tested carbohydrates. Cell-free FTF was found either at the interface layers with the solid surface or at the layers interfacing with the solution. These layers contained limited amount of bacteria thus it may be assumed that the FTF is embedded in the cell-free polysaccharide. The other layers had a mixture of cell-free FTF, cells expressing FTF, and cells not expressing FTF.

The predicted MW of *S. mutans* FTF is 87.6 kDa.[4] However, *S. mutans* were found to secret FTF's with various MW's ranging from 59 to 106 kDa.[9,21,22] Processing of a single *ftf* gene product by post-translation modifications may account for the MW diversity. Formation of FTF complexes may also explain the relatively high MW FTF (160, 125, 120, and 100 kDa) detected by antibodies in our study. It has been shown by native gel electrophoresis that adsorption of purified *S. mutans* FTF onto HA surfaces induced the formation of high MW FTF complexes.[23] The high MW FTF bands found in our study may be also FTF complexes derived from *S. mutans* immobilized on HA surfaces. In this study the FTF complexes were detected using SDS gel electrophoresis emphasizing the stability of the complexes formed in situ by bacteria harboring the biofilms. The smaller FTF band (50 kDa) may result from protein degradation.

Our results show that carbohydrates regulate expression and secretion of FTF from *S. mutans* GS-5 biofilms and that the distribution of cell-free and cell-associated FTF depends on the exposure to the sugars and to the depth of the biofilm.

Acknowledgements

This study is part of the Ph.D. dissertation of R. Rozen.

References

1. Kuramitsu, H. K.; Smorawinska, M.; Yamashita, Y. Molecular Biology of *Streptococcus mutans* Virulence. In *Cariology for the Nineties*; Bowen, W. H., Tabak, L. A., Eds.; University of Rochester Press: Rochester, 1994; pp 301–308.
2. Liljemark, W. F.; Bloomquist, C. *Crit. Rev. Oral Biol. Med.* **1996**, *7*, 180–198.
3. Bowden, G. H.; Hamilton, I. R. *Crit. Rev. Oral Biol. Med.* **1998**, *9*, 54–85.
4. Shiroza, T.; Kuramitsu, H. K. *J. Bacteriol.* **1988**, *170*, 810–816.
5. Burne, R. A. Oral Ecological Disaster: The Role of Short-Term Extracellular Storage Polysaccharides. In *Cariology for the Nineties*; Bowen, W. H., Tabak, L. A., Eds.; University of Rochester Press: Rochester, 1994; pp 351–364.
6. Milward, C. P.; Jacques, N. A. *J. Gen. Microbiol.* **1990**, *136*, 165–169.
7. Townsend-Lawman, P.; Bleiweis, A. S. *J. Gen. Microbiol.* **1991**, *137*, 5–13.
8. Rolla, G.; Ciardi, J. E.; Eggen, K. H.; Bowen, W. H.; Afseth, J. Free glucosyl- and fructosyltransferase in human saliva and adsorption of these enzymes to teeth in vivo. In *Glucosyltransferases, Glucans, Sucrose and Dental Caries*; Doyle, R. J., Ciardi, J. E., Eds.; Chemical Senses, IRL Press: Washington, DC, 1983; pp 21–30.
9. Munro, C.; Michalek, S. M.; Macrina, F. L. *Infect. Immun.* **1991**, *59*, 2316–2323.
10. Rozen, R.; Bacharach, G.; Bronstheyn, M.; Gedalia, I.; Steinberg, D. *FEMS Microbiol. Lett.* **2001**, *195*, 205–210.
11. Wexler, D. L.; Hudson, M. C.; Burne, R. A. *Infect. Immun.* **1993**, *61*, 1259–1267.
12. Hudson, M. C.; Curtiss, R. *Infect. Immun.* **1990**, *58*, 464–470.
13. Kiska, D. L.; Macrina, F. L. *Infect. Immun.* **1994**, *62*, 1241–1251.
14. Burne, R. A.; Chen, Y. M. M.; Penders, J. E. C. *Adv. Dent. Res.* **1997**, *11*, 100–109.
15. Rozen, R.; Steinberg, D.; Bachrach, G. *FEMS Microbiol. Lett.* **2004**, *232*, 39–43.
16. Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
17. Banas, J. A.; Hazlett, K. R. O.; Mazurkiewicz, J. E. An in vitro model for studying the contributions of the *Streptococcus mutans* glucan-binding protein A to biofilm structure In *Methods in Enzymology*; Conn, P. M., Ed.; Academic: San Dieago, 2001; Vol. 337, pp 425–433.
18. Hazlett, K. R. O.; Mazurkiewicz, J. E.; Banas, J. A. *Infect. Immun.* **1999**, *67*, 3909–3914.
19. Hardy, L.; Jacques, N. A.; Forester, H.; Campbell, L. K.; Knox, K. W.; Wicken, A. J. *Infect. Immun.* **1981**, *31*, 78–87.
20. Jacques, N. J.; Morrey-Jones, J. G.; Walker, G. J. *J. Gen. Microbiol.* **1985**, *131*, 1625–1633.
21. Sato, S.; Koga, T.; Inoue, M. *Carbohydr. Res.* **1984**, *134*, 293–304.
22. Sato, S.; Kuramitsu, H. K. *Infect. Immun.* **1986**, *52*, 166–170.
23. Bronstheyn, M.; Steinberg, D. *Lett. Appl. Microbiol.* **2002**, *34*, 205–209.