Structural and Molecular Basis of the Role of Starch and Sucrose in *Streptococcus mutans* Biofilm Development^{∇†}

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The interaction of sucrose and starch with bacterial glucosyltransferases and human salivary amylase may enhance the pathogenic potential of *Streptococcus mutans* within biofilms by influencing the structural organization of the extracellular matrix and modulating the expression of genes involved in exopolysaccharide synthesis and specific sugar transport and two-component systems.

Oral diseases related to biofilms, such as dental caries, affect the majority of the world's population (27, 34). Dental caries results from the interaction of specific bacteria and salivary constituents with dietary carbohydrates in the oral cavity; the appearance of biofilms on the tooth surface is the first clinical evidence of the diet-bacterium interaction. Sucrose is considered the “arch criminal” from the dietary standpoint because it is fermentable and also serves as a substrate for synthesis of extracellular polysaccharides (EPS) in dental biofilm (2, 28). Starches are an important source of fermentable carbohydrate and are usually consumed simultaneously with sucrose in modern societies. The combination of starch and sucrose is highly cariogenic in vivo (1, 10, 33) and may enhance the pathogenicity of biofilms in humans (30).

Streptococcus mutans is a key contributor to the formation of cariogenic biofilms; this bacterium (i) synthesizes large amounts of extracellular glucans and fructans from sucrose using several glucosyltransferases (Gtfs) and a fructosyltransferase, (ii) adheres tenaciously to glucan-coated surfaces, and (iii) is highly acidogenic and acid tolerant, which are critical virulence properties in the pathogenesis of dental caries (2, 29, 31). Glucans provide specific binding sites for bacterial colonization on the tooth surface and bulk and structural integrity to the extracellular matrix; thus, they are essential for the formation and accumulation of dental biofilms (2). In addition, starches can be digested by α -amylases to maltose, maltodextrins, and other oligosaccharides, some of which can be acceptors during glucan synthesis by Gtfs (11, 12, 35). Starch hydrolysates produced by salivary α -amylases bound to saliva-coated hydroxyapatite (sHA) increased, in the presence of sucrose, the synthesis of structurally distinctive glucans by surface-adsorbed GtfB (35).

Moreover, maltose and maltodextrins resulting from starch hydrolysis can be catabolized in acids by *S. mutans* (4).

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In this study, we investigated whether biochemical reactions involving interactions between specific host (α -amylase) and bacterium-derived (Gtfs) enzymes and dietary carbohydrates (starch and sucrose) influence (i) the biochemical and structural properties of the EPS matrix and (ii) trigger specific adaptive responses by *S. mutans* at the transcriptional level, resulting in a biofilm with enhanced virulence.

Biofilms of *S. mutans* UA159 (= ATCC 700610) were formed using our amylase-active sHA disk model (18). Biofilms were grown in buffered tryptone yeast extract broth (pH 7.0) containing (i) 1% (wt/vol) starch (soluble starch [80% amylopectin and 20% amylose]; Sigma Chemical Company, St. Louis, MO), (ii) 1% sucrose, (iii) 1% starch plus 1% sucrose, (iv) 1% starch plus 0.5% glucose plus 0.5% fructose, or (v) 1% sucrose plus 1% glucose. The structural organization of the biofilms was examined by laser scanning confocal fluorescence imaging using a Leica TCS SP1 microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) with a 40 $\times$, 0.8-numerical-aperture water immersion objective. The bacterial cells were labeled by using 2.5 μ M SYTO 9 green fluorescent nucleic acid stain (480/500 nm; Molecular Probes Inc., Eugene, OR). The polysaccharides were labeled with 2.5 μ M Alexa Fluor 647-dextran conjugate (molecular weight, 10,000; maximum absorbance wavelength, 647 nm; maximum fluorescence emission wavelength, 668 nm; Molecular Probes Inc., Eugene, OR). Fluorescently labeled dextran serves as a primer for Gtfs and can be simultaneously incorporated during EPS matrix synthesis over the course of biofilm development (37). Figure 1 shows that Alexa Fluor 647-dextran was incorporated into the glucans by GtfB but did not stain the bacterial cells at the concentrations used in this study. The biofilm structure was quantified and visualized using COMSTAT (16) and Amira 4.1.1 software (Mercury Computer Systems Inc., Chelmsford, MA). The amount and structure of the EPS were determined by colorimetric assays (20) and linkage analysis using gas chromatography-mass spectrometry (15).

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FIG. 1. (A) Water-insoluble glucans synthesized by *S. mutans* GtfB labeled with Alexa Fluor 647-dextran conjugate with a maximum absorbance wavelength of 647 nm and a maximum fluorescence emission wavelength of 668 nm. (Panel A-1) Phase-contrast image of glucans before excitation with a laser at 633 nm (20 $\times$ oil objective; numerical aperture, 0.7). (Panel A-2) Fluorescence image of glucans. (B) *S. mutans* cells grown in culture medium containing 2.5 μ M Alexa Fluor 647-dextran conjugate. (Panel B-1) Differential contrast image of bacterial cells. (Panel B-2) Bacterial cells with laser excitation at 633 nm for Alexa Fluor 647-dextran detection. (Panel B-3) Bacterial cells with laser excitation at 488 nm for SYTO 9 detection.

S. mutans growing in the presence of sucrose and starch formed a distinctive three-dimensional biofilm structure on the surface of amylase-active sHA (Table 1 and Fig. 2); the presence of starch alone or in combination with glucose plus fructose resulted in negligible biofilm formation. Measurements of the biovolume obtained by using COMSTAT revealed that biofilms formed in the presence of sucrose plus starch contained more EPS and had higher EPS-to-bacterium biovolume ratios than biofilms formed in the presence of sucrose or in the presence of sucrose plus glucose (Table 1) ($P < 0.05$), especially in the deeper and outer layers of the biofilm ($>90 \mu$ m from the substratum) (Fig. 2A, panel A-1, and 2B, panel B-1). Rendered three-dimensional images revealed that almost all bacteria in the outer layers of biofilms formed in the presence of sucrose plus starch were associated with or were in contact with EPS (Fig. 2, panel A-2, and 2B, panel B-2). Biochemical

analyses showed that the EPS matrix in biofilms formed in the presence of sucrose plus starch contained larger amounts of highly branched insoluble glucans (branch points, 3,4-, 3,6-, and 3,4,6-linked glucose) and consequently had more biomass than biofilms formed in the presence of sucrose or in the presence of sucrose plus glucose (data not shown). The synthesis of the modified polysaccharide could be partially explained by previous observations that the presence of oligosaccharides resulting from starch digestion by surface-adsorbed amylase increased the synthesis of 3-linked branched insoluble glucans by GtfB bound to an sHA surface (21, 35). Furthermore, the architectural and structural differences of the biofilms might also be related to environmental changes (e.g., the availability of oligosaccharides) which could trigger *S. mutans* responses at the transcriptional level, including modulation of the expression of genes associated with EPS formation.

Thus, the expression of genes encoding the synthesis (*gtfB*, *gtfC*, *gtfD*) (38) and degradation (*dexA*) (13) of glucans by *S. mutans* within biofilms was determined by real-time quantitative reverse transcriptase PCR. At selected time points (48, 72, 96, and 120 h), the RNA were extracted from the biofilms and purified as described previously (6). The reverse transcriptase PCR real-time amplification conditions and the gene-specific primers were similar to those described previously by Koo et al. (19); relative expression was calculated by normalizing each gene of interest to the 16S rRNA (19). As shown in Fig. 3, biofilms formed in the presence of sucrose plus starch expressed higher levels of *gtfB* mRNA than biofilms formed in the presence of sucrose during the entire biofilm developmental process, especially between 48 and 96 h ($P < 0.05$); the Gtf encoded by *gtfB* synthesizes mostly water-insoluble α (1,3)-linked glucans (38). The increased availability of metabolizable carbohydrates (such as maltose and maltotriose) and conse-

TABLE 1. Biovolume and average thickness of *S. mutans* UA159 biofilms determined by COMSTAT analysis^a

Carbohydrate(s)	Biofilm component	Biovolume ($\mu\text{m}^3/\mu\text{m}^2$)	Avg thickness (μm)
Sucrose + starch	EPS	53.1 (8.5) a	119.1 (33.5) a
	Bacteria	29.9 (6.4) A	91.2 (37.8) A
Sucrose	EPS	33.4 (8.2) b	74.0 (27.4) b
	Bacteria	38.1 (8.5) B	73.5 (25.2) AB
Sucrose + glucose	EPS	23.1 (6.6) c	45.0 (16.5) c
	Bacteria	37.5 (9.9) B	57.1 (18.2) B

^a Growing cells with starch and with starch plus fructose plus glucose resulted in minimal biofilm formation. The values in parentheses are standard deviations ($n = 15$). Values for each parameter (biovolume or average thickness) followed by the same letter are not significantly different ($P > 0.05$, as determined by an analysis of variance for all pairs using Tukey's test).

FIG. 2. (A) Biofilm formation in the presence of sucrose plus starch. (Panel A-1) Distribution of bacteria and EPS in the biofilms. (Panel A-2) Representative three-dimensional images of the structural organization of the biofilms: rendered images of the outer layers of biofilms. Green, bacteria; red, EPS. (B) Biofilm formation in the presence of sucrose. (Panel B-1) Distribution of bacteria and EPS in the biofilms. (Panel B-2) Representative three-dimensional images of the structural organization of the biofilms: rendered images of the outer layers of biofilms. Green, bacteria; red, EPS.

FIG. 3. Real-time PCR analysis of *gtfB*, *gtfC*, *gtfD* and *dexA* gene expression by *S. mutans* growing in the presence of sucrose or sucrose plus starch. The mRNA level of each gene in each sample was normalized to that of 16S rRNA. The values were then compared to those for sucrose-grown biofilms (which were assigned an arbitrary value of 1) to determine the change (fold) in *gtf* gene expression. The data are the means $\pm$ standard deviations for triplicate determinations in at least three separate experiments. An asterisk indicates that a value is significantly different from the value for the sucrose-grown biofilms ($P < 0.05$, Tukey's test).

quently the enhanced acidification of the biofilms may influence the expression of *gtf* genes (25). However, we and other workers have shown that addition of excess glucose or other simple sugars actually reduces the synthesis of EPS (8, 9, 14), which may involve downregulation of the expression of *gtf* genes (32). Thus, it is conceivable that the presence of additional acceptors sensed by *S. mutans* resulted in increased *gtfB* expression since the starch hydrolysates could act as primers for glucan synthesis (35). In contrast, the *dexA* mRNA levels in biofilms formed in the presence of sucrose plus starch were less than the *dexA* mRNA levels in biofilms formed in the presence of sucrose at 120 h ($P < 0.05$). The dextranase encoded by *dexA* is upregulated when biofilm formation reaches a steady state after a certain amount of glucans is produced. Thus, it is apparent that glucan degradation/remodeling initiates at earlier stages in biofilms grown in the presence of sucrose than in biofilms grown in the presence of sucrose plus starch, which suggests that the latter biofilms may be more persistent and metabolically active for prolonged periods. Furthermore, our initial transcriptome analysis of the biofilms also revealed that in the presence of starch and sucrose, (i) the expression of specific genes related to sugar transport systems (e.g., multiple sugar metabolism; *msm* genes) was enhanced and (ii) two-component systems (TCS), such as *comE* and *covR/sncR*, were downregulated (see Table S1 in the supplemental material for a complete list of genes and microarray procedures).

Overall, the results of this study revealed that the interaction of salivary α -amylase and streptococcal Gtfs with dietary carbohydrates influenced the development and virulence of *S. mutans* biofilms in at least two interconnected ways: (i) by changing the structural organization of the EPS matrix and (ii) by triggering specific transcriptome responses by *S. mutans*. Elevated amounts of highly branched insoluble glucans occupying most of the biovolume across the depth of a biofilm that

formed in the presence of starch plus sucrose and enmeshing the bacterial cells could (i) increase the physical integrity or stability of the biofilm (5), (ii) influence the diffusion properties (7), and (iii) provide increased protection to inimical influences of antimicrobials and other environmental assaults (22, 24). Moreover, the structural differences between glucan made with starch hydrolysates and glucan made without starch hydrolysates may also provide distinct bacterial binding sites (35). The induction of expression of *gtfB* mRNA may play a critical role in altering the biofilm structure. GtfB secreted by *S. mutans* binds not only to the apatitic surface but also on the bacterial membrane in an active form (36). The insoluble glucans synthesized in situ could contribute to the overall increase in the polysaccharide content and explain the higher EPS/bacterium ratio across the biofilm depth. The overproduction of GtfB could be advantageous to the organism for persistent colonization of tooth surfaces (3, 31). Furthermore, mutant strains of *S. mutans* defective in *gtfB* are far less cariogenic than parent strains in vivo (38); a higher level of insoluble EPS in the matrix is associated with increased cariogenicity of biofilms in humans (17). Thus, the combination of starch and sucrose would result in a more virulent and more adherent biofilm. From a global perspective, the effects on sugar uptake systems, including upregulation of the multiple sugar metabolism system, may explain the increased acidogenicity of biofilms formed in the presence of starch plus sucrose (8). The *comE* and *covR/sncR* genes are response regulators of TCS-13 and TCS-3 in *S. mutans* UA159, which are associated with biofilm formation and morphology, development of genetic competence, and acid tolerance (23, 26). Inactivation of *comE* affected both the formation and the architecture of *S. mutans* biofilms (26). It is apparent that the presence of starch, sucrose, and salivary amylase modulates the expression of spe-

cific genes that may enhance the fitness of, competence of, and biofilm formation by *S. mutans*.

In summary, our data provide insight into how starch and sucrose in combination are potentially more cariogenic than either compound alone in vivo (1, 10, 30) and also show that the composition of diet in association with specific host-pathogen interactions can modulate the development of biofilms by *S. mutans* with enhanced virulence. Further in vitro and in vivo studies using both parental strains of *S. mutans* and mutant strains of *S. mutans* (defective in *gtfB* or TCS) in the presence of microorganisms that bind amylase (e.g., *Streptococcus gordonii*) should elucidate in more detail the structural and molecular mechanisms in a multispecies system.

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REFERENCES

- Bowen, W. H., S. M. Amsbaugh, S. Monell-Torrens, J. Brunelle, H. Kuzniak-Jones, and M. F. Cole. 1980. A method to assess cariogenic potential of foodstuffs. *J. Am. Dent. Assoc.* **100**:677-681.
- Bowen, W. H. 2002. Do we need to be concerned about dental caries in the coming millennium? *J. Am. Dent. Assoc.* **133**:1405-1407.
- Burne, R. A., B. Rubinfeld, W. H. Bowen, and R. E. Yasbin. 1986. Tight genetic linkage of a glucosyltransferase and dextranase of *Streptococcus mutans* GS-5. *J. Dent. Res.* **65**:1392-1401.
- Clarkson, C. H., D. Krell, J. S. Wefel, J. Crall, and F. F. Feagin. 1987. *In vitro* caries-like lesion production by *Streptococcus mutans* and *Actinomyces viscosus* using sucrose and starch. *J. Dent. Res.* **66**:795-798.
- Cross, S. E., J. Kreth, L. Zhu, R. Sullivan, W. Shi, F. Qi, and J. K. Gimzewski. 2007. Nanomechanical properties of glucans and associated cell-surface adhesion of *Streptococcus mutans* probed by atomic force microscopy under in situ conditions. *Microbiology* **153**:3124-3132.
- Cury, J. A., and H. Koo. 2007. Extraction and purification of total RNA from *Streptococcus mutans* biofilms. *Anal. Biochem.* **365**:208-214.
- Dibdin, G. H., and R. P. Shellis. 1988. Physical and biochemical studies of *Streptococcus mutans* sediments suggest new factors linking the cariogenicity of plaque with its extracellular polysaccharide content. *J. Dent. Res.* **67**:890-895.
- Duarte, S., M. I. Klein, C. P. Aires, J. A. Cury, W. H. Bowen, and H. Koo. 2008. Influences of starch and sucrose on *Streptococcus mutans* biofilms. *Oral Microbiol. Immunol.* **23**:206-212.
- Ellwood, D. C., P. J. Phipps, and I. R. Hamilton. 1979. Effect of growth rate and glucose concentration on the activity of the phosphoenolpyruvate phosphotransferase system in *Streptococcus mutans* Ingbritt grown in continuous culture. *Infect. Immun.* **23**:224.
- Firestone, A. R., R. Shmid, and H. R. Muhlemann. 1982. Cariogenic effects of cooked wheat starch alone or with sucrose and frequency-controlled feedings in rats. *Arch. Oral Biol.* **27**:759-763.
- Fu, D. T., and J. F. Robyt. 1991. Maltodextrin acceptor reactions of *Streptococcus mutans* 6715 glucosyltransferases. *Carbohydr. Res.* **217**:201-211.
- Fukui, K., and T. Moriyama. 1983. Effect of maltose on glucan synthesis by glucosyltransferases of *Streptococcus mutans*. *Microbiol. Immunol.* **27**:917-927.
- Guggenheim, B., and J. J. Burckhardt. 1974. Isolation and properties of a dextranase from *Streptococcus mutans* OMZ 176. *Helv. Odontol. Acta* **18**:101-113.
- Hamilton, I. R., P. J. Phipps, and D. C. Ellwood. 1979. Effect of growth rate and glucose concentration on the biochemical properties of *Streptococcus mutans* Ingbritt in continuous culture. *Infect. Immun.* **26**:861-869.
- Hayacibara, M. F., H. Koo, A. M. Vacca-Smith, L. K. Kopec, K. Scott-Anne, J. A. Cury, and W. H. Bowen. 2004. The influence of mutanase and dextranase on the production and structure of glucans synthesized by streptococcal glucosyltransferases. *Carbohydr. Res.* **339**:2127-2137.
- Heydorn, A., A. T. Nielsen, M. Hentzer, C. Sternberg, M. Givskov, B. K. Ersbøll, and S. Molin. 2000. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* **146**:2395-2407.
- Hotz, P., B. Guggenheim, and R. Schmid. 1972. Carbohydrates in pooled dental plaque. *Caries Res.* **6**:103-121.
- Koo, H., B. D. Schobel, K. Scott-Anne, G. Watson, W. H. Bowen., J. A. Cury, P. L. Rosalen, and Y. K. Park. 2005. Apigenin and *tt*-farnesol with fluoride effects on *S. mutans* biofilms and dental caries. *J. Dent. Res.* **84**:1016-1020.
- Koo, H., J. Seils, J. Abranches, R. A. Burne, W. H. Bowen, and R. G. Quivey, Jr. 2006. Influence of apigenin on *gtf* gene expression in *Streptococcus mutans* UA159. *Antimicrob. Agents Chemother.* **50**:542-546.
- Koo, H., M. F. Hayacibara, B. D. Schobel, J. A. Cury, P. L. Rosalen, Y. K. Park, A. M. Vacca-Smith, and W. H. Bowen. 2003. Inhibition of *Streptococcus mutans* biofilm accumulation and polysaccharide production by apigenin and *tt*-farnesol. *J. Antimicrob. Chemother.* **52**:782-789.
- Kopec, L. K., A. M. Vacca-Smith, and W. H. Bowen. 1997. Structural aspects of glucans formed in solution and on the surface of hydroxyapatite. *Glycobiology* **7**:929-934.
- Kreth, J., L. Zhu, J. Merritt, W. Shi, and F. Qi. 2008. Role of sucrose in the fitness of *Streptococcus mutans*. *Oral Microbiol. Immunol.* **23**:213-219.
- Lévesque, C. M., R. W. Mair, J. A. Perry, P. C. Lau, Y. H. Li, and D. G. Cvitkovitch. 2007. Systemic inactivation and phenotypic characterization of two-component systems in expression of *Streptococcus mutans* virulence properties. *Lett. Appl. Microbiol.* **45**:398-404.
- Lewis, K. 2001. Riddle of biofilm resistance. *Antimicrob. Agents Chemother.* **45**:999-1007.
- Li, Y., and R. A. Burne. 2001. Regulation of the *gtfBC* and *ftf* genes of *Streptococcus mutans* in biofilms in response to pH and carbohydrate. *Microbiology* **147**:2841-2848.
- Li, Y. H., P. C. Lau, N. Tang, G. Svensater, R. P. Ellen, and D. G. Cvitkovitch. 2002. Novel two-component regulatory system involved in biofilm formation and acid resistance in *Streptococcus mutans*. *J. Bacteriol.* **184**:6333-6342.
- Marsh, P. D. 2003. Are dental diseases examples of ecological catastrophes? *Microbiology* **149**:279-294.
- Paes Leme, A. F., H. Koo, C. M. Bellato, G. Bedi, and J. A. Cury. 2006. The role of sucrose in cariogenic dental biofilm formation—new insight. *J. Dent. Res.* **85**:878-887.
- Quivey, R. G., W. L. Kuhnert, and K. Hahn. 2001. Genetics of acid adaptation in oral streptococci. *Crit. Rev. Oral. Biol. Med.* **12**:301-314.
- Ribeiro, C. C., C. P. Tabchoury, A. A. Del Bel Cury, L. M. Tenuta, P. L. Rosalen, and J. A. Cury. 2005. Effect of starch on the cariogenic potential of sucrose. *Br. J. Nutr.* **94**:44-50.
- Schilling, K. M., and W. H. Bowen. 1992. Glucans synthesized in situ in experimental salivary pellicle function as specific binding sites for *Streptococcus mutans*. *Infect. Immun.* **60**:284-295.
- Shemesh, M., A. Tam, and D. Steinberg. 2007. Differential gene expression profiling of *Streptococcus mutans* cultured under biofilm and planktonic conditions. *Microbiology* **153**:1307-1317.
- Thurnheer, T., E. Giertsen, R. Gmür, and B. Guggenheim. 2008. Cariogenicity of soluble starch in oral in vitro biofilm and experimental rat caries studies: a comparison. *J. Appl. Microbiol.* **105**:829-836.
- U.S. Department of Health and Human Services National Center for Health Statistics. 2007. Trends in oral health status: United States, 1988-1994 and 1999-2004. Vital Health Statistics, series 11, number 248. National Center for Health Statistics, Hyattsville, MD.
- Vacca-Smith, A. M., A. R. Venkitaraman, R. G. Quivey, Jr., and W. H. Bowen. 1996. Interactions of streptococcal glucosyltransferases with alpha-amylase and starch on the surface of saliva-coated hydroxyapatite. *Arch. Oral Biol.* **41**:291-298.
- Vacca-Smith, A. M., and W. H. Bowen. 1998. Binding properties of streptococcal glucosyltransferases for hydroxyapatite, saliva-coated hydroxyapatite, and bacterial surfaces. *Arch. Oral Biol.* **3**:103-110.
- Venkitaraman, A. R., A. M. Vacca-Smith, L. K. Kopec, and W. H. Bowen. 1995. Characterization of glucosyltransferases B, GtFC, and GtFD in solution and on the surface of hydroxyapatite. *J. Dent. Res.* **74**:1695-1701.
- Yamashita, Y., W. H. Bowen, R. A. Burne, and H. K. Kuramitsu. 1993. Role of the *Streptococcus mutans gtf* genes in caries induction in the specific-pathogen-free rat model. *Infect. Immun.* **61**:3811-3817.