

LETTER TO EDITOR

Extracellular Polysaccharides Matrix — An Often Forgotten Virulence Factor in Oral Biofilm Research**Hyun Koo*, Jin Xiao, Marlise I. Klein**

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Oral diseases related to dental biofilms continue to afflict the majority of the world's population. Among them, dental caries continues to be the single most prevalent and costly oral infectious disease (Marsh, 2003; Dye *et al.*, 2007). Dental caries results from the interaction of specific bacteria with constituents of the diet within a dental biofilm known as plaque (Bowen, 2002). Sucrose is considered to be the "arch criminal" from the dietary aspect because it serves as a substrate for synthesis of extracellular (EPS) and intracellular (IPS) polysaccharides in dental biofilm and is also fermentable (Bowen, 2002). However, it is important to emphasize that additional sugars and starch can certainly contribute to the pathogenesis (Bowen *et al.*, 1980; Firestone *et al.*, 1982; Thurnheer *et al.*, 2008). *Streptococcus mutans* (*S. mutans*), a member of the oral microbial community, is generally regarded as the primary microbial culprit although additional microorganisms may be involved (Hamada and Slade, 1980; Loesche, 1986; Beighton, 2005). This bacterium (i) effectively utilizes dietary sucrose (and possibly starch) to synthesize large amounts of EPS through glucosyltransferases (Gtfs) and a fructosyltransferase (Ftfs), (ii) adheres tenaciously to glucan-coated surfaces, and (iii) is also acidogenic and acid-tolerant, which are critical virulence properties involved in the pathogenesis of dental caries.

Biofilms and dental caries — the role of exoenzymes and EPS synthesis

In nature, most of the biofilms develops from initial microbial attachment on a surface followed by formation of highly structured cell clusters (or microcolonies) and further development and stabilization of the microcolonies, which are occurring in a complex extracellular matrix (Branda *et al.*, 2005). The majority of biofilms matrices are rich in polysaccharides, and dental biofilms are no exception; up to 40% of the dry-weight of dental biofilm is composed of polysaccharides (as reviewed in Paes Leme *et al.*, 2006). All the available evidence shows clearly that the primary sources of EPS in dental biofilms are products from the interaction of Gtfs and Ftfs with sucrose and starch (as reviewed in Vacca-Smith *et al.*, 1996; Kopec *et al.*, 1997; Hayacibara *et al.*, 2004; van Hijum *et al.*, 2006; Klein *et al.*, 2009).

S. mutans is a key contributor to the formation of exopolysaccharide-matrix in dental biofilms. This bacterium produces three Gtfs, products of *gtfB*, *gtfC* and *gtfD* genes (Kuramitsu, 2003): GtfB, which synthesizes mostly insoluble glucans containing elevated amounts of α 1,3-linked glucose; GtfC, which synthesizes a mixture of insoluble and soluble glucans [rich in α (1,6) linkages]; and GtfD which synthesizes predominantly soluble glucans. In addition, *S. mutans* produces a Ftfs, the

product of single *ftf* gene, which catalyzes the synthesis of fructans composed primarily of $\beta(2,1)$ linkages. All of these exoenzymes and their polysaccharide products have been implicated in various roles in biofilm formation and dental caries process. For example, GtfB and GtfC are associated with bacterial adherence on tooth surface and structural stability/integrity of the extracellular matrix, and have been shown to be essential for the expression of virulence of *S. mutans* in rat caries model (Tanzer *et al.*, 1985; Munro *et al.*, 1991; Yamashita *et al.*, 1993). Fructans are used as extracellular carbohydrate reservoir, which can be metabolized by bacteria during periods of nutrient deprivation (Burne *et al.*, 1996). These observations show very clearly all these enzymes could be primary targets for therapeutic intervention to prevent biofilm formation and dental caries (Koo *et al.*, 2006).

The surface-adsorbed Gtfs and initial bacterial adherence on apatitic surfaces

S. mutans cells can attach initially to saliva coated surfaces through sucrose-independent mechanisms mediated primarily by lectin-like interactions between specific pellicle proteins and bacterial adhesins (Gibbons, 1996). However, this bacterium binds to the glucan-coated surfaces, especially those synthesized by GtfB and GtfC, in larger numbers and with higher adhesion strength than to uncoated or saliva-coated apatitic surfaces (Kuramitsu, 1974; Schilling and Bowen, 1992; Cross *et al.*, 2007). The Gtfs secreted by *S. mutans*, particularly GtfC, bind avidly to the pellicle formed on the tooth surface in an active form; a layer of polysaccharides is formed rapidly on the surfaces in the presence of sucrose (Rolla *et al.*, 1983; Scheie *et al.*, 1987; Schilling and Bowen, 1988; Vacca-Smith and Bowen, 1998; Hannig *et al.*, 2008). In addition, starches can be digested by salivary α -amylases to maltose, maltodextrins and other oligosaccharides, some of which can be acceptors during glucan synthesis by Gtfs increasing the overall exopolysaccharide production (Fukui and Moriyama, 1983; Fu and Robyt, 1991; Vacca-Smith *et al.*, 1996). The polysaccharides on the surface provide binding site for colonization by *S. mutans* (Schilling and Bowen, 1992) through several surface-proteins capable of binding glucans,

including the Gtfs and specific non-enzymatic glucan binding proteins (Banas and Vickerman, 2003). Moreover, it is apparent that glucan synthesized *in situ* by Gtf in pellicle provides enhanced binding for several oral microorganisms, including other oral streptococci, and *Lactobacillus* and *Actinomyces* species (Vacca-Smith *et al.*, 1996; Bowen, 2002).

The Gtfs, especially GtfB, also adhere to bacterial surfaces, and furthermore adhere to surfaces of bacteria that do not make enzyme, thereby converting them into *de facto* glucan producers (McCabe and Donkersloot, 1977; Hamada *et al.*, 1978; Vacca-Smith and Bowen, 1998). Thus, glucans, and at a lesser extent fructans, formed *in situ* promote the accumulation of microorganisms on the tooth surface and to each other, and may explain the electron micrographs of even early dental biofilm-plaque, which display microorganisms enmeshed in and attached to polysaccharide on the surface of saliva-coated hydroxyapatite (Vacca-Smith and Bowen, 2000). The attachment of bacterial cells on surfaces and formation of cell-cluster (or microcolonies) within an extracellular matrix are critical steps for the initial formation and further development of pathogenic biofilms.

The role of EPS in the development of cariogenic biofilms

If the initial biofilm is allowed to remain on tooth surfaces with a frequent consumption of a high carbohydrate diet (especially sucrose and starch), *S. mutans*, a constituent of the biofilm community, will continue to synthesize exopolysaccharides and metabolize the sugars to organic acids. The elevated amounts of EPS, which may involve up-regulation of *gtf* genes in response to pH and carbohydrate availability (Li and Burne, 2001), increase the accumulation of bacterial cells on the surface and the bulk and stability of the biofilm, enhancing its virulence (Bowen, 2002). Recently, we examined the structural relationships between the bacterial cells and EPS using a novel fluorescence imaging technique (Klein *et al.*, 2009; Xiao and Koo, 2009), which allows for visualization and quantification of exopolymers and bacterial cells simultaneously within intact biofilms (see Figure 1). The rendered images showed EPS closely associated with bacterial cells and microcolonies

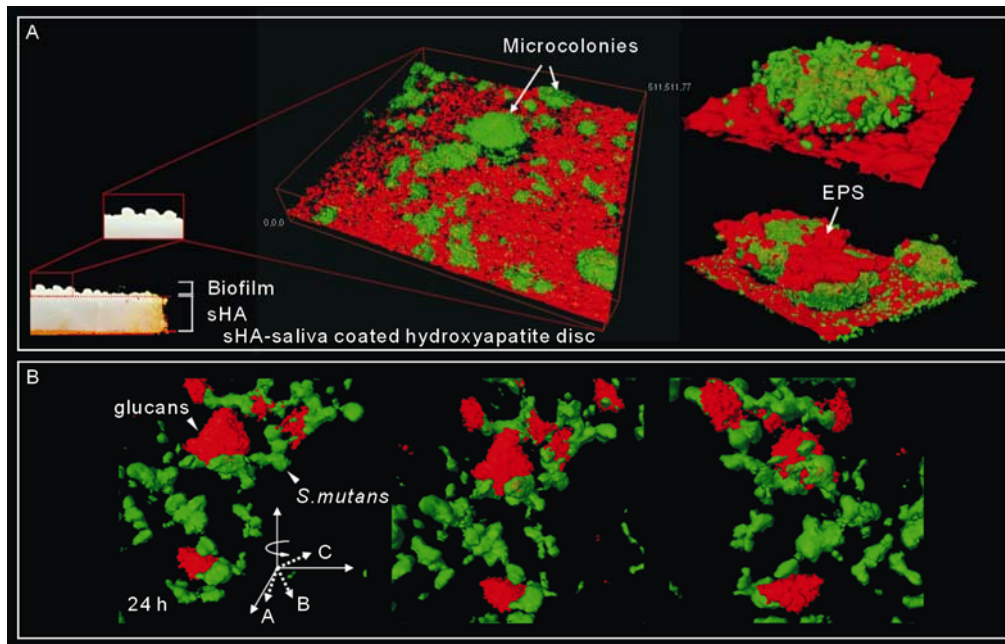


Figure 1 Fluorescence imaging showed the structural relationships between the bacterial cells and EPS

(A): Rendered 3-D images of the structural organization of the biofilms. Green: bacteria; Red: EPS. (B): Close-up view of the EPS: bacteria structural relationship.

throughout the biofilm development process (1) serving as a matrix holding bacterial cells on the surface allowing for the initial clusterization, and further development into microcolonies, and (2) providing supporting frame for continuous growth of the microcolonies (by enmeshing and filling the spaces between bacterial cells). The formation of specific EPS domains could be explained by the presence of enzymatically active Gtfs (particularly GtfB and GtfC) bound to saliva-coated apatitic and bacterial surfaces (Schilling and Bowen, 1988; Venkitaraman *et al.*, 1995; Vacca-Smith and Bowen, 1998; Hannig *et al.*, 2008). The production of exopolysaccharides by Gtfs acting in concert at different sites is a highly efficient mechanism by which *S. mutans* colonize the surface and maintain the microcolony structure, allowing them to persist on the tooth surface for prolonged period. This EPS-dependent mechanism could explain why *S. mutans*, either alone or mixed with other species, can only build up microcolonies when sucrose is available (Kreth *et al.*, 2008; Xiao and Koo, 2009); strongly suggesting that sucrose may increase the competitiveness of this bacterium in a mixed species environment. Considering that bacterial fitness in biofilms may be linked with microcolonies formation, EPS may modulate the

colonization and survival of *S. mutans* within complex biofilms in a context not previously considered.

The presence of these structured EPS microdomains within and surrounding microcolonies across the biofilm depth may influence the diffusion properties and also protect the bacteria from inimical influences of antimicrobials and other environmental assaults (Dibdin and Shellis, 1988; Thurnheer *et al.*, 2003; Reese and Guggenheim, 2007; Kreth *et al.*, 2008; Xiao and Koo, 2009). In addition, the ability of *S. mutans* to utilize some exopolysaccharides (soluble glucans and fructans) as short term storage compounds offers an additional ecological benefit, and simultaneously, increases the amount of acid production and the extent of acidification. The persistence of this acidic condition leads to selection of highly acid tolerant (and acidogenic) flora (Quivey *et al.*, 2001; Marsh, 2003; Marquis *et al.*, 2003); the low pH microenvironment in the biofilm's matrix results in dissolution of enamel of tooth surface, thus contributing to the pathogenesis of dental caries disease. Furthermore, glucans synthesized by other oral streptococci (*e.g.* *S. oralis* and *S. sanguinis*) may also contribute to the overall polysaccharide synthesis in the extracellular matrix

(Tamesada *et al.*, 2004; Reese and Guggenheim, 2007). Clearly, EPS, particularly glucans synthesized by Gtfs, are essential for the formation and establishment of cariogenic biofilms.

A number of *in vivo* studies investigated the influence of Gtfs in sucrose-dependent colonization of teeth and in the pathogenesis of dental caries disease using animal models (Tanzer *et al.*, 1985; Munro *et al.*, 1991; Yamashita *et al.*, 1993). Among them, Yamashita *et al.* (1993) conducted a comprehensive study using *S. mutans* mutants defective of each of the three *gtf* genes in a glucan-dependent rat model system to determine the precise role of these enzymes in the expression of virulence *in vivo*. Mutants defective in *gtfB*, *gtfC* and *gtfBC* exhibited marked reductions (up to 90%) in smooth-surface caries relative to that from parental organism. The *gtfD* mutant also induced significantly fewer smooth-surface lesions than did the parental organism albeit with less pronounced effect than *gtfBC* mutants; GtfD enzyme appears to be important for maximum development of smooth-surface carious lesions in the oral cavities of rats (Yamashita *et al.*, 1993). Furthermore, several clinical studies have demonstrated that insoluble glucans produced by GtfB and GtfC in the extracellular matrix augment the cariogenicity of dental biofilms in humans (as reviewed in Paes Leme *et al.*, 2006). Recently, it was shown that GtfB levels in saliva correlate strongly with presence of clinical caries and with number of carious lesions in young children (Vacca-Smith *et al.*, 2007). Overall, the observations from *in vitro*, *in vivo* and clinical studies are highly supportive of the importance of the Gtfs and EPS in the pathogenesis of dental caries. Therefore, biofilm-control strategies based on disruption of EPS offer an attractive and alternative approach to the traditional chemotherapeutic approaches based on use of broad spectrum microbiocides, which require elevated concentration of agents due to reduced susceptibility of microorganisms in biofilms (Koo and Jeon, 2009).

Summary and future directions

The presence, composition and structure of the EPS-matrix influence the physical and biochemical properties of biofilm; the matrix may be as variable

as the microbial constituents and is influenced by local environmental conditions and even changes with time. All the available evidences indicate that EPS, particularly glucans produced by *S. mutans* Gtfs, can contribute to the pathogenesis of dental caries in animals and humans by at least 6 distinct routes: (1) enhancing tight adherence and further accumulation of *S. mutans* and other oral species on the surface, (2) providing mechanical integrity/stability to the extracellular matrix allowing the microbial cells to establish microcolonies (and increasing the bulk of the biofilms), (3) protecting microorganisms from inimical influences of antimicrobials and other environmental assaults, (4) acting as reserve source of energy, (5) limiting diffusion of substances into and out of biofilm, and (6) helping to concentrate metal ions and other physiological nutrients within a microenvironment. Thus, elucidation of how the exopolymers synthesized by these enzymes affects the biophysical, structure, and diffusion properties of the matrix would advance our understanding of the exact mechanisms by which *S. mutans* influences the formation and pathogenicity of oral biofilms, and possibly identify novel therapeutic targets for effective anti-biofilm therapies.

References

- Banas JA, Vickerman MM (2003). Glucan-binding proteins of the oral streptococci. *Crit Rev Oral Biol Med*, 14(2): 89–99.
- Beighton D (2005). The complex oral microflora of high-risk individuals and groups and its role in the caries process. *Community Dent Oral Epidemiol*, 33(4): 248–255.
- Bowen WH (2002). Do we need to be concerned about dental caries in the coming millennium? *J Am Dent Assoc*, 133(10): 1405–1407.
- Bowen WH, Amsbaugh SM, Monell-Torrens S, Brunelle J, Kuzmiak-Jones H, Cole MF (1980). A method to assess cariogenic potential of foodstuffs. *J Am Dent Assoc*, 100(5): 677–681.
- Branda SS, Vik S, Friedman L, Kolter R (2005). Biofilms: the matrix revisited. *Trends Microbiol*, 13(1): 20–26.
- Burne RA, Chen YY, Wexler DL, Kuramitsu H, Bowen WH (1996). Cariogenicity of *Streptococcus mutans* strains with defects in fructan metabolism assessed in a program-fed specific-pathogen-free rat model. *J Dent*

- Res*, 75(8): 1572–1577.
- Cross SE, Kreth J, Zhu L, Sullivan R, Shi W, Qi F, et al. (2007). Nanomechanical properties of glucans and associated cell-surface adhesion of *Streptococcus mutans* probed by atomic force microscopy under *in situ* conditions. *Microbiology*, 153(Pt 9): 3124–3132.
- Dibdin GH, Shellis RP (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(6): 890–895.
- Dye BA, Tan S, Smith V, Lewis BG, Barker LK, Thornton-Evans G, et al. (2007). Trends in oral health status: United States, 1988-1994 and 1999-2004. *Vital Health Stat*, 11(248): 1–92.
- Firestone AR, Schmid R, Mühlemann HR (1982). Cariogenic effects of cooked wheat starch alone or with sucrose and frequency-controlled feedings in rats. *Arch Oral Biol*, 27(9): 759–763.
- Fu DT, Robyt JF (1991). Maltodextrin acceptor reactions of *Streptococcus mutans* 6715 glucosyltransferases. *Carbohydr Res*, 217: 201–211.
- Fukui K, Moriyama T (1983). Effect of maltose on glucan synthesis by glucosyltransferases of *Streptococcus mutans*. *Microbiol Immunol*, 27(11): 917–927.
- Gibbons RJ (1996). Role of adhesion in microbial colonization of host tissues: a contribution of oral microbiology. *J Dent Res*, 75(3): 866–870.
- Hamada S, Slade HD (1980). Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol Rev*, 44(2): 331–384.
- Hamada S, Tai S, Slade HD (1978). Binding of glycosyltransferase and glucan synthesis by *Streptococcus mutans* and other bacteria. *Infect Immun*, 21(1): 213–220.
- Hannig C, Ruggeri A, Al-Khayer B, Schmitz P, Spitzmüller B, Deimling D, et al. (2008). Electron microscopic detection and activity of glucosyltransferase B, C, and D in the *in situ* formed pellicle. *Arch Oral Biol*, 53(11): 1003–1010.
- Hayacibara MF, Koo H, Vacca-Smith AM, Kopec LK, Scott-Anne K, Cury JA, et al. (2004). The influence of mutanase and dextranase on the production and structure of glucans synthesized by streptococcal glucosyltransferases. *Carbohydr Res*, 339(12): 2127–2137.
- Klein MI, Duarte S, Xiao J, Mitra S, Foster TH, Koo H (2009). Structural and molecular basis of the role of starch and sucrose in *Streptococcus mutans* biofilms development. *Appl Environ Microbiol*, 75(3): 837–841.
- Koo H, Jeon JG (2009). Naturally occurring molecules as alternative therapeutic agents against cariogenic biofilms. *Adv Dent Res*, 21(1): 63–68.
- Koo H, Seils J, Abranches J, Burne RA, Bowen WH, Quivey RG Jr (2006). Influence of apigenin on *gtf* gene expression in *Streptococcus mutans* UA159. *Antimicrob Agents Chemother*, 50(2): 542–546.
- Kopec LK, Vacca-Smith AM, Bowen WH (1997). Structural aspects of glucans formed in solution and on the surface of hydroxyapatite. *Glycobiology*, 7(7): 929–934.
- Kreth J, Zhu L, Merritt J, Shi W, Qi F (2008). Role of sucrose in the fitness of *Streptococcus mutans*. *Oral Microbiol Immunol*, 23(3): 213–219.
- Kuramitsu HK (2003). Molecular genetic analysis of the virulence of oral bacterial pathogens: an historical perspective. *Crit Rev Oral Biol Med*, 14(5): 331–344.
- Li Y, Burne RA (2001). Regulation of the *gtfBC* and *fff* genes of *Streptococcus mutans* in biofilms in response to pH and carbohydrate. *Microbiology*, 147(10): 2841–2848.
- Loesche WJ (1986). Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev*, 50(4): 353–380.
- Marquis RE, Clock SA, Mota-Meira M (2003). Fluoride and organic weak acids as modulators of microbial physiology. *FEMS Microbiol Rev*, 26(5): 493–510.
- Marsh PD (2003). Are dental diseases examples of ecological catastrophes? *Microbiology*, 149(2): 279–294.
- McCabe RM, Donkersloot JA (1977). Adherence of *Veillonella* species mediated by extracellular glucosyltransferase from *Streptococcus salivarius*. *Infect Immun*, 18(3): 726–734.
- Munro C, Michalek SM, Macrina FL (1991). Cariogenicity of *Streptococcus mutans* V403 glucosyltransferase and fructosyltransferase mutants constructed by allelic exchange. *Infect Immun*, 59(7): 2316–2323.
- Paes Leme AF, Koo H, Bellato CM, Bedi G, Cury JA. (2006). The role of sucrose in cariogenic dental biofilm formation--new insight. *J Dent Res*, 85(10): 878–887.
- Quivey RG, Kuhnert WL, Hahn K (2001). Genetics of acid adaptation in oral streptococci. *Crit Rev Oral Biol Med*, 12(4): 301–314.
- Reese S, Guggenheim B (2007). A novel TEM contrasting technique for extracellular polysaccharides in *in vitro* biofilms. *Microsc Res Tech*, 70(9): 816–822.
- Rolla G, Ciardi JE, Schultz SA (1983). Adsorption of glucosyltransferase to saliva coated hydroxyapatite. Possible mechanism for sucrose dependent bacterial colonization of teeth. *Scand J Dent Res*, 91(2): 112–117.
- Scheie AA, Eggen KH, Rølla G (1987). Glucosyltransferase

- activity in human *in vivo* formed enamel pellicle and in whole saliva. *Scand J Dent Res*, 95(3): 212–215.
- Schilling KM, Bowen WH (1988). The activity of glucosyltransferase adsorbed onto saliva-coated hydroxyapatite. *J Dent Res*, 67(1): 2–8.
- Schilling KM, Bowen WH (1992). Glucans synthesized *in situ* in experimental salivary pellicle function as specific binding sites for *Streptococcus mutans*. *Infect Immun*, 60(1): 284–295.
- Tamasada M, Kawabata S, Fujiwara T, Hamada S (2004). Synergistic effects of streptococcal glucosyltransferases on adhesive biofilm formation. *J Dent Res*, 83(11): 874–879.
- Tanzer JM, Freedman ML, Fitzgerald RJ (1985). Virulence of mutants defective in glucosyltransferase, dextran-mediated aggregation, or dextranase activity//Mergenhagen SE, Rosan B. *Molecular Basis of Oral Microbial Adhesion*. Washington DC: American Society of Microbiology: 204–211.
- Thurnheer T, Giertsen E, Gmür R, Guggenheim B (2008). Cariogenicity of soluble starch in oral *in vitro* biofilm and experimental rat caries studies: a comparison. *J Appl Microbiol*, 105(3): 829–836.
- Vacca-Smith AM, Bowen WH (1998). Binding properties of streptococcal glucosyltransferases for hydroxyapatite, saliva-coated hydroxyapatite, and bacterial surfaces. *Arch Oral Biol*, 43(2): 103–110.
- Vacca-Smith AM, Venkitaraman AR, Quivey RG Jr, Bowen WH (1996). Interactions of streptococcal glucosyltransferases with alpha-amylase and starch on the surface of saliva-coated hydroxyapatite. *Arch Oral Biol*, 41(3): 291–298.
- van Hijum SA, Kralj S, Ozimek LK, Dijkhuizen L, van Geel-Schutten IG (2006). Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiol Mol Biol Rev*, 70(1): 157–176.
- Venkitaraman AR, Vacca-Smith AM, Kopec LK, Bowen WH (1995). Characterization of glucosyltransferase B, GtfC, and GtfD in solution and on the surface of hydroxyapatite. *J Dent Res*, 74(10): 1695–1701.
- Xiao J, Koo H (2009). Structural organization and dynamics of exopolysaccharide matrix and microcolonies formation by *Streptococcus mutans* in biofilms. *J Appl Microbiol*, [Epub ahead of print].
- Yamashita Y, Bowen WH, Burne RA, Kuramitsu HK (1993). Role of the *Streptococcus mutans* *gtf* genes in caries induction in the specific-pathogen-free rat model. *Infect Immun*, 61(9): 3811–3817.

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