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LETTER TO EDITOR

Extracellular Polysaccharides Matrix — An Often Forgotten Virulence Factor in Oral Biofilm Research

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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 α1,3-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 β(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 *Actynomyces* 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

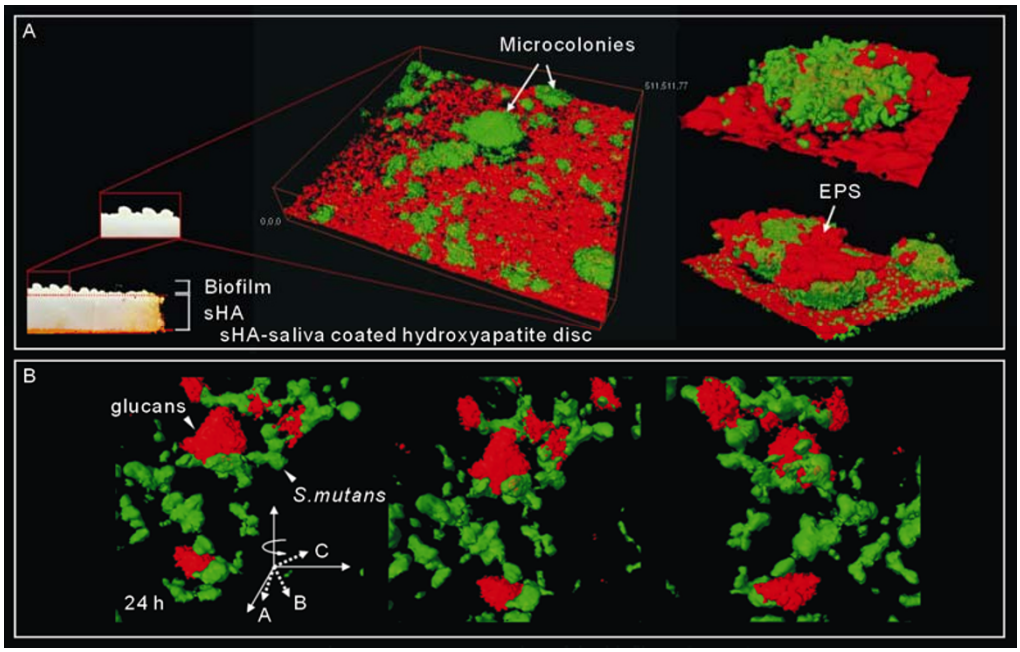

Figure 1 Fluorescence imaging showed the structural relationships between the bacterial cells and EPS

(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 micro-colonies 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 micro-domains 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.

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