CRITICAL REVIEWS IN ORAL BIOLOGY & MEDICINE

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The Exopolysaccharide Matrix: A Virulence Determinant of Cariogenic Biofilm

ABSTRACT

Many infectious diseases in humans are caused or exacerbated by biofilms. Dental caries is a prime example of a biofilm-dependent disease, resulting from interactions of microorganisms, host factors, and diet (sugars), which modulate the dynamic formation of biofilms on tooth surfaces. All biofilms have a microbial-derived extracellular matrix as an essential constituent. The exopolysaccharides formed through interactions between sucrose- (and starch-) and *Streptococcus mutans*-derived exoenzymes present in the pellicle and on microbial surfaces (including non-mutans) provide binding sites for cariogenic and other organisms. The polymers formed *in situ* enmesh the microorganisms while forming a matrix facilitating the assembly of three-dimensional (3D) multicellular structures that encompass a series of microenvironments and are firmly attached to teeth. The metabolic activity of microbes embedded in this exopolysaccharide-rich and diffusion-limiting matrix leads to acidification of the milieu and, eventually, acid-dissolution of enamel. Here, we discuss recent advances concerning spatio-temporal development of the exopolysaccharide matrix and its essential role in the pathogenesis of dental caries. We focus on how the matrix serves as a 3D scaffold for biofilm assembly while creating spatial heterogeneities and low-pH microenvironments/niches. Further understanding on how the matrix modulates microbial activity and virulence expression could lead to new approaches to control cariogenic biofilms.

KEY WORDS: dental caries, *Streptococcus mutans*, glucosyltransferases, extracellular matrix, pH microenvironment, heterogeneity.

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INTRODUCTION

In general, biofilms are structured communities of microbial cells that are attached to a surface and enmeshed in a three-dimensional (3D) extracellular matrix. The extracellular matrix provides an essential scaffold for biofilm development, promoting microbial adhesion to surfaces and cohesion as well as hindering diffusion (Flemming and Wingender, 2010). It is comprised of a variety of hydrated extracellular polymeric substances. Exopolysaccharides, proteins, lipids, nucleic acids, and lipooligosaccharides have been identified in the matrices of a variety of biofilms formed in nature and at various sites in humans (Flemming and Wingender, 2010). The matrix is considered essential for the existence of the biofilm lifestyle and full expression of virulence by several bacterial pathogens (Branda *et al*., 2005).

Polysaccharides produced by exoenzymes from *Streptococcus mutans* are the main constituents in the matrix of cariogenic plaque-biofilms and are recognized as essential virulence factors associated with dental caries (Bowen and Koo, 2011). It is conceivable that the primary role of *S. mutans* in the pathogenesis of the disease resides with its ability to assemble an insoluble polymeric matrix and not simply with numerical superiority or acidogenicity. Many organisms ensnared in dental plaque-biofilms are equally (or more) potent acid-producers and are acid-tolerant, including other streptococci (*e.g., Streptococcus vestibularis/Streptococcus salivarius*), *Lactobacillus* spp, *Bifidobacterium dentium, Candida* spp, and *Scardovia* spp (Aas *et al.*, 2008; Palmer *et al.*, 2010; Takahashi and Nyvad, 2011; Gross *et al*., 2012).

This article focuses on recent discoveries which serve to emphasize the critical role of the matrix in the expression of virulence of plaque-biofilms and future research directions. A detailed literature review about extracellular matrix in cariogenic biofilms can be found elsewhere (Bowen and Koo, 2011).

Exopolysaccharide Matrix: 3D Scaffold for Biofilm Assembly and Spatial Heterogeneity

In the mouth, the tooth surfaces are coated with pellicle, which is a proteinaceous film derived from mammalian and microbial sources, such as salivary proteins and bacterial exoenzymes (Siqueira *et al.*, 2012). Within the complex oral microbiome (Dewhirst *et al*., 2010), only a small group of bacteria (mostly *Streptococci* and *Actinomyces* spp) can adhere in low numbers to uncoated apatitic surfaces and even fewer to pellicle-coated surfaces *via* adhesin/receptors and/or charge interactions (Nobbs *et al*., 2009). *Streptococcus mutans* is not always the most abundant organism in the initial colonizing community on the tooth surface. However, it can orchestrate the development of cariogenic biofilms through exoenzymes, such as glucosyltransferases (Gtfs), which are constituents of the pellicle and also bind to microbial

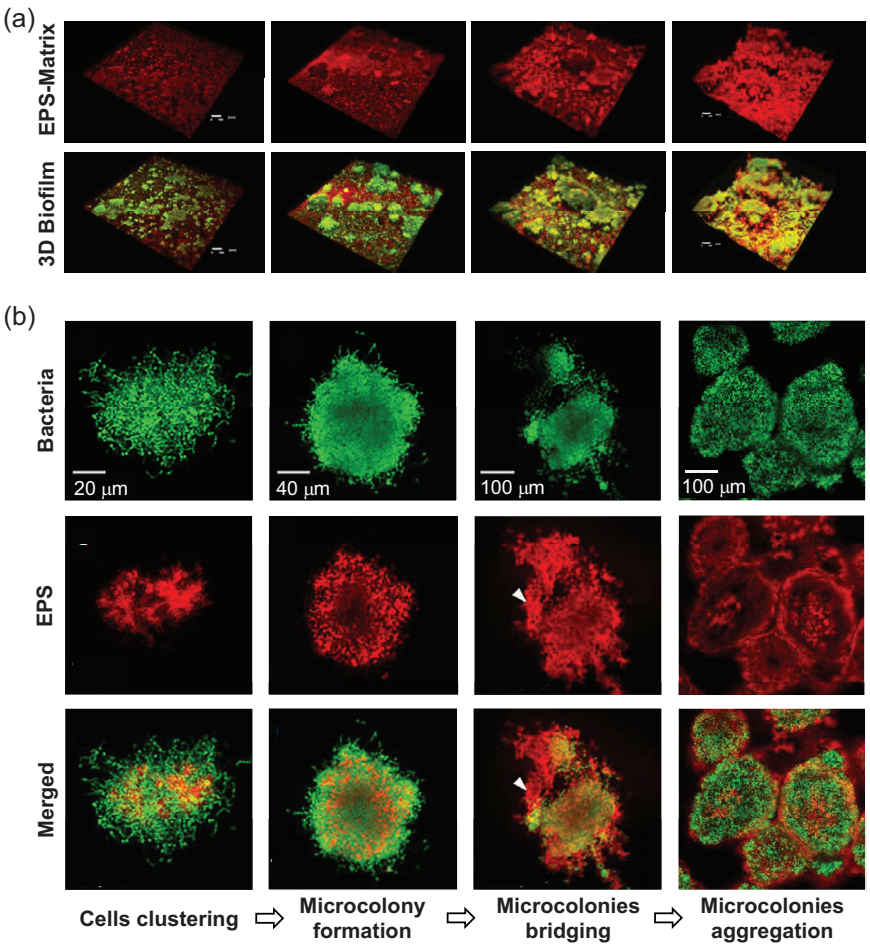

Figure 1. Assembly of an EPS-rich matrix and 3D biofilm architecture. This Fig. highlights the developmental process of EPS-matrix assembly and morphological and structural changes that occur to produce a mature biofilm structure. Panel **(a)** displays representative 3D renderings of the matrix in red and the bacterial microcolonies in green. These *in vitro* mixed-species (*S. mutans, S. oralis*, and *A. naeslundii*) biofilms were grown in culture medium with 1% sucrose on saliva-coated hydroxyapatite (sHA) surfaces for the times depicted (43, 67, 91, and 115 hrs). Panel **(b)** provides close-up cross-sectional images of the structural organization of bacterial cells, EPS, and overlay of both components. The arrow denotes an area where EPS connect 2 microcolonies. These images are adapted from Xiao *et al.* (2012).

surfaces. The surface-adsorbed enzymes synthesize glucans *in situ* from sucrose (and starch). These exopolysaccharides (EPS) provide an abundance of primary binding sites and form the core of the matrix-scaffold in cariogenic biofilms (Bowen and Koo, 2011). Because of the proven role of Gtfs from *S. mutans* in the pathogenesis of dental caries (Yamashita *et al.*, 1993), we focus on EPS produced by these exoenzymes, while recognizing that other organisms (*e.g.*, non-mutans streptococci, *Actinomyces* and *Lactobacillus* species) are also capable of producing soluble glucans and/or fructans (Klein *et al.*, 2013).

EPS Synthesis *in situ* – Priming Surfaces for Enhanced Bacterial Adhesion-Cohesion

Although numerous and distinct microbial species are found in plaque-biofilms, most of them do not contribute to the synthesis of insoluble polysaccharides (Klein *et al.*, 2013) until they are coated by Gtfs. *S. mutans* appears to be the main source of Gtfs, particularly those producing insoluble glucans (Klein *et al.*, 2013). At least 3 genetically separate enzymes (GtfB, GtfC, and GtfD) are produced by the bacterium, each of which synthesizes a structurally distinct EPS (glucan) using sucrose as substrate; starch hydrolysates can also be incorporated during glucan synthesis by Gtfs *via* acceptor reactions (Bowen and Koo, 2011). GtfC is primarily found in the pellicle in an enzymatically active form. The surface-formed polymers provide additional non-mammalian bacterial binding sites in the pellicle for enhanced accumulation of microorganisms, particularly *S. mutans* (Schilling and Bowen, 1988, 1992; Vacca-Smith and Bowen, 1998). Interestingly, Gtfs (particularly GtfB) also bind many oral microbes (*e.g., Actinomyces viscosus, Lactobacillus casei*, and *Candida albicans*), even those that do not synthesize Gtfs, thereby converting them into glucan producers (Vacca Smith and Bowen, 1998; Gregoire *et al.*, 2011). GtfD forms soluble glucans that serve as primers for GtfB and as a reserve source of energy. The EPS formed on microbial surfaces enhance adhesive interactions between *S. mutans* and other organisms while increasing overall cell cohesion (Cross *et al.*, 2007; Gregoire *et al.*, 2011). These phenomena explain the electron micrographs of plaque-biofilms revealing organisms enmeshed in EPS (Reese and Guggenheim, 2007). However, the knowledge of how exopolysaccharides are assembled three-dimensionally and establish spatial heterogeneities, which may be critical for development of acidic microenvironments, is limited.

EPS Synthesis *in situ* – Developing a 3D Matrix Scaffold

We have devised an innovative method of incorporating a fluorescent probe during the synthesis of exopolysaccharides by *S. mutans*-derived Gtfs (Klein *et al.*, 2009; Koo *et al.*, 2010). This pioneering technique allowed us to examine the spatio-temporal development of the EPS matrix in 3D and the structural organization of EPS with bacterial cells within intact mixed-species biofilms (Fig. 1) (Xiao *et al.*, 2012).

Based on EPS location, the polysaccharide-based matrix construction mediates biofilm assembly as follows: (i) The EPS formed on apatitic and bacterial surfaces form an initial polymeric matrix promoting initial microbial colonization and cell clustering; (ii) continuous EPS production *in situ* further expands

the matrix in 3 dimensions while forming a core of EPS-enmeshed bacterial cells; (iii) this core provides a supporting framework facilitating the development of microcolonies (*i.e.*, structured 3D cell clusters) (Xiao *et al*., 2012). The transition from initial cell clustering to microcolony formation bears some similarities to the transition mediated by exopolymers in other biofilm-forming organisms, such as *Pseudomonas aeruginosa, Bacillus subtilis*, and *Vibrio cholerae* (Branda *et al*., 2005; Berk *et al.*, 2012; Mann and Wozniak, 2012). Such spatial arrangements between EPS and bacterial cells may explain why microcolonies are formed in the presence of sucrose (Kreth *et al*., 2008b). As the biofilm matures, the EPS also surround individual microcolonies and appears to bridge them one to another, resulting in the formation of multi-microcolony aggregates. Such compartmentalized architecture confers highly heterogeneous yet cohesive environments within a 3D matrix scaffold (Xiao *et al*., 2012).

Recently, Klein *et al.* (2012) revealed how *S. mutans* assembles the EPS-rich matrix over time in the presence of other organisms using a combination of transcriptional analysis and quantitative proteomics. As the biofilm develops, *S. mutans* within mixed-species biofilms increases the expression of specific genes associated with EPS (glucan) synthesis (*gtfB* and *gtfC*), remodeling (*dexA*), and glucan-binding (*gbpB*). Multidimensional protein identification technology (MudPIT), a high-throughput large-scale quantitative proteomic method, confirmed that the abundance of the encoded proteins was enhanced at the early-biofilm phase, particularly GtfB and GtfC. The presence of other species (*Streptococcus oralis* and *Actinomyces naeslundii*) within a biofilm could influence the gene expression of *S. mutans* within the same biofilm. The expression of *gtfB* and *gtfC* was highly induced in mixed-species biofilms (*vs*. single-species *S. mutans* biofilms), likely through interspecies signaling involving autoinducer molecules (Yoshida *et al*., 2005). When *gtfB* and *gtfC* were deleted simultaneously from *S. mutans*, the mutant strain was unable to assemble an EPS-rich matrix and form EPS-microcolony complexes (Xiao *et al*., 2012).

Interestingly, the digestion of surface-formed glucans by glucanohydrolases, such as dextranase, prevented or reduced bacterial adherence (Schilling and Bowen, 1992). Furthermore, treating the glucans produced by surface-adsorbed GtfB and GtfC (rich in α1,3-linked glucosyl linkages) using α1,3 glucanase (mutanase) dismantled the 3D matrix scaffold causing the disassembly of the EPS microcolonies (Xiao *et al.*, 2012). Clearly, the absence or removal of GtfB- and GtfC-derived glucans eliminates any cell-to-matrix bridging, and thereby causes the collapse of the EPS-microcolony complexes. Analysis of the data agrees well with earlier chemical examination of human dental plaque revealing that α1,3 and α1,3/6 are major linkages of the glucan polymer in the matrix (Hotz *et al*., 1972). Deletion of *gtfB* and *gtfC* was associated with reduced virulence *in vivo* in a rodent model of dental caries (Yamashita *et al*., 1993).

The spatial heterogeneities shaped by EPS synthesis *in situ* form a complex 3D matrix architecture, which may create environmental and biological niches within biofilms that can directly modulate the pathogenesis of dental caries.

Relevance of 3D Biofilm Matrix: Creating pH Microenvironments

In the context of causing disease, acid production alone may not be the key determinant of virulence, but rather of how and where acidic microenvironments are formed, maintained, and protected within the 3D biofilm architecture. Usually there is an abundance of buffering saliva capable of neutralizing acids produced in the mouth. However, EPS and other matrix materials could restrict access by saliva by affecting the diffusion of substances into and out of the biofilm (Flemming and Wingender, 2010). It was initially assumed that a simple concentration gradient exists within most biofilms, because the matrix could act as a physical barrier that causes diffusion limitation. However, the matrix architecture is highly complex, since polysaccharides are not evenly distributed and/or have a different structure within intact plaque-biofilms (Reese and Guggenheim, 2007; Xiao *et al*., 2012). Results from previous studies have shown that average pH values are variable across the biofilm (Werner *et al*., 2004; Rani *et al.*, 2007). Using a two-photon excitation microscopy, Vroom *et al.* (1999) showed microzonal variations of pH within intact mixed-species oral biofilms, indicating heterogeneous microenvironments.

The formation of a compartmentalized architecture protects particular areas of the biofilm from neutralization by buffering the saliva that surrounds the teeth. We have mapped the spatial distribution of pH within undisturbed biofilms by means of a fluorescent pH indicator incorporated into the 3D matrix scaffold (Xiao *et al*., 2012). Low-pH environments were detected in the interior of the EPS-microcolony complexes and at the microcolony/sHA (saliva-coated hydroxyapatite) interface, despite incubation in neutral pH (7.0) buffer (Fig. 2). The appearance of delineated regions of low pH values (4.5-5.5) reveals that the acids accumulated and confined in these specific areas are not rapidly neutralized; more than 2 hrs of neutral pH buffer exposure are required before neutralization ensues. Biofilms formed with the *gtfB* and/or *gtfC* null mutants (lacking a fully developed 3D matrix and defective in forming EPS-microcolony complexes) or treated with glucanohydrolases (that disassemble the matrix) were readily neutralized upon exposure to (pH 7.0) buffer and failed to generate any acidic microenvironments. Clearly, there is a substantial limitation to diffusion that is associated with the presence of an insoluble EPS-rich matrix and EPS-enmeshed microcolonies.

The precise mechanisms involved in limiting diffusion are unclear. It is possible that the presence of glucans produced by oral streptococci limits diffusion of charged ions in and out of the plaque-biofilm, whereas uncharged substances, such as sucrose, may diffuse readily (Melvaer *et al*., 1972; Melsen *et al*., 1979; Tatevossian, 1990; Hope and Wilson, 2004). In contrast, others have noted that soluble polysaccharides have little effect on acid diffusion with artificial plaque *in vitro* (Dibdin and Shellis, 1988; Hata and Mayanagi, 2003). However, none of these studies evaluated intact biofilms. The study conducted by Xiao *et al*. (2012) clearly showed the importance of the manner by which the matrix is assembled three-dimensionally and how it is spatially arranged with the bacterial cells to create highly compartmentalized pH microenvironments.

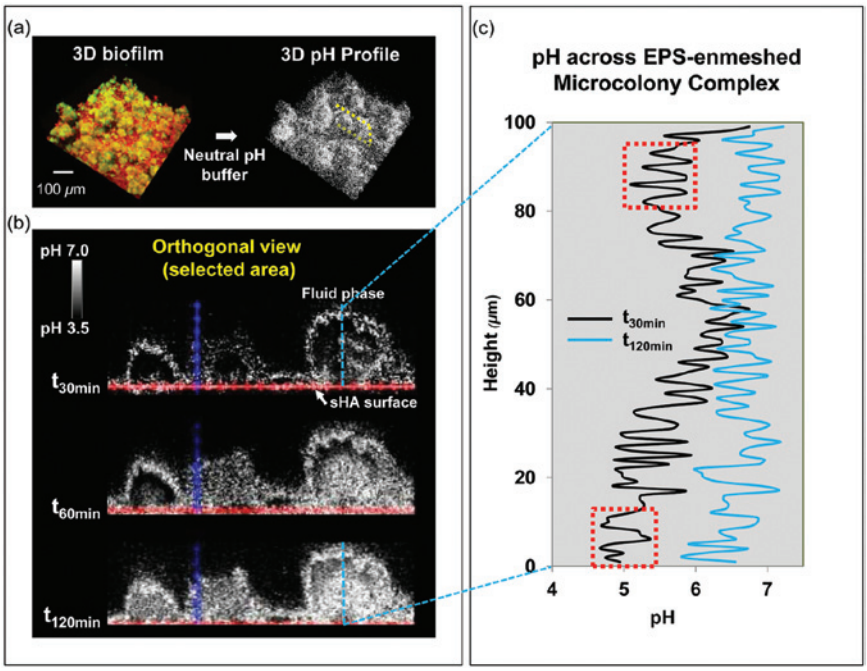

Figure 2. Three-dimensional *in situ* pH mapping of intact mixed-species biofilm. This Fig. provides representative 3D renderings of an *in vitro* mixed-species biofilm that has been subjected to 3D pH mapping. In panel **(a)**, bacteria are highlighted in green and EPS in red, while the yellow box depicts an area of detailed pH mapping. Panel **(b)** is a set of orthogonal views, illustrating temporal changes of pH across the selected area following incubation in neutral sodium-phosphate-based buffer (pH 7.0). The dark areas indicate regions of low pH, while white or light areas indicate regions of pH that are more neutral, defined by the scale bar. In panel **(c)**, a graph shows the distribution of pH values across the selected EPS-microcolony complex after 30 and 120 min of exposure to the buffer. The red boxes highlight the acidic pH regions. sHA: saliva-coated hydroxyapatite surface. This Fig. is adapted from Xiao *et al.* (2012).

The sugar-based metabolic activity of such a high density of acidogenic-aciduric bacteria clustered within the EPS-microcolony complexes can produce copious amounts of acids locally. In addition to external influx of fermentable carbohydrates, the matrix itself and intracellular polysaccharides may also serve as local sugar reservoirs. Soluble fructans and glucans produced by mutans streptococci and other organisms present in the matrix can be degraded by dextranase and fructanases (Reese and Guggenheim, 2007; Bowen and Koo, 2011), increasing the availability of glucose and fructose that could be converted into acids. Recently, Guo *et al.* (2013) found that heterogeneous pH distribution may also be linked with differences in the metabolic activity of *S. mutans* within the biofilm. Thus, the combination of the physical and metabolic factors could, at least in part, explain the creation of numerous and spatially heterogeneous acidic niches across the EPS-microcolony complex and low-pH values at the microcolony/sHA interface (Fig. 3).

Low pH at the Surface of Attachment

Acids present at the biofilm/enamel interface increase local demineralization of the tooth surface. Cross-sectional images of the biofilm at the sHA surface and pH mapping in the same field of view illustrate the distribution of surface-attached microcolonies and pH values (Fig. 3). It is readily apparent that the presence of microcolonies (white line) corresponds with acidic pH on the sHA surface (darker areas highlighted with red lines). The pH values at the microcolony/sHA interface were below 5.5 (which may lead to the dissolution of enamel) and were significantly more acidic compared with areas without such particular 3D structures (highlighted in red in the pH map image) (Fig. 3). The presence of localized and elevated concentrations of acid trapped close to the apatitic surface has clinical relevance. Dental caries is characterized by the appearance of defined regions of demineralization on the tooth enamel or "white spots" under plaque, which eventually coalesce into a broader lesion, rather than a diffuse and homogeneous layer of erosion. The distribution of acidic pools mediated by the matrix may explain the pattern of caries lesion appearance during the onset of the disease.

Creation of Acidic Niches within Biofilms

The development of highly structured and localized acidic-microenvironments has additional biological relevance. Niches displaying low-pH values would activate the acid-stress-adaptive responses of particular organisms clustered in these EPS-sheltered acidic niches. As the pH value declines, dynamic shifts in the local microbial population could be triggered according to the ecological plaque model (Marsh, 1994). At the same time, EPS production by *S. mutans* GtfB and GtfC could be induced under acidic pH (Li and Burne, 2001). Assembly of the EPS-rich matrix and the concomitant development of acidic niches appear to modulate the regulation of stress survival pathways by *S. mutans* (Klein *et al.*, 2012). *S. mutans* up-regulates specific adaptation mechanisms to cope with acidic environments (F1F0-ATPase system, fatty acid biosynthesis, branched-chain amino acid metabolism, and molecular chaperones; Lemos and Burne, 2008). GtfB, GtfC, and glucan-binding proteins are all induced, leading to continuous biofilm accretion and the development of a favorable milieu for the survival-growth of cariogenic organisms. As the environmental acidic stress further increases, the microbial diversity is dramatically reduced in favor of a highly aciduric and acidogenic complex flora, as recently demonstrated in microbiome studies of plaque samples collected from caries-active populations (Palmer *et al.*, 2010; Gross *et al.*, 2012).

Altogether, the creation of site-specific microenvironments or niches, sheltered by a diffusion-limiting extracellular matrix, has profound effects on the architecture, metabolism, and

expression of virulence of biofilm as a whole. Although the immediate cause of enamel dissolution is certainly acid production, the absence of the cloistering effect of the biofilm matrix would minimize the ability of acids to demineralize in the presence of saliva.

MANY QUESTIONS REMAIN TO BE ADDRESSED

The synthesis of EPS on surfaces and the assembly of an insoluble matrix are critical for the existence of cariogenic biofilms. However, many areas remain either unexplored or poorly understood.

Gtf Adsorption and Activity on Surfaces

The adsorption of Gtfs to pellicle and to bacterial surfaces results in substantial enhancement of activity compared with the same enzymes in solution (Bowen and Koo, 2011). The exact identity of components in the pellicle and bacterial membrane, as well as the amino acid motifs in the Gtf structure that mediate the enzyme-surface interactions, remains largely unknown. When adsorbed to surfaces, Gtfs display unique enzymatic kinetics and increased resistance to inhibitors, and produce structurally distinct glucans *vs*. solution-phase enzymes (Bowen and Koo, 2011). The reasons for these phenomena are unclear, although they may be related to conformational changes that these enzymes undergo during adsorption (Fears and Latour, 2009). It is also unclear whether there are compounds in saliva or bacterially derived molecules that may enhance or hinder enzyme activity. Recent elucidation of the Gtf structure (Ito *et al.*, 2011) may help to determine the conformational changes of Gtfs when adsorbed onto saliva-coated apatitic and bacterial surfaces. Understanding how the reactions of Gtfs affect the production and structure of EPS *in situ*, provision of microbial binding sites, and subsequent matrix formation will advance our knowledge about the mechanisms of cariogenic biofilm assembly.

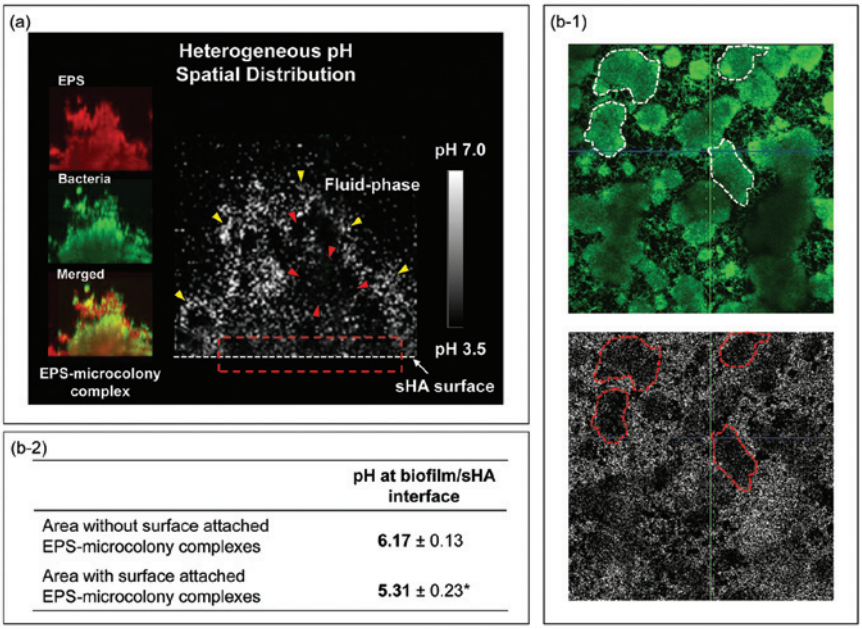

Figure 3. Acidic microenvironments across an EPS-microcolony complex and at the surface of biofilm attachment. This Fig. illustrates the heterogeneous pH distribution within the selected EPS-microcolony complex and at the biofilm/sHA interface. Panel **(a)** gives a detailed image of an EPS-microcolony complex attached to the sHA surface. EPS are depicted in red, bacteria are in green, and dark areas indicate acidic pH, with white areas indicating more neutral pH. The red arrows highlight acidic pH regions within the microcolony structure. Yellow arrows point out pH values close to neutral, which tend to occur at the microcolony/fluid phase interface. The red box denotes acidic pH at the interface between the microcolony complex and the surface of attachment (sHA). Panel **(b-1)** gives representative cross-sectional images at the sHA surface. White marks indicate the areas with surface-attached microcolonies, while red marks show the corresponding area in the pH channel. The table in Panel **(b-2)** lists the pH values at the biofilm/sHA interface in areas with and without surface-attached microcolonies following a 30-minute exposure to neutral buffer. The asterisk (*) indicates the values significantly different from each other ($p < .05$). This Fig. is adapted from Xiao *et al.* (2012).

EPS Matrix and Mechanical Stability of Biofilms

Well-established biofilms are difficult to remove from surfaces. Results from a previous study showed that *S. mutans* biofilms display viscoelastic properties that are similar to those of synthetic polymers (Vinogradov *et al*., 2004). The EPS formed on surfaces (Cross *et al*., 2007) and further development of a polymeric matrix may be responsible for the mechanical properties of biofilms, such as adhesive strength on apatitic surfaces and cohesiveness. However, it is yet to be determined whether (and how) EPS modulate adhesive and cohesive forces of the matrix, which are essential properties for the mechanical stability of biofilms. Enhanced understanding about the biomechanical properties of the EPS matrix may lead to innovative chemical or biological approaches to remove or disassemble cariogenic biofilms.

The Role of Other Matrix Constituents in Biofilm Virulence

The charge, composition, and structure of the biofilm matrix can change with time and are influenced by local microbial populations and environmental conditions (Branda *et al.*, 2005; Flemming and Wingender, 2010). Microbial products, host saliva proteins, and even lipids have been detected in human dental plaque (Paes Leme *et al.*, 2006; Bowen and Koo, 2011). How extracellular constituents other than Gtf-derived EPS influence the structure and biology of the matrix remains poorly understood. For example, soluble EPS produced by other organisms could also contribute to biofilm development and architecture by serving as alternative acceptors and reserve sources of

energy. Perhaps an avenue can be developed whereby the structure of glucan in the matrix could be rendered non-virulent.

Extracellular DNA (eDNA) from bacteria enhances *S. mutans* adhesion and surface aggregation *in vitro* (Das *et al*., 2010). eDNA also appears to be incorporated into the EPS matrix, contributing to the scaffold and development of *S. mutans* biofilms (Klein *et al.*, 2010). Lipoteichoic acid (LTA) synthesis by *S. mutans* is enhanced in mixed-species biofilms formed *in vitro*, particularly during active EPS-matrix assembly (Klein *et al*., 2012). This observation may explain why the concentration of LTA is elevated in sucrose-induced plaque *in vivo* (Rölla *et al*., 1980). Because it is negatively charged, the presence of LTA in high amounts could influence the electrostatic interactions and diffusion properties of the matrix. Clearly, additional matrix constituents could influence the biological and structural properties of cariogenic biofilms.

Matrix and Niche Biology

The distribution of a variety of oral bacteria in biofilms is clearly variable (Zijnge *et al*., 2010; Valm *et al*., 2011). The matrix provides protection to embedded bacteria while creating tailored niches for microorganisms to interact with each other and adapt to or even modify the local environment. Thus, the adaptation of a particular species could be facilitated in response to a changing ecological niche. For example, the positioning of the microorganisms and their stress response mechanisms may correlate with their susceptibility to, or affinity for, low-pH environments. The presence of alkali-generating bacteria within oral biofilms may influence the local pH and change the dynamics of the microenvironment (Liu *et al*., 2012). Persistent low-pH values within niches and increased sugar availability appear to induce the expression of *S. mutans gtfBC* (Li and Burne, 2001). These and other genes would therefore likely be modulated locally, coinciding with areas that are undergoing active matrix remodeling and acidogenesis.

The matrix could also affect the availability of nutrients, metabolites, and signaling compounds, influencing the metabolic state of cells as well as microbial interactions (Mann and Wozniak, 2012; Phelan *et al.*, 2012). By offering a variety of chemical milieus, the matrix may locally modulate the complex interspecies competition, synergism, and/or mutualism that takes place between *S. mutans* and other co-habitants (such as the early colonizers *Streptococcus gordonii* and *Veillonella parvula*; Kreth *et al*., 2008a; Liu *et al.*, 2011; Merritt and Qi, 2012). How the matrix promotes cell heterogeneity and influences signaling and localized acid/alkali and antimicrobial substance production within biofilms remains to be fully elucidated. The possibility of creating structured microenvironments within microfluidic devices (Connell *et al*., 2011; Son *et al*., 2012) may help, in part, to elucidate how the matrix modulates niche biology.

Matrix Remodeling and Biofilm Dispersal

Available evidence demonstrates that there is significant turnover in the matrix of biofilm. During active biofilm growth, the matrix can be continuously produced at specific locations, but also degraded. This dynamic remodeling not only produces byproducts that can be utilized as acceptors for EPS synthesis and metabolized by bacterial cells, but may also change the topography and biomechanical properties of the matrix (Flemming and Wingender, 2010; Bowen and Koo, 2011; McDougald *et al.*, 2011). In cariogenic biofilms, glucanohydrolases and fructanases encoded by specific oral species may be participating in the localized degradation and remodeling of EPS matrix (Bowen and Koo, 2011); the oral microbiome database shows at least 17 species encoding "dextranase" genes. The expression of *S. mutans dexA* is induced within a mixed-species community during later stages of biofilm development (Klein *et al*., 2012). Thus, a dynamic interaction of the enzymes responsible for glucan synthesis with those cleaving the glucosidic linkages could be occurring concomitantly in the biofilm matrix. The presence of glucanohydrolases during glucan synthesis by *S. mutans* Gtfs dramatically alters the structure and bacterial binding sites (Hayacibara *et al.*, 2004). How matrix degradation-remodeling is activated and whether it leads to the dispersal of its microbial constituents remain to be elucidated.

We are currently making strides to study intact matrix architecture in plaque-biofilms formed on teeth using a rodent model of caries that more accurately simulates the conditions encountered in the mouth, including exposure to diet and salivary components in the presence of complex resident flora and hydrodynamic-abrasive forces. Further research with human dental plaque is warranted for a full understanding of EPS-matrix assembly *in vivo*.

CONCLUDING REMARKS

Dental caries represents one of the most prevalent and costly biofilm-dependent diseases worldwide. The EPS matrix is an essential virulence determinant in cariogenic biofilms by providing avid microbial-binding sites on the attachment surfaces, a cohesive diffusion-limiting 3D scaffold, and a source for fermentable sugars and by facilitating the creation of acidic environments (Fig. 4). Nevertheless, most studies have focused on microbial components, either enumerating them or identifying new organisms. Clearly, *S. mutans* does not need to dominate numerically within plaque-biofilms, because the Gtfs released, even at the time of biofilm initiation, are incorporated into tooth pellicle and adsorbed to other organisms, producing EPS *in situ*. The EPS formed on surfaces promote local colonization and accumulation of microbes on the teeth, leading to the formation of microcolonies and the assembly of an intricate polymeric matrix. Upon biofilm establishment, the resident microorganisms enmeshed and protected in the matrix are recalcitrant to antimicrobials and are highly acidogenic/acid-tolerant, making them difficult to remove, thereby becoming reservoirs for pathogens and toxins.

Novel anticaries agents can be designed to disrupt glucan-based priming of apatitic and bacterial surfaces by targeting specific binding sites on the pellicle or even preventing further glucan synthesis by blocking the catalytic activity of surface-adsorbed enzymes. Early attempts to use dextranase to disrupt plaque matrix and prevent dental caries have generated mixed results *in vivo* (Balakrishnan *et al.*, 2000). The combination of dextranase and mutanase appears to be more effective *in vitro* than either enzyme alone (Hayacibara *et al.*, 2004). Lack of

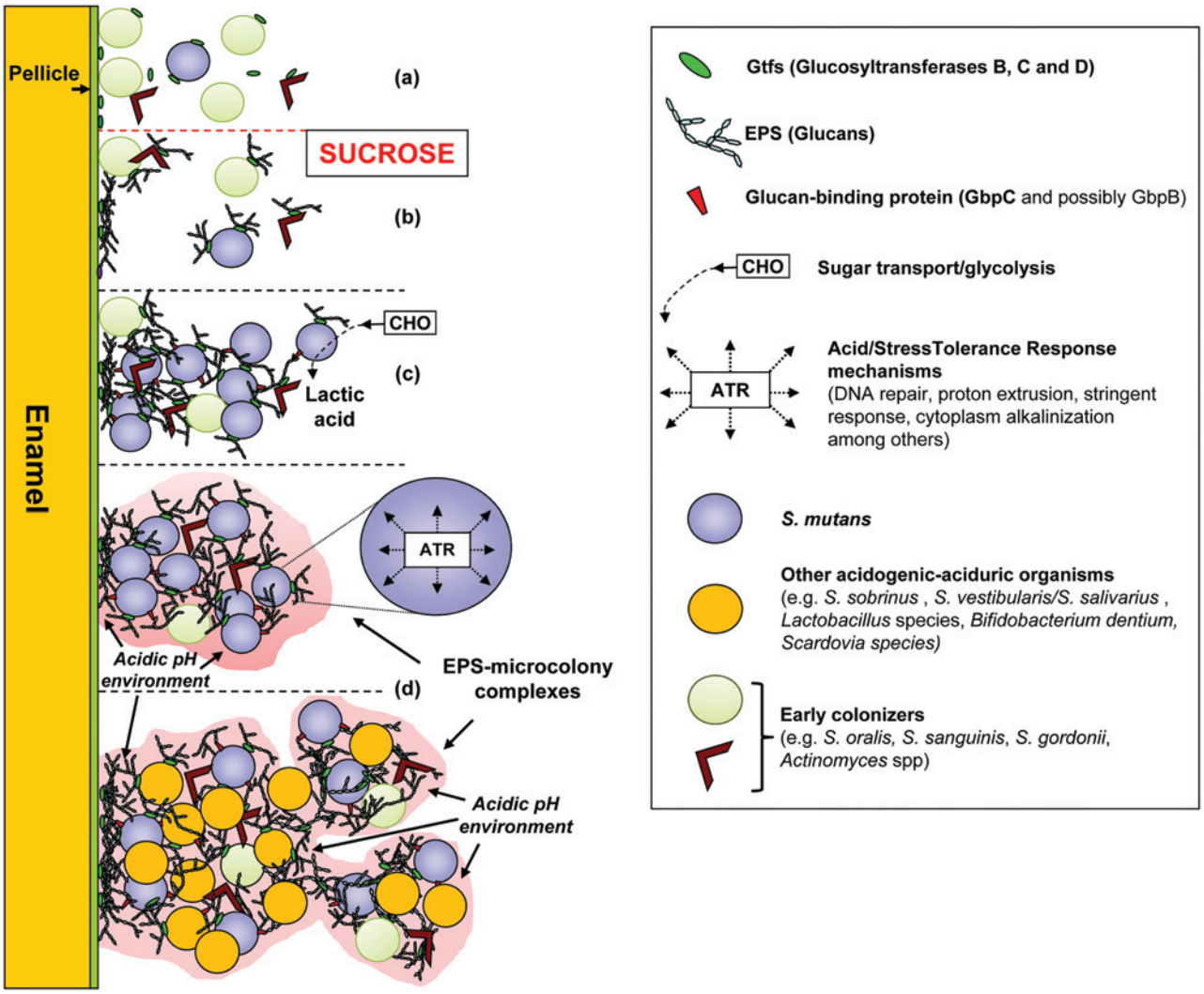

Figure 4. Exopolysaccharide synthesis *in situ* mediates assembly of matrix scaffold and cariogenic biofilm formation. This Fig. depicts the sequential assembly of the matrix scaffold of cariogenic biofilms. In panel **(a)**, Gtf enzymes secreted by *S. mutans* become incorporated into the pellicle (particularly GtfC) and/or are adsorbed to bacterial surfaces (mainly GtfB). GtfB also absorbs to microorganisms that do not produce Gtfs (*e.g., Actinomyces* spp). In panel **(b)**, GtfB and GtfC that are absorbed to surfaces within the oral cavity can rapidly utilize dietary sucrose (and starch hydrolysates). As a result, insoluble and soluble glucans are synthesized *in situ*. GtfD also produces soluble glucans that can serve as primers for GtfB to augment total insoluble EPS synthesis. In panel **(c)**, the glucan molecules formed on surfaces provide avid binding sites for various resident microorganisms and especially *S. mutans*, which mediates bacterial clustering and adherence to the tooth enamel. This process occurs primarily through glucan-glucan and glucan-Gbp interactions (Bowen and Koo, 2011). Furthermore, bacteria coated by the Gtfs themselves become *de facto* glucan producers, so they could bind to tooth and microbial surfaces by mechanisms similar to those used by *S. mutans*. The surface-adsorbed Gtfs produce an insoluble matrix for dental plaque-biofilm *in situ*. Concomitantly, dietary carbohydrates are metabolized into acids by acidogenic/aciduric organisms, including *S. mutans*. In panel **(d)**, once the EPS-rich matrix and the biofilm have been established, ecological pressures (*e.g.*, pH, nutrient availability) determine which and how bacteria survive, facilitating the dominance of certain species within plaque, namely, cariogenic species (*e.g., S. mutans*) under frequent sucrose or other fermentable carbohydrate exposure. The presence of soluble polysaccharides in the matrix also provides additional sources of fermentable sugar. When the biofilm remains on tooth surfaces and the consumption of a carbohydrate-rich diet (especially sucrose) persists, the amount of EPS and extent of acidification of the matrix increase (*e.g.*, as EPS-microlony complexes form). Such conditions elicit biochemical, ecological, and structural changes that favor the survival and dominance of highly acid-stress-tolerant organisms (Aas *et al.*, 2008; Palmer *et al.*, 2010; Gross *et al.*, 2012) in these cohesive and firmly attached biofilms. The low-pH environment at the tooth-biofilm interface promotes demineralization of enamel. This model may explain the rapid accumulation of cariogenic plaque in the presence of sucrose (and starch hydrolysates), even if the initial *S. mutans* population is numerically low.

retention in the mouth of topically applied glucanohydrolases, possible degradation by proteolysis in saliva, and slow diffusion of the enzymes into plaque may explain the mixed results. Nevertheless, the concept of specifically digesting the EPS matrix is certainly attractive. Thus, new approaches should also be developed to deliver and retain therapeutic agents on-site in active form for sufficient duration to exert full therapeutic potential *in vivo*.

Precise inhibition of EPS-matrix and microcolony assembly would impede biofilm development and the creation of acidic microenvironments and thereby the formation of selective ecological niches and demineralization sites on the tooth surface. Such strategies may be highly effective and specific, since even partial inhibition of surface-Gtf activity can greatly reduce the development of dental caries *in vivo* without disturbing the resident oral flora (Jeon *et al*., 2011). Clearly, the outcome of these studies could have relevance in other biofilm-dependent infectious diseases, since matrix is inherent to most, if not all, biofilms.

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