

# The dental plaque biofilm matrix

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## 1 | INTRODUCTION

Advances in deoxyribonucleic acid (DNA) sequencing have given us unprecedented insights into the identity of microbial cells within complex consortia, such as dental plaque biofilms. Our understanding of the other key component of microbial biofilms, the extracellular matrix, has not benefited to such an extent from this technological revolution. Nevertheless, a great deal of progress has been made in recent years toward defining the structure and function of biofilm matrices. The roles of macromolecules, such as nucleic acids, proteins, and lipids, in addition to polysaccharides, are beginning to be unraveled. A more thorough characterization of these components may lead to new approaches to control biofilms based on inhibition of matrix function.

Studies of biofilm matrices had been ongoing for many years before the term “biofilm” was first coined to describe interface-associated microbial communities in 1975.<sup>1</sup> Early work on freshwater biofilms described the matrix as a “slime layer” and revealed polysaccharide-like material by electron microscopy.<sup>2</sup> Intercellular material was also observed in dental plaque and was identified as polysaccharide based on reactions with osmium-black visualized under the electron microscope.<sup>3</sup> The extracellular polysaccharide layer surrounding both prokaryotic and eukaryotic cells, and present in tissues as a basement membrane,

was termed “glycocalyx” by Bennett in 1963.<sup>4</sup> Glycocalyx means “sweet husk” and was considered appropriate since the layer always contained carbohydrate.<sup>5</sup> In bacteria, two types of glycocalyx were identified: rigid paracrystalline S-layers surrounding bacterial cells, and more flexible capsules that may remain cell associated or be shed into the wider environment and form a casing for microcolonies.<sup>6,7</sup> Thus, from an early stage, the same terminology was used to describe bacterial and eukaryotic extracellular matrices. The use of the term “glycocalyx” to describe the biofilm matrix has largely been dropped, since it overemphasizes the importance of carbohydrates. Nevertheless, it is clear that there are similarities in both structure and function between eukaryotic and bacterial extracellular matrices (Figure 1).

Arguably the most thoroughly characterized biofilm matrix polymers in dental plaque are the glucan and fructan polysaccharides that are produced by the action of extracellular glucosyltransferase and fructosyltransferase enzymes on sucrose.<sup>8</sup> These polysaccharides are considered to be important virulence factors in the pathogenesis of dental caries.<sup>9,10</sup> There is also an extensive extracellular matrix in subgingival dental plaque of patients with periodontitis (Figure 2).<sup>11,12</sup> The chemical composition of this matrix is not well understood at present. As with many microbial biofilms,<sup>13</sup> the structural components of the matrix likely include a complex mixture of

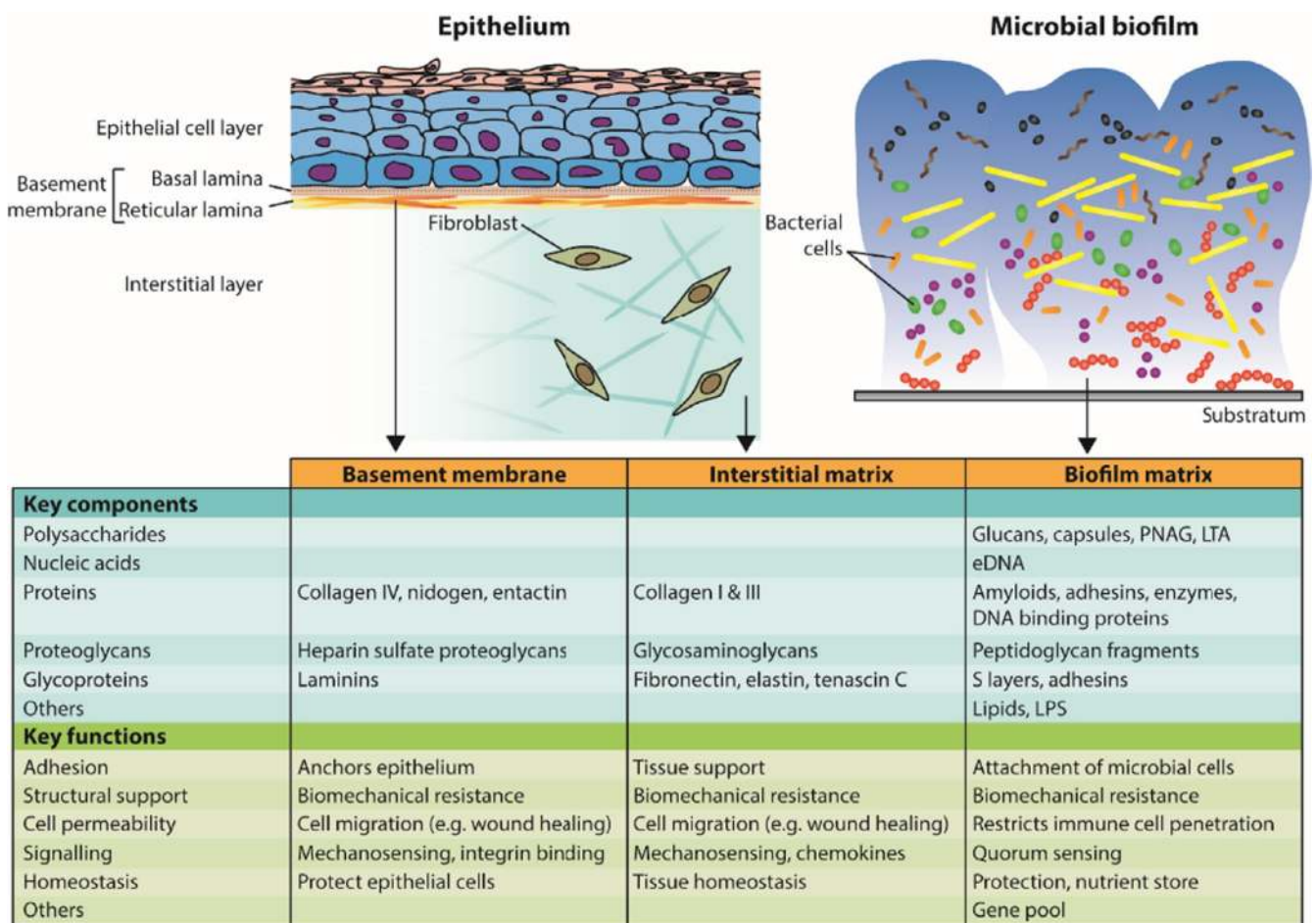
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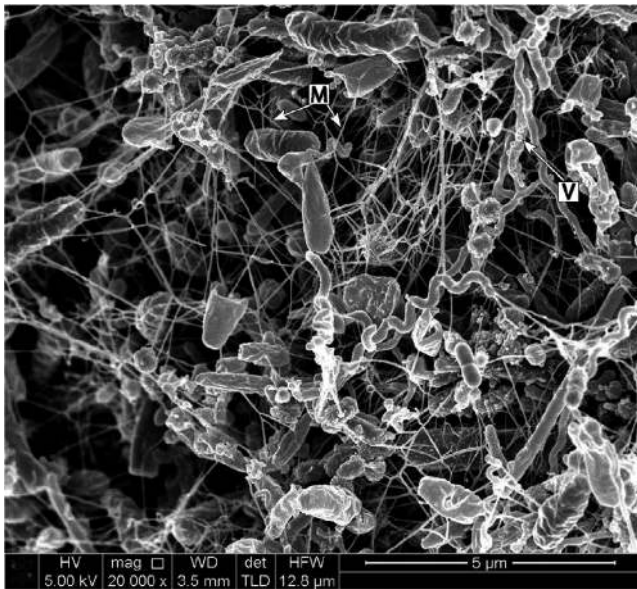
carbohydrates, proteins, nucleic acids, lipids, and other macromolecules derived from both the resident microorganisms in the biofilm and from the host. These polymers accumulate once the surface has become colonized with microbial cells. In the first 1-2 hours of colonization, bacterial cells are sparsely scattered over the enamel surface and there is little evidence of biofilm matrix.<sup>14</sup> Small amounts of matrix material can be observed by electron microscopy from 2 hours onward.<sup>15</sup> It is likely that the matrix undergoes changes during the transition from biofilms at the gum margins in health to subgingival dental plaque associated with periodontal disease. Identifying these changes and the key macromolecules that contribute to the function of pathogenic dental plaque may provide new targets for oral biofilm control. This article discusses the current state of knowledge regarding the dental plaque matrix. Where possible, we will focus on the microbial-derived matrix of subgingival dental plaque and its potential role in the pathogenesis of periodontal disease.

## 2 | KEY COMPONENTS OF THE MATRIX

The presence of a matrix is one of the defining features of microbial biofilms and is responsible for many of their emergent characteristics (the properties of the system that only appear when the cells are together in a biofilm).<sup>16</sup> The major classes of extracellular polymeric substances that form the matrix are common to most biofilms and comprise carbohydrates, proteins, nucleic acids, and cell wall polymers, such as peptidoglycans and lipids.<sup>13</sup> However, within these classes, there is extensive variation in the specific types and proportions of macromolecules between different types of biofilm. Analysis of the bulk chemical composition of the biofilm matrix requires large amounts of material, which is difficult or impossible to obtain for subgingival dental plaque. Therefore, much of our understanding of the biofilm matrix comes from *in vitro* studies on individual species or on targeted detection of specific macromolecules.<sup>17</sup> Together,



**FIGURE 1** Comparison of the structure and function of host vs biofilm extracellular matrix. Host tissue contains a variety of different extracellular matrices. Shown here are the basement membrane and interstitial matrix underlying a layer of keratinized epithelium. Each of these contains proteins, proteoglycans, and glycoproteins that serve a variety of functions in adhesion, sensing, and protection. The basement membrane consists of the basal lamina, which forms the underlying layer for the epithelial cells, and the reticular lamina, composed primarily of collagenous fibers that serve to anchor the basal lamina. The interstitial matrix contains a variety of different cells, including fibroblasts. The biofilm matrix contains macromolecules primarily derived from the resident bacterial cells. These also function in adhesion, sensing, and protection, in addition to acting as a store of nutrient and a potential source of DNA for natural transformation. eDNA, extracellular DNA; LPS, lipopolysaccharide; LTA, lipoteichoic acid; PNAG, poly-N-acetylglutamic acid



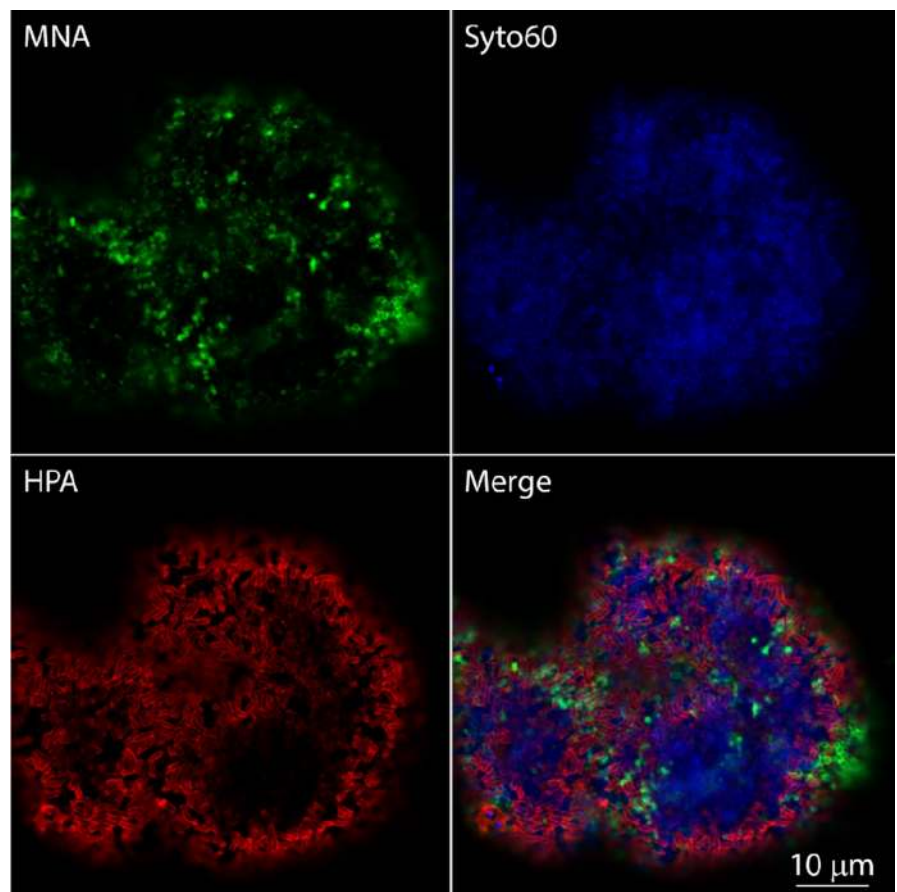
**FIGURE 2** Scanning electron micrograph of subgingival dental plaque on the surface of a tooth extracted due to periodontal disease. Microbial cells are connected by a meshwork of fibrous material (M), apparently the collapsed remains of a hydrated polymeric matrix. Some microbial cells are associated with small particles or vesicles (V) that may provide a source for matrix polymers

these methods are starting to reveal the complex macromolecular composition of the dental plaque extracellular matrix.

## 2.1 | Carbohydrates

Carbohydrates constitute approximately 20% of the dry weight of supragingival dental plaque, and around two-thirds of these are water insoluble.<sup>18,19</sup> A significant proportion of this biomass consists of intracellular storage polysaccharides and other intracellular carbohydrates.<sup>20</sup> In addition, approximately 2%-10% of the dry weight of dental plaque consists of glucans, which are homopolymers of glucose that are produced extracellularly from sucrose by glucosyltransferase enzymes.<sup>19</sup> Sucrose can also be converted to fructose polymers (fructans) by fructosyltransferases.<sup>21</sup> Fructans tend to be present at lower amounts than glucans in dental plaque.<sup>10</sup> Glucans and fructans can be observed macroscopically in sucrose-fed laboratory monocultures of oral streptococci as crystalline structures on surfaces of agar plates or suspended in planktonic cultures. When grown in the presence of sucrose, dental plaque has a significantly increased wet weight and increased concentration of alkali-soluble polysaccharide due to the production of glucans and fructans.<sup>22,23</sup> The ubiquity of these polysaccharides, particularly in cariogenic oral biofilms, has led to them becoming the focus of intensive research

**FIGURE 3** Fluorescent lectin binding analysis of supragingival dental biofilm, grown in situ in the mouth of a volunteer for 48 hours in the absence of dietary sucrose. The biofilm was stained with Morniga-G lectin (MNA-G-fluorescein isothiocyanate, green, recognizes galactose), *Helix pomatia* lectin (HPA-tetramethylrhodamine, red, recognizes *N*-acetyl- $\alpha$ -galactosamine), and Syto60 (blue, stains DNA). Maximum projection images are shown; scale bar: 10  $\mu$ m. The target carbohydrates appear to be primarily associated with microbial cell walls. Image kindly provided by Thomas R. Neu, Pune N. Paqué, and Sebastian Schlafer



for several decades, and they have been the subject of several comprehensive reviews.<sup>8,21,24</sup> Water-insoluble glucans and fructans promote the attachment of bacteria to dental plaque, thus driving an increase in complexity of the population as plaque ages.<sup>25-27</sup> However, subgingival dental plaque will become cut off from dietary nutrient sources, including sucrose, and therefore extracellularly synthesized glucans and fructans are not considered to play a major role in periodontal disease. Nevertheless, glucose is available in gingival crevicular fluid,<sup>28</sup> and carbohydrates as a whole can be observed in dental plaque even in the absence of dietary sucrose. Using fluorescent lectin-binding analysis, a wide range of carbohydrate structures were detected in 48-hour supragingival dental biofilm grown in situ in the mouths of volunteers who abstained from ingesting sucrose.<sup>29</sup> Some of these glycoconjugates appeared to be closely associated with the surfaces of microbial cells (Figure 3), whereas others were distributed more diffusely in the biofilm matrix.<sup>29</sup>

### 2.1.1 | Protein-linked bacterial glycans: S-layers, outer membrane proteins and flagella

Though in its infancy compared with other areas of glycobiology, it is becoming increasingly obvious that attachment of glycans to surface proteins in bacteria is a common event rather than being a rare oddity.<sup>30</sup> For example, since its initial discovery in Archaea, there are now examples of biologically important glycosylation events of bacterial surface proteins with import for biofilm formation that, given their surface location, should be considered part of the biofilm matrix. This is even more pertinent when considering our increasing awareness of bacterially derived vesicles that carry proteins and other surface molecules derived from the outermost membrane of either gram-positive or gram-negative bacteria.

As outlined later (Section 2.3), bacterial species contain a wide range of protein-based adhesins; these include well-characterized pili and fimbriae proteins, as well as bacterial flagella and amyloid-type proteins, such as the leucine-rich repeat family proteins of *Streptococcus* spp and *Actinomyces* spp, which are key species in oral biofilms.<sup>31-33</sup> Indeed, it is now becoming clear that correct glycosylation is essential to the correct folding and function of these proteins.<sup>34,35</sup> For example, loss of glycosylation of the Fap1 serine-rich repeat adhesin with GalNAc, GlcNAc, and rhamnose of the oral commensal *Streptococcus parasanguinis* influences biofilm-forming ability.<sup>36-38</sup> Similarly, the leucine-rich-repeat adhesins of other oral primary colonizing organisms, such as *Actinomyces oris* GspA, lose biofilm adhesion capability if the glycosylation machinery that adds their glycan moiety is deleted.<sup>39</sup> In addition to these extended-repeat-containing adhesins, there are also examples of shared glycosylation pathways between surface lipopolysaccharide glycosylation pathways and flagella proteins that are key in biofilms.<sup>40</sup> These include well-studied flagellin glycosylation pathways, such as those in *Campylobacter* spp<sup>41,42</sup> and *Aeromonas* spp.<sup>34,43</sup> In fact, glycosylation of surface-biofilm-contributing proteins and structures in other gram-negative bacteria is also becoming increasingly

understood, with numerous examples particularly evident for the keystone periodontal pathogen *Porphyromonas gingivalis*, such as the minor fimbriae (Mfal),<sup>44</sup> the OmpA proteins,<sup>45</sup> and the major gingipain virulence factors.<sup>46</sup> However, this is by no means the only example in periodontitis virulence, with the EmaA collagen adhesin of *Aggregatibacter actinomycetemcomitans*<sup>47</sup> key in biofilm adhesion, but also, one imagines, in adhesion involving the human collagen-rich extracellular matrix present on the hard and soft surfaces in the mouth.

Finally, bacterial proteinaceous surface-layers (S-layers) that exist across Archaea, gram-positive bacteria, and gram-negative bacteria are well known to be glycosylated.<sup>48-50</sup> These are prominent in the biology of several oral bacteria, most notably for several *Campylobacter* spp<sup>51</sup> and the periodontal pathogen *Tannerella forsythia*.<sup>52</sup> Indeed, the S-layer of *T. forsythia* has been shown not only to be upregulated in biofilm cells,<sup>53</sup> but also to be critical for adhesion to abiotic (plastic) surfaces<sup>54</sup> and to cellular<sup>55</sup> and mucin-coated surfaces.<sup>56</sup> The interaction with mucins is strongly linked to its glycan modifications, with the host-mimicking nonulosonic sugars pseudaminic and legionaminic acid that terminate these glycans being key. Overall, it seems that the protein-based glycome of bacterial cells might be a critical part of the picture of surface interactions, with these glycans often defining hydrophilicity/hydrophobicity<sup>57</sup> and charge characteristics of bacterial cells that may also be key for nucleation of other components, such as extracellular DNA (see later).<sup>58</sup>

### 2.1.2 | Capsular and related polysaccharides

Many bacteria produce polysaccharide capsules that provide a variety of functions, including desiccation tolerance and protection from the immune system. Sometimes, the capsular polysaccharides remain tightly associated with bacterial cells. Alternatively, the polysaccharides may be released and become part of a more diffuse matrix. In either case, these polysaccharides are important extracellular components that contribute to the structure and function of biofilms. Genetic loci encoding capsular polysaccharide biosynthesis pathways similar to those of *Streptococcus pneumoniae* are present in the genomes of many oral streptococci, and immunodiffusion studies have shown that these are widely expressed.<sup>59</sup> In general, the capsular polysaccharides form very thin layers on the surface of cells and are not easily visualized by microscopy. One exception is the *Streptococcus mitis* type strain (NCTC 12261), which produces a capsule that can be observed by electron or atomic force microscopy.<sup>60</sup> Expression of the capsule reduces biofilm formation, cell-cell aggregation (autoaggregation), and epithelial cell binding, protects cells against phagocytosis and clearance in a mouse model of lung infection, and modulates sensitivity to host antimicrobial peptides.<sup>60-62</sup> The thin layers of capsular polysaccharides present in *Streptococcus oralis*, *Streptococcus gordonii*, and *Streptococcus sanguinis* strains appear to be primarily involved in cell-cell adhesive interactions and have been termed "coaggregation receptor polysaccharides."<sup>63</sup> Many

different streptococcal receptor polysaccharides have been characterized biochemically and shown to fall into different groups with distinct immunological and coaggregation specificity profiles.<sup>63–65</sup> Species such as *A. oris* also produce polysaccharides involved in coaggregation, although the structure and synthesis of these are not well understood.<sup>66</sup> In *Streptococcus mutans*, related polysaccharides known as rhamnose-glucose polymers are responsible for serotype specificity and biofilm formation.<sup>67,68</sup> However, *S. mutans* undergoes very few coaggregation interactions, indicating that rhamnose-glucose polymers are not strong receptors for microbial cell-surface adhesins.

Capsules are important virulence factors for periodontal pathogens, such as *P. gingivalis*. At least six capsular (K) serotypes have been identified that vary in the extent to which they induce responses in dendritic cells and T-lymphocytes.<sup>69,70</sup> In *P. gingivalis* W50, expression of the capsule reduced the activation of the host immune system, reduced phagocytosis, and increased virulence in a mouse abscess model compared with an unencapsulated mutant.<sup>71</sup> Knocking out capsule production in *P. gingivalis* W83 led to enhanced autoaggregation and biofilm formation compared with the isogenic wild type.<sup>72</sup> However, the presence of capsule is required for coaggregation with *Fusobacterium nucleatum* and for synergistic virulence in a mixed infection murine model of periodontitis.<sup>73</sup> It is not yet clear whether capsules are strongly expressed within dental plaque. However, it is noteworthy that capsular biosynthesis genes in model commensal subgingival biofilms were upregulated in response to tobacco exposure.<sup>74</sup> It is possible that the regulation of these extracellular carbohydrates may affect coaggregation interactions and drive the biofilm toward a more pathogenic state. Indeed, a DNABII protein regulates capsule expression in *P. gingivalis* intracellularly.<sup>75</sup> This protein has an interesting dual role in the biofilm matrix, since it is also a structural component of the *P. gingivalis* extracellular matrix, as described later.

### 2.1.3 | Poly-N-acetyl-D-glucosamine

Poly-N-acetyl-D-glucosamine was first identified as the polysaccharide intercellular adhesin that is an important component of the biofilm matrix of *Staphylococcus epidermidis*.<sup>76</sup> More recently, poly-N-acetyl-D-glucosamine was identified as the primary adhesion-mediating polymer in biofilms formed by the periodontal pathogen *A. actinomycetemcomitans*.<sup>77,78</sup> A proportion of poly-N-acetyl-D-glucosamine remains associated with the *A. actinomycetemcomitans* cell surface due to charge interactions with lipopolysaccharide. A de-N-acetylase, PgaB, is required for this interaction, and disruption of the catalytic domain of PgaB leads to reduced retention of poly-N-acetyl-D-glucosamine on the cell surface and decreased gene expression of the poly-N-acetyl-D-glucosamine biosynthesis gene locus.<sup>79</sup> Further, this mutant did not form tenacious biofilms on the surface of glass tubes. Therefore, association of poly-N-acetyl-D-glucosamine with the cell surface appears to be important for attachment and colonization. Mutants that do not produce

poly-N-acetyl-D-glucosamine show reduced virulence in a rat model of periodontitis, indicating that this polysaccharide is important for pathogenesis.<sup>80</sup> In addition, the turnover of poly-N-acetyl-D-glucosamine modulates the positioning of *A. actinomycetemcomitans* in mixed-species biofilms. When co-cultured with *S. gordonii*, *A. actinomycetemcomitans* cells upregulated the *dspB* gene encoding the poly-N-acetyl-D-glucosamine-degrading enzyme Dispersin B.<sup>81</sup> This resulted in cells becoming positioned at a distance from *S. gordonii* where they could benefit from metabolic cross-feeding by scavenging *S. gordonii*-derived lactate without succumbing to high concentrations of hydrogen peroxide in the close vicinity of *S. gordonii* cells. Therefore, production and turnover of the biofilm matrix appears to be an important factor in intermicrobial competition by *A. actinomycetemcomitans*.

### 2.1.4 | Teichoic acids and lipoteichoic acids

Wall teichoic acids and lipoteichoic acids are highly charged glycopolymers that are present in the cell walls of many gram-positive bacteria.<sup>82</sup> Wall teichoic acids are covalently linked to peptidoglycan, whereas lipoteichoic acids are anchored in the cell membrane. The teichoic acid backbone consists of repeating units of negatively charged polyols, such as ribitol phosphate or glycerol phosphate, linked by phosphodiester bonds. In some cases, the repeating monomers are substituted with cationic D-alanyl esters, resulting in zwitterionic polymers. In *Staphylococcus aureus*, disruption of the *dltA* gene that is essential for D-alanine incorporation resulted in reduced colonization of inert surfaces, presumably due to the increased negative charge of the cell wall.<sup>83</sup> In *S. epidermidis*, teichoic acids were identified in the extracellular fraction of cultures, and they were shown to be a critical component of the biofilm matrix.<sup>84,85</sup> More recently, teichoic acids were identified as the major polysaccharides present in the matrix of *Listeria monocytogenes* biofilms.<sup>86</sup>

There is relatively little information at present on the role of extracellular teichoic acids or lipoteichoic acids in mixed-species dental plaque, even though lipoteichoic acids were shown to be abundant in sucrose-grown in vivo plaque as long ago as 1980.<sup>87</sup> Studies on *S. mutans* demonstrated that lipoteichoic acids are present outside the cell and associated with glucosyltransferases.<sup>88</sup> In fact, lipoteichoic acids inhibited glucosyltransferase activity, potentially leading to alteration in the glucan composition of biofilms. Lipoteichoic acids from *S. gordonii* were shown to associate with extracellular glucan polymers and to enhance the binding of *S. gordonii* cells to glucan aggregates.<sup>89</sup> In addition, lipoteichoic acids from *Lactobacillus plantarum* inhibited the formation of *S. mutans* biofilms through suppression of exopolysaccharide production, and they reduced the formation of mixed-species oral biofilms in vitro.<sup>90,91</sup> Interestingly, *S. mutans* proteins encoded by the *dltABCD* operon and responsible for D-alanylation of lipoteichoic acids were identified in biofilms by quantitative proteomics and were shown to be upregulated in three-species biofilms compared with monospecies biofilms.<sup>92</sup> Lipoteichoic acids were detected in the matrix of both mixed-species and *S. mutans*

monospecies biofilms.<sup>93</sup> *S. mutans dltA* or *dltD* knockout mutants produced biofilms that were structurally distinct from those of the isogenic wild type and contained increased levels of lipoteichoic acids.<sup>93</sup> These data indicate that lipoteichoic acids are important in the development of biofilms, particularly over the later stages during maturation of the matrix. It will be of interest to determine whether (lipo)teichoic acids are produced and secreted by gram-positive bacteria that are associated with the progression to periodontal disease, such as *Filifactor alocis* or *Peptoanaerobacter stomatis*.<sup>94</sup> More generally, there is a need to characterize the extent and the origin of teichoic acids and lipoteichoic acids in the matrix of oral biofilms. It is not clear whether concentrations are sufficiently high to mediate intermicrobial competition, or whether certain species in the biofilm are particularly active in secreting these molecules. This information will provide a better understanding of the contribution of gram-positive bacteria to the structure and composition of dental plaque.

### 2.1.5 | Fungal polysaccharides

Fungi are commonly present in the mouth, including in subgingival dental plaque.<sup>95,96</sup> Of these, *Candida albicans* and other *Candida* spp have been most intensively studied, although molecular methods have detected many other genera.<sup>97</sup> The biofilm matrix of *C. albicans* monocultures in RPMI medium consists of approximately 55% proteins, 25% carbohydrate, 15% lipids, and 5% nucleic acids.<sup>98</sup> The polysaccharide component is largely composed of  $\alpha$ -mannan,  $\beta$ -1,6 glucan, and  $\beta$ -1,3 glucan, which are major polysaccharides in the fungal cell wall.<sup>99</sup> Interestingly, the glucans and mannan appear to be arranged in a complex that is assembled extracellularly.<sup>100</sup> Similar extracellular mannan-glucan complexes are produced by a wide range of *Candida* spp, indicating that these are important components for *Candida* biofilm formation.<sup>101</sup>

## 2.2 | Extracellular DNA

Though the role of extracellular DNA in the extracellular matrix of oral biofilms is understudied, the work to date indicates that extracellular DNA acts as an architectural material in the maintenance of the structural integrity of biofilms. Indeed, there is evidence that at least the periodontal pathogens *A. actinomycetemcomitans*, *Prevotella* spp, and *P. gingivalis* can rely on extracellular DNA for the maintenance of their extracellular matrices.<sup>12,102-104</sup>

Extracellular DNA has been known to be part of the extracellular bacterial milieu for over 90 years, ever since Griffith<sup>105</sup> discovered the *transforming principle* and Avery et al<sup>106</sup> showed that extracellular DNA was horizontally transferred between *S. pneumoniae* strains. Later, several investigators discovered that extracellular DNA was a natural component of microbial mats (the ecologic precursor term for biofilms).<sup>107-109</sup> However, it is only since 2002 that it has become clear that extracellular DNA is not only omnipresent in the extracellular matrix of bacterial biofilms but possesses a critical structural

function as well. This was demonstrated when Whitchurch et al<sup>110</sup> showed that deoxyribonuclease I could be used to prevent *Pseudomonas aeruginosa* biofilm formation. Human recombinant deoxyribonuclease I (dornase alpha) was already used as a mucolytic to alleviate the symptoms of cystic fibrosis,<sup>111,112</sup> a condition that is often associated with chronic *P. aeruginosa* pulmonary infection. The observation that deoxyribonuclease can also control *P. aeruginosa* biofilms has opened the door to optimizing deoxyribonuclease as a therapeutic to control the microbial infection.<sup>113</sup> Though the removal of biofilms by deoxyribonuclease enzymes has been recapitulated in multiple single and mixed-species biofilms, including dental plaque,<sup>12,102,107,108,114-116</sup> the biofilms eventually become resistant to deoxyribonuclease treatment as they age. Initially, this was interpreted to mean that the DNA had turned over in favor of other matrix materials or that extracellular DNA was only required for initial biofilm formation. More recently, it has become clear that the extracellular DNA is consistently present in the biofilm matrix as the biofilm matures and that, where examined, it is even more critical to biofilm stability.<sup>117</sup> Indeed, as the biofilm matures, the extracellular DNA enters a nuclease-recalcitrant state, likely to further protect the resident bacteria in the biofilm. Importantly, DNA has several relevant qualities that make it a versatile matrix material. First, it is omnipresent in eubacteria<sup>118</sup> and microbial fungi,<sup>98,119</sup> and is therefore accessible to all microorganisms whether in single or mixed-species biofilms. Second, it is relatively stable at varying pH extremes,<sup>120</sup> like those that occur in the oral cavity. Third, it has a significant persistence length of ~50 nm, which means it is stiff over this short distance but can bend over longer distances.<sup>121</sup> Fourth, since DNA can base pair between strands, it has the capacity to form complex secondary structures, including fibers.<sup>122,123</sup> Further, as already mentioned, extracellular DNA becomes resistant to nuclease digestion as biofilms mature, thus protecting the resident bacteria from external hazards.

The presence of extracellular DNA has been documented in multiple single and mixed-species oral biofilms.<sup>11,12,102,103,107-110,116,124-127</sup> Indeed, many oral streptococci rely on extracellular DNA for biofilm matrix structural stability,<sup>12,116,127</sup> and in some cases extracellular DNA improves adherence to the tooth surface.<sup>128,129</sup> In contrast, few periodontal pathogen biofilms have been thoroughly examined for their reliance on extracellular DNA. Of those that have been examined, *A. actinomycetemcomitans*, *Prevotella* spp, and *P. gingivalis* have been shown to rely on extracellular DNA for wild-type matrix formation.<sup>102,114,116</sup> In addition, *Enterococcus faecalis*, which is associated with secondary endodontic infections, is also reliant on extracellular DNA.<sup>124</sup> Other "red complex" pathogens, like *Treponema denticola* and *T. forsythia*, have yet to be examined.

The apparent reliance of biofilm matrix stability on extracellular DNA indicates that extracellular DNA is a universal matrix material. This makes sense, as it is one of the few structural commodities that is available to all microorganisms (ie, eubacteria and fungi). According to this hypothesis, when microorganisms need to enter or be inclusive in a multispecies biofilm, they could use extracellular DNA as the common structural material. This would also imply that

the structure of the extracellular DNA would need to be sufficiently conserved. Indeed, images detailing the structure of extracellular DNA from multiple species are remarkably similar,<sup>125,130</sup> showing a three-dimensional lattice of DNA. Though this could imply a random self-forming structure, it is more likely that this structure would need to be sufficiently robust so as to be stable under varied conditions. In this regard, other ubiquitous components would need to help shape and stabilize the extracellular DNA architecture. Indeed, it has recently been shown that the vertices of these scaffolds are functionally related to Holliday junction recombination intermediates, structures that are both created and turned over by known DNA repair pathways in the cell.<sup>131</sup> It has yet to be determined how the extracellular structures form, but they are critical for the integrity of the matrix.

### 2.3 | Proteins

A wide range of proteins have been identified in the matrix of microbial biofilms. For example, studies on *Vibrio cholerae* and *P. aeruginosa* biofilms have identified secreted proteins, cell surface adhesins, and subunits of pili and flagella in the biofilm matrix (reviewed by Fong and Yildiz<sup>132</sup>). Proteins are estimated to contribute greater than 50% of the biomass of *C. albicans* biofilms, and include enzymes in metabolic pathways for carbohydrates, amino acids, and energy metabolism.<sup>98</sup> Proteomic analysis of plaque-like biofilm grown in vivo identified a variety of bacterial stress-response proteins in the extracellular matrix.<sup>133</sup> Sucrose-grown biofilms contained higher levels of certain carbohydrate metabolism proteins, including pyruvate kinase and components of a mannose-specific phosphotransferase system, whereas control (no sucrose) biofilms contained higher levels of calcium-binding proteins. Amyloid fibers are important structural components of biofilms.<sup>134,135</sup> Several proteins produced by *S. mutans* can form amyloids including the antigen I/II adhesin (P1 or PAc), WapA, and SMU\_63c.<sup>136</sup> It is not yet clear how these proteins affect the structure of the dental plaque matrix, or which other amyloid proteins are present in the matrix.

Amyloid-forming proteins, such as antigen I/II, also play important roles in adhesion of streptococcal cells to bacterial or host receptors.<sup>137</sup> A number of additional adhesins are produced by oral streptococci, including serine-rich repeat proteins, lipoproteins, and pili.<sup>138</sup> These proteins are critical for adhesion and colonization, the first steps of biofilm formation. Type IV pili have recently been discovered in *S. sanguinis* and have been shown to mediate twitching motility in some strains.<sup>139</sup> *S. sanguinis* SK36 produces short hair-like type IV pili that do not confer motility but are important for adhesion to host cells.<sup>32,140</sup> Gram-negative oral bacteria also produce a variety of adhesins that contribute to mixed-species biofilm formation and host cell interactions. For example, long (FimA) and short (Mfal) pili of *P. gingivalis* are involved in coaggregation and adhesion to host cells.<sup>141-143</sup> In addition, leucine-rich repeat proteins, such as BspA of *T. forsythia*, are important adhesins for host receptors, such as glycoprotein340, and contribute to bone loss in animal models

of periodontitis.<sup>31,144</sup> However, BspA is downregulated in biofilms, possibly in order to evade immune recognition.<sup>145</sup>

Extracellular enzymes are key components of biofilm matrices, including dental plaque. Enzymes that catalyze biosynthetic processes are rare due to the lack of an energy source outside the cytoplasmic environment. The major exceptions in oral biofilms are the glucosyltransferases and fructosyltransferases, described earlier, that harness the energy of the glycosidic linkage in sucrose to synthesize glucan or fructan polymers.<sup>146</sup> Degradative enzymes, such as proteases, deoxyribonucleases, and glycosidases, are secreted by many bacteria and play a variety of different roles, including turnover of the matrix, scavenging of nutrients, and modulating host immune responses. For example, cysteine proteases of periodontal pathobionts (such as *P. gingivalis*, *T. denticola*, and *T. forsythia*) target host proteins (including complement components, cytokines, and matrix metalloproteinases), leading to the destruction of host tissues and the progression of periodontal disease.<sup>147,148</sup> Bacterial proteases have also been implicated in processing cell-surface proteins and in bacterial cell-cell sensing.<sup>147,149</sup> However, the role of microbial proteases in processing structural biofilm matrix proteins, including functional amyloids, is not yet clear. It is interesting to note that *P. gingivalis* proteases (gingipains) have been detected in association with amyloid plaques in the brain tissue of Alzheimer's patients, and it is possible that they play a role in stimulating amyloid formation.<sup>150</sup> Extracellular deoxyribonucleases are produced by many oral bacteria and may be important in biofilm matrix turnover, particularly in the early stages of biofilm formation when extracellular DNA is most sensitive to degradation.<sup>104</sup> Extracellular glycosidases, such as Dispersin B and fructanases, target microbially synthesized polysaccharides,<sup>151,152</sup> whereas enzymes such as neuraminidases (sialidases) primarily target host components.<sup>153,154</sup> Several distinct glycosidase activities for nutrient scavenging have been identified in oral bacteria.<sup>153,155-159</sup> A range of glycosidase activities from the pooled resources of several species in the oral biofilm, acting in concert with proteases, are required for the degradation of complex glycoproteins in saliva, which serve as a key nutrient source during the development of dental plaque.<sup>160,161</sup>

Many proteins interact with other molecules in the biofilm matrix, such as extracellular DNA. Proteins that would manipulate the extracellular DNA structure fall into one of three categories: inducing structure, stabilizing structure, or altering structure. Proteins that could affect extracellular DNA structure would be those that naturally bind, induce, and/or maintain DNA structure. Eubacteria possess nucleoid-associated proteins that make up the intracellular chromatin. Though nucleoid-associated proteins vary between genera, one family of nucleoid-associated proteins is absolutely conserved: the DNABII proteins.<sup>162</sup> All eubacteria possess at least one allele of the DNABII family.<sup>162</sup> All DNABII proteins are homologous and function as homo or heterodimers. Importantly, the DNABII proteins bind with high affinity to DNA that is bent and, as a consequence, stabilize the DNA structure. Recently, it was shown that multiple single and mixed-species biofilms not only possess extracellular DNABII, but also that DNABII proteins are bound to the

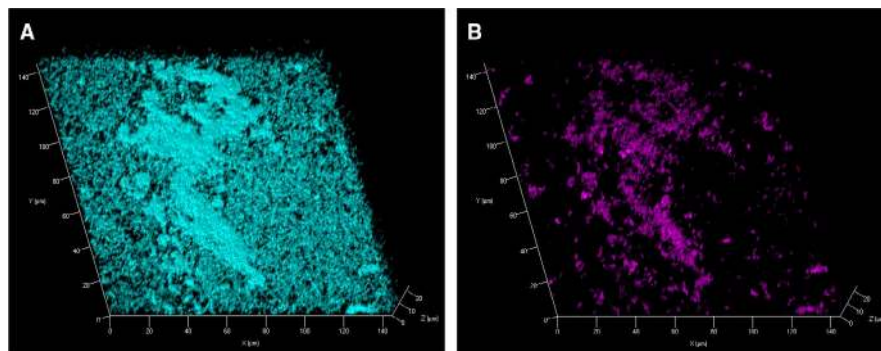
vertices of the lattice.<sup>130</sup> Indeed, it was recently shown that these structures are functionally equivalent to Holliday junction recombination intermediates,<sup>131</sup> where DNABII proteins are known to bind and stabilize these structures.<sup>163</sup> Subsequent work showed that, in each case, biofilms could be disrupted and the resident bacteria released by titrating the DNABII protein from the matrix with an antibody directed against DNABII.<sup>117,130,164–166</sup> Included in the biofilms tested were *A. actinomycetemcomitans*, *S. mutans*, and *P. gingivalis*, indicating that not only do potentially pathogenic oral bacteria possess extracellular DNA and DNABII proteins but they can also be targeted for biofilm resolution.<sup>164,166</sup> One novelty is that all DNABII proteins examined to date are sufficiently antigenically similar such that an antibody directed to one will bind with high affinity to any other. The only exception so far is the DNABII protein PG0121 from *P. gingivalis*, encoding histone-like protein  $\beta$ -subunit, which is antigenically unique.<sup>166</sup> Despite this distinctiveness, PG0121 can be complemented extracellularly by the DNABII protein from *S. gordonii*, showing that DNABII proteins appear to function in the biofilm matrix in an identical manner.<sup>166</sup> This means that antibodies directed against PG0121 will only affect *P. gingivalis*. Indeed, PG0121 is abundant in dual-species biofilms with *S. gordonii* (Figure 4), and anti-PG0121 effectively prevents *P. gingivalis* from entering extant biofilms.<sup>165</sup> This further proves that the nucleoprotein complex formed by extracellular DNA and DNABII proteins creates a common inclusive structure recognizable by bacteria.

Though it is formally possible that other nucleoid-associated proteins play a role in inducing or stabilizing, or perhaps simply altering, the extracellular DNA-dependent matrix (nucleoid), few candidates have emerged so far. Indeed, for nontypeable *Haemophilus influenzae*, a survey of extracellular nucleoid-associated proteins showed that, even when present, only the DNABII proteins affected the structural integrity of extant biofilms.<sup>167</sup> There are exceptions, however. For example, beta toxin is released from *S. aureus* and binds to extracellular DNA. Though not a nucleoid-associated protein, it does facilitate formation of extracellular DNA fibers by cross-linking DNA and enhancing biofilm formation.<sup>126</sup> A second exception is curli fibers from *Escherichia coli* and *Salmonella*.<sup>168</sup> These proteins are

synthesized extracellularly to create an amyloid that binds to extracellular DNA that facilitates biofilm formation. Examples of similar proteins from oral bacteria have yet to be discovered, but the aforementioned examples posit the possibility that each bacterium may have a means to manipulate the extracellular DNA nucleoid. Finally, though proteins that induce and maintain extracellular DNA-dependent matrix structure beyond the DNABII protein are rare, nucleases that cleave DNA are not. Indeed, many bacteria release nucleases, including oral pathogens such as *Prevotella* spp, *S. mutans*, *P. gingivalis*, *T. forsythia* and *F. nucleatum*.<sup>104,169,170</sup> Interestingly, the role of these nucleases has been ascribed not to altering the bacteria's own extracellular DNA but to disrupting neutrophil extracellular traps.<sup>171</sup> This suggests that the extracellular DNA lattice possessed by bacterial biofilms differs sufficiently from that of neutrophil extracellular traps that only the neutrophil extracellular trap DNA is susceptible. Indeed, DNABII proteins are only found in eubacteria and, as such, appear to facilitate a recalcitrant extracellular DNA structure of just biofilm extracellular DNA. This tête-à-tête at the interface between biofilm and neutrophil extracellular trap DNA looms as the front line in host-pathogen interactions.

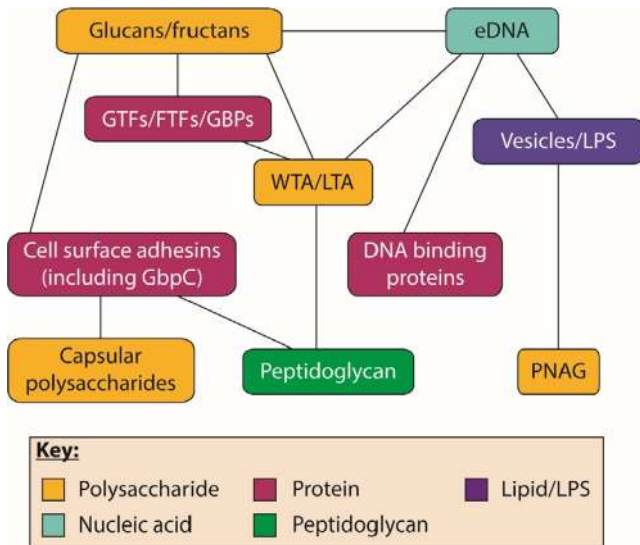
## 2.4 | Cell wall fragments

The outer layers of bacteria contain macromolecules, such as peptidoglycan, phospholipids, and, in the case of gram-negative bacteria, lipopolysaccharides, that may integrate into the biofilm matrix following cell lysis or the production of vesicles (see later). It is relatively difficult to study these molecules in the extracellular milieu; consequently, their role in biofilms is not well characterized. Wall-less (L-form) *Enterococcus faecium* are capable of attaching to solid substrata, indicating that peptidoglycan is not essential for the initial attachment phase of biofilm formation.<sup>172</sup> However, peptidoglycan fragments may play roles in more mature biofilms as environmental cues, since it has been demonstrated that they regulate processes such as the germination of spores in *Bacillus*, the production of antimicrobial compounds by *P. aeruginosa*, and



**FIGURE 4** *P. gingivalis* histone-like protein PG0121 is abundant in dual-species biofilms with *Streptococcus gordonii* (*Sg*). *Porphyromonas gingivalis* (*Pg*) was seeded into extant *S. gordonii* biofilms and immunofluorescence was performed to determine the distribution of PG0121 present in dual-species biofilms of a 1:1 ratio of *Pg*:*Sg*. All bacterial cells were labeled with the membrane stain FM4-64 and pseudocolored cyan (A), and PG0121 was detected with antibodies directed against PG0121 followed by the addition of secondary antibodies conjugated to Alexa Fluor-488 and pseudocolored magenta (B)





**FIGURE 5** Interactions between macromolecules in the matrix of dental plaque. Glucans and fructans are associated with the enzymes that produce them (glucosyltransferases, GTFs/fructosyltransferases, FTFs), and insoluble glucans are recognized by glucan-binding proteins (GBPs), including the wall-anchored protein GbpC. Glucans and GTFs associate with extracellular DNA (eDNA) and lipoteichoic acid (LTA), which also binds extracellular DNA. In addition, wall teichoic acids (WTAs) and certain gram-positive cell-surface proteins are covalently linked to peptidoglycan. Therefore, these will remain attached to peptidoglycan fragments in the biofilm matrix. Cell-surface adhesins mediate coaggregation through binding to capsular polysaccharides. Proteins are also associated with extracellular DNA, and extracellular DNA is present on the surface of gram-positive membrane vesicles. Gram-negative outer membrane vesicles contain lipopolysaccharides (LPS), which bind to poly-*N*-acetyl-*D*-glucosamine (PNAG) through charge interactions

the yeast-hyphal transition in *C. albicans*.<sup>173-175</sup> In addition, peptidoglycan is sensed by host cells through toll-like receptor 2 and nucleotide-binding oligomerization domain-like receptors and acts synergistically with lipopolysaccharides to induce bone resorption and osteoclastogenesis in mouse models of periodontitis.<sup>176,177</sup> Lipids and lipopolysaccharides will likely aggregate into vesicles or associate with cell membranes in the hydrophilic environment of the biofilm matrix. Vesicles have been observed in dental plaque, and their role in delivery of macromolecules to the biofilm matrix is discussed later.<sup>178</sup> The polysaccharide O-antigen of lipopolysaccharide has been shown to inhibit biofilm formation by a range of enteric gram-negative bacteria, and it is possible that this component may influence periodontal biofilms that are typically enriched in gram-negative species.<sup>179</sup>

## 2.5 | Host molecules

Although this article focuses on microbial-derived biofilm matrix molecules, it is important to note that natural dental plaque will also contain macromolecules of host origin. Components of saliva,

such as proteins and glycoproteins, are continuously adsorbed onto surfaces. On the surface of enamel, these form the acquired enamel pellicle.<sup>180</sup> More recently, it has been shown that there is also an extensive mucosal pellicle on the surface of oral soft tissues.<sup>181</sup> Many of the (glyco)proteins that adsorb to oral surfaces also interact with oral microorganisms, including glycoprotein340, secretory immunoglobulin A, mucins, proline-rich proteins, amylase, and statherin.<sup>182,183</sup> When dental plaque grows below the gumline, it becomes isolated from saliva and, instead, is exposed to gingival crevicular fluid, a serum exudate. This is rich in protein and bound glycans that can be released and bound by bacteria and is likely a source of molecules in the matrix of subgingival dental plaque.<sup>184</sup> Periodontal pockets contain a variety of host inflammatory cells that may provide an additional source of proteins, glycoproteins, nucleic acids, and lipids for dental plaque.

## 2.6 | Interactions between macromolecules

Throughout this review, multiple matrix molecules are described and discussed. Many of these macromolecules interact covalently or noncovalently to form complexes (Figure 5). For example, peptidoglycan of gram-positive bacteria is covalently linked to wall teichoic acids and to a variety of cell-surface proteins that contain conserved C-terminal motifs that are recognized and processed by sortase enzymes.<sup>185,186</sup> Peptidoglycan fragments in the biofilm matrix will likely retain these interactions. Certain cell-surface adhesins, such as antigen I/II, WapA, and GbpC, form amyloids. It is not clear whether these amyloids remain bound to fragments of peptidoglycan in the matrix. Electrostatic interactions are responsible for many of the complexes that are formed, including between teichoic acids and proteins/polysaccharides/extracellular DNA and between poly-*N*-acetyl-*D*-glucosamine and lipopolysaccharides. Extracellular DNA is a key component of many biofilms and undergoes a number of interactions with other macromolecules. However, as already described, though periodontal pathogens make exopolysaccharides, for most of them it is unclear at present if they interact with or are exclusive of extracellular DNA. When grown in the presence of sucrose, the large quantities of exopolysaccharides produced by streptococci rival extracellular DNA in proportion and could, in principle, dominate, add to, or synergize with the extracellular nucleoid. For *S. mutans*, it is clear that insoluble glucan interacts productively with extracellular DNA to contribute to the extracellular matrix.<sup>127</sup> Lipoteichoic acids are a large component of the gram-positive cell wall, and at least for *S. mutans* appear to interact with extracellular DNA in concert with glucans.<sup>127</sup> More generally, extracellular DNA has been shown to associate with the surface of membrane vesicles.<sup>187</sup> It is possible that other components of the outer layers of microbial cells, including lipids and lipopolysaccharides, may bind to extracellular DNA or other biofilm matrix components and alter the function of the matrix. As the field of the extracellular nucleoid evolves and mixed-species biofilms are analyzed, other

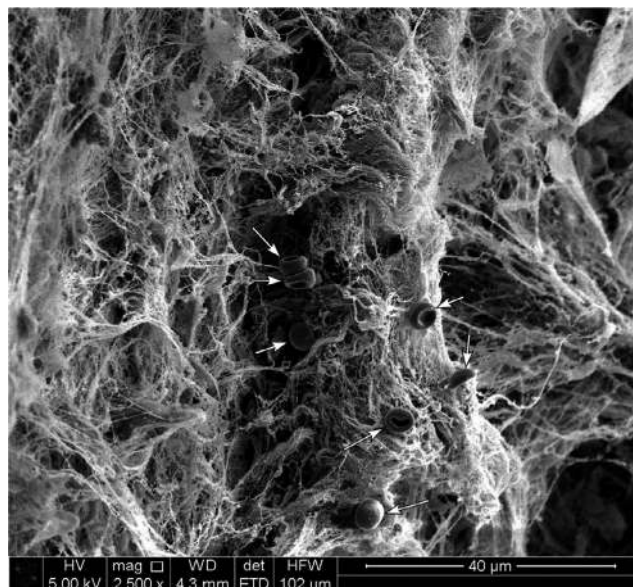
matrix molecules that are exclusive to single species may not only be found to interact with extracellular DNA but, in the context of the mixed-species community, may be shown to interact with the extracellular DNA-dependent matrix at large.

### 3 | ORIGINS OF THE MATRIX

Many components of the dental plaque extracellular matrix are originally synthesized within microbial cells and actively secreted into the surrounding milieu. Microbial vesicles and cell lysis also play important roles in the accumulation of matrix material. Bacteriophages are abundant within dental plaque, and virus-like particles can be observed in association with microbial cells by transmission electron microscopy.<sup>188,189</sup> These may provide a source of extracellular nucleic acids or proteins. In addition, molecules from the host diet, such as fiber, polysaccharides, and proteins, may become entrapped within dental plaque. These components, along with debris from nondietary sources, can be identified in dental calculus from archaeological specimens.<sup>190</sup> Saliva is particularly important during the early phases of dental plaque growth, since molecules from saliva adsorb onto tooth surfaces and promote microbial attachment and biofilm formation.<sup>191</sup> Depending on the location of dental plaque (above or below the gumline), macromolecules from saliva and/or gingival crevicular fluid may attach to dental plaque and integrate into the biofilm.<sup>180</sup> In addition, host cells are commonly found in dental plaque and may contribute to the plaque matrix when they degrade. Epithelial cells from the tongue, oral mucosa, or gingiva can be found in dental plaque within 1 hour after introducing a clean tooth surface into the mouth.<sup>192</sup> Periodontal disease and gingival bleeding lead to the accumulation of erythrocytes at or close to sites of dental plaque (Figure 6). For some immune cells, lysis of cellular contents appears to be an important process to generate extracellular “traps” containing nucleic acids, antimicrobial peptides, and proteins that catch invading microorganisms. The process of extracellular trap formation was first identified in neutrophils, and this has been shown more recently in macrophages.<sup>171,193</sup> Neutrophil extracellular traps have been identified in purulent exudate from periodontal pockets of patients with chronic periodontitis.<sup>194</sup> Proteins associated with neutrophil extracellular traps have also been identified in supragingival plaque during an experimental gingivitis study.<sup>195</sup> The impact of nonmicrobial components of dental plaque matrices may be missed when dental plaque is modelled and studied *in vitro*. Future studies are needed to elucidate the complex network of microbial and non-microbial products in dental plaque *in vivo*.

#### 3.1 | Secretion

Polysaccharides and proteins are actively secreted from microbial cells through a number of different export pathways. In some cases, DNA may also be exported through the type IV secretion system.<sup>196</sup> For example, the *Neisseria gonorrhoeae* type IV secretion system



**FIGURE 6** Scanning electron micrograph showing erythrocytes and fibrous material on the surface of a tooth extracted due to periodontal disease. Erythrocytes (arrows) are associated with fibrous noncellular material overlying the dental plaque on the tooth surface. Bacterial cells are not visible in this image

exports single-stranded DNA into the surrounding milieu and is required for biofilm formation.<sup>197</sup> Similarly, nontypeable *Haemophilus influenzae* has been shown to release DNA through a competence-mediated T4SS-like complex. In this case, the export of DNA was shown to contribute directly to the DNA-based extracellular polymeric material.<sup>198</sup> However, it is not yet clear whether type IV secretion systems, or even whether single-stranded extracellular DNA, are important for oral biofilms. A brief overview of carbohydrate and protein export pathways will be presented here. For further information, the reader is referred to excellent reviews on the secretion of polysaccharides<sup>199,200</sup> or proteins.<sup>201–203</sup>

Three major pathways are responsible for the export of polysaccharides in both gram-positive and gram-negative bacteria: (a) Wzx/Wzy dependent, (b) adenosine triphosphate-binding cassette dependent, and (c) synthase dependent. The Wzx/Wzy-dependent pathway mediates the export of lipopolysaccharide O-antigen polysaccharides and capsular polysaccharides.<sup>204,205</sup> Central to this pathway are the Wzx flippase and the Wzy polymerase that elongate the polysaccharide chain on the outer surface of the cell. The polysaccharide is delivered to Wzx attached to the lipid acceptor moiety undecaprenyl phosphate.<sup>206</sup> By contrast, adenosine triphosphate-binding cassette transporter systems translocate the fully formed polysaccharide using energy from ATP hydrolysis. This pathway is responsible for export of *S. mutans* rhamnose-glucose polymers.<sup>207</sup> The synthase-dependent pathway involves the simultaneous polymerization and translocation via a membrane-embedded glycosyl transferase, and is employed for the export of poly-N-acetyl-D-glucosamine from bacterial cells.<sup>200</sup> A similar pathway mediates the export of glucans in *C. albicans*.<sup>208</sup>

As in other eukaryotes, protein secretion in *C. albicans* is driven by vesicle-mediated trafficking between cellular compartments, and out to the cell surface.<sup>209</sup> However, the secretory apparatus differs between yeast and hyphal cells, indicating that different morphologic forms may play distinct contributions to the extracellular proteome. Numerous protein secretion systems have been described in bacteria, and there is a great deal of confusion regarding their nomenclature in the literature.<sup>202</sup> Bacteria with a gram-negative structure must transport proteins across two membranes to reach the extracellular milieu, whereas gram-positive bacteria only have to secrete proteins through the cytoplasmic membrane. Therefore, the Sec system, which is present in both gram-negative and gram-positive bacteria and transports proteins across the cytoplasmic membrane, is only a true secretion system in gram-positive organisms. In gram-negative bacteria, additional secretion apparatus is required to translocate proteins across the periplasmic membrane. The Sec and Tat systems are dependent on signal peptides that are present on the N-terminus of proteins.<sup>210</sup> The key difference between these pathways is that Sec translocates unfolded proteins whereas Tat recognizes folded proteins. Signal peptides that are cleaved by the prepilin-specific signal peptidase PilD direct the transport of type IV pilus subunits in certain gram-positive bacteria, including *S. pneumoniae*.<sup>211</sup> In gram-negative bacteria, nine different secretion systems have been identified that mediate export across the periplasmic membrane (type I–IX secretion system, designated T1SS–T9SS).<sup>202,203</sup> Of these, T1SS, T3SS, T4SS, and T6SS span both membranes and transport proteins directly from the cytosol to the extracellular environment. The other systems traverse the periplasmic membrane only and require the prior export of proteins to the periplasm by Sec, Tat, or holins. T9SS was only discovered relatively recently and has been characterized in particular in periodontal pathobionts such as *P. gingivalis*, where it is responsible for secretion of a range of virulence factors including cysteine proteases,<sup>203</sup> and *T. forsythia*, which employs T9SS for the secretion and assembly of S-layers.<sup>52,212</sup>

### 3.2 | Vesicles

Extracellular vesicles are abundant in dental plaque (Figure 2) and are produced by many species of bacteria and fungi. There are marked differences between vesicles from gram-negative and gram-positive bacteria. Gram-negative outer membrane vesicles, including those of *P. gingivalis*, contain lipopolysaccharides and protect DNA within them.<sup>213,214</sup> By contrast, gram-positive vesicles, such as those from *S. mutans*, are associated with extracellular DNA on the external surface.<sup>215</sup> The fungus *C. albicans* also produces extracellular vesicles that play a central role in biofilm matrix production by transporting proteins and polysaccharides (glucans and mannans) out of the cell.<sup>216</sup> Approximately 45% of the proteins identified in the *C. albicans* biofilm matrix were also present in the proteome of biofilm extracellular vesicles, indicating that extracellular vesicles may be a major route for secretion of proteins into the matrix. Proteomics has also been extensively employed to characterize bacterial extracellular

vesicles, and the proteins identified have been cataloged in a database (<http://evpedia.info>).<sup>217,218</sup> In the gram-negative model biofilm organism *P. aeruginosa*, the extracellular vesicle proteome contained approximately 20% of the proteins found in the biofilm matrix, again indicating that extracellular vesicles may play a key role in delivering proteins to the matrix.<sup>219</sup>

Proteomics has been employed to analyze the proteins present in extracellular vesicles from several gram-negative periodontal pathobionts. For example, extracellular vesicles of *A. actinomycetemcomitans* are enriched in virulence factors, including the leukotoxin LtxA and the tight adhesion proteins TadA, TadD, TadE, TadF, TadG, and TadZ.<sup>220,221</sup> Virulence factors are also abundant in *P. gingivalis* extracellular vesicles, which are enriched in T9SS substrates, including arginine and lysine-specific gingipains.<sup>222</sup> *T. forsythia* extracellular vesicles are also enriched in substrates of T9SS, including S-layer proteins TfsA and TfsB.<sup>212</sup> Interestingly, vesicles of *A. actinomycetemcomitans*, *P. gingivalis*, and *T. denticola* also contain a range of small ribonucleic acids that can be delivered directly to host cells and may play roles in immunomodulation.<sup>223</sup>

### 3.3 | Cell lysis

As with extracellular vesicles, electron microscopy studies have provided evidence of cell lysis within dental plaque.<sup>11,12</sup> Lysis may occur as a natural consequence of cell senescence or may be induced by molecules that degrade the cell wall—including exogenous compounds, such as antibiotics or bacteriocins—or enzymes (autolysins) present within the bacterial cell. It appears that cell lysis is an important mechanism for delivery of extracellular DNA into the biofilm matrix. For example, competence-stimulating peptide-mediated induction of bacteriocins in *S. mutans* leads to lysis of a proportion of cells in a population and enhancement of the biofilm matrix by extracellular DNA.<sup>224</sup> Competence-inducing peptide also triggers cell lysis in *S. mutans*, which will release DNA.<sup>225</sup> The autolysin AtIA is critical for *S. mutans* extracellular DNA release and biofilm matrix production in vivo in a rat model of endocarditis.<sup>226</sup> Fratricide is also responsible for extracellular DNA production and biofilm development by *E. faecalis*, an important endodontic pathogen.<sup>227,228</sup> However, cell lysis is not essential for extracellular DNA in this species, as an abundant extracellular DNA matrix can be detected in early biofilms, where cell lysis is not detectable.<sup>124</sup> In *P. aeruginosa*, explosive cell lysis of a subpopulation of cells, driven by the bacteriophage-like endolysin Lys, rapidly releases extracellular vesicles and liberates extracellular DNA into the biofilm matrix.<sup>229</sup> It remains to be determined whether a similar process occurs in bacteria within dental plaque.

## 4 | FUNCTIONS OF THE MATRIX

The biofilm matrix is important for adhesion to substrata and for maintaining a homeostatic environment for the resident microbial cells.<sup>134</sup>

The matrix helps to position cells at a distance where mutually beneficial interactions are optimized and competition is minimized.<sup>81,230</sup> Although the development of the biofilm matrix is considered to be a process that follows the initial colonization of an interface, there is evidence that matrix macromolecules are important for initial attachment of microorganisms. For example, cell-surface protein adhesins that are found within biofilm matrices are important for attachment to receptors in the saliva pellicle or for coaggregation interactions between microbial cells.<sup>183</sup> In addition, extracellular DNA has been shown to promote the attachment of *S. mutans* to surfaces.<sup>231</sup> In more mature biofilms, the matrix provides physicochemical forces for adhesion of the biofilm to the substratum and cohesion of the biofilm biomass.

#### 4.1 | Adhesion/cohesion and mechanical resistance

The biofilm matrix acts as a focus for interactions between macromolecules (Figure 5), which help to retain cells within the biofilm and to stabilize the overall structure. For example, glucan binding proteins of *S. mutans* promote adherence to matrix glucans and shape the overall architecture of the biofilm.<sup>25,26,232,233</sup> Biofilms are typical examples of multicomponent materials and exhibit viscoelastic behavior when subjected to external stress factors.<sup>234</sup> Viscoelasticity consists of an elastic component that does not lose energy when a stress is applied and then removed, and a viscous component that undergoes molecular rearrangement in response to a stress and dissipates energy in the process. This means that the biofilm will deform under a given stress (eg, shear stress) but will return to a state that is similar to, but not necessarily identical to, the initial state after this given stress is removed.<sup>234</sup> The mechanical properties of a biofilm and its sturdiness against detachment forces are further influenced by the shear forces that the biofilm experiences during growth.<sup>13,234,235</sup> Biofilms grown under higher shear stress exhibit stronger attachment and stronger cohesive forces than biofilms grown under lower shear.<sup>236,237</sup> This may be due to structural changes in the matrix; for example, with regard to the physical arrangement and structure of extracellular polymers, or due to a selection favoring subpopulations that produce biofilms with increased strength of their structural matrix under high-shear conditions.<sup>13,238–240</sup> Although some of these studies were performed on biofilms from environmental pathogens, such as *P. aeruginosa*, the results may be translated to periodontal biofilms that grow under rather constant mechanical challenge exerted through the flow of naturally occurring fluids, like saliva or gingival crevicular fluid, or due to tongue movements.<sup>234</sup> Furthermore, Paramonova et al<sup>236</sup> showed that biofilms of typical oral colonizers *S. oralis* and *Actinomyces naeslundii* adapted to changes in hydrodynamic conditions by changing their architecture.

#### 4.2 | Regulation of mass transfer and cell migration

The macromolecular biofilm matrix forms a scaffold that can impede the transfer of molecules and cells. Thus, poly-*N*-acetyl-*D*-glucosamine

has been shown to restrict fluid convection and to retard the penetration of the quaternary ammonium compound cetylpyridinium chloride.<sup>241</sup> Thurnheer et al<sup>242</sup> found that penetration depths of macromolecules decreased linearly for molecular weights up to 240 000 Da in their Zurich biofilm model of supragingival plaque. The authors suggested that the discrepancy found between diffusion of these macromolecules in a biofilm compared with diffusion in bulk water most likely may be explained by the phenomenon of tortuosity. This means that a given molecule will be delayed in diffusion throughout a biofilm because its pathway is determined by the interstitial voids in the biofilm structure; consequently, the route will be a three-dimensional one rather than the direct path found for free diffusion in bulk water.<sup>242</sup> It was also shown that the penetration of poly(ethylene glycol) with molecular weight of 10 000 Da through *S. mutans* biofilms was dependent on the density of the polymeric matrix of the biofilms, which was in turn influenced by the sucrose concentration in the culture medium.<sup>243</sup> These authors concluded that steric exclusion is likely responsible for the decreased penetration. Although this concept seems quite intuitive, times needed for penetration throughout biofilms do not always increase with increasing molecular weight of the respective agents, even for chemically inert compounds. In fact, some studies have reported that even large antibiotics are able to penetrate in vitro biofilms within a few minutes.<sup>244</sup> For instance, Oubekka et al<sup>245</sup> using time-lapse microscopy and fluorescence imaging, showed that BODIPY-labeled vancomycin penetrated to the deepest layers (~30  $\mu$ m) of *S. aureus* biofilms in 8 minutes or less. On the other hand, Jefferson et al<sup>246</sup> observed that penetration of BODIPY-labeled vancomycin through their *S. aureus* biofilms occurred rather slowly over the course of 60 minutes, which, however, may be related to the particular density of the extracellular polymeric substances in these biofilms due to overproduction of poly-*N*-acetyl-*D*-glucosamine by the respective *S. aureus* strain. This example demonstrates the difficulties with interpretation of in vitro studies on penetration of antimicrobials and other chemicals. In addition, the situation becomes more complex for charged molecules that undergo electrostatic interactions with biofilm matrix components.<sup>247</sup> In this case, the biofilm matrix acts as an ion-exchange resin to reduce the rate of movement through the biofilm.<sup>248</sup> For some molecules, such as strongly oxidizing agents, reaction with the outer layers of the biofilm can impede diffusion to the center of the structure. Therefore, models of mass transfer through biofilms need to account for both reaction and diffusion.<sup>249</sup>

Subgingival dental plaque is bathed in gingival crevicular fluid and is in contact with the gingival epithelium. Therefore, it is exposed to host immune mediators, including antibodies, complement, and immune cells. Mixed-species biofilms cultured in vitro retard the penetration of immunoglobulin G (IgG) and immunoglobulin M antibodies, to the point where IgG does not reach the center of clusters a few hundred micrometers in diameter.<sup>242,250</sup> However, the diffusion of *S. mutans*-specific IgG was not affected by the presence of exopolysaccharides in single-species biofilms.<sup>251</sup> It is possible that more complex matrix components or tighter cell-cell interactions in mixed-species biofilms are

responsible for reduced levels of IgG penetration in these systems. Proteases present in the biofilm matrix may cleave antibodies, acting as “shared goods” to protect multiple species of bacteria within the biofilm. Immunoglobulin A-specific proteases are well known in oral streptococci, such as *S. mitis* and *S. sanguinis*,<sup>252,253</sup> and *P. gingivalis* gingipains have been shown to cleave IgG.<sup>254</sup> Similarly, proteases of periodontal pathogens cleave multiple complement proteins (reviewed by Damgaard et al<sup>255</sup>), which will potentially protect biofilm bacteria from host immunity.

Cell migration through host tissue extracellular matrices is critical for a wide range of physiologic processes and for responses to tissue damage (Figure 1).<sup>256</sup> In a similar manner, bacteria can potentially migrate within biofilms. Motility mediated by type IV pili and flagella is required for the formation of three-dimensional cap structures in *P. aeruginosa* biofilms.<sup>257</sup> Flagella-driven motility is also important for *T. denticola* to form mixed-species biofilms with *P. gingivalis*.<sup>258</sup> Two small ribonucleic acids that repress the expression of a type IV pilus subunit in *S. sanguinis* ATCC10556 also suppress biofilm formation.<sup>259</sup> However, twitching motility was not observed in this strain, and it is not yet clear whether twitching motility is important in oral biofilms. However, gliding motility driven by *Campytophaga gingivalis* has been shown to contribute to the organization of polymicrobial oral biofilms.<sup>260</sup> In fact, gliding *C. gingivalis* cells can transport nonmotile bacteria to other areas of the biofilm.

### 4.3 | Signaling and host interactions

The biofilm matrix helps to position microbial cells in close proximity where cell-cell sensing and signaling are optimized.<sup>230</sup> Signaling between taxonomically distinct bacteria is mediated by autoinducer-2,<sup>261</sup> whereas signaling between closely related strains of oral *Streptococcus* spp involves peptides, such as competence-stimulating peptide or *S. mutans* comX-inducing peptide.<sup>262</sup> Extracellular proteases may also be important for cell-cell signaling. For example, the *S. gordonii* serine protease Challisin can interfere with *S. mutans* cell-cell communication by degrading competence-stimulating peptide<sup>263</sup> or can scavenge amino acids from the surface of *A. oris* cells that are then sensed by *S. gordonii*.<sup>149</sup>

There is evidence that oral biofilms elicit responses in epithelial cells that are distinct from responses to planktonic cells of the same species.<sup>264</sup> In addition, responses of epithelial cells to multispecies biofilms are different from the sum of the effects of individual species.<sup>265,266</sup> Many biofilm matrix molecules, including lipopolysaccharides, peptidoglycans, and extracellular DNA, are pathogen-associated molecular patterns that are recognized by immune cells, such as neutrophils and macrophages. However, there is evidence that immune responses are attenuated by bacteria in biofilms. For example, *S. aureus* biofilms have been shown to dampen host immune responses and to induce macrophage dysfunction and cell death.<sup>267</sup> Similarly, a biofilm-forming strain of *Prevotella intermedia* was shown to resist phagocytosis by polymorphonuclear

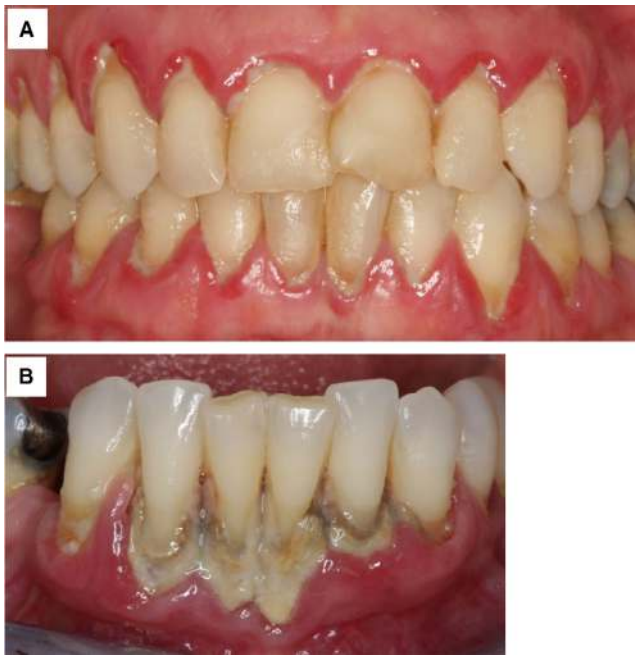
leukocytes, and a mannose-rich exopolysaccharide was essential for resistance.<sup>268</sup>

### 4.4 | Extracellular pool of nutrients and genes

The matrix provides an important source of extracellular nutrients for bacteria within the biofilm. Macromolecules, such as polysaccharides, proteins, and extracellular DNA, may be broken down into monomers or oligomers that can then be internalized by bacteria. Thus, fructans are thought to serve primarily as extracellular nutrients, since they are produced rapidly by oral bacteria but do not accumulate to high levels in dental plaque.<sup>269</sup> Further, *P. aeruginosa* has been shown to use extracellular DNA as a source of nutrients.<sup>270</sup> The biofilm matrix also traps small molecules, including micronutrients such as metal cations. *P. gingivalis* vesicles are highly enriched in proteins IhtB and HmuY, which are involved in the acquisition of heme iron, indicating that vesicles may scavenge and concentrate iron from the host.<sup>222</sup> Iron has been identified as a growth-rate-limiting nutrient in *P. aeruginosa* biofilms<sup>249</sup> and may also limit the growth of black-pigmented oral anaerobes, such as *P. gingivalis*, which are highly dependent on iron acquisition for porphyrin pigment production. As well as providing nutrients for biofilm bacteria, the extracellular DNA component of the matrix may serve as a pool of genes for oral bacteria. As noted earlier, transformation was first discovered in *S. pneumoniae*, and many oral streptococci are naturally transformable.<sup>271</sup> More recently, it has been shown that gram-negative periodontal pathogens *P. gingivalis* and *T. forsythia* undergo natural transformation.<sup>272,273</sup> Genes for antimicrobial resistance are common in the oral microbiome, and extracellular DNA-mediated gene transfer has been demonstrated in oral biofilms.<sup>274-276</sup> In addition, there is evidence from genome sequence analysis that mosaic genes encoding antibiotic-resistant forms of penicillin-binding proteins have arisen through horizontal gene transfer between oral streptococci and *S. pneumoniae*.<sup>277</sup> In view of the current global antimicrobial resistance crisis, it is critical to elucidate the role of extracellular DNA in horizontal gene transfer and to develop approaches to minimize the spread of genes between bacteria.

## 5 | IMPLICATIONS FOR PERIODONTITIS CONTROL

Accumulation of subgingival biofilms initiates development of clinical signs of gingivitis (see Figure 7A for a clinical example). As a result of complex interactions between subgingival biofilms and the host immune response, gingivitis can further develop to periodontitis with concomitant loss of periodontal supportive tissues (Figure 7B).<sup>239,278,279</sup> Periodontal treatment approaches are first and foremost based on complete removal of subgingival biofilms and associated calculus deposits, but they may also comprise adjunctive use of antimicrobials, either applied as local dressings or systemically.<sup>240</sup> In the following, the implications of our current understanding of the



**FIGURE 7** Clinical images of dental plaque. A, A 49-year-old female patient with insufficient oral hygiene, presenting massive amounts of subgingival plaque and clinical signs of plaque-associated gingivitis. B, A 53-year-old female patient with insufficient oral hygiene, presenting massive amounts of subgingival plaque and calculus and clinical signs of advanced periodontitis



**FIGURE 8** Dental plaque and calculus on the surface of extracted teeth. Teeth 37, 36, and 35 were extracted due to periodontal disease. All teeth present massive amounts of subgingival plaque and calculus

structure and the characteristics of the subgingival biofilm matrix on clinical periodontal treatment are discussed with regard to mechanical removal of subgingival biofilms, as well as to the delivery of antimicrobials to these biofilms.

## 5.1 | Mechanical removal

The logical first step in every periodontal treatment is mechanical removal of subgingival biofilms and calculus deposits.<sup>239,280,281</sup>

Although this subgingival debridement most often shows successful clinical outcomes in terms of reductions in probing pocket depth, it still proves to be a tough job in a clinical environment. This is because it is technically demanding and further impeded by limited access and impaired visibility into deeper periodontal pockets.<sup>282</sup> In a classic study, Rateitschak-Plüss et al<sup>283</sup> investigated the possibilities and limitations of manual subgingival debridement in 10 single-rooted teeth from four patients by scanning electron microscopy. Eleven out of 40 instrumented root surfaces exhibited residual plaque and “islands” of calculus. These plaque and calculus residues were detected in irregularities of the root surfaces such as fine grooves, ridges, or lacunae, and in areas where the operator may have changed from one curette to another.<sup>283</sup> Therefore, removal of calculus and plaque in furcation sites of multirooted teeth may be limited,<sup>281</sup> even when subgingival debridement is aided by endoscopy.<sup>284</sup> Residual dental plaque and calculus are sometimes clearly visible on teeth extracted for periodontal disease (Figure 8).

Biofilms are generally able to resist mechanical challenges to a certain extent due to the physical protection provided by the biofilm matrix, and in particular by its exopolysaccharide components.<sup>13,234</sup> Removal of biofilms can therefore only be accomplished by overcoming the cohesive and adhesive forces provided by the biofilm matrix.<sup>13</sup> When a mature biofilm is subjected to mechanical forces, it shows complex viscoelastic properties, as described earlier.<sup>234,285</sup> Depending on the strength of the force acting on the biofilm matrix, it will either undergo reversible elastic responses or irreversible deformation. When the detaching forces exceed the cohesive and adhesive forces provided by the biofilm matrix, failure will occur in the biofilm (cohesive failure) or between the substrate and the biofilm (adhesive failure).<sup>234</sup> This viscoelastic nature of a biofilm may not be a crucial factor when its removal is accomplished by forces applied directly (eg, by means of a curette), but it is of vital importance when hydrodynamic forces are applied in a noncontact mode (eg, by means of sonic scalers during subgingival debridement or by brushing with powered toothbrushes).<sup>234,286,287</sup> Busscher et al<sup>286</sup> investigated the noncontact effects of powered toothbrushes on removal of biofilms formed in vitro from *S. oralis* and *A. naeslundii*. They proposed that a biofilm reacts to the absorption of “brush energy” (hydrodynamic force) by viscoelastic expansion of the whole biofilm structure. If enough energy is absorbed and the deformation of the biofilm exceeds a given yield point, biofilm removal will occur. On the other hand, if the absorbed energy is not sufficient, deformation will occur and the biofilm is expanded but not removed.<sup>234,286</sup> Fabbri et al<sup>288</sup> documented the formation of ripples and wrinkles and their migration throughout single-species biofilms from *S. mutans*, *S. epidermidis*, and *P. aeruginosa* in a high-shear environment. They further suggested that these may form from so-called Kelvin-Helmholtz instabilities at the interfaces between two fluids and indicate the onset of turbulence in these biofilms.<sup>288</sup> Further studies on these phenomena are required to improve noncontact removal of biofilms and delivery of antimicrobials throughout their matrices.

## 5.2 | Antimicrobial therapy

Several distinct classes of antimicrobial agents are routinely used in clinics adjunctively to mechanical biofilm removal, both in oral care products for home use and in the course of professional periodontal treatment.<sup>289,290</sup> Some oral care products comprise antimicrobial agents, such as chlorhexidine, cetylpyridinium chloride, or natural compounds.<sup>290,291</sup> Chlorhexidine rinses and gels are also routinely applied concomitantly with periodontal treatment; for example, within full-mouth disinfection concepts.<sup>292</sup> Furthermore, antibiotics can be used adjunctively, either administered systemically in severe cases of periodontitis or locally in persistent or recurrent active periodontal pockets.<sup>289,291,293</sup> Finally, alternative antimicrobial approaches, such as antimicrobial photodynamic therapy, have been proposed in view of the rising threats from antimicrobial resistance.<sup>291</sup>

There is general consensus that thorough subgingival debridement needs to be performed preceding adjunctive antimicrobial therapy in order to disrupt the subgingival biofilm structure.<sup>289</sup> This is because bacteria embedded in biofilms can be up to 1000 times more tolerant toward antimicrobials than their planktonic counterparts.<sup>294,295</sup> For instance, it was found that antibiotics commonly used in periodontics caused reductions only of approximately one log<sub>10</sub> step in the Zurich biofilm model of subgingival plaque comprising 10 periodontitis-associated bacterial species when applied in concentrations that can typically be reached in gingival crevicular fluid.<sup>296</sup> Likewise, Wang et al<sup>297</sup> reported biofilm eradication concentrations of 800 µg/mL metronidazole for an in vitro double-species biofilm of *F. nucleatum* and *P. gingivalis*, whereas minimum bactericidal concentrations for the same bacterial species in planktonic cultures were 25 µg/mL and 10 µg/mL, respectively. This, however, is not a new finding; on the contrary, it was first described as early as 1683 in the famous letter written by Antony van Leeuwenhoek: "From hence I conclude, that the Vinegar with which I washt my Teeth, kill'd only those Animals which were on the outside of the scurf, but did not pass thro the whole substance of it."<sup>298</sup> Though it is now understood that the etiology behind this enhanced tolerance of biofilm-embedded bacteria toward antimicrobials is multifactorial,<sup>299</sup> we will focus our attention in this review on those aspects associated with the biofilm matrix, its structure, or its individual components.

First of all, a given antimicrobial needs to penetrate the biofilm (and its matrix) in order to reach bacteria in the deeper layers of the biofilm. The degree of penetration thereby depends on the thickness and the sorptive capacities of the biofilm, as well as on factors associated with the respective antimicrobial, such as its effective diffusivity within the biofilm, its reactivity with biofilm components, and its concentration and period of application.<sup>244,300</sup> Consequently, distinct antimicrobial agents will show differential patterns of distribution within a biofilm.<sup>301</sup> In a comprehensive review,<sup>244</sup> Stewart combined data from in vitro studies on antimicrobial penetration through biofilms and plotted the penetration times of antimicrobials that had been experimentally

measured in in vitro biofilms vs the molecular weight of the respective antimicrobials. He found two groups of agents exhibiting retarded penetration: (a) reactive oxidants, such as chlorine and hydrogen peroxide; and (b) cationic molecules, including some antibiotics, chlorhexidine, or quaternary ammonium compounds (eg, cetylpyridinium chloride). Stewart attributed their limited penetration characteristics to reactions and sorption of the agents with matrix components.<sup>244</sup> Accordingly, De Beer et al<sup>302</sup> showed that the limited antimicrobial activity of chlorine in biofilms from *Klebsiella pneumoniae* and *P. aeruginosa* was due to limited penetration stemming from reaction-diffusion interactions. They further suggested that the biofilm matrix with its extracellular polymeric substances may be the substrate for neutralization of chlorine. Tseng et al<sup>303</sup> found that the positively charged antibiotic tobramycin was sequestered in the outer layers of *P. aeruginosa* biofilms, whereas the neutral antibiotic ciprofloxacin readily penetrated the biofilms. As the penetration of tobramycin could be improved by addition of cations, the authors concluded that the reduced penetration may be due to ionic interactions of tobramycin with negatively charged components of the biofilm matrix. It has also been demonstrated by confocal microscopy that positively charged chlorhexidine only affected the outer layers of bacteria within biofilms formed in situ.<sup>304</sup> However, not only positive charges, but also hydrophobic interactions involving alkyl chains, such as those in quaternary ammonium compounds, may play a considerable role in binding or retention of antimicrobials during diffusion through biofilms.<sup>243</sup> Last but not least, the viscoelastic properties of a biofilm may play an important role not only in its mechanical stability, as discussed earlier, but also regarding penetration of antimicrobials throughout its structure due to relaxation-structure-composition relationships.<sup>305</sup>

Another important factor with regard to enhanced tolerance of biofilm-bacteria toward antimicrobials is their inactivation by enzymes that either originate from lysed bacteria owing to antimicrobial exposure or from active secretion by the bacteria in the biofilm via membrane vesicles.<sup>299,306</sup> In a landmark study, Anderl et al<sup>307</sup> showed that ampicillin was unable to penetrate *K. pneumoniae* biofilms owing to production of the ampicillin-degrading enzyme β-lactamase, whereas, conversely, ampicillin was able to readily penetrate biofilms formed by a mutant not producing β-lactamase. Interestingly, the biofilms formed by the mutant still showed enhanced tolerance to ampicillin, which clearly demonstrates the multifactorial mechanisms of enhanced tolerance exhibited by bacteria in biofilms.<sup>299,307</sup>

The majority of studies on antimicrobial treatment of biofilms reviewed herein have not been conducted on subgingival biofilms, and not even on biofilms formed by oral bacteria. With the emergence of in vitro models that allow culture of microcosm biofilms resembling complex subgingival microbial communities,<sup>308-311</sup> it should be a major goal for the future to use these models to investigate the effects of the subgingival biofilm matrix on penetration and inactivation of antimicrobials. For now, however, results from studies on other biofilms can only be extrapolated to subgingival biofilms: The

biofilm matrix with its respective structural components protects the embedded bacteria (at least in the inner layers of the biofilm) from penetration of antimicrobials, in particular if they carry positive charge. Furthermore, some agents may be inactivated during their diffusion throughout the biofilm structure. In consequence, this may lead to subinhibitory concentration of antimicrobials in the deeper layers of biofilms, which may pose the risk of inducing drug resistances in bacteria.<sup>280,312,313</sup>

## 6 | FUTURE PERSPECTIVES—TARGETING THE BIOFILM MATRIX?

As discussed already, it is clear that the biofilm matrix is a key factor with regard to the enhanced tolerance of biofilm-embedded bacteria toward mechanical removal, as well as toward antimicrobial approaches. The optimal approach would be to prevent the matrix forming in order to arrest the transition to a pathogenic biofilm state. Inhibition of streptococcal glucosyltransferases has been investigated for many years. Simple sugars, such as maltose, have been shown to inhibit enzyme activity.<sup>314</sup> More recently, small-molecule inhibitors have been identified that can reduce dental caries in animal models.<sup>315</sup> Once the matrix has formed, targeting the biofilm matrix and disintegrating its bonds and shielding effects will lead not only to dispersal of biofilms and release of planktonic cells but also to changes in gene expression in bacteria, potentially making them more susceptible toward antimicrobials.<sup>316</sup>

In this regard, matrix-degrading or biofilm-dispersing enzymes have been discussed as potential therapeutic agents.<sup>317</sup> Among these, Dispersin B and deoxyribonucleases have been the focus of recent research.<sup>301,318,319</sup> As already noted, Dispersin B is a glycoside hydrolase produced by *A. actinomycetemcomitans*<sup>319</sup> that has been shown to degrade the glycosidic linkages of polymeric  $\beta$ -1,6-*N*-acetyl-glucosamine (poly-*N*-acetyl-D-glucosamine).<sup>318</sup> Dispersin B detaches preformed biofilms from *A. actinomycetemcomitans*<sup>320</sup> and other poly-*N*-acetyl-D-glucosamine-producing bacteria, such as *S. epidermidis*.<sup>321,322</sup> Recently, it was shown that Dispersin B significantly inhibited colonization of *S. epidermidis* on porcine skin and detached preformed biofilms.<sup>323</sup> Although a combination of Dispersin B and triclosan has shown successful prevention of *S. aureus* colonization when used as a catheter coating in a rabbit model in vivo,<sup>324</sup> Dispersin B must still be considered to be in its preclinical stages of development.<sup>325</sup> In view of the wide range of polysaccharides that contribute to the biofilm matrix, it is likely that matrix degradation would require a combination of glycosyl hydrolases with different specificities.

Since the discovery by Whitchurch et al<sup>110</sup> that extracellular DNA is an important structural component within the biofilm matrix, the dispersing effects of exogenously applied deoxyribonucleases on bacterial biofilms have been investigated in many in vitro studies.<sup>115,116,326</sup> Younger biofilms seem to be more prone to dispersal by deoxyribonuclease than more mature ones are, whereas the latter may still be affected by increasing their antimicrobial

susceptibility.<sup>115,317,327,328</sup> Human recombinant deoxyribonuclease has already been employed clinically in the course of treating cystic fibrosis for at least two decades,<sup>111</sup> and is also emerging as a therapy for bacterial vaginosis.<sup>115,329</sup> Whereas application of human deoxyribonuclease on a wider scale is still limited by its high costs,<sup>115,317</sup> bacterially derived deoxyribonucleases, like NucB from *Bacillus licheniformis*, can be produced cost-effectively and have also already shown high potential in vitro.<sup>12</sup> As matrix-degrading enzymes do not kill bacteria, there should be little or no risk of induction of resistance to these agents.<sup>317</sup> However, potential immunogenic properties must be ruled out before any clinical application, such as in mouthwashes or toothpastes, for control of subgingival biofilms.<sup>316</sup>

Similarly, antibodies derived against the DNABII family that maintain the structural integrity of the extracellular DNA-dependent extracellular polymeric substances have been shown to disrupt biofilms of multiple bacterial species and have no apparent limitation due to the age of the biofilm.<sup>117</sup> Likewise, these same antibodies disrupt biofilms without killing the resident bacteria. Importantly, the newly released bacteria are four to eightfold more sensitive to antibiotics than the original planktonic bacteria that seeded the biofilm.<sup>117</sup> Since anti-DNABII antibodies are effective both in vitro and in vivo on *A. actinomycetemcomitans* periodontal biofilms,<sup>164</sup> using a cocktail of these antibodies and antimicrobials may be an interesting approach for adjunctive use in clinics.

Other interesting approaches targeting the biofilm matrix include bacteriophages, D-amino acids, modulation of cyclic dimeric guanosine monophosphate signaling pathways, or inhibitors of extracellular metabolic enzymes.<sup>316,317</sup> Bacteriophages have been shown to play important roles in biofilm development, and particularly in the phase of detachment. Some bacteriophages have been shown to incorporate polysaccharide depolymerase enzymes that can degrade the biofilm matrix of susceptible biofilms, leading to biofilm dispersal.<sup>330,331</sup> A bacteriophage engineered to express Dispersin B for simultaneously attacking bacterial cells and the biofilm matrix, showed a reduction of viable cell counts of about 4.5 log<sub>10</sub> units.<sup>332</sup> Kolodkin-Gal et al<sup>333</sup> showed that treatment of biofilms formed by *Bacillus subtilis* with D-amino acids (D-leucine, D-methionine, D-tyrosine, D-tryptophan) caused release of amyloid fibers that linked bacterial cells in biofilms together and suggested that production of D-amino acids in biofilms may be a general strategy for biofilm dispersal. This D-amino acid mixture has recently been shown to be effective in dispersing biofilms in dental unit waterlines.<sup>334</sup> Furthermore, D-leucine could effectively disperse biofilms of *E. faecalis* on human dentine slabs.<sup>335</sup> Cyclic dimeric guanosine monophosphate is one of the most important bacterial second messengers, playing a key role in the transition from the planktonic to the biofilm lifestyle of bacteria, whereby intracellular levels of cyclic dimeric guanosine monophosphate induce biofilm dispersal.<sup>336</sup> Therefore, inhibition of diguanylate cyclase, the enzyme synthesizing cyclic dimeric guanosine monophosphate, should promote biofilm dispersal.<sup>317,337</sup> As the cyclic dimeric guanosine monophosphate signaling system is found in bacteria but not in eukaryotic cells, it may be an attractive target for antibiofilm therapies.<sup>338</sup> However, the effects of diguanylate



cyclase inhibition are not easy to predict, as diguanylate cyclase comprises a large superfamily of enzymes with many homologues in some bacteria.<sup>317</sup> The protein component of the biofilm matrix includes a number of active enzymes that contribute to pathogenicity. Neuraminidase inhibitors, such as zanamivir, are employed in the treatment of influenza, and they have also shown potential for controlling bacterial pathogens, such as *Gardnerella vaginalis*.<sup>339</sup> It is possible that the selective inhibition of microbial biofilm matrix enzymes could modulate biofilm functions, such as the ability to stimulate inflammation.

Though biochemical approaches to control oral biofilms are important, it is likely that these will always be an adjunct to mechanical removal approaches. Brushing and flossing are the mainstay of oral hygiene, but they have limited efficacy in difficult-to-reach areas of the teeth. In addition, people with physical disabilities may find manual cleaning measures difficult to use. New approaches designed to overcome these limitations include high-velocity water microdroplets, which can physically remove biofilms in interproximal spaces.<sup>287</sup> More controlled physical cleaning may eventually be performed by robots. A recent report has described dual-function catalytic antimicrobial robots that are driven by magnets and are highly effective at eradicating biofilms *in vitro*.<sup>340</sup> It remains to be seen whether such sophisticated systems can be adapted for controlling dental plaque.

Overall, targeting the biofilm matrix seems to be a very elegant and exciting way to disrupt bacterial biofilms, such as those present in periodontal pockets, and additionally enhance the efficacy of concomitantly applied antimicrobials. Nevertheless, for most of the approaches described herein, research is still in its infancy, and many questions still need to be answered before these agents can be applied clinically. In addition, there are still many unanswered questions regarding the role of the biofilm matrix in the complex interplay between bacteria and host. In multispecies biofilms, the matrix will consist of many different classes of polymers, and numerous variants of each. Therefore, simplified models of oral biofilms are still critical to further unravel the structure and function of biofilm matrix components.

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## REFERENCES

- Mack WN, Mack JP, Ackerson AO. Microbial film development in a trickling filter. *Microb Ecol.* 1975;2:215-226.
- Jones HC, Roth IL, Sanders WM III. Electron microscopic study of a slime layer. *J Bacteriol.* 1969;99:316-325.
- Schroeder HE, de Boever J. The structure of microbial dental plaque. In: McHugh WD, ed. *Dental Plaque*. Dundee, UK: E. & S. Livingstone; 1969:49-74.
- Bennett HS. Morphological aspects of extracellular polysaccharides. *J Histochem Cytochem.* 1963;11:14-23.
- Ito S. Form and function of the glycocalyx on free cell surfaces. *Philos Trans R Soc Lond B Biol Sci.* 1974;268:55-66.
- Costerton JW, Irvin RT, Cheng KJ. The bacterial glycocalyx in nature and disease. *Annu Rev Microbiol.* 1981;35:299-324.
- Hoyle BD, Jass J, Costerton JW. The biofilm glycocalyx as a resistance factor. *J Antimicrob Chemother.* 1990;26:1-5.
- Bowen WH, Burne RA, Wu H, Koo H. Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. *Trends Microbiol.* 2018;26:229-242.
- Koo H, Falsetta ML, Klein MI. The exopolysaccharide matrix: a virulence determinant of cariogenic biofilm. *J Dent Res.* 2013;92:1065-1073.
- Klein MI, Falsetta ML, Xiao J, Bowen WH, Koo H. The role of extracellular polysaccharides matrix in virulent oral biofilms. In: Jakubovics NS, Palmer RJ Jr, eds. *Oral Microbial Ecology: Current Research and New Perspectives*. Wymondham, UK: Caister Academic Press; 2013:63-84.
- Holliday R, Preshaw PM, Bowen L, Jakubovics NS. The ultrastructure of subgingival dental plaque, revealed by high-resolution field emission scanning electron microscopy. *BDJ Open.* 2015;1:15003.
- Rostami N, Shields RC, Yassin SA, et al. A critical role for extracellular DNA in dental plaque formation. *J Dent Res.* 2017;96:208-216.
- Flemming HC, Wingender J. The biofilm matrix. *Nat Rev Microbiol.* 2010;8:623-633.
- Hannig C, Hannig M, Rehmer O, Braun G, Hellwig E, Al-Ahmad A. Fluorescence microscopic visualization and quantification of initial bacterial colonization on enamel *in situ*. *Arch Oral Biol.* 2007;52:1048-1056.
- Jung DJ, Al-Ahmad A, Follo M, et al. Visualization of initial bacterial colonization on dentine and enamel *in situ*. *J Microbiol Methods.* 2010;81:166-174.
- Flemming HC, Wuertz S. Bacteria and archaea on Earth and their abundance in biofilms. *Nat Rev Microbiol.* 2019;17:247-260.
- Schlafer S, Meyer RL. Confocal microscopy imaging of the biofilm matrix. *J Microbiol Methods.* 2017;138:50-59.
- Hotz P, Guggenheim B, Schmid R. Carbohydrates in pooled dental plaque. *Caries Res.* 1972;6:103-121.
- Wood JM. The state of hexose sugar in human dental plaque and its metabolism by the plaque bacteria. *Arch Oral Biol.* 1969;14:161-168.
- Busuioc M, Mackiewicz K, Buttaro BA, Piggot PJ. Role of intracellular polysaccharide in persistence of *Streptococcus mutans*. *J Bacteriol.* 2009;191:7315-7322.
- van Hijum SA, Kralj S, Ozimek LK, Dijkhuizen L, van Geel-Schutten IG. Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiol Mol Biol Rev.* 2006;70:157-176.
- Cury JA, Rebelo MA, Del Bel Cury AA, Derbyshire MT, Tabchoury CP. Biochemical composition and cariogenicity of dental plaque formed in the presence of sucrose or glucose and fructose. *Caries Res.* 2000;34:491-497.
- Critchley P, Wood JM, Saxton CA, Leach SA. The polymerisation of dietary sugars by dental plaque. *Caries Res.* 1967;1:112-129.

24. Bowen WH, Koo H. Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. *Caries Res.* 2011;45:69-86.
25. Banas JA, Vickerman MM. Glucan-binding proteins of the oral streptococci. *Crit Rev Oral Biol Med.* 2003;14:89-99.
26. Miehler JL, Larson MR, Schormann N, et al. Glucan binding protein C of *Streptococcus mutans* mediates both sucrose-independent and sucrose-dependent adherence. *Infect Immun.* 2018; e00146-18.
27. Rozen R, Bachrach G, Bronshteyn M, Gedalia I, Steinberg D. The role of fructans on dental biofilm formation by *Streptococcus sobrinus*, *Streptococcus mutans*, *Streptococcus gordonii* and *Actinomyces viscosus*. *FEMS Microbiol Lett.* 2001;195:205-210.
28. Yamaguchi M, Kawabata Y, Kambe S, et al. Non-invasive monitoring of gingival crevicular fluid for estimation of blood glucose level. *Med Biol Eng Comput.* 2004;42:322-327.
29. Tawakoli PN, Neu TR, Busck MM, et al. Visualizing the dental biofilm matrix by means of fluorescence lectin-binding analysis. *J Oral Microbiol.* 2017;9:1345581.
30. Nothhaft H, Szymanski CM. Bacterial protein N-glycosylation: new perspectives and applications. *J Biol Chem.* 2013;288:6912-6920.
31. Loimaranta V, Hytönen J, Pulliainen AT, et al. Leucine-rich repeats of bacterial surface proteins serve as common pattern recognition motifs of human scavenger receptor gp340. *J Biol Chem.* 2009;284:18614-18623.
32. Chen Y-YM, Chiang Y-C, Tseng T-Y, et al. Molecular and functional analysis of the type IV pilus gene cluster in *Streptococcus sanguinis* SK36. *Appl Environ Microbiol.* 2019;85:e02788-18.
33. Haiko J, Westerlund-Wikström B. The role of the bacterial flagellum in adhesion and virulence. *Biology.* 2013;2:1242-1267.
34. Parker JL, Lowry RC, Couto NA, Wright PC, Stafford GP, Shaw JG. Maf-dependent bacterial flagellin glycosylation occurs before chaperone binding and flagellar T3SS export. *Mol Microbiol.* 2014;92:258-272.
35. Siegel SD, Amer BR, Wu C, et al. Structure and mechanism of LcpA, a phosphotransferase that mediates glycosylation of a gram-positive bacterial cell wall-anchored protein. *MBio.* 2019;10:e01580-01518.
36. Peng Z, Fives-Taylor P, Ruiz T, et al. Identification of critical residues in Gap3 of *Streptococcus parasanguinis* involved in Fap1 glycosylation, fimbrial formation and in vitro adhesion. *BMC Microbiol.* 2008;8:52.
37. Wu H, Zeng M, Fives-Taylor P. The glycan moieties and the N-terminal polypeptide backbone of a fimbria-associated adhesin, Fap1, play distinct roles in the biofilm development of *Streptococcus parasanguinis*. *Infect Immun.* 2007;75:2181-2188.
38. Zhu F, Zhang H, Yang T, Haslam SM, Dell A, Wu H. Engineering and dissecting the glycosylation pathway of a streptococcal serine-rich repeat adhesin. *J Biol Chem.* 2016;291:27354-27363.
39. Siegel SD, Wu C, Ton-That H. A type I signal peptidase is required for pilus assembly in the gram-positive, biofilm-forming bacterium *Actinomyces oris*. *J Bacteriol.* 2016;198:2064-2073.
40. Serra DO, Richter AM, Klauck G, Mika F, Hengge R. Microanatomy at cellular resolution and spatial order of physiological differentiation in a bacterial biofilm. *MBio.* 2013;4:e00103-13.
41. Young NM, Brisson J-R, Kelly J, et al. Structure of the N-linked glycan present on multiple glycoproteins in the gram-negative bacterium, *Campylobacter jejuni*. *J Biol Chem.* 2002;277:42530-42539.
42. Merino S, Tomás JM. Gram-negative flagella glycosylation. *Int J Mol Sci.* 2014;15:2840-2857.
43. Parker JL, Day-Williams MJ, Tomas JM, Stafford GP, Shaw JG. Identification of a putative glycosyltransferase responsible for the transfer of pseudaminic acid onto the polar flagellin of *Aeromonas caviae* Sch3N. *Microbiologyopen.* 2012;1:149-160.
44. Zeituni AE, McCaig W, Scisci E, Thanassi DG, Cutler CW. The native 67-kilodalton minor fimbria of *Porphyromonas gingivalis* is a novel glycoprotein with DC-SIGN-targeting motifs. *J Bacteriol.* 2010;192:4103-4110.
45. Murakami Y, Hasegawa Y, Nagano K, Yoshimura F. Characterization of wheat germ agglutinin lectin-reactive glycosylated OmpA-like proteins derived from *Porphyromonas gingivalis*. *Infect Immun.* 2014;82:4563-4571.
46. Rangarajan M, Hashim A, Aduse-Opoku J, Paramonov N, Hounsell EF, Curtis MA. Expression of Arg-gingipain RgpB is required for correct glycosylation and stability of monomeric Arg-gingipain RgpA from *Porphyromonas gingivalis* W50. *Infect Immun.* 2005;73:4864-4878.
47. Tang G, Mintz KP. Glycosylation of the collagen adhesin EmaA of *Aggregatibacter actinomycetemcomitans* is dependent upon the lipopolysaccharide biosynthetic pathway. *J Bacteriol.* 2010;192:1395-1404.
48. Schuster B, Sleytr UB. Relevance of glycosylation of S-layer proteins for cell surface properties. *Acta Biomater.* 2015;19:149-157.
49. Sleytr UB, Schuster B, Egelseer E-M, Pum D. S-layers: principles and applications. *FEMS Microbiol Rev.* 2014;38:823-864.
50. Messner P, Steiner K, Zarschler K, Schäffer C. S-layer nanoglycobiology of bacteria. *Carbohydr Res.* 2008;343:1934-1951.
51. Thompson SA. *Campylobacter* surface-layers (S-layers) and immune evasion. *Ann Periodontol.* 2002;7:43-53.
52. Posch G, Sekot G, Friedrich V, et al. Glycobiology aspects of the periodontal pathogen *Tannerella forsythia*. *Biomolecules.* 2012;2:467-482.
53. Pham TK, Roy S, Noirel J, Douglas I, Wright PC, Stafford GP. A quantitative proteomic analysis of biofilm adaptation by the periodontal pathogen *Tannerella forsythia*. *Proteomics.* 2010;10:3130-3141.
54. Narita Y, Sato K, Yukitake H, et al. Lack of a surface layer in *Tannerella forsythia* mutants deficient in the type IX secretion system. *Microbiology.* 2014;160:2295-2303.
55. Sakakibara J, Nagano K, Murakami Y, et al. Loss of adherence ability to human gingival epithelial cells in S-layer protein-deficient mutants of *Tannerella forsythensis*. *Microbiology.* 2007;153:866-876.
56. Friedrich V, Janesch B, Windwarder M, et al. *Tannerella forsythia* strains display different cell-surface nonulosonic acids: biosynthetic pathway characterization and first insight into biological implications. *Glycobiology.* 2017;27:342-357.
57. Renner LD, Weibel DB. Physicochemical regulation of biofilm formation. *MRS Bull.* 2011;36:347-355.
58. Ostapska H, Howell PL, Sheppard DC. Deacetylated microbial biofilm exopolysaccharides: it pays to be positive. *PLoS Pathog.* 2018;14:e1007411.
59. Skov Sørensen UB, Yao K, Yang Y, Tettelin H, Kilian M. Capsular polysaccharide expression in commensal *Streptococcus* species: genetic and antigenic similarities to *Streptococcus pneumoniae*. *MBio.* 2016;7:e01844-16.
60. Rukke HV, Hegna IK, Petersen FC. Identification of a functional capsule locus in *Streptococcus mitis*. *Mol Oral Microbiol.* 2012;27:95-108.
61. Rukke HV, Engen SA, Schenck K, Petersen FC. Capsule expression in *Streptococcus mitis* modulates interaction with oral keratinocytes and alters susceptibility to human antimicrobial peptides. *Mol Oral Microbiol.* 2016;31:302-313.
62. Rukke HV, Kalluru RS, Repnik U, et al. Protective role of the capsule and impact of serotype 4 switching on *Streptococcus mitis*. *Infect Immun.* 2014;82:3790-3801.
63. Yang J, Yoshida Y, Cisar JO. Genetic basis of coaggregation receptor polysaccharide biosynthesis in *Streptococcus sanguinis* and related species. *Mol Oral Microbiol.* 2014;29:24-31.
64. Cisar JO, Sandberg AL, Reddy GP, Abeygunawardana C, Bush CA. Structural and antigenic types of cell wall polysaccharides from

- viridans group streptococci with receptors for oral actinomyces and streptococcal lectins. *Infect Immun.* 1997;65:5035-5041.
65. Yang J, Cisar JO, Bush CA. Structure of type 3Gn coaggregation receptor polysaccharide from *Streptococcus cristatus* LS4. *Carbohydr Res.* 2011;346:1342-1346.
  66. Back CR, Douglas SK, Emerson JE, Nobbs AH, Jenkinson HF. *Streptococcus gordonii* DL1 adhesin SspB V-region mediates coaggregation via receptor polysaccharide of *Actinomyces oris* T14V. *Mol Oral Microbiol.* 2015;30:411-424.
  67. Nakano K, Ooshima T. Serotype classification of *Streptococcus mutans* and its detection outside the oral cavity. *Future Microbiol.* 2009;4:891-902.
  68. Kovacs CJ, Faustoferri RC, Quivey RG Jr. RgpF is required for maintenance of stress tolerance and virulence in *Streptococcus mutans*. *J Bacteriol.* 2017;199:e00497-00417.
  69. Vernal R, Diaz-Guerra E, Silva A, Sanz M, Garcia-Sanz JA. Distinct human T-lymphocyte responses triggered by *Porphyromonas gingivalis* capsular serotypes. *J Clin Periodontol.* 2014;41:19-30.
  70. Vernal R, Leon R, Silva A, van Winkelhoff AJ, Garcia-Sanz JA, Sanz M. Differential cytokine expression by human dendritic cells in response to different *Porphyromonas gingivalis* capsular serotypes. *J Clin Periodontol.* 2009;36:823-829.
  71. Singh A, Wyant T, Anaya-Bergman C, et al. The capsule of *Porphyromonas gingivalis* leads to a reduction in the host inflammatory response, evasion of phagocytosis, and increase in virulence. *Infect Immun.* 2011;79:4533-4542.
  72. Davey ME, Duncan MJ. Enhanced biofilm formation and loss of capsule synthesis: deletion of a putative glycosyltransferase in *Porphyromonas gingivalis*. *J Bacteriol.* 2006;188:5510-5523.
  73. Polak D, Ferdman O, Hourri-Haddad Y. *Porphyromonas gingivalis* capsule-mediated coaggregation as a virulence factor in mixed infection with *Fusobacterium nucleatum*. *J Periodontol.* 2017;88:502-510.
  74. Shah SA, Ganesan SM, Varadharaj S, Dabdoub SM, Walters JD, Kumar PS. The making of a miscreant: tobacco smoke and the creation of pathogen-rich biofilms. *NPJ Biofilms Microbiomes.* 2017;3:26.
  75. Alberti-Segui C, Arndt A, Cugini C, Priyadarshini R, Davey ME. HU protein affects transcription of surface polysaccharide synthesis genes in *Porphyromonas gingivalis*. *J Bacteriol.* 2010;192:6217-6229.
  76. Mack D, Fischer W, Krokotsch A, et al. The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis. *J Bacteriol.* 1996;178:175-183.
  77. Izano EA, Sadovskaya I, Wang H, et al. Poly-N-acetylglucosamine mediates biofilm formation and detergent resistance in *Aggregatibacter actinomycetemcomitans*. *Microb Pathog.* 2008;44:52-60.
  78. Kaplan JB, Velliyagounder K, Ragunath C, et al. Genes involved in the synthesis and degradation of matrix polysaccharide in *Actinobacillus actinomycetemcomitans* and *Actinobacillus pleuropneumoniae* biofilms. *J Bacteriol.* 2004;186:8213-8220.
  79. Shanmugam M, Oyeniyi AO, Parthiban C, Gujjarlupudi SK, Pier GB, Ramasubbu N. Role of de-N-acetylase PgaB from *Aggregatibacter actinomycetemcomitans* in exopolysaccharide export in biofilm mode of growth. *Mol Oral Microbiol.* 2017;32:500-510.
  80. Shanmugam M, Gopal P, El Abbar F, et al. Role of exopolysaccharide in *Aggregatibacter actinomycetemcomitans*-induced bone resorption in a rat model for periodontal disease. *PLoS One.* 2015;10:e0117487.
  81. Stacy A, Everett J, Jorth P, Trivedi U, Rumbaugh KP, Whiteley M. Bacterial fight-and-flight responses enhance virulence in a polymicrobial infection. *Proc Natl Acad Sci USA.* 2014;111:7819-7824.
  82. Brown S, Santa Maria JP Jr, Walker S. Wall teichoic acids of gram-positive bacteria. *Annu Rev Microbiol.* 2013;67:313-336.
  83. Gross M, Cramton SE, Gotz F, Peschel A. Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. *Infect Immun.* 2001;69:3423-3426.
  84. Sadovskaya I, Vinogradov E, Li J, Jabbouri S. Structural elucidation of the extracellular and cell-wall teichoic acids of *Staphylococcus epidermidis* RP62A, a reference biofilm-positive strain. *Carbohydr Res.* 2004;339:1467-1473.
  85. Sadovskaya I, Vinogradov E, Flahaut S, Kogan G, Jabbouri S. Extracellular carbohydrate-containing polymers of a model biofilm-producing strain, *Staphylococcus epidermidis* RP62A. *Infect Immun.* 2005;73:3007-3017.
  86. Brauge T, Sadovskaya I, Faïlle C, et al. Teichoic acid is the major polysaccharide present in the *Listeria monocytogenes* biofilm matrix. *FEMS Microbiol Lett.* 2016;363:fnv229.
  87. Rolla G, Oppermann RV, Bowen WH, Ciardi JE, Knox KW. High amounts of lipoteichoic acid in sucrose-induced plaque in vivo. *Caries Res.* 1980;14:235-238.
  88. Kuramitsu HK, Wondrack L, McGuinness M. Interaction of *Streptococcus mutans* glucosyltransferases with teichoic acids. *Infect Immun.* 1980;29:376-382.
  89. Vickerman MM, Jones GW. Adhesion of glucosyltransferase phase variants to *Streptococcus gordonii* bacterium-glucan substrata may involve lipoteichoic acid. *Infect Immun.* 1992;60:4301-4308.
  90. Ahn KB, Baik JE, Park OJ, Yun CH, Han SH. *Lactobacillus plantarum* lipoteichoic acid inhibits biofilm formation of *Streptococcus mutans*. *PLoS One.* 2018;13:e0192694.
  91. Kim AR, Ahn KB, Yun CH, et al. *Lactobacillus plantarum* lipoteichoic acid inhibits oral multispecies biofilm. *J Endod.* 2019;45:310-315.
  92. Klein MI, Xiao J, Lu B, Delahunty CM, Yates JR 3rd, Koo H. *Streptococcus mutans* protein synthesis during mixed-species biofilm development by high-throughput quantitative proteomics. *PLoS One.* 2012;7:e45795.
  93. Castillo Pedraza MC, Novais TF, Faustoferri RC, et al. Extracellular DNA and lipoteichoic acids interact with exopolysaccharides in the extracellular matrix of *Streptococcus mutans* biofilms. *Biofouling.* 2017;33:722-740.
  94. Lamont RJ, Koo H, Hajishengallis G. The oral microbiota: dynamic communities and host interactions. *Nat Rev Microbiol.* 2018;16:745-759.
  95. Canabarro A, Valle C, Farias MR, Santos FB, Lazera M, Wanke B. Association of subgingival colonization of *Candida albicans* and other yeasts with severity of chronic periodontitis. *J Periodontol Res.* 2013;48:428-432.
  96. Diaz PI, Hong BY, Dupuy AK, Strausbaugh LD. Mining the oral mycobiome: methods, components, and meaning. *Virulence.* 2017;8:313-323.
  97. Hong B-Y, Hoare A, Cardenas A, et al. The salivary mycobiome contains 2 ecologically distinct mycotypes. *J Dent Res.* 2020;99:730-738.
  98. Zarnowski R, Westler WM, Lacmbouh GA, et al. Novel entries in a fungal biofilm matrix encyclopedia. *MBio.* 2014;5:e01333-14.
  99. Mitchell KF, Zarnowski R, Andes DR. Fungal super glue: the biofilm matrix and its composition, assembly, and functions. *PLoS Pathog.* 2016;12:e1005828.
  100. Mitchell KF, Zarnowski R, Sanchez H, et al. Community participation in biofilm matrix assembly and function. *Proc Natl Acad Sci USA.* 2015;112:4092-4097.
  101. Dominguez E, Zarnowski R, Sanchez H, et al. Conservation and divergence in the *Candida* species biofilm matrix mannan-glucan complex structure, function, and genetic control. *MBio.* 2018;9:e00451-18.
  102. Ali Mohammed MM, Nerland AH, Al-Haroni M, Bakken V. Characterization of extracellular polymeric matrix, and treatment

- of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* biofilms with DNase I and proteinase K. *J Oral Microbiol.* 2013;5:20015.
103. Hisano K, Fujise O, Miura M, Hamachi T, Matsuzaki E, Nishimura F. The *pga* gene cluster in *Aggregatibacter actinomycetemcomitans* is necessary for the development of natural competence in Ca<sup>2+</sup>-promoted biofilms. *Mol Oral Microbiol.* 2014;29:79-89.
  104. Palmer LJ, Chapple IL, Wright HJ, Roberts A, Cooper PR. Extracellular deoxyribonuclease production by periodontal bacteria. *J Periodontol Res.* 2012;47:439-445.
  105. Griffith F. The significance of pneumococcal types. *J Hyg (Lond).* 1928;27:113-159.
  106. Avery OT, Macleod CM, McCarty M. Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. *J Exp Med.* 1944;79:137-158.
  107. Murakawa T. Slime production by *Pseudomonas aeruginosa*. 3. Purification of slime and its physicochemical properties. *Jpn J Microbiol.* 1973;17:273-281.
  108. Murakawa T. Slime production by *Pseudomonas aeruginosa*. IV. Chemical analysis of two varieties of slime produced by *Pseudomonas aeruginosa*. *Jpn J Microbiol.* 1973;17:513-520.
  109. Smithies WR, Gibbons NE. The deoxyribose nucleic acid slime layer of some halophilic bacteria. *Can J Microbiol.* 1955;1:614-621.
  110. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. *Science.* 2002;295:1487.
  111. Shak S, Capon DJ, Hellmiss R, Marsters SA, Baker CL. Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. *Proc Natl Acad Sci USA.* 1990;87:9188-9192.
  112. Yang C, Montgomery M. Dornase alfa for cystic fibrosis. *Cochrane Database Syst Rev.* 2018;9:CD001127.
  113. Baelo A, Levato R, Julian E, et al. Disassembling bacterial extracellular matrix with DNase-coated nanoparticles to enhance antibiotic delivery in biofilm infections. *J Control Release.* 2015;209:150-158.
  114. Ohta H, Kato K, Kokeguchi S, Hara H, Fukui K, Murayama Y. Nuclease-sensitive binding of an *Actinobacillus actinomycetemcomitans* leukotoxin to the bacterial cell surface. *Infect Immun.* 1991;59:4599-4605.
  115. Okshevsky M, Regina VR, Meyer RL. Extracellular DNA as a target for biofilm control. *Curr Opin Biotechnol.* 2015;33:73-80.
  116. Jakubovics NS, Burgess JG. Extracellular DNA in oral microbial biofilms. *Microbes Infect.* 2015;17:531-537.
  117. Brockson ME, Novotny LA, Mokrzan EM, et al. Evaluation of the kinetics and mechanism of action of anti-integration host factor-mediated disruption of bacterial biofilms. *Mol Microbiol.* 2014;93:1246-1258.
  118. Ibáñez de Aldecoa AL, Zafra O, González-Pastor JE. Mechanisms and regulation of extracellular DNA release and its biological roles in microbial communities. *Front Microbiol.* 2017;8:1390.
  119. Martins M, Uppuluri P, Thomas DP, et al. Presence of extracellular DNA in the *Candida albicans* biofilm matrix and its contribution to biofilms. *Mycopathologia.* 2010;169:323-331.
  120. Lindahl T, Nyberg B. Rate of depurination of native deoxyribonucleic acid. *Biochemistry.* 1972;11:3610-3618.
  121. Shore D, Langowski J, Baldwin RL. DNA flexibility studied by covalent closure of short fragments into circles. *Proc Natl Acad Sci USA.* 1981;78:4833-4837.
  122. Wells RD. Unusual DNA structures. *J Biol Chem.* 1988;263:1095-1098.
  123. Bloomfield VA. Condensation of DNA by multivalent cations: considerations on mechanism. *Biopolymers.* 1991;31:1471-1481.
  124. Barnes AM, Ballering KS, Leibman RS, Wells CL, Dunne GM. *Enterococcus faecalis* produces abundant extracellular structures containing DNA in the absence of cell lysis during early biofilm formation. *MBio.* 2012;3:e00193-12.
  125. Böckelmann U, Janke A, Kuhn R, et al. Bacterial extracellular DNA forming a defined network-like structure. *FEMS Microbiol Lett.* 2006;262:31-38.
  126. Huseby MJ, Kruse AC, Digre J, et al. Beta toxin catalyzes formation of nucleoprotein matrix in staphylococcal biofilms. *Proc Natl Acad Sci USA.* 2010;107:14407-14412.
  127. Klein MI, Hwang G, Santos PHS, Campanella OH, Koo H. *Streptococcus mutans*-derived extracellular matrix in cariogenic oral biofilms. *Front Cell Infect Microbiol.* 2015;5:10.
  128. Das T, Sharma PK, Busscher HJ, van der Mei HC, Krom BP. Role of extracellular DNA in initial bacterial adhesion and surface aggregation. *Appl Environ Microbiol.* 2010;76:3405-3408.
  129. Regina VR, Lokanathan AR, Modrzynski JJ, Sutherland DS, Meyer RL. Surface physicochemistry and ionic strength affects eDNA's role in bacterial adhesion to abiotic surfaces. *PLoS One.* 2014;9:e105033.
  130. Goodman SD, Obergfell KP, Jurcisek JA, et al. Biofilms can be dispersed by focusing the immune system on a common family of bacterial nucleoid-associated proteins. *Mucosal Immunol.* 2011;4:625-637.
  131. Devaraj A, Buzzo JR, Mashburn-Warren L, et al. The extracellular DNA lattice of bacterial biofilms is structurally related to Holliday junction recombination intermediates. *Proc Natl Acad Sci USA.* 2019;116:25068-25077.
  132. Fong JN, Yildiz FH. Biofilm matrix proteins. *Microbiol Spectr.* 2015;3. <https://doi.org/10.1128/microbiolspec.MB-0004-2014>
  133. Paes Leme AF, Bellato CM, Bedi G, Cury AA, Koo H, Cury JA. Effects of sucrose on the extracellular matrix of plaque-like biofilm formed in vivo, studied by proteomic analysis. *Caries Res.* 2008;42:435-443.
  134. Hobley L, Harkins C, MacPhee CE, Stanley-Wall NR. Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. *FEMS Microbiol Rev.* 2015;39:649-669.
  135. Blanco LP, Evans ML, Smith DR, Badtke MP, Chapman MR. Diversity, biogenesis and function of microbial amyloids. *Trends Microbiol.* 2012;20:66-73.
  136. Besingi RN, Wenderska IB, Senadheera DB, et al. Functional amyloids in *Streptococcus mutans*, their use as targets of biofilm inhibition and initial characterization of SMU\_63c. *Microbiology.* 2017;163:488-501.
  137. Jakubovics NS, Strömberg N, van Dolleweerd CJ, Kelly CG, Jenkinson HF. Differential binding specificities of oral streptococcal antigen I/II family adhesins for human or bacterial ligands. *Mol Microbiol.* 2005;55:1591-1605.
  138. Nobbs AH, Lamont RJ, Jenkinson HF. *Streptococcus* adherence and colonization. *Microbiol Mol Biol Rev.* 2009;73:407-450.
  139. Gurung I, Spielman I, Davies MR, et al. Functional analysis of an unusual type IV pilus in the gram-positive *Streptococcus sanguinis*. *Mol Microbiol.* 2016;99:380-392.
  140. Berry JL, Gurung I, Anonsen JH, et al. Global biochemical and structural analysis of the type IV pilus from the gram-positive bacterium *Streptococcus sanguinis*. *J Biol Chem.* 2019;294:6796-6808.
  141. Amano A, Fujiwara T, Nagata H, et al. *Porphyromonas gingivalis* fimbriae mediate coaggregation with *Streptococcus oralis* through specific domains. *J Dent Res.* 1997;76:852-857.
  142. El-Awady A, de Sousa RM, Meghil MM, et al. Polymicrobial synergy within oral biofilm promotes invasion of dendritic cells and survival of consortia members. *NPJ Biofilms Microbiomes.* 2019;5:11.
  143. Park Y, Simionato MR, Sekiya K, et al. Short fimbriae of *Porphyromonas gingivalis* and their role in coadhesion with *Streptococcus gordonii*. *Infect Immun.* 2005;73:3983-3989.
  144. Sharma A, Inagaki S, Honma K, Sfintescu C, Baker PJ, Evans RT. *Tannerella forsythia*-induced alveolar bone loss in mice involves leucine-rich-repeat BspA protein. *J Dent Res.* 2005;84:462-467.

145. Inagaki S, Kuramitsu HK, Sharma A. Contact-dependent regulation of a *Tannerella forsythia* virulence factor, BspA, in biofilms. *FEMS Microbiol Lett.* 2005;249:291-296.
146. Robyt JF, Martin PJ. Mechanism of synthesis of D-glucans by D-glucosyltransferases from *Streptococcus mutans* 6715. *Carbohydr Res.* 1983;113:301-315.
147. Potempa J, Banbula A, Travis J. Role of bacterial proteinases in matrix destruction and modulation of host responses. *Periodontol 2000.* 2000;24:153-192.
148. Yongqing T, Potempa J, Pike RN, Wijeyewickrema LC. The lysine-specific gingipain of *Porphyromonas gingivalis*: importance to pathogenicity and potential strategies for inhibition. *Adv Exp Med Biol.* 2011;712:15-29.
149. Mohammed WK, Krasnogor N, Jakubovics NS. *Streptococcus gordonii* Challisin protease is required for sensing cell-cell contact with *Actinomyces oris*. *FEMS Microbiol Ecol.* 2018;94:fiy043.
150. Dominy SS, Lynch C, Ermini F, et al. *Porphyromonas gingivalis* in Alzheimer's disease brains: evidence for disease causation and treatment with small-molecule inhibitors. *Sci Adv.* 2019;5:eaau3333.
151. Manuel SG, Ragunath C, Sait HB, Izano EA, Kaplan JB, Ramasubbu N. Role of active-site residues of dispersin B, a biofilm-releasing  $\beta$ -hexosaminidase from a periodontal pathogen, in substrate hydrolysis. *FEBS J.* 2007;274:5987-5999.
152. Ogawa A, Furukawa S, Fujita S, et al. Inhibition of *Streptococcus mutans* biofilm formation by *Streptococcus salivarius* FruA. *Appl Environ Microbiol.* 2011;77:1572-1580.
153. Roy S, Honma K, Douglas CW, Sharma A, Stafford GP. Role of sialidase in glycoprotein utilization by *Tannerella forsythia*. *Microbiology.* 2011;157:3195-3202.
154. Wong A, Grau MA, Singh AK, Woodiga SA, King SJ. Role of neuraminidase-producing bacteria in exposing cryptic carbohydrate receptors for *Streptococcus gordonii* adherence. *Infect Immun.* 2018;86:e00068-18.
155. Byers HL, Homer KA, Beighton D. Utilization of sialic acid by viridans streptococci. *J Dent Res.* 1996;75:1564-1571.
156. Harty DW, Chen Y, Simpson CL, et al. Characterisation of a novel homodimeric N-acetyl- $\beta$ -D-glucosaminidase from *Streptococcus gordonii*. *Biochem Biophys Res Commun.* 2004;319:439-447.
157. Roy S, Phansopa C, Stafford P, et al. Beta-hexosaminidase activity of the oral pathogen *Tannerella forsythia* influences biofilm formation on glycoprotein substrates. *FEMS Immunol Med Microbiol.* 2012;65:116-120.
158. Frey AM, Satur MJ, Phansopa C, et al. Evidence for a carbohydrate-binding module (CBM) of *Tannerella forsythia* NanH sialidase, key to interactions at the host-pathogen interface. *Biochem J.* 2018;475:1159-1176.
159. Megson ZA, Koerdts A, Schuster H, et al. Characterization of an  $\alpha$ -L-fucosidase from the periodontal pathogen *Tannerella forsythia*. *Virulence.* 2015;6:282-292.
160. Wickström C, Hamilton IR, Svensäter G. Differential metabolic activity by dental plaque bacteria in association with two preparations of MUC5B mucins in solution and in biofilms. *Microbiology.* 2009;155:53-60.
161. Wickström C, Herzberg MC, Beighton D, Svensäter G. Proteolytic degradation of human salivary MUC5B by dental biofilms. *Microbiology.* 2009;155:2866-2872.
162. Dey D, Nagaraja V, Ramakumar S. Structural and evolutionary analyses reveal determinants of DNA binding specificities of nucleoid-associated proteins HU and IHF. *Mol Phylogenet Evol.* 2017;107:356-366.
163. Tjokro NO, Rocco CJ, Priyadarshini R, Davey ME, Goodman SD. A biochemical analysis of the interaction of *Porphyromonas gingivalis* HU PG0121 protein with DNA. *PLoS One.* 2014;9:e93266.
164. Freire MO, Devaraj A, Young A, et al. A bacterial-biofilm-induced oral osteolytic infection can be successfully treated by immunotargeting an extracellular nucleoid-associated protein. *Mol Oral Microbiol.* 2017;32:74-88.
165. Rocco CJ, Bakaletz LO, Goodman SD. Targeting the HU $\beta$  protein prevents *Porphyromonas gingivalis* from entering into preexisting biofilms. *J Bacteriol.* 2018;200:e00790-00717.
166. Rocco CJ, Davey ME, Bakaletz LO, Goodman SD. Natural antigenic differences in the functionally equivalent extracellular DNABII proteins of bacterial biofilms provide a means for targeted biofilm therapeutics. *Mol Oral Microbiol.* 2017;32:118-130.
167. Devaraj A, Buzzo J, Rocco CJ, Bakaletz LO, Goodman SD. The DNABII family of proteins is comprised of the only nucleoid associated proteins required for nontypeable *Haemophilus influenzae* biofilm structure. *Microbiologyopen.* 2018;7:e00563.
168. Tursi SA, Lee EY, Medeiros NJ, et al. Bacterial amyloid curli acts as a carrier for DNA to elicit an autoimmune response via TLR2 and TLR9. *PLoS Pathog.* 2017;13:e1006315.
169. White PC, Chicca IJ, Cooper PR, Milward MR, Chapple IL. Neutrophil extracellular traps in periodontitis: a web of intrigue. *J Dent Res.* 2016;95:26-34.
170. Liu J, Sun L, Liu W, et al. A nuclease from *Streptococcus mutans* facilitates biofilm dispersal and escape from killing by neutrophil extracellular traps. *Front Cell Infect Microbiol.* 2017;7:97.
171. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. *Science.* 2004;303:1532-1535.
172. Jass J, Phillips LE, Allan EJ, Costerton JW, Lappin-Scott HM. Growth and adhesion of *Enterococcus faecium* L-forms. *FEMS Microbiol Lett.* 1994;115:157-162.
173. Shah IM, Laaberki M-H, Popham DL, Dworkin J. A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. *Cell.* 2008;135:486-496.
174. Xu XL, Lee RT, Fang HM, et al. Bacterial peptidoglycan triggers *Candida albicans* hyphal growth by directly activating the adenylyl cyclase Cyr1p. *Cell Host Microbe.* 2008;4:28-39.
175. Korgaonkar A, Trivedi U, Rumbaugh KP, Whiteley M. Community surveillance enhances *Pseudomonas aeruginosa* virulence during polymicrobial infection. *Proc Natl Acad Sci USA.* 2013;110:1059-1064.
176. Kishimoto T, Kaneko T, Ukai T, et al. Peptidoglycan and lipopolysaccharide synergistically enhance bone resorption and osteoclastogenesis. *J Periodontol Res.* 2012;47:446-454.
177. Janket S-J, Javaheri H, Ackerson LK, Ayilavarapu S, Meurman JH. Oral infections, metabolic inflammation, genetics, and cardiometabolic diseases. *J Dent Res.* 2015;94:1195-1275.
178. Frank RM, Houver G. An ultrastructural study of human supragingival dental plaque formation. In: McHugh WD, ed. *Dental Plaque: A Symposium of the University of Dundee*. Dundee, UK: D. C. Thomson; 1970:85-108.
179. Lee KJ, Lee MA, Hwang W, Park H, Lee KH. Deacylated lipopolysaccharides inhibit biofilm formation by gram-negative bacteria. *Biofouling.* 2016;32:711-723.
180. Heller D, Helmerhorst EJ, Oppenheim FG. Saliva and serum protein exchange at the tooth enamel surface. *J Dent Res.* 2017;96:437-443.
181. Hannig C, Hannig M, Kensche A, Carpenter G. The mucosal pellicle—an underestimated factor in oral physiology. *Arch Oral Biol.* 2017;80:144-152.
182. Carpenter G. Salivary factors that maintain the normal oral commensal microflora. *J Dent Res.* 2020;99:644-649.
183. Nobbs AH, Jenkinson HF, Jakubovics NS. Stick to your gums: mechanisms of oral microbial adherence. *J Dent Res.* 2011;90:1271-1278.
184. Cross BW, Ruhl S. Glycan recognition at the saliva-oral microbiome interface. *Cell Immunol.* 2018;333:19-33.

185. Liang X, Liu B, Zhu F, et al. A distinct sortase SrtB anchors and processes a streptococcal adhesin AbpA with a novel structural property. *Sci Rep*. 2016;6:30966.
186. Nobbs AH, Vajna RM, Johnson JR, et al. Consequences of a sortase A mutation in *Streptococcus gordonii*. *Microbiology*. 2007;153:4088-4097.
187. Bitto NJ, Chapman R, Pidot S, et al. Bacterial membrane vesicles transport their DNA cargo into host cells. *Sci Rep*. 2017;7:7072.
188. Pride DT, Salzman J, Haynes M, et al. Evidence of a robust resident bacteriophage population revealed through analysis of the human salivary virome. *ISME J*. 2012;6:915-926.
189. Halhoul N, Colvin JR. Virus-like particles in association with a microorganism from human gingival plaque. *Arch Oral Biol*. 1975;20:833-836.
190. Radini A, Nikita E, Buckley S, Copeland L, Hardy K. Beyond food: the multiple pathways for inclusion of materials into ancient dental calculus. *Am J Phys Anthropol*. 2017;162(Suppl 63):71-83.
191. Jakubovics NS, Kolenbrander PE. The road to ruin: the formation of disease-associated oral biofilms. *Oral Dis*. 2010;16:729-739.
192. Tinanoff N, Gross A. Epithelial cells associated with the development of dental plaque. *J Dent Res*. 1976;55:580-583.
193. Doster RS, Rogers LM, Gaddy JA, Aronoff DM. Macrophage extracellular traps: a scoping review. *J Innate Immun*. 2018;10:3-13.
194. Vitkov L, Klappacher M, Hannig M, Krautgartner WD. Extracellular neutrophil traps in periodontitis. *J Periodontol Res*. 2009;44:664-672.
195. Hirschfeld J, Dommisch H, Skora P, et al. Neutrophil extracellular trap formation in supragingival biofilms. *Int J Med Microbiol*. 2015;305:453-463.
196. Grohmann E, Christie PJ, Waksman G, Backert S. Type IV secretion in gram-negative and gram-positive bacteria. *Mol Microbiol*. 2018;107:455-471.
197. Zweig M, Schork S, Koerdt A, et al. Secreted single-stranded DNA is involved in the initial phase of biofilm formation by *Neisseria gonorrhoeae*. *Environ Microbiol*. 2014;16:1040-1052.
198. Jurgisick JA, Brockman KL, Novotny LA, Goodman SD, Bakaletz LO. Nontypeable *Haemophilus influenzae* releases DNA and DNABII proteins via a T4SS-like complex and ComE of the type IV pilus machinery. *Proc Natl Acad Sci USA*. 2017;114:E6632-E6641.
199. Zeidan AA, Poulsen VK, Janzen T, et al. Polysaccharide production by lactic acid bacteria: from genes to industrial applications. *FEMS Microbiol Rev*. 2017;41:S168-S200.
200. Whitney JC, Howell PL. Synthase-dependent exopolysaccharide secretion in gram-negative bacteria. *Trends Microbiol*. 2013;21:63-72.
201. Gunasinghe SD, Webb CT, Elgass KD, Hay ID, Lithgow T. Super-resolution imaging of protein secretion systems and the cell surface of gram-negative bacteria. *Front Cell Infect Microbiol*. 2017;7:220.
202. Desvaux M, Hebraud M, Talon R, Henderson IR. Secretion and subcellular localizations of bacterial proteins: a semantic awareness issue. *Trends Microbiol*. 2009;17:139-145.
203. Lasica AM, Ksiazek M, Madej M, Potempa J. The type IX secretion system (T9SS): highlights and recent insights into its structure and function. *Front Cell Infect Microbiol*. 2017;7:215.
204. Yoshida Y, Yang J, Peaker PE, Kato H, Bush CA, Cisar JO. Molecular and antigenic characterization of a *Streptococcus oralis* coaggregation receptor polysaccharide by carbohydrate engineering in *Streptococcus gordonii*. *J Biol Chem*. 2008;283:12654-12664.
205. Shoji M, Yukitake H, Sato K, et al. Identification of an O-antigen chain length regulator, WzzP, in *Porphyromonas gingivalis*. *Microbiol Open*. 2013;2:383-401.
206. Islam ST, Lam JS. Wzx flippase-mediated membrane translocation of sugar polymer precursors in bacteria. *Environ Microbiol*. 2013;15:1001-1015.
207. Shibata Y, Yamashita Y, Ozaki K, Nakano Y, Koga T. Expression and characterization of streptococcal *rgp* genes required for rhamnan synthesis in *Escherichia coli*. *Infect Immun*. 2002;70:2891-2898.
208. Sheppard DC, Howell PL. Biofilm exopolysaccharides of pathogenic fungi: lessons from bacteria. *J Biol Chem*. 2016;291:12529-12537.
209. Fonzi WA. The protein secretory pathway of *Candida albicans*. *Mycoses*. 2009;52:291-303.
210. Natale P, Bruser T, Driessen AJ. Sec and Tat-mediated protein secretion across the bacterial cytoplasmic membrane—distinct translocases and mechanisms. *Biochim Biophys Acta*. 2008;1778:1735-1756.
211. Balaban M, Battig P, Muschiol S, et al. Secretion of a pneumococcal type II secretion system pilus correlates with DNA uptake during transformation. *Proc Natl Acad Sci USA*. 2014;111:E758-E765.
212. Veith PD, Chen YY, Chen D, et al. *Tannerella forsythia* outer membrane vesicles are enriched with substrates of the type IX secretion system and TonB-dependent receptors. *J Proteome Res*. 2015;14:5355-5366.
213. Dorward DW, Garon CF. DNA is packaged within membrane-derived vesicles of gram-negative but not gram-positive bacteria. *Appl Environ Microbiol*. 1990;56:1960-1962.
214. Ho MH, Chen CH, Goodwin JS, Wang BY, Xie H. Functional advantages of *Porphyromonas gingivalis* vesicles. *PLoS One*. 2015;10:e0123448.
215. Liao S, Klein MI, Heim KP, et al. *Streptococcus mutans* extracellular DNA is upregulated during growth in biofilms, actively released via membrane vesicles, and influenced by components of the protein secretion machinery. *J Bacteriol*. 2014;196:2355-2366.
216. Zarnowski R, Sanchez H, Covelli AS, et al. *Candida albicans* biofilm-induced vesicles confer drug resistance through matrix biogenesis. *PLoS Biol*. 2018;16:e2006872.
217. Lee J, Kim OY, Gho YS. Proteomic profiling of gram-negative bacterial outer membrane vesicles: current perspectives. *Proteomics Clin Appl*. 2016;10:897-909.
218. Kim DK, Lee J, Kim SR, et al. EVpedia: a community web portal for extracellular vesicles research. *Bioinformatics*. 2015;31:933-939.
219. Couto N, Schooling SR, Dutcher JR, Barber J. Proteome profiles of outer membrane vesicles and extracellular matrix of *Pseudomonas aeruginosa* biofilms. *J Proteome Res*. 2015;14:4207-4222.
220. Kato S, Kowashi Y, Demuth DR. Outer membrane-like vesicles secreted by *Actinobacillus actinomycetemcomitans* are enriched in leukotoxin. *Microb Pathog*. 2002;32:1-13.
221. Kieselbach T, Zijngje V, Granström E, Oscarsson J. Proteomics of *Aggregatibacter actinomycetemcomitans* outer membrane vesicles. *PLoS One*. 2015;10:e0138591.
222. Veith PD, Chen YY, Gorasia DG, et al. *Porphyromonas gingivalis* outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors. *J Proteome Res*. 2014;13:2420-2432.
223. Choi JW, Kim SC, Hong SH, Lee HJ. Secretable small RNAs via outer membrane vesicles in periodontal pathogens. *J Dent Res*. 2017;96:458-466.
224. Perry JA, Cvitkovitch DG, Levesque CM. Cell death in *Streptococcus mutans* biofilms: a link between CSP and extracellular DNA. *FEMS Microbiol Lett*. 2009;299:261-266.
225. Wenderska IB, Lukenda N, Cordova M, Magarvey N, Cvitkovitch DG, Senadheera DB. A novel function for the competence inducing peptide, XIP, as a cell death effector of *Streptococcus mutans*. *FEMS Microbiol Lett*. 2012;336:104-112.
226. Jung CJ, Hsu RB, Shun CT, Hsu CC, Chia JS. AtIA mediates extracellular DNA release, which contributes to *Streptococcus mutans* biofilm formation in an experimental rat model of infective endocarditis. *Infect Immun*. 2017;85:e00252-17.
227. Thomas VC, Hiromasa Y, Harms N, Thurlow L, Tomich J, Hancock LE. A fratricidal mechanism is responsible for eDNA release and

- contributes to biofilm development of *Enterococcus faecalis*. *Mol Microbiol*. 2009;72:1022-1036.
228. Thomas VC, Thurlow LR, Boyle D, Hancock LE. Regulation of autolysis-dependent extracellular DNA release by *Enterococcus faecalis* extracellular proteases influences biofilm development. *J Bacteriol*. 2008;190:5690-5698.
  229. Turnbull L, Toyofuku M, Hynen AL, et al. Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat Commun*. 2016;7:11220.
  230. Kolenbrander PE, Palmer RJ Jr, Periasamy S, Jakubovics NS. Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol*. 2010;8:471-480.
  231. Das T, Sharma PK, Krom BP, van der Mei HC, Busscher HJ. Role of eDNA on the adhesion forces between *Streptococcus mutans* and substratum surfaces: influence of ionic strength and substratum hydrophobicity. *Langmuir*. 2011;27:10113-10118.
  232. Couvigny B, Kulakauskas S, Pons N, et al. Identification of new factors modulating adhesion abilities of the pioneer commensal bacterium *Streptococcus salivarius*. *Front Microbiol*. 2018;9:273.
  233. Lynch DJ, Fountain TL, Mazurkiewicz JE, Banas JA. Glucan-binding proteins are essential for shaping *Streptococcus mutans* biofilm architecture. *FEMS Microbiol Lett*. 2007;268:158-165.
  234. Peterson BW, He Y, Ren Y, et al. Viscoelasticity of biofilms and their recalcitrance to mechanical and chemical challenges. *FEMS Microbiol Rev*. 2015;39:234-245.
  235. Stoodley P, Cargo R, Rupp CJ, Wilson S, Klapper I. Biofilm material properties as related to shear-induced deformation and detachment phenomena. *J Ind Microbiol Biotechnol*. 2002;29:361-367.
  236. Paramonova E, Kalmykova OJ, van der Mei HC, Busscher HJ, Sharma PK. Impact of hydrodynamics on oral biofilm strength. *J Dent Res*. 2009;88:922-926.
  237. Stoodley P, Jacobsen A, Dunsmore BC, et al. The influence of fluid shear and AlCl<sub>3</sub> on the material properties of *Pseudomonas aeruginosa* PAO1 and *Desulfovibrio* sp. EX265 biofilms. *Water Sci Technol*. 2001;43:113-120.
  238. Cieplik F, Kara E, Muehler D, et al. Antimicrobial efficacy of alternative compounds for use in oral care toward biofilms from caries-associated bacteria in vitro. *MicrobiologyOpen*. 2019;8:e00695.
  239. Darveau RP. Periodontitis: a polymicrobial disruption of host homeostasis. *Nat Rev Microbiol*. 2010;8:481-490.
  240. Flemmig TF, Beikler T. Control of oral biofilms. *Periodontol*. 2011;55:9-15.
  241. Ganeshnarayan K, Shah SM, Libera MR, Santostefano A, Kaplan JB. Poly-N-acetylglucosamine matrix polysaccharide impedes fluid convection and transport of the cationic surfactant cetylpyridinium chloride through bacterial biofilms. *Appl Environ Microbiol*. 2009;75:1308-1314.
  242. Thurnheer T, Gmür R, Shapiro S, Guggenheim B. Mass transport of macromolecules within an in vitro model of supragingival plaque. *Appl Environ Microbiol*. 2003;69:1702-1709.
  243. Sandt C, Barbeau J, Gagnon M-A, Lafleur M. Role of the ammonium group in the diffusion of quaternary ammonium compounds in *Streptococcus mutans* biofilms. *J Antimicrob Chemother*. 2007;60:1281-1287.
  244. Stewart PS. Antimicrobial tolerance in biofilms. *Microbiol Spectrum*. 2015;3. <https://doi.org/10.1128/microbiolspec.MB-0010-2014>
  245. Oubekka SD, Briandet R, Fontaine-Aupart MP, Steenkeste K. Correlative time-resolved fluorescence microscopy to assess antibiotic diffusion-reaction in biofilms. *Antimicrob Agents Chemother*. 2012;56:3349-3358.
  246. Jefferson KK, Goldmann DA, Pier GB. Use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms. *Antimicrob Agents Chemother*. 2005;49:2467-2473.
  247. Marcotte L, Therien-Aubin H, Sandt C, Barbeau J, Lafleur M. Solute size effects on the diffusion in biofilms of *Streptococcus mutans*. *Biofouling*. 2004;20:189-201.
  248. Kurniawan A, Yamamoto T, Tsuchiya Y, Morisaki H. Analysis of the ion adsorption-desorption characteristics of biofilm matrices. *Microbes Environ*. 2012;27:399-406.
  249. Stewart PS, Zhang T, Xu R, et al. Reaction-diffusion theory explains hypoxia and heterogeneous growth within microbial biofilms associated with chronic infections. *NPJ Biofilms Microbiomes*. 2016;2:16012.
  250. Takenaka S, Pitts B, Trivedi HM, Stewart PS. Diffusion of macromolecules in model oral biofilms. *Appl Environ Microbiol*. 2009;75:1750-1753.
  251. Zhu M, Takenaka S, Sato M, Hoshino E. Extracellular polysaccharides do not inhibit the reaction between *Streptococcus mutans* and its specific immunoglobulin G (IgG) or penetration of the IgG through *S. mutans* biofilm. *Oral Microbiol Immunol*. 2001;16:54-56.
  252. Mitchell J. *Streptococcus mitis*: walking the line between commensalism and pathogenesis. *Mol Oral Microbiol*. 2011;26:89-98.
  253. Zheng W, Tan MF, Old LA, Paterson IC, Jakubovics NS, Choo SW. Distinct biological potential of *Streptococcus gordonii* and *Streptococcus sanguinis* revealed by comparative genome analysis. *Sci Rep*. 2017;7:2949.
  254. Vincents B, Guentsch A, Kostolowska D, et al. Cleavage of IgG1 and IgG3 by gingipain K from *Porphyromonas gingivalis* may compromise host defense in progressive periodontitis. *FASEB J*. 2011;25:3741-3750.
  255. Damgaard C, Holmstrup P, Van Dyke TE, Nielsen CH. The complement system and its role in the pathogenesis of periodontitis: current concepts. *J Periodontol Res*. 2015;50:283-293.
  256. Charras G, Sahai E. Physical influences of the extracellular environment on cell migration. *Nat Rev Mol Cell Biol*. 2014;15:813-824.
  257. Barken KB, Pamp SJ, Yang L, et al. Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. *Environ Microbiol*. 2008;10:2331-2343.
  258. Vesey PM, Kuramitsu HK. Genetic analysis of *Treponema denticola* ATCC 35405 biofilm formation. *Microbiology*. 2004;150:2401-2407.
  259. Ota C, Morisaki H, Nakata M, et al. *Streptococcus sanguinis* non-coding *cia*-dependent small RNAs negatively regulate expression of type IV pilus retraction ATPase PilT and biofilm formation. *Infect Immun*. 2018;86:e00894-17.
  260. Shrivastava A, Patel VK, Tang Y, Yost SC, Dewhirst FE, Berg HC. Cargo transport shapes the spatial organization of a microbial community. *Proc Natl Acad Sci USA*. 2018;115:8633-8638.
  261. Wright CJ, Burns LH, Jack AA, et al. Microbial interactions in building of communities. *Mol Oral Microbiol*. 2013;28:83-101.
  262. Shanker E, Federle MJ. Quorum sensing regulation of competence and bacteriocins in *Streptococcus pneumoniae* and *mutans*. *Genes*. 2017;8:E15.
  263. Wang BY, Kuramitsu HK. Interactions between oral bacteria: inhibition of *Streptococcus mutans* bacteriocin production by *Streptococcus gordonii*. *Appl Environ Microbiol*. 2005;71:354-362.
  264. Ebersole JL, Peyyala R, Gonzalez OA. Biofilm-induced profiles of immune response gene expression by oral epithelial cells. *Mol Oral Microbiol*. 2019;34:14-25.
  265. Peyyala R, Kirakodu SS, Novak KF, Ebersole JL. Oral epithelial cell responses to multispecies microbial biofilms. *J Dent Res*. 2013;92:235-240.
  266. Ramage G, Lappin DF, Millhouse E, et al. The epithelial cell response to health and disease associated oral biofilm models. *J Periodontol Res*. 2017;52:325-333.

267. Thurlow LR, Hanke ML, Fritz T, et al. *Staphylococcus aureus* biofilms prevent macrophage phagocytosis and attenuate inflammation in vivo. *J Immunol*. 2011;186:6585-6596.
268. Yamanaka T, Furukawa T, Matsumoto-Mashimo C, et al. Gene expression profile and pathogenicity of biofilm-forming *Prevotella intermedia* strain 17. *BMC Microbiol*. 2009;9:11.
269. Burne RA, Chen YY, Wexler DL, Kuramitsu H, Bowen WH. Cariogenicity of *Streptococcus mutans* strains with defects in fructan metabolism assessed in a program-fed specific-pathogen-free rat model. *J Dent Res*. 1996;75:1572-1577.
270. Mulcahy H, Charron-Mazenod L, Lewenza S. *Pseudomonas aeruginosa* produces an extracellular deoxyribonuclease that is required for utilization of DNA as a nutrient source. *Environ Microbiol*. 2010;12:1621-1629.
271. Jakubovics NS, Yassin SA, Rickard AH. Community interactions of oral streptococci. *Adv Appl Microbiol*. 2014;87:43-110.
272. Tribble GD, Rigney TW, Dao D-H, et al. Natural competence is a major mechanism for horizontal DNA transfer in the oral pathogen *Porphyromonas gingivalis*. *MBio*. 2012;3:e00231-11.
273. Nishikawa K, Tanaka Y. A simple mutagenesis using natural competence in *Tannerella forsythia*. *J Microbiol Methods*. 2013;94:378-380.
274. Hannan S, Ready D, Jasni AS, Rogers M, Pratten J, Roberts AP. Transfer of antibiotic resistance by transformation with eDNA within oral biofilms. *FEMS Immunol Med Microbiol*. 2010;59:345-349.
275. Roberts AP, Kreth J. The impact of horizontal gene transfer on the adaptive ability of the human oral microbiome. *Front Cell Infect Microbiol*. 2014;4:124.
276. Sukumar S, Roberts AP, Martin FE, Adler CJ. Metagenomic insights into transferable antibiotic resistance in oral bacteria. *J Dent Res*. 2016;95:969-976.
277. Chi F, Nolte O, Bergmann C, Ip M, Hakenbeck R. Crossing the barrier: evolution and spread of a major class of mosaic *bbp2x* in *Streptococcus pneumoniae*, *S. mitis* and *S. oralis*. *Int J Med Microbiol*. 2007;297:503-512.
278. Kilian M, Chapple ILC, Hannig M, et al. The oral microbiome—an update for oral healthcare professionals. *Br Dent J*. 2016;221:657-666.
279. Meyle J, Chapple I. Molecular aspects of the pathogenesis of periodontitis. *Periodontol*. 2015;69:7-17.
280. Block C, Furman M. Association between intensity of chlorhexidine use and micro-organisms of reduced susceptibility in a hospital environment. *J Hosp Infect*. 2002;51:201-206.
281. Fleischer HC, Mellonig JT, Brayer WK, Gray JL, Barnett JD. Scaling and root planing efficacy in multirooted teeth. *J Periodontol*. 1989;60:402-409.
282. Petersilka GJ, Ehmke B, Flemmig TF. Antimicrobial effects of mechanical debridement. *Periodontol*. 2002;28:56-71.
283. Rateitschak-Plüss EM, Schwarz JP, Guggenheim R, Düggelin M, Rateitschak KH. Non-surgical periodontal treatment: where are the limits? An SEM study. *J Clin Periodontol*. 1992;19:240-244.
284. Michaud RM, Schoolfield J, Mellonig JT, Mealey BL. The efficacy of subgingival calculus removal with endoscopy-aided scaling and root planing: a study on multirooted teeth. *J Periodontol*. 2007;78:2238-2245.
285. Grumbein S, Werb M, Opitz M, Lieleg O. Elongational rheology of bacterial biofilms in situ. *J Rheol*. 2016;60:1085-1094.
286. Busscher HJ, Jager D, Finger G, Schaefer N, van der Mei HC. Energy transfer, volumetric expansion, and removal of oral biofilms by non-contact brushing. *Scand J Dent Res*. 2010;118:177-182.
287. Rmaile A, Carugo D, Capretto L, et al. Removal of interproximal dental biofilms by high-velocity water microdrops. *J Dent Res*. 2014;93:68-73.
288. Fabbri S, Li J, Howlin RP, et al. Fluid-driven interfacial instabilities and turbulence in bacterial biofilms. *Environ Microbiol*. 2017;19:4417-4431.
289. Jepsen K, Jepsen S. Antibiotics/antimicrobials: systemic and local administration in the therapy of mild to moderately advanced periodontitis. *Periodontol*. 2016;71:82-112.
290. Sanz M, Serrano J, Iniesta M, Santa Cruz I, Herrera D. Antiplaque and antigingivitis toothpastes. *Monogr Oral Sci*. 2013;23:27-44.
291. Cieplik F, Deng D, Crielaard W, et al. Antimicrobial photodynamic therapy—what we know and what we don't. *Crit Rev Microbiol*. 2018;44:571-589.
292. Teughels W, Dekeyser C, Van Essche M, Quirynen M. One-stage, full-mouth disinfection: fiction or reality? *Periodontol*. 2009;50:39-51.
293. Pretzl B, Sälzer S, Ehmke B, et al. Administration of systemic antibiotics during non-surgical periodontal therapy—a consensus report. *Clin Oral Investig*. 2018;93:1-13.
294. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol*. 1999;37:1771-1776.
295. Marsh PD. Dental plaque as a microbial biofilm. *Caries Res*. 2004;38:204-211.
296. Belibasakis GN, Thurnheer T. Validation of antibiotic efficacy on in vitro subgingival biofilms. *J Periodontol*. 2014;85:343-348.
297. Wang H-Y, Cheng J-W, Yu H-Y, Lin L, Chih Y-H, Pan Y-P. Efficacy of a novel antimicrobial peptide against periodontal pathogens in both planktonic and polymicrobial biofilm states. *Acta Biomater*. 2015;25:150-161.
298. Fred EB. Antony van Leeuwenhoek: on the three-hundredth anniversary of his birth. *J Bacteriol*. 1933;25:iv.2-18.
299. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol*. 2001;9:34-39.
300. Stewart PS. Theoretical aspects of antibiotic diffusion into microbial biofilms. *Antimicrob Agents Chemother*. 1996;40:2517-2522.
301. Marsh PD. Plaque as a biofilm: pharmacological principles of drug delivery and action in the sub and supragingival environment. *Oral Dis*. 2003;9:16-22.
302. De Beer D, Srinivasan R, Stewart PS. Direct measurement of chlorine penetration into biofilms during disinfection. *Appl Environ Microbiol*. 1994;60:4339-4344.
303. Tseng BS, Zhang W, Harrison JJ, et al. The extracellular matrix protects *Pseudomonas aeruginosa* biofilms by limiting the penetration of tobramycin. *Environ Microbiol*. 2013;15:2865-2878.
304. Zaura-Arite E, Marle J, ten Cate JM. Confocal microscopy study of undisturbed and chlorhexidine-treated dental biofilm. *J Dent Res*. 2001;80:1436-1440.
305. He Y, Peterson BW, Jongsma MA, et al. Stress relaxation analysis facilitates a quantitative approach towards antimicrobial penetration into biofilms. *PLoS One*. 2013;8:e63750.
306. Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents*. 2010;35:322-332.
307. Anderl JN, Franklin MJ, Stewart PS. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob Agents Chemother*. 2000;44:1818-1824.
308. Cieplik F, Zaura E, Brandt BW, et al. Microcosm biofilms cultured from different oral niches in periodontitis patients. *J Oral Microbiol*. 2019;11:1551596.
309. Edlund A, Yang Y, Hall AP, et al. An in vitro biofilm model system maintaining a highly reproducible species and metabolic diversity approaching that of the human oral microbiome. *Microbiome*. 2013;1:25.



310. Fernandez y Mostajo M, Exterkate RAM, Buijs MJ, et al. A reproducible microcosm biofilm model of subgingival microbial communities. *J Periodontol Res.* 2017;52:1021-1031.
311. Velsko IM, Shaddox LM. Consistent and reproducible long-term in vitro growth of health and disease-associated oral subgingival biofilms. *BMC Microbiol.* 2018;18:70.
312. Roberts AP, Mullany P. Oral biofilms: a reservoir of transferable, bacterial, antimicrobial resistance. *Expert Rev Anti Infect Ther.* 2010;8:1441-1450.
313. Cieplik F, Jakubovics NS, Buchalla W, Maisch T, Hellwig E, Al-Ahmad A. Resistance toward chlorhexidine in oral bacteria—is there cause for concern? *Front Microbiol.* 2019;10:587.
314. Sharma M, Dhillion AS, Newbrun E. Cell-bound glucosyltransferase activity of *Streptococcus sanguis* strain 804. *Arch Oral Biol.* 1974;19:1063-1072.
315. Garcia SS, Blackledge MS, Michalek S, et al. Targeting of *Streptococcus mutans* biofilms by a novel small molecule prevents dental caries and preserves the oral microbiome. *J Dent Res.* 2017;96:807-814.
316. McDougald D, Rice SA, Barraud N, Steinberg PD, Kjelleberg S. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nat Rev Microbiol.* 2012;10:39-50.
317. Kaplan JB. Therapeutic potential of biofilm-dispersing enzymes. *Int J Artif Organs.* 2009;32:545-554.
318. Itoh Y, Wang X, Hinnebusch BJ, Preston JF, Romeo T. Depolymerization of  $\beta$ -1,6-*N*-acetyl- $\beta$ -D-glucosamine disrupts the integrity of diverse bacterial biofilms. *J Bacteriol.* 2005;187:382-387.
319. Kaplan JB, Raguath C, Ramasubbu N, Fine DH. Detachment of *Actinobacillus actinomycetemcomitans* biofilm cells by an endogenous  $\beta$ -hexosaminidase activity. *J Bacteriol.* 2003;185:4693-4698.
320. Izano EA, Wang H, Raguath C, Ramasubbu N, Kaplan JB. Detachment and killing of *Aggregatibacter actinomycetemcomitans* biofilms by dispersin B and SDS. *J Dent Res.* 2007;86:618-622.
321. Izano EA, Amarante MA, Kher WB, Kaplan JB. Differential roles of poly-*N*-acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *Appl Environ Microbiol.* 2008;74:470-476.
322. Kaplan JB, Raguath C, Velliyagounder K, Fine DH, Ramasubbu N. Enzymatic detachment of *Staphylococcus epidermidis* biofilms. *Antimicrob Agents Chemother.* 2004;48:2633-2636.
323. Kaplan JB, Mlynek KD, Hettiarachchi H, et al. Extracellular polymeric substance (EPS)-degrading enzymes reduce staphylococcal surface attachment and biocide resistance on pig skin in vivo. *PLoS One.* 2018;13:e0205526.
324. Darouiche RO, Mansouri MD, Gawande PV, Madhyastha S. Antimicrobial and antibiofilm efficacy of triclosan and DispersinB combination. *J Antimicrob Chemother.* 2009;64:88-93.
325. Kaplan JB. Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *J Dent Res.* 2010;89:205-218.
326. Jakubovics NS. Extracellular proteins and DNA in the matrix of oral biofilms. In: Jakubovics NS, Palmer RJ, eds. *Oral Microbial Ecology: Current Research and New Perspectives.* Wymondham, UK: Caister Academic Press; 2013:85-95.
327. Kaplan JB, LoVetri K, Cardona ST, et al. Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in staphylococci. *J Antibiot (Tokyo).* 2012;65:73-77.
328. Schlafer S, Meyer RL, Dige I, Regina VR. Extracellular DNA contributes to dental biofilm stability. *Caries Res.* 2017;51:436-442.
329. Hymes SR, Randis TM, Sun TY, Ratner AJ. DNase inhibits *Gardnerella vaginalis* biofilms in vitro and in vivo. *J Infect Dis.* 2013;207:1491-1497.
330. Hughes KA, Sutherland IW, Clark J, Jones MV. Bacteriophage and associated polysaccharide depolymerases—novel tools for study of bacterial biofilms. *J Appl Microbiol.* 1998;85:583-590.
331. Hughes KA, Sutherland IW, Jones MV. Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. *Microbiology.* 1998;144:3039-3047.
332. Lu TK, Collins JJ. Dispersing biofilms with engineered enzymatic bacteriophage. *Proc Natl Acad Sci USA.* 2007;104:11197-11202.
333. Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kolter R, Losick R.  $\beta$ -Amino acids trigger biofilm disassembly. *Science.* 2010;328:627-629.
334. Ampornaramveth RS, Akeathichod N, Lertnukkhid J, Songsang N. Application of  $\beta$ -amino acids as biofilm dispersing agent in dental unit waterlines. *Int J Dent.* 2018;2018:1-7.
335. Rosen E, Tsesis I, Elbahary S, Storzi N, Kolodkin-Gal I. Eradication of *Enterococcus faecalis* biofilms on human dentin. *Front Microbiol.* 2016;7:2055.
336. Yildiz FH. Cyclic dimeric GMP signaling and regulation of surface-associated developmental programs. *J Bacteriol.* 2008;190:781-783.
337. Wu H, Moser C, Wang H-Z, Høiby N, Song Z-J. Strategies for combating bacterial biofilm infections. *Int J Oral Sci.* 2015;7:1-7.
338. Sambanthamoorthy K, Sloup RE, Parashar V, et al. Identification of small molecules that antagonize diguanylate cyclase enzymes to inhibit biofilm formation. *Antimicrob Agents Chemother.* 2012;56:5202-5211.
339. Govinden G, Parker JL, Naylor KL, Frey AM, Anumba DOC, Stafford GP. Inhibition of sialidase activity and cellular invasion by the bacterial vaginosis pathogen *Gardnerella vaginalis*. *Arch Microbiol.* 2018;200:1129-1133.
340. Hwang G, Paula AJ, Hunter EE, et al. Catalytic antimicrobial robots for biofilm eradication. *Sci Robot.* 2019;4:eaaw2388.

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