

**MINI-
REVIEW****Biofilm exopolysaccharides: a strong and sticky framework**

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Biofilms probably comprise the normal environment for most microbial cells in many natural and artificial habitats, and as such are complex associations of cells, extracellular products and detritus either trapped within the biofilm or released from cells which have lysed as the biofilm ages (Christensen, 1989). The main 'cement' for all these cells and products is the mixture of polysaccharides secreted by the cells established within the biofilm. Probably the nearest analogy is processed food, in which a mixture of macromolecules of all types interact in various ways to form a recognizable structure. Within such a structure, cells, water, ions and soluble low- and high-molecular-mass products are trapped. In many biofilms, as in food, the hydrated polysaccharides may be in a semi-solid state. The major component in the biofilm matrix is water – up to 97% (Zhang *et al.*, 1998), and the characteristics of the solvent are determined by the solutes dissolved in it. The exact structure of any biofilm is probably a unique feature of the environment in which it develops. As pointed out by Stoodley *et al.* (1999a), nutritional and physical conditions greatly affect the nature of laboratory biofilms and this is equally true for other types. Wimpenny & Colasanti (1997) have also suggested that biofilm structure is largely determined by the concentration of substrate. They further postulated that such differences also validate at least three conceptual models of biofilms – heterogeneous mosaics, structures penetrated by water channels, and dense confluent biofilms.

Any study of biofilms must accept that biofilms may develop in an enormous number of environments, and that the structural intricacies of any single biofilm formed under any specific set of parameters may well be unique to that single environment and microflora. The enormous number of microbial species capable of forming biofilms or interacting with others to do so, together with the very great range of polysaccharides produced, gives rise to an infinite number of permutations. In natural conditions, monospecies bio-

films are relatively rare; thus most biofilms are composed of mixtures of micro-organisms. This adds to the interspecies and intraspecies interactions and to the general complexity of the macromolecular mixture present.

The exopolysaccharides (EPS) synthesized by microbial cells vary greatly in their composition and hence in their chemical and physical properties. Some are neutral macromolecules, but the majority are polyanionic due to the presence of either uronic acids (D-glucuronic acid being the commonest, although D-galacturonic and D-mannuronic acids are also found) or ketal-linked pyruvate. Inorganic residues, such as phosphate or rarely sulphate, may also confer polyanionic status (Sutherland, 1990). A very few EPS may even be polycationic, as exemplified by the adhesive polymer obtained from strains of *Staphylococcus epidermidis* strains associated with biofilms (Mack *et al.*, 1996). The composition and structure of the polysaccharides determines their primary conformation. Further, ordered secondary configuration frequently takes the form of aggregated helices. In some of these polymers, the backbone composition of sequences of 1,4- β - or 1,3- β -linkages may confer considerable rigidity, as is seen in the cellulosic backbone of xanthan from *Xanthomonas campestris*. Other linkages in polysaccharides may yield more flexible structures. These can be exemplified by the 1,2- α - or 1,6- α -linkages found in many dextrans. The transition in solution from random coil to ordered helical aggregates is often greatly influenced by the presence or absence of acyl substituents such as O-acetyl or O-succinyl esters or pyruvate ketals (Sutherland, 1997). In most natural and experimental environments, the EPS will be found in the ordered configurations which are found at lower temperatures and in the presence of salts. The polysaccharides are essentially very long, thin molecular chains with molecular mass of the order of $0.5\text{--}2.0 \times 10^6$ Da, but they can associate in a number of different ways. In several preparations, the polysaccharides have been visualized as fine strands attached to the bacterial cell surface and forming a

complex network surrounding the cell. Mayer *et al.* (1999) suggested that electrostatic and hydrogen bonds are the dominant forces involved. Ionic interactions may be involved, but more subtle chain-chain complex formation in which one macromolecule 'fits' into the other may result in either floc formation or networks which are very poorly soluble in aqueous solvents. Another result may be the formation of strong or weak gels. The polysaccharides can thus form various types of structures within a biofilm. However, in biofilms the polysaccharides do not exist alone but may interact with a wide range of other molecular species, including lectins, proteins, lipids etc., as well as with other polysaccharides. The resultant tertiary structure comprises a network of polysaccharide and other macromolecules, in which cells and cell products are also trapped.

Are there specific biofilm polysaccharides?

Despite some claims for the existence of biofilm-specific polysaccharides, there is little, if any, conclusive evidence to support such claims. A major problem is to obtain sufficient EPS which is truly biofilm-derived for detailed study. The EPS present in biofilms almost certainly resemble closely the corresponding polymers synthesized by planktonic cells. This has been demonstrated by the use of antibodies prepared against EPS from planktonic cells and also by comparison of the enzymic products following digestion of planktonic and biofilm EPS using highly specific polysaccharases. There may be production of increased amounts of polysaccharide as part of a stress response, as is seen in colanic acid synthesis by *Escherichia coli* and other enterobacterial species. Those bacteria capable of forming several different polysaccharides may produce more of one found in lower amounts in planktonic cultures; this again is probably part of a stress response. One result of this effect has been the report of variations in polysaccharide composition, almost certainly due to the varying proportions of the different polysaccharides synthesized within the biofilm. This is also true of biofilms containing a mixture of microbial species. In these, it must be remembered that the relative amounts of different polysaccharides and the proportions of the microbial cells present will depend greatly on the physiological state of the biofilm. Such biofilms are also unlikely to be uniform; consequently any sample taken for analysis will only represent a single 'snapshot' of the EPS composition. Thus, apparent variations in composition of the mixtures are the result of differential synthesis of the component polymers. It is also quite possible that within extensive biofilms, different subpopulations might have miscellaneous micro-environments leading to production of different mixtures of polysaccharides.

The amount of EPS synthesis within the biofilm will depend greatly on the availability of carbon substrates (both inside and outside the cell) and on the balance between carbon and other limiting nutrients. The

presence of excess available carbon substrate and limitations in other nutrients, such as nitrogen, potassium or phosphate, will promote the synthesis of EPS. The slow bacterial growth observed in most biofilms would also be expected to enhance EPS production. In organisms such as colanic-acid-producing *Es. coli*, the production of EPS forms part of the stress response under control of the *rpoS* gene. A similar response might account for the starvation-specific formation of an adhesive EPS observed in cultures of a marine bacterium, *Pseudomonas* sp. strain S9 (Wrangstadh *et al.*, 1990). Increased EPS synthesis has indeed now been observed as part of a major change in gene expression in the biofilm state for a number of bacterial species. These include both colanic acid production in *Es. coli* (Prigent-Combaret *et al.*, 1999) and alginate synthesis in *Pseudomonas aeruginosa* (Davies & Geesey, 1995), as well as secretion of a galactoglucan EPS of unknown structure in *Vibrio cholerae* El Tor (Watnick & Kolter, 1999). Is it possible that, because of the soluble EPS within the biofilm, localized high osmolarity within the biofilm could be one of the signals? It is known to act as a signal for enhanced transcription of the *algD* promoter in *P. aeruginosa* (Berry *et al.*, 1989) and was earlier shown to enhance colanic acid synthesis.

It is clear from a number of studies that mutants unable to synthesize the EPS are unable to form biofilms (Allison & Sutherland, 1987; Watnick & Kolter, 1999), although they may still attach to surfaces and form micro-colonies to a limited extent. However, in our study of a natural biofilm isolate attaching to glass, most of the EPS⁻ mutant bacteria were seen as well-separated cells; under calcium-limiting conditions, where little EPS was synthesized, the effect was very similar. However, when the bacteria are components of mixed biofilms, the presence of one species producing copious amounts of EPS may enhance the stability of other cell types even if they do not themselves synthesize EPS. Such stabilizing effects were considered by James *et al.* (1995) to be commensal interactions. As pointed out by Skillman *et al.* (1999), the proportions of different EPS in mixed biofilms do not necessarily reflect the proportions of the cells present, nor do the EPS contribute equally to the structure and properties of the resulting biofilms.

What do we know of the structure and properties of the biofilm polysaccharides?

As only small amounts of biofilm-derived EPS are normally available, we must mainly use data derived from planktonic cultures and extrapolate this to biofilms. Such extrapolation is probably sustainable for the most part, although some differences in properties such as molecular mass might well be expected. Unfortunately, because of the widespread study of biofilms produced by alginate-synthesizing strains of *P. aeruginosa*, the concept has perhaps arisen that all biofilms contain very highly charged uronic-acid-containing polysaccharides and all biofilm polysaccharides are similar. At the other extreme, the major components of

many oral biofilms are neutral homopolymers such as dextrans of the 'mutan' type or levans. Most biofilm polysaccharides appear to resemble the general types found associated with all classes of microbial cells, being either homopolysaccharides or heteropolysaccharides. Some of the homopolysaccharides possess regular structures, but dextrans and levans do not. With the exception of bacterial alginates, the heteropolysaccharides are composed of regular repeat units of 2–8 monosaccharides. The bacterial alginates, like their algal counterparts, are formed from irregular sequences of D-mannuronic acid and L-guluronic acid residues, but differ from the algal material in being heavily acetylated on many of the mannuronosyl residues.

Many of these polysaccharides are relatively soluble, and because of their large molecular mass, yield highly viscous aqueous solutions. A few will form weak gels, which dissolve in excess solvent, thus sloughing off the exposed surface of biofilms. Changes may occur when ions are present. Some ions may specifically interact with exposed carboxylic acid groups on the EPS to yield networks of macromolecules which show increased viscosity or even gelation. Various cations may compete for the same binding site, as was shown by Loaçc *et al.* (1997); alternatively, ion binding may be less specific. The ionic radii of the cations may sometimes be important in determining the extent of interaction between the polymer chains and the ultimate extent of aggregation of helices. In most cases, the presence of multivalent cations such as Ca^{2+} will lead to more extensive formation of ordered helices than monovalent ions, although some polysaccharides resemble kappa carrageenan and reveal aggregates of double helices in the presence of K^+ .

The EPS contribute directly to the properties of biofilms in that they normally permit considerable amounts of water to be bound. This is not a feature which has been extensively examined. However, polysaccharides such as hyaluronic acid can bind up to 1 kg water (g polysaccharide)⁻¹. It is probable that many of the EPS in biofilms bind lesser quantities whilst some, like bacterial cellulose, mutan or curdlan, manage to exclude most water from their tertiary structure. The EPS will also contribute to the mechanical stability of the biofilms (Mayer *et al.*, 1999), enabling them to withstand considerable shear forces. In some polymers, the interaction with ions may yield relatively rigid gels which are less readily deformed by shear, thus producing a much more stable biofilm. Mayer *et al.* (1999) suggested that biofilms might indeed represent gel-like structures, but these may be very weak and consequently may be readily destroyed by shear or dissolution of the polysaccharides. It should not be forgotten that a small number of EPS, because of their composition and tertiary structure, might actually be hydrophobic (Neu & Poralla, 1988). Others possess localized hydrophilic and hydrophobic regions. They thus confer very different properties on the matrices in which they are found and account for the wide differences in properties found in different biofilms.

What is the relationship of structure to function?

A comparison of bacterial and algal alginates gives a clear indication of the relationship of polysaccharide structure and function. The algal polysaccharides readily form rigid, non-deformable gels due to the highly specific interaction with either Ca^{2+} or Sr^{2+} , a property which is widely used in biotechnology for the immobilization of cells and enzymes. This is not seen in bacterial alginates from *Azotobacter vinelandii*, even though these EPS closely resemble the algal alginates in possessing sequences of polyguluronic acid blocks producing the characteristic egg-box structure (Ertesvag & Valla, 1998). The bacterial polysaccharides are acetylated and the acetyl groups strongly inhibit the interaction between polymer chains and cations and resultant gel formation. Some binding of cations does occur and there is also some specificity towards Ca^{2+} . *P. aeruginosa* alginates totally lack sequences of guluronosyl residues, are normally incapable of gelling with divalent cations but do still bind them to a more limited extent (Geddie & Sutherland, 1994). The resultant polysaccharides yield highly viscous aqueous solutions with viscoelastic properties. The acetylated polysaccharides are also fairly resistant to most of the alginate lyases which can degrade the algal products (Sutherland, 1997). In both types of bacterial alginate, chemical removal of the O-acetyl residues significantly alters their physical properties and leads to increased binding of divalent cations. The solubility of the macromolecules is also much reduced.

Many bacterial EPS possess backbone structures in which there exist sequences of 1,3- or 1,4- β -linked hexose residues. When such sequences are present, the polymers tend to be much more rigid in structure, less deformable and, in the case of neutral polysaccharides such as mutan or those from some strains of *Enterobacter agglomerans*, either poorly soluble or effectively insoluble. These EPS molecules may be very robust. The long chains of stiff macromolecules may be present as gels due to the entanglements found within the long chains and also, in some polymers, to the ionic environment (Ross-Murphy & Shatwell, 1993; Ross-Murphy, 1995). The stability of the gel state will depend on the effective polysaccharide concentration, the ionic status and the other macromolecules present. Those EPS molecules, which are effectively in solution, may well dissolve with dilution, thus accounting in part for the observed 'sloughing off' of biofilm material. It has to be remembered that enzymes will also contribute to this (Boyd & Chakrabarty, 1994). Another aspect, which has received relatively little study, is the possibility of interaction of EPS with proteins and other excreted or surface-associated macromolecules. Either association or segregation may occur.

Other EPS, of which curdlan is an example, form triple helices in which the strands are very firmly held together by hydrogen bonds. Such tight bonding together of the linear molecules may effectively exclude water

molecules. Any substituents present may very greatly affect the conformation, as is seen in the commercial product 'gellan' from a *Sphingomonas elodea* strain. The native, acylated polymer forms weak gels, whereas the deacylated material yields brittle, rigid gels (Chandrasekaran & Thailambal, 1990). Similarly, comparison of the polysaccharides from *Klebsiella aerogenes* K54 and *Enterobacter aerogenes* XM6 indicates the role of acetyl groups. Both EPS have the same tetrasaccharide repeat units containing D-glucose, L-fucose and D-glucuronic acid in the molar ratio 2:1:1, and both yield viscous aqueous solutions (O'Neill *et al.*, 1986). However, the non-acetylated XM6 polymer presents a highly crystalline structure recognizable by X-ray fibre diffraction (Atkins *et al.*, 1987), whereas the K54 polymers carrying either 0.5 or 1 acetate per repeat unit are amorphous. Deacetylation of the latter converts its pattern to one similar to XM6. XM6 and deacetylated K54 gel in the presence of various ions, whilst native K54 does not (Nisbet *et al.*, 1984). Possibly many biofilm EPS form either highly viscous solutions or localized gels, the latter being deformable under shear but recovering to something like their original state after the shear is removed.

How do biofilm polysaccharides interact?

In some biofilms, the solutions of EPS act totally independently of each other and are devoid of any major interaction. However, the majority of EPS present in biofilms can interact in a wide variety of ways. Several of these are ion-dependent, including many of the chain-chain interactions leading to gelation either of single polymers or of mixtures. Computer-derived models of many of the EPS of which the structure has been totally elucidated commonly reveal that charged groups are all on the exterior of the molecular chains and can thus interact readily with ions and other charged molecules (Chandrasekaran *et al.*, 1994). In oral biofilms, the EPS structures can also provide binding epitopes for lectins present on the surface of other oral bacteria, thus permitting accretion of cells and further EPS. The poorly water-soluble α -D-glucans are first secreted and attach to the oral surfaces. Subsequently, oral streptococci can bind to these (Jenkinson & Lamont, 1997). The glucans thus act as intramolecular bridges. Whilst co-aggregation involving EPS, lectins and other cell-surface molecules has been demonstrated in oral systems by Kolenbrander & London (1993), it has not yet been shown to play such a significant role in other types of biofilm, although it is known to occur also in aquatic bacteria (Rickard *et al.*, 2000). In experimental systems such as that examined by Skillman *et al.* (1999), pre-coating of surfaces with EPS from several enterobacterial species did indicate that whilst some of the polysaccharides promoted adhesion of cells of heterologous strains, others had little, if any, effect. Where promotion of adhesion was observed, it could be reversed by enzymic degradation of the EPS. Much also depends on whether the EPS are firmly bound to the cells which produce them, thus also binding the micro-

organisms firmly into the matrix of the biofilm. It is also not clear whether the EPS interact directly at the interface with inert surfaces of different chemical nature or whether the conditioning layer acts as an intermediary. Although EPS may promote adherence to a wide variety of solid substrata, EPS production is not, in itself, synonymous with adhesion. Atomic force microscopy is now providing new insights into the molecular determinants of microbial adhesion. A recent study of *Es. coli* adhesion by Razatos *et al.* (1998) indicated that the adhesion forces were affected by the production of colanic acid and also by the length of the core of the lipopolysaccharide molecules. It is not yet clear to what extent the viscosity of the colanic acid influences the forces, yet colanic acid from different strains varies very greatly in its solution viscosity.

EPS may also interact with protein molecules and micelles, i.e. effectively with microbial cells and their associated surface proteins within the biofilm matrix, as well as with soluble proteins, including enzymes. The two types of biopolymer may interact in different ways. They may be associative, but are more commonly segregative or incompatible. In the latter example, the EPS concentration near the protein molecule is reduced; there may even be phase separation into polysaccharide-rich and protein-rich phases. Polysaccharides may also adsorb onto more than one protein surface to cause coacervation (Tuinier, 1999). The effect of such polymer-polymer interactions on biofilm structure and stability has not yet received attention.

As yet, relatively little is known about the interactions which occur between biofilm EPS and enzymes (Sutherland, 1999b). Whilst proteases would certainly affect those proteins which interact with EPS within biofilms, polysaccharases and polysaccharide lyases can have a much greater effect. A wide range of highly specific polysaccharases and polysaccharide lyases are available from lysates of EPS-producing bacteria, following infection with virulent bacteriophage (Sutherland, 1999a; Hughes *et al.*, 1998a). In experimental systems, it is clear that the effect of any enzyme degrading any one EPS will depend on the other EPS and microbial cells present in the biofilm. Thus in a *Pseudomonas fluorescens/En. agglomerans* mixed biofilm, addition of an alginate lyase released many of the *P. fluorescens* cells from the surface whilst attached cell numbers of the enteric species increased (V. Morton & I. W. Sutherland, unpublished results). Hughes *et al.* (1998b) observed a rapid decline in the number of attached *En. agglomerans* cells following the addition of a phage-induced polysaccharide depolymerase. This was also confirmed by scanning electron microscopy. Using GFP-labelled *En. agglomerans* cells, Skillman *et al.* (1999) noted that treatment with either the polysaccharase or a protease reduced the adhesion of these bacteria to a monolayer of *Klebsiella pneumoniae* cells. This indicated roles for both the polysaccharide and proteins in the adhesion process and suggests that considerable differences can be expected when different combinations of microbial species are examined.

Some bacteria secrete esterases with wide specificity; these can remove acyl groups from bacterial polymers as well as from other esters (Cui *et al.*, 1999). Such enzymes could alter the physical properties of a biofilm structure, either locally or to a greater extent. Deacylation of the bacterial polysaccharide succinoglycan improved pseudoplasticity in aqueous solution as well as increasing the cooperativity of the order-disorder transition (Ridout *et al.*, 1997). On the other hand, deacylation of some polysaccharides may lead to loss of any ordered conformation (Villain-Simonnet *et al.*, 2000). Other polysaccharides, such as XM6 and gellan, can readily form gels when freed of acyl substituents (Sutherland, 1997); this would lead to strengthening of portions of biofilms containing such polymers.

Many bacteria are capable of synthesizing and excreting surfactants, some of which, such as emulsan, resemble lipopolysaccharides (LPS), whilst rhamnolipids are products of *Pseudomonas* spp. Al-Tahhan *et al.* (2000) pointed out that even very low levels of a rhamnolipid biosurfactant could render the cell surface more hydrophobic, causing loss of LPS in the process. It has also been suggested that biosurfactants might be involved in the horizontal transfer of exopolymer from one bacterial species to another (Osterreicher-Ravid *et al.*, 2000). This could take place much more efficiently within the matrix of a biofilm where cells are in close proximity to each other. The production of these biosurfactants also enables the component cells within biofilms to solubilize and utilize substrates which would otherwise be inaccessible.

Do the biofilm polysaccharides offer any protection to the cells within the biofilm?

This again is one of the aspects where generalization is impossible. Some reports do suggest that the EPS in biofilms interact with antimicrobial agents and protect the cells, either by preventing access of the compounds or by effectively reducing their concentration. However, the protective effects are probably limited. By maintaining a highly hydrated layer surrounding the biofilm, the EPS will prevent lethal desiccation in some natural biofilms and may thus protect against diurnal variations in humidity. This would be typically encountered in many natural biofilms, including the mixed algal/cyanobacterial biofilm studied in our laboratory (Sutherland, 1996). This hydrated network of polysaccharide molecules may be quite extensive, with relatively few microbial cells within it. Alternatively there may only be a thin layer surrounding the microorganisms. In either case, the outermost layer of the EPS may lose water and harden to form an effective film providing protection against further desiccation. EPS may offer little protection against bacteriophage or bacteriocins when these are present in appreciable concentrations. Many lytic bacteriophage acting on EPS-synthesizing bacteria produce polysaccharases degrading capsular and other EPS and permitting access to

the cell surface. The release of more phage particles and enzyme activity on completion of the lytic cycle will further damage or remove the biofilm, as was demonstrated in biofilms of enteric species by Hughes *et al.* (1998b). The outcome of interaction with phage will possibly depend on whether the virus particles carry associated polysaccharase activity and on whether the EPS plays a major or minor role in the biofilm structure. In natural systems, bacteria within a biofilm may also be exposed to *Bdellovibrio*. Again, those bacteria at the periphery of the biofilm probably become infected whereas penetration of the cells in the depth of the system is less likely. If phage with no polysaccharase activity selectively destroy the cells of one of the bacterial components in a mixed biofilm it may have little effect on total cell numbers. Indeed, the remaining bacteria may even show enhanced growth in the absence of a competitor for available nutrients (L. Napier & I. W. Sutherland, unpublished results).

As any biofilm is unlikely to comprise a single type of EPS, the effect of enzyme action will depend on whether its substrate plays a major role in maintaining the biofilm structure. Thus, Skillman *et al.* (1999) observed that in biofilms composed of mixed enteric species, hydrolysis of one EPS caused greater destruction of the biofilm than did removal of the other. This would indicate that, as in cell walls, certain polymers may provide a fairly rigid scaffolding onto or into which other polymers attach to fill the interstices. In oral biofilms, many of the component bacteria are capable of synthesizing several different EPS, including dextrans (α -D-glucans) and levans (β -D-fructans). In addition, both dextranases and fructan hydrolases may be secreted. Little is known of the effects such polysaccharide hydrolases have on oral biofilms, but recent studies on regulation of expression of the fructan-degrading enzyme in *Streptococcus mutans* may start to provide an insight (Burne *et al.*, 1999). The effects of polysaccharases released by senescent bacterial cells have also not been studied.

The environments in which biofilms are found vary greatly. In some, the aqueous milieu is effectively stagnant with no shear exerted on the biofilm and its components. Others, including oral biofilms, are subjected to repeated, and sometimes high, shear forces. These will inevitably displace or destroy sections of the biofilm. Where the shear is constant, the eventual structure will again be different. As pointed out by Ross-Murphy & Shatwell (1993), large deformations lead to rupture of strong gels similar to agarose, whereas weak gels like xanthan will recover and can even flow during the shear. Both types of behaviour can be expected from biofilm EPS. This was observed in mixed species biofilms studied by Stoodley *et al.* (1999b). Under laminar flow, roughly circular micro-colonies were separated by water channels, whereas in turbulent flow, filamentous streamers were seen with ripple-like structures after prolonged growth. Unfortunately, although cell counts for the different component species were determined,

the nature and composition of the polysaccharides was not analysed.

Conclusion

Biofilms provide an almost infinite range of EPS and it is unlikely that any of these are specifically and uniquely synthesized in biofilms. They do, however, confer on biofilms many of their physical characteristics. Their ability to interact with other polysaccharides and with other macromolecules and cells, as well as with ions and low-molecular-mass solutes, provide a multitude of microenvironments within any biofilm. Currently, many of these effects can only be speculated upon, although application of novel probes and improved analytical methods will gradually expand on our current, rather limited, and perhaps blinkered view of what biofilm structures really are and the extent to which they are determined by EPS.

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