Biofilm exopolysaccharides: a strong and sticky framework

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Overview

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 Soodley 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 cellullosic 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 ketalts (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 0.5–2.0 × 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 Escherichia 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 galactogluconan 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.

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
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 algal 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-$\beta$-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.
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 of acetyl groups. Both EPS have the same tetrasaccharide repeat units containing D-glucose, 1-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.
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 succino-glycan 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 micro-organisms. 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 different EPS, including dextrins (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.

**References**


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