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The biofilm matrix – an immobilized but dynamic microbial environment

Ian W. Sutherland

The biofilm matrix is a dynamic environment in which the component microbial cells appear to reach homeostasis and are optimally organized to make use of all available nutrients. The major matrix components are microbial cells, polysaccharides and water, together with excreted cellular products. The matrix therefore shows great microheterogeneity, within which numerous microenvironments can exist. Although exopolysaccharides provide the matrix framework, a wide range of enzyme activities can be found within the biofilm, some of which will greatly affect structural integrity and stability.

Ian W. Sutherland
Institute of Cell & Molecular Biology, Edinburgh University, Rutherford Building, Mayfield Road, Edinburgh, UK EH9 3JH.
e-mail: i.w.sutherland@ed.ac.uk

With the realization that many, perhaps even the majority of microorganisms exist naturally as biofilms, interest in these phenomena has increased considerably. Not only are biofilms found in a very wide range of natural and artificial environments, they also provide their component microbial cells with an almost infinite range of constantly changing microenvironments. The matrix can almost be considered as an immobilized enzyme system in which the milieu and the enzyme activities are constantly changing and evolving to an approximately steady state. This steady state can then be radically altered by applying physical forces such as high shear, or via external or internal reactions that cause the detachment and loss of regions of the biofilm. It must also be remembered that, because of the wide range of environments in which biofilms are found, it is extremely difficult to generalize about their structure and physiological activities[1]. The nature of the matrix, as exemplified by Wimpenny[2], is thus dependent on both intrinsic and extrinsic factors. Intrinsic factors arise in accordance with the genetic profile of the component microbial cells; extrinsic factors include the physico-chemical environment in which the biofilm and its matrix are located, which, inevitably, is constantly influenced by solute transport and solute diffusion gradients.

Sufficient information on biofilms and their structure is now available to permit the construction of realistic models[3]. Three variants have been suggested – heterogeneous mosaic, dense confluent and penetrated water channel – and all could be correct given the wide variety of biofilms that have been studied. Biofilms are found in the majority of environments, natural or artificial, where a surface is

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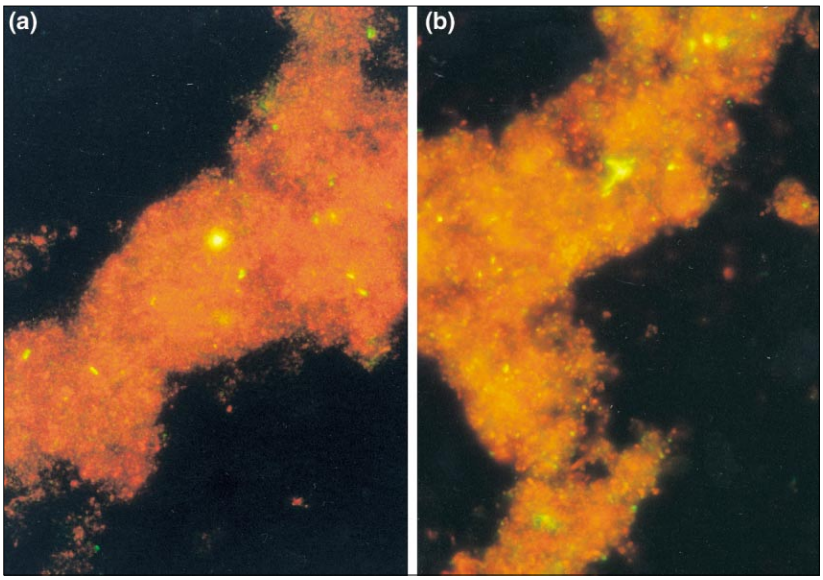

Fig. 1. Microcolonies from a 16 h dual enteric species biofilm on a glass coverslip showing the heterogeneity found within even a small part of a biofilm. Cultures were treated with 10 mM cetyl pyridinium chloride followed by 25 μg ml^{-1} propidium iodide. Cells expressing green fluorescent protein (GFP) appear green or yellow, and the second species is red. Staining reveals large amounts of exopolysaccharide (EPS) associated with the cells and contributing to the biofilm heterogeneity. Figure courtesy of Dr L.C. Skillman.

exposed to adequate moisture. They are of as great significance in natural systems as they are in many disease syndromes and industrial processes, and we rely on biofilm-based systems for the purification of drinking water and the safe disposal of waste water. Biofilms therefore represent both nutrient-rich and oligotrophic environments. Many laboratory studies have concentrated either on oral systems or on laboratory models in which *Pseudomonas aeruginosa* has been the microbial species of choice. This has perhaps led to the misconception that all biofilm matrices resemble these systems, and ignores the complexity and diversity of the environments in which the majority of biofilms are found.

Composition of the biofilm matrix

Much of the biofilm matrix – perhaps up to 97% – is actually water[4], although, as with all aspects of biofilms, this will depend on the specific system under examination. The water can be bound within the capsules of microbial cells or can exist as a solvent whose physical properties such as viscosity are determined by the solutes dissolved in it. Water binding and mobility within the biofilm matrix are integral to the diffusion processes that occur within the biofilm; water is therefore organized within the fine structure of the biofilm[5]. Apart from water and microbial cells, the biofilm matrix is a complex of secreted polymers, absorbed nutrients and metabolites, products from cell lysis and even particulate material and detritus from the immediate surrounding environment. Thus, all major classes of macromolecule – proteins, polysaccharides, DNA and RNA – can be present in addition to peptidoglycan, lipids, phospholipids and other cell components. The amount of cellular material within a biofilm can itself vary greatly (Fig. 1); values for total organic carbon suggest that cellular material might represent as little as 2–15%, with the remainder being extracellular in nature[6]. Because of its inherent complexity, analysis of the biofilm matrix has proved difficult. One can either attempt to extract the matrix components singly or in mixtures and analyse them, or one can use specific probes to identify the presence of certain epitopes, macromolecules or monomers. Both approaches have been used with varying degrees of success. Jahn *et al.*[7] extracted a mixture of polymers from *Pseudomonas putida* biofilm material and found it to be very heterogeneous. In contrast to the gross composition of typical biofilms (Table 1), proteins comprised the largest fraction (75%) of the extract; polysaccharide and DNA were also present.

Chemical, biological and physical methods have been used to probe the matrix structure. Costerton *et al.*[8] prepared antibodies against a planktonically synthesized polymer and used them to reveal interaction with material in a biofilm matrix. This indicated that some components of the biofilm exopolysaccharide (EPS) had the same composition as the planktonic product. Further confirmation of the close similarity and probable identity of biofilm and planktonic polysaccharides has been obtained by using highly specific, phage-induced polysaccharases[9]. The apparent complexity of composition reported for some materials obtained from biofilm isolates is explicable if only very small amounts of one polymer are produced and are impossible to separate from large quantities of a second polysaccharide.

Although new methods have been developed for probing the composition of the biofilm matrix, not all have proved successful. Fluorescently labelled lectins were used by Johnsen *et al.*[10] as non-invasive probes to localize carbohydrate-containing polymers within biofilms. Binding of the lectins was not necessarily proof of the presence of specific targets in the biofilms as the lectins also bound non-specifically or adhered to other components present in the biofilm matrix. However, identification of specific cell types within the matrix through expression of green fluorescent protein (GFP) and its variants has been extremely valuable[11]. Other microsensor probes can be used to determine dissolved oxygen and pH, and pH mapping throughout the matrix has indicated that, in dental biofilms, pH heterogeneity is extensive both before and after the addition of a carbon substrate such as sucrose.

Confocal scanning laser microscopy (CLSM) has provided some new information on the structural complexity of biofilms and has confirmed their heterogeneity[12]. The consensus from such studies is that biofilms comprise aggregates of microbial cells within a matrix of EPS and interstitial voids and channels separate the microcolonies. Several studies indicate that EPS is not necessarily required for the initial attachment of microbial cells to surfaces[13], but the production of EPS is essential for the development

Table 1. Range of composition of biofilm matrices

Component	% of matrix
Water	up to 97%
Microbial cells	2–5% (Many species)
Polysaccharides (homo- and heteropolysaccharides	1–2% (Neutral and polyanionic)
Proteins (extracellular and resulting from lysis)	<1–2% (Many, including enzymes)
DNA and RNA	<1–2% (From lysed cells)
Ions	? (Bound and free)

of the architecture of any biofilm matrix[14]. The EPS molecules provide the framework into which microbial cells are inserted. As EPS synthesis continues following cell attachment and as EPS might even provide a nutrient source for some of the cells, these macromolecules are dynamic components of the biofilm[15]. When combined with molecular probes, the distribution of individual microbial species within the matrix can be plotted[16], but it is not yet possible to distinguish different EPSs within mixed biofilms. Use of dual labelling with GFP followed by CLSM located the positions of bacterial cells within a biofilm matrix and confirmed the heterogeneity of their distribution[16]. Interestingly, in the system examined, cells of *Escherichia coli* were seen near the solid (poly-lysine coated) substratum whereas *P. aeruginosa* formed a thick layer superimposed on the other species. Although no data on its composition or structure were reported, this study also indicated that the matrix was sufficiently fluid to permit redistribution of the two cell types within its structure during growth and development. This is probably a feature of matrices in which the polysaccharides are relatively fluid, whereas in others in which the polymers form more rigid gels, cells can remain effectively immobilized.

The biofilm matrix structure

The actual structure of the biofilm matrix will vary greatly depending on the microbial cells present, their physiological status, the nutrients available and the prevailing physical conditions. The cells within the matrix can be dispersed and can form a thin layer with surrounding EPS or, as in the case of dental plaque, can form a thick adherent covering on oral surfaces. As pointed out by Lawrence *et al*.[17], even the initial microcolonies can develop in distinct ways with resultant differences in the final matrix architecture. It has also become clear from CLSM that, even in the architecture of dental plaque, channels and fluid-filled voids were detectable[18]. Thus, although it can possess a more dense structure than some other types of biofilm matrix, as was suggested by earlier studies using electron microscopy, dental plaque also has some similarities to *P. aeruginosa* monocultures. In all these systems, the channels permit the flow of nutrients, enzymes, metabolites, waste products and other solutes, throughout the biofilm complex. Sections through the matrix of any biofilm will also reveal differences in density. This parameter will depend on the nature of the communities of microbial cells found within the matrix, their metabolic activity, secretion of extracellular polymers and other activities. Cell density in *E. coli* biofilms has also been shown to be dependent on the expression of *rpoS*, a gene which, among its other functions, is expressed during slow growth[19].

Although EPSs are regarded as the major structural components of the biofilm matrix, various interactions maintain its structural integrity. Polysaccharide molecules can interact with themselves or with heterologous molecules to yield gels, often with multivalent cations playing a significant role in the process. Polysaccharides also interact with proteins and glycoprotein molecules both as solutes and when attached to the surface of the microbial cells. In oral biofilms, the reactions between cell-bound bacterial lectins and suitable epitopes on the surface of other cells have been shown to be an important feature in maintaining the structure of plaque[20]. The lectins permit intergeneric aggregation with high specificity and some of the bacterial cells act as bridges between different cell groups. They also allow selective recruitment of new cells to the peripheral areas of the matrix. The epitopes that lectins bind can be either on the cell surface or can form part of excreted EPS and they can thus strengthen the matrix structure and appear to be especially significant in the early stages of its establishment, through co-aggregation of various bacterial types found in the oral cavity. The microheterogeneity of the surface of the oral biofilm matrix is constantly changing as cells attach (and detach). As a result of this, the lectins on the bacterial cell surfaces, or the epitopes to which they bind, are occluded (or exposed). We still do not know how other interactions – protein–protein, protein–polysaccharide or ionic – affect the overall structure and properties of the biofilm matrix.

Physiological determination of matrix composition

Matrix composition and architecture for any specific system are subject to physiological determinants including substrates and metabolites. In a recent study of oral biofilms, supplementation with sucrose yielded significantly denser and thicker biofilms[21]. This is almost certainly caused by the ability of many oral bacteria to synthesize dextrans (including the insoluble 1,3-α-D-glucan mutan) and levans using sucrose as a substrate. In general, higher levels of nutrients lead to much denser biofilms than those observed under oligotrophic conditions. However, some of the natural biofilms involving both prokaryotic and eukaryotic cells can be both extensive and relatively dense[22]. Møller *et al*.[15] observed that if a mixed biofilm was grown on a poorly used substrate then switched to a rich medium, the appearance of the matrix changed considerably. Initially, the biofilm revealed mounds of cells but became much more uniform on alteration of the nutrient by one of the strains and utilization of the products by the other.

Further studies on dual-species biofilms grown in the presence of chlorobiphenyl indicated that microcolonies comprising both species were formed, whereas use of a substrate utilizable by both species yielded separate microcolonies[23]. On shifting back to the original substrate, the bacteria were able to move through the matrix and re-associate with the other species. Other studies have indicated that both single- and double-species biofilms altered their appearance as the level of a toxic metabolite (*p*-cresol) was increased, thus further supporting the concept that the architecture of the biofilm matrix is strongly substrate dependent[24].

Biofilms in differing environments are subject to a very wide range of hydrodynamic conditions, which will greatly affect the matrix[25]. The transport of microbial cells and of nutrients and waste products is subjected to these conditions. The shear rate will determine the rate of erosion of cells and regions of the matrix from the biofilm. The shear stresses to which a biofilm is exposed will also affect the physical morphology and dynamic behaviour[25,26]. Polysaccharide solutions will exhibit flow and elastic recovery; because of the flexibility of the matrix its shape will also change in response to an applied force. Under turbulent flow, the biofilm, consisting mainly of polysaccharide sols or weak gels, flows like a viscous fluid. The combination of turbulent flow and increased carbon source changes the appearance of an established biofilm from ripples and streamers to mounds of closely packed cells. There is also less of a tendency for the biofilm to move downstream. Under higher flow rates, the matrix appears to be more firmly attached.

In the natural environment, bacterial cells normally have to adapt rapidly to alterations in the surrounding medium. However, it has been suggested that for cells within the biofilm matrix this might not be necessary and that the matrix provides a buffer against changing organic nutrients[27]. As many of the matrix polymers are anionic in nature, they might also bind cations and provide a reserve of these essential nutrients. The matrix itself can also act as a carbon and energy reserve. Normally, microbial cells are unable to utilize EPSs that they have synthesized, although some of those found in dental biofilm matrices can be exceptional in this respect. However, heterologous species within the matrix can degrade and utilize the EPS, thus also altering the composition and structure of the biofilm.

Changes within the matrix

Within a biofilm matrix, the microbial cells are in close proximity to one another and competition for available nutrients is likely to be intense. This also means that any antimicrobial compounds released by one cell type, such as bacteriocins, microcins, antibiotics or phage, have a good chance of successfully attacking and possibly destroying neighbouring heterologous cell types. These same compounds entering from the external environment can also target cells either at the periphery or within the matrix. The presence of relatively large channels and pores within the matrix structure might allow entry of colonizing cells and their establishment within the biofilm. It has been suggested that hydrophobic cells would attach both on the surface and within a floc structure whereas hydrophilic cells would not[28]. Thus, the microbial and macromolecular composition of the matrix changes over time.

Many bacteria are now known to secrete surfactants. These molecules will alter the internal matrix of the biofilm, although their precise effect is hard to determine. Al-Tahhan *et al*.[29] suggested that even very low levels of a rhamnolipid biosurfactant could render the cell surface more hydrophobic, and lead to loss of lipopolysaccharide (LPS) from the surface of Gram-negative cells. Biosurfactants might be involved in the horizontal transfer of exopolymer from one bacterial species to another[30] as part of the degradation process. This process could take place more efficiently within the matrix of a biofilm where cells are in close proximity to each other. Production of surfactants also enables the microbial cells within biofilms to solubilize and utilize substrates that would otherwise be inaccessible. The surfactants can either be bound to the surface of the microbial cell or excreted from it. Thus, their effect might either be highly localized or occur distant from the cells that produce them. Neu[31] suggested that if amphiphilic polymers were anchored in the cell surface such that the hydrophilic region was exposed, it might enhance the interaction with hydrophilic surfaces. The converse might also apply if anchoring occurred via the hydrophilic moiety. The excreted biosurfactants could also lead to loss of matrix material from a surface through alteration of the conditioning film by which it is attached.

In oral biofilms, the presence of glycosyltransferases capable of synthesizing levans and dextrans[32] means that when sucrose is available for EPS synthesis the matrix composition is constantly changing. Enzymes degrading both these polymers can also be present. Thus, new surfaces are created and others masked. In turn, new species can then adhere; for example, glucans mediate the binding of *Veillonella* spp. and *Streptococcus mutans*. In this type of biofilm, there are likely to be much greater changes in the matrix architecture within a relatively shorter time scale than might be found in other types. Enzymes altering the macromolecules within the matrix will also have a very marked influence on its physical properties. Polysaccharide-degrading enzymes of microbial or phage origin will cause localized destruction[33], with possible weakening of the community structure and loss of both cells and macromolecules. At higher concentrations, almost complete removal of the matrix can occur. Esterases with wide specificity excreted by some bacteria can remove acyl groups

Questions for future research

- Can we determine exactly how the matrix is ordered? The application of atomic force microscopy has revealed networks of bacterial polysaccharide gels. Can it also reveal how these polymers are structured within the biofilm matrix?
- Which are the 'key' polymers involved in maintaining matrix structure? In 1990, Neu and Marshall posed the question 'What is the structure of the true adhesive polymer'? We still cannot answer that question. As systems vary so much, EPS could be most important in maintaining matrix structure in one type of biofilm, whereas proteins and lectins could be more significant in another.
- Can we make probes sensitive enough to tell us which cells within the matrix are producing the signals and excreting products such as homoserine lactones?
- Which cells are producing the polysaccharide skeleton, and indeed for how long do cells within the biofilm matrix excrete EPS?
- Can we trace the flow of solutes more effectively into, through and from the matrix?
- Can we also determine which cells and which enzymes within the matrix are active in changing its composition and structure? If so, should we regard the biofilm matrix as a primitive type of developmental biology in which the spatial organization of the cells within the matrix optimizes the utilization of the nutritional resources available?

from bacterial polymers as well as from other esters[34]. If enzymes of this type acted on the structural polysaccharides of a biofilm matrix, they could alter the physical properties of the biofilm structure either locally or perhaps more extensively. Deacylation of the bacterial polysaccharides might cause improved pseudoplasticity in aqueous solution and increase the cooperativity of the polymer strands as they undergo a transitional change from random coils to ordered helices[35]. Alternatively, deacylation of some polysaccharides might lead to loss of any ordered conformation[36]. A third possibility is that loss of acyl substituents from polysaccharides enhances gel formation, thus strengthening portions of the biofilm structure[37].

Muramidases active against the peptidoglycan and capable of degrading adjacent bacterial cells have been observed as components of membrane vesicles released from the surface of *P. aeruginosa*[38] along with other enzyme activities. As other bacterial cells are lysed by such autolysins, they would markedly affect matrix structure via the degradation of neighbouring cells. Cell lysis would also release intracellular nutrients, enhancing the growth of the lysin-producing bacteria.

Functionality of the biofilm matrix

In addition to its structural complexity, the biofilm matrix can be regarded as a functional community of microbial cells, often achieving as a community what the individual species cannot: in other words, the properties of the community of microbial cells within the matrix are greater than that of the sum of the individual species. This can be a direct result of the high cell densities found within the biofilm matrix. Examples of this can be seen in the co-metabolism of xenobiotics and other complex substrates. Within the matrix, the molecules required for cell–cell communication and community behaviour might also accumulate at high enough concentrations to be effective. In Gram-negative bacteria, one such mechanism – quorum sensing – involves homoserine lactones as signal molecules. Once a critical population density has been reached, the bacteria respond by activating the expression of a range of specific genes[39]. In a study of a three-species enteric biofilm, a nitrogen-fixing *Klebsiella* sp. fixed nitrogen only in mature biofilms, indicating that initially either sufficient fixed nitrogen was available or, more probably, that anaerobiosis was not induced until the matrix was thicker[40]. Another example of the matrix functionality achieving results impossible for the individual components is the generation of alkaline conditions through ammonia production in oral biofilms, leading to pH homeostasis[41].

Possible ways forward

Inevitably, simple models such as monocultures will provide both further information on the biofilm matrix and a means of evaluating new techniques, although microbiologists will have to look beyond *P. aeruginosa* and *E. coli*. However, eventually, complex systems more closely resembling natural biofilms must also be examined. Neu *et al.*[42] recently pointed out that lectins provide valuable tools for examining the glycoconjugates found in biofilm systems. We must also determine accurately how much of each component polysaccharide is present in the matrix and the extent to which removal or modification of these polysaccharides alters the structure and integrity of the biofilm under a wide range of physiological conditions. Specific enzymes such as the phage-induced polysaccharases used by Hughes *et al.*[8] could prove valuable for this. It must be remembered that although different strains can apparently synthesize the same EPS, there can be differences in physical properties especially with respect to viscosity and gel formation. Several recent biofilm studies have used colanic acid-producing *E. coli*[43,44], yet this polymer can vary greatly in mass and viscosity, as can bacterial alginates. Improved chemical and physical microanalytical methods can provide better definition of these systems.

The effects of changing metabolites including xenobiotics have already been discussed, but few studies have examined the enzyme activities associated with the matrix of biofilms or sludge flocs. In one study, peptidase was the major enzyme activity detected but esterases were also present[5]. The activities were immobilized in the matrix but could be released into the aqueous phase by disassociating the floc. Chromogenic substrates linked to CFSM might indicate whether enzyme activities are uniformly distributed in the matrix.

As suggested by Tait[45], bacterial warfare, that is, excretion of products such as bacteriocins and microcins from one species affecting cells of others, does occur within biofilms. However, despite initial antagonism between related species, a *modus vivendi* might eventually be established, resulting in biofilms that allow both the producing and target species to co-exist. This means that the structure of the matrix will change considerably as the equilibrium between the species is established and a balance between competition and commensalism is achieved within the microbial community. Bacteriocins, microcins and bacteriophages also provide very specific tools for the selective attack of bacterial cells within mixed biofilms.

Conclusions

The biofilm matrix is a highly complex array of microenvironments. The different components within the biofilm – water, polysaccharides and other macromolecules – offer a range of localized and constantly changing effects that generate osmotic and nutrient gradients. In any biofilm, these will contribute to the heterogeneous composition of the matrix, while also contributing to its multicellular function.

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