The role of hydrophobicity and exopolymers in initial adhesion and biofilm formation

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2.1 INTRODUCTION

The formation of a biofilm includes several steps but a prerequisite is the adhesion of microbial cells to a solid surface. Studies of bacterial adhesive properties have indicated that a number of cell surface physico-chemical factors contribute to the process of adhesion. Such factors include cell surface hydrophobicity (Busscher et al., 1990; Oliveira et al., 2001), the presence of extracellular polymers (Allison and Sutherland, 1987; Azeredo and Oliveira, 2000a) and cell surface charge. The latter determines the electrostatic interaction between the cell and the substratum (van Loosdrecht et al., 1990). However, in most common situations, i.e. aqueous media with pH near neutrality, the microbial cells and solid substrata are negatively charged. This means that surface charge normally has a repulsive effect and acts contrary to adhesiveness. The extracellular polymers are not only important for the adhesion process but also determine the structure of the biofilm. Due to the importance of hydrophobicity in the first stage of biofilm formation and the role of extracellular polymers in the final establishment of a biofilm, they will be the subjects of the following overview.
2.2 THE IMPORTANCE OF HYDROPHOBICITY IN INITIAL MICROBIAL ADHESION

In the last two decades, many studies have been referring to the effect of hydrophobicity in microbial adhesion. Most of these studies have focused on the effect of substrate on bacterial adhesion, but there is now evidence that microorganisms and viral particles have evolved ways to use the hydrophobic effect to adhere to solid substrata (Doyle, 2000). Albeit the recognised importance of hydrophobicity in the adhesion process, it has been very difficult to have an acceptable definition of hydrophobicity, especially because different experimental methodologies have been used for its measurement. In the case of solid substrata, the most common methods are contact angle measurement for flat surfaces and thin-layer wicking for particulate materials (Teixeira et al., 1998). For microbial cells, there is a higher diversity of methods (van der Mei et al., 1987; Doyle, 2000), with microbial adhesion to hydrocarbons (MATH), hydrophobic interaction chromatography (HIC), salt aggregation test (SAT) and microsphere adhesion being the most commonly used. All these methods have some intrinsic drawbacks, especially those based on the adhesion of cells to some liquid or solid material because they are dependent on factors as temperature, time, pH, ionic strength and relative concentration of interacting species, which can all combine to influence the adhesive event (Ofek and Doyle, 1994).

Presently, it is almost generally accepted that the contact angle method is probably the most reliable way to determine cell surface hydrophobicity (Doyle, 2000). According to this method, hydrophobicity is usually expressed in terms of the contact angle formed by a sessile drop of pure water – a contact angle higher than 90° means a hydrophobic surface. However, it is not very easy to obtain a flat non-porous cell layer enabling reproducible measurements. Thus, there are still many situations where other techniques are used and each one leads to a specific way to express hydrophobicity. On account of this, it is not correct to define the surface ‘hydrophobicity’ of a microbial strain other than on a comparative level with closely related strains (van der Mei et al., 1987). Moreover, these approaches do not enable the calculation of the hydrophobic interaction established between the microbial cells and the supporting substratum.

2.2.1 Quantification of hydrophobicity

The above-mentioned limitations are circumvented in the approach proposed by van Oss (1995), which is based on the definition of hydrophobicity of a given entity (i) (macromolecule, microbial cell or solid surface) as the free energy of interaction between two entities (i) when immersed in water (w) – \( \Delta G_{\text{int}} \). If \( \Delta G_{\text{int}} < 0 \), there is a preferential interaction between entities (i) rather than between an entity (i) and water and the substance (i) is considered hydrophobic. By the same reasoning, if \( \Delta G_{\text{int}} > 0 \), the substance (i) is hydrophilic. It must be stressed that \( \Delta G_{\text{int}} \) can be expressed in SI units, and thus, considered in calculations involving the contribution of other forms of energy of interaction.
Biofilm characteristics

\[ \Delta G_{\text{wai}} \text{ is simply related to the interfacial tension between } i \text{ and water, } \gamma_{iw}, \text{ as:} \]

\[ \Delta G_{\text{wai}} = -2 \gamma_{iw} \]  
(2.1)

However, the determination of the surface free energy (i.e. surface tension) of solids can only be obtained by indirect measurements. Therefore, \( \gamma_{iw} \) can be determined by contact angle measurements (van Oss et al., 1988) or thin-layer wicking (Teixeira et al., 1998). The latter is appropriate when the solid material is in particulate form (Teixeira et al., 1998).

Considering the approach of van Oss et al. (1988) and van Oss (1991), the surface free energy of a solid or a liquid, \( \gamma^\text{TOT} \) is the sum of apolar Lifshitz–van der Waals (LW) \( \gamma^\text{LW} \), and polar acid–base (AB) interactions, \( \gamma^\text{AB} \):

\[ \gamma^\text{TOT} = \gamma^\text{LW} + \gamma^\text{AB} = \gamma^\text{LW} + 2(\gamma_i^- \gamma_i^+)^{1/2} \]  
(2.2)

The polar interactions are mainly due to London dispersion interactions, but induction (Debye) and orientation (Keesom) interactions may also be involved (van Oss et al., 1988). In many situations, the polar AB interactions consist entirely in hydrogen bonding; and in the most general sense, they are electron donor, \( \gamma_i^- \), and electron acceptor, \( \gamma_i^+ \), interactions. Thus, the interfacial free energy between entity \( i \) and water (w) can be expressed as:

\[ \gamma_{iw} = \gamma_i^\text{LW} + \gamma_w^\text{LW} - 2(\gamma_i^\text{LW} \gamma_w^\text{LW})^{1/2} \]

\[ + 2((\gamma_i^- \gamma_i^+)^{1/2} + (\gamma_i^- \gamma_w^-)^{1/2} - (\gamma_i^+ \gamma_w^+)^{1/2} - (\gamma_i^- \gamma_w^+)^{1/2} \]  
(2.3)

The surface free energy components of water are known, but the corresponding values for the entity \( i \) have to be determined. For a solid substratum or microbial cells, the surface tension components can be determined by measuring the contact angles (\( \theta \)) formed by three different liquids (for which apolar, \( \gamma_i^\text{LW} \) and polar components \( \gamma_i^- \), \( \gamma_i^+ \) are known) on its surface. Thereafter, three forms of the following equation, resulting from Young’s equation, are obtained and solved simultaneously to calculate, \( \gamma_i^\text{LW}, \gamma_i^- \) and \( \gamma_i^+ \):

\[ \gamma_i (1 + \cos \theta) = 2\sqrt{\gamma_i^\text{LW} \gamma_i^-} + 2\sqrt{\gamma_i^+ \gamma_i^-} + 2\sqrt{\gamma_i^+ \gamma_i^+} \]  
(2.4)

In the set of liquids used (Table 2.1), one has to be non-polar for the determination of \( \gamma_i^\text{LW} \).

According to van Oss (1997), in biological systems, hydrophobic interactions are usually the strongest of all long-range non-covalent interactions. Its sole driving force is the hydrogen bonding (also designated AB forces or Lewis AB) energy of cohesion between the surrounding water molecules. This means that the AB forces, if strongly asymmetrical or monopolar, are responsible for the orientation of water molecules adsorbed on the surfaces. Moreover, water molecules oriented on the surface of one particle will repel water molecules oriented in the same manner on the surface of an adjacent particle (Parsegian et al., 1985; van
Table 2.1  Surface tension parameters (mJ/m²) of the liquids commonly used in contact angle measurements for the determination of solids surface tension.

<table>
<thead>
<tr>
<th>Liquid</th>
<th>$\gamma_{\text{TOT}}$</th>
<th>$\gamma_{\text{LW}}$</th>
<th>$\gamma^+$</th>
<th>$\gamma^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>72.8</td>
<td>21.8</td>
<td>25.5</td>
<td>25.5</td>
</tr>
<tr>
<td>Glycerol</td>
<td>64.0</td>
<td>34.0</td>
<td>3.9</td>
<td>57.4</td>
</tr>
<tr>
<td>Formamide</td>
<td>58.0</td>
<td>39.0</td>
<td>2.3</td>
<td>39.6</td>
</tr>
<tr>
<td>Di-iodomethane</td>
<td>50.8</td>
<td>50.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$\iota$-Decane</td>
<td>23.8</td>
<td>23.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$\alpha$-Bromonaphthalene</td>
<td>44.4</td>
<td>44.4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Oss, 1994). If the orientation of the water molecules is sufficiently strong, the two particles will not approach each other. On the other hand, if the surface is more weakly apolar, its capacity for orienting the most closely adsorbed water molecules is less pronounced and the particles will approach each other under the influence of their net LW attraction. Thus, ‘hydrophobic’ compounds or surfaces do not repel water: they attract water with rather substantial binding energies, albeit not quite strongly as very hydrophilic ones (van Oss, 1995). It should be stressed that hydrophobic attractions can prevail between one hydrophobic and one hydrophilic site immersed in water, as well as between two hydrophobic entities. Summarising, the so-called hydrophobic interaction is a Lewis AB interaction and for two entities (i) immersed in water, it can be expressed by (see Equation (2.1)):

$$\Delta G_{\text{AB}}^{\text{LW}} = -4 \left[ \sqrt{\gamma_i^+ \gamma_i^-} + \sqrt{\gamma_w^+ \gamma_w^-} - \sqrt{\gamma_i^+ \gamma_w^-} - \sqrt{\gamma_i^- \gamma_w^+} \right]$$ (2.5)

with $\gamma_i^+$ and $\gamma_i^-$ being calculated from a system of Equations (2.4). It is also possible to calculate the AB free energy of interaction between an entity 1 and an entity 2 when immersed in water:

$$\Delta G_{1w2}^{\text{AB}} = 2 + \left[ \sqrt{\gamma_1^+ \gamma_2^-} + \sqrt{\gamma_1^- \gamma_2^+} - \sqrt{\gamma_1^+ \gamma_2^-} \right]$$

$$+ \left[ (\gamma_1^+ \gamma_2^-)^{1/2} - (\gamma_1^- \gamma_2^+)^{1/2} \right]$$ (2.6)

This means that it is possible to extend the DLVO theory (named after Derjaguin, Landau, Verwey and Overbeek (Oliveira, 1997)) to account for ‘hydrophobic interactions’. The DLVO theory (Oliveira, 1997), that has been applied to explain microbial adhesion, considers that the total energy of interaction is the balance between the energy of interaction due to LW forces ($\Delta G_{1w2}^{\text{LW}}$ – usually attractive) and the electrostatic energy of interaction arising from the interpenetration of the electrical double layers of the two interacting surfaces ($\Delta G_{1z2}^{\text{LZ}}$ – normally
repulsive). In its extended version (XDLOV), the total energy of interaction ($\Delta G_{iw}^{TOT}$) is given by:

$$\Delta G_{iw}^{TOT} = \Delta G_{iw}^{LW} + \Delta G_{iw}^{EL} + \Delta G_{iw}^{AB}$$  \hspace{1cm} (2.7)

### 2.2.2 Hydrophobic effect in initial adhesion

In a study on the attachment of *Staphylococcus epidermidis* to cellulose diacetate (Oliveira et al., 2001; Fonseca et al., 2001) three different strains were used: *S. epidermidis* ATCC, 35984 (RP62A) and the strains M187 and M187-Sn3 kindly offered by Gerald B. Pier (Channing Laboratory, Harvard Medical School, Boston, MA, USA). The strains RP62A and M187 have a capsule and are polysaccharide/adhesin positive (PS/A+), while M187-Sn3 is an isogenic mutant of M187 and is polysaccharide/adhesin negative (PS/A−). The polymeric material and the bacterial cells were characterised in terms of their surface tension (hydrophobicity) by contact angle measurements (Table 2.2).

As $\Delta G_{iw}$ is positive, the three strains are hydrophilic, but a higher degree of hydrophilicity means a lower degree of hydrophobicity and directly correlates with the number of attached cells. It is interesting to note that the strain devoid of the PS/A is the one with less ability to attach, as could be expected, but it is also the less hydrophobic.

In the sequence of the same study, four polymeric materials, commonly used in indwelling devices, were assayed to test their ability to be colonised by the strain RP62A: polyethylene (PE), silicone (SI), expanded polytetrafluorethylene (ePTFE) and cellulose diacetate (CDA). The results are presented in Figure 2.1.

In this case, the materials can all be considered hydrophobic ($\Delta G_{iw} < 0$) and an increase in the degree of hydrophobicity linearly correlates with the number of attached cells. A similar behaviour of linear correlation between the hydrophobicity of the substrata and the number of cells attached was encountered for an anaerobic consortium (Alves et al., 1999) and in the attachment of *Alcaligenes denitrificans* to polymeric supports (Teixeira and Oliveira, 1999).

The hydrophobic effect is also an important factor in yeast cells attachment. An interesting example is the comparison between the binding ability of two

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of cells adhered (CFU/mm² × 10⁵)</th>
<th>$\Delta G_{iw}$ (mJ/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP62A</td>
<td>3.31 ± 0.17</td>
<td>17.5</td>
</tr>
<tr>
<td>M187</td>
<td>3.33 ± 0.39</td>
<td>17.4</td>
</tr>
<tr>
<td>M187-Sn3</td>
<td>2.08 ± 0.40</td>
<td>31.9</td>
</tr>
</tbody>
</table>
Saccharomyces cerevisiae strains to different types of materials (Nakari-Setälä et al., 2002). One strain was a transformant able to express an hydrophobin of the filamentous fungus Trichoderma reesei (HFBI) on the yeast cell surface, the other was the parent strain. The materials used were strained, siliconised strain and immobasil. The first two are hydrophobic (\(\Delta G_{w} = 120 \text{ mJ/m}^2\) and \(\Delta G_{w} = 21 \text{ mJ/m}^2\), respectively) and immobasil is hydrophobic (\(\Delta G_{w} = -29 \text{ mJ/m}^2\)). The strain expressing the hydrophobin displayed a higher hydrophobicity and also had a higher-binding ability to all materials, which increased with the increasing degree of material hydrophobicity. A point to note is that the immobasil surface became less hydrophobic when coated with pure HFBI protein and a decrease in yeast cells attachment was concomitantly detected. This alteration of the coated surface is supposed to be due to the exposure of the hydrophilic sites of the macromolecule on account of the hydrophobic ones being in direct contact with the solid surface. However, for longer periods of contact with the hydrophobin, the hydrophobicity of the immobasil surface raised again. Such a phenomenon can be explained by the self-assembly of the protein on the surface to form bilayered structures (van der Vegt et al., 1995). This is one of the possible effects of extracellular polymers in the process of microbial adhesion as will be outlined in the next section.

2.3 THE IMPORTANCE OF EXOPOLYMERS IN INITIAL MICROBIAL ADHESION AND BIOFILM FORMATION

2.3.1 Exopolymers in initial adhesion

The involvement of exopolymers in the advents of initial adhesion has been recognised for a long time. Marshall et al. (1971) suggested that exopolymers
were involved in a time-dependent irreversible phase of adhesion. This hypothesis was later supported by Fletcher and Floodgate (1973), who demonstrated that the production of a secondary acidic polysaccharide-mediating irreversible adhesion of a marine bacterium to a solid was a time-dependent process.

The observation of the complementary microbial cell surface pattern on a surface (footprint) in which adhering cells were removed by shear force (Marshall et al., 1971), enzymes (Paul and Jeffre, 1985) or sonication (Neu and Marshall, 1991) was a clear evidence that extracellular polymers are somehow involved in the interaction between microorganisms and surfaces. The term footprint was first used to designate polymeric materials that are left onto a surface after the bacterial cells were removed by a shear force (Marshall et al., 1971). Later, it was suggested to extend the term footprint to all molecules by which bacteria are able to label an interface (Neu, 1992). Thus, adsorption footprints (true adhesive polymers obtained when removing bacteria artificially from interfaces) or desorption footprints (molecules released by bacteria to detach themselves from interfaces) were distinguished. Nevertheless, these molecules are extracellular polymers (polysaccharides, biosurfactants, proteins, glycoproteins, lipids, etc.) that have an important role in the interaction between microorganisms and surfaces. Another important feature of microbial footprints is that they may influence the adhesion of microorganisms to a surface. In a recent survey, it was shown that footprints of Pseudomonas aeruginosa strains detached by passing air bubbles had a negative influence on the adhesion of newly redepositing cells (Gómez-Suárez et al., 2002).

Studies of microbial footprints have given an important contribution to the knowledge of the role of exopolymers in microbial adhesion. Other studies based on the utilisation of mutants with different capabilities to secrete exopolymers have also enlightened the contribution of exopolymers in adhesion (Flemming et al., 1998; Azeredo and Oliveira, 2000a; Gómez-Suárez et al., 2002). The adhesion to glass of three mutants of Sphingomonas paucimobilis having different gellan production abilities was studied in the presence and absence of solutions of their exopolymers (Azeredo and Oliveira, 2000a). The results revealed that the extent of cell adhesion in the absence of the solution of exopolymers was very similar for the three mutants, whereas in the presence of exopolymers the highest-producing mutant was able to adhere in a larger extent followed by the intermediate producer. These results have pointed out to an important feature of the involvement of exopolymers in microbial adhesion. The exopolymers released by the suspended cells can easily coat a surface, altering its surface tension and making adhesion favourable (Azeredo and Oliveira, 2001) or unfavourable (Leriche and Carpentier, 2000). This aspect is commonly found when the exopolymers have surface-active properties (Neu, 1996). Surface-active compounds (SACs) are molecules formed by a hydrophilic part and a hydrophobic one, which tend to interact with interfaces. Synthetic SACs have been used to reduce the adherence of cells to hydrophobic surfaces (Paul and Jeffre, 1985; Stelmack et al., 1999). The assembly of microbial SACs onto surfaces can either inhibit adhesion to hydrophobic surfaces (Velthuijs et al., 1996), or enhance attachment to hydrophilic
ones (Neu, 1996). The physico-chemical properties of the attachment surface play an important role on the way microbial polymers assemble onto the surface (linked by the hydrophilic part, exposing the hydrophobic one or the opposite) and on the amount assembled. Capsular exopolymers of *Pseudomonas* sp. were able to cover in larger extent hydrophilic surfaces than hydrophobic ones (Kalaji and Neal, 2000).

Attempts to interpret microbial adhesion through colloidal theories, such as DLVO and more recently the XDLVO, have also revealed another valuable aspect of the contribution of exopolymers to microbial adhesion. The adhesion of *Lactococcus lactis* to glass and polystyrene could not be explained by DLVO theory, neither by the influence of surface hydrophobicity, meaning that interactions beyond physico-chemical ones have played an important role in this study (Boonaert *et al.*, 2001). The authors hypothesised that the release of macromolecular compounds, such as peptides and polysaccharides, may have a bridging or repelling action between the cells and the adhesion surface (Boonaert *et al.*, 2001). The adhesion of a high gellan-producing mutant of *Sphingomonas paucimobilis* to glass could not be explained by XDLVO theory. The explanation can be given considering that polymeric bridging between the exopolymeric layer that surrounds the cell wall and the exopolymers coating the glass surface was necessary to overcome the energy barrier between the adhesion surface and the microbial cell (Azeredo *et al.*, 1999a).

The examples presented so far only stressed the non-specific involvement of exopolymers in adhesion, either by coating the adhesion surface (by assemble or through footprints) or by bridging the cells to the substratum. The establishment of specific interactions (of adhesin-receptor type) between cells and adhesion substratum can be seen as a specific contribution of exopolymers to adhesion. Bacterial lipopolysaccharides (LPSs), capsules and polymeric slime layers (slime) undertake specific interactions with biotic and abiotic surfaces.

LPSs molecules have been suggested to function as adhesins that mediate the binding of *Campylobacter jejuni* to epithelial cells (McSweegan and Walker, 1986). LPS have also been shown to mediate the interaction of bacteria with phagocytic cells (Perry and Ofek, 1984; Wright *et al.*, 1989).

Capsules are usually acidic polysaccharides secreted by bacteria that remain cell bound following their secretion. They differ from the slime polymeric layer, which, after secretion, remains loosely associated with the cell surface. The term glyocalyx has also been used to describe both capsule and slime polymers (Ofek and Doyle, 1994). An example of the involvement of polymeric capsules in the adhesion event is the interaction of *Klebsiella pneumoniae* with macrophages. This bacterium undergoes phagocytosis mediated by capsular polysaccharides recognised by the mannose/N-acetylgalcosamine-specific lectin of macrophages (Athanana *et al.*, 1991). In the case of *Staphylococcus epidermidis*, the expression of a capsular polysaccharide adhesin termed PS/A has been shown to be determinant for initial attachment to abiotic surfaces (Shiro *et al.*, 1994). Conversely, capsules may act as anti-adhesive structures due to their uniformly high density of negative charges (Gotschlich, 1983). Furthermore, capsules may mask potential
adhesins located at the cell surface (Favre-Bonte et al., 1999). The dual role of capsules (stimulation and inhibition of bacterial adhesion) can be well exemplified by *Klebsiella pneumoniae*. The initial adhesion of these bacteria to epithelium is mediated by the specific interaction between their capsules and the mucous layer. Following initial interaction, capsule expression is downregulated to facilitate interactions between bacterial cell surface adhesins and the underlying epithelial cell (Taylor and Roberts, 2002).

The ability of slime to promote adhesion through the establishment of specific interactions with the substratum is now well established. Ofek and Doyle (1994) proposed the word 'slimecins' to denominate slime-like materials that are directly involved in microbial adhesive events. Slimecins is, e.g. the polymeric slime, produced by some oral streptococci (α-1,3- and α-1,6-glucans) that participate as receptors for glucan-binding lectins.

Exopolymers are also responsible for the detachment of bacteria from surfaces. Enzymes may be able to break down different types of bonding in proteins and polysaccharides (Sutherland, 1999). The secretion of polymers at the interface is another mechanism with which bacteria can detach from a surface. This is the case of bacteria degrading hydrocarbons that can release themselves from oil droplets by producing an emulsifying substance (Rosenberg, 1986). Another example is the case of hydrophobic bacteria that can detach from hydrophilic surfaces by excreting hydrophilic polymers (Fattom and Shilo, 1985).

### 2.3.2 Biofilm exopolymers

Exopolymers are constituents of the polymeric matrix of biofilms and are understood as all the polymeric material, i.e. produced and secreted by microbial cells, which comprise polysaccharides, proteins, glycoproteins, LPS, lipids, among others. Apart from exopolymers, the biofilm matrix also comprises cellular debris and the products of extracellular hydrolytic activity as well as adsorbed chemicals and particles. These materials are commonly designated by the acronym EPS meaning extracellular polymeric substances (Wingender et al., 1999).

Polysaccharides are considered the major constituents of the exopolymeric fraction of EPS. There are many publications referring that the exopolysaccharide fraction of the exopolymers found in the biofilm matrix is of different composition than that produced by planktonic cells. Allison et al. (1998) when studying the activity of a polysaccharide lyase of *P. fluorescens*, found that while lyases recovered from planktonic supernatants were only active against planktonic-derived exopolysaccharides, lyases recovered from biofilm were active against both planktonic- and biofilm-derived exopolysaccharides. This suggests that the composition of biofilm exopolymers is different from that of planktonic exopolymers. In another study, the characterisation of the exopolymers recovered from a planktonic culture of a marine *Pseudomonas* sp. and the ones obtained from the biofilm matrix of the same strain by Fourier-transform IR spectroscopy demonstrated that clear differences between these two exopolymers – namely, O- and N-acetylglucosamines – were greater in the biofilm exopolymers (Beech et al., 1999).
Despite this and other evidences, one cannot straightforwardly withdraw the conclusion that biofilm-derived exopolysaccharides are different from planktonic-derived polysaccharides. In fact, a major problem is to obtain sufficient amount of exopolysaccharides having the certainty that they are really from the exopolymeric fraction of the EPS biofilm matrix. The method used to extract the EPS fraction of the biofilm clearly influences the amount and composition recovered. Aggressive methods, such as NaOH, vapour extraction or prolonged sonication, lead to contamination of the fraction recovered with intracellular material released during the extraction procedure. On the other hand, smooth methods (like EDTA, heating at 70°C and centrifugation) extract only a small portion of the EPS matrix (Azeredo et al., 1999b; Nielsen and Jahn, 1999). Sutherland (2001) considers that the exopolysaccharides present in biofilms resemble closely the corresponding polymers synthesised by planktonic cells. This has been demonstrated through the utilisation of non-destructive and *in situ* techniques, such as the use of antibodies or specific lectins (Sutherland, 2001; Leriche et al., 2000).

### 2.3.3 Exopolymers in biofilm formation

#### 2.3.3.1 Cell–substratum binding

Once attached to the surface, bacteria must maintain contact with the adhesion surface and grow in order to develop a mature biofilm. The hallmark between bacteria in a biofilm is the exopolymers produced by the cells. The presence of exopolymers helps maintaining the integrity of the biofilm, allowing large numbers of bacteria to coexist even under turbulent flow conditions (Melo and Vieira, 1999). It is clear from a large number of studies, that mutants unable to synthesise EPS are unable to form biofilms (Allison and Sutherland, 1987; Watnick and Kolter, 1999), which definitely enlightens the importance of EPS in biofilm formation. This was evident in a study performed with mutants of *Sphingomonas paucimobilis* having different capabilities to secrete exopolymers. The greatest exopolymer-producing mutant was able to form low dense and thick biofilms (1 mm in average); the intermediate producer gave rise to biofilms denser and ten times thinner and the lowest-producing mutant was biofilm negative (Azeredo and Oliveira, 2000b). In this case, the exopolymers were both responsible for cell attachment and biofilm growth. However, there are cases in which exopolymers are secreted after cell attachment, thus, only influencing biofilm growth. Allison and Sutherland (1987) demonstrated that two strains of freshwater bacteria only synthesised significant amounts of exopolymers after attachment. This is also the case for alginate-producing *P. aeruginosa* strains. A study performed with *P. aeruginosa* 8830 (a stable mucoid derivative of a clinical isolate) showed that the activation of alginate production occurs after cell attachment by a mechanism in which bacteria are capable of sensing the presence of the surface. Adhered bacteria are upregulated for the production of alginate. If and when there is a change in the environment (osmolarity and ethanol presence), bacteria respond by
downregulating alginate production and ultimately detach from the surface (Davies et al., 1993; Davies and Geesey, 1995; Davies, 1999). Alginate presumably enhances the ability of P. aeruginosa to remain attached to the surface. It has been reported that a non-alginate-producing mutant sticks better to glass surfaces than a producing one. However, the non-producing mutant is not able to form a thick biofilm and only develops as a monolayer of cells. This indicates that alginate production leads to the formation of cell clusters with bacteria embedded in the alginate matrix (Davies, 1999). In another study, it was demonstrated that alginate plays an important role in the biofilm structure of P. aeruginosa, required for the formation of a thicker three-dimensional biofilm (Nivens et al., 2001).

2.3.3.2 Cell-to-cell binding

Exopolymers are also directly responsible for cell-to-cell binding, which is a *sine qua non* condition for biofilm development. Several works have revealed that the formation of S. epidermidis biofilms is dependent on the presence of a polysaccharide intercellular adhesin (PIA). PIA consists of two polysaccharide species that mediate cell-to-cell adhesion of the proliferating cells (Mack et al., 1994; 2000). Cell-to-cell adhesion of S. aureus is also dependent of a PIA being determinant in biofilm formation (Cramton et al., 1999).

In addition to the involvement of exopolymers in biofilm formation, exopolymers are also determinant for biofilm architecture. In a recent review of the basic structure of microbial biofilms, Wimpenny and Colasanti (1997) postulated a unifying hypothesis in which the three conceptual models revealed that three types of biofilm structure exist, namely, a heterogeneous mosaic biofilm, a penetrated water-channel biofilm and a dense confluent biofilm. According to these authors, biofilm architecture is a consequence of nutrients availability. van Loosdrecht et al. (1997) pointed out to the importance of biomass detachment in biofilm structure. Biofilm architecture is, thus, very dependent of the balance between biomass accumulation and detachment, and this is undoubtedly influenced by biofilm exopolymers. Because, besides being important for biofilm formation (as already seen), exopolymers determine the mechanical stability of biofilms, mediated by non-covalent interactions (Mayer et al., 1999). The influence of exopolymers in biofilm architecture can be exemplified in a study in which a mutant of E. coli defective in colanic acid production showed a different biofilm structure from the original strain, whereas initial adhesion was not affected in this mutant, suggesting that colanic acid is not acting as an adhesin during the early attachment events but influences biofilm structure (Danese et al., 2000). The biofilm produced by a high gellan-secreting mutant of Sphingomonas paucimobilis presented a different structure from the one formed by an intermediate gellan-secreting mutant (Azeredo and Oliveira, 2000b). In both studies, biofilms formed by high polysaccharide-producing strains were less dense and packed. Kreft and Wimpenny (2001) simulated a dual species nitrifying biofilm, elucidating the effect of exopolymer production in biofilm architecture. In this study, exopolymers lowered the density of the biofilm and the roughness of biofilm surface.
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Several other reports have also emphasised the influence of exopolymers in biofilm structure. However, their specific role is still not clear, partly due to analytical limitations. For instance, there are uncertainties of binding specificities to exopolymers if antibodies or lectins are used (Neu and Lawrence, 1999).

2.4 CONCLUSIONS

In most situations, there is a strong correlation between the number of microbial cells adhered to solid surfaces and the degree of hydrophobicity of either the solid substratum and/or the cells surface. Concerning the effect of exopolymers, conversely to hydrophobicity, they can enhance or inhibit the process of cell adhesion.

The importance of exopolymers in microbial adhesion, biofilm formation and structure has been mostly elucidated by studies based on pure and well-defined cultures. However, in multispecies biofilms the interaction between the exopolymers of the different strains can strongly influence biofilm formation and structure (Skillman et al., 1999; Gideon et al., 1999). This can be seen as a challenging field for future work.

REFERENCES


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