THE ROLE OF EXOPOLYMERS IN *SPHINGOMONAS PAUCIMOBILIS* ATTACHMENT AND BIOFILM FORMATION

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Exopolymers have an important role in biofilm formation, being involved in the initial adhesion events and influencing the biofilm characteristics. The results of a study with a strain of ***Sphingomonas paucimobilis*** *that excretes a polysaccharide gum (gellan) has been used to exemplify the role of exopolymers in cell attachment and biofilm formation. The attachment results were interpreted through the XDLVO theory and they revealed that exopolymers have a dual role in cell attachment by both coating the substratum making adhesion favourable and by strengthening adhesion through the establishment of polymeric bridges. Exopolymers were also essential to the formation of* ***Sphingomonas paucimobilis*** *biofilms acting as scaffolds for bacterial proliferation. The physicochemical properties, the composition of the biofilm matrix and the cohesion forces within the biofilm were also conditioned by the presence of exopolymers.*

Introduction

Biofilms are formed by microbial cells attached to a surface and enveloped in a matrix of extracellular polymeric substances (EPS). EPS comprise macromolecules produced and excreted by microbial cells (exopolymers) as well as cellular debris and products of extracellular hydrolytic activity. In addition, EPS can also contain adsorbed chemicals and particles. EPS encompass 50% to 90% of the total organic carbon of biofilms (Christensen & Characklis 1990, Nielsen *et al.* 1997), depending on the extraction method used. Aggressive methods such as NaOH extraction, vapour extraction or prolonged sonication lead to an overestimation of the amount of EPS, on account of the contamination by intracellular material released during the extraction procedure. On the other hand, smooth methods such as EDTA treatment, heating at 70° C and centrifugation extract only a small portion of the exopolymeric matrix (Azeredo *et al.* 1999a). Nevertheless, EPS are important constituents of biofilms and have three main attributes: they are directly involved in biofilm formation, they influence many important properties of the biofilm (strength, elasticity and sorption capacity) and have a protective effect against adverse environmental conditions (Morton *et al.* 1998).

The results of a study with a strain of *Sphingomonas paucimobilis* will be used to exemplify the role of EPS in bacterial attachment and biofilm formation. *S.*

Table 1 The number of cells per mm² of *Sphingomonas paucimobilis* mutants TR, CV and F72 attached to glass when immersed in PBS (0.1M) and in their extracted exopolymer for 2h at 30°C.

mutant	Attached cell mm⁻²	
	in PBS (0.1 M)	in exopolymer
TR	323±36	2513±215
CV	539±72	1508±144
F72	646±72	826±72

paucimobilis is ubiquitous in soil, water and sediments and has a great potential in soil bioremediation and waste water treatment due to its capacity of degrading aromatic compounds (Fredrickson *et al.* 1995). Strain ATCC 31461 (Yabuuchi *et al.* 1990) is able to produce large amounts of the anionic exopolymer gellan. This substance is composed by repeated units of a tetrasaccharide with two molecules of D-glucose, one of D-glucoronic acid and one of L-rhamnose (Chandrasekaren & Radha 1995, Kang & Veeder 1982, Pollock 1993). Moreover, a series of well-defined mutants defective in EPS production (Richau *et al.* 1997) are available that constitute a powerful tool to investigate the importance of EPS in bacterial attachment and biofilm formation.

Exopolymers in Cell Attachment

In a simplistic form, biofilm formation begins with transport of microorganisms towards a substratum surface and the establishment of an interaction with the surface which is generally reversible and is governed by weak interaction forces (Norde & Lyklema 1989). Subsequently, a firmer attachment occurs which is often associated with the production of EPS. Finally the adherent cells grow and multiply, giving rise to a mature biofilm.

Initial microbial adhesion can be interpreted in terms of the XDLVO theory, recently proposed by Van Oss (1994), which is an extension of the classic DLVO theory. This new approach considers that the total free energy of interaction between two surfaces immersed in an aqueous medium is the sum of the Lifshitz-van der Waals forces, polar interactions (or Lewis acid-base forces), electrical double layer interactions and Brownian movement forces. This balance gives rise to an energy profile characterised by a secondary minimum of energy, normally positioned at 10-20 nm of the interacting surfaces (this is dependent on the ionic strength of the medium) where reversible attachment can occur (Busscher & van der Mei 2000).

Three mutants of *Sphingomonas paucimobilis* (TR, CV and F72) having decreasing capabilities to excrete exopolymers (10, 7 and 3gL⁻¹ respectively) were incubated with hydrophilic glass coupons immersed in a solution of phosphate buffer saline (PBS), 0.1M; pH 7.0. The number of cells attached after 2 hours of incubation at 30 °C were counted automatically using an image

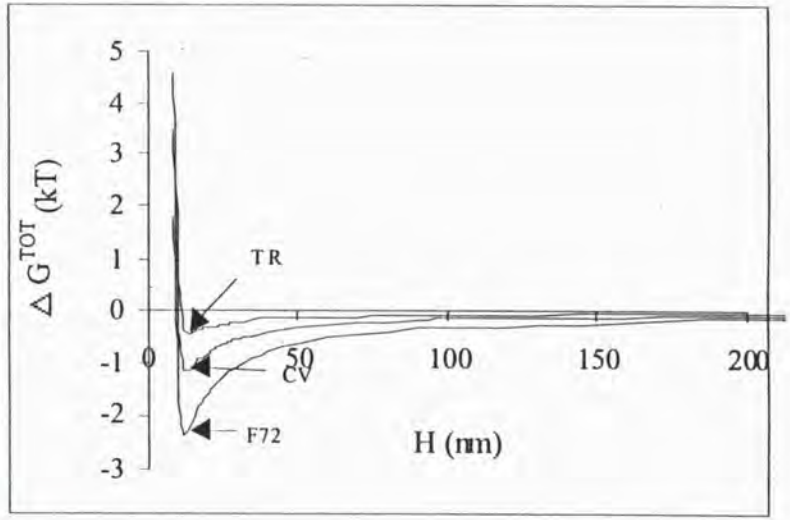

Figure 1 Variation of the free energy of interaction (ΔG^{TOT}) between the mutants TR, CV and F72 and glass when immersed in PBS (0.1M) as a function of the separation distance (H).

analysis program according to the methods of Azeredo *et al.* (1997) and are shown in Table 1. From these data, the mutant F72 was shown to be able to adhere to the greatest extent followed by CV and finally TR. By examining surface properties (surface tension and zeta potential) it was possible to build an energy profile of the three mutants, according to the XDLVO theory (Figure 1). From this figure, an energy minimum for each mutant was established at 12-15 nm from the surface where theoretically reversible attachment would take place. The number of cells attached (Table 1) in these conditions is in accordance with the depth of these energy minima. Thus, the XDLVO theory is suitable to interpret the initial adhesion phenomena (Azeredo *et al.* 1999b).

The formation of a more tenacious anchoring has been associated with the production of extracellular polymers. Indeed, the presence of polymeric "footprints" after cell detachment (Neu & Marshall 1991) and SEM observations showing exopolymers anchoring cells to the adhesion substratum (Fletcher & Floodgate 1972) have revealed that exopolymers play an adhesive role. The cell attachment process has been described in terms of a reversible adsorption step followed by an irreversible firm attachment (Fletcher & Floodgate 1972). It has also been speculated that EPS are involved in initial adhesion by acting as polymeric bridges (van Loosdrecht *et al.* 1990).

Table 2 Surface tension (γ) and surface hydrophobicity (ΔG_{sw}) of glass, clean and coated with exopolymers produced by the mutants TR, CV and F72 (± SD).

Glass surface properties	γ (mJ/m²)	ΔG_{sw} (mJ/m²)
Clean	48.9±2.8	+45.0±3.1
coated with TR exopolymer	21.4±2.3	-62.8±0.3
coated with CV exopolymer	30.6±2.5	-26.0±1.2
coated with F72 exopolymer	48.5±3.4	+44.0±4.5

In the present example, the same mutants (TR, CV and F72) were incubated with glass coupons immersed in their respective exopolymers at concentrations of 5.3gL^{-1}, 4.3gL^{-1} and 2.5gL^{-1} respectively for 2h. The polymers were isolated from cells grown in a nutrient rich medium designed to enhance polymer production (Kang & Veeder 1981), purified and used for adhesion assays. The numbers of cells attached to glass in the presence of EPS are presented in Table 1. In all instances the number of attached cells was greater than occurred in the presence of PBS. Furthermore, TR was able to attach to glass to a larger extent than the other two mutants and about 8-fold more than in the absence of its exopolymer.

This mutant is able to excrete large amounts of exopolymers in batch cultures (about 10 g/l) while CV produces intermediate amounts (about 7g/l) and F72 smaller quantities (about 3g/l) (Richau *et al.* 1997). It must be noted that the mutants used in this study are able to produce the same exopolysaccharide, however in different amounts and having different degrees of polymerisation (Richau *et al.* 1997). From the comparison of the values presented in Table 1 it becomes clear that the exopolymer enhances cell attachment to glass.

The exopolymer produced by *S. paucimobilis* is able to lower the surface tension of water, so is expected to possess surface-active properties (Azeredo & Oliveira 2000a). Surface-active compounds (SACs) are molecules formed by a hydrophilic part and a hydrophobic one, which tend to interact with interfaces. Polysaccharides containing 6–deoxy sugars such as rhamnose, which is the case for gellan, are considered polyphilic polymers with hydrophobic groups distributed across the entire molecule (Neu 1996). Synthetic surface-active compounds have been used to reduce the adherence of cells to hydrophobic surfaces (Paul & Jeffrey 1985, Stelmack *et al.* 1999). Microbial SACs have been reported to play an important role in bacterial attachment as they can either inhibit attachment to hydrophobic surfaces (Velraeds *et al.* 1996), or enhance attachment to hydrophilic surfaces (Neu 1996). It is likely, therefore, that the EPS produced by *S. paucimobilis* would influence its attachment to surfaces.

Table 2 shows the surface properties of glass after being coated with

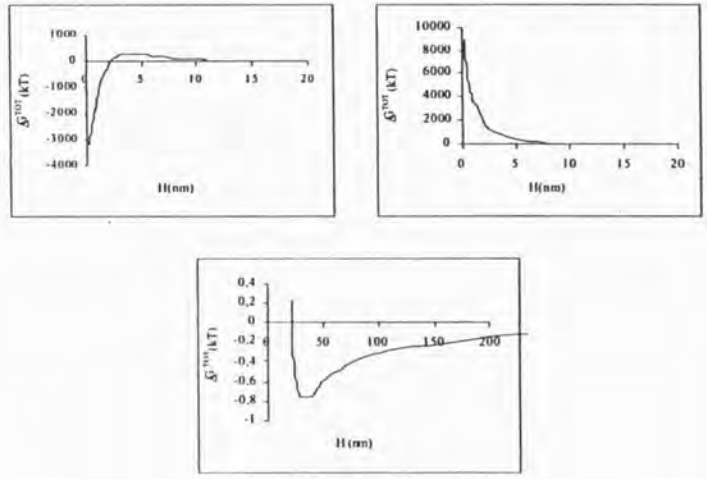

Figure 2 Variation of the free energy of interaction (ΔG^{TOT}) between the mutants TR (A), CV (B) and F72 (C) and glass when immersed in their excreted exopolymer as a function of the separation distance (H).

exopolymers (as per attachment assay but minus cells) produced by the mutants. Following incubation, the slides were removed carefully from the EPS solutions, dried at 30°C before measuring contact angles (Azeredo *et al.* 2000a). According to these data, exopolymers excreted by CV and TR are able to lower the surface tension of glass. Consequently, the degree of glass hydrophobicity is changed. Using the approach proposed by van Oss & Giese (1995) in which the degree of hydrophobicity can be expressed as the free energy of interaction between two surfaces of the same material when immersed in water (ΔG_{sws}), glass becomes hydrophobic when coated with the exopolymer produced by TR ($\Delta G_{sws} < 0$). It should be noted that if $\Delta G_{sws} > 0$ the surface is hydrophilic, while $\Delta G_{sws} < 0$ means that the surface is hydrophobic.

In order to verify if the XDLVO theory is also able to explain the great increase in cell adherence in the presence of exopolymers, the respective energy profiles were built (Figure 2). The mutant F72 presents the same energy profile as in PBS, with a secondary minimum followed by an energy barrier (Figure 2c). The total free energy of interaction between the mutant CV and the glass in EPS is always repulsive (Figure 2b). According to this energy profile, adhesion would not occur. The number of CV cells attached in the presence of exopolymer was however three times greater than the number adhered in PBS (Table 1). A possible explanation for this fact is that the polymers adsorbed to the glass

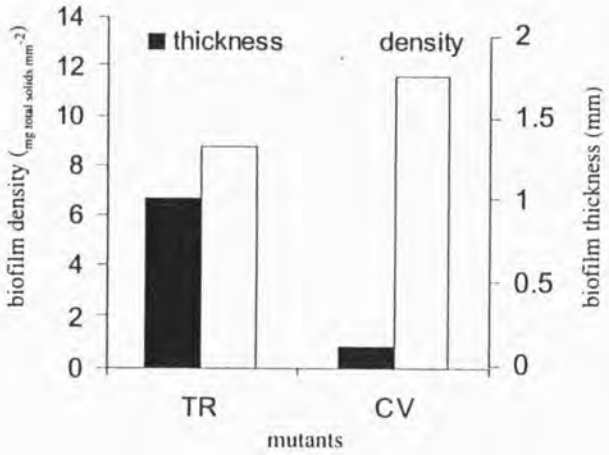

Figure 3 Physical characteristics of the biofilms produced by the mutants TR and CV.

surface can bind to the polymers that surround the CV cells by polymeric bridges. This is supported by the existence of a slime layer around the cells, which was observed by fluorescence microscopy after binding calcofluor white and a fluorescent lectin (ConA). The thickness of the slime layer is greater for TR, followed by CV and is almost non-existent in F72 (Azeredo & Oliveira 2000a).

The energy balance diagram obtained for the mutant TR (Figure 2a) presents an energy barrier with a maximum at 4 nm followed by a minimum of energy close to the substratum surface. van Loosdrecht and Zehnder (1990) showed that the energy barrier could be overcome by polymeric extensions present at the cell surface, due to the increase of the effective radius of the cell. It is possible therefore that the polymeric extensions present at the cell surface may eventually be able to overcome the energy barrier and therefore an effective and strong adhesion can take place at the primary minimum.

From this study it can be concluded that the exopolymer from *S. paucimobilis* has a dual role in attachment. It can coat the surface, thereby strengthening initial attachment and enhancing attachment by the establishment of polymeric bridges.

Table 3 Composition of TR and CV biofilm matrices per cell (Pol–polysaccharides; Prot-proteins).

Composition	Pol/cell (10^{-11}g/cell)	Prot/cell (10^{-11}g/cell)	DNA/cell (10^{-16} g/cell)
TR	2.5	4.6	11.0
CV	1.9	2.2	6.0

Exopolymers in Biofilm Formation and Composition

Once attached to the surface, bacteria must maintain contact with the adhesion surface and grow in order to develop into a mature biofilm. The glue separating and linking bacteria in a biofilm are the EPS. The presence of EPS helps maintaining the integrity of the biofilm, allowing large numbers of bacteria to coexist even under turbulent flow conditions (Melo & Vieira 1999). It is clear from a large number of studies that mutants unable to synthesise EPS are unable to form biofilms (Allison & Sutherland 1987, Watnick & Kolter 1999). This points to their critical importance in biofilm formation.

In the case of *S. paucimobilis*, the mutant F72 was not able to produce a biofilm. Although this mutant is able to excrete exopolymers, the amount produced may be not sufficient enough for the biofilm development. The physical characteristics of the biofilms formed by TR and CV are presented in Figure 3. TR, the greatest producer of exopolymers was able to form thick biofilms (1 mm in average), while CV, the intermediate EPS producer gave rise to biofilms 10-times thinner. In terms of biofilm density, however, CV biofilms were denser than those of TR. These results reveal that the capability to produce great amounts of exopolymers affects the thickness of the biofilm and thus its physical properties.

The composition of biofilm matrices is very heterogeneous: the major component is water up to 97% (Zhang *et al.* 1998). The organic fraction is mainly composed of proteins and polysaccharides, although other compounds can be found in smaller amount such as glycoproteins, DNA and humic substances (Jahn & Nielsen 1998).

The amount, composition and properties of EPS vary in response to the availability of nutrients, especially on the balance between carbon and other limiting nutrients (Wrangstadh *et al.* 1990) and to other environmental conditions.

In the case of *S. paucimobilis*, the amount of exopolymer excreted also influenced the composition of the biofilm matrix, as can be seen in Table 3. Curiously, the cells within TR biofilm produced 1.3-fold more exopolysaccharide than the cells in CV biofilm, which is the same ratio as that excreted by planktonic cells. Proteins are also important constituents of biofilms (Jahn *et al.*, 1999). Since *S. paucimobilis* is able to excrete proteins in batch culture, the presence of proteins in the polymeric matrix of these biofilms must be expected.

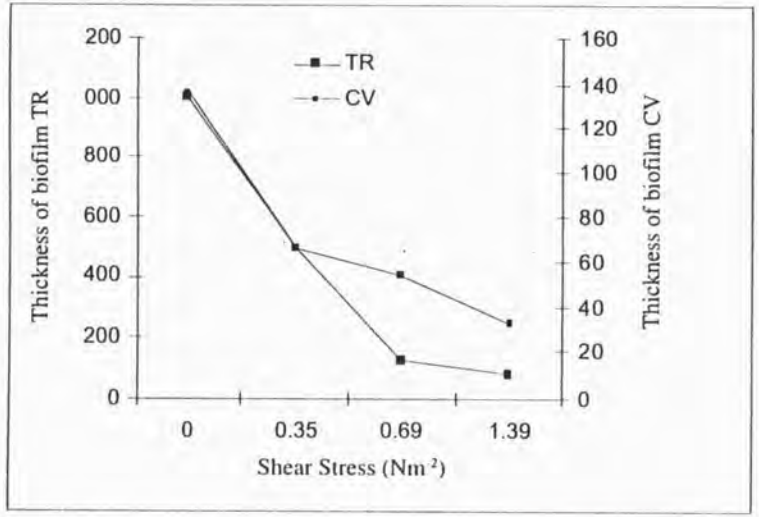

Figure 4 Decrease of the biofilm thickness after the application of increasing shear stresses.

Cell lysis together with proteolytic activity, as a result of nutrient limitation in the inner layers of the thick biofilms formed by TR, are both responsible for the high content of protein (Azeredo & Oliveira 2000b).

Another important attribute of EPS is their involvement in biofilm stability and cohesiveness. Flemming et al. (2000) pointed out the importance of electrostatic interactions for biofilm cohesion. Mayer *et al.* (1999) suggested that biofilms represent gel-like structures that may be readily destroyed by shear or dissolution of the polysaccharides. These authors also enhance the contribution of the EPS to the mechanical stability of biofilms, enabling them to withstand considerable shear forces.

In order to examine this, biofilms for mutants TR and CV were grown on four glass cylinders immersed in a 500ml reactor fed continuously with aerated S medium (gL^{-1}: 12.5g $Na_2HPO_4.2H_2O$, 3g KH_2PO_4, 1g NaCl, 1g K_2SO_4, 0.2g $MgSO_4.7H_2O$, 0.01g $CaCl_2.2H_2O$, 0.001 $FeSO_4.7H_2O$, 5g Glucose, 1g yeast extract, 1g casein). Inoculated cultures were left for 2h after which the reactor was fed with increasing dilution rates ($0.03h^{-1}$, $0.06h^{-1}$ and $0.2h^{-1}$). After 10 days the glass cylinders were removed and the attached biofilm subjected to increasing shear stresses by immersing the cylinders in PBS and submitting them to different rotational velocities, promoting the detachment of three uniform layers. Figure 4 shows the fractions of biofilms detached as a function of shear stress. According to this data, at low shear stresses the same percentage of biofilm TR

and CV is detached. When increasing the shear stress, it became easier to detach biofilm TR than to remove biofilm CV. These results suggest that the greatest content of exopolymers in the matrix of biofilm TR (Table 3) does not contribute to the improvement of the cohesion forces within this biofilm. The mutants used in this study produce gellan with different viscosities, corresponding to different degrees of polymerization. The exopolymer excreted by CV is much more viscous than that produced by the mutant TR (Richau *et al.* 1997). So, it is possible that the exopolymer excreted by CV is much more cohesive, explaining the results obtained.

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