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Exopolymers in bacterial adhesion: interpretation in terms of DLVO and XDLVO theories

J. Azeredo, J. Visser, R. Oliveira *

Centro de Engenharia Biológica/IBQF-Universidade do Minho, Campus de Gualtar, 4710 Braga, Portugal

Abstract

Exopolymers have an important role in bacterial adhesion and are associated with irreversible adhesion. Moreover, they can coat surfaces enhancing or avoiding bacterial colonisation. To study the role of exopolymers in the adhesion of bacteria to glass, three mutants of *Sphingomonas paucimobilis* (which are high (TR), medium (CV) and low (F72) exopolymer producers), were used. The adhesion tests were performed in phosphate saline buffers and in solutions of the exopolymer produced by each mutant. The DLVO theory was able to explain the results in phosphate saline buffers, although this theory could not explain the results obtained in the presence of the exopolymer. The XDLVO theory enabled the interpretation of the results in the presence of the exopolymer, where hydrophobic interactions played an important role. However, polymeric interactions that are not taken into account in these two theories are also expected to be determinant in the adhesion process.

Keywords: Bacterial adhesion; DLVO theory; XDLVO theory; Exopolymers

1. Introduction

A surface immersed in water can be easily colonised. As adhesion of bacteria to surfaces is ubiquitous in nature, health and technological processes, this phenomenon has been intensively studied.

The first theory used to explain the interactions involved in bacterial adhesion was the DLVO theory, developed for macromolecules and particles. This theory considers that the total energy of adhesion is the result of the van der Waals attractive forces (LW) and the generally repulsive interactions due to the interpenetration of the electrical double layers (DL) [1].

The DLVO theory does not consider short-range interactions that are also important for adhesion, mainly Brownian movement forces and polar interactions (e.g. hydrophobic interactions). Hydrophobicity can originate forces that are very important in aqueous media. Nikawa et al. [2] studied adhesion of *Candida* spp. and they showed that the forces between the more hydrophobic species are predominantly due to hydrophobic interactions. It was also shown that hydrophobicity plays an important role in initial adhesion of pathogenic bacteria [3]. Hydrophobicity has also been associated with fouling in marine

* Corresponding author. Tel.: +351-53-604409; fax: +351-53-678986.

E-mail address: roliveira@deb.uminho.pt (R. Oliveira)

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environments [4], the adhesion of microorganisms to minerals [5] and to polymeric supports [6].

Recently, van Oss [7] proposed an extension of the DLVO theory, generally known as XDLVO 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 (LW) forces, polar interactions (AB), electrical double layer (DL) interactions and Brownian movement forces (BR):

$$\Delta G^{TOT} = \Delta G^{LW} + \Delta G^{AB} + \Delta G^{DL} + \Delta G^{BR} \quad (1)$$

All these interactions are dependent on the distance of separation (H) between the interacting entities and also on their geometry. In the case of bacterial adhesion to a solid surface a sphere/flat plate geometry is generally assumed, because the dimensions of the surface are usually several orders of magnitude above those of an individual cell.

The equations for the calculation of the free energy due to the LW and DL interactions are the same as those used in DLVO calculations [1].

The polar free energy of interaction (AB) between a sphere (1) and a flat infinite plate (2) both immersed in a liquid (3) along the distance of separation (H), is given by:

$$\Delta G_H^{AB} = 1.257 \times 10^{-8} r[\sqrt{\gamma_3^+}(\sqrt{\gamma_1^-} + \sqrt{\gamma_2^-} - \sqrt{\gamma_3^-}) + \sqrt{\gamma_3^-}(\sqrt{\gamma_1^+} + \sqrt{\gamma_2^+} - \sqrt{\gamma_3^+}) - \sqrt{\gamma_1^+\gamma_2^-} - \sqrt{\gamma_1^-\gamma_2^+}]\exp\left(\frac{0.157 \times 10^{-9} - H}{1 \times 10^{-9}}\right) \quad (2)$$

where r is the radius of the sphere and γ_i^- and γ_i^+ are the electron donor and electron acceptor parameters, respectively, of the polar component of the surface tension, that can be determined by contact angle measurements [8].

According to van Oss [7], when LW, AB and DL interactions are measured separately, the total free energy of interaction (ΔG^{TOT}) can be obtained by summing the values for those entities and adding $+1$ kT for ΔG^{BR} (for a system with two degrees of freedom). If the ΔG^{TOT} is measures as a whole (e.g. by interfacial tension determination), then ΔG^{BR} is already included.

Adhesion of microorganisms to surfaces is a very complex phenomenon that might restrict the applicability of both DLVO and XDLVO theories. Bacteria are living organisms capable of producing extracellular specific structures which are quite important in adhesion [9]. Most bacteria are able to excrete polymeric substances (EPS) that can be associated with the cell wall like a capsule or can be released in the medium, involving the cell like a slime [10]. The EPS are normally constituted by polysaccharides, glycoproteins, lypopolysaccharides and uronic acids [11,12]. Some of the EPS produced by bacteria have surfactant properties that can inhibit adhesion, which is the case of the EPS produced by a *Lactobacillus*, that avoids the adhesion of *A. faecalis* to teflon [13]. Other EPS can enhance adhesion, like the polymeric substance produced by *P. fluorescens* responsible for adhesion of these bacteria to hydrophilic substrata [14].

The aim of this work was to study the role of the exopolymer produced by *Sphingomonas paucimobilis* in the adhesion of these bacteria to glass. The results were interpreted in terms of DLVO and XDLVO theories.

2. Materials and methods

2.1. Bacterial strains

In this study we used three mutants of *S. paucimobilis* (ATCC 31461), a gelan (polysaccharide) producer, kindly supplied by Instituto Superior Técnico, Portugal.

2.2. Bacterial cultivation and preparation

The three mutants, TR, CV and F72 (in decreasing order of gelan production), were grown in S medium designed to enhance exopolysaccharide production [15]. The cells were harvested in the exponential growth phase by centrifugation for 20 min at 9000 g and washed three times with ultra-pure water and twice with chilled 0.1 M phosphate saline buffer (PBS). The cells were

preserved in a filtered (0.2 mm pore size) phosphate saline buffer solution (PBS 0.1 M, pH 7.0) at −70°C.

2.3. Adhesion medium

The adhesion assays were performed in two different media: in phosphate saline buffer (PBS 0.1 M, pH 7.0) and in solutions of the excreted and isolated exopolymers of each mutant. The supernatants from cell harvesting were used as the exopolymers solutions after filtration through a 0.2 mm nitro-cellulose membrane, followed by 2 days of dialysis against ultra-pure water using a cellulose membrane with a MWCO of 14 KDa.

2.4. Physicochemical characterisation of bacterial surfaces

The surface tension and hydrophobicity of the bacterial surfaces were determined by sessile drop contact angle measurements on bacterial lawns prepared as described by Busscher et al. [16]. The measurements were carried out at room temperature using as reference liquids: water, di-iodomethane and glycerol. The determination of the contact angle was made automatically with the aid of an image analysis system (Kruss-GmbH, Hamburg). The images were received by a video camera connected to a 486 DX4 100 MHz personal computer, with an automatic measuring system (G2/G40).

The zeta potential of the cells was determined by electrophoretic mobility measured in phosphate saline buffer and in the exopolymer solutions by means of a Zeta-Meter 3.0+.

2.5. Adhesion surface

Glass microscope slides were cut into squares of about 1 cm^2 and carefully cleaned by immersion in concentrated chromosulfuric acid for 24 h, after which the slides were well rinsed with distilled water. The slide surfaces were then degreased by cleaning with a detergent followed by alternately rinsing with methanol and demineralised water.

2.6. Physicochemical characterisation of glass surfaces

The surface tension and surface hydrophobicity were determined by contact angle measurements with water, glycerol and di-iodomethane on non-coated (bare) glass slides and glass slides coated (preconditioned) with the exopolymers produced by each mutant. To coat the surface, the glass slides were immersed into the exopolymer solutions for 30 min. The slides were carefully withdrawn from the solutions and allowed to dry for 48 h at 30°C in a Petri dish.

The zeta potential was determined by electrophoretic mobility using fine particles of crushed glass immersed in phosphate saline buffer and in the solutions of each bacterial exopolymer (TR, CV and F72), using a Zeta-Meter 3.0+.

2.7. Adhesion studies

The adhesion assays were performed for each mutant on non-coated glass slides and on preconditioned glass slides with the respective exopolymer solutions. For each mutant, eight glass slides were immersed for 0.5 h in phosphate saline buffer and another eight glass slides in the corresponding solution of exopolymer. Two bacterial cell suspensions (0.5×10^8 cells per ml) were prepared by resuspending a pellet of bacterial cells in phosphate saline buffer and in the exopolymer solution. The previously preconditioned glass slides, immersed in each type of cell suspension, were incubated at 30°C under gentle agitation (100 rpm). After 2 h of incubation, the glass slides were withdrawn from the suspensions and rinsed for 30 s in demineralised water to remove loosely adhering cells. The rinsed slides were dried in Petri dishes at 30°C for 24 h. The adhering cells were enumerated by an automatic image analysis system connected to a microscope (Zeiss-Germany), as described elsewhere [17]. For each experiment, eight plates were analysed and 10 images of each plate were taken, giving a total number of 80 images analysed.

3. Results and discussion

Some of the results that are presented are physico–chemical properties of the bacteria, glass and liquid medium, which were used to calculate the different types of interaction forces. Others are presented in order to support the hypotheses assumed in the interpretation of the adhesion phenomenon.

The electrostatic (double layer) energy of interaction (DL) is dependent on the surface potential and the ionic strength of the medium, the latter being directly correlated with the thickness of the double layer. As the surface potential cannot be experimentally determined, this parameter is often replaced by the zeta potential of the surface that can be measured by electrokinetic methods. The values of zeta potential and ionic strength that were used in the calculations of electrostatic double layer interactions are shown in Table 1.

The surface tension of bacteria was determined by contact angle measurements and the values of its components are displayed in Table 2.

The surface tension and the hydrophobicity of bare glass coated with EPS were also determined by contact angle measurements. The values are displayed in Table 3. The hydrophobicity is expressed in terms of ΔG_{sws}^{tot} [18], assuming that the hydrophobicity increases as the free energy between two entities of the same kind immersed in

Table 1
Zeta potential (ζ) of the cells TR, CV and F72 and glass when immersed in PBS and EPS and the ionic strength of the liquid media

Surfaces	ζ (mV)			
	PBS	EPS		
		TR	CV	F72
TR	-88.5 ± 12	-38.2 ± 5		
CV	-93.2 ± 12		-40.7 ± 5	
F72	-88.3 ± 13			-20.7 ± 5
Glass	-32.13 ± 10	-13.9 ± 1	-14.1 ± 2	-7.5 ± 1
Ionic strength (mM)	100	5.0	5.9	4.73

Table 2
Surface tension of the mutants TR, CV and F72 (γ_b^{tot}) and the respective apolar component (γ_b^{LW}) and the electron donor (γ_b^-) and electron acceptor (γ_b^+) parameter of the polar component

Mutant	γ_b^{LW} (mJ m^{-2})	γ_b^+ (mJ m^{-2})	γ_b^- (mJ m^{-2})	γ_b^{tot} (mJ m^{-2})
TR	22.9	5.8	49.0	56.6
CV	24.6	6.2	49.0	59.5
F72	27.4	6.0	49.0	61.7

Table 3
The apolar (γ_s^{LW}) and polar (γ_s^{AB}) components and the parameters γ_s^+ and γ_s^- of the surface tension and the hydrophobicity (ΔG_{sws}^{tot}) of bare and coated glass with EPS produced by TR, CV and F72

Glass	γ_s^{LW} (mJ m^{-2})	γ_s^+ (mJ m^{-2})	γ_s^- (mJ m^{-2})	γ_s^{AB} (mJ m^{-2})	ΔG_{sws}^{tot} (mJ m^{-2})
Bare	33.0	1.0	63.5	19.9	45.0
Coated with EPS_{TR}	19.0	1.3	1.1	2.4	−62.8
Coated with EPS_{CV}	20.7	2.4	10.2	9.9	−26.0
Coated with EPS_{F72}	32.7	1.0	62.5	15.9	44.0

Table 4
The number of bacteria adhered to glass when immersed in PBS and in EPS

Mutant	Adhering cells in PBS		Adhering cells in EPS	
	By screen	By mm^2	By screen	By mm^2
TR	9 ± 1	323 ± 36	70 ± 6	2513 ± 215
CV	15 ± 2	539 ± 72	42 ± 4	1508 ± 144
F72	18 ± 2	646 ± 72	23 ± 2	826 ± 72

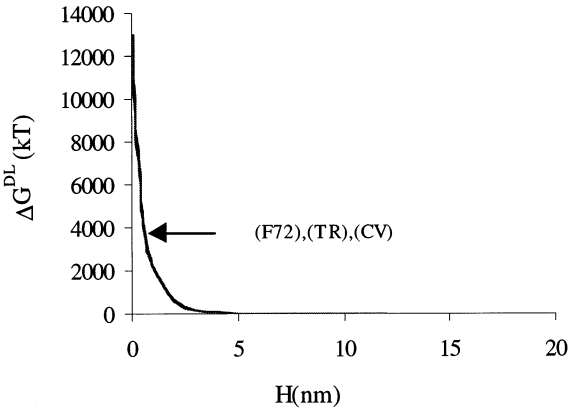

Fig. 1. Variation of the free energy of electrostatic interactions (ΔG^{DL}) between the mutants TR, CV and F72 and glass immersed in PBS as a function of the distance of separation (H).

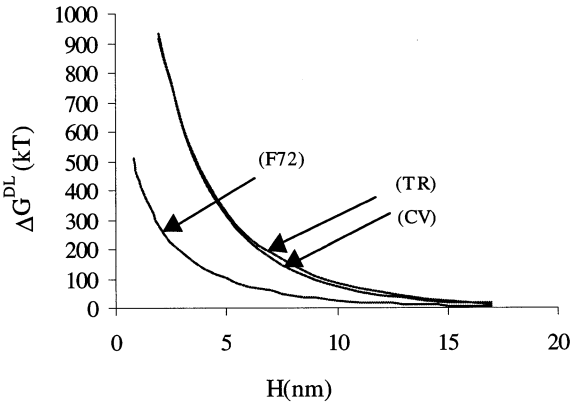

Fig. 2. Variation of the free energy of the electrostatic interactions (ΔG^{DL}) between the mutants TR, CV and F72 and glass immersed in EPS as a function of the distance of separation (H).

water becomes more negative. This means that they establish a stronger interaction between themselves than with water.

The surface tension of bare glass ($\gamma^{LW} + \gamma^{AB}$) is very similar to the value reported by van Oss and Giese [18]. When coated with the exopolymers produced by TR and CV, it decreases, resulting in an increase of the surface hydrophobicity. The polymer produced by F72 has no significant effect on the surface tension of glass. In a previous study it was demonstrated that the exopolymers produced by the mutants TR and CV have surfactant properties that were responsible for the hydrophobization of the glass surface [19].

The adhesion assays were performed by putting into contact the cells of each mutant and the glass slides immersed in PBS and in the respective solution of EPS. The number of adhered cells was determined automatically with a computer aided image analysis; the values are given in Table 4. This technique also enables the determination of the average equivalent radius of the cells (0.73 μm).

3.1. Understanding adhesion through DLVO and XDLVO theory

3.1.1. DLVO theory

The electrostatic interaction energy between the three mutants and glass in the presence of PBS (0.1 M, pH 7.0) as a function of the distance is presented in Fig. 1. It can be observed that the high ionic strength of the medium and therefore the thickness of the double layer gives rise to an energy profile that decays rapidly with the distance of separation.

In solutions of exopolymers the electrostatic repulsion between cells and glass is one order of magnitude lower than in PBS, for distances smaller than 2 nm. This is due to the decrease of the zeta potential of both interacting entities when immersed in EPS (Table 1). For greater separation distances the opposite is obtained. Moreover, the electrostatic interactions decay more slowly with the separation distance, due to the lower ionic strength of the EPS solutions (Fig. 2).

The profile of the van der Waals free energy of interaction is displayed in Fig. 3. It is apparent

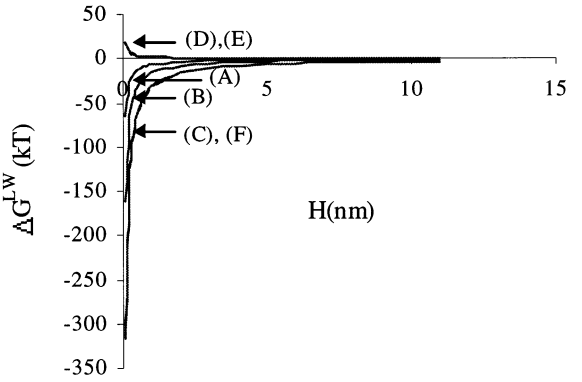

Fig. 3. Variation of the free energy of the van der Waals interaction between the mutants TR (A), CV (B) and F72 (C) and glass when immersed in PBS and between the mutants TR (D), CV (E) and F72 (F) and glass when immersed in EPS as a function of the distance of separation (H).

that the intensity of the attractive van der Waals interactions decays with the interacting distance and becomes zero for distances above 8 nm. The van de Waals interactions between the mutants CV and TR in the presence of EPS are repulsive, due to the low polarity of the glass when coated with the respective exopolymers.

The total free energy of interaction defined by the DLVO theory is the sum of the van der Waals and electrostatic interactions. The resultant profile of the total free energy (Fig. 4) shows that in PBS van der Waals interactions play a more important role than the electrostatic interactions, because the former are longer range than the DL forces.

In the case of the interactions between the mutants TR and CV with glass, when immersed in the solution of exopolymers both electrostatic and van der Waals interactions were repulsive, resulting in a positive total energy profile (Fig. 5).

According to the DLVO energy profiles in EPS adhesion of TR and CV to glass would not be possible. However, in both cases adhesion occurred to a large extent.

3.1.2. XDLVO theory

The great innovation of the XDLVO theory is the inclusion of polar interactions. Usually the polar interactions are repulsive, creating an energy barrier near the surface [7]. In the present case, due to the great hydrophobicity of the glass when coated with the EPS from TR, the polar interactions between the coated glass and the mutant TR are attractive (Fig. 6).

The great intensity of the polar interactions at small separation distances leads (in the case of adhesion in PBS) to an energy profile with a

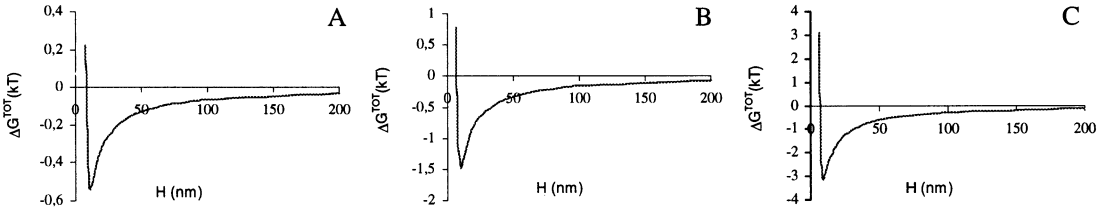

Fig. 4. Variation of the total DLVO free energy of interaction between the mutants TR (A), CV (B) and F72 (C) and glass when immersed in PBS as a function of the distance of separation (H).

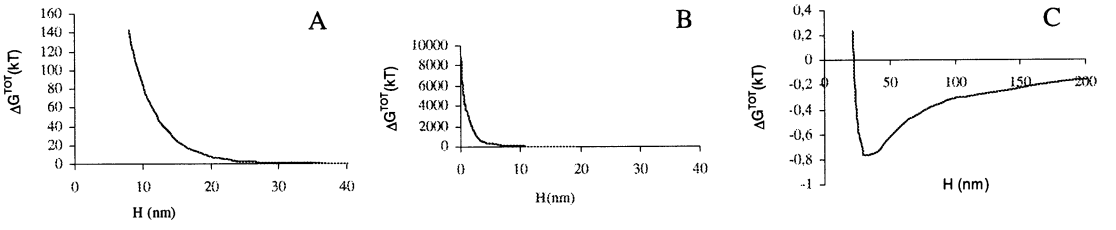

Fig. 5. Variation of the total DLVO free energy of interaction between the mutants TR (A), CV (B) e F72 (C) and the glass when immersed in EPS as a function of the distance of separation (H).

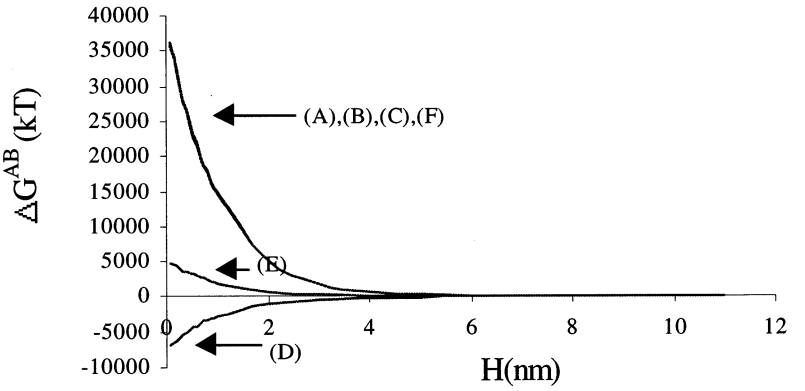

Fig. 6. Variation of the polar free energy of interaction between the mutants TR (A), CV (B) and F72 (C) and glass when immersed in PBS and between the mutants TR (D), CV (E) and F72 (F) and glass when immersed in EPS as a function of the distance of separation (H).

minimum of 12–15 nm from the surface (Fig. 7). It can be assumed that in PBS (0.1 M) some cells adhered in this minimum by weak interacting forces. Actually, this minimum acts as a secondary minimum and an irreversible adhesion would not be expected, due to the energy barrier at the minimum distance of separation. It is also possible that some of the adhering cells in the secondary minimum were detached by the shear stress during the removal of non-adhering cells, resulting in the small number of cells observed (Table 4). Moreover, the number of adhered cells is in accordance with the depths of the secondary minima.

In the solution of exopolymer, the mutant F72 has the same energy profile as in PBS, with a secondary minimum followed by an energy barrier (Fig. 8). The total free energy of interaction between the mutant CV and the glass in EPS is always repulsive. According to this energy profile adhesion would not occur. However, the number of adhering CV cells in PBS was three times greater than the number adhered in PBS. A possible explanation for this fact is that the polymers adsorbed to the glass surface can bind to the polymers that surround the CV mutant 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 slime layer is thickest for TR, followed by CV and is almost non-existent in F72 [19].

The energy balance diagram obtained for the mutant TR (Fig. 8(A)) presents an energy barrier with a maximum at 4 nm followed by a minimum of energy at small distances. Van Loosdrecht and Zehnder [20] showed that the energy barrier can be overcome by polymeric extensions present at the cell surface, due to the increase of the effective radius of the cell. This originates an increase of the van der Waals attractive interactions and the establishment of polymeric bridges. The number of TR cells adhering to glass in the presence of exopolymers was seven times greater than the number of cells adhering in PBS (0.1 M). So, it is

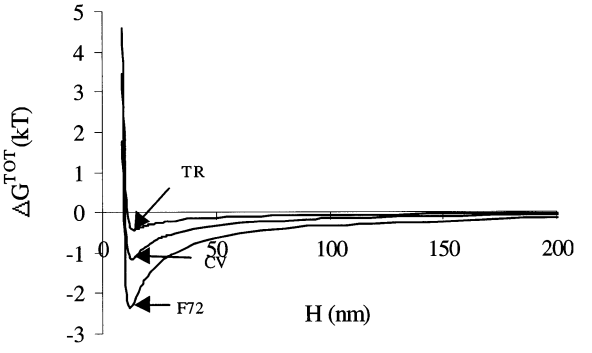

Fig. 7. Variation of the XDLVO total free energy of interaction between the mutants TR, CV e F72 and glass when immersed in PBS as a function of the distance of separation (H).

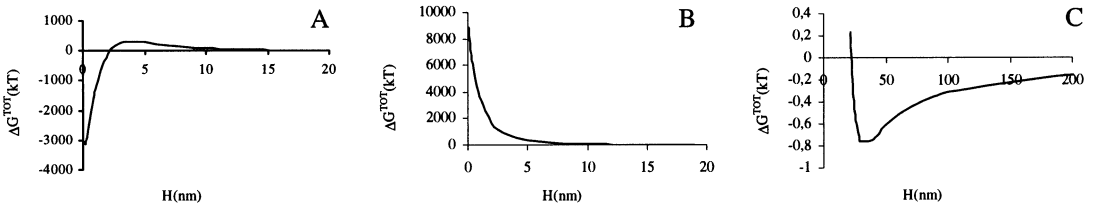

Fig. 8. Variation of the XDLVO total free energy of interaction between the mutants TR (A), CV (B) and F72 (C) and glass when immersed in EPS as a function of the distance of separation (H).

possible that the polymeric extensions present at the cell surface may eventually be able to overcome the energy barrier and an effective and strong adhesion can take place at the primary minimum.

4. Conclusions

In PBS the adhesion of the mutants is mainly governed by DLVO forces. The energy profiles showed a primary minimum followed by an energy barrier. The depth of the primary minimum is in accordance with the number of adhering cells. DLVO theory was not able to explain the adhesion of the mutants TR and CV in EPS.

With the inclusion of the polar interactions, the energy diagrams obtained by XDLVO theory for the mutant TR in EPS presented an energy barrier followed by a minimum at short separation distances, where adhesion should be irreversible. In this situation hydrophobic interactions are expected to play a major role.

XDLVO was not able to explain the results obtained with CV in EPS because the energy profile was always repulsive. Polymeric interactions may have been determinant in this case.

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