Antonie van Leeuwenhoek (2010) 98:317–329
DOI 10.1007/s10482-010-9444-2

ORIGINAL PAPER

Adhesion and biofilm formation on polystyrene by drinking water-isolated bacteria

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Received: 3 February 2010 / Accepted: 6 April 2010 / Published online: 20 April 2010

Abstract This study was performed in order to characterize the relationship between adhesion and biofilm formation abilities of drinking water-isolated bacteria (*Acinetobacter calcoaceticus*, *Burkholderia cepacia*, *Methylobacterium* sp., *Mycobacterium mucogenicum*, *Sphingomonas capsulata* and *Staphylococcus* sp.). Adhesion was assessed by two distinct methods: thermodynamic prediction of adhesion potential by quantifying hydrophobicity and the free energy of adhesion; and by microtiter plate assays. Biofilms were developed in microtiter plates for 24, 48 and 72 h. Polystyrene (PS) was used as adhesion substratum. The tested bacteria had negative surface charge and were hydrophilic. PS had negative surface charge and was hydrophobic. The free energy of adhesion between the bacteria and PS was > 0 mJ/m^2 (thermodynamic unfavorable adhesion). The thermodynamic approach was inappropriate for modelling adhesion of the tested drinking water bacteria, underestimating adhesion to PS. Only three (*B. cepacia*, *Sph. capsulata* and *Staphylococcus* sp.) of the six bacteria were non-adherent to PS. *A. calcoaceticus*, *Methylobacterium* sp. and *M. mucogenicum* were weakly adherent. This adhesion ability was correlated with the biofilm formation ability when comparing with the results of 24 h aged biofilms. *Methylobacterium* sp. and *M. mucogenicum* formed large biofilm amounts, regardless the biofilm age. Given time, all the bacteria formed biofilms; even those non-adherents produced large amounts of matured (72 h aged) biofilms. The overall results indicate that initial adhesion did not predict the ability of the tested drinking water-isolated bacteria to form a mature biofilm, suggesting that other events such as phenotypic and genetic switching during biofilm development and the production of extracellular polymeric substances (EPS), may play a significant role on biofilm formation and differentiation. This understanding of the relationship between adhesion and biofilm formation is important for the development of control strategies efficient in the early stages of biofilm development.

Keywords Adhesion · Biofilm formation · Hydrophobicity · Opportunistic drinking water bacteria · Surface charge

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Introduction

Many problems in drinking water distribution systems (DWDS) are related with the presence of

microorganisms, including biofilm growth, nitrification, microbially mediated corrosion, and the occurrence and persistence of pathogens (Regan et al. 2003; Camper 2004; Emtiazi et al. 2004; Bauman et al. 2009). DWDS are known to harbour biofilms, even though these environments are oligotrophic and often contain a disinfectant. By adopting this sessile mode of life, biofilm-embedded microorganisms enjoy a number of advantages over their planktonic counterparts, namely the increased resistance to antimicrobials (Gilbert et al. 2002). Microbial adhesion will initiate biofilm formation, exacerbating contamination of drinking water, reducing the aesthetic quality of potable water, increasing the corrosion rate of pipes and reducing microbiological safety through increased survival of pathogens (Percival and Walker 1999; Niquette et al. 2000). The development of a biofilm is believed to occur in a sequential process that includes transport of microorganisms to surfaces, initial reversible/irreversible adhesion, cell–cell communication, formation of microcolonies, extracellular polymeric substances (EPS) production and biofilm maturation (Doyle 2000; Sauer and Camper 2001; Bryers and Ratner 2004; Dobretsov et al. 2009). Accordingly, the adhesion of bacteria to the surface is one of the prime steps in biofilm formation.

Several theoretical approaches have been applied to describe bacteria-surface adhesion, such as the classical Derjaguin–Landau–Verwey–Overbeek (DLVO) theory (Rutter and Vincent 1984; van Loosdrecht et al. 1988, 1990), the extended DLVO (XDLVO) theory (van Oss 1989; Meinders et al. 1995), and the thermodynamic approach (surface Gibbs energy) (Absolom et al. 1983; Busscher et al. 1984). When a microorganism and a surface in aqueous solution enter in direct contact the water film present between the interacting entities has to be removed. This is in accordance with the thermodynamic theory of adhesion and is expressed by the Dupré equation which states that the Gibbs free energy of interaction can be calculated assuming that the interfaces between bacteria/liquid medium and solid/liquid medium are replaced by a bacteria/solid interface (Absolom et al. 1983). The interaction between a microbial cell and a solid substratum is only possible from a thermodynamic point of view if it leads to a decrease in the surface Gibbs free energy (Absolom et al. 1983; Busscher et al. 1984). Those approaches consider bacteria as colloids. However, important biological factors have been largely ignored in those models. Walker et al. (2004, 2005) have found that the heterogeneity of active sites from cell surface macromolecules, such as proteins and lipopolysaccharide-associated functional groups, controls the adhesion process.

Bacterial adhesion is a complex process that is affected by many factors, including the physicochemical characteristics of bacteria (hydrophobicity, surface charge), the material surfaces properties (chemical composition, surface charge, hydrophobicity, roughness and texture) and by the environmental factors (temperature, pH, time of exposure, bacterial concentration, chemical treatment or the presence of antimicrobials and fluid flow conditions). The biological properties of bacteria, such as the presence of fimbriae and flagella, and the production of EPS also influence the attachment to surface (An and Friedman 1998). Recently, adhesion has been described as a two-phase process including an initial, instantaneous, and reversible physicochemical phase and a time-dependent and irreversible molecular and cellular phase (Pavithra and Doble 2008). In the first phase, planktonic bacteria move or are moved to a surface through and by the effects of physical forces, such as Brownian motion, van der Waals attraction forces, gravitational forces, the effect of surface electrostatic charge, and hydrophobic interactions. These physical interactions are further classified as long-range (non-specific, distances > 150 nm) and short-range interactions (distances < 3 nm). Bacteria are first transported to the surface by the long-range interactions and at closer proximity the short-range interactions become more important. In the second phase, molecular reactions between bacterial surface structures and substratum surfaces become predominant. This implies a firmer adhesion of bacteria to a surface by the bridging function of bacterial surface polymeric structures.

The understanding of the overall biofilm formation process depends on the deep understanding of the main aspects regulating biofilm development, such as the initial adhesion. However, there is a lack of information regarding the behavior of cells in the earlier stages of biofilm formation, and its relationship with the biofilm development process. This study was performed in order to characterize the adhesion and biofilm formation abilities of drinking

water-isolated bacteria to polystyrene (PS) and to assess the possible relationships between adhesion and biofilm results.

Materials and methods

Bacteria isolation and identification

The microorganisms used throughout this work were isolated from a model laboratory DWDS, as described previously by Simões et al. (2006). Identification tests, by determination of 16S rDNA gene sequence, were performed for putative bacteria according to the method described by Simões et al. (2007a).

Planktonic bacterial growth

Assays were performed with 6 representative (above 80% of the total bacterial genera isolated and identified) drinking water bacteria: *Acinetobacter calcoaceticus*, *Burkholderia cepacia*, *Methylobacterium* sp., *Mycobacterium mucogenicum*, *Sphingomonas capsulata* and *Staphylococcus* sp.

Bacterial cells were grown overnight in batch culture using 100 ml of R2A (Merck, Portugal) broth, at room temperature (23°C ± 2), under agitation (150 rpm). Cells were harvested by centrifugation (20 min at 13,000×*g*), washed three times in saline phosphate buffer (0.1 M PBS, pH 7.2) and resuspended in a certain volume of sterile tap water (pH 6.7 ± 0.2) or R2A broth (biofilm studies) necessary to achieve the bacterial concentration required for each assay.

Substratum

The material assayed was PS. In order to prepare PS for further analysis, it was immersed in a solution of commercial detergent (Sonasol Pril, Henkel Ibérica S. A.) and ultrapure water for 30 min. In order to remove any remaining detergent, the material was rinsed in ultrapure water and subsequently immersed in ethanol at 96% (v/v) for 10 s. After being rinsed three times with ultrapure water, it was dried at 65°C for 3 h before being used for contact angle measurements, zeta potential assessment and adhesion assays.

Zeta potential

Zeta potential experiments were performed with the cells resuspended in sterile tap water at a final concentration of 10^9 cells/ml. The zeta potential of PS was also assessed. The experiments were determined using a Malvern Zetasizer instrument (Zetasizer Nano ZS ZEN3600, Malvern). Before measuring the electrostatic values, the zeta potential cell (DTS1060, Malvern) was rinsed three times with each suspension using a disposable syringe. All experiments were carried out at room temperature. The zeta potential was derived from the electrophoretic mobility using the Smoluchowski approximation (Hunter 1981). The experiments were performed in triplicate and repeated three times.

Surface contact angles

Bacterial lawns for contact angle measurements were prepared as described by Busscher et al. (1984). The surface tension of the bacterial surfaces and of the adhesion surface were then determined using the sessile drop contact angle method. The measurements were carried out at room temperature (23°C ± 2) using three different liquids: water, formamide and α-bromonaphtalene (Sigma, Portugal). Determination of contact angles was performed automatically using a model OCA 15 Plus (DATAPHYSICS, Germany) video based optical contact angle measure instrument, allowing image acquisition and data analysis.

Contact angle measurements (at least 25 determinations for each liquid and for each microorganism and PS) were performed at three independent experiments for each condition tested. The reference liquids surface tension components were obtained from literature (Janczuk et al. 1993).

Surface hydrophobicity and free energy of adhesion

Hydrophobicity was assessed after contact angle measurements and using the approach of van Oss et al. (1987, 1988, 1989). In this approach, the degree of hydrophobicity of a given material (1) is expressed as the free energy of interaction between two entities of that material when immersed in water (w)—ΔG_{1w1}. If the interaction between the two entities is stronger than the interaction of each entity with water

$\Delta G_{1w1} < 0$ mJ/m^2 the material is considered hydrophobic. Conversely, if $\Delta G_{1w1} > 0$ mJ/m^2 the material is hydrophilic. ΔG_{1w1} can be calculated through the surface tension components of the interacting entities, according to:

$$\Delta G_{1w1} = -2\left(\sqrt{\gamma_1^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_1^+\gamma_w^-} + \sqrt{\gamma_1^-\gamma_w^+} - \sqrt{\gamma_1^+\gamma_1^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \quad (1)$$

where γ^{LW} accounts for the Lifshitz–van der Waals component of the surface free energy and γ^+ and $\gamma-$ are the electron acceptor and electron donor parameters, respectively, of the Lewis acid–base component (γ^{AB}), with $\gamma^{AB} = 2\times\sqrt{\gamma^+\gamma^-}$.

The surface tension components of a surface (s) (bacteria or substratum) are obtained by measuring the contact angles of three pure liquids (l) (one apolar—α-bromonaphtalene and two polar—water and formamide), with well known surface tension components, followed by the simultaneous resolution of three equations of the form:

$$(1+\cos\theta)\gamma_l^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + \sqrt{\gamma_s^+\gamma_l^-} + \sqrt{\gamma_s^-\gamma_l^+}\right) \quad (2)$$

where θ is the contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$.

The free energy of adhesion was calculated through the surface tension components of the entities involved in the adhesion process by the thermodynamic theory expressed by Dupré equation (3). When studying the interaction between one bacteria (b) and a substratum (s) that are immersed or dissolved in water (w), the total interaction energy, ΔG_{bws}^{TOT}, can be expressed by the interfacial tensions components as:

$$\Delta G_{bws}^{TOT} = \gamma_{bs} - \gamma_{bw} - \gamma_{sw} \quad (3)$$

For instance, the interfacial tension for one diphasic system of interaction (bacteria/substratum—γ_{bs}) can be defined by the thermodynamic theory according to the following equations:

$$\gamma_{bs} = \gamma_{bs}^{LW} + \gamma_{bs}^{AB} \quad (4)$$

$$\gamma_{bs}^{LW} = \gamma_b^{LW} + \gamma_s^{LW} - 2\times\sqrt{\gamma_b^{LW}\times\gamma_s^{LW}} \quad (5)$$

$$\gamma_{bs}^{AB} = 2\times\left(\sqrt{\gamma_b^+\times\gamma_b^-} + \sqrt{\gamma_s^+\times\gamma_s^-} - \sqrt{\gamma_b^+\times\gamma_s^-} - \sqrt{\gamma_b^-\times\gamma_s^+}\right) \quad (6)$$

The other interfacial tension components, γ_{bw} (bacteria/water) and γ_{sw} (substratum/water), were calculated in the same way. The value of the free energy of adhesion was obtained by the application of Eqs. 3–6, which allowed the assessment of thermodynamic adhesion. Thermodynamically, if $\Delta G_{bws}^{TOT} < 0\,mJ/m^2$ the adhesion of one bacteria to substratum is favourable. On the contrary, adhesion is not expected to occur if $\Delta G_{bws}^{TOT} > 0\,mJ/m^2$.

Adhesion

Coupons of PS with 8 mm × 8 mm, prepared as indicated previously, were inserted in the bottom of 24-wells (15 mm diameter each well) microtiter plates (polystyrene, Orange Scientific, USA) and 2 ml of each cell suspension (10^9 cells/ml in sterile tap water), was added to each well. Adhesion to each material was allowed to occur for 2 h at room temperature, in an orbital shaker at 150 rpm, according to the methods of Simões et al. (2007a). Negative controls were obtained by placing PS in sterile tap water without bacterial cells. At the end of the assay each well was washed twice with sterile distilled water, by pipetting carefully only the liquid above the coupon to remove reversibly adherent bacteria. After the last wash, the coupons were used for biomass quantification by crystal violet (CV) staining. All the experiments were performed in triplicate with three repeats.

Biofilm formation

Biofilms were developed according to the modified microtiter plate test proposed by Stepanović et al. (2000). Briefly, for each bacterium at least sixteen wells of a sterile 96-well flat tissue culture plates (polystyrene, Orange Scientific, USA) were filled under aseptic conditions with 200 µl of cell suspension (1×10^8 cells/ml in R2A broth). To promote biofilm formation, the plates were incubated aerobically on a shaker at 150 rpm, at room temperature,

for 24, 48 and 72 h. Each 24 h the growth medium was carefully discarded and replaced by fresh one. After each biofilm formation period, the content of each well was removed and the wells were washed three times with 250 µl of sterile distilled water to remove reversibly adherent bacteria. The plates were air dried for 30 min, and the remaining attached bacteria were analysed in terms of biomass adhered on the surfaces of the microtiter plates. Negative controls were obtained by incubating the wells only with R2A broth without adding any bacterial cells. All the experiments were repeated three times.

Biomass quantification by CV

The coupons with adhered bacteria in the 24-wells plates were removed from each well and immersed in a new microtiter plate containing 1 ml of methanol 98% (v/v) in each well for biomass quantification by crystal violet (CV—Gram colour-staining set for microscopy, Merck) (Simões et al. 2007a). Methanol was withdrawn after 15 min of contact and the coupons were allowed to dry at room temperature. Aliquots (600 µl) of CV were then added to each well and incubated for 5 min. After gently washing in water the coupons were left to dry, before being immersed in 1 ml of acetic acid 33% (v/v) to release and dissolve the stain.

The bacterial biofilms in the 96-wells plates were fixed with 250 µl of 98% methanol (Vaz Pereira, Portugal) per well for 15 min. Afterwards, the plates were emptied and left to dry. Then, the fixed bacteria were stained for 5 min with 200 µl of CV per well. Excess stain was rinsed off by placing the plate under running tap water (Stepanović et al. 2000). After the plates were air dried, the dye bound to the adherent cells was resolubilized with 200 µl of 33% (v/v) glacial acetic acid (Merck, Portugal) per well.

The optical density (OD) of the obtained solutions were measured at 570 nm using a microtiter plates reader (BIO-TEK, Model Synergy HT) and adhesion and biofilm mass were presented as $OD_{570\ nm}$ values.

Adherent/biofilm bacteria classification

Bacteria were classified using the scheme of Stepanović et al. (2000) as follow: non-adherent/non-biofilm producer (0): $OD \leq ODc$; weakly adherent/weak biofilm producer (+): $ODc < OD \leq 2 \times ODc$; moderately adherent/moderate biofilm producer (++): $2 \times ODc < OD \leq 4 \times ODc$; strongly adherent/strong biofilm producer (+++): $4 \times ODc < OD$. This classification was based upon the cut-off of the optical density (ODc) value defined as three standard deviation values above the mean OD of the negative control.

Statistical analysis

The data were analysed using the statistical program SPSS version 14.0 (Statistical Package for the Social Sciences). Because low samples numbers contributed to uneven variation, the adhesion results were analyzed by the nonparametric Wilcoxon test. Statistical calculations were based on a confidence level $\geq 95\%$ ($P < 0.05$ was considered statistically significant).

Results

Surface physicochemical properties and free energy of adhesion

Bacterial adhesion can be influenced by the surface physicochemical properties of both bacteria and substratum. Consequently, the drinking water-isolated bacteria and the PS surface were characterized in terms of surface properties—hydrophobicity and surface charge (zeta potential). All the tested isolates had negative zeta potential. The bacteria with the highest zeta potential was *A. calcoaceticus* (-6.7 ± 0.4 mV) and *M. mucogenicum* (-31 ± 3 mV) had the lowest zeta potential (Table 1). PS surface had a zeta potential of -32 ± 2 mV (Table 1).

The surface hydrophobicity was determined as a quantitative result using the approach proposed by van Oss (1995, 1997), which allows the assessment of the absolute degree of hydrophobicity of any surface in comparison with their interaction with water. Based on this approach the surfaces of the tested bacteria are hydrophilic ($\Delta G_{bwb}^{TOT} > 0\,mJ/m^2$) (Table 2). Conversely, the PS surface is hydrophobic ($\Delta G_{sws}^{TOT} = -44\,mJ/m^2$) (Table 2). Bacteria had similar hydrophobicity values ($P > 0.05$), with the exception of *Sph. capsulata*. According to the surface tension parameters (Table 2), the Lifshitz–van der Waals (γ^{LW}) component of the bacteria had similar values and all the bacteria were predominantly

Table 1 Zeta potential (mV) values of drinking water-isolated bacteria and PS

Zeta potential (mV)	
Bacteria	
Acinetobacter calcoaceticus	−6.7 ± 0.4
Burkholderia cepacia	−7.7 ± 0.3
Methylobacterium sp.	−9.0 ± 0.5
Mycobacterium mucogenicum	−31 ± 3
Sphingomonas capsulata	−27 ± 0.6
Staphylococcus sp.	−10 ± 0.3
Substratum	
PS	−32 ± 2

Values are means ± SDs of three independent experiments

electron donors (γ^-). Moreover, all the bacteria had the ability to accept electrons (γ^+). On the other hand, PS had only an electron donating character ($\gamma^+ = 0$ mJ/m^2).

In order to predict the ability of the microorganisms to adhere to PS surfaces, the free energy of interaction between the bacteria and the surface, when immersed in water, was calculated according to the thermodynamic approach. Based on this approach, all the bacteria had no theoretical thermodynamic ability to adhere to PS ($\Delta G^{TOT}_{bws} > 0\,mJ/m^2$). *B. cepacia*, had the smallest ΔG^{TOT}_{bws} and *Sph. capsulata* had the highest ΔG^{TOT}_{bws} (less prone to adhere to PS).

Adhesion

Adhesion assays were performed with the drinking water-isolated bacteria and PS surfaces, using a modified microtiter-plate assay methodology (Stepanović et al. 2000) and CV staining for biomass assessment of the adhered bacteria. The tested bacteria adhered to PS surfaces (Fig. 1) with different potentials ($P < 0.05$). *A. calcoaceticus* and *Sph. capsulata* had the highest and lowest adhesion ability, respectively. *Methylobacterium* sp. and *M. mucogenicum* adhered to similar extents ($P > 0.05$). The degree of bacterial adhesion was found to follow the sequence *A. calcoaceticus* > *Methylobacterium* sp. > *M. mucogenicum* > *Staphylococcus* sp. > *B. cepacia* > *Sph. capsulata*. However, only *A. calcoaceticus*, *Methylobacterium* sp. and *M. mucogenicum* were weakly adherent to PS. The remaining bacteria were classified as non-adherent (Table 3).

Biofilm formation

In order to assess the biofilm formation ability of the several drinking water-isolated bacteria, a standard 96-wells microtiter plates with CV staining was used to characterize biofilms (Fig. 2). The tested bacteria formed biofilms, with *Methylobacterium* sp. producing the highest biomass amount for all the sampling times. *M. mucogenicum* was the second stronger

Table 2 Contact angles (in degrees) with water (θ_W), formamide (θ_F), α-bromonaphtalene (θ_B), surface tension parameters, free energy of interaction (ΔG^{TOT}_{bwb} or ΔG^{TOT}_{sws}) of the bacteria (b) and PS (s) when immersed in water (w); free energy of adhesion (ΔG^{TOT}_{bws}) between the bacteria (b) and PS (s) when immersed in water (w). Values are means ± SDs of three independent experiments

	Contact angle (°)			Surface tension parameters (mJ/m^2)			Hydrophobicity (mJ/m^2)	Free energy of adhesion (mJ/m^2)
	θ_W	θ_F	θ_B	γ^{LW}	γ^+	γ^-	ΔG^{TOT}_{bwb} or ΔG^{TOT}_{sws}	ΔG^{TOT}_{bws}
Bacteria								
Acinetobacter calcoaceticus	28 ± 1	31 ± 1	43 ± 0.8	33	1.3	51	30	2.3
Burkholderia cepacia	38 ± 2	43 ± 2	47 ± 1	32	0.5	49	32	0.3
Methylobacterium sp.	20 ± 1	20 ± 2	42 ± 2	34	2.1	51	28	4.1
Mycobacterium mucogenicum	27 ± 1	25 ± 1	58 ± 8	26	4.4	46	20	5.3
Sphingomonas capsulata	31 ± 5	53 ± 2	73 ± 4	19	1.2	69	51	19
Staphylococcus sp.	28 ± 0.9	27 ± 1	51 ± 2	30	2.8	47	23	3.0
Substratum								
PS	83 ± 3	71 ± 2	28 ± 1	39	0.0	9.9	−44	–

ΔG^{TOT}_{bwb} or $\Delta G^{TOT}_{sws} < 0\,mJ/m^2$—hydrophobic surface; ΔG^{TOT}_{bwb} or $\Delta G^{TOT}_{sws} > 0\,mJ/m^2$—hydrophilic surface. $\Delta G^{TOT}_{bws} < 0\,mJ/m^2$—thermodynamic favourable adhesion; $\Delta G^{TOT}_{bws} > 0\,mJ/m^2$—thermodynamic unfavorable adhesion

biofilm producer. A directly proportional time—biomass formation was found for the various bacteria ($P < 0.05$), except for *B. cepacia* ($P > 0.05$). Only for sampling times higher than 48 h, *Sph. capsulata* formed biofilms. The degree of biofilm formation was found to follow the sequence—24 h biofilms: *Methylobacterium* sp. > *M. mucogenicum* > *A. calcoaceticus* > *Staphylococcus* sp. > *B. cepacia* > *Sph. capsulata*; 48 h biofilms: *Methylobacterium* sp. > *M. mucogenicum* > *B. cepacia* > *Staphylococcus* sp. > *A. calcoaceticus* > *Sph. capsulata*; 72 h biofilms: *Methylobacterium* sp. > *M. mucogenicum* > *A. calcoaceticus* > *Staphylococcus* sp. > *Sph. capsulata* > *B. cepacia*.

According to the rank of biofilm formation (Table 3), *Methylobacterium* sp. and *M. mucogenicum* showed a strong biofilm producing ability for the several sampling times. *Sph. capsulata* and *Staphylococcus* sp. only presented biofilm formation ability (moderate) for the 72 h sampling time. *B. cepacia* formed weak biofilms after 48 h, while *A. calcoaceticus* showed variability in the biofilm formation ability by forming weak biofilms at 24 h, being classified as non-biofilm producer at 48 h, and as a strong biofilm producer at the 72 h sampling time.

Table 3 Adhesion and biofilm formation ability of drinking water-isolated bacteria to PS according to the classification proposed by Stepanović et al. (2000) and used by Simões et al. (2007b)

Bacteria	Adhesion	Biofilm		
		24 h	48 h	72 h
Acinetobacter calcoaceticus	+	+	0	+++
Burkholderia cepacia	0	0	+	+
Methylobacterium sp.	+	+++	+++	+++
Mycobacterium mucogenicum	+	+++	+++	+++
Sphingomonas capsulata	0	0	0	++
Staphylococcus sp.	0	0	0	++

(0) non-adherent/non-biofilm producer; (+) weakly adherent/weak biofilm producer; (++) moderately adherent/moderate biofilm producer; (+++) strongly adherent/strong biofilm producer

Discussion

The dynamics of the microbial growth and biofilm formation in drinking water networks is very complex, as a large number of interacting processes are involved (Simões et al. 2007b, 2008b; Liu et al. 2009). Biofilms are suspected to be the primary source of microorganisms in DWDS that are fed with treated water and have no pipeline breaches, and are of particular concern in older DWDS (LeChevalier et al. 1987). Bacterial adhesion to surfaces, the first step in the formation of a biofilm, has been studied extensively over the past decades in many diverse areas. However, to our knowledge this is the first study reporting the relationship between adhesion and biofilm formation by autochthonous drinking water bacteria. Microorganisms isolated from any given niche, whether medical, environmental, water, or industrial, will have different mechanisms of

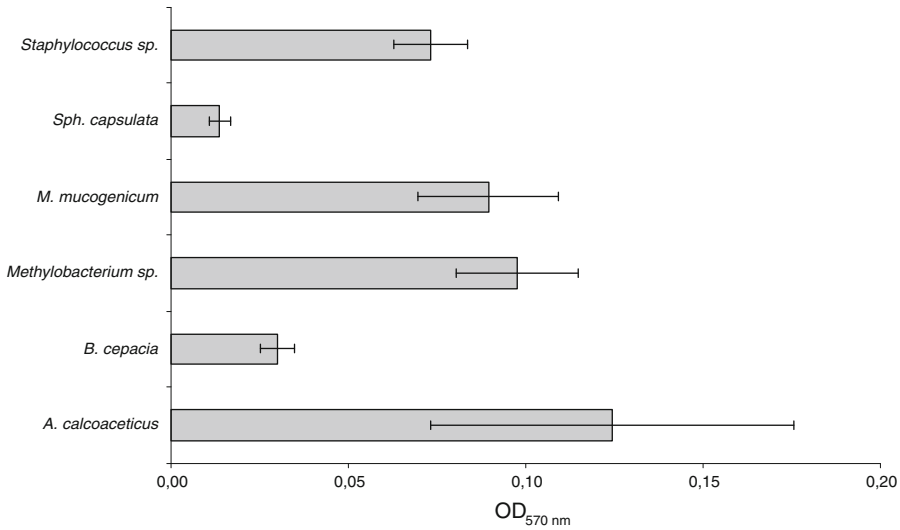

Fig. 1 Values of $OD_{570\ nm}$ as a measure of bacteria adhesion to PS during 2 h. The means ± SDs for three independent experiments are illustrated

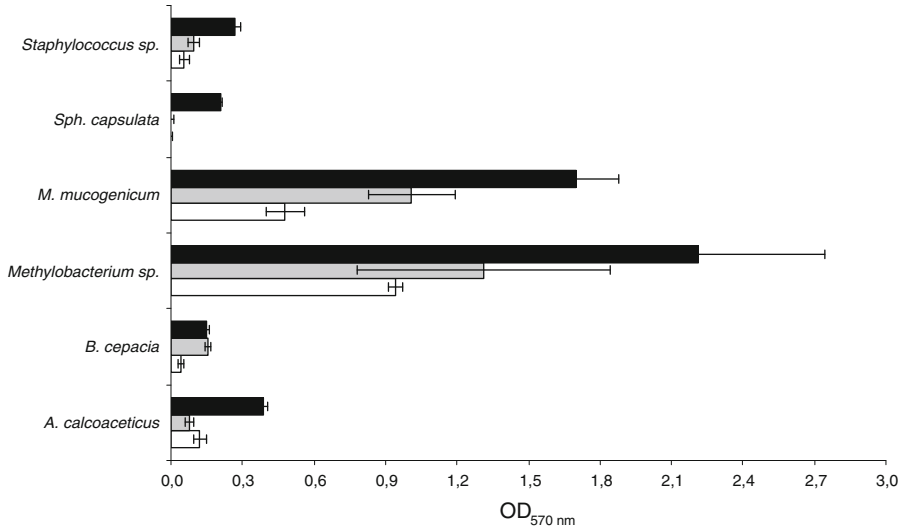

Fig. 2 Values of $OD_{570\ nm}$ as a measure of mass of 24 h (□), 48 h (▨) and 72 h (■) aged biofilms. The means ± SDs for three independent experiments are illustrated

adhesion and retention, not only because the substrata, nutrients, ionic strength, pH values, and temperatures differ, but also because their phenotype and genotype (expression of structural components and adhesive surface proteins) have adapted differently over time through selective pressures (Thomas et al. 2002). Bakker et al. (2004) also reported that bacterial strains isolated from different niches can exhibit different patterns of adhesion to substrata. The bacteria used in this study are recognized as problematic opportunistic bacteria with the potential to cause public health problems (Bifulco et al. 1989; Rusin et al. 1997; Szewzyk et al. 2000; Zanetti et al. 2000; Conway et al. 2002; Pavlov et al. 2004; Stelma et al. 2004). Similarly to other studies, PS was used as a model surface for adhesion and biofilm formation under laboratorial conditions (Simões et al. 2007b; Pompilio et al. 2008; Silva et al. 2008; Johansen et al. 2009). The PS microtiter plates are commonly used as the standard bioreactor system for adhesion and biofilm formation of bacteria isolated from many different environments, providing reliable comparative data (Djordjevic et al. 2002; Andersson et al. 2008; Cotter et al. 2009). PS has physico-chemical surface properties (hydrophobicity) similar to those of other materials used in water distribution systems such as stainless steel and polyvinylchloride (Simões et al. 2007a). Understanding the relationship between adhesion and biofilm formation is crucial to understand the role microorganisms may play in the system and to develop reliable preventive and control strategies efficient in the early stages of biofilm development.

The influence of the surface free energies of the substratum and the bacterium can be modelled using a thermodynamic approach (Bos et al. 1999). The XDLVO theory accounts for Lifshitz–Van der Waals, electrostatic, and short range acid–base interaction energies between the surface and the bacterium as a function of their separation distance (Van Oss et al. 1986). This mechanistic knowledge of bacterial adhesion obtained from the XDLVO theory provides guidelines for the development of surface coatings exhibiting propensity for minimal bacterial adhesion (Genzer and Efimenko 2006; Webster et al. 2007; Bennett et al. 2010). However, the initial microbial adhesion, as governed by physicochemical interaction forces, is only one of the steps in the development of a mature biofilm. After adsorption of conditioning film components and adhesion of initial colonizers, many subsequent biological, ecological and environmental events determine the ultimate microbial composition and structure of a mature biofilm (Bryers and Ratner 2004; Simões et al. 2009).

Bacterial characteristics known to influence adhesion are hydrophobicity, surface charge, motility, and release of extracellular substances, such as polysaccharides, proteins and metabolite molecules (Dufrene et al. 1996; Kogure et al. 1998; Azeredo et al. 1999; Bos et al. 1999; van Hoogmoed et al. 2000). Relevant properties of the substratum surface are hydrophobicity, charge, and texture (Holland et al. 1998; Bos et al. 1999; Gottenbos et al. 1999; Akesso et al. 2009). Based on the surface properties studied all the bacteria had negative zeta potential and are

hydrophilic. According to Rijnaarts et al. (1999), at physiological pH (pH 7) bacterial cells generally have a net negative charge on their cell wall. In this study, the bacteria had similar hydrophobicity (exception—*Sph. capsulata*) and zeta potential (exceptions—*M. mucogenicum* and *Sph. capsulata*) values. It is not surprising that the surface properties of *M. mucogenicum* were considerably different from the other bacteria due to the presence of a waxy cell wall. PS had also negative zeta potential, but had a hydrophobic character. Furthermore, it was observed that all bacteria were predominantly electron donors, with low electron acceptor parameters. This polar character can be due to the presence of residual water of hydration or polar groups (van Oss 1994).

A comparison between the theoretical thermodynamic adhesion evaluation and the adhesion assays shows that adhesion was underestimated when based on thermodynamic approaches. In fact, no agreement between thermodynamic approaches and the adhesion assays were obtained for the tested bacteria. Even if for all the bacteria $\Delta G_{bws}^{TOT} > 0$ mJ/m^2 they adhered to PS. The lack of agreement between thermodynamic and adhesion results proposes that bacterial adhesion on PS surfaces is not influenced by the surface physicochemical properties. *Sph. capsulata* physicochemical properties revealed the highest hydrophilicity, consequently, being the less prone to adhere to PS according to the thermodynamic approach. This bacterium had also the lowest ability to adhere to PS according to the adhesion assays. This demonstrates that the physicochemical properties account apparently for the low adhesion ability of *Sph. capsulata*. However, for the other bacteria, no correlation was found between cell surface hydrophobicity and their ability to adhere to PS. This fact is corroborated by other studies (Oliveira et al. 2007; Sousa et al. 2009), likely due to the multiplicity of parameters involved in the adhesion process being influenced both by biological and environmental factors. Also, it is perceptible that the zeta potential differences do not influence the adhesion process. PS, *M. mucogenicum* and *Sph. capsulata* had highly negatively charged surfaces (zeta potential < -25 mV), while the other bacteria had surfaces with moderate negatively charged. However, there is no clear relationship between the zeta potential data and adhesion. Flint et al. (1997) were unable to assess any relationship between the numbers of *Streptococci* cells attaching to stainless steel and cell surface charge. Previous studies already reported the lack of a correlation between the bacterial surface properties and attachment. The attachment process was strongly influenced by the presence of extracellular biological molecules (Li and Logan 2004; Chae et al. 2006). Barton et al. (1996), however, found that surface growth of *Pseudomonas aeruginosa* on diverse polymers correlated with the free energy of adhesion, while no such correlation was found for *Staphylococcus epidermidis* and *Escherichia coli*. Simões et al. (2008b) found a correlation between the thermodynamic approaches and biofilm formation of a *Bacillus cereus* strain forming biofilms with low EPS content. In the current study, the lack of agreement between thermodynamic approaches and adhesion assays reinforces that biological mechanisms, such as the expression of extracellular appendages—adhesins that mediate specific interactions with substrata at a nanometer scale, during the irreversible phase of microbial adhesion, in addition to the physicochemical ones, are the plausible aspects mediating the entire adhesion process (Flint et al. 1997; Doyle 2000; Sinde and Carballo 2000; Donlan 2002; Rodrigues and Elimelech 2009).

The importance of initial events in biofilm development still remains unknown due to the multitude of subsequent events taking place on a much longer time scale (Busscher and van der Mei 1997). There are some evidences indicating initial adhesion may be an important aspect in final biofilm formation, particularly for systems under fluctuating shear conditions (Quirynen et al. 1993; Busscher and Van der Mei 1997). Drinking water distributing systems are usually subjected to variable hydraulic situations, ranging from no-flow (stagnant water) to steady-state hydrodynamic conditions. In this study, the magnitude of the initial bacterial adhesion on the subsequent biofilm formation was compared for the drinking water-isolated bacteria (under constant shear conditions) being found that only for *Methylobacterium* sp. and *M. mucogenicum*, both weakly adherent bacteria, are good biofilm producers regardless the biofilm age. Also, adhesion and biofilm formation are correlated when analyzing the 24 h aged biofilms. Non-adherent bacteria (*B. cepacia*, *Sph. capsulata* and *Staphylococcus* sp.) are non-biofilm producers or produce low biofilm amounts only for low aged biofilms (24 or 48 h). However, after a certain period

of time all the bacteria had the ability to develop biofilms. When increasing the biofilm formation period the relationship between adhesion and biofilm formation decreases. This time-dependent effects are evident when characterizing the *A. calcoaceticus* biofilms. This bacterium develops weak biofilms for a 24 h period, 24 h later (48 h aged biofilms) the biofilm formation ability decreases and 24 h (72 h aged biofilms) after the bacteria forms large biofilm amounts. This result indicates that the biofilm maturation process increases the system complexity and decreases the possibility of making reliable correlations with the early biofilm development stages. A recent report demonstrated the autoaggregation ability of *A. calcoaceticus* (Simões et al. 2008a). This bacterial ability provides an increased opportunity for metabolic cooperation in the early biofilm development process, being important not only for colonization, but also for biofilm development (Rickard et al. 2003, 2004). Some authors (Fox et al. 1990; Petrozzi et al. 1993) already questioned the significance of the effect of the initial bacterial adhesion on biofilm formation because the number of bacterial cells involved in the initial biofilm formation process is much smaller than that in mature biofilms. However, other researchers have suggested that there is a link between the initially adhering bacteria and the biofilms that subsequently are formed (Busscher et al. 1995). Motility is another important cellular aspect in the early stages of biofilm formation and development. Pratt and Kolter (1998) demonstrated that surface motility is an important factor in the initial interaction with an abiotic surface. Also, Kogure et al. (1998) have shown that motility increases adhesion to a bare glass substratum. This has been attributed to the increased collision frequency with the solid surface (Morisaki et al. 1999). Comparing the current results with a previous study, it is evident that the motility of the tested drinking water isolates does not regulate adhesion and biofilm formation (Simões et al. 2007b). *B. cepacia* has the highest motility, however, this bacterium is non-adherent and non- (24 h) or low biofilm producer (48 and 72 h). The remaining species had low motility values and similar between then (Simões et al. 2007b). Roosjen et al. (2006) observed that the motility and zeta potential were not distinctive for adhesive and non-adhesive strains, and could therefore not be the reason for the difference in adhesion behavior. In other study, no correlation between motility, adhesion and biofilm formation was found (Pompilio et al. 2008). Also, those authors found a strong relationship between the extent of initial adhesion of *Stenotrophomonas maltophilia* to PS surfaces and biofilm formation.

In conclusion, controlling and preventing the adverse impact of the bacterial deposition on the aquatic environment needs an in-depth understanding about the mechanisms regulating this process. The XDLVO theory has been used extensively to describe the deposition of bacteria in many current researches. However, physicochemical approaches based on the XDLVO theory were inappropriate for modelling adhesion of the tested drinking water bacteria to PS. The adhesion results suggest that mechanisms other than physicochemical surface properties may play a determinant role on bacterial adherence ability. Bacteria themselves produce extracellular molecules with sufficient surface activity to play a role in the bacterial adhesion process. However, the adhesion step does not provide conclusive information on the formation of mature biofilms. Adhesion ability was only correlated when comparing the results of the 24 h biofilms. Given time, all the bacteria had the ability to form biofilms even if considered non-adherent. *A. calcoaceticus*, *Methylobacterium* sp. and *M. mucogenicum* were classified as weakly adherent to PS and formed large biofilm amounts. The remaining bacteria were non-adherent, however, had the ability to form biofilms. This identification of the main bacteria forming more complex biofilms (*A. calcoaceticus*, *Methylobacterium* sp. and *M. mucogenicum*), probably more resistant to disinfection, due to their high biomass amount, may provide new information necessary for improving water quality for the consumers. Furthermore, these biofilms can act as a harbour and/or substrate for other microorganisms less prone to biofilm formation, increasing the probability of pathogen survival and further dissemination in the DWDS.

Acknowledgments The authors acknowledge the financial support provided by the Portuguese Foundation for Science and Technology (SFRH/BD/31661/2006—Lúcia C. Simões).

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