Research Paper

Potential of the adhesion of bacteria isolated from drinking water to materials

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Heterotrophic bacteria (11 genera, 14 species, 25 putative strains) were isolated from drinking water, identified either biochemically or by partial 16s rDNA gene sequencing and their adherence characteristics were determined by two methods: i. thermodynamic prediction of adhesion potential by measuring hydrophobicity (contact angle measurements) and ii. by measuring adherence to eight different substrata (ASI 304 and 316 stainless steel, copper, polyvinyl chloride, polypropylene, polyethylene, silicone and glass). All the test organisms were hydrophilic and inter-species variation in hydrophobicity occurred only for *Comamonas acidovorans*. Stainless steel 304 (SS 304), copper, polypropylene (PP), polyethylene (PE) and silicone thermodynamically favoured adhesion for the majority of test strains (>18/25), whilst adhesion was generally less thermodynamically favorable for stainless steel 316 (SS 316), polyvinyl chloride (PVC) and glass. The predictability of thermodynamic adhesion test methods was validated by comparison with 24-well microtiter plate assays using nine reference strains and three adhesion surfaces (SS 316, PVC and PE). Results for *Acinetobacter calcoaceticus*, *Burkolderia cepacia* and *Stenotrophomonas maltophilia* sp. 2 were congruent between both methods whilst they differed for the other bacteria to at least one material. Only *A. calcoaceticus* had strongly adherent properties to the three tested surfaces. Strain variation in adhesion ability was detected only for *Sphingomonas capsulata*. Analysis of adhesion demonstrated that in addition to physicochemical surface properties of bacterium and substratum, biological factors are involved in early adhesion processes, suggesting that reliance on thermodynamic approaches alone may not accurately predict adhesion capacity.

Keywords: Autochthonous microflora / Bacterial adhesion / Drinking water / Interaction energy / Surface hydrophobicity

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Introduction

Bacterial adhesion to surfaces is one of the initial steps leading to biofilm formation and is therefore an important microbiological event in medicine (Costerton *et al.* 1987), industry (Simões *et al.* 2005) and the environment (Bayoudh *et al.* 2005, Simões *et al.* 2006a). In drinking water distribution systems, 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, Tsai 2005, Simões *et al.* 2006a). Microorganisms are generally less of a problem in planktonic phase since due to increased disinfection efficiency (Simões *et al.* 2003. 2005, 2006b). Considerable resources have therefore been directed towards technologies designed to inhibit the microbial attachment with the aim of deriving colonization-free surfaces (Thouvenin *et al.* 2003, Tang *et al.* 2005). Microbial adhesion to surfaces is a complex process, influenced by several physicochemical properties of both microorganism and substratum, the most significant of which are hydrophobicity and surface charge (Donlan 2002, Gallardo-Moreno *et al.* 2002a, 2002b). The initial adhe-

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sion step can be interpreted in terms of Lifshitz-van de Waals forces (LW) and acid-base forces (AB) (Smets *et al.* 1999, Gallardo-Moreno *et al.* 2002a). When a microorganism and a surface enter into 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). Accordingly, hydrophobicity has been considered the most important short-range interaction force in bacterial attachment, playing a determinant role in bacterial adhesion (van Oss 1997). Other cellular and support material associated inherent factors can also significantly account for the adhesion process (Flint *et al.* 1997, Sinde and Carballo 2000). For example, the production of polysaccharides, lipopolysaccharide chemistry, and other factors may also affect adhesion (Li and Logan 2004) even if their contribution is not incorporated into predictive models. The purpose of the present study was to characterize the physicochemical properties of surfaces of a selection of numerically important drinking water isolates and to evaluate their potential for adhesion to a variety of materials with potential use in drinking water distribution pipes, comparing predicted adhesion based on thermodynamic approaches with adhesion assays.

Materials and methods

Bacteria isolation and growth

The microorganisms used throughout this work were isolated from a model laboratory drinking water distribution system, as described by Simões *et al.* (2006a). Briefly, the system consisted of a Perspex vessel (volume, 1.6 l; diameter, 16.8 cm) fed with normal tap water in Braga, Portugal. The system was sterile until filled with potable water and operated so as to prevent immigration of microorganisms other than via the tap water feed. The flow rate of tap water gave a dilution rate of 3.125 h^{-1}. Microorganisms were isolated by collecting 100 μl chemostat water and plating on trypticase soy agar – TSA (Merck, VWR, Portugal) and R2A (Oxoid, UK) aerobically at room temperature for 15 days. TSA and R2A were selected since in validation studies (data not shown) they supported the optimal growth, successfully recovering heterotrophic bacteria from drinking water. R2A has been previously validated as an effective isolation medium for aquatic bacteria (Reasoner and Geldrich 1985, Simões *et al.* 2006a).

Bacterial identification

Preliminary, presumptive bacteria identification was done using selective medium Chromocult® TBX agar (Merck); *Pseudomonas* isolation agar (Difco); Metanol minimum medium, according to Kim *et al.* (1999), Gram staining and biochemical methods (API 20 NE and API ID32 GN systems (Biomerieux)), according to the manufacturer's instructions. Further identification tests, by determination of 16S rDNA gene sequence, were performed for putative *Acinetobacter* sp., *Burkolderia* spp., *Methylobacterium* spp., *Pseudomonas* spp., *Sphingomonas* spp. and *Stenotrophomonas* spp. As follows: Genomic DNA was extracted and purified by applying an isolated colony, collected from pure plate cultures resuspended in 20 μl of TE buffer, in indicating FTA Classic Cards (WB120206, Whatman) and proceeding according to manufacturer's instructions. 16S rDNA was amplified with universal primers pA (5′- AGA GTT TGA TCC TGG CTC AG) and pH (5′- AAG GAG GTG ATC CAG CCG CA-3′) (ULRIKE *et al.* 1989) or 109F (5′- ACG GGT GMG TAACKC GT-3′) and 1392R (5′- ACG GGC GGT GTG TRC-3′) (LANE, 1991). PCR was performed in a Thermocycler (Uno II, Biometra) and the reaction (mixtures containing the template DNA in the FTA disc) occurred in 35 cycles with 1 min denaturation at 96 °C, 1 min annealing at 50 °C and 1 min extension at 72 °C, after a previous step of denaturation (96 °C for 4 min) and followed by a final extension step (72 °C for 5 min). PCR products were visualized using ethidium bromide staining after electrophoresis through a 1% (w/v) agarose gel and their sizes were determined by comparison with a molecular weight standard (1 kb plus DNA ladder; GibcoBRL). The PCR products were purified using Jet Quick-PCR Purification Kit (Genomed, Germany) as described by the manufacturer. Sequecing was done using an automated DNA capillary sequencer CEQ 2000-XL (Beckman Coulter, USA, in ICAT-Lisbon Faculty of Sciences Sequencing Services) by a dye-labeled dideoxy termination method (DTCS, Dye Terminator Cycle sequencer start kit, Beckman Coulter). Five sequencing reactions were performed using the two primers for PCR amplification and internal primers 534R (5′- ATT ACC GCG GCT GCT GG-3′), 907R (5′- CCG TCA ATT CMT TTR AGT TT-3′) and 926F (5′-AAA CTY AAA KGA ATT GAC GG-3′) (LANE 1991). For each strain, the partial 16S rDNA sequence was assembled by combining the sequences generated by each primer, using the CEQ Investigator program (software CEQ 8000, Beckman Coulter). The sequences were compared with National Centre for Biotechnology Information GenBank entries using BLAST algorithm (Altschul *et al.* 1990).

Adhesion substrata

The materials assayed were ASI 304 stainless steel (SS 304), ASI 316 stainless steel (SS 316), copper, glass, polyvinyl chloride (PVC), polypropylene (PP), polyethylene (PE) and silicone (Neves & Neves, Muro, Portugal). Some of these materials (SS 304, SS 316, PVC, PP, PE, copper) are commonly used in drinking water distribution networks, while other materials were used (glass, silicone) for comparative purposes (glass, silicone). In order to prepare the materials for further analysis, they were 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 materials were rinsed in ultrapure water and subsequently immersed in ethanol at 96% (v/v) for 30 min, except for PVC, PP, PE and silicone that were immersed for 10 s. After being rinsed three times with ultrapure water, the materials were dried at 65 °C for 3 h before being used in contact angle measurements and for adhesion assays.

Bacterial cell growth and preparation

Bacterial cells were grown in batch culture using TSB medium (Merck, VWR, Portugal), at room temperature (23 ± 2 °C), under agitation (150 rpm), until reaching the stationary phase of growth as assessed by spectrometry. Cells were harvested by centrifugation (10 min at 7000 rpm), washed three times in phosphate buffer saline (0.1 M PBS, pH 7.2) and resuspended in PBS (200 ± 10 ml) in order to achieve the bacterial concentration required for each assay.

Surface contact angle measurements

Bacterial lawns for contact angle measurements were prepared as described by Busscher *et al.* (1984). The surface tension of the bacterial surface and of the tested materials were then determined using the sessile drop contact angle method. The measurements were carried out at room temperature (23 ± 2 °C) 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 material) were performed. 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 $\Delta G_{1w1} < 0$ the material is considered hydrophobic. Conversely, if $\Delta G_{1w1} > 0$ 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 γ^- are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ^{AB}), with $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$.

The surface tension components of a solid material are obtained by measuring the contact angles of three pure liquids (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:

$$(1+\cos\theta)\gamma_1^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_1^{LW}} + \sqrt{\gamma_s^+\gamma_1^-} + \sqrt{\gamma_s^-\gamma_1^+}\right) \quad (2)$$

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

When studying the interaction between substances 1 and 2 that are immersed or dissolved in water (w), the total interaction energy, ΔG_{1w2}^{TOT}, can be expressed as:

$$\Delta G_{1w2}^{TOT} = \gamma_{12}^{LW} - \gamma_{1w}^{LW} - \gamma_{2w}^{LW} + 2\left[\sqrt{\gamma_w^+}\left(\sqrt{\gamma_1^-} + \sqrt{\gamma_2^-} - \sqrt{\gamma_w^-}\right) + \sqrt{\gamma_w^-}\left(\sqrt{\gamma_1^+} + \sqrt{\gamma_2^+} - \sqrt{\gamma_w^+}\right) - \sqrt{\gamma_1^+\gamma_2^-} - \sqrt{\gamma_1^-\gamma_2^+}\right] \quad (3)$$

Thermodynamically, if $\Delta G_{1w2}^{TOT} < 0$, adhesion is favorable. On the contrary, adhesion is not expected to occur if $\Delta G_{1w2}^{TOT} > 0$.

Adhesion assays

Adhesion assays were performed with 9 representative bacteria, respectively, *A. calcoaceticus*, *B. cepacia, Methylobacterium* sp., *M. mucogenicum, Sph. capsulata* sp. 1 and sp. 2, *Staphylococcus* sp., and *S. maltophilia* sp. 1 and sp. 2 using PE, PVC and SS 316 as representative adhesion surfaces. Coupons of materials 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 PBS), was added to each well. Adhesion to each material was allowed to occur for 2 h at room temperature, in a shaker at 150 rpm, according to the methods of Cerca (2006). Negative controls were obtained by placing materials in PBS without bacterial cells. The experiments were performed in triplicate and repeated three times. At the end of the assay each well was washed twice with PBS, by pipetting carefully only the liquid above the coupon. After the last wash, the coupons were removed from each well and immersed in a new microtiter plate containing 1 ml of methanol 98% (v/v) in each well (Henriques *et al.* 2005). Methanol was withdrawn after 15 min of contact and the coupons were allowed to dry at room temperature. Aliquots (600 μl) of crystal violet 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 optical density (OD) of the obtained solution was measured at 570 nm using a microtiter plate reader (BIO-TEK, Model Synergy HT).

Bacteria were classified using the scheme of Stepanović *et al.* (2000) as follows: Non-adherent (0): $OD \leq OD_c$; weakly adherent (+): $OD_c < OD \leq 2 \times OD_c$; moderately adherent (++): $2 \times OD_c < OD \leq 4 \times OD_c$; strongly adherent (+++): $4 \times OD_c < OD$. This classification was based upon the cut-off OD (OD_c) value defined as three standard deviation values above the mean OD of the negative control.

Statistical analysis

The data were analyzed 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 ≥95% ($P < 0.05$ was considered statistically significant).

Results and discussion

Characterisation of drinking water bacteria

In this study, 25 phenotypically distinct autochthonous drinking water bacteria were isolated (Table 1), belonging to 14 different bacterial species. Several of the isolates (*Acinetobacter* spp., *Burkholderia* spp., *Comamonas* spp., *Methylobacterium* spp., *Mycobacterium* spp., *Pseudomonas* spp., *Sphingomonas* spp., *Stenotrophomonas* spp.) have previously been detected in drinking water (Kuhn *et al.* 1997, Norton and LeChevallier 2000, Zanetti *et al.* 2000, Rickard *et al.* 2004, Stelma Jr. *et al.* 2004). The test organisms were analyzed in terms of hydrophobic surface characteristics. All the bacteria had a water contact angle lower than 65° and a $\Delta G_{bwb}^{TOT} > 0$ (Table 1), fitting the hydrophilic classification. According to Vogler (1998), surfaces that have a contact angle higher than 65° are classified as hydrophobic; conversely, hydrophilic surfaces are the ones with water contact angle values lower than 65°. However, based on the water contact angle approach, hydrophobicity can only be qualitatively analyzed (Oliveira *et al.* 2001). The application of the van Oss (1997) approach, which allows the assessment of the absolute degree of hydrophobicity of any substance in comparison with water as a quantitative result, seems to be more accurate than the assessment of the water contact angles, since it comprises the contact angle values of three different liquids (water, formamide and α-bromonaphtalene).

Comparisons of the surface characteristics of bacteria within the same species, detected the existence of significant inter-strain differences in water contact angles and ΔG_{bwb}^{TOT} values, although these were variations in the extent of hydrophilic properties rather than between hydrophilic and hydrophobic. These differences are more pronounced for the *Comamonas acidovorans* strains, where ΔG_{bwb}^{TOT} ranged from 26 to 115 mJ/m^2. Van der Mei *et al.* (1998), Teixeira *et al.* (2005) and Chae *et al.* (2006) also observed a large variation in the degree of hydrophobicity among strains of the same species, emphasizing that generalization concerning the surface properties of bacterial cells, based on their identity should be made with caution. The water contact angle measurements did not allow the grouping of strains according to their taxonomy as reported previously by van der Mei *et al.* (1998) and Teixeira *et al.* (2005).

Comparing the results obtained with those reported in the literature, *A. calcoaceticus* gave water contact angle values in agreement with those reported by van der Mei *et al.* (1998). Concerning the other test organisms, no previous reports were found concerning cell surface hydrophobicity characterization.

C. acidovorans sp. 1, sp. 2, *M. mucogenicum* and *Pseudomonas* sp., had formamide contact angles above 55°, meaning that the polar characteristics of the bacteria were different from the other tested bacteria. These bacteria, with exception of the *Pseudomonas* sp., were only electron donors and the acid base component of the surface free energy approaches zero (Bos *et al.* 1999). It is not surprising that cell surface properties of *M. mu-*

Table 1. Values of contact angle (in degrees) with water (θ_W), formamide (θ_F), α-bromonaphtalene (θ_B), surface tension parameters and free energy of interaction (ΔG_{bwb}^{TOT}) of the isolated microorganisms (b) when immersed in water (w). Values are means ± SDs.

Bacteria		Contact angle (°)			Surface tension parameters (mJ/m^2)			Hydrophobicity
		θ_W	θ_F	θ_B	γ_b^{LW}	γ_b^+	γ_b^-	ΔG_{bwb}^{TOT} (mJ/m^2)
Acinetobacter calcoaceticus		50.9 ± 2.6	45.5 ± 3.5	64.4 ± 2.4	22.8	3.1	31.2	7.0
Burkholderia sp.		35.8 ± 2.1	38.0 ± 1.5	66.6 ± 1.5	21.7	4.0	45.6	20.8
Burkholderia cepacia		22.8 ± 1.3	37.2 ± 4.5	54.2 ± 3.0	27.9	1.4	60.9	42.0
CDC gr. IV C-2	sp. 1	19.2 ± 1.6	39.2 ± 3.3	51.9 ± 4.7	29.0	0.8	66.6	51.0
	sp. 2	21.8 ± 3.2	34.8 ± 4.1	56.7 ± 2.4	26.7	2.0	59.5	38.2
Comamonas acidovorans	sp. 1	52.0 ± 3.6	100.2 ± 8.2	51.2 ± 2.6	29.4	0.0	117.1	115.4
	sp. 2	52.5 ± 4.4	57.8 ± 4.7	39.2 ± 2.6	35.0	0.0	42.1	26.0
	sp. 3	40.0 ± 2.0	50.0 ± 2.1	75.5 ± 2.0	17.4	3.0	51.2	27.5
	sp. 4	28.2 ± 2.3	38.9 ± 2.0	57.9 ± 1.4	26.0	1.8	56.5	36.4
Methylobacterium sp.		14.0 ± 3.6	25.9 ± 3.5	47.0 ± 3.3	31.4	1.8	58.9	37.1
Methylobacterium mesophilicum	sp. 1	23.1 ± 3.3	30.3 ± 3.2	34.6 ± 4.5	36.9	0.6	55.6	37.3
	sp. 2	26.6 ± 3.4	36.9 ± 3.7	46.3 ± 2.1	31.7	0.8	57.1	39.8
	sp. 3	16.8 ± 3.1	24.5 ± 2.9	48.4 ± 2.3	30.7	2.3	56.0	32.9
	sp. 4	35.0 ± 2.3	44.3 ± 3.3	54.9 ± 2.4	27.5	0.9	53.7	36.7
	sp. 5	16.8 ± 1.9	27.8 ± 1.7	48.4 ± 2.2	30.7	1.8	58.3	36.7
	sp. 6	30.7 ± 2.5	33.4 ± 1.5	55.8 ± 2.3	27.1	2.6	48.8	26.0
Moraxella lacunata		41.9 ± 2.5	44.7 ± 2.4	78.3 ± 1.9	16.1	5.5	42.6	15.2
Mycobacterium mucogenicum		37.4 ± 3.2	71.4 ± 2.9	62.5 ± 3.6	23.7	0.0	89.4	88.9
Pseudomonas sp.		56.0 ± 2.0	63.8 ± 2.5	88.4 ± 2.1	11.7	3.0	40.4	14.3
Pseudomonas reactans		28.0 ± 2.1	36.2 ± 2.4	63.3 ± 2.9	23.3	3.1	53.9	30.0
Sphingomonas capsulata	sp. 1	47.7 ± 2.6	47.1 ± 4.5	51.3 ± 7.9	29.3	0.8	38.0	17.5
	sp. 2	40.4 ± 1.9	46.5 ± 1.7	86.9 ± 1.7	12.3	7.1	46.0	13.8
Staphylococcus sp.		24.9 ± 1.9	22.9 ± 2.9	78.6 ± 1.4	15.9	10.6	46.2	11.6
Stenotrophomonas maltophilia	sp. 1	48.8 ± 1.8	47.0 ± 2.8	53.6 ± 3.2	28.2	1.1	36.2	14.7
	sp. 2	32.8 ± 3.0	32.0 ± 1.6	72.0 ± 2.1	19.0	6.8	44.1	15.4

cogenicum were considerably different from the other bacteria due to the presence of a waxy cell wall. The differences found between the *C. acidovorans* strains may be related to the different surface properties of the bacterial cell wall such as the ability to produce different exopolymeric substances (Li and Logan 2004) and surface proteins (Schär-Zammaretti *et al.* 2005).

The Lifshitz-van der Waals surface tension component of the microorganisms was comprised between 11.7 mJ/m^2 and 36.9 mJ/m^2, the lowest value being that of *Pseudomonas* sp. and the highest the one for *M. mesophilicum* sp.1. All the bacteria were predominantly electron donors (electron-donating component between 31.2 and 117.1 mJ/m^2) and, except *C. acidovorans* sp. 1, sp. 2 and *M. mucogenicum* (electron-accepting component –0 mJ/m^2), had the ability to accept electrons (electron-accepting component between 0.6 mJ/m^2 and 10.6 mJ/m^2). *Staphylococcus* sp. was one of the less hydrophilic bacteria and had the highest ability for accepting electrons. *A. calcoaceticus* was the less hydrophilic bacteria but had a very low electron donor capacity. *C. acidovorans* sp. 1 and *M. mucogenicum* were the more hydrophilic and were solely electron donors.

Characterization of colonisable materials

A range of materials were characterized in terms of surface properties (Table 2). The water contact angle values of all the materials analyzed were higher than 65° and $\Delta G_{sws}^{TOT} < 0$, meaning that all the surfaces analyzed were hydrophobic with an electron donating character. In agreement with previous studies, (Flint *et al.* 2000, Teixeira *et al.* 2005) energy interaction values revealed that glass was the least hydrophobic of the test materials, while the most hydrophobic were PE and silicone. The remaining materials had water contact angle values that ranged between 92° and 107°. It was found that for SS 316 and SS 304 the water contact angle values (93° and 101°, respectively) were slightly higher than those reported in other studies (Flint *et al.* 2000, Teixeira *et al.* 2005), with a difference of approximately 10° for SS 316 and 15° for SS 304. Such differences could arguably be related to variations in surface finishing or the cleaning treatment. In accordance with the work performed by Sinde and Carballo (2000), it was found that SS 304 was more hydrophobic than SS 316, according to both the water contact angle and by the ΔG_{sws}^{TOT} value.

Table 2. Contact angle (in degrees), with water (θ_W), formamide (θ_F), α-bromonaphtalene (θ_B), surface tension parameters and free energy of interaction (ΔG_{sws}^{TOT}) of the support materials (s) when immersed in water (w). Values are means ± SDs.

Support Material	Contact angle (°)			Surface tension parameters (mJ/m^2)			Hydrophobicity
	θ_W	θ_F	θ_B	γ_b^{LW}	γ_b^+	γ_b^-	ΔG_{sws}^{TOT} (mJ/m^2)
SS 316	92.9 ± 1.8	80.0 ± 1.9	51.0 ± 2.7	29.5	0.0	5.7	−55.1
SS 304	101 ± 2.0	84.4 ± 2.0	46.5 ± 1.3	31.7	0.0	2.6	−71.5
Copper	98.6 ± 3.5	77.7 ± 2.2	45.4 ± 2.0	32.1	0.0	1.5	−79.6
PVC	95.4 ± 2.9	84.0 ± 1.6	41.6 ± 2.0	33.9	0.0	5.8	−55.9
PP	107 ± 3.0	91.8 ± 2.4	53.2 ± 2.3	28.4	0.0	1.7	−76.6
PE	102 ± 2.4	78.0 ± 2.8	35.8 ± 1.8	36.4	0.0	0.6	−90.3
Silicone	122 ± 1.8	112 ± 1.1	86.6 ± 1.9	12.4	0.0	0.9	−85.6
Glass	73.5 ± 3.1	68.9 ± 3.1	50.8 ± 1.6	29.6	0.0	20	−13.8

Prediction of adhesion

In order to predict the ability of the microorganisms to adhere to surfaces, the free energy of interaction between the isolated microorganisms and the materials, when immersed in water, was calculated (Table 3) according to the approach of van Oss *et al.* (1987, 1988, 1989). Based on this approach, *C. acidovorans* sp. 1, *M. mucogenicum* and *Staphylococcus* sp. had no theoretical thermodynamic ability to adhere to the test materials, whilst adhesion was thermodynamically favorable at least to one material for the other bacteria. Comparing the thermodynamic ability for adhesion between test materials, it is noticeable that the adhesion is thermodynamically less favorable for glass due to its hydrophobic properties. Teixeira *et al.* (2005), also conclude that glass was a thermodynamically less favorable substrate for bacterial adhesion using a variety of dairy isolates in conjunction with materials commonly used in a dairy industry.

Adhesion to SS 316 and PVC, were thermodynamically favorable for six bacteria, SS 304 for 18 bacteria, copper, PP and PE for 21 bacteria, whilst silicone supported the theoretical adhesion of 22 bacteria. These data demonstrate that adhesion is dependent on the

Table 3. Free energy of adhesion (ΔG_{bws}^{TOT}) between the isolated microorganisms (b) and the different support materials (s) when immersed in water (w).

Bacteria		ΔG_{bws}^{TOT} (mJ/m^2)							
		SS 316	SS 304	Copper	PVC	PP	PE	Silicone	Glass
Acinetobacter calcoaceticus		−12.3	−17.5	−20.0	−12.2	−19.4	−23.1	−21.4	1.2
Burkholderia sp.		1.0	−3.8	−6.2	1.2	−5.6	−8.9	−7.9	13.5
Burkholderia cepacia		6.1	−0.1	−3.2	5.9	−2.1	−7.1	−2.7	22.0
CDC gr. IV C-2	sp. 1	8.0	1.3	−2.1	7.8	−0.8	−6.3	−1.2	25.1
	sp. 2	6.8	0.9	−2.0	6.6	−1.0	−5.6	−1.8	21.6
Comamonas acidovorans	sp. 1	30.2	22.0	18.0	29.9	19.4	12.9	18.5	50.8
	sp. 2	−14.3	−22.6	−26.7	−14.9	−24.9	−32.1	−24.1	6.4
	sp. 3	4.2	−0.7	−3.3	4.9	−3.0	−5.9	−7.2	17.9
	sp. 4	4.4	−1.5	−4.5	4.4	−3.5	−8.1	−4.6	19.6
Methylobacterium sp.		5.3	−0.8	−3.8	4.9	−2.5	−7.8	−1.8	20.5
Methylobacterium mesophilicum	sp. 1	−0.7	−7.9	−11.4	−1.5	−9.6	−16.2	−7.6	16.8
	sp. 2	1.6	−5.2	−8.6	1.2	−7.1	−13.0	−6.7	18.6
	sp. 3	4.3	−1.6	−4.4	3.9	−3.2	−8.2	−2.6	18.8
	sp. 4	0.2	−6.4	−9.7	0.0	−8.6	−13.8	−9.5	17.0
	sp. 5	5.0	−1.1	−4.1	4.6	−2.8	−8.0	−2.3	20.1
	sp. 6	0.4	−5.2	−7.9	0.2	−6.9	−11.4	−7.5	14.4
Moraxella lacunata		1.5	−2.5	−4.5	2.2	−4.5	−6.5	−8.8	12.6
Mycobacterium mucogenicum		17.2	9.3	5.3	17.4	6.4	0.7	3.5	37.9
Pseudomonas sp.		−2.7	−7.4	−9.9	−1.5	−10.1	−11.9	−17.0	11.0
Pseudomonas reactans		5.4	0.2	−2.3	5.5	−1.6	−5.4	−3.4	18.8
Sphingomonas capsulata	sp. 1	−12.1	−18.8	−22.1	−12.3	−20.9	−26.4	−21.2	5.0
	sp. 2	6.6	3.3	1.6	7.7	1.2	0.3	−4.7	16.3
Staphylococcus sp.		9.1	6.6	5.3	9.8	5.2	4.2	1.4	16.5
Stenotrophomonas maltophilia	sp. 1	−12.6	−19.1	−22.3	−12.9	−21.2	−26.4	−21.7	3.8
	sp. 2	3.5	−0.2	−2.1	3.9	−1.8	−4.0	−4.7	13.5

physicochemical properties of the bacterial surface and of the materials. Comparing the predicted adhesion (Table 3) with the surface characteristics of the materials (Table 2), it can be seen that adhesion is favored as the surface hydrophobicity increases (linear correlation $-R^2 > 0.9$). Additionally, adhesion was favored when both surfaces are hydrophobic. This result is in agreement with other reports (Busscher *et al.* 1990, Erner and Douglas 1992, Flint *et al.* 1997, Panagoda *et al.* 1998, Chen and Strevett 2001, Chavant *et al.* 2002, Chen and Zhu 2005). These investigations demonstrated a relationship between the surface properties and the extent of adhesion to solid materials. The current investigation showed that, based on surface tension parameters values, smaller γ_b^- values of both bacteria and surfaces lead to a thermodynamically favored adhesion (Tables 1–3). Considering the surface tension properties (Tables 1 and 2), both bacteria and support materials are electron donors. However, no apparent relationship between other surface tension parameters and thermodynamic adhesion was evident ($R^2 < 0.9$).

Adhesion to materials

Adhesion assays were performed with representative test bacteria and material surfaces, using a modified microtiter-plate assay methodology (Stepanović *et al.* 2000). The bacteria used were *A. calcoaceticus*, *B. cepacia*, *Methylobacterium* sp., *M. mucogenicum*, *Sph. capsulata*, *Staphylococcus* sp., *S. maltophilia* and the control materials tested were SS 316, PVC and PE (Fig. 1). Strain variation on adherence ability was assessed by using two distinct strains of *Sph. capsulata* (sp. 1 and sp. 2) and *S. maltophilia* (sp. 1 and sp. 2). Figure 1 shows that all tested bacteria adhered to the three distinct surfaces with different adhesion potentials. The tested bacteria adhered in a significant higher extent to PE ($P < 0.05$), with the exception of *Methylobacterium* sp. which adhered most strongly to SS 316) and *Staphylococcus* sp. (SS 316 and PVC). Furthermore, SS 316 was the material displaying the smaller number of adhered cells ($P < 0.05$), except for *Methylobacterium* sp. and *Staphylococcus* sp. Also, the adherence ability of *Sph. capsulata* sp. 1 to SS 316 and PVC, *M. mucogenicum* to SS 316 and *Methylobacterium* sp. to PE was almost negligible. *A. calcoaceticus* had the highest ability to adhere to the three control materials, while *Sph. capsulata* sp. 1 was the bacterium with the lowest adherence ability. Comparing adhesion ability within the same species, a significant difference ($P < 0.05$) was detected for *Sph. capsulata* strains, whilst for *S. maltophilia* strains the differences were smaller ($P > 0.05$), although *S. maltophilia* sp. 1 possessed higher ability to adhere to the three control materials.

A rank of adherence was produced according to Stepanović *et al.* (2000), classifying test bacteria as non-adherent, weakly adherent, moderately adherent and strongly adherent bacteria (Table 4). *A. calcoaceticus* was the only strongly adherent microorganism to PVC and PE. Moderate adherence was detected for *A. calcoaceticus* to SS 316, *Staphylococcus* sp. to PVC and *S. maltophilia* sp. 1 to PE. Weak adherence was observed for *Methylobacterium* sp. and *Staphylococcus* sp. to SS 316, *S. maltophilia* sp. 1 and *Sph. capsulata* sp. 2 to PVC, *B. cepacia*, *M. mucogenicum*, *Sph. capsulata* sp. 2, *Staphylococcus* sp. and *S. maltophilia* sp. 2 to PE. The remaining bacterial/material situations analyzed demonstrated that bacteria were non-adherent to the three materials. The use of distinct *S. maltophilia* and *Sph. capsulata* strains showed the existence of varying ability of adherence for the distinct strains, suggesting that individual strains are not reliable predictive paradigms (Fux *et al.* 2005).

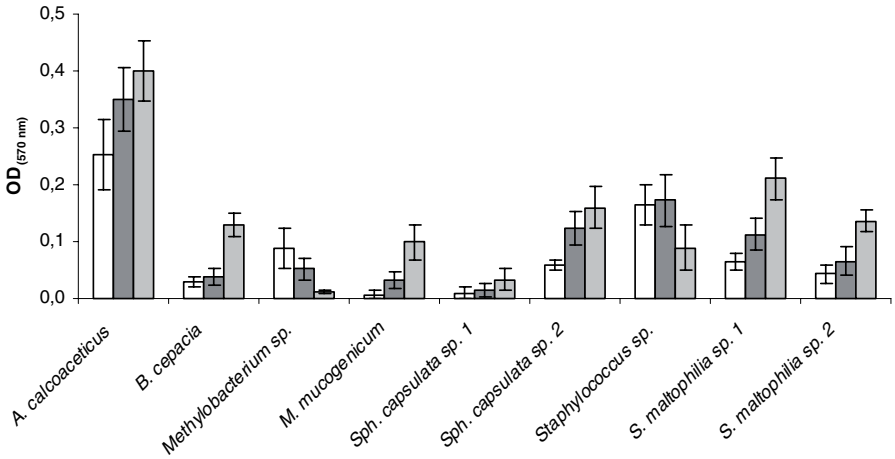

Figure 1. Values of $OD_{570\ nm}$ as a measure of adhesion of representative bacteria isolated from drinking water to SS 316 (□), PVC (■) and PE (▣). The means ± SD for at least three replicates are illustrated.

Table 4. Adhesion ability of representative bacteria isolated from drinking water to SS 316, PVC and PE, according to the adherence classification proposed by Stepanović *et al.* (2000): (0) non-adherent; (+) weakly adherent; (++) moderately adherent; (+++) strongly adherent.

Bacteria		Adhesion surfaces		
		SS 316	PVC	PE
Acinetobacter calcoaceticus		++	+++	+++
Burkholderia cepacia		0	0	+
Methylobacterium sp.		+	0	0
Mycobacterium mucogenicum		0	0	+
Sphingomonas capsulata	sp. 1	0	0	0
	sp. 2	0	+	+
Staphylococcus sp.		+	++	+
Stenotrophomonas maltophilia	sp. 1	0	+	++
	sp. 2	0	0	+

Comparison between thermodynamic prediction and adhesion assay

Comparison between the theoretical thermodynamic prediction of adhesion (Table 3) and laboratory adhesion assays (Fig. 1) shown that adhesion is underestimated when based on thermodynamic approaches. In fact, the results were only in agreement for *A. calcoaceticus*, *B. cepacia* and *S. maltophilia* sp. 2 (Table 4). No agreement between thermodynamic approaches and the adhesion assays was obtained for *Sph. capsulata* sp. 1 and *Staphylococcus* sp. A similar trend between thermodynamic prevision and adhesion results was found for *M. mucogenicum* when assayed with SS 316 and PVC, *S. maltophilia* sp. 1 when assayed with PVC and PE, *Methylobacterium* sp. when assayed with PVC and *Sph. capsulata* sp. 2 when assayed with SS 316.

The apparent agreement between thermodynamic and adhesion results detected for *A. calcoaceticus*, *B. cepacia* and *S. maltophilia* sp. 2, suggests the existence of a strong influence of surface physicochemical properties on bacterial adhesion for the referred bacteria. Slight correlation was found between the bacterial surface properties and the adhesion results ($R^2 < 0.85$). For all the bacteria, the extent of adhesion correlated with all surface properties of the materials ($R^2 \geq 0.85$), verifying that adhesion increases with the decrease of γ_s^{LW} and with the increase of γ_s^- and hydrophobicity (ΔG_{sws}^{TOT}) of the materials, with the exception of *Methylobacterium* sp. and *Staphylococcus* sp. Several previous studies have reported the lack of a correlation between hydrophobicity of the bacteria and bacterial attachment; the attachment process was strongly influenced by the presence of extracellular molecules (Li and Logan 2004, Chae *et al.* 2006). Sardin *et al.* (2004), however, reported a correlation between bacterial adherence with the non-polar surface tension component of the materials and with bacterial hydrophobicity. In the current study, the lack of agreement between thermodynamic approaches and adhesion assays reinforces that biological mechanisms, such as the expression of adhesins that mediate specific interactions with substrata at a nanometer scale (during the irreversible phase of microbial adhesion) in addition to the physicochemical ones, mediate the entire microbial adhesion process (Flint *et al.* 1997, Doyle 2000, Sinde and Carballo 2000).

In conclusion, whilst the prediction of adhesion potential on the basis of physicochemical properties gives useful information about the possible real-life microbial behavior, adhesion results suggest that mechanisms other than cellular physicochemical surface properties may play a determinant role on bacterial adherence ability. These will include microbial flagella, pili or fimbrae, prothecae and production of extracellular polymeric substances (Flint *et al.* 1997, Doyle 2000, Sinde and Carballo 2000, Donlan 2002, Simões 2005). Prediction based on surface physicochemical properties and thermodynamic approaches therefore did not provide conclusive results. Furthermore, because multispecies interactions prevail in the environment, strongly adherent bacteria may play a determinant role in the primary colonization of surfaces, seeding biofilms which will then develop by cellular proliferation and immigration.

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