Adhesion potential of bacteria isolated from tap water to several materials using a modified microtiter-plate test

[1]Lúcia C. Simões, [1]M. Simões and [1]Maria J. Vieira

[1]Centro de Engenharia Biológica – CEB, Universidade do Minho, 4710-057 Braga, Portugal
(luciachaves@deb.uminho.pt; mjvsimoes@deb.uminho.pt; mjv@deb.uminho.pt)

Abstract

Autochthonous heterotrophic aerobic bacteria from drinking water were isolated, identified by growth on selective media, biochemical tests and 16S rDNA gene sequence. From 25 different isolated bacteria, 8 representative bacteria were selected in order to test their adhesion ability to four different support materials. The bacteria selected were *Acinetobacter calcoaceticus*, *Burkholderia cepacia*, *Methylobacterium* sp., *Mycobacterium mucogenicum*, *Sphingomonas capsulata*, *Staphylococcus* sp., *Stenotrophomonas maltophilia* and the materials used for adhesion were stainless steel ASI 316 (SS), polyethylene (PE), polyvinyl chloride (PVC) and polystyrene (PS). 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). The adhesion assays were performed during 2 h using a modified microtiter-plate test. The results obtained revealed that the bacteria adhered in a higher extent to PE ($P < 0.05$), with the exception of *Methylobacterium* sp. (higher colonization of SS) and *Staphylococcus* sp. (higher colonization of PS). Strong and moderate adherence were detected for *A. calcoacticus* and *Staphylococcus* sp. - adhered to the four materials used, while only *Sph. capsulata* sp. 1 was non-adherent to the tested materials. Furthermore, it is expected that some of the strong and moderately (*A. calcoaceticus*, *Staphylococcus* sp. and *S. maltophilia* sp. 1) adherent bacteria will play a determinant role in the primary colonization of the surfaces. The use of distinct *S. maltophilia* and *Sph. capsulata* strains showed the existence of varying ability of adherence for the distinct strains, demonstrating that no strain can effectively represent its species.

This study provides information about a rapid and reliable methodology for bacteria adherence ability assessment and gives useful clues about the behaviour of drinking water autochthonous bacteria when exposed to potential adhesion surfaces.

Keywords: Autochthonous microflora; Bacterial adhesion; Drinking water; Modified microtiter-plate test; Strain variation

Introduction

Bacterial adhesion to material surfaces is an important aspect in a wide number of areas, such as biomedical, environmental and industrial (Bayoudh *et al.*, 2005), as it is the first step on the biofilm formation process. In drinking water distribution systems, microbial adhesion may lead to the contamination of the water resources and biofilm formation. Biofilms reduce the aesthetic quality of distribution water, increasing the corrosion rate of pipes, and favouring the survival of pathogenic microorganisms (Percival and Walker, 1999; Niquette *et al.*, 2000).

Many current technological systems require a better understanding of the factors controlling biofilm formation or, more specifically, the initial attachment process (Medilansky *et al.*, 2003). For example, several attempts within many industries tried to create a non-stick surface to prevent microbial attachment and consequent biofilm

formation (Tang *et al.,* 2005). In many technical processes microorganisms are not a problem as long as they remain in planktonic state, since disinfection procedures are often more efficient against planktonic cells then when they are adhered to surfaces (Simões *et al.,* 2005; 2006b). So, the evaluation of the ability of microorganisms to adhere and form biofilms is a subject that needs adequate research, since measurements online are often impracticable, as found out in a complex drinking water network (Simões *et al.,* 2006a). Thus, if we are to find means to control biofilms, more knowledge about the environmental factors and microbial pioneers that influence and improve biofilm formation is needed. In fact, the initial microbial adhesion to surfaces is a complicated and multivariable process that needs reliable research (Stepanović *et al.,* 2000).
The purpose of the present study was to evaluate, by using a rapid and reliable adhesion assay, the adhesion potential of drinking water autochthonous bacteria to materials with potential application in drinking water distribution networks and devices.

Materials and Methods

Bacteria isolation and growth

The microorganisms used throughout this work were isolated from a laboratorial drinking water distribution system, as described by Simões *et al.* (2006a), consisting in a 1.6 L Perspex vessel (diameter = 16.8 cm) fed with tap water from a drinking water distribution system in the North of Portugal. The system was maintained at aseptic conditions and worked at a dilution rate of 3.125 h^{-1}. The cells were isolated in the planktonic state. Aerobic, heterotrophic bacteria were selected for further work by culture plating in trypticase soy agar - TSA (Merck) and R2A (Oxoid) (both media tested successfully in the recovery of heterotrophic bacteria from drinking water) at room temperature for 15 d.

Bacterial identification

Bacteria identification was carried out by selective medium Chromocult® TBX agar (Merck); *Pseudomonas* isolation agar (Difco); Metanol minimum medium, according to Kim *et al.* (1999) - Gram staining; 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 *Acinetobacter* sp., *Burkolderia* spp., *Methylobacterium* spp., *Sphingomonas* spp. and *Stenotrophomonas* spp.

Materials

The materials assayed were ASI 316 stainless steel (SS), polyethylene (PE), polyvinyl chloride (PVC), and polystyrene (PS). Some of these materials are commonly used in drinking water distribution networks, while other materials were used with comparative purposes. 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 detergent remaining the materials were rinsed in ultrapure water and subsequently immersed in ethanol at 96 % (v/v) during 30 min, except for PE and PVC that were only immersed for 10 s. After rinsed with ultrapure water, the materials were dried at 65 ºC for 3 h before being used for adhesion assays.

Bacterial cell growth and preparation

Bacterial cells were cultured under batch conditions on TSB medium (Merck), at room temperature (23 ± 2 ºC) and under agitation (150 rpm), until reaching stationary phase.

Cells were harvested by centrifugation (10 min at 7000 rpm), washed three times in phosphate buffer saline (PBS) and resuspended in a certain volume of PBS necessary to achieve the bacterial concentration required for adhesion assays.

Adhesion assays with modified microtiter-plate test

Adhesion assays were performed with the bacteria of main biofilm formation impact, denominated as 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. Coupons of the four distinct 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), were added to each well. Each bacteria and material were tested using independent microtiter-plates. Adhesion to each material was allowed to occur for 2 h at room temperature, in a shaker at 150 rpm, according to Cerca *et al.* (2004). 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 pipeting carefully only the liquid above the coupon. After the last washing, the coupons were removed from each well and immersed in a new microtiter-plate containing 1 ml of methanol 98 % (Vaz Pereira, Portugal) in each well, based on the procedure described by Henriques *et al.* (2005). Methanol was withdrawn after 15 min of contact and the coupons were allowed to dry at room temperature. Aliquots (600µl) of crystal violet (Merck) 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 plates reader (BIO-TEK, Model Synergy HT) and the correspondent value provided the amount of adhered cells.

Bacteria were classified as follows (Stepanović *et al.*, 2000): 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 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 ≥ 95 % ($P < 0.05$ was considered statistically significant).

Results and Discussion

A total of 25 phenotypically distinct drinking water autochthonous bacteria were isolated, belonging to 13 different bacterial species and to 2 strains of the group IV class II of the CDC. From the bacteria isolated, were selected 8 representative bacteria based on their potential impact in biofilm formation events, respectively *A. calcoaceticus*, *B. cepacia*, *Methylobacterium* sp., *M. mucogenicum*, *Sph. capsulata*, *Staphylococcus* sp., *S. maltophilia.* 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). The materials tested were SS 316, PE, PVC and PS. Several of the isolated bacteria (*Acinetobacter* spp., *Burkholderia* spp., *Methylobacterium* spp., *Mycobacterium* spp.,

Sphingomonas spp., *Stenotrophomonas* spp.) were already detected in drinking water (Norton and LeChevallier, 2000; Rickard *et al.,* 2004). Adhesion assays were performed using the modified microtiter-plate methodology. This approach for assessment of microbial adhesion is simple and easy to perform providing reliable results (Stepanović *et al.,* 2000). Fig. 1 shows the adhesion results of the several isolated bacteria when exposed to the four adhesion materials.

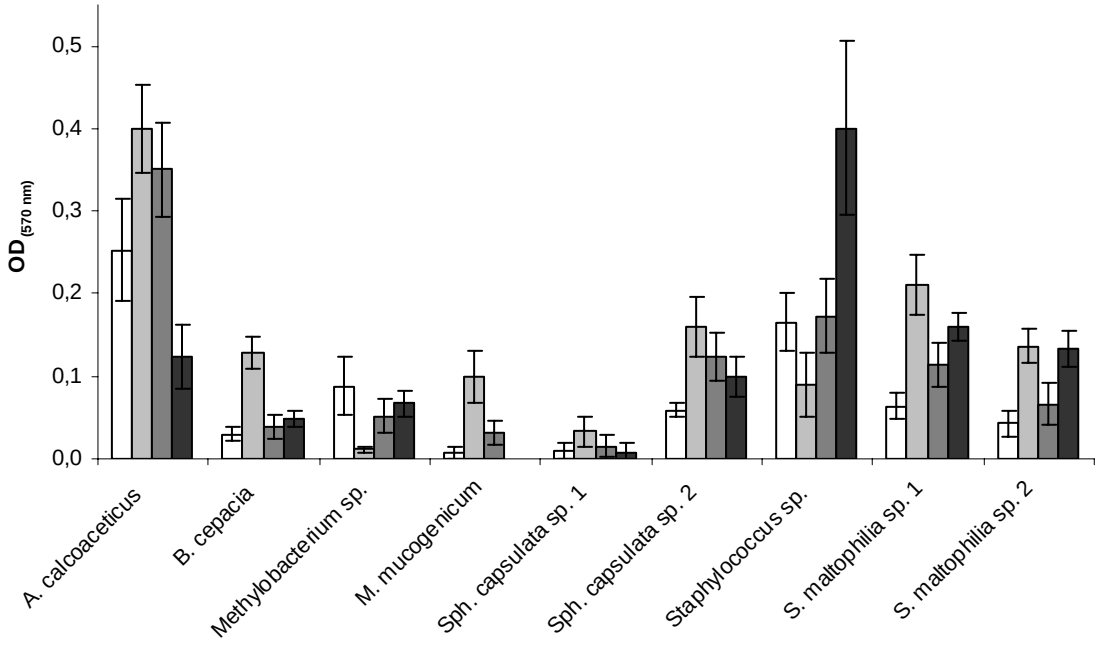

Fig. 1 Values of OD $_{(570\ nm)}$ as a measure of adhesion of representative bacteria isolated from drinking water to SS (□), PE (▫), PVC (▪) and PS (■). The means ± SDs for at least three replicates are illustrated.

Fig. 1 shows that almost all tested bacteria adhered to the four distinct surfaces with different adhesion potential. The bacteria adhered in a significant higher extent to PE ($P < 0.05$), with the exception of *Methylobacterium* sp. (higher extent of adhesion to SS) and *Staphylococcus* sp. (higher colonization of PS). Furthermore, analyzing the adhesion to the four surfaces, with the exception of *Methylobacterium* sp. and *Staphylococcus* sp., a lower adhesion ability was detected for SS 316 ($P < 0.05$) by *B. cepacia*, *Sph. capsulata* sp. 2, *S. maltophilia* sp. 1 and sp. 2 and for PS by *A. calcoaceticus*, *M. mucogenicum* and *Sph. capsulata* sp. 1. Also, the adherence ability of *Sph. capsulata* sp. 1 to SS, PVC and PS, *M. mucogenicum* to SS and PS and *Methylobacterium* sp. to PE was almost negligible. *A. calcoaceticus* had the highest ability to adhere to the four materials, while *Sph. capsulata* sp. 1 was the bacteria with the lowest adherence ability. Comparing adhesion ability within the same species, a significant difference ($P < 0.05$) was detected for *Sph. capsulata* strains. Regarding the *S. maltophilia* strains the differences are not so evident ($P > 0.05$), even with *S. maltophilia* sp. 1 having a higher ability to adhere to the four surfaces.
A rank of adherence was assessed according to Stepanović *et al.* (2000), showing the existence of non-adherent, weakly adherent, moderately adherent and strongly adherent bacteria (Table 1).

Table 1 Adhesion ability of the isolated bacteria to SS, PE, PVC and PS, according to the adherence classification proposed by Stepanović *et al.* (2000): (0) non-adherent; (+) weakly adherent; (++) moderately adherent; (+++) strongly adherent

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

A. calcoaceticus demonstrated to be the only strongly adherent microorganism, this property being expressed when exposed to PVC and PE. Moderated adherence was detected for *A. calcoaceticus* to SS, *Staphylococcus* sp. to PVC and PS and *S. maltophilia* sp. 1 to PE. Weak adherence was found for *Methylobacterium* sp. and *Staphylococcus* sp to SS, *B. cepacia*, M. mucogenicum, *Sph. capsulata* sp. 2, *Staphylococcus* sp. and *S. maltophilia* sp. 2 to PE, *Sph. capsulata* sp. 2 and *S. maltophilia* sp. 1 to PVC and *A. calcoaceticus*, *S. maltophilia* sp. 1 and sp. 2 to PS. The remaining situations analyzed demonstrated that the bacteria were non-adherent to the four materials. These results reinforces, by using of distinct *S. maltophilia* and *Sph. capsulata* strains, the varying ability of adherence for the distinct strains, demonstrating that no strain can effectively represent its species (Fux *et al.*, 2005). This study also demonstrates that some bacteria adhered similarly on PS and the other tested materials, thus, the modified microtiter-plate assay seems to be a convenient tool for evaluation of bacterial adhesion potential of drinking water autochthonous bacteria.

Conclusions

This study provides information about a reliable methodology for bacteria adherence ability assessment and gives useful clues about the behaviour of microorganisms when exposed to a potential adhesion surface. From the autochthonous microflora used, the extreme adherence situations were detected for *A. calcoacticus* and *Staphylococcus* sp. - adhered to the four materials used, while only *Sph. capsulata* sp. 1 was non-adherent. Furthermore, since multi-species interactions and coexistence prevail in a real process, probably some of the strong and moderately (*A. calcoaceticus*, *Staphylococcus* sp. and *S. maltophilia* sp. 1) adherent bacteria will play a determinant role in the primary colonization of the surfaces and will work as biofilm pioneers and bridging bacteria for the other microorganisms, as found out in previous coaggregation studies (results not shown).

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