

***Acinetobacter calcoaceticus* plays a bridging function in drinking water biofilms**

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ABSTRACT

Intergeneric coaggregation of six drinking water autochthonous heterotrophic bacteria isolated from a model laboratory system were tested for their ability to coaggregate by a visual assay and by two microscopic techniques (epifluorescence and scanning electron microscopies). One isolate, identified as *Acinetobacter calcoaceticus*, was found not only to autoaggregate, but also to coaggregate with four of the five other isolates (*Burkholderia cepacia*, *Methylobacterium sp.*, *Mycobacterium mucogenicum*, *Sphingomonas capsulata* and *Staphylococcus sp.*) to different degrees as assessed by the visual assay, highlighting a possible bridging function in a biofilm consortium. In its absence, no coaggregation was found. Microscopic observations revealed a higher degree of interaction for all the aggregates than did the visual assay. Heat and protease reversed autoaggregation and coaggregation, suggesting that interactions were lectin-saccharide mediated. The increase/decrease in the level of extracellular proteins and polysaccharides produced during intergeneric bacteria association was not correlated with coaggregation occurrence, but probably with coaggregation strength. The bridging function of *A. calcoaceticus* was evidenced by multispecies biofilm studies through a strain exclusion process.

RESUMO

Este trabalho investiga a co-agregação intergenérica de seis bactérias heterotróficas autóctones de água potável isoladas de um sistema laboratorial modelo, testando assim a sua capacidade de co-agregação através do ensaio visual e de duas técnicas microscópicas (microscopia de epifluorescência e microscopia electrónica de varrimento). Para o isolado identificado como *Acinetobacter calcoaceticus*, foi detectado através do ensaio visual que não só auto-agrega, mas também co-agrega, a diferentes intensidades, com quatro dos outros cinco isolados (*Burkholderia cepacia*, *Methylobacterium sp.*, *Mycobacterium mucogenicum*, *Sphingomonas capsulata* and *Staphylococcus sp.*), realçando a possível função de ligação em biofilmes multi-espécie. Na sua ausência não foi detectada co-agregação. As observações microscópicas revelaram um maior grau de interacção para todos os agregados do que a detectada pelo ensaio visual. O tratamento com calor e protease reverteram a auto-agregação e a co-agregação, sugerindo que as interacções são mediadas por lectinas-açúcares. O aumento/diminuição no nível de proteínas e polissacarídeos extracelulares produzidos durante os fenómenos de co-agregação não estão relacionados com a sua ocorrência, mas provavelmente com a sua força de interacção. A função de ligação da *A. calcoaceticus* nos consórcios microbianos foi evidenciada pela formação de biofilmes multi-espécie, através de um processo de exclusão bacteriana.

Keywords: Autochthonous microflora; Bridging bacteria; Coaggregation; Drinking water; Multispecies biofilm

INTRODUCTION

Biofilm formation in drinking water distribution systems improves drinking water contamination by reducing the microbiological safety through the increased survival of pathogens [1, 2, 3]. The knowledge of the main mechanisms promoting drinking water biofilm formation is of great interest as it can contribute to their understanding and control.

The development of microbial biofilm communities results from a series of processes including: initial surface association and adherence, subsequent multiplication of the constituent organisms, adherence of additional species and production of extracellular polymeric substances [4]. Many of these processes leading to biofilm development, such as primary colonization, the production of extracellular polymeric substances and gross phenotypic changes, are well described [5, 6]. The bacterial surface properties, coaggregation and coadhesion of bacteria and interspecies relationships are processes that are believed to play a determinant role in the formation of single and multispecies biofilms in drinking water distribution

systems [7]. Nevertheless, the function of coaggregation in the initial development of biofilm communities still remains unclear.

Coaggregation, the specific recognition and adherence of genetically distinct bacteria to one another, occurs in a variety of ecosystems [7, 8] and was first demonstrated from dental plaque bacteria [9]. This mechanism of adhesion is highly specific being recognized as a mechanism that allows specific association between collaborating bacteria species [10, 11, 12]. Aggregation conveys advantages to microorganisms. These include transfer of chemical signals, exchange of genetic information, protection from adverse environmental conditions, metabolic cooperation between different species, as well as cell differentiation in some populations [13]. The coaggregation between pairs of bacteria is typically mediated by a protein “adhesin” on one cell type and a complementary saccharide “receptor” on the other. These protein-saccharide interactions could be blocked by the addition of simple sugars [14, 15]. Coaggregation interactions contribute to the development of biofilms by two routes. The first route is by single cells in suspension specifically recognizing and adhering to genetically distinct cells in the developing biofilm. The second is by the prior coaggregation in suspension of secondary colonizers followed by the subsequent adhesion of this coaggregate to the developing biofilm [12]. In both cases, bacterial cells in suspension specifically adhere to biofilm cells in a coadhesion process [16].

The purpose of the present study was to study the intergeneric coaggregation of six heterotrophic bacteria isolated from a laboratorial drinking water distribution system by visual coaggregation detection, epifluorescence microscopy and scanning electron microscopy (SEM). Extracellular proteins and polysaccharides (EPS) were assessed over time and correlated with coaggregation ability. The surface-associated molecules involved in the coaggregation process were investigated by heat and protease treatment, and by sugar reversal tests. The role of the isolated *A. calcoaceticus* strain as bridging organism in drinking water biofilms was assessed by multispecies biofilms experiments, through a strain exclusion process.

MATERIALS and METHODS

Bacteria isolation and identification

The microorganisms used throughout this work were isolated from a model laboratory drinking water distribution system, as described previously by Simões *et al.* [3]. Trypticase soy agar – TSA (Merck, VWR, Portugal) and R2A (Oxoid, UK) were used for heterotrophic drinking water bacteria recovery and growth.

Bacteria were identified by 16S rRNA gene sequencing according to the method described by Simões *et al.* [17].

Culture conditions and preparation of cell suspensions

The assays were performed with 6 representative drinking water isolated bacteria, *Acinetobacter calcoaceticus*, *Burkholderia cepacia*, *Methylobacterium sp.*, *Mycobacterium mucogenicum*, *Sphingomonas capsulata* and *Staphylococcus sp.*, respectively.

Cells were grown in batch culture using 200 mL of R2A broth in 500 mL glass flasks (Schott, Duran), at room temperature ($23\text{ }^{\circ}\text{C} \pm 2$), under agitation (150 rpm), until reaching the stationary growth phase as assessed by spectrometry (Spectronic 20 Genesys, Spectronic Instruments) at 640 nm. The stationary phase of growth was selected because numerous works [7, 12, 18, 19] refereed that coaggregation is growth-phase-dependent, being maximum when both partner bacteria are in stationary phase. Cells were harvested by centrifugation (20 min at 13000 g), washed three times in sterile tap water and resuspended in a certain volume of sterile tap water or R2A broth necessary to achieve the bacterial concentration needed for each assay.

Visual coaggregation assay

A visual coaggregation assay, with some modifications from the method of Cisar *et al.* [14], was used to assess the ability of bacteria to coaggregate. Bacterial suspensions prepared as described above were resuspended in sterile tap water to an optical density at 640 nm of 1.5 and mixed together in pair by putting equal volumes (2 mL) of each cell suspension at room temperature in 10 mL roll glass tubes. The mixtures were then vortexed for 10 s, and the tubes were rolled gently for 30 s. The degree of coaggregation between each pair was assessed visually in a semiquantitative assay with the scoring scheme originally described by Cisar *et al.* [14]. If specific cell-to-cell recognition occurs, the cells flocculate (coaggregate) together and settle out. The scoring criteria were as follows: 0, no visible coaggregates in the cell suspension; 1, very small uniform coaggregates in a turbid suspension; 2, easily visible small coaggregates in a turbid suspension; 3, clearly visible coaggregates which settle, leaving a clear supernatant; 4, very large flocs of coaggregates that settle almost instantaneously, leaving a clear supernatant. Control tubes of each isolate on their own were also included to assess autoaggregation and

scored by the same criteria. The coaggregation and autoaggregation scores were evaluated over time (0, 2, 24 and 48 h), staying this mixtures, during this period, at room temperature. Coaggregation was considered to be present when the score in the reaction mixtures was greater than the autoaggregation score of either strain.

Microscopy visualizations

Bacterial coaggregates were observed (2 and 24 h) by epifluorescence microscopy using a DNA binding stain, 4,6-diamino-2-phenylindole (DAPI; Sigma, Portugal), and by scanning electron microscopy (SEM). For epifluorescence microscopy visualizations, aliquots (15 μ L) of bacterial autoaggregates and coaggregates were fixed using 2 % (v/v) formaldehyde (Merck, Germany) and then filtrated through a 25 mm black Nuclepore® polycarbonate membrane with a pore size of 0.2 μ m (Whatman, UK). After filtration, bacterial aggregates were stained with 100 μ g/mL DAPI for 5 min and preparations were stored at 4 °C in the dark until their visualization. Bacterial coaggregates were observed under an epifluorescence microscope (Carl Zeiss, Germany) equipped with a filter sensitive to DAPI fluorescence (359 nm excitation filter in combination with a 461 nm emission filter). Several microphotographs of the stained samples were obtained using an AxioCam HRC camera (Carl Zeiss) and a program path (AxioVision, Carl Zeiss Vision) involving image acquisition and image processing.

Prior to SEM observations, 100 μ L of bacterial autoaggregates and coaggregates were fixed with 3% (v/v) glutaraldehyde (Riedel-de-Haën, Germany) in microtiter dishes (polystyrene, Orange Scientific, USA) for 1 h at room temperature and then 15 μ L were placed in glass coverslips, dehydrated by heat (60 °C, 2 h) and stored in a desiccator for 3 d. The samples were sputter-coated with gold and examined with a Leica S360 scanning electron microscope at 10-15 kV. Microscopy visualizations were documented through the acquisition of at least 20 representative microphotographs.

Inhibition of coaggregation with simple sugars

The reversal or inhibition of coaggregation was determined by the addition of simple sugars: D(+) – galactose, N-acetyl-D-glucosamine, D(+) – fucose and D(+) – lactose (Sigma) to the coaggregating pairs. Filter-sterilized solutions of each simple sugar (500 mM in sterile deionised water) were added independently to the coaggregating pairs, to a final concentration of 50 mM. Mixtures were then vortexed and analyzed by the visual coaggregation assay. Inhibition or reversal of coaggregation was determined as a reduction in the coaggregation score.

Inhibition of coaggregation by heat treatment

The inhibition of coaggregation by heat pre-treatment of members of coaggregating pairs was performed using a modified method from Kolenbrander *et al.* [20]. Bacterial isolates suspensions were resuspended in sterile tap water to an optical density at 640 nm of 1.5 and heated for 30 min at 80 °C. Heat-treated and untreated bacterial cells were then combined in reciprocal pairs, and the capacity for the bacterial cells to coaggregate assessed by the visual coaggregation assay. Inhibition or reversal of coaggregation was detected if coaggregation score decrease.

Inhibition of coaggregation by protease treatment

The protease sensitivity of the biopolymers mediating coaggregation on each element of the coaggregating pair was assessed using a modification of the method used by Cookson *et al.* [21]. Briefly, bacterial suspension prepared as described above were resuspended to an optical density at 640 nm of 1.5 in sterile tap water. Protease type XIV from *Streptomyces griseus* (P5147, Sigma) was added to the bacterial cell suspension to a final concentration of 2 mg/mL. Protease pre-treatment of bacteria was carried out at 37 °C, and cells were harvested after 2 h by centrifuging, and washing three times in sterile tap water. The bacterial suspensions were then readjusted to an optical density at 640 nm of 1.5. Protease treated and untreated cells were mixed and their ability to coaggregate determined using the visual assay. Afterwards, inhibition or reversal of coaggregation was detected by coaggregation score decrease.

Extracellular proteins and polysaccharides extraction and quantification

Extraction of the EPS of the coaggregation partnerships was carried out (0 and 24 h) using Dowex resin (50X 8, NA⁺ form, 20-50 mesh, Aldrich-Fluka 44445) according to the procedure described by Frølund *et al.* [22]. Prior to extraction, Dowex resin was washed with extraction buffer (2 mM Na₃PO₄, 2 mM NaH₂PO₄, 9 mM NaCl and 1mM KCl, pH 7). The suspensions containing the bacterial coaggregates were resuspended in 20 ml of extraction buffer and 50 g of Dowex resin per g of volatile solids were added to the coaggregation partnerships suspension and the extraction took place at 400 rpm for 4 h at 4 °C. The extracellular components were separated from the cells through a centrifugation (13000 g, 20 min).

Total proteins were determined using the Lowry modified method (Protein Kit n° P5656, Sigma) using bovine serum albumin as standard and total polysaccharides content by the phenol-sulphuric acid method of Dubois *et al.* [23] using glucose as standard.

Multispecies species biofilm formation in microtiter plates

Multispecies biofilm formation was performed with all the representative drinking water bacteria used in this study. Thus, biofilms were developed at seven different bacterial combinations, one mixture of all six bacteria and six combinations with a mixture of five distinct bacteria, through a strain exclusion process (biofilm formation in the absence of a specific strain, obtaining distinct species combinations).

Biofilms were developed according to the modified microtiter plate test proposed by Stepanović *et al.* [24] using R2A broth as growth medium. For each condition 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 a cell suspension mixture (10^8 cells/mL). Biofilms were developed with equal initial cell densities of each isolate. To promote biofilm formation, plates were incubated aerobically on an orbital shaker, at 150 rpm and room temperature, for 24, 48 and 72 h. The growth medium was discarded and freshly added every 24 h. Negative controls were obtained by incubating the wells with R2A broth without adding any cells. All experiments were performed in triplicate with three repeats.

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 non-adherent and weakly adherent bacteria. The plates were air dried for 30 min, and the remaining attached bacteria were analysed in terms of the amount of biomass adhered on the microtiter plates surfaces using crystal violet stain according to Simões *et al.* [25].

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, total proteins and polysaccharides and biofilm 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

Visual coaggregation ability of drinking water bacteria

Six numerically dominant heterotrophic bacteria (*Acinetobacter calcoaceticus*, *Burkholderia cepacia*, *Methylobacterium* sp., *Mycobacterium mucogenicum*, *Sphingomonas capsulata*, *Staphylococcus* sp.) isolated from tap water coming from a drinking water distribution system in Braga, Portugal, were identified by 16S ribosomal RNA gene sequencing and their coaggregation partnerships were determined immediately after dual bacteria mixture, 2, 24 and 48 h later, using a visual coaggregation assay (Table 1).

0 h						
Bacteria	<i>Sph. capsulata</i>	<i>B. cepacia</i>	<i>M. mucogenicum</i>	<i>Methylobacterium</i> sp.	<i>A. calcoaceticus</i>	<i>Staphylococcus</i> sp.
<i>Sph. capsulata</i>	0	0	0	0	2/3	0
<i>B. cepacia</i>		0	0	0	3	0
<i>M. mucogenicum</i>			0	0	2/3	0
<i>Methylobacterium</i> sp.				0	1/2	0
<i>A. calcoaceticus</i>					2	3/4
<i>Staphylococcus</i> sp.						0
2 h						
Bacteria	<i>Sph. capsulata</i>	<i>B. cepacia</i>	<i>M. mucogenicum</i>	<i>Methylobacterium</i> sp.	<i>A. calcoaceticus</i>	<i>Staphylococcus</i> sp.
<i>Sph. capsulata</i>	0	0	0	0	3/4	0
<i>B. cepacia</i>		0	0	0	2/3	0
<i>M. mucogenicum</i>			0	0	3	0
<i>Methylobacterium</i> sp.				0	2	0
<i>A. calcoaceticus</i>					2	3/4
<i>Staphylococcus</i> sp.						0

24 h						
Bacteria	<i>Sph. capsulata</i>	<i>B. cepacia</i>	<i>M. mucogenicum</i>	<i>Methylobacterium</i> sp.	<i>A. calcoaceticus</i>	<i>Staphylococcus</i> sp.
<i>Sph. capsulata</i>	0	0	0	0	4	0
<i>B. cepacia</i>		0	0	0	2	0
<i>M. mucogenicum</i>			0	0	3	0
<i>Methylobacterium</i> sp.				0	2	0
<i>A. calcoaceticus</i>					2	3/4
<i>Staphylococcus</i> sp.						0
48 h						
Bacteria	<i>Sph. capsulata</i>	<i>B. cepacia</i>	<i>M. mucogenicum</i>	<i>Methylobacterium</i> sp.	<i>A. calcoaceticus</i>	<i>Staphylococcus</i> sp.
<i>Sph. capsulata</i>	0	0	0	0	4	0
<i>B. cepacia</i>		0	0	0	2	0
<i>M. mucogenicum</i>			0	0	3	0
<i>Methylobacterium</i> sp.				0	2	0
<i>A. calcoaceticus</i>					2	3/4
<i>Staphylococcus</i> sp.						0

Table 1. Coaggregation scores over time (0, 2, 24 and 48 h) of drinking water heterotrophic isolated bacteria using the visual assay. Bold numbers indicate the bacterial interactions with effective coaggregation

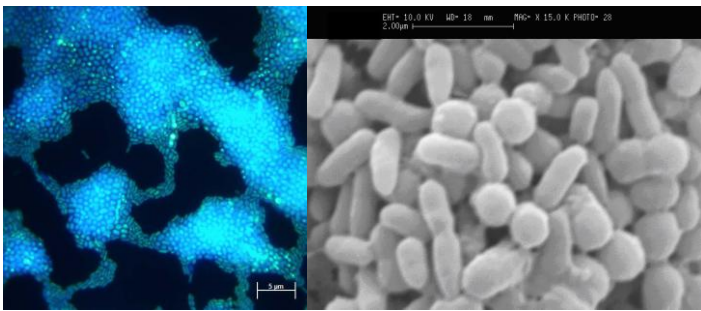
A. calcoaceticus coaggregated with four of the five other bacteria, the exception being *Methylobacterium* sp. The other bacteria did not coaggregate in *A. calcoaceticus* absence. Coaggregation, after immediate bacteria association, was higher for *A. calcoaceticus*/*Staphylococcus* sp. (coaggregation score of 3/4) with an invariable score throughout the 48 h of experiment. *A. calcoaceticus*/*B. cepacia* was the only interaction that decreased the coaggregation score after incubation. The remaining interactions increased (*A. calcoaceticus*/*Methylobacterium* sp., *A. calcoaceticus*/*M. mucogenicum*, *A. calcoaceticus*/*Sph. capsulata*) coaggregation scores over time. Comparing coaggregation scores over time, it is shown that the maximum score was achieved 24 h after bacteria-bacteria contact, as this value was similar 48 h after. Analyzing the coaggregation scores after 24 or 48 h using a score increasing factor it is found the following order: *A. calcoaceticus*/*B. cepacia* = *A. calcoaceticus*/*Methylobacterium* sp. < *A. calcoaceticus*/*M. mucogenicum* < *A. calcoaceticus*/*Staphylococcus* sp. < *A. calcoaceticus*/*Sph. capsulata*. *A. calcoaceticus*/*Sph. capsulata* is the coaggregation partnership with the highest time-coaggregation score increment.

Autoaggregation experiments revealed that only *A. calcoaceticus* had autoaggregation ability, with easily visible small aggregates (score 2). Moreover, this score was constant along the experiment. The other bacteria did not autoaggregate (Table 1).

Coaggregation detection by microscopic methods

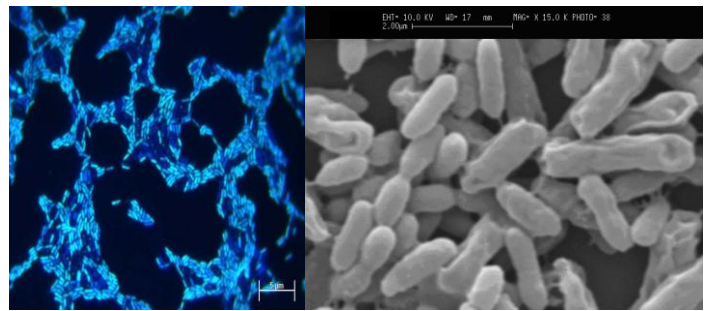
The coaggregates were observed over time (2 and 24 h – time required for maximum aggregation scores) by epifluorescence microscopy using DAPI and by SEM. Fig. 1 shows several representative microphotographs concerning various interactions between the distinct drinking water bacteria with and without visual coaggregation. Both epifluorescence microscopy and SEM analysis revealed a higher degree of interaction than did the visual coaggregation assay. This feature was evident for all the interactions, even for autoaggregation.

With visual coaggregation

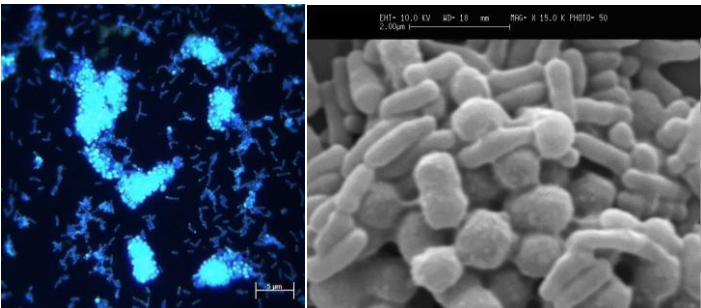


A. calcoaceticus/B. cepacia

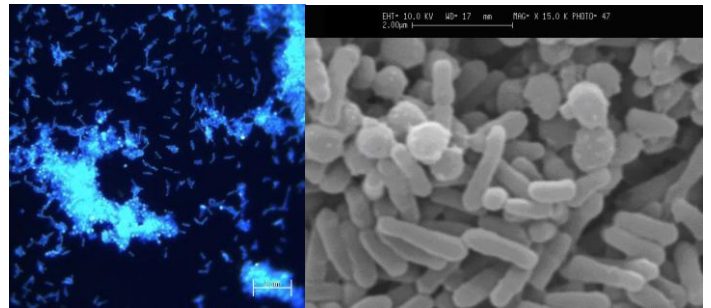
Without visual coaggregation



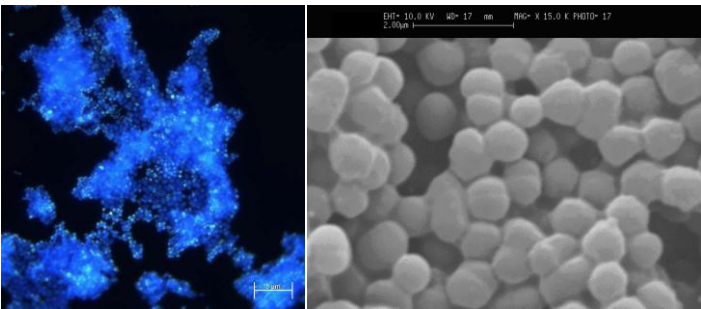
B. cepacia/Methylobacterium sp.



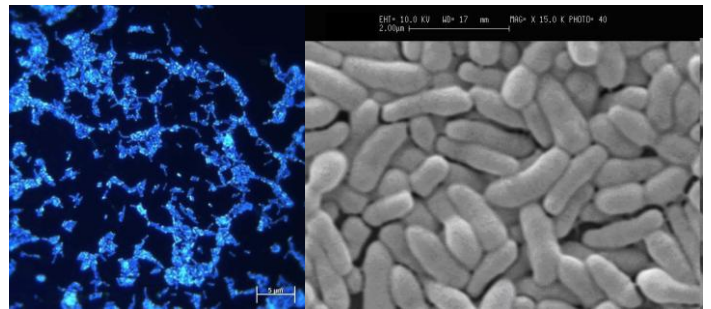
A. calcoaceticus/M. mucogenicum



M. mucogenicum/Staphylococcus sp.



A. calcoaceticus/Staphylococcus sp.



B. cepacia/M. mucogenicum

Fig. 1. Microscopy visualizations by epifluorescence microscopy and SEM of the distinct interacting drinking water bacteria with and without visual coaggregation. X1320; bar = 5 µm (epifluorescence photomicrographs); X8000; bar = 5 µm (SEM photomicrographs).

Effect of simple sugars, heat and protease treatment on coaggregation

In order to determine the surface-associated molecules involved in coaggregation several inhibition assays were performed, by the addition of simple sugars, heat and protease treatment. Inhibition or reversal of coaggregation was determined as a reduction in the coaggregation score.

Coaggregation between several pairs of bacteria was inhibited by the addition of some simple sugars (Table 2). N-acetyl-D-glucosamine was the only simple sugar unable of reverse any coaggregation partnership. The others sugars reversed two coaggregating pairs, *A. calcoaceticus/Sph. capsulata* and *A. calcoaceticus/Methylobacterium sp.* For the other situations no disaggregation was detected. The simple sugars only caused a partial disaggregation, not able to completely reverse coaggregation (score 0). No autoaggregation (*A. calcoaceticus/A. calcoaceticus*) inhibition was detected by simple sugars.

Coaggregation partnership	Sugars			
	D (+) - Galactose	N-acetyl-D-glucosamine	D(+) - Fucose	D(+) - Lactose
<i>A. calcoaceticus/Sph. capsulata</i>	+	-	+	+
<i>A. calcoaceticus/B. cepacia</i>	-	-	-	-
<i>A. calcoaceticus/M. mucogenicum</i>	-	-	-	-
<i>A. calcoaceticus/Methylobacterium</i> sp.	+	-	+	+
<i>A. calcoaceticus/A. calcoaceticus</i>	-	-	-	-
<i>A. calcoaceticus/Staphylococcus</i> sp.	-	-	-	-

++ Complete disaggregation; + partial disaggregation; - no disaggregation.

Table 2. The reversal of coaggregation using simple sugars

Table 3 shows the effect of heat and protease treatment on coaggregation scores when each partner was pretreated separately by those two kinds of treatments and then mixed with either a treated or an untreated partner. Heat and protease treatment, when applied to both partners, lead to complete coaggregation inhibition for all coaggregation partnerships, with the exception of *A. calcoaceticus/Sph. capsulata* with a partial inhibition for heat treatment (score 1). When only one partner was treated, and if it was *A. calcoaceticus*, the results were similar to those observed when both partners were treated, except for protease treatment where it was only observed almost complete inhibition for *A. calcoaceticus/Sph. capsulata* and *A. calcoaceticus/M. mucogenicum* (score 0/1). No disaggregation was verified if the treated partner was one of the other bacterium, with the exception of *A. calcoaceticus/Sph. capsulata* and *A. calcoaceticus/M. mucogenicum* (heat and protease) and *A. calcoaceticus/B. cepacia* (protease). For these cases, the interaction score decreased lightly, translated in a partial disaggregation. Protease treatment was more efficient than heat treatment on coaggregation inhibition of *A. calcoaceticus/Sph. capsulata* and *A. calcoaceticus/B. cepacia*. For the other coaggregation partnerships it were obtained the same results with the two treatments.

Analyzing the effect of heat and protease treatment on autoaggregation, it was verified *A. calcoaceticus* autoaggregation inhibition. No inhibition was detected when treated cells were mixed 1:1 with untreated cells (Table 3).

Bacteria		Coaggregation scores											
		<i>Sph. capsulata</i>		<i>B. cepacia</i>		<i>M. mucogenicum</i>		<i>Methylobacterium</i> sp.		<i>A. calcoaceticus</i>		<i>Staphylococcus</i> sp.	
		UT	T	UT	T	UT	T	UT	T	UT	T	UT	T
Heat Treatment													
<i>A. calcoaceticus</i>	U												
	T	4	3	2	2	3	2	2	2	2	2	3/4	3/4
	T	1	1	0	0	0	0	0	0	2	0	0	0
Protease Treatment													
<i>A. calcoaceticus</i>	U												
	T	4	2	2	1/2	3	2	2	2	2	2	3/4	3/4
	T	0/1	0	0	0	0/1	0	0	0	2	0	0	0

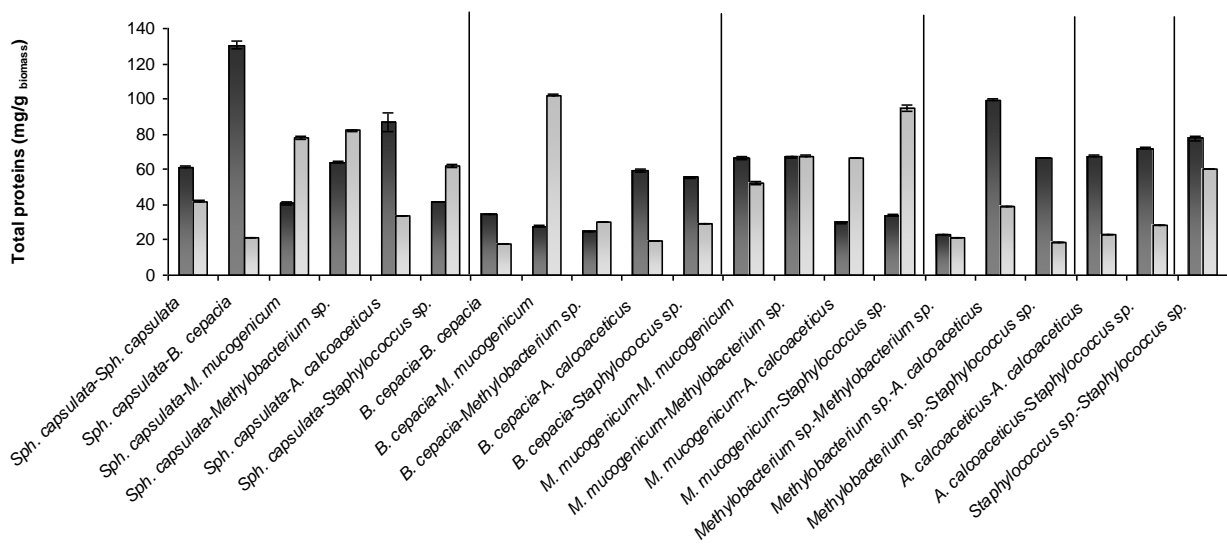
Table 3. The effect of heat and protease treatment on coaggregation scores when each partner was pretreated separately with heat and protease and then mixed with either a treated or an untreated partner.

Bold numbers indicate untreated control scores. UT, untreated partner; T, treated partner

Extracellular proteins and polysaccharides production by bacterial aggregates

With the aim to correlate the EPS production with coaggregation ability of drinking water bacteria, it was assessed their production over time (0 and 24 h) for all coaggregation partnerships (Fig. 2). An increase on total proteins content was found for *Sph. capsulata*/*M. mucogenicum*, *Sph. capsulata*/*Methylobacterium* sp., *Sph. capsulata*/*Staphylococcus* sp., *B. cepacia*/*M. mucogenicum*, *B. cepacia*/*Methylobacterium* sp., *A. calcoaceticus*/*M. mucogenicum*, *M. mucogenicum*/*Staphylococcus* sp (Fig. 2a). Maintenance on proteins content was found for *M. mucogenicum*/*Methylobacterium* sp. and *Methylobacterium* sp. autoaggregates. Statistical equivalence ($P > 0.05$) of proteins level for the two sampling time was found for *B. cepacia*/*Methylobacterium* sp., *M. mucogenicum*/*Methylobacterium* sp., and *Methylobacterium* sp./*Methylobacterium* sp. For the other interactions the proteins content decreased over time. Regarding over time variation of total polysaccharides content, Fig. 2b shows that an increase on polysaccharides content occurs for the interactions between *Sph. capsulata*/*M. mucogenicum*, *Sph. capsulata*/*Methylobacterium* sp., *Sph. capsulata*/*Staphylococcus* sp., *B. cepacia*/*M. mucogenicum*, *B. cepacia*/*Methylobacterium* sp., *M. mucogenicum*/*Methylobacterium* sp., *A. calcoaceticus*/*M. mucogenicum*, *M. mucogenicum*/*Staphylococcus* sp., *A. calcoaceticus*/*Methylobacterium* sp., *Methylobacterium* sp./*Staphylococcus* sp., *A. calcoaceticus* /*Staphylococcus* sp. and *A. calcoaceticus* autoaggregates. Maintenance on polysaccharides content was found for *Methylobacterium* sp. and *Staphylococcus* sp. autoaggregates. For the other situations the polysaccharides content decreased over time. Statistical equivalence ($P > 0.05$) on polysaccharides level for the two sampling times were found for *B. cepacia*/*Methylobacterium* sp., *Methylobacterium* sp./*Methylobacterium* sp., and *Staphylococcus* sp./*Staphylococcus* sp. Thus, the number of the coaggregation partnerships with reduction on the total extracellular proteins and polysaccharides content along time are far more than that increasing. All coaggregation partnerships had a similar over time variation in total proteins and polysaccharides ($P > 0.05$).

a)



b)

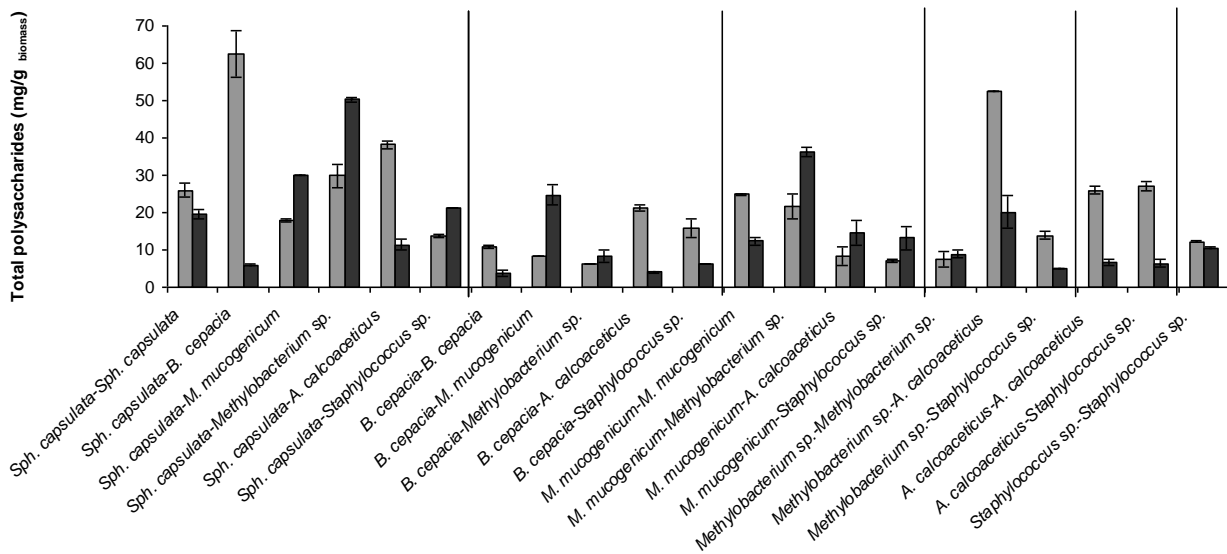


Fig. 2. Extracellular proteins (a) and polysaccharides (b) of the coaggregation partnerships over time, 0 h (□) and 24 h (■). The means \pm SD of at least three replicates are illustrated.

Multispecies species biofilm formation in microtiter plates

In order to ascertain the putative bridging function of *A. calcoaceticus*, in drinking water bacterial interactions, mixed biofilm formation was carried out (24, 48 and 72 h), with the six isolates.

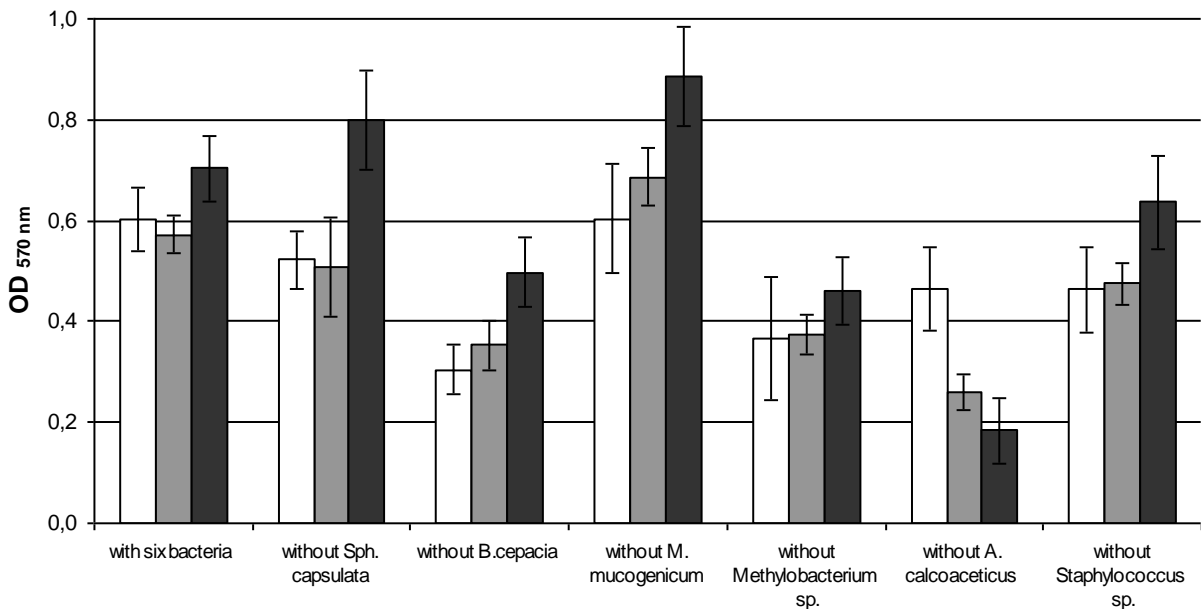


Fig. 3. Values of OD_{570 nm} as a measure of multispecies biofilm mass for 24 h (□), 48 h (■) and 72 h (■). The means \pm SD of at least three replicates are illustrated.

Fig. 3 shows that all tested combinations formed biofilms on microtiter plates. Biofilm mass over time increase was found for the various situations, except for multispecies biofilms without *A. calcoaceticus*. *M. mucogenicum* was the only bacterium that, when not present, leads to a relative over time biofilm mass increase comparatively to biofilm formation with all six bacteria. The remaining bacteria reduced biofilm formation. The decrease of biofilm mass formation was less significant ($P > 0.05$) for biofilms in the absence of *Sph. capsulata* (24 and 48 h) and *Staphylococcus* sp. (72 h), and more significant ($P <$

0.05) in the absence of *B. cepacia* (24 h) and *A. calcoaceticus* (48 and 72 h). However, taking into account all sampling times, biofilms without *A. calcoaceticus* lead to the highest reduction on multispecies biofilm development ($P < 0.05$).

DISCUSSION

Biofilm formation and development is a consequence of several types of cell-cell interactions between different pairs and groups of bacteria [15]. Coaggregation has been pointed as one of the main mechanisms of adhesion that can enhance the potential for biofilm development [12]. This adhesion mechanism is highly specific and is thought to have a role in the development of multispecies biofilms in many diverse environments [8, 12]. Consequently, it is important to study the involvement of coaggregation in multispecies bacterial formation and behavior. As suggested and demonstrated by Rickard *et al.* [7], coaggregation is a phenomenon that occurs most frequently between bacteria from natural multispecies biofilms than from planktonic populations. This phenomenon is likely to enhance the development of freshwater multispecies biofilms and may influence biofilm species diversity in the natural environment [7]. In this study, six heterotrophic drinking water isolated bacteria [17], belonging to different genera, were analyzed in terms of coaggregation ability by a visual assay and by microscopic techniques. Coaggregation was detected between several pairs of the autochthonous water flora. However, this phenomenon had the particularity of occurring only in the presence of *A. calcoaceticus* as assessed by the visual assay. The other bacteria did not coaggregate in its absence. Moreover, *A. calcoaceticus* was also the only tested bacteria with the ability to autoaggregate.

Microscopy analyses revealed some degree of interaction, not detected by the visual assay. According to Buswell *et al.* [15], low visual coaggregation scores are not necessarily indicators of weak interaction between the cells. The scores detected with this assay are not an accurate measure of the relative interaction strength between individual ligands on different cells. Furthermore, these authors, furthermore, proposed that visual coaggregation will depend on the relative size and morphology of the bacteria involved, and may depend on the density of interacting ligands on the bacterial surface. A lack of sensitivity associated with the visual assay was also proposed by Elliott *et al.* [26]. Nevertheless, the rapid and simple visual assay provided reproducible results with enough sensitive to detect significant interactions [15].

Coaggregation is a highly specific process involving interactions between bacterial surface molecules that act as adhesins and complementary receptors, including proteins and carbohydrates. In most of the situations, heat and protease treatments of coaggregating pairs, totally inhibited coaggregation. The interactions between the tested coaggregation partnerships are apparently mediated by heat and protease sensitive adhesins of *A. calcoaceticus* and heat and protease stable interactive sites in the surface of the other bacterium. However, for *A. calcoaceticus* with *Sph. capsulata* and *A. calcoaceticus* with *M. mucogenicum* the results suggest the existence of other type of interactions between heat and protease stable receptors in *A. calcoaceticus* and heat and protease sensitive adhesins in *Sph. capsulata* and *M. mucogenicum*. Heat and protease treatment inhibited *A. calcoaceticus* autoaggregation. However, no inhibition was detected when treated cells were mixed 1:1 with untreated cells. This result demonstrates that not only heat and protease sensitive proteins (lectins) mediate aggregation between the tested bacteria, but also that other molecules may be involved, such as saccharides, that can bind to lectins of untreated cells. Moreover, this also suggests that *A. calcoaceticus* binding molecules are apparently constituted by lectins and saccharides, therefore, increasing the interaction potential with other bacteria [12]. In fact, many bacteria have been found to possess proteinaceous adhesins on their surfaces which bind in a stereochemically specific manner to complementary molecules or receptors (often saccharides), on the surface of other bacterial cells of the same or different species [12, 27]. The ability of simple sugars to reverse the coaggregation process was not verified for all coaggregating pairs and for those with reversed coaggregation, interactions were only partially inhibited. The addition of simple sugars was expected to reverse the lectin-saccharide (protein-carbohydrate)-like interactions. Nevertheless, such interactions are known to be very specific [28]. It is possible that either the selected sugars, or the tested concentrations, were not appropriate. Kolenbrander *et al.* [29] found that depending upon the involved bacterial pairs, a varied response to sugar addition was observed in the case of potential lectin-saccharide-like coaggregation of oral pathogens. Other authors also found that protein-carbohydrate-like interactions between *Candida albicans* and *Actinomyces* species was not reversed by sugars [30]. Based on Malik *et al.* [8], reversibility by simple sugars is not an essential feature of lectin-like interactions. Although the present study could not elucidate the exact nature of the surface molecules involved in coaggregation, the results suggest the possibility of lectin-saccharide-like interactions involvement. This finding is in agreement with the conclusions of Rickard *et al.* [7, 18] about the interactions mediating

coaggregation on freshwater bacteria belonging generally to different species from the ones used in this study.

The exact function of EPS, which are secreted by microorganisms during growth, are not completely elucidated because of their extremely heterogeneous nature. It has been reported that EPS may play a significant role in the formation and function of microbial aggregates, including matrix structure formation and microbial physiological processes [31, 32]. The increased production of EPS is an important early physiological event that can occur during the development of a biofilm, and which might be important in the adhesion of secondary colonizers. These biopolymers envelop the attached cells within the biofilm, strengthen their adhesion and can act as receptors for coaggregation interactions [12]. In this study, it was found that, in some cases, the amount of proteins and polysaccharides increased over time. It is tempting to speculate that the bacterial community organization may be explained on the basis of weak and strong cell-to-cell interactions combined with bacterial metabolic and chemotactic properties. In some SEM inspections it is also perceptible that EPS-like structures were present in the intercellular spaces. However, besides coaggregation being lectin and saccharin dependent, it was not correlated with EPS content. The over time variation of the extracellular proteins and polysaccharides content seemed not to account for the coaggregation phenomenon, but could be arguably involved in the coaggregation strength. Further studies, such as those based on atomic force microscopy, are required in order to provide more evidences on the role of EPS level on coaggregation.

Under natural environments, monospecies biofilms are rare. Conversely, microorganisms are associated as a complex multispecies sessile communities. Diversity in microbial communities leads to a variety of complex relationships involving interspecies and intraspecies interactions which can influence profoundly biofilm formation and development [27]. Multispecies biofilms formed by the isolated bacteria were performed in order to identify the role of *A. calcoaceticus* as bridging microorganism in the tested microflora. In fact, *A. calcoaceticus* coaggregated with the other tested drinking water bacteria, suggesting the ability of forming multigeneric coaggregates and a potential bridging function, similarly to *Fusobacterium* sp. and *Prevotella* sp. in dental plaque accretion [20, 28]. Rickard *et al.* [18] reported similar findings for *Blastomonas natatoria* in freshwater bacteria communities. An *A. johnsonii* strain has also been proposed as bridging bacterium in an activated sludge microflora [8]. Such bridging microorganisms are believed to carry complementary receptors recognized by functionally similar adhesins on cells from distinct genera [8]. The role of *A. calcoaceticus* as bridging bacteria is reinforced by studies of strain exclusion in multispecies biofilms. Biofilm formation decreased for five of the six strain exclusion scenarios. Nevertheless, it was significant and decreased over time only for biofilms without *A. calcoaceticus*. This result provides additional evidences concerning the role of *A. calcoaceticus* in drinking water microbial ecosystems.

The overall results demonstrate that *A. calcoaceticus* plays a bridging function in drinking water biofilm formation. This bacterium coaggregates with almost all other tested bacteria and their presence in a multispecies community represents a colonization advantage. Probably, this bacterium may facilitate the association of the other species which do not coaggregate directly each other, increasing the opportunity for metabolic cooperation. The presence and absence of *A. calcoaceticus* in multispecies biofilms can, therefore, enhance or decrease, respectively, biofilm formation by drinking water bacteria.

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