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ORIGINAL ARTICLE

Interference in adhesion of bacteria and yeasts isolated from explanted voice prostheses to silicone rubber by rhamnolipid biosurfactants

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Abstract

Aims: The effects and extent of adhesion of four different bacterial and two yeast strains isolated from explanted voice prostheses to silicone rubber with and without an adsorbed rhamnolipid biosurfactant layer obtained from *Pseudomonas aeruginosa* DS10-129 was studied.

Methods and Results: The ability of rhamnolipid biosurfactant to inhibit adhesion of micro-organisms to silicone rubber was investigated in a parallel-plate flow chamber. The anti-adhesive activity of the biosurfactant at different concentrations was significant against all the strains and depended on the micro-organism tested. The results showed an effective reduction in the initial deposition rates, and the number of bacterial cells adhering after 4 h, for all micro-organisms tested at the 4 g l^{-1} undiluted rhamnolipid solution. Maximum initial reduction of adhesion rate (an average of 66%) occurred for *Streptococcus salivarius* GB 24/9 and *Candida tropicalis* GB 9/9. The number of cells adhering after 4 h on silicone rubber conditioned with biosurfactant was reduced to 48% for *Staphylococcus epidermidis* GB 9/6, *Strep. salivarius* GB 24/9, *Staphylococcus aureus* GB 2/1 and *C. tropicalis* GB 9/9 in comparison to controls. Perfusing the flow chamber with biosurfactant containing solution followed by the passage of a liquid–air interface, to investigate detachment of micro-organisms adhering to silicone rubber, produced high detachment (96%) of adhered cells for all micro-organisms studied, except for *Staph. aureus* GB 2/1 (67%).

Significance and Impact of the Study: It is concluded that biosurfactant represent suitable compounds that should be considered in developing future strategies to prevent the microbial colonization of silicone rubber voice prostheses.

Introduction

Silicone rubber has several advantageous properties that have led to its use in voice prostheses (Neu *et al.* 1993), urinary catheters (Farber and Wolff 1993) and contact lens materials (Holly and Owen 1983). Patients who have undergone a laryngectomy because of a malignant laryngeal tumour need to breathe through a tracheostoma and receive voice prostheses for speech rehabilitation (Neu *et al.* 1993). A serious problem in laryngectomized patients with a voice prosthesis inserted is the limited lifetime of the prosthesis of about 3–4 months. Voice prostheses that are covered with a biofilm (Neu *et al.* 1993) cause leakage of food and liquid or an increased airflow resistance (Mahieu *et al.* 1986) that result in the frequent replacement of the prosthesis. This biofilm formation immediately starts once the device has been placed in the fistula and is usually composed of various bacterial and yeast strains. The type of environment of the neopharynx and the presence of a tracheostoma are ideal for microbial adherence to the surface of the voice prosthesis (Busscher *et al.* 2000). Frequent replacement of the prosthesis

is uncomfortable, costly, time consuming and may lead to damage of the shunt with scar tissue formation, insufficiency or stenosis. Less frequent replacements of voice prosthesis therefore would be advantageous and has resulted in general interest in finding techniques to inhibit biofilm formation and prolonging the lifetime of voice prostheses (Busscher *et al.* 2000; Elving *et al.* 2000; Rodrigues *et al.* 2004a,b). Everaert *et al.* (1999) studied, *in vivo*, the influence of perfluoroalkylsiloxane (PA) surface modification of silicone rubber voice prostheses on biofouling, with 18 consecutive patients with laryngectomies, and found that chemisorption of long-chain PAs reduces biofilm formation. Additionally, Rodrigues *et al.* (2004a) developed a promising strategy to lengthen lifetime of the voice prostheses as it was demonstrating that the use of biosurfactants obtained from probiotic bacteria inhibit biofilm formation and the occurrence of increased airflow resistances.

Surfactants of microbial origin, referred to as biosurfactants, are surface-active compounds that have some influence on interfaces. With regard to an anti-adhesive effect of biosurfactants, hypotheses have been forwarded in which adsorption of biosurfactants to a substratum surface alters the hydrophobicity of the surface and causes interference in microbial adhesion and desorption processes (Bai *et al.* 1997; Desai and Banat 1997). Rhamnolipid biosurfactants mainly produced by *Pseudomonas aeruginosa* are the most well known and characterized biosurfactant molecules (Banat *et al.* 2000, Rahman *et al.* 2002a,b,2003a,b). Because of their diversity, environmental-friendly nature, possibility of large-scale production, selectivity, effectiveness under extreme conditions and at low concentrations, in addition to the possibility for production on renewable sources, they have been essentially applied in environmental protection (Banat *et al.* 2000, Rahman *et al.* 2002a,b,2003a,b). Despite their potential, only a few studies were done on application in biomedical sciences. Moreover, as they are biological and safe (Flasz *et al.* 1998; Makkar and Cameotra 2002; Benincasa *et al.* 2004), rhamnolipid biosurfactant are a suitable alternative to synthetic medicines and antimicrobial agents, and may be used as safe and effective therapeutic agents (Singh and Cameotra 2004).

The aim of this study was to investigate the effects and extent of adhesion of four different bacterial and two yeast strains isolated from explanted voice prostheses to silicone rubber with and without an adsorbed rhamnolipid biosurfactant layer in a parallel-plate flow chamber. Additionally, the influence of the biosurfactant on the detachment of these micro-organisms adhering to silicone rubber was studied. In order to determine the best dilution of the rhamnolipid with an anti-adhesive effect a quick screening was performed in a 96-well plate, before the adhesion experiments in the parallel-plate flow chamber were started.

Materials and methods

Biosurfactant production and extraction

Biosurfactants were produced in batch culture in 250 ml conical flasks containing 100 ml of medium with the following composition in grams per litre (g l^{-1}) Na_2HPO_4, 2·2; KH_2PO_4, 1·4; $MgSO_4{\cdot}7H_2O$, 0·6; $(NH_4)_2SO_4$, 3·0, glucose, 2·0 and 1 ml trace element solution (Banat *et al.* 1991). The culture medium pH was maintained at 7·5. The flasks were inoculated with *P. aeruginosa* DS10-129 and incubated in a shaker at 200 rev min^{-1} at 30 °C. Glycerol was added to the culture at 10 g l^{-1} after 48 h growth to induce biosurfactant production (Rahman *et al.* 2002b) and flasks re-incubated for another 48–72 h. After removing the cells by centrifugation (8000 × ***g***, 15 min) the whole culture broth was sterilized (121 °C, 15 min) and used to extract the rhamnolipid biosurfactant. The supernatant was mixed with chloroform: methanol (2 : 1) solution shaken well, allowed to stand for 2 h after which the chloroform solvent layer containing the rhamnolipid was separated and left at 40 °C overnight to complete evaporation and dissolving in water.

Derivatization and HPLC analysis of rhamnolipid

The derivatization and HPLC analysis were carried out using a modified method described by Schenk *et al.* (1995) and Mata-Sandoval *et al.* (1999) to determine the rhamnolipid-containing solution concentration. One millilitre rhamnolipid-containing water solution was added to 1 ml acetonitrile containing 2-bromoacetophenone and triethylamine at a molar ratios 1 : 8 : 2 (glycolipid : 2-bromoacetophenone triethylamine). The mixture was incubated at 80 °C for 1 h. The mixture was filtered with Minisart 0·22 μm syringe filters prior to HPLC analysis. The derivatized phenacyl esters were separated by HPLC using an isocratic mobile phase of CH_3CN – 3·3 mmol l^{-1} H_3PO_4 [80 : 20 (v/v)] at a flow rate of 1 ml min^{-1}. The column used was a phenomenex Kromasil C-18 (150 × 4·6 mm) and absorbance measured at 254 nm. The rhamnolipid-containing solution used in further studies had a 4 g l^{-1} rhamnolipid concentration.

Surface-activity determination

The surface activity of the rhamnolipid was determined by measuring the surface tension by the ring method (Kim *et al.* 2000) using a KRUSS tensiometer equipped with a 1·9 cm De Noüy platinum ring at room temperature

(25 ± 1 °C). The concentration at which micelles began to form was represented as the critical micelle concentration (CMC). The CMC was determined by plotting the surface tension as a function of the biosurfactant concentration. Dilutions of the rhamnolipid-containing solution were prepared with phosphate-buffered saline (PBS: 10 mmol l^{-1} KH_2PO_4/K_2HPO_4 and 150 mmol l^{-1} NaCl with pH adjusted to 7·0). Measurements were done in triplicate.

Contact angle measurements

Advancing type contact angles with Millipore water on silicone rubber with and without an adsorbed rhamnolipid layer at several concentrations were measured with an optical contact-measuring KRUSS device using the sessile drop technique. On each sample, at least six droplets were placed at different positions and results of three separately prepared surfaces with adsorbed biosurfactant were averaged. Conditioned silicone rubber was prepared through overnight immersion in a rhamnolipid solution (several rhamnolipid dilutions were tested) at 4 °C. In a second set of experiments after conditioning the silicone rubber with rhamnolipid it was rinsed in PBS solution for half an hour. All the samples were left to air dry before contact angle measurements.

Microbial strains and growth conditions

Four bacterial strains, *Staphylococcus epidermidis* GB 9/6, *Streptococcus salivarius* GB 24/9, *Staphylococcus aureus* GB 2/1 and *Rothia dentocariosa* GBJ 52/2B and two yeast strains: *Candida albicans* GBJ 13/4A and *Candida tropicalis* GB 9/9 isolated from explanted voice prostheses were used in this study (Elving *et al.* 2002). All strains were first grown overnight at 37 °C in ambient air on agar plates from frozen stocks, the agar plates were kept at 4 °C, never longer than 2 weeks. Several colonies were used to inoculate 10 ml of brain heart infusion broth (BHI, OXOID, Basingstoke, England) for all the bacterial and yeast strains in use. This preculture was incubated at 37 °C in ambient air for 24 h and used to inoculate a second culture of 200 ml that was grown statically for 18 h. The micro-organisms from the second culture were harvested by centrifugation for 5 min at 10 000 × ***g*** and washed twice with demineralized water. Subsequently, bacterial cells were suspended in 200 ml PBS solution (PBS: 10 mmol l^{-1} KH_2PO_4/K_2HPO_4 and 150 mmol l^{-1} NaCl with pH adjusted to 7·0), after sonication on ice (10 s), to a concentration of 3×10^8 ml^{-1}. The sonication procedure did not promote cell lysis. Yeasts were suspended in PBS to a concentration of 3×10^6 ml^{-1}. A Bürker–Türk counting chamber was used to count the cells.

Adhesion assay in 96-well plate

The anti-adhesive activity of the rhamnolipid biosurfactant against four bacterial strains, *S. epidermidis* GB 9/6, *S. salivarius* GB 24/9, *S. aureus* GB 2/1 and *R. dentocariosa* GBJ 52/2B and two yeast strains: *C. albicans* GBJ 13/4A and *C. tropicalis* GB 9/9 isolated from explanted voice prostheses was quantified according to a previously reported adhesion assay (Heinemann *et al.* 2000, Stepanovic *et al.* 2000). Briefly, the wells of a sterile 96-well flat-bottomed plastic tissue culture plate with a lid were filled with 200 μl of the rhamnolipid solution to be tested for anti-adhesive activity. Several rhamnolipid dilutions were tested. The undiluted rhamnolipid-containing solution used had a 4 g l^{-1} rhamnolipid concentration. The plate was incubated for 18 h at 4 °C and subsequently washed twice with PBS. Control wells contained buffer (PBS) only. An aliquot of 200 μl of a washed bacterial or yeast suspension was added and incubated in the wells for 4 h at 25 °C. Unattached organisms were removed by washing the wells three times with PBS. The adherent micro-organisms were fixed with 200 μl of 99% methanol per well, and after 15 min the plates were emptied and left to dry. Then the plates were stained for 5 min with 200 μl of 2% crystal violet used for Gram staining per well. Excess stain was rinsed of by placing the plate under running tap water. After the plates were air dried, the dye bound to the adherent micro-organisms was resolubilized with 200 μl of 33% (v/v) glacial acetic acid per well and the optical density readings of each well were taken at 595 nm. The microtitre-plate anti-adhesion assay allows the estimation of the rhamnolipid concentration that is effective in inhibiting adhesion of the micro-organisms studied and constitutes a quick screening for the dilution to use in the flow experiments.

Adhesion experiments in the parallel-plate flow chamber

The flow chamber and image analysis system have been described in detail before (Sjollema *et al.* 1989). The parallel-plate flow chamber consists of a thin sheet of silicone rubber (with or without an adsorded rhamnolipid layer) affixed to a polymethylmethacrylate (PMMA) bottom plate and a top plate made of glass, both with dimensions 5·5 × 3·8 cm. The top and bottom plates were cleaned by sonicating for 3 min in a commercial detergent (2% Sonazol Pril in water, Henkel Ibérica, Alverca, Portugal), rinsed thoroughly with tap water, and then rinsed with demineralized water. Top and bottom plates were subsequently mounted in the housing of the flow chamber, separated by 0·06 cm spacers. Images were taken from the bottom plate with a 3CCD video camera (Axiocam HRC, Zeiss, Heidenheim, Germany) mounted on a phase-contrast microscope

(Zeiss Axioscop, Zeiss) equipped with a 40× ultra-long working distance objective for experiments with bacteria and with a 10× objective for experiments with yeasts. The camera was coupled to an image analyser (Image Pro Plus, Media Cybernetics, Silver Spring, MD, USA). Each life image (768 × 576 pixels with 8-bit resolution) was obtained after summation of five consecutive images (time interval 500 ms) in order to eliminate moving bacteria and to enhance the signal-to-noise ratio and to eliminate moving micro-organisms from the analysis.

Conditioned silicone rubber was prepared through overnight immersion in a rhamnolipid solution at 4 °C. Two different sets of experiments were performed, the first with rhamnolipid solution without any dilution and the second with a 1 : 1000 dilution of the rhamnolipid solution.

Prior to each experiment, all tubes and the flow chamber were filled with PBS, while care was taken to remove air bubbles from the system. Flasks, containing microbial suspension and buffer, were positioned at the same height with respect to the chamber to ensure that immediately after the flow was started, all fluids would circulate (each fluid at the time) by hydrostatic pressure through the chamber at the desired shear rate of 10 s^{-1} (0·025 ml s^{-1}) that yields a laminar flow (Reynolds number 0·6). The microbial suspension was circulated through the system for 4 h and images were obtained from silicone rubber with or without the adsorbed rhamnolipid. The number of micro-organisms per unit area was plotted *vs* time and the initial linear increase in the number of adhering micro-organisms with time was expressed in a so-called initial deposition rate j_0 (micro-organisms cm^{-2} s^{-1}), i.e. the number of adhering micro-organisms per unit area and time. The number of adhering micro-organisms after 4 h was also determined (n_{4h}). All values presented in this work are the averages of triplicate measurements on silicone rubber surfaces with or without the adsorbed rhamnolipid layer and were carried out with separately grown micro-organisms.

Detachment protocol

After the 4 h adhesion period, flow was switched for 30 min to PBS in order to remove nonadhering cells from the tubes and chamber. Then, 15 ml of the rhamnolipid solution (dilution 1 : 15) was perfused once through the flow chamber, at a flow rate of 0.025 ml s^{-1}, by a second pump connected to the system by a valve downstream of the flow chamber, followed by 30 ml of PBS to clean the system from rinsing components. The time required to pass one dosage (15 ml) of test agent through the system was nearly 4 min. Subsequently, the number of organisms still adhering to the silicone rubber was determined. Finally, the flow was switched back to PBS for 1 h in order to remove possibly detached cells and the chamber was emptied by hydrostatic pressure, so that a liquid–air interface could pass over the surface. Finally, the number of adhering organisms withstanding this extremely high removal force was counted again. All experiments were done in duplicate with separate microbial cultures at room temperature.

Statistical analysis

All the adhesion experiments were compared using one-way analysis of variance (ANOVA) by applying the Levene's test of homogeneity of variances, the Tukey multiple-comparisons test, and also paired samples *t*-test, using SIGMAPLOT 8·0 software. Student's *t*-test was applied to all the experimental data, for rejection of some experimental values. All tests were performed with a confidence level of 95% (Altman 1991).

Results

Surface-activity and biosurfactant concentration

The quantity of rhamnolipid obtained after chloroform: methanol extraction and used for the preparation of the dilutions was 4 g l^{-1} of culture broth. Surface tension measurements at tenfold dilution factors up to 10^{-9} of the rhamnolipid-containing solution measured by the ring method is shown in Fig. 1. The rhamnolipid reached the lowest surface tension of 32 ±0.5 mN m^{-1} with a dilution up to 10^{-5}, which means that the solution had a strong effect on surface tension at low concentrations. The CMC is achieved with a 10^{-5} dilution of the rhamnolipid solution therefore, no matter how much we

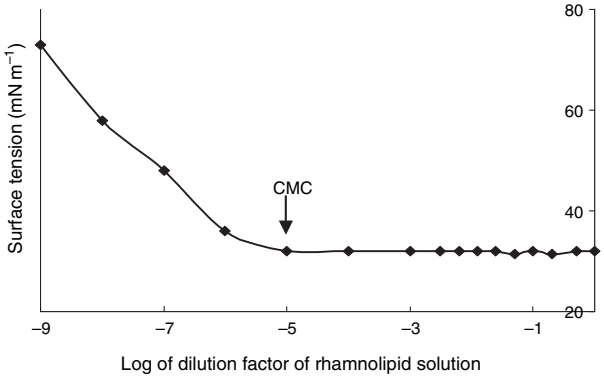

Figure 1 Surface tension *vs* dilution factor as a measure of the concentration of rhamnolipid solution. The undiluted rhamnolipid-containing solution used for the preparation of the dilutions had a 4 g l^{-1} rhamnolipid concentration.

Table 1 Microbial inhibition percentages obtained from the microtiter-plate anti-adhesion assay with several rhamnolipid concentrations. The dilution factor is a direct measure of the rhamnolipid concentration. The undiluted rhamnolipid-containing solution used had a 4 g l^{-1} rhamnolipid concentration. PBS was used as control and set at 0% as no microbial inhibition occurs. Experiments were carried out performed in triplicate and correspond within 15%

	Microbial inhibition (%)							
	Rhamnolipid dilution factor							
Micro-organism	PBS control	Undiluted	2	5	10	100	1000	100 000
Staphylococcus epidermidis GB 9/6	0·0	53·1	48·8	28·2	21·0	16·9	2·7	0·6
Streptococcus salivarius GB 24/9	0·0	58·2	55·2	45·1	36·8	19·0	14·3	1·4
Staphylococcus aureus GB 2/1	0·0	33·8	25·1	22·5	21·5	19·0	4·8	1·3
Rothia dentocariosa GBJ 52/2B	0·0	60·9	55·2	54·6	49·7	48·1	42·0	31·0
Candida albicans GBJ 13/4A	0·0	38·2	32·0	26·4	24·8	23·3	17·1	7·1
Candida tropicalis GB 9/9	0·0	35·3	16·9	14·3	13·3	11·8	8·7	2·7

reduce the dilution factor, surface tension obtained will be nearly the same.

Anti-adhesive activity of the rhamnolipid

The anti-adhesive activity of the rhamnolipid was evaluated at several dilutions and compared against a variety of bacterial and yeast strains isolated from explanted voice prostheses (Table 1). The undiluted biosurfactant had an anti-adhesive effect against all micro-organisms tested but the anti-adhesive effect is depending on the dilution factor and micro-organism tested. The rhamnolipid biosurfactant showed the highest anti-adhesive activity against *R. dentocariosa* GBJ 52/2B and worked even at a dilution of 100 000. *R. dentocariosa* GBJ 52/2B, *S. epidermidis* GB 9/6 and *S. salivarius* GB 24/9 were inhibited till more than 50%. The combined results from surface tension measurements and microtitre-plate anti-adhesion assay permitted the selection of the rhamnolipid concentration to use in the flow experiments. In the adhesion experiments with the parallel-plate flow chamber, undiluted and 1 : 1000 dilution rhamnolipid solutions were used. In the detachment protocol, a 1 : 15 dilution of the rhamnolipid solution was used.

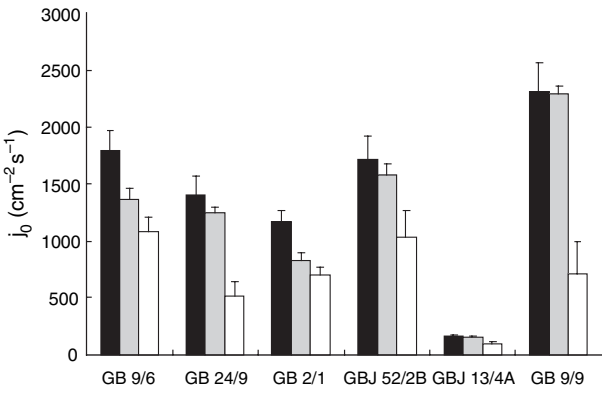

Figure 2 The initial deposition rates (j_0) of the bacterial (*Staph. epidermidis* GB 9/6, *Strep. salivarius* GB 24/9, *Staph. aureus* GB 2/1 and *R. dentocariosa* GBJ 52/2B) and yeast (*C. albicans* GBJ 13/4A and *C. tropicalis* GB 9/9) strains isolated from explanted voice prostheses on silicone rubber with and without an adsorbed rhamnolipid layer. The undiluted rhamnolipid-containing solution used had a 4 g l^{-1} rhamnolipid concentration. Black column, without rhamnolipid; grey column, with rhamnolipid dilution 1 : 1000; and white column, with undiluted rhamnolipid. Results are averages of triplicate experiments varying within 10–15% (anova) and the standard deviation represented by the error bars.

Microbial adhesion in the parallel-plate flow chamber

Initial deposition rates of the tested bacterial and yeast strains on silicone rubber, before and after coating with the 1 : 1000 or undiluted rhamnolipid solution, as measured in buffer (PBS) in a parallel-plate flow chamber are presented in Fig. 2. For the microbial strains *R. dentocariosa* GBJ 52/2B, *Staph. epidermidis* GB 9/6 and *C. tropicalis* GB 9/9 the initial deposition rates on silicone rubber were relatively high between 1716 ± 206, 1793 ± 179 and 2312 ± 254 micro-organisms $cm^{-2}\,s^{-1}$, respectively, whereas *Strep. salivarius* GB 24/9 and *Staph. aureus* GB 2/1 had a lower deposition rate (from 1167 ± 105 to 1398 ± 168 micro-organisms $cm^{-2}\,s^{-1}$). *C. albicans* GBJ 13/4A has the lowest initial deposition rate (163 ± 13 micro-organisms $cm^{-2}\,s^{-1}$). The adsorption of undiluted rhamnolipid solution on the silicone rubber reduces the initial deposition rate about 40% for *Staph. epidermidis* GB 9/6, *Staph. aureus* GB 2/1, *R. dentocariosa* GBJ 52/2B and *C. albicans* GBJ 13/4A, whereas a higher reduction of about 66% on average for *Strep. salivarius* GB 24/9 and *C. tropicalis* GB 9/9 was achieved. When coating the silicone rubber with the 1 : 1000 rhamnolipid dilution a minor effect was observed.

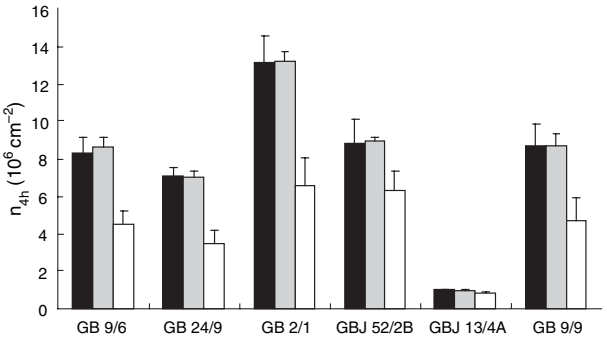

Figure 3 The number of micro-organisms adhering after 4 h (n_{4h}) on silicone rubber with and without an adsorbed rhamnolipid layer (1 : 1000 dilution and undiluted rhamnolipid). The codification of the micro-organisms is presented in Fig. 2. The undiluted rhamnolipid-containing solution used had a 4 g l^{-1} rhamnolipid concentration. Black column, without rhamnolipid; grey column, with rhamnolipid dilution 1 : 1000; and white column, with undiluted rhamnolipid. Results are averages of triplicate experiments varying within 10–15% (anova) and the standard deviation represented by the error bars.

The number of attached micro-organisms after 4 h on biosurfactant-coated and uncoated silicone rubber is shown in Fig. 3. the number of micro-organisms adhering to bare silicone rubber after 4 h (n_{4h}) was between 7×10^6 and 13×10^6 cm^{-2} for all micro-organisms studied except for *C. albicans* GBJ 13/4A that exhibit the lowest number of adhering micro-organisms (1×10^6 cm^{-2}). Coating the silicone rubber with a 1 : 1000 dilution of rhamnolipid solution resulted in a number of adhered cells after 4 h similar to the control. Reductions of 50% on the number of cells adhering after 4 h on the silicone rubber conditioned with undiluted rhamnolipid solutions was achieved for *Staph. epidermidis* GB 9/6, *Strep. salivarius* GB 24/9, *Staph. aureus* GB 2/1 and *C. tropicalis* GB 9/9. The yeast strain *C. albicans* GBJ 13/4A and the bacterial strain *R. dentocariosa* GBJ 52/2B showed a lower decrease in the number of attached cells after 4 h (20–28%).

Although the surface tension measurements give a clear indication of the surface activity of the compound it is not sufficient to determine whether it would produce an anti-adhesive effect against the selected micro-organisms. Table 2 summarizes contact angles measured with water on silicone rubber with and without an adsorbed rhamnolipid layer at several concentrations. The water contact angle on bare silicone rubber decreased from 115 ± 1 to 35 ± 1° after adsorption of the undiluted rhamnolipid. The adsorption of a rhamnolipid dilution up to 10^{-5} decreased the silicone rubber contact angle to a much lesser extent; to 89 ± 1°. From Table 2 it is possible to observe a concentration effect as the water contact angle of silicone rubber increases with the dilution factor of the rhamnolipid solution. Moreover, higher water contact angles values were achieved when a rinsing of the conditioned silicone rubber in PBS was performed. The adsorption of dilutions from 10^{-5} to 10^{-2} followed by a PBS rinsing did not produce any effect on the silicone rubber contact angle. The water contact angles results permitted the confirmation of the rhamnolipid concentration to use in the flow experiments.

The adherence of all the strains studied to the silicone rubber with the rhamnolipid biosurfactant was significantly different ($P < 0{\cdot}05$, ANOVA and Tukey's multiple comparison test) from the adhesion to the silicone rubber without the rhamnolipid, as proved by the *F*-values that are fivefold higher than the critical *F*-values (critical value extracted from the *f*-distribution in statistical tables). The replicates of adhesion experiments with and without adsorbed rhamnolipid for each strain were compared and found to be statistically similar. Adhesion (j_0, n_{4h}) on silicone rubber occurred in a larger extent and was several times faster than the adhesion on silicone rubber with adsorbed rhamnolipid ($P < 0{\cdot}05$, paired samples *t*-test).

Detachment protocol

Figure 4 shows the deposition kinetics of all the microbial strains tested and the subsequent effects of perfusing the rhamnolipid solution (1 : 15 dilution) through the parallel-plate flow chamber and then followed by the passage

Table 2 Contact angles measured at 25 °C on silicone rubber with and without an adsorbed rhamnolipid layer as determined by sessile drop technique. Mean ± SD were determined over three separate measurements

	Contact angles (°)	
Surface	Without rinsing in PBS	Rinsing in PBS
Silicone rubber	115 ± 1	115 ± 1
Silicone rubber + PBS	113 ± 1	113 ± 1
Silicone rubber + undiluted rhamnolipid	35 ± 1	55 ± 1
Silicone rubber + rhamnolipid (1 : 2 dilution)	39 ± 1	59 ± 1
Silicone rubber + rhamnolipid (1 : 5 dilution)	45 ± 1	86 ± 1
Silicone rubber + rhamnolipid (1 : 10 dilution)	57 ± 1	89 ± 1
Silicone rubber + rhamnolipid (1 : 15 dilution)	60 ± 1	94 ± 1
Silicone rubber + rhamnolipid (1 : 100 dilution)	65 ± 1	113 ± 2
Silicone rubber + rhamnolipid (1 : 1000 dilution)	71 ± 1	113 ± 2
Silicone rubber + rhamnolipid (1 : 100 000 dilution)	89 ± 1	113 ± 1

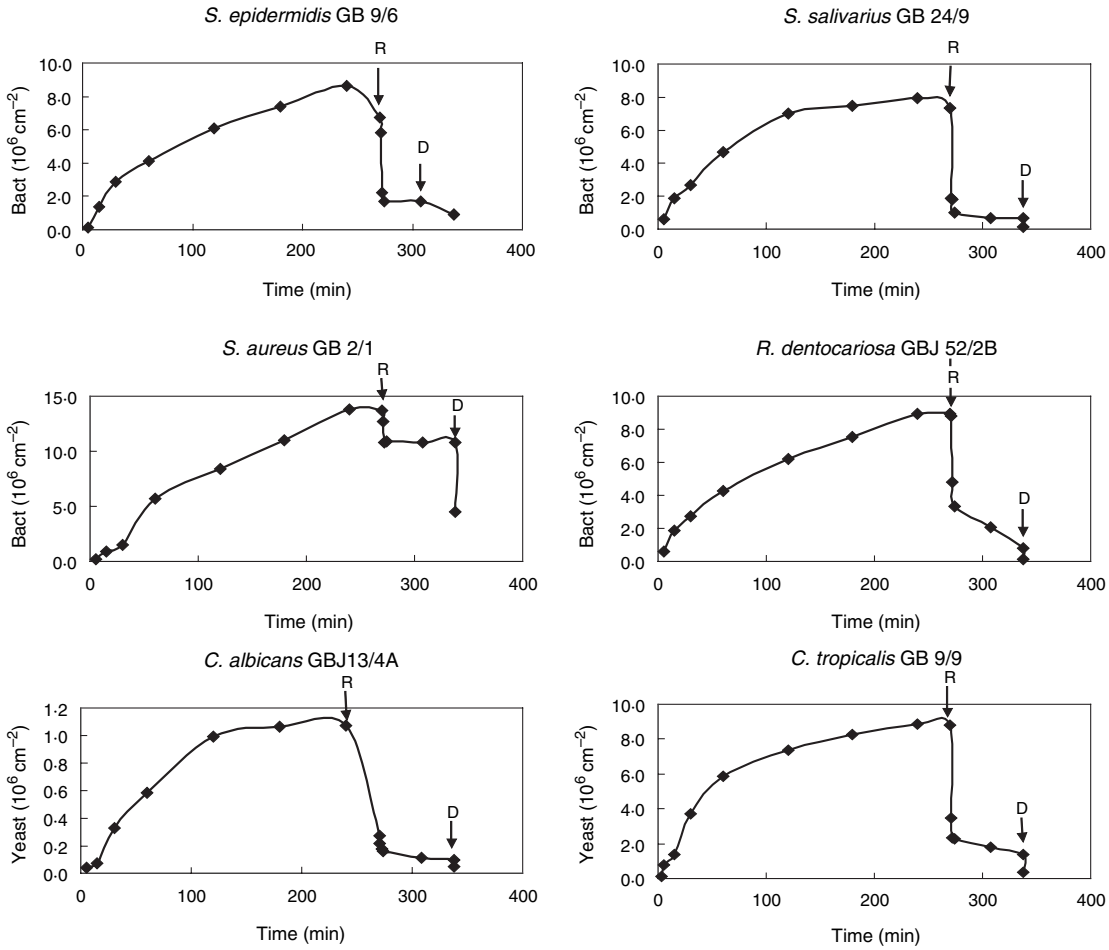

Figure 4 Deposition kinetics observed for the bacterial (*Staph. epidermidis* GB 9/6, *Strep. salivarius* GB 24/9, *Staph. aureus* GB 2/1 and *R. dentocariosa* GBJ 52/2B) and yeast (*C. albicans* GBJ 13/4A and *C. tropicalis* GB 9/9) strains isolated from explanted voice prostheses adhering to silicone rubber, and the subsequent effects of perfusing a rhamnolipid solution (dilution 1 : 15) through the chamber followed by the passage of a liquid–air interface. R denotes the perfusion of the flow chamber with the rhamnolipid solution. D denotes the passage of a liquid–air interface. Results are averages of duplicates experiments varying within 10–15% (anova).

of a liquid–air interface. Perfusion of the rhamnolipid resulted in 81% desorption on average of adhered cells for *Staph. epidermidis* GB 9/6, *Strep. salivarius* GB 24/9, *C. albicans* GBJ 13/4A and *C. tropicalis* GB 9/9. The bacterial strains *Staph. aureus* GB 2/1 and *R. dentocariosa* GBJ 52/2B showed a lower reduction in the number of adhering cells after 4 h, 21% and 63%, respectively. The combined effect of rinsing with the rhamnolipid solution and passing a liquid–air interface through the flow chamber produced maximum desorption percentages (96%) for all micro-organisms studied, except for *Staph. aureus* GB 2/1 that had the lowest percentage (67%).

Statistical analysis was conducted to compare adhesion experiments with and without rinsing rhamnolipid solution through the flow chamber followed by the passage of a liquid–air interface and it was found that the differences obtained are statistically significant (F-values > critical F-values), as proved by the F-values that are threefold higher than the critical F-values. Additionally, the comparison of duplicate experiments using one-way analysis of variance (ANOVA) resulted in a 10–15% variation in the results.

Discussion

The extent of adhesion of several microbial strains associated with biofilm formation on voice prostheses, to silicone rubber in the presence and absence of an adsorbed rhamnolipid layer obtained from *P. aeruginosa* DS10-129 were compared using a parallel-plate flow chamber system under defined hydrodynamic shear conditions. It is well known that the voice prostheses major weakness relies on

the fact that the hydrophobic silicone rubber surface becomes rapidly colonized with a thick biofilm and in this perspective the anti-fouling improvement of the silicone rubber material is desirable. New technologies for preventing or retarding biofilm formation on voice prostheses have to be developed as the long-term use of antimycotic or antibiotic agents may induce the growth of resistant strains with all associated risks. The use of biosurfactants as antimicrobial agents seems to be promising as a method of prolonging lifetimes of voice prostheses (Rodrigues *et al.* 2004a,b). Biosurfactants antimicrobial activity against various microbes, has been described before. Surfactin, for example, a cyclic lipopeptide produced by *B. subtilis* strains, is a biosurfactant with well-known antimicrobial properties (Ahimou *et al.* 2000). A new antibiotic from *Pseudomonas fluorescens*, with surface active properties different from those of the known biosurfactant viscosin from the same species, was also reported to have antifungal properties (Singh and Cameotra 2004). Additionally, a biosurfactant obtained from *Strep. thermophilus* A showed a significant antimicrobial activity against *C. tropicalis* GB 9/9 at low biosurfactant concentrations (Rodrigues *et al.* 2004a) and it has been reported that *C. tropicalis* is implicated in premature failure of the prostheses (Elving *et al.* 2002).

In the present case, the extent of bacterial adhesion to silicone rubber conditioned with a rhamnolipid produced by *P. aeruginosa* DS10-129 in a parallel-plate flow chamber was significantly reduced in comparison to the untreated silicone rubber. However, coating the silicone rubber with a 1 : 1000 dilution of the rhamnolipid solution resulted in a lower reduction in the initial deposition rates (lower than 26%) and the number of adhered cells was not affected. From the surface tension measurements it was expected that a 1 : 1000 rhamnolipid dilution would have an enormous anti-adhesive effect as the lowest surface tension of 32 ± 0.5 mN m^{-1} was achieved; however, in the parallel-plate flow chamber we did not observe this effect. The number of micro-organisms adhering after coating the silicone rubber with undiluted rhamnolipid solution was reduced between 40% and 66% for all strains studied that is less effective than the biosurfactant from *Lactococcus lactis* 53 described previously by Rodrigues *et al.* (2004b) that inhibited 90% the microbial adhesion for all strains studied, except for *R. dentocariosa* GBJ 52/2B, *C. albicans* GBJ 13/4A and *C. tropicalis* GB 9/9 that inhibited between 56% and 78%.

Although a very low concentration of the rhamnolipid solution was necessary to produce a high reduction in the surface tension it was observed that a relative high concentration is needed to coat the silicone rubber in order to achieve an effect in the adhesion. We believe that these results are a consequence of a washing out of the rhamnolipid solution layer adsorbed to the silicone rubber because the rhamnolipid is bond to the surface by Lifshitz–van der Waals forces that are relatively weak. This conclusion is based on the results obtained from the water contact angle measurements as we can clearly see a concentration effect as a result of the rhamnolipid adsorption onto the silicone rubber surface by the lowering of the contact angle with the rhamnolipid concentration increase. Moreover, a rinsing effect was observed as it is possible to see that for all rhamnolipid concentrations studied the rinsing procedure produced higher contact angles values than before.

The initial deposition rates and numbers of micro-organisms adhering in a stationary state are determined by a complicated interplay of hydrophobicity (interfacial free energies), electrostatic interactions, the relative prevalence of specific receptor sites on the microbial cell surfaces and possible biosurfactants produced (Landa *et al.* 1996). Often, high initial deposition rates of a given strain are found on hydrophobic substrates (for example, silicone rubber), presumably because of easy removal of interfacial water from in between interacting surfaces, facilitating close approach and adhesion of an organism. Biofilm formation on solid surfaces, for most micro-organisms, occurs in direct proportion to the hydrophobicity of the surface, provided that the suspended medium is a simple buffer. Surface-active agents reduce hydrophobic interactions and by doing so reduce microbial adhesion to silicone rubber (Klotz 1990). Clearly, in the detachment protocol, rinsing the flow chamber with the rhamnolipid solution appears effective in disrupting the bond between initially adhering micro-organisms and the silicone rubber. Interestingly, the combined effect of rinsing with the rhamnolipid solution and passing a liquid–air interface through the flow chamber produced 96% reduction on the number of adhered cells for five out of six micro-organisms studied, except for *Staph. aureus* GB 2/1 that exhibit only 67%. It has been demonstrated that the effect of passing an air–liquid interface through adherent cells in the parallel flow chamber induces a shear force of about 10^{-7} N, which is in the range of the adhesion strength evaluated by atomic force microscopy and results in removal of a large proportion of the adherent organisms (Fang *et al.* 2000).

Microbial detachment stimulated by the rhamnolipid solution seems to be more likely because of a proper detergent system rather than an antimicrobial effect. Surface active agents as incorporated into oral hygiene products have not received the attention they merit and have mostly been included into oral hygiene products for their ability to create foam (Landa *et al.* 1996). Davey *et al.* (2003) proposed that rhamnolipids are able to modulate cell-to-cell and cell-to-surface interactions, thus inhibiting planktonic cells from attaching to a performed

biofilm and inducing the detachment of the biofilm. Rhamnolipids are among the best known biosurfactants and it is well known that there are different structural variants of rhamnose lipids, as the type produced depends on the *Pseudomonas* strain, the carbon source used and the strategy of production (Desai and Banat 1997, Banat *et al.* 2000, Rahman *et al.* 2002b). Also, rhamnolipids are known as potent antimicrobial compounds against several micro-organisms (Bai *et al.* 1997, Abalos *et al.* 2001, Benincasa *et al.* 2004). In the cosmetic industry, rhamnolipids have been useful because they are compatible with skin and produce extremely low irritation (Haba *et al.* 2000). Benincasa *et al.* (2004) have demonstrated that the rhamnolipid obtained from *P. aeruginosa* LBI has antimicrobial activity against several micro-organisms, thus suitable for application in the pharmaceutical and cosmetic industries. Additionally, rhamnolipids are food grade as they are a source of stereospecific L-rhamnose that is used commercially in the production of high-quality flavour compounds (Makkar and Cameotra 2002). Nevertheless, some rhamnolipids have been implicated as virulence factors and influence the immune response (Cosson *et al.* 2002, Rossolini and Mantengoli 2005). Low concentrations of purified *P. aeruginosa* rhamnolipids were found to induce changes in fluid transport across airway surface epithelia, thus encouraging colonization by increasing the number of binding sites for *P. aeruginosa* (Graham *et al.* 1993, Evans *et al.* 1998). Moreover, rhamnolipids were found to be ciliostatic factors that may enable *P. aeruginosa* to more easily colonize the respiratory tract predisposing the lung to infection (Hingley *et al.* 1986, Read *et al.* 1992). Therefore, further work has to be done in order to guarantee that the studied rhamnolipid will not produce undesirable effects in the oropharyngeal environment. As the detachment results obtained in this study were very promising it would be advantageous to evaluate the biocompatibility of the rhamnolipid for therapeutical application.

In conclusion, we consider that biosurfactants may have the potential to be used as one of the preventive strategies to delay the onset of biofilm growth on catheters and other implant materials; thus decreasing the large number of hospital infections without increased use of synthetic drugs or chemicals. They may also be used in pulmonary immunotherapy and incorporated into probiotic preparations to combat urogenital tract infections. It has been demonstrated in this study that using a rinse of rhamnolipid from *P. aeruginosa* DS10-129 through a flow chamber significantly reduces the adhesion of a variety of bacterial and yeast strains isolated from explanted voice prostheses to silicone rubber, and therefore may be used as a biodetergent solution for prostheses cleaning, prolonging their lifetime and directly benefiting laryngectomized patients.

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