Inhibition of microbial adhesion to silicone rubber treated with biosurfactant from *Streptococcus thermophilus* A

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Abstract

Microbial adhesion of four bacterial and two yeast strains isolated from explanted voice prostheses to silicone rubber before and after conditioning with a biosurfactant obtained from the probiotic bacterium *Streptococcus thermophilus* A was investigated in a parallel plate flow chamber. The silicone rubber with and without an adsorbed biosurfactant layer was characterized using contact angle measurements. Water contact angles indicated that the silicone rubber surface with adsorbed biosurfactant was more hydrophilic (58°) than bare silicone rubber (109°). The results obtained showed that the biosurfactant was effective in decreasing the initial deposition rates, and the number of bacterial cells adhering after 4 h, for all microorganisms tested. A decrease in the initial deposition rate was observed for *Rothia dentocariosa* GBJ 52/2B and *Staphylococcus aureus* GB 2/1 from 1937 ± 194 to 179 ± 21 microorganisms $cm^{-2} s^{-1}$ and from 1255 ± 54 to 233 ± 26 microorganisms $cm^{-2} s^{-1}$, respectively, accounting for an 86% reduction of the initial deposition rate for both strains. The number of bacterial cells adhering to the silicone rubber with preadsorbed biosurfactant after 4 h was further reduced by 89% and 97% by the two strains, respectively. The two yeast strains tested showed less reduction in adhesion after 4 h, to values between 67% and 70%. Such a pretreatment with surface-active compounds may constitute a promising strategy to reduce the microbial colonization rate of silicone rubber voice prostheses.

Introduction

Patients who have undergone a laryngectomy due to a malignant laryngeal tumor need to breath through a tracheostoma and receive voice prostheses for speech rehabilitation (van der Mei *et al.*, 1999). Silicone rubber is an ideal material for manufacturing voice prostheses because of its ease of molding and excellent mechanical properties, but in laryngectomized patients, the hydrophobic silicone rubber surface becomes colonized rapidly with a thick biofilm, consisting of a variety of bacterial and yeast strains (Neu *et al.*, 1993). Biofilm formation poses a severe problem once the biofilm has extended towards the valve of the prosthesis, leading to either blocking or leakage with the consequent loss of function of the device and inevitable need for replacement on average every 3–4 months (Busscher *et al.*, 1997). It has been suggested by laryngectomized patients that the consumption of dairy products may positively influence the lifetime of their prostheses (van der Mei *et al.*, 1999). Active bioyogurt containing active *Streptococcus thermophilus* has been suggested to have beneficial effects in prolonging the lifetime of indwelling voice prostheses. The mechanism by which this occurs has not been investigated, but it is hypothesized that the presence of *S. thermophilus* and *Lactobacillus bulgaricus*, two well-known probiotic bacterial strains, in active bioyogurt may interfere with the adhesion of yeast to the silicone rubber (van der Mei *et al.*, 1999, 2000). In addition, it has been demonstrated that dairy *S. thermophilus* strains produce biosurfactants causing their own desorption (Busscher *et al.*, 1994) and reduction of yeasts adhesion to silicone rubber in a parallel plate flow chamber system (Busscher *et al.*, 1997), as well as interference with the formation of a mixed fungal/bacterial biofilm on silicone rubber voice prostheses in the modified Robbins device (Busscher *et al.*, 1999).

Biosurfactants are biological surface-active compounds produced by some microorganisms and can have some influence on interfaces. They include a wide variety of

chemical structures such as glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipids (Desai & Banat, 1997). With regard to an antiadhesive 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 (Desai & Banat, 1997). Biosurfactants have also been reported to have various degrees of antimicrobial activity (Banat *et al.*, 2000). Several biosurfactants have strong antibacterial, antifungal and antiviral activity (Singh & Cameotra, 2004).

In this paper, we report on the use of a biosurfactant produced by the probiotic bacterium *S. thermophilus* A in the reduction of the adhesion ability of four bacterial and two yeast strains isolated from explanted voice prostheses to silicone rubber.

Materials and methods

Biosurfactant production

An overnight subculture (10 mL) of *S. thermophilus* A grown in optimized M17 broth was used to inoculate a second culture (600 mL). Cells were harvested by centrifugation (10 000 *g*, 5 min, 10 °C), washed twice in demineralized water and resuspended in 100 mL of phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 pH 7.0, 150 mM NaCl). Crude biosurfactant extract was produced by gently stirring this suspension for 2 h at room temperature. Subsequently, the bacteria were removed by centrifugation and the remaining supernatant was filtered through a 0.22-μm pore-size filter (Millipore, Billerica, USA). The supernatant was dialyzed against demineralized water at 4 °C using a Spectrapor membrane tube (molecular weight cut-off 6000–8000, Spectrum Medical Industries Inc., Houston, CA, USA), freeze-dried, and then used in the experiments.

Biosurfactant surface-activity determination

Axisymmetric drop shape analysis by profile (ADSA-P) is a technique for determining liquid surface tensions based on the shape of an axisymmetric droplet on a solid substratum. This technique was used as an indirect yet a reliable estimation of the presence of a suitable biosurfactant concentration. In order to measure the surface-activity of the biosurfactant obtained by ADSA-P, a 100-μL droplet of a biosurfactant solution was placed on fluoroethylene-propylene (FEP)-Teflon (Fluorplast, Raamsdonkveer, The Netherlands) in an enclosed chamber to prevent evaporation from the droplet. The shape of the droplet was monitored for 2 h at room temperature and the surface tension of the droplet was calculated from its shape as a function of time as described by van der Vegt *et al.* (1991). All values presented are the averages of at least triplicate 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. All strains were first grown overnight at 37 °C in ambient air on an agar plate from a frozen stock. The agar plate was kept at 4 °C for a maximum of 2 weeks. Several colonies were used to inoculate 10 mL of brain heart infusion broth (BHI, OXOID, Basingstoke, UK) 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 for 18 h. The microorganisms from the second culture were harvested by centrifugation at 10 000 *g* for 5 min and washed twice with demineralized water. Subsequently, bacterial cells were resuspended in 200 mL PBS, after sonication on ice (10 s), to a concentration of 3×10^8 mL^{-1}. Yeast cells were resuspended in PBS to a concentration of 3×10^6 mL^{-1}. A Bürker–Türk counting chamber was used to count the cells.

Contact angle measurements

Advancing type contact angles with Millipore water on silicone rubber with and without an adsorbed biosurfactant layer were measured with a locally manufactured contour monitor using the sessile drop technique (van Oss *et al.*, 1988). On each sample, at least five droplets were placed at different positions and results of three separately prepared surfaces with adsorbed biosurfactant were averaged.

Adhesion experiments

The flow chamber and image analysis system have been described in detail elsewhere (Sjollema *et al.*, 1989). Images were taken from the bottom plate (5.5×3.8 cm) of the parallel plate flow chamber, which consisted of a thin sheet of silicone rubber (with or without an adsorbed biosurfactant layer) affixed to a polymethylmethacrylate (PMMA) plate. The top plate of the chamber was made of glass.

Conditioned silicone rubber was prepared through overnight immersion in a 3 mg mL^{-1} biosurfactant solution at 4 °C. This concentration was estimated by ADSA-P as described by van der Vegt *et al.* (1991) as the one that allowed for a reasonable reduction in the surface tension (18.6 mN m^{-1}) and that would be expected to interfere in adhesion.

Prior to each experiment, all tubes and the flow chamber were filled with PBS, and 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 flows were started, all fluids would circulate by hydrostatic pressure through the chamber at the desired shear rate of $10\,s^{-1}$ ($0.025\,mL\,s^{-1}$), which 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 biosurfactant layer. The initial increase in the number of adhering microorganisms with time was expressed in a so-called initial deposition rate j_0 (microorganisms $cm^{-2}\,s^{-1}$), i.e. the number of adhering microorganisms per unit area and time. The number of adhering microorganisms after 4 h was also determined (n_{4h}). All values presented in this work are the averages of at least three measurements on silicone rubber surfaces with or without the adsorbed biosurfactant layer, and were carried out with separately grown microorganisms.

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%.

Results

The water contact angle measured at 25 °C on untreated silicone rubber decreased from $109 \pm 2^{\circ}$ to $58 \pm 5^{\circ}$ after adsorption of the biosurfactant, which means that the biosurfactant makes the silicone rubber surface less hydrophobic.

The liquid surface tensions of the freeze-dried biosurfactant were measured using ADSA-P as a dilution series from 50 to 0.1 mg mL^{-1}. The surface tension measured reached after 2 h is plotted against the biosurfactant concentration in Fig. 1. The biosurfactant reached the lowest surface tension of $40\,mJ\,m^{-2}$ with a concentration of $10\,mg\,mL^{-1}$. Although the surface tension measurements give a clear indication of the surface activity of the compound, they are not sufficient to determine whether it would produce an antiadhesive effect against the selected microorganisms. Therefore, previous results of an inhibition test (Rodrigues *et al.*, 2004a) were considered for the selection of the biosurfactant concentration to use in the flow experiments. Rodrigues *et al.* (2004a) evaluated the antimicrobial activity

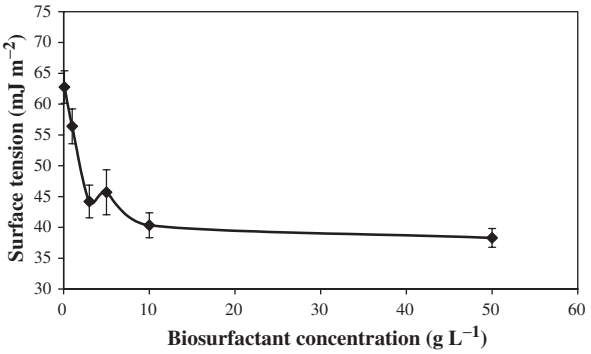

Fig. 1. Surface tension of crude biosurfactant concentrations obtained from *Streptococcus thermophilus* A in PBS (pH 7.0) after 2 h as measured by axisymmetric drop shape analysis by profile. Results are averages of triplicate experiments varying within 3%–8% (ANOVA) and the standard deviation is represented by error bars.

of the biosurfactant at several concentrations against a variety of bacterial and yeast strains isolated from explanted voice prostheses, and found that the biosurfactant is an antimicrobial agent, but there are different effective concentrations depending on the microorganism. Thus, in the adhesion experiments a biosurfactant concentration of 3 mg mL^{-1} was used because a surface tension decrease of 19 mJ m^{-2} was assumed to be enough to give an effect in adhesion.

The initial deposition rates (Fig. 2) of *R. dentocariosa* GBJ 52/2B, *S. epidermidis* GB 9/6 and *C. tropicalis* GB 9/9 on silicone rubber were relatively high, between 1937 ± 194, 2105 ± 234 and 2598 ± 227 microorganisms $cm^{-2}\,s^{-1}$, whereas *S. salivarius* GB 24/9 and *S. aureus* GB 2/1 deposit at a slower rate onto silicone rubber (1200–1600 micro-

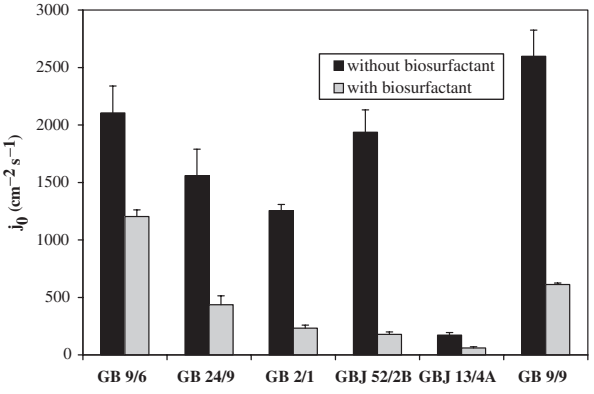

Fig. 2. The initial deposition rates (j_0) of the bacterial (*Staphylococcus epidermidis* GB 9/6, *Streptococcus salivarius* GB 24/9, *Staphylococcus aureus* GB 2/1 and *Rothia dentocariosa* GBJ 52/2B) and yeast (*Candida albicans* GBJ 13/4A and *Candida tropicalis* GB 9/9) strains isolated from explanted voice prostheses on silicone rubber with and without an adsorbed biosurfactant layer. Results are averages of triplicate experiments varying within 10%–15% (ANOVA) and the standard deviation is represented by error bars.

organisms $cm^{-2} s^{-1}$) and *C. albicans* GBJ 13/4A has by far the lowest initial deposition rate (172 ± 22 microorganisms $cm^{-2} s^{-1}$). Adsorption of biosurfactant on the silicone rubber reduces the initial deposition rate to about 86% for *R. dentocariosa* GBJ 52/2B and *S. aureus* GB 2/1. A reduction of about 75% on average for *S. salivarius* GB 24/9 and *C. tropicalis* GB 9/9 was achieved. The initial deposition rates of *S. epidermidis* GB 9/6 and *C. albicans* GBJ 13/4A are far less reduced (43% and 65%, respectively) in the presence of the biosurfactant than the initial deposition rates of the other strains.

The numbers of microorganisms adhering to silicone rubber after 4 h, (n_{4h}, Fig. 3) varied between 7×10^6 and 15×10^6 cm^{-2} for all the microorganisms studied except for *C. albicans* GBJ 13/4A, which exhibited the lowest value of adhering microorganisms (1×10^6 cm^{-2}). The numbers of bacterial and yeast cells adhering to silicone rubber treated with biosurfactant after 4 h were significantly reduced by 89–97% and 67–70%, respectively.

The replicates of adhesion experiments with and without adsorbed biosurfactant for each strain were compared and found to be statistically similar ($P < 0.05$, ANOVA and Tukey's multiple comparison test). Differences in mean values by group that resulted in P-values < 0.05 were considered to be statistically significant. The adherence of all the strains studied was significantly different ($P < 0.05$, ANOVA and Tukey's multiple comparison test), when comparing the adhesion achieved after 4 h and also the initial deposition rate, for experiments done with and without adsorbed biosurfactant. The adhesion (j_0, n_{4h}) on silicone rubber was several times higher than the adhesion on silicone rubber with adsorbed biosurfactant ($P < 0.05$, paired samples t-test).

Furthermore, adhesion of all the strains studied was significantly different when comparing experiments done with and without adsorbed biosurfactant, with the F-values being five-fold higher than the critical F-values (critical value extracted from the f-distribution in statistical tables).

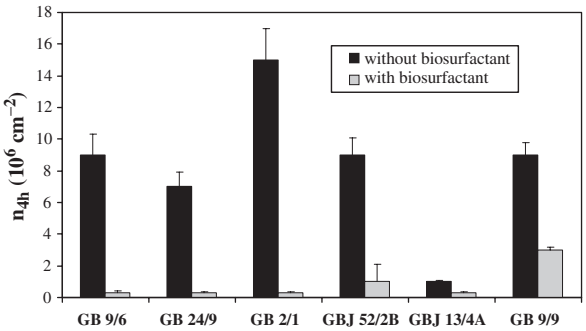

Fig. 3. The number of microorganisms adhering after 4 h (n_{4h}) on silicone rubber with and without an adsorbed biosurfactant layer. The microorganisms used are as described in Fig. 2. Results are averages of triplicate experiments varying within 10–15% (ANOVA) and the standard deviation is represented by the error bars.

Discussion

Our aim was to evaluate the extent of adhesion of several microbial strains involved in the biofilm formation on voice prostheses to silicone rubber in the absence and presence of an adsorbed biosurfactant layer obtained from *S. thermophilus* A using a parallel plate flow chamber system.

The two main reasons for the frequent replacement of silicone rubber voice prostheses are patients' complaints about leakage of esophageal contents into the trachea and increasing efforts needed to produce phonation. Both are signs of failure of voice prostheses and are due to biofilm formation (Neu *et al.*, 1993). An antifouling improvement of the silicone rubber material is therefore a highly desirable outcome. In the case of laryngectomized patients, the voice prostheses' lifetimes are usually less than 2 months and employing 'antibiofilm' therapy at the time of insertion of the voice prostheses therefore is usually necessary. Using antimycotics or antibiotics is undesirable due to the risk of inducing the production of resistant strains (Mahieu *et al.*, 1986; van Weissenbruch *et al.*, 1997). Research into the development of new methods for preventing or retarding biofilm formation on voice prostheses is therefore increasing.

Biosurfactants can exhibit antimicrobial activity against various microorganisms (Singh & Cameotra, 2004). One of the earliest noted antimicrobial activities of biosurfactants was that of iturin A, a potential antifungal lipopeptide produced by strains of *Bacillus subtilis*. Surfactin, a cyclic lipopeptide produced by *B. subtilis* strains, is another 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 later identified and named viscosinamide, which was also found to have antifungal properties (Singh & Cameotra, 2004). Interestingly, Rodrigues *et al.* (2004a) found that a biosurfactant obtained from *S. thermophilus* A showed a significant antimicrobial activity against *C. tropicalis* GB 9/9 at low biosurfactant concentrations and it has been demonstrated that *C. tropicalis* contributes to the premature failure of the prostheses (Elving *et al.*, 2002). The use of biosurfactants as antimicrobial agents may represent therefore a promising technique for prolonging the lifetime of voice prostheses.

In the present study, it has been demonstrated that the extent of bacterial adhesion to silicone rubber conditioned with a biosurfactant produced by *S. thermophilus* A in a parallel plate flow chamber during the first 4 h was significantly decreased with respect to untreated silicone rubber.

Similarly, the number of adhered yeast cells was also decreased in the presence of the biosurfactant to a lesser extent in comparison to bacteria. This reduction of adhesion may have been improved if higher concentrations of biosurfactant were used. The ADSA-P results demonstrated that a 23 mJ m^{-2} reduction on surface tension is possible with a biosurfactant concentration of 10 mg mL^{-1} compared with the 19 mJ m^{-2} reduction obtained with the concentration (3 mg mL^{-1}) used in these experiments. The involvement of biosurfactants in microbial adhesion, as demonstrated in this study, has been described previously. The use of a biosurfactant from *Lactococcus lactis* 53 as an antimicrobial and/or antiadhesive agent and its ability to inhibit adhesion in a parallel plate flow chamber of various microorganisms isolated from explanted voice prostheses has been demonstrated by Rodrigues *et al.* (2004b). Given that inhibition of initial microbial adhesion occurs in the first hours, we only considered an exposure time of 4 h for the adhesion experiments. However, in a previous work performed in the artificial throat model, the exposure time was up to 8 days (Rodrigues *et al.*, 2004a) and the cycle of nutrients provided during this experiment was essential for growing biofilms with features as found on explanted prostheses. The performance of the biosurfactant in the artificial throat model confirmed the results presented in this study, and although the biosurfactant was not tested for exposure times longer than 8 days, the results achieved allow us to conclude that this is a coating that resists body fluids, as no biological degradation was observed.

Biosurfactants have the potential to be used as a preventive strategy 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 can also be used in pulmonary immunotherapy and incorporated into probiotic preparations to combat urogenital tract infections (Singh & Cameotra, 2004). Biofilm formation on solid surfaces, for most microorganisms, 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 as a consequence diminish microbial adhesion to silicone rubber (Klotz, 1990). The use of preadsorbed biosurfactants to silicone rubber is believed to interfere with the formation of the conditioning film and adhesion of the first micro-organisms, thus preventing or retarding biofilm formation (Busscher *et al.*, 1995; Rodrigues *et al.*, 2004b). Consequently, biofilm formation can be influenced by adjusting the properties of the voice prostheses material or by surface modification. It has been demonstrated in this study that the adsorption of the biosurfactant from *S. thermophilus* A to silicone rubber significantly reduces the water contact angle, thus reducing the hydrophobic interactions and the ability of microbial adhesion to silicone rubber and its general surface properties. As a consequence, adsorbing biosurfactants to the voice prostheses may increase their lifetime, which would directly benefit laryngectomized patients.

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