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Streptococcus thermophilus and Its Biosurfactants Inhibit Adhesion by *Candida* spp. on Silicone Rubber

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The adhesion of yeasts, two *Candida albicans* and two *Candida tropicalis* strains isolated from naturally colonized voice prostheses, to silicone rubber with and without a salivary conditioning film in the absence and presence of adhering *Streptococcus thermophilus* B, a biosurfactant-releasing dairy isolate, was studied. Coverage of 1 to 4% of the surface of silicone rubber substrata with adhering *S. thermophilus* B gave significant reductions in the initial yeast adhesion regardless of the presence of a conditioning film. Mechanistically, this interference in yeast adhesion by *S. thermophilus* B was not due to direct physical effects but to biosurfactant release by the adhering bacteria, because experiments with *S. thermophilus* B cells that had released their biosurfactants prior to adhesion to silicone rubber and competition with yeasts did not show interference with initial yeast adhesion. The amounts of biosurfactants released were highest for mid-exponential- and early-stationary-phase bacteria (37 mg · g of cells^{-1} [dry weight]), but biosurfactants released by stationary-phase bacteria (14 mg · g of cells^{-1} [dry weight]) were the most surface active. The crude biosurfactants released were mixtures of various components, with a glycolipid-like component being the most surface active. A lipid-enriched biosurfactant fraction reduced the surface tension of an aqueous solution to about 35 mJ · m^{-2} at a concentration of only 0.5 mg · ml^{-1}. The amount of biosurfactant released per *S. thermophilus* B cell was estimated to be sufficient to cover approximately 12 times the area of the cross section of the bacterium, making biosurfactant release a powerful defense weapon in the postadhesion competition of the bacterium with microorganisms such as yeasts. Preadsorption of biosurfactants to the silicone rubber prior to allowing yeasts to adhere was as effective against *C. albicans* GB 1/2 adhesion as covering 1 to 2% of the silicone rubber surface with adhering *S. thermophilus* B, but a preadsorbed biosurfactant layer was less effective against *C. tropicalis* GB 9/9.

Patients after laryngectomy due to a malignant laryngeal tumor have to breathe through a tracheostoma and receive a voice prosthesis for speech rehabilitation. Voice prostheses are implanted as a shunt-valve between the digestive tract and the trachea. By closing the tracheostoma with a finger, patients can direct an airflow through the valve into the oropharyngeal region, where remaining muscular structures act as pseudovocal cords. Silicone rubber is an ideal material for manufactured 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 (17, 20). Bacteria isolated from voice prostheses originate from either the skin (staphylococci) or the oral cavity (streptococci), while *Candida albicans* and *Candida tropicalis* are the main yeast strains isolated (25, 28). Especially in the case of indwelling voice prostheses, biofilm formation poses a severe and costly problem since once the biofilm has extended towards the valve of the prosthesis, either blocking or leakage occurs, with the consequent loss of function of the device and making replacement inevitable. On explanted prostheses, bacteria have been found predominantly on the valve side, but yeasts also grow into the silicone rubber (6, 26, 27), thereby greatly increasing their adhesive forces; this makes them difficult to detach by the naturally occurring shear forces in the esophagus. Biofilm formation limits the average lifetime of indwelling silicone rubber voice prostheses to 3 to 4 months on average (40). Attempts to increase the lifetime of indwelling voice prostheses are for this reason mainly aimed at reducing the adhesion of yeasts to the silicone rubber (21).

There is anecdotal evidence among patients that the consumption of buttermilk, which contains antimycotic-releasing *Lactococcus lactis* spp. (1), prolongs the lifetime of indwelling voice prostheses. Recently, suggestions that the consumption of 2 kg of Turkish yogurt per day effectively eliminates biofilm formation on indwelling voice prostheses have been raised. The mechanism by which this occurs has never been investigated, but it is hypothesized that the presence of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, two well-known probiotic bacterial strains (13, 38), in Turkish yogurt may interfere with the adhesion of yeasts to the silicone rubber. Lactobacilli have long been known for their capacity to interfere with the adhesion of uropathogens to epithelial cells (3, 33) and catheter materials (14), and the mechanisms of this interference have been demonstrated to include, among others, the release of proteinaceous biosurfactants (43). Also, *S. thermophilus* releases biosurfactants (8, 9) that are suggested to interfere with their own adhesion to substratum surfaces (5). Biosurfactants released by *S. thermophilus* strains have not been extensively studied, but thin-layer chromatography (TLC) has indicated that the crude product is a mixture of various components. One of the components of the product released by *S. thermophilus* B decreased the surface tension of water from 72 mJ · m^{-2} to approximately 42 mJ · m^{-2} at a concentration of only 0.1 mg · ml^{-1} (9), which makes it a powerful surfactant compared with many synthetic surfactants. The release of biosur-

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factants by *S. thermophilus* isolates is maximal when sucrose is added to the growth medium (8).

The aim of the present study was to determine whether biosurfactant release by *S. thermophilus* might constitute a mechanism by which the lifetime of indwelling, silicone rubber voice prostheses can become prolonged. To this end, the adhesion of different yeast strains, isolated from Groningen button voice prostheses, to silicone rubber in the absence and presence of biosurfactant-releasing *S. thermophilus* B cells was studied. Also, the effects of isolated biosurfactants adsorbed to silicone rubber on adhesion of yeasts to silicone rubber were studied. The biophysical and biochemical nature of the biosurfactants was identified by axisymmetric drop shape analysis by profile (ADSA-P), Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), and TLC.

MATERIALS AND METHODS

Yeast strains. The yeast strains used in this study were isolated from an explanted voice prostheses, the Groningen button, and identified on the basis of API ID 32C (BioMérieux SA, Marcy-l'Etoile, France) (28). Two *C. albicans* and two *C. tropicalis* strains were cultured overnight in brain heart infusion broth at 37°C (Oxoid, Basingstoke, United Kingdom) from an agar plate. This culture was used to inoculate a second culture, which was grown for 16 h prior to harvesting. Strains were cultured in ambient air at 37°C.

The microorganisms were harvested by centrifugation (5 min at 10,000 × *g*), washed twice with Millipore-filtered, distilled water, and resuspended in adhesion buffer (2 mM potassium phosphate, 50 mM potassium chloride, 1 mM calcium chloride [pH 6.8]) to a concentration of 10^6 per ml as determined directly in a Bürker-Türk counting chamber. Chemicals were from Merck (Darmstadt, Germany).

***S. thermophilus*, growth conditions, and biosurfactant release.** *S. thermophilus* B, isolated from heat exchanger plates in the downward section of a pasteurizer (4), was kindly provided by A. H. Weerkamp (NIZO, Ede, The Netherlands) and additionally identified by us by the method of Bergey. Bacteria were stored in M17 broth (Oxoid), supplemented with 1% sucrose, containing 7% (vol/vol) dimethyl sulfoxide at −60°C. For each experiment, subcultures (10 ml) were prepared by inoculating M17 broth, supplemented with 1% sucrose, with bacteria from a frozen stock (1% inoculum) and incubated overnight at 37°C.

For biosurfactant production, an overnight subculture (10 ml) was used to inoculate a second culture (1,400 ml). Cells were harvested in the mid-exponential, early stationary, and stationary phases (16 h of growth) by centrifugation at 4,000 × *g*, washed twice in water, and resuspended in 200 ml of water. Crude biosurfactant was produced by gently stirring this suspension for 2 h at room temperature. Subsequently, the microorganisms and the biosurfactants released were separated by centrifugation at 10,000 × *g*. To ensure the complete removal of all cell remnants, the supernatant was centrifuged twice at 10,000 × *g*. After the final centrifugation, both the cellular pellet and the crude biosurfactant were freeze-dried and weighed, and the crude biosurfactant was stored at −20°C for further experiments.

For deposition experiments, a second culture (200 ml) was incubated with 10 ml of an overnight subculture. After 16 h, cells were harvested by centrifugation at 4,000 × *g*, washed two times in water, and resuspended in water. To break bacterial chains, the bacterial suspension was sonicated for 30 s at 30 W (Vibra Cell model 375; Sonics and Materials Inc., Danbury, Conn.). Sonication was done intermittently while cooling in an ice-water bath. Finally, cells were suspended in adhesion buffer. The cell concentration was fixed at a density of 3×10^8 cells · ml of buffer^{-1} with the aid of a Bürker-Türk counting chamber.

Silicone rubber. A Silastic medical-grade silicone rubber (Q7-4750; Nusil, Anglet, France) kit was purchased, and 0.4-mm-thick plates (50 by 76 mm) were produced by the procedures suggested by the manufacturer. Briefly, equal proportions of part A and part B were thoroughly blended and injected into a mold at room temperature through a 3-mm-diameter opening with a force of 3 tons. Subsequently, the silicone rubber was immediately cured at 200°C for 50 min. Finally, samples were cleaned in a 2% RBS 35 detergent solution (Societé des Traitements Chimiques de Surface, Lambersat, France) under simultaneous sonication (5 min, 150 W) and thoroughly rinsed in water, absolute methanol (>96%), and water again.

Saliva. From 10 healthy volunteers of both sexes, human whole saliva was collected in ice-chilled cups. Saliva was stimulated by having the volunteers chew Parafilm (3M-Company, Minneapolis, Minn.). After the saliva was pooled and centrifuged at 10,000 × *g* for 5 min at 10°C, phenylmethylsulfonyl fluoride (0.2 M; Merck) was added to a final concentration of 1 mM as a protease inhibitor. The solution was again centrifuged, dialyzed overnight at 4°C against water, and freeze-dried for storage. A solution of 1.5 mg of freeze-dried stock ml^{-1} in adhesion buffer (see above) was designated (reconstituted human whole) saliva.

Characterization of biosurfactants. Lipids were extracted from the freeze-dried biosurfactants by the method of Bligh and Dyer (2). Briefly, crude stationary-phase biosurfactants released by *S. thermophilus* B were mixed with 4 ml of water, 5 ml of chloroform, and 10 ml of methanol. After shaking for 5 min, the mixture was filtered through Whatman no. 1 filter paper with slight suction. The residue on the filter was homogenized with 5 ml of chloroform and filtered once more. The residue on the filter was dissolved in water, freeze-dried, and weighed.

The two filtrates were combined, 5 ml of water was added, and the mixture was shaken thoroughly before separation of the layers was allowed. The upper methanol-water layer was removed by aspiration, rotoevaporated until an aqueous solution was left, and subsequently freeze-dried and weighed. The lower chloroform layer, containing the lipid fraction, was rotoevaporated, resuspended in chloroform-methanol-water (4:3:1, vol/vol), and again rotoevaporated until a cloudy aqueous emulsion was formed. This emulsion was freeze-dried and weighed. All freeze-dried material was stored at −20°C.

ADSA-P was performed as described by Noordmans and Busscher (30) to determine the surface tensions of biosurfactant solutions. Briefly, ADSA-P involves digitizing the circumference of a liquid droplet on a solid surface by means of a contour monitor. The circumference of the droplet is fitted to the Laplace equation of capillarity (36), yielding the surface tension of the biosurfactant solution. Droplets with biosurfactant dissolved in water with a volume of approximately 100 μl were placed on fluoroethylenepropylene (FEP-Teflon). Measurements on one solution droplet were done after 2 h to allow equilibration of the interface in an enclosed chamber at room temperature. To prevent evaporation, a small water reservoir was placed in the chamber to create conditions of saturated vapor. One liquid profile was recorded twice with a minimal time interval between (<0.5 s), and the ADSA-P surface tensions were averaged. This procedure was carried out in duplicate with separate liquid droplets. ADSA-P was done on the crude biosurfactant released and on the three fractions obtained after lipid extraction as a function of biosurfactant concentration.

For FTIR, 2 mg of the crude biosurfactant or 0.1 mg of the fractions obtained after lipid extraction was combined with 100 mg of KBr and pressed with 7,500 kg of weight for 30 s to obtain translucent tablets. Infrared absorption spectra were recorded on a FTS-175 spectrometer from Bio-Rad Laboratories (Richmond, Calif.) with a spectral resolution and a wave number accuracy of 4 and 0.01 cm^{-1}, respectively. All measurements consisted of 100 scans, with a KBr tablet as background.

For XPS, 100-μl droplets of crude biosurfactants or extracted fractions dissolved in water (±10 mg · ml^{-1}) were placed on gold-coated glass slides (1 by 1 cm). After air drying, the glass slides were inserted into the chamber of the spectrometer (S-probe; Surface Science Instruments, Mountain View, Calif.). The residual pressure in the spectrometer during operation was approximately 10^{-9} Pa. A magnesium anode was used for X-ray production (10 kV, 22 mA) at a spot size of 250 by 1,000 μm. After scans of the overall spectrum in the binding energy range of 1 to 1,200 eV at low resolution (150-eV pass energy) had been taken, peaks over a 20-eV binding energy range were recorded at high resolution (50-eV pass energy) in the order C_{1s} (four scans), O_{1s} (four scans), N_{1s} (eight scans), P_{2p} (eight scans), and C_{1s} again to be able to account for contamination or deterioration under X-rays of the samples.

The carbon peak was split by a least-squares fitting program into four Gaussian components at 284.8 (C_1), 286.2 (C_2), 287.8 (C_3), and 289.2 (C_4) eV by imposing a constant full width at a half-maximum of 1.35 eV and thought to be representative for carbon involved in C—C bonds, C—O and C—N bonds, O═C—N and O═C—O^- bonds, and O═C—OH bonds, respectively. The oxygen peak was split into two components at 531.0 (O_1) and 532.4 (O_2) eV by imposing a constant full width at a half-maximum of 1.70 eV and thought to be representative for oxygen involved in O═C and C—O bonds, respectively.

For TLC, 20 mg of freeze-dried crude biosurfactants was dissolved in a mixture of chloroform, methanol, and water (1:1:0.3, vol/vol), while 2 mg of the chloroform fraction was dissolved in 1 ml of chloroform. TLC was carried out on Silica Gel 60 plates (0.25-mm thick) with concentration zones (Merck) by use of a mixture of chloroform, methanol, and water (65:25:4, vol/vol).

For visualizing, the following stains were sprayed: water (water-repellent spots including lipids become visible as opaque bands or spots on a translucent background), iodine vapor (glycolipids and phospholipids appear as brown spots in daylight and very dark spots under UV light), 0.2% 2′,7′-dichlorofluorescein in 95% ethanol (neutral lipids are visible as yellow spots on a purple background under UV light), 0.2% ninhydrin in 96% ethanol (free-amino-acid-containing material including amino lipids appears as purple-mauve spots after heating at 110°C for 10 min), and 0.2% orcinol in 70% H_2SO_4 (carbohydrates including glycolipids become visible as blue spots after heating at 100°C for 5 to 10 min).

***S. thermophilus* B cell surface properties.** The cell surface hydrophobicity and zeta potential of *S. thermophilus* B prior to and after biosurfactant release were measured to rule out that the components released were cell surface components whose release affected the adhesive properties of the cell surface (29). Bacterial cell surface hydrophobicity was assessed by water contact angle measurements on bacterial lawns, prepared by depositing about 50 layers of bacteria suspended in water on membrane filters (pore diameter, 0.45 μm). Wet filters with deposited organisms were fixed on sample holder plates with double-sided sticky tape and air dried, while water contact angles were measured as a function of drying time. Water contact angles became independent of drying time after 20 min, yielding plateau contact angles (42). Contact angles were measured by image analysis techniques at 25°C with an accuracy of ±2 degrees, employing sessile droplets of water. At least three different filters from separate cultures were prepared.

TABLE 1. Adhesion of *Candida* strains to silicone rubber with and without a salivary conditioning film and their detachment by passage of an air bubble[a]

Conditioning film on silicone rubber	Yeast strain	Initial cell deposition rate ($cm^{-2} \cdot s^{-1}$)	No. of cells adhering at 4 h (10^4 cm^{-2})	% Detachment[b]
Absent	*C. albicans* GB 1/2	103 ± 26	55 ± 15	88 ± 10
	C. albicans GB 8/1	175 ± 22	87 ± 3	96 ± 2
	C. tropicalis GB 9/9	132 ± 37	56 ± 29	16 ± 12
	C. tropicalis GB 19/4	158 ± 20	83 ± 2	62 ± 6
Present	*C. albicans* GB 1/2	78 ± 28	37 ± 15	90 ± 1
	C. albicans GB 8/1	23 ± 6	23 ± 2	95 ± 2
	C. tropicalis GB 9/9	100 ± 15	50 ± 12	85 ± 11
	C. tropicalis GB 19/4	130 ± 24	87 ± 5	91 ± 3

[a] Results are means ± standard deviations of triplicate runs with separately cultured microorganisms.

[b] Percentage of adhering yeasts detached by passing an air bubble over the adhering cells. Percentages were calculated with respect to the number of cells adhering at 4 h.

Zeta potentials were determined in adhesion buffer from the speed of suspended bacteria in an applied electric field of 150 V by use of the Helmholtz-Smoluchowski equation (18). Six readings with the instrument, a Lazer Zee Meter 501 (PenKem, Bedford Hills, N.Y.), were taken per filling of the electrophoresis chamber, yielding an average standard deviation of ±2 mV.

Adhesion experiments. The flow chamber (dimensions, 7.6 by 3.8 by 0.06 cm [length by width by height]) and image analysis system have been described in detail before (39). Images were taken from the bottom plate (5.8 by 3.8 cm) of the parallel plate flow chamber that consisted of a thin sheet of silicone rubber affixed to a thicker (1.5-mm) Perspex plate. The top plate of the chamber was made of glass.

Prior to each experiment, all tubes and the flow chamber were filled with buffer, with care taken to remove air bubbles from the system. Flasks, containing microbial suspensions, buffer, and saliva when appropriate, were positioned at the same height with respect to the chamber, so that immediately after the flows were switched, all fluids would circulate through the chamber under the influence of hydrostatic pressure at the desired shear rate of 10 s^{-1} (0.025 ml · s^{-1}), which yields a laminar flow (Reynolds no. 0.6).

The first flow was switched to saliva, when appropriate, for 1.5 h to create a salivary conditioning film, after which the flow was switched for 30 min to buffer to remove all remnants of saliva from the tubing and the flow chamber. Thereafter, the flow was switched, again when appropriate, to the *S. thermophilus* B suspension until the desired surface coverage by adhering *S. thermophilus* of 1 to 4% was attained as measured in real time with an image analysis system (39). In the experiments carried out to determine the effects of preadsorbed biosurfactants, biosurfactants were adsorbed overnight to the silicone rubber from a 30-mg · ml^{-1} solution. Between the two flow steps, buffer was delivered through the system to remove unbound material from the tubes and chamber. Finally, a yeast suspension was circulated through the system for 4 h.

The initial increase in the number of adhering yeasts with time was expressed in a so-called initial deposition rate of j_0 (in square centimeters per second), i.e., the number of microorganisms initially adhering per area and unit time. The number of yeasts adhering after 4 h, $n_{4\ h}$, was taken as an estimate of microbial adhesion in a more advanced state of the adhesion process.

Finally, while focus was maintained on the same spot of the substratum and the fluid flow was switched from the yeast suspension to the buffer, the number of adhering organisms in this field of view was compared with the number of organisms that remained adhering after an air bubble was passed through the chamber to obtain an indication of the adhesive forces (19). The detachment forces accompanying the passage of an air-liquid interface, i.e., an air bubble, over adhering micron-sized particles can be easily estimated to be around 10^{-7} N per adhering microorganism, which is in the order of magnitude of the adhesive forces of microorganisms on solid substrata (37).

All adhesion experiments were done in triplicate with separately cultured organisms at room temperature.

RESULTS

Adhesion of yeasts to silicone rubber with and without a salivary conditioning film. Table 1 summarizes the adhesion of the yeast strains to silicone rubber with and without a salivary conditioning film. The initial deposition rates of the *C. albicans* strains to silicone rubber were not different from those of the *C. tropicalis* strains. Also, the numbers of yeasts adhering after 4 h, which actually represents adhesion in a stationary end point under the present conditions, were not systematically different for the two species. However, when a high detachment force was exerted over the adhering yeasts by the passage of an air bubble through the parallel plate flow chamber, almost all adhering *C. albicans* strains detached, whereas adhering *C. tropicalis* strains were far better able to withstand this detachment force.

The presence of a salivary conditioning film on the silicone rubber reduced the adhesion of both *C. albicans* strains but hardly affected the adhesion of the *C. tropicalis* strains. However, *C. tropicalis* cells adhering to the conditioning film also detached in high percentages as a result of the passage of an air bubble, although this is due, presumably, to the cohesive failure of the conditioning film.

Competition of yeasts with biosurfactant-releasing *S. thermophilus* B in adhesion to silicone rubber. Table 2 shows the adhesion of the yeasts in the presence of adhering, biosurfactant-releasing *S. thermophilus* B cells. The presence of the bacteria at the extremely low surface coverage of only 2% greatly reduced the adhesion of both *C. albicans* and *C. tropicalis* strains. The presence of adhering *S. thermophilus* B cells slightly increased the ability of the yeasts to withstand the passage of an air bubble by approximately 9% on silicone rubber without a conditioning film. When a salivary conditioning film was present, an average of 5% more adhering yeasts were stimulated to detach.

Competition of yeasts with non-biosurfactant-releasing *S. thermophilus* B in adhesion to silicone rubber. To establish whether the reduction in yeast adhesion in the presence of adhering *S. thermophilus* B was due to simple physical effects or to biosurfactant release by the adhering bacteria, experiments were carried out with biosurfactant-releasing *S. thermophilus* cells and a nonreleasing *S. thermophilus* variant as a function of the degree of surface coverage. Nonreleasing cells were prepared by allowing the *S. thermophilus* cells to release their biosurfactants overnight, after which they were used in competition experiments with *C. albicans* GB 1/2 and *C. tropicalis* GB 9/9 testing adhesion to silicone rubber without a conditioning film. With ADSA-P, it was established first that the non-biosurfactant-releasing variant had completely lost its ability to release biosurfactants and to reduce the surface tension of an aqueous suspension (Table 3), while neither the

TABLE 2. Adhesion of *Candida* strains to silicone rubber with and without a salivary conditioning film in competition with adhering *S. thermophilus* B (2% surface coverage)[a]

Conditioning film on silicone rubber	Yeast strain	Relative initial cell deposition rate (%)	Relative % of cells adhering at 4 h
Absent	*C. albicans* GB 1/2	35 ± 7	77 ± 26
	C. albicans GB 8/1	35 ± 16	49 ± 16
	C. tropicalis GB 9/9	51 ± 7	40 ± 17
	C. tropicalis 19/4	64 ± 6	76 ± 12
Present	*C. albicans* GB 1/2	68 ± 10	41 ± 9
	C. albicans GB 8/1	25 ± 11	46 ± 3
	C. tropicalis GB 9/9	64 ± 33	58 ± 28
	C. tropicalis 19/4	36 ± 17	31 ± 2

[a] Results are expressed as percentages with respect to the control (surface coverage by *S. thermophilus* B = 0%). Results are means of triplicate runs ± standard deviations, with the results in the absence and presence of adhering *S. thermophilus* B cells always paired.

TABLE 3. Zeta potentials, intrinsic cell surface hydrophobicities, and surface tension decrease measurements before and after biosurfactant release[a]

Condition	ζ (mV)	θ (degrees)	$\Delta\gamma_{1v}$, 2 h ($mJ \cdot m^{-2}$)
As cultured	−6.4	21	25
After 2 h of biosurfactant release	−6.5	20	6
After 24 h of biosurfactant release	−7.3	29	0

[a] Zeta potentials (ζ) in adhesion buffer and intrinsic cell surface hydrophobicities (θ) by water contact angles of *S. thermophilus* B before and after surfactant release, together with the surface tension decrease brought about by a bacterial suspension within 2 h ($\Delta\gamma_{1v}$, 2 h), measured by ADSA-P.

intrinsic cell surface hydrophobicity nor the zeta potentials had changed after the release of biosurfactants (Table 3).

Direct physical effects of adhering non-biosurfactant-releasing bacteria caused an increase in the initial deposition rates of the yeasts (Table 4). In contrast, the presence of biosurfactant-releasing bacteria yielded a reduction in the initial deposition rates of the yeasts. For *C. albicans* GB 1/2, for instance, 4% coverage of the silicone rubber surface by non-biosurfactant-releasing *S. thermophilus* B cells increased the initial deposition rate by 23%, whereas a similar surface coverage by biosurfactant-releasing bacteria decreased the initial deposition rate by 85%. Also, the number of adhering yeasts after 4 h was more reduced by the presence of biosurfactant-releasing *S. thermophilus* cells than by the presence of non-biosurfactant-releasing variants. Generally, the effects of biosurfactant-releasing *S. thermophilus* B were greater for *C. albicans* GB 1/2 than for *C. tropicalis* GB 9/9.

Adhesion of yeasts to silicone rubber with preadsorbed *S. thermophilus* B biosurfactants. The amounts of purified biosurfactant components that could be obtained were too small to permit experiments with preadsorbed purified components. However, since significant effects were observed when biosurfactant-releasing *S. thermophilus* B was adhered to the silicone rubber, it was considered worthwhile to study whether preadsorption of crude biosurfactants had any effect on the adhesion of two of the yeast strains employed, *C. albicans* GB 1/2 and *C. tropicalis* GB 9/9. Preadsorption of crude, stationary-phase biosurfactants had a reducing effect on the adhesion of *C. albicans* GB 1/2 similar to that observed when 1 to 2% of the silicone rubber was covered with adhering *S. thermophilus* B (Table 4). The percentage of detachment by the passage of an air bubble of *C. albicans* GB 1/2 remained at a similarly high level in the presence of preadsorbed biosurfactants and in their absence. The effects of preadsorbed biosurfactants upon the adhesion of *C. tropicalis* GB 9/9 were not expressed in their initial deposition rates nor in the number of adhering yeast cells after 4 h. For *C. tropicalis* GB 9/9, however, an effect on the detachment characteristics of this strain was clearly measured. Whereas only 18% of the adhering *C. tropicalis* GB 9/9 cells detached upon the passage of an air bubble through the flow chamber, 51% of the adhering yeasts detached in the presence of preadsorbed biosurfactants, indicating that the preadsorbed biosurfactants weakened the bond between this yeast strain and the silicone rubber.

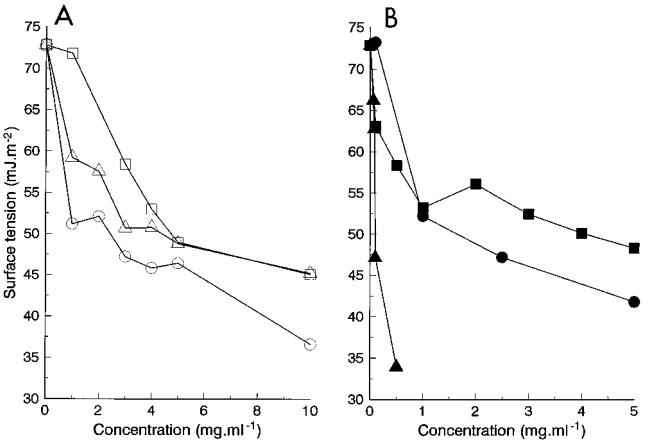

FIG. 1. Surface tensions of crude biosurfactants (A) and extracted components (B) released by *S. thermophilus* B as a function of their concentration in water, measured by ADSA-P 2 h after a solution droplet was positioned. (A) Biosurfactants from the mid-exponential (□), early stationary (△), and stationary (○) phases were assessed. (B) Methanol-water (●) and chloroform (▲) fractions and the filter residue (■) were assessed.

Characterization of *S. thermophilus* B biosurfactants. The amount of biosurfactant released was largest per gram of cells (dry weight) for cells in their mid-exponential (37.3 mg · g of $cells^{-1}$ [dry weight]) and early stationary (37.0 mg · g of $cells^{-1}$ [dry weight]) phases. Biosurfactants released in the stationary phase (14.0 mg · g of $cells^{-1}$ [dry weight]) are more surface active than biosurfactants released in the mid-exponential and early stationary phases since stationary-phase biosurfactants give a larger reduction in the surface tension of an aqueous solution (Fig. 1A). After lipid extraction, the surface tensions of the upper methanol-water fraction and the filter residue of stationary-phase biosurfactants were essentially unaltered from those of the crude biosurfactant (Fig. 1B), but the chloroform fraction, containing lipids, possessed an extremely surface-active component with a surface tension reduction to 35 $mJ \cdot m^{-2}$ at a concentration of only 0.5 mg · ml^{-1}. The chloroform fraction constituted approximately 0.9% of the crude biosurfactant.

Table 5 summarizes the chemical composition of the biosurfactants as determined by XPS. Although all biosurfactants

TABLE 4. Adhesion of *Candida* strains to silicone rubber in competition with adhering *S. thermophilus* B and to silicone rubber with preadsorbed stationary-phase biosurfactants only[a]

Yeast strain	Surface coverage by *S. thermophilus* (%)	Relative initial cell deposition rate (%)[b]		Relative % of cells adhering at 4 h[b]	
		b^-	b^+	b^-	b^+
C. albicans GB 1/2	0	100	100	100	100
	1	113 ± 37	60 ± 20	121 ± 20	67 ± 35
	2	114 ± 20	35 ± 7	80 ± 16	77 ± 26
	4	123 ± 40	15 ± 4	78 ± 31	32 ± 4
	—[c]	NA[d]	69 ± 20	NA	77 ± 14
C. tropicalis GB 9/9	0	100	100	100	100
	1	125 ± 14	64 ± 25	96 ± 11	89 ± 37
	2	109 ± 6	51 ± 7	84 ± 20	40 ± 17
	4	107 ± 5	51 ± 2	118 ± 10	43 ± 10
	—	NA	103 ± 28	NA	124 ± 32

[a] Results are expressed in percentages with respect to the control (coverage by *S. thermophilus* B = 0%). Results are means of triplicate runs ± standard deviations, with the results in the absence and presence of adhering *S. thermophilus* B cells or preadsorbed biosurfactants always paired.

[b] b^-, variants of *S. thermophilus* B that have released all their biosurfactants prior to being engaged in the experiments; b^+, the original, biosurfactant-releasing strain.

[c] —, coverage with biosurfactants only.

[d] NA, not applicable.

TABLE 5. Chemical composition data by XPS of *S. thermophilus* B biosurfactants released in different growth phases, the lipid-enriched chloroform fraction obtained from stationary-phase biosurfactants, and reference compounds (for comparison)

Biosurfactant, fraction, or compound	Elemental composition ratio			Fractions of C and O atoms					
	N/C	O/C	P/C	C_1	C_2	C_3	C_4	O_1	O_2
Biosurfactant released in:									
Mid-exponential phase	0.154	0.64	0.042	0.46	0.35	0.19	0.01	0.55	0.45
Early-stationary phase	0.158	0.63	0.016	0.46	0.33	0.20	0.01	0.44	0.56
Stationary phase	0.126	0.42	0.024	0.55	0.26	0.16	0.02	0.55	0.45
Chloroform fraction	0.002	0.33	0.020	0.82	0.12	0.04	0.02	0	1
Reference compounds									
Glycosidic residue[a]	0	0.83	0	0	0.83	0.17[e]		0	1
Phospholipid[b]	0.009	0.21	0.034	0.82	0.13	0.05	0.01	0.37	0.63
Cholesterol	0	0.03	0	0.87	0.12	0	0	0	1
LTA[c]	0.031	0.63	0.066	0.41	0.44	0.44	0.05	0.24	0.76
Protein[d]	0.270	0.32	0	0.41	0.32	0.28[e]		0.86	0.14

[a] Glycosidic residue $C_6H_{10}O_5$ (24).
[b] L-α-Phosphatidyl-DL-glycerol dimyristoyl.
[c] Lipoteichoic acid from *S. mutans* (Sigma).
[d] Average protein, calculated for a collection of bacterial, fungal, and mammalian proteins (24).
[e] C_3 and C_4 together.

contained sizeable amounts of nitrogen, the N/C ratios measured were too low for the biosurfactants to be identified as pure protein. The high O/C ratios were also too high to indicate the presence of pure proteins and point to the presence of polysaccharides. This was in agreement with the FTIR spectra presented in Fig. 2, where the so-called amide I (1,655 cm^{-1}) and amide II (1,550 cm^{-1}) absorption bands indicative of proteins were not detected. The absorption bands between 1,000 and 1,200 cm^{-1} were attributed to ethereal and hydroxylic C—O stretch vibrations in polysaccharides. However, despite the fact that TLC indicated the presence of more than 10 components in the crude biosurfactant, only one spot indicative of carbohydrate was found (see Fig. 3). Possibly, the polysaccharide-containing molecules were too big to move over the TLC plate or were not all dissolved in the solvent used. It is important to note that the crude biosurfactant also contained an insoluble fraction.

The XPS data (Table 5) demonstrated that the chloroform fraction contained a higher percentage of carbon involved in C_1 bonds and of oxygen in O_2 bonds, while nitrogen was nearly absent. In fact, the percentage of oxygen involved in O_2 bonds was too high for the material to be identified as a phospholipid, but on the basis of the XPS data and the infrared absorption bands present around 2,965, 2,930, 2,860, 1,465, 1,420, and 1,380 cm^{-1} (—CH_3—) and between 1,000 and 1,200 cm^{-1} (C—O stretch vibration in sugars), the material probably con-

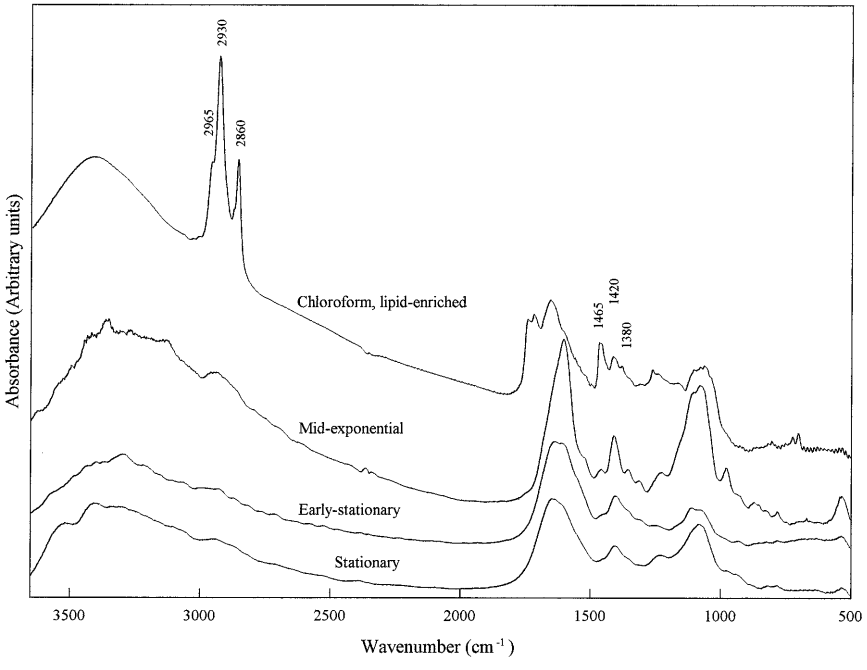

FIG. 2. Infrared spectra of crude biosurfactants released in different growth phases by *S. thermophilus* B and of the chloroform, lipid-enriched fraction of the stationary-phase biosurfactant.

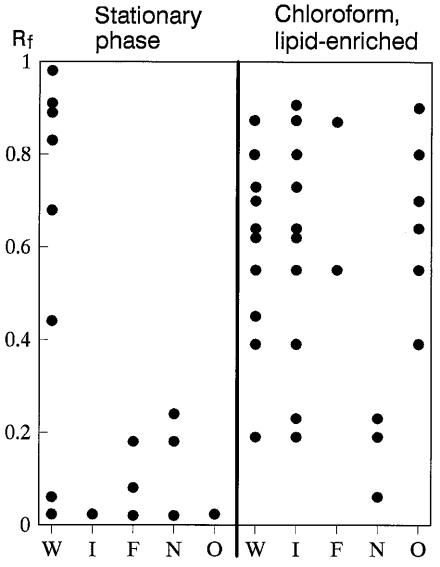

FIG. 3. Schematic presentation of the stainable spots on TLC plates with crude *S. thermophilus* B biosurfactants and of a chloroform, lipid-enriched fraction. Abbreviations: W, water; I, iodine vapor; F, 2′,7′-dichlorofluorescein; N, ninhydrin; O, orcinol.

tains glycolipids. This suggestion was in line with the TLC results showing six glycolipid-, two neutral lipid-, and three lipid-containing amino acid spots in the chloroform fraction.

DISCUSSION

We measured the adhesion of yeasts, isolated from indwelling voice prostheses, to silicone rubber in the absence and presence of adhering thermophilic dairy streptococci by use of a parallel plate flow chamber system. The parallel plate flow chamber system is a highly reproducible and versatile system to measure microbial adhesion to surfaces under controlled flow conditions. As a consequence, mass transport in the parallel plate flow chamber is well controlled. With respect to mass transport, it is interesting to note that in the presence of non-biosurfactant-releasing *S. thermophilus* B cells on the silicone rubber, the initial deposition rates of the yeasts were higher than those without adhering bacteria (Table 4). This increased mass transport to silicone rubber with adhering non-biosurfactant-releasing bacteria was due to a convective flow parallel to the substratum surface, yielding collisions between flowing yeasts and adhering bacteria with an associated increased mass transport in addition to convective diffusion perpendicular to the substratum (11). Actually, the increased deposition resulting from the physical presence of non-biosurfactant-releasing streptococci enforces the conclusion that the decreased initial deposition rates observed in the presence of adhering, biosurfactant-releasing streptococci were indeed due to the biosurfactants. Biosurfactant release by these dairy streptococci reduced yeast adhesion not only to silicone rubber without a salivary conditioning film but also to silicone rubber with a salivary conditioning film, despite the fact that adsorbed salivary components themselves had already decreased adhesion of *C. albicans* and stimulated detachment of *C. tropicalis* after the passage of an air bubble (7).

Whereas the control of mass transport and hydrodynamic conditions in the parallel plate flow chamber offers advantages from a fundamental point of view, real-life situations are generally less controlled. In the oropharyngeal cavity, the detachment forces operative may vary widely, from situations in which shear is virtually absent to periods of excessively high detachment forces such as during swallowing, eating, drinking, and speaking (44). Therefore, it is important to study not only microbial adhesion but also the ability of adhering microorganisms to withstand detachment forces as exerted by a passing air bubble (31). The presence of biosurfactant-releasing *S. thermophilus* B cells on the silicone rubber was effective in reducing adhesion of both *C. albicans* and *C. tropicalis* strains. Preadsorbed biosurfactants reduced the adhesion only of *C. albicans*, not of *C. tropicalis*. However, *C. tropicalis* detached more readily from silicone rubber in the presence of preadsorbed biosurfactants. Thus, the conclusion that *S. thermophilus* B utilizes released biosurfactants as a defense weapon in postadhesion competition on silicone rubber with *Candida* species seems justified. Similar conclusions have been drawn recently for the postadhesion competition between lactobacilli and uropathogens (22, 43) and between *Streptococcus mitis* and cariogenic *Streptococcus mutans* (32, 41).

It is not clear why the presence of preadsorbed biosurfactants had such a different effect on *C. tropicalis* adhesion than the presence of biosurfactant-releasing streptococci themselves did while the effects were roughly similar on *C. albicans* adhesion. Most likely, the essential difference between preadsorbing biosurfactants onto the silicone rubber and covering the surface with biosurfactant-releasing streptococci is the fact that adsorbed biosurfactants are not available for adsorption onto the yeast cell surfaces, while biosurfactants released by adhering *S. thermophilus* can adsorb either to the yeast cell surface or to the silicone rubber. *C. tropicalis* is much more hydrophobic than *C. albicans* (23), which may have a potential impact on adsorption of amphiphilic biosurfactants.

Biosurfactant release by adhering bacteria has been implicated before in their detachment from substratum surfaces, as in the detachment of *S. thermophilus* from glass (5) or of *Acinetobacter calcoaceticus* from oil droplets (35). A role for biosurfactants as defense weapons in postadhesion competition with other strains or species has to date been suggested only for biosurfactants released by *S. mitis* strains against *S. mutans* adhesion and for biosurfactants released by lactobacilli against adhesion of uropathogens. Recently, it was demonstrated that not only a culture of *Lactobacillus casei* GG but also filtered spent culture supernatant impeded invasion by a *Salmonella* strain into enterocyte-like Caco-2 cells, suggesting a role for released, biosurfactive products (16).

The most commonly isolated biosurfactants are glycolipids. Glycolipids released by *Pseudomonas aeruginosa*, the so-called rhamnolipids, are well studied (15) since they are released in relatively large amounts (34). The surface tension of spent culture supernatant was less than 30 $mJ \cdot m^{-1}$ after growth of *P. aeruginosa*. A similarly low surface tension of spent culture supernatant was found for surfactin, released by *Bacillus subtilis* strains (10). Proteinaceous biosurfactants, released by *Lactobacillus* species and named surlactin (43), reduced the surface tension of water to around 39 $mJ \cdot m^{-1}$, but their release per liter of culture medium (approximately 100 $mg \cdot liter^{-1}$) was orders of magnitude smaller than that of rhamnolipids. The amounts released by *S. thermophilus* B in this study were even smaller than those released by *Lactobacillus* species, but a simple calculation is instructive to demonstrate that even small amounts may have a dramatic effect on adhesion to substratum surfaces. Assuming that the molecular size of the biosurfactant released is 1,000 Da, it can be estimated that *S. thermophilus* B cells adhering at a surface coverage of around 8% release enough biosurfactants to fully coat the silicone rubber.

Since the first studies on probiotics by Metchnikoff, published in 1907, there has been a growing awareness that the consumption of fermented dairy products "is good for you" (12). The many claims that have been made for probiotics include suppression of diarrhea, antitumor activity, stimulation of immunity, and relief of lactose intolerance, but development of probiotics slowed in the 1930s and 1940s with the introduction of chemotherapy and penicillin. Now that the limits of antibiotics may almost be reached, the interest in the use of probiotics for health benefits has renewed. The results of this study provide evidence in support of rumors circulating among laryngectomized patients and certain groups of ear-nose-throat clinicians that the consumption of dairy products with active bacteria may prolong the lifetime of indwelling silicone rubber voice prostheses. The time scale over which reduced yeast adhesion to silicone rubber was observed, i.e., 4 h after a single introduction of *S. thermophilus* B in the flow chamber, is clinically relevant since the consumption of, for instance, a glass of drinking yogurt at a 4- to 6-h time interval during the day does not pose an inconvenience to most patients (extensive consumption of fermented dairy products is a lifelong pattern in many countries). Therefore, the health benefits to be gained by the consumption of probiotics extend to patients with biomaterial implants through the prevention or delay of implant failure from infectious biofilms. Since it is not considered feasible, based on the current biosurfactant release by *S. thermophilus*, to manufacture biosurfactant-coated voice prostheses, health benefits for laryngectomized patients with indwelling voice prostheses can be obtained only by the consumption of bio-yogurts, yogurts with active probiotics, enabling these patients to simultaneously profit from the possible other health benefits of the probiotic.

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